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Different Metal-Binding Properties of the Two Sites of Human Transferrin†

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ABSTRACT: Transferrin, the serum iron-transport protein which can bind two metal ions at physiologic pH, binds just one Fe^{3+}, VO^{2+}, or Cr^{3+} ion at pH 6.0. Fe^{3+} and VO^{2+} appear to be bound at the same site, designated A, based on electron paramagnetic resonance (EPR) spectra of VO^{2+}-transferrin and $(Fe^{3+})_1(VO^{2+})_1$-transferrin. The EPR spectra of $(Cr^{3+})_1(VO^{2+})_1$-transferrin and of $(Cr^{3+})_1(Fe^{3+})_1$-transferrin indicate that Cr^{3+} is bound to site B at pH 6.0. Transferrin was labeled at site A with ^{59}Fe at pH 6.0 and at site B with ^{55}Fe at pH 7.5. When the pH of the resulting preparation was lowered to 6.3 and the dissociated iron was separated by gel filtration, about ten times as much ^{55}Fe as ^{59}Fe was lost. The same EPR and isotopic-labeling experiments showed that Fe^{3+} added to transferrin at pH 7.5 binds to site A with about 90% selectivity.

Transferrin is the protein responsible for carrying iron in the blood (Aisen and Brown, 1975; Morgan, 1974; Aisen, 1973). Each molecule of weight near 80 000 consists of a single peptide chain containing two metal-binding sites of remarkably similar thermodynamic and spectroscopic properties (Aasa et al., 1963; Aisen et al., 1966; Binford and Foster, 1974; Gafni and Steinberg, 1974). Since the suggestion of Fletcher and Huehns (1967, 1968; Fletcher, 1969) that each site of transferrin may have a different physiologic function, there has been a great deal of interest in any distinguishing properties of the two sites. The interpretation of the EPR[1] spectrum of diferric transferrin has been somewhat controversial (Aasa and Aisen, 1968; Aasa, 1972), but there appear to be two kinds of spectroscopic behavior in the presence of perchlorate (Price and Gibson, 1972). A very clear distinction between the sites is evident in the EPR spectra of the vanadyl (VO^{2+}) (Cannon and Chasteen, 1975) and Cr^{3+} (Aisen et al., 1969; Harris et al., 1975) complexes of transferrin.

It has recently been demonstrated by Princiotto and Zapolski (1975) and by Lestas (1976) that the two iron-binding constants of transferrin have significantly different pH dependences. It was shown that one site loses its iron as the pH is reduced to near six, but the other site retains its iron until the pH is reduced to approximately five.

It was our hope that using the effect of pH upon metal binding, it would be possible to label transferrin at each site with a different radioisotope of iron. Protein so labeled would

† From the Department of Chemistry, University of California, Davis, California 95616. *Received July 15, 1976.* Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; EPR, electron paramagnetic resonance.

then be a valuable tool in the study of the physiologic function of the two sites of transferrin. The present work was undertaken to study the feasibility of such double labeling and to correlate the EPR spectra of VO^{2+} and Cr^{3+} with the particular site they occupy.

Experimental Procedure

Glassware was acid rinsed and buffers were extracted with dithizone in carbon tetrachloride to remove traces of iron. Solutions were boiled to remove carbon tetrachloride.

Transferrin (Aisen et al., 1966; Laurell and Ingelman, 1947; Gelotte et al., 1962). Serum pooled from five healthy adults was saturated with iron by stirring in the air for 1 h with ferrous ammonium sulfate and a 20-fold excess of bicarbonate, assuming an iron-binding capacity of 3 μg of Fe/mL. The fraction precipitated by ammonium sulfate between 50 and 70% saturation was dissolved in water and dialyzed against several changes of 0.05 M Tris, pH 8.0. The red solution was applied to a column of DEAE-Sephadex A-50 and eluted with a Tris gradient ranging from 0.05 to 0.20 M, pH 8.0. Fractions exhibiting an absorbance ratio (A_{470}/A_{410}) greater than 1.25 were dialyzed against 0.05 M Tris, pH 8.0, and passed through a second column of DEAE-Sephadex as above, or through a column of Bio-Rad Bio-Gel A, eluted with 0.05 M Tris, pH 8.0. Transferrin used in this work exhibited a ratio A_{470}/A_{410} in the range 1.35–1.41. Apoprotein was prepared as described previously (Harris et al., 1974).

The molecular weight of transferrin was assumed to be 80 000 and the absorbance at 280 nm of a solution containing 10 mg of protein/mL was taken as 10.9 for apoprotein and 14.0 for diferric transferrin. It was assumed that the absorbance varied linearly between these two extremes as the iron content increased.

Chromium Transferrin. Dichromium transferrin was prepared at pH 7.7 as described by Harris et al. (1975) using a 6:1 molar ratio of chromium to transferrin. Excess metal was removed by gel filtration at pH 7.5. Monochromium transferrin was prepared by the exact same procedure, except that the pH was left at 5.9 and excess metal was removed by gel filtration at pH 6.1 in 0.01 M succinate.

Other Materials. Radioactively labeled iron was purchased as $FeCl_3$ in 0.5 M HCl from New England Nuclear. Iron nitrilotriacetate was prepared and standardized spectrophotometrically (Harris and Aisen, 1975a). Chemicals were reagent grade whenever available. The concentration of $VOSO_4 \cdot 2H_2O$ (Eastman) was estimated using a molar extinction coefficient at 750 nm of 18.0 (Fitzgerald and Chasteen, 1974). The absorbance was constant for several weeks in a solution unprotected from the air.

EPR Spectra. Spectra were recorded at liquid nitrogen temperature using a Varian E-4 spectrometer operating at 9.05 GHz, with a modulation amplitude of 10 G and a power of 20 mW. Vanadyl transferrin solutions were $1\text{–}4 \times 10^{-4}$ M in protein and ~3 mM in $NaHCO_3$. All samples containing VO^{2+} were prepared and maintained under a pure nitrogen atmosphere (Cannon and Chasteen, 1975). Solutions were chilled to 0 °C and degassed in vacuo using a single Thunberg tube (Aisen et al., 1967). VO^{2+} solution was then added with a stream of nitrogen flowing out of the tube. pH adjustment was accomplished with an electrode inserted into the Thunberg tube while nitrogen was flowing out of the tube. The EPR spectra of VO^{2+}-transferrin solutions prepared in this manner were stable at room temperature for at least 5 days.

Many of our early EPR studies were complicated by the presence of nitrilotriacetate or succinate, both of which bind VO^{2+} and yield spectra at 77 K similar to the site B transferrin spectrum in Figure 4. VO^{2+} bound to these chelates gives a characteristic eight-line spectrum at room temperature, which is quite different from the anisotropic spectrum of protein-bound VO^{2+} (Cannon and Chasteen, 1975). The solution used for the spectrum in Figure 1 was buffered only with bicarbonate. As expected (Francavilla and Chasteen, 1975; Iannuzzi and Rieger, 1975), VO^{2+} in 0.1 M Hepes buffer at pH 7.5 gave a negligible signal at 77 K.

Liquid Scintillation Counting. Protein containing both ^{55}Fe and ^{59}Fe was analyzed by liquid scintillation counting on a Beckman LS-250 counter using the equations given previously (Harris and Aisen, 1975a). Solutions were virtually colorless so no special precautions were taken to reduce quenching. All scintillation samples contained 1 mL of aqueous solution and 10 mL of Aquasol.

Double-Label Experiments: Initial Labeling at pH 6.0. A solution containing 13 mg (0.16 μmol) of apotransferrin in 1.7 mL of water was treated with 0.10 mL of 0.1 M $NaHCO_3$ and 0.20 mL of 0.5 M succinate buffer (pH 6.0). The pH was adjusted to 6.0 with a few μL of 1 M HCl with stirring and 50 μL (0.48 μmol) of ^{59}Fe-nitrilotriacetate was added. The solution was concentrated to 0.5 mL through a collodion membrane and passed through a 1.5 × 10 cm column of Sephadex G-25 equilibrated with 0.1 M succinate (pH 6.0) at 23 °C. Prior to the experiment, the column was washed with 100 mL of 1 mM ethylenediaminetetraacetic acid, followed by 300 mL of succinate buffer. The void volume fraction had a ratio of iron-to-protein of 0.89, as estimated by the ratio of ^{59}Fe counts to absorbance at 280 nm. Maintaining the approximate ratio of Sephadex G-25 to protein given above is critical. The column appears to compete with the protein for iron at pH 6.0. At greater Sephadex-to-protein ratios, we consistently observed product with much less than one iron per protein molecule.

One milligram (0.0125 μmol) of the ^{59}Fe-transferrin in 263 μL of 0.01 M succinate (pH 6.0) was treated with 70 μL of 0.5 M Hepes (pH 7.5), 10 μL of 0.1 M $NaHCO_3$, and 1.6 μL (0.014 μmol) of ^{55}Fe-nitrilotriacetate. After 1 h at room temperature, the solution was passed through a 0.7 × 2.5 cm column of Bio-Rad AG 1-X4 anion-exchange resin equilibrated with 0.05 M Hepes, pH 7.5. The protein was eluted with 3 mL of buffer and a portion of the eluate was analyzed for iron and protein. The remainder was concentrated to 100 μL through a collodion membrane. To the concentrated solution at 23 °C was added 60 μL of 0.5 M succinate buffer (pH 6.0) and the solution was immediately applied to a 1.0 × 6 cm column of Sephadex G-25 eluted with 0.01 M succinate, pH 6.2–6.3. (The amount of succinate buffer added to the protein prior to gel filtration was found to lower the pH to 6.3.) The void volume fraction was analyzed for radioactivity and protein absorbance.

Double-Label Experiments: Labeling at pH 7.5. The experiment was similar to that described above, except that both isotopes of iron were added to transferrin at pH 7.5 in 0.1 M Hepes with vigorous magnetic stirring. ^{59}Fe was added first and after 30 min at 23 °C ^{55}Fe was added. After an additional 30 min at 23 °C, the protein was passed through the anion-exchange resin and the experiment proceeded exactly as described above.

Labeling Monoferric Transferrin With VO^{2+}. To 5 mL of solution containing 18.5 mg (0.231 μmol) of apotransferrin in 0.1 M $NaClO_4$–0.01 M Hepes–0.02 M KCl at pH 7.7 was added 0.231 μmol of ^{59}Fe-nitrilotriacetate. After 30 min at 23 °C, the solution was concentrated through a collodion membrane to 0.5 mL and passed through a 1.5 × 10 cm column of

TABLE I: Results of Double-Label Experiments.

^{59}Fe added at pH	% Fe Lost Upon Lowering pH to 6.2–6.3		
	^{55}Fe	^{59}Fe	$\frac{^{55}\text{Fe lost}}{^{59}\text{Fe lost}}$
6.0	66	11	6.0
6.0	46	4.1	11
6.0[a]	28	6.3	4.4
7.5	54	8.6	6.3
7.5	38	3.0	13
7.6[b]	46	5.0	9.2
7.5[c]	20	20	1.0

[a] In this experiment, the doubly labeled protein was incubated for 80 min at 37 °C at pH 7.5 in the presence of 10^{-3} M citrate prior to passage through the anion-exchange column. [b] In this case, ^{59}Fe-nitrilotriacetate was added to a buffer at pH 7.6 prior to addition to the apoprotein. [c] In this control experiment, ^{59}Fe and ^{55}Fe were mixed together before addition to the transferrin.

Sephadex G-25 eluted with 0.01 M Hepes–0.02 M KCl–0.1 M $NaClO_4$ at pH 7.5 to remove nitrilotriacetate (Bates and Schlabach, 1973). The void volume fraction exhibited the molar ratio Fe/protein = 0.99. To a degassed solution containing 0.126 μmol of this protein plus 1 μmol of $NaHCO_3$ in 0.36 mL was added 0.126 μmol of $VOSO_4$. After shaking for 15 min at 37 °C, the EPR spectrum in Figure 4 was recorded.

Results

The Binding of Iron to Transferrin. When apotransferrin was titrated with iron nitrilotriacetate at pH 5.0, a plot of A_{470} vs. iron added exhibited a clear end point at an iron-to-protein ratio of 1:1, similar to that reported by Lestas (1976). The solution, about 1×10^{-4} M in protein and buffered with 0.05 M acetate, originally contained bicarbonate sufficient to be 5 mM. However, at equilibrium at pH 5 the total concentration of species derived from dissolved carbon dioxide is only about 10^{-5} M (Stumm and Morgan, 1970). After the excess iron was removed by gel filtration through Sephadex G-25 at pH 5.0, the EPR spectrum showed that the iron was present as the ternary iron transferrin–nitrilotriacetate complex (Aisen et al., 1974). When the same experiment was carried out at pH 6.0 in succinate buffer, an end point was again observed at an iron-to-protein ratio of 1:1, but the EPR spectrum was that of the iron transferrin–carbonate complex (Aisen et al., 1974).

To see if each of the two binding sites of transferrin could be labeled with different isotopes of iron, the following experiment was performed. Apotransferrin was labeled at pH 6.0 with 1 equiv of ^{55}Fe, as described under Experimental Procedures. The pH was raised to 7.5, a second equivalent of ^{55}Fe was added, and any excess iron nitrilotriacetate was removed by anion-exchange chromatography. The pH was then lowered to 6.3 by addition of succinate buffer and the solution was immediately subjected to gel filtration at pH 6.2–6.3. As shown in the top two entries of Table I, most of the iron which dissociated from transferrin under these conditions was ^{55}Fe. This indicates that the original labeling scheme did indeed place ^{59}Fe at one site (designated A) and ^{55}Fe at the other site (designated B), with at least about 90% specificity.

We then repeated the double-label experiment with both isotopes of iron added sequentially at pH 7.5. Entries 4–6 in Table I indicate that the labeling at physiologic pH was just as specific as the labeling at pH 6.0. As a control, the isotopes were premixed and then added to the protein. The last entry in Table I indicates completely random occupation of the sites in this case.

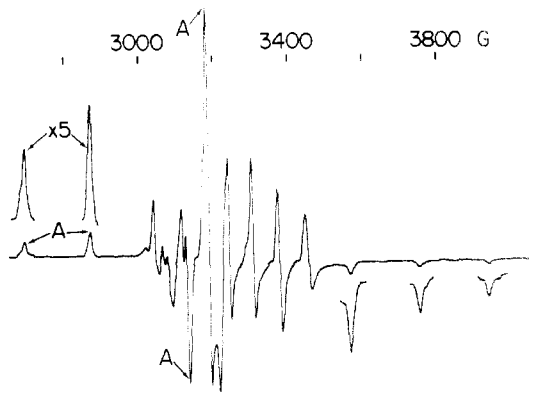

FIGURE 1: EPR spectrum of transferrin at pH 6.0 with a VO^{2+}/transferrin molar ratio of 2.0. This is mainly a site A spectrum. The site B component is nearly completely absent in a preparation in which VO^{2+}/transferrin = 1.0.

It seems possible that the iron added at pH 7.5 might go to site A because the iron nitrilotriacetate solution has an initial pH of 4.0. If the pH of protein solution in the immediate vicinity of a droplet of iron nitrilotriacetate was lowered to, say, 6, then only site A would be occupied. To eliminate this possibility, ^{59}Fe-nitrilotriacetate was first added to a Hepes solution buffered to pH 7.6, and then it was mixed with transferrin. The results were the same as in other experiments: the iron went into site A with about 90% specificity (entry 6, Table I).

The results of these double-label experiments place a lower limit on the specificity of iron binding at site A. To remove some iron from diferric transferrin, the pH was lowered to 6.3 by addition of pH 6.0 buffer and the solution was immediately subjected to gel filtration. If the pH was lowered to 6.0 for 30 min prior to gel filtration at pH 6.0, the two isotopes were completely randomized. It was essential to observe very mild conditions for selective removal of one isotope. Since even the conditions we employed might yield some scrambling of iron between sites, the results in Table I represent the minimum ratio of isotopes at each site. It should be pointed out that the amount of ^{59}Fe lost in these experiments was often very small (e.g., 3%), and comparable to the experimental error in measuring the iron content. Since the amount of ^{59}Fe lost contains a good deal of uncertainty, the ratios of ^{55}Fe lost to ^{59}Fe lost may therefore contain significant experimental error. Comparison of the several results in Table I, however, suggests to us that the specificity of binding to site A is indeed large, on the order of 90%.

Use of VO^{2+} as a Site Marker. Cannon and Chasteen (1975) have shown by spectrophotometric titration that only one vanadyl ion is bound to transferrin at pH 6.1, but two cations are bound at pH 7.5 or 9.0. They interpreted the EPR spectrum of divanadyl transferrin as a superposition of the spectra of VO^{2+} at two different sites in the protein. Figure 1 shows the spectrum of vanadyl-saturated protein at pH 6.0. By the nomenclature of Cannon and Chasteen (1975), this is the spectrum of VO^{2+} mostly at site A of transferrin. We will argue below that this is the same site A at which Fe^{3+} binds at pH 6.0. For comparison, the spectrum of divanadyl transferrin at pH 7.5 (Figure 2) in 0.1 M Hepes buffer clearly exhibits lines from sites A and B. Some of the lines which we found to be most useful in identifying the occupied sites are labeled. The resolution of these two overlapping spectra is particularly clear in Hepes buffer, but was not as clear in the absence of Hepes.

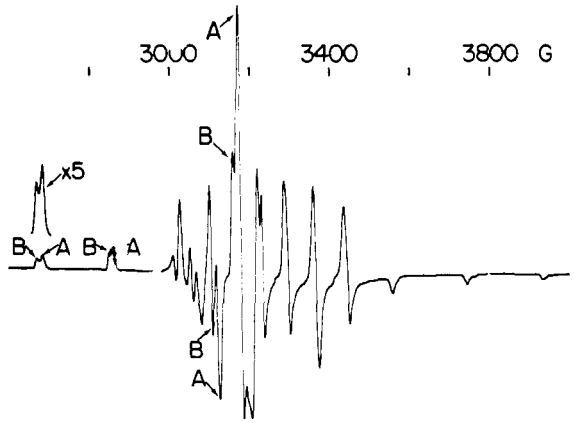

FIGURE 2: EPR spectrum of transferrin at pH 7.5 in 0.1 M Hepes with a VO^{2+}/transferrin molar ratio of 2.0. This is a site A plus site B spectrum.

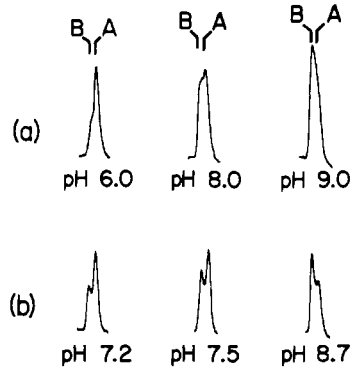

FIGURE 3: EPR spectra showing the shape of the low-field peak of vanadyl transferrin (VO^{2+}/transferrin = 2.0) as a function of pH in (a) the absence of Hepes and (b) the presence of 0.1 M Hepes buffer.

The spectra in Figure 3 show that the apparent ratio of VO^{2+} in sites A and B changes with pH, despite the fact that two ions are bound throughout the pH range. It therefore appears that the VO^{2+} spectrum is sensitive to conformational changes of the protein, as well as the specific site which is occupied (N. D. Chasteen, L. K. White, R. F. Campbell, and J. C. Cannon, submitted for publication). Bearing this in mind, we still found the VO^{2+} spectrum to be a useful qualitative marker for the two sites of transferrin when one site was occupied by another metal.

As shown in the double-label experiments above, 1 mol of Fe-nitrilotriacetate added to the protein at either pH 6.0 or 7.5 occupies mainly one of the two metal-binding sites. We were unable to demonstrate binding of VO^{2+} to monoferric transferrin at pH 6.0. However, when a preparation of $^{59}Fe_1$-transferrin free of nitrilotriacetate (Bates and Schlabach, 1973) was treated with 1 mol of VO^{2+} at pH 7.7, the spectrum in Figure 4 resulted. This shows vanadyl ion mainly at site B, and identifies the site at which iron is bound as site A. The spectrum of this solution at 23 °C showed only signals attributable to transferrin-bound VO^{2+} (Cannon and Chasteen, 1975) and none of the eight-line isotropic spectrum characteristic of VO^{2+} bound to a small chelate, such as nitrilotriacetate. An aliquot of the $^{59}Fe_1$-transferrin used for Figure 4 was treated with 1.0 mol of ^{55}Fe-nitrilotriacetate and any excess Fe-nitrilotriacetate was removed by anion exchange at pH 7.5. Upon lowering the pH to 6.3, 18% of the ^{55}Fe was lost, but only 3% of the ^{59}Fe was lost. The appearance of the B-site spectrum in Figure 4 plus our inability to demonstrate binding of VO^{2+} to monoferric transferrin at pH 6.0 strongly suggest that iron occupies site A at pH 6.0.

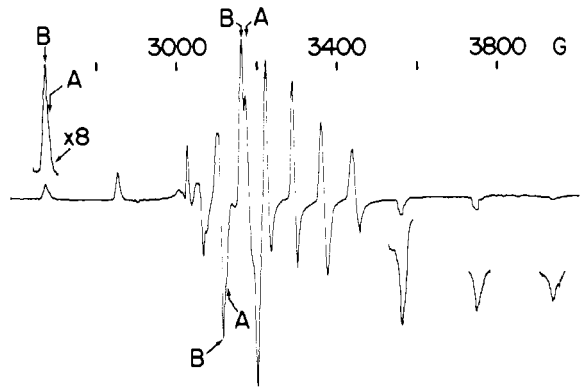

FIGURE 4: EPR spectrum which results when 1.0 mol of VO^{2+} is added to chelate-free monoferric transferrin prepared at pH 7.7. This shows VO^{2+} mainly at site B. Details appear under Experimental Procedure.

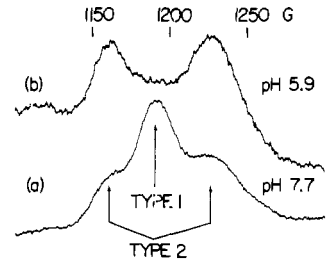

FIGURE 5: EPR spectra of Cr^{3+}-transferrin prepared at (a) pH 7.7 and (b) pH 5.9.

Binding of Chromium to Transferrin. Transferrin was treated with 6 molar equiv of Cr^{3+} at pH 7.7 for 5 days and the excess metal was removed by gel filtration. The resulting dichromium transferrin exhibited the EPR signals of both type 1 and 2 chromium, using the nomenclature of Aisen et al. (1969) (Figure 5a). When a sample was prepared in the same manner, but at pH 5.9, only the type 2 spectrum was observed (Figure 5b). When 0.9 mol of VO^{2+} was added to this second preparation (in 0.01 M succinate, pH 6.1), a spectrum indicative of mainly A-site VO^{2+} appeared. Type 2 chromium is therefore at site B of transferrin. This is in contrast to Fe^{3+} and VO^{2+}, which selectively bind to site A at low pH. That the chromium was bound at site B was also supported by the observation that iron added to this monochromium transferrin at pH 6.0 was readily bound. It is also known that iron added to dichromium transferrin at pH 7.4 selectively displaces type 1 chromium from the protein (Harris et al., 1975; Aisen and Brown, 1975). This is consistent with the affinity of iron being greater for site A than for site B. A sample of type 2 monochromium transferrin left at 4 °C for 10 days at pH 6.0 in 0.01 M succinate exhibited a spectrum with metal at both sites.

Summary. The most important EPR results are summarized in Table II.

Discussion

The double-labeling and chromium and vanadyl EPR experiments indicate that iron occupies site A of transferrin at pH 6.0. We were very surprised that 1 mol of iron added to apotransferrin at pH 7.5 also went almost entirely to site A. This contradicts the EPR results reported by Chasteen, as well as our expectation based on the equilibrium dialysis experiments of Aasa et al. (1963). In the latter work, it was found that the two intrinsic binding constants of transferrin for iron were equal, but the stated experimental uncertainty in this ratio spanned an order of magnitude. In the protein conalbumin,

TABLE II: Summary of EPR Results.

Metal Ions[a]	Mol of Metal/ Mol of Transferrin	pH	VO^{2+} Spectrum[b]	Cr^{3+} Spectrum[c]	Site Occupied Fe^{3+}[d]
VO^{2+}	1[e,f]	6.0	A		
VO^{2+}	2[f]	7.2–9.0	A + B		
Fe^{3+} + VO^{2+}	1 + 1	7.7	B		A
Cr^{3+}	2[g]	7.7		Type 1 + 2	
Cr^{3+}	1[g]	5.9		Type 2	
Cr^{3+} + VO^{2+}	1 + 1[g]	6.1	A	Type 2	
Cr^{3+} + Fe^{3+}	1 + 1[g]	6.0 or 7.5[h]		Type 2	A

[a] For double entries, the metal listed first was added first. [b] The nomenclature is that of Cannon and Chasteen (1975). [c] The nomenclature is that of Aisen et al. (1969). [d] This is inferred from the VO^{2+} and Cr^{3+} spectra. Under no conditions in these experiments did we see any difference between the EPR spectra of Fe^{3+} at site A or at both sites. [e] Only one VO^{2+} is bound at pH 6.0. Unbound metal gives no signal (Cannon and Chasteen, 1975). [f] Samples with less than 2 equiv of VO^{2+} also exhibited A + B spectra. [g] Samples were prepared with excess Cr^{3+}, the unbound portion of which was removed by gel filtration. [h] At pH 7.5, Fe^{3+} displaces Cr^{3+} from dichromium transferrin, leaving a Type 2 Cr^{3+} spectrum (Harris et al., 1975).

which appears to be very similar to transferrin in its iron-binding properties, the two intrinsic binding constants differ by about a factor of three, as indicated by equilibrium dialysis (Aisen and Leibman, 1968a). The difference between our results and previous results might be attributable to the fact that equilibrium may not have been reached in our experiments. If iron binds first to site A, and then slowly redistributes itself between the sites, we would only have seen the iron at site A. Experiments are planned to investigate this possibility.

The hypothesis of Fletcher and Huehns (1967; 1968; Fletcher, 1969) states that the two iron-binding sites of transferrin possess different physiologic functions, and their occupancy therefore serves to regulate iron metabolism. The transferrin of a healthy adult is only about 30% saturated with iron (Giovanniello and Peters, 1963). If the results of our in vitro studies accurately reflect the case in vivo, then serum iron should behave as a homogeneous pool under normal conditions, with most iron at site A. Only under conditions of iron excess would the function of site B be important.

We have shown that it is possible to selectively label transferrin at each site with a different isotope of iron, and that the two isotopes are not extensively scrambled by 1 mM citrate at pH 7.5 at 37 °C after 80 min (entry 3, Table I; cf. Aisen and Leibman, 1968b). If it can be shown that other components of serum, besides citrate, do not lead to isotopic scrambling, it should be feasible to use such doubly labeled protein for a more decisive test of the Fletcher–Huehns hypothesis than has thus far been possible (Awai et al., 1975; Brown et al., 1975; Beamish et al., 1975; Harris and Aisen, 1975a,b).

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