

THE STABILITY CONSTANTS OF THE IRON-TRANSFERRIN COMPLEX¹Ben Davis², Paul Saltman³, and Sidney BensonDepartments of Biochemistry and Chemistry
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Since the discovery of the plasma iron-binding, β_1 -globulin, transferrin (Holmberg and Laurell, 1945, Schade and Caroline, 1946) an intensive investigation has been made of the chemistry and biochemistry of this important protein. Results of these efforts are succinctly described in the excellent review by Laurell (1960). Recently, both in this laboratory (Saltman, 1961) and in that of Hallberg and Solvell (1960), it has been demonstrated that transferrin participates directly in the regulation and control of iron absorption in the mammalian organism. Further, it has been shown that the transport of the iron from the plasma to liver cells is regulated by a series of equilibrium-binding reactions in which the transport across cell membranes is facilitated by low molecular weight chelates (Charley, et al., 1960). It became of prime importance to determine accurately the stability constants of the iron for the protein.

Transferrin binds 2 moles of Fe^{+++} per mole of protein (Laurell, 1960). There is a close chemical similarity between the metal-binding behaviour of transferrin and that of conalbumin found in eggs. Both exhibit a high specificity and affinity for Fe^{+++} , although Cu^{++} and Zn^{++} are loosely bound.

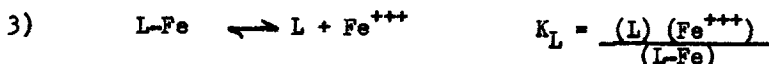
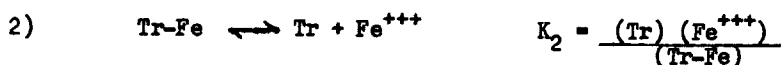
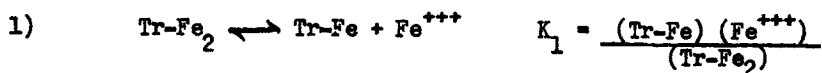
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It has been proposed (Laurell, 1960) that iron exists on the protein in a bicarbonate complex, probably linked with tyrosine and other specific amino acids. However, intimate knowledge of the nature of the binding site is unknown at this time. It is interesting that carbohydrates and sialic acid constitute about 4% of the purified protein, since it is now recognized that sugars can effectively chelate trace metals (Saltman et al., 1962).

In a preliminary study of whole plasma, using the techniques of equilibrium dialysis, Rubin et al. (1960) showed that the iron was bound with an affinity greater than 10^{27} . Because of the key role of the protein in the regulation of iron metabolism and the participation of equilibrium-binding in this regulation, we felt that it was important to determine the binding constants of both iron atoms with a greater degree of precision.

Theoretical

One of the most rapid and accurate means of estimating the stability constants of a metal for a protein is by the equilibrium dialysis of the metal-protein against a solution of a chelate, whose affinity constant for the metal has been previously determined. Equations relating to binding of Fe^{+++} with transferrin (Tr) and a low molecular weight competing ligand (L) are:



Since pH can directly influence the dissociation, it was maintained constant. No attempt was made to determine the effect of pH on the dissociation and we do not stipulate in equations 1, 2 or 3 the charge on the various

complexes. The apparent dissociation constants are for conditions closely approximating the physiological environment. We know that the total transferrin is restricted within the dialysis sac and occurs as:

$$4) \quad (\text{Tr})_{\text{total}} = (\text{Tr}) + (\text{Tr-Fe}) + (\text{Tr-Fe}_2)$$

Total iron inside the sac is present as:

$$5) \quad (\text{Fe})_{\text{in}} = (\text{Fe}^{+++}) + (\text{L-Fe}) + (\text{Tr-Fe}) + (\text{Tr-Fe}_2)$$

The only iron present in the external solution is:

$$6) \quad (\text{Fe})_{\text{out}} = (\text{Fe}^{+++}) + (\text{L-Fe})$$

It can be shown (see data below) that $K_1 \gg K_2$ so that when the protein is less than 50% saturated $(\text{Tr-Fe}_2) = 0$, and:

$$7) \quad (\text{Tr})_{\text{total}} = (\text{Tr}) + (\text{Tr-Fe})$$

Methods

Transferrin was isolated from pooled human serum, following the removal of the globulins by precipitation at 50% saturated ammonium sulfate, by chromatography on DEAE cellulose using a modification of the technique of Sober *et al.* (1956). It was homogeneous on paper electrophoresis and its extinction coefficient was comparable to that of the transferrin prepared by Schade and Caroline (1946). Manipulations and dialysis of the transferrin were carried out in a 4°C room to minimize protein denaturation. Endogenous iron was removed by acidifying to pH 3.5 in the presence of excess citrate. The iron-citrate complex was then removed with IRA 401 ion exchange resin. The apo-transferrin solution was decanted and brought to pH 7.4 with bicarbonate buffer. A slight excess of the calculated amount of Fe^{59} -citrate solution containing 34 µg Fe/ml, specific activity 5,200 c.p.m./µg Fe, needed to saturate the protein with iron was added. Excess iron-citrate was removed with the exchange resin. Protein concentrations were determined using the Biuret method. In the experiments described here, the concentration of

protein was $5.5 \mu\text{M}$. Radioactivity was measured with a scintillation well counter and a pulse height analyzer.

Two ml aliquots of the radioactive transferrin solution were brought to the desired concentration of EDTA and securely tied into sacs of washed Visking dialysis tubing. The sacs were rinsed and placed in 100 ml of the EDTA solution at the concentration indicated. All solutions were 0.04 M NaHCO_3 , pH 7.4, and were kept under constant aeration with 5% CO_2 -95% O_2 . At periodic intervals samples of the external solutions were withdrawn and counted. A plateau in the efflux of radioactivity was reached in 30 hrs. Dialysis was continued for another 15 hrs. to be certain that equilibration was achieved. The sacs were removed, rinsed rapidly with distilled water, blotted and weighed. The protein solution was removed and the bag weighed. The volume of solution was determined by difference of the weighings. Radioactivity in an aliquot of the protein solution and in the external solution was measured. From the known specific activity of the iron and the radioactivity, the molar concentration of iron was determined. Schwartzbach and Heller (1951) give the value, $K_{\text{EDTA}} = 10^{-25.1}$. Data from a typical experiment are presented in Table I. The value of pK_1 was obtained by using the value of pK_2

Table I

Equilibrium Dialysis of Fe^{59} -Transferrin
Against Increasing Concentrations of EDTA

EDTA (M)	0	10^{-3}	10^{-2}	10^{-1}
c.p.m./ml in sac	3400	1750	1280	465
(Fe) _{in} (μM)	10.6	5.7	4.0	1.4
c.p.m./ml outside	0	38	41	59
(Fe) _{out} (μM)	0	0.12	0.13	0.19
(Tr) _{total} (μM)	5.5	5.5	5.5	5.5
pK_1	-	27.7	-	-
pK_2	-	-	30.3	30.2

found experimentally. The values for pK_2 were calculated assuming that Tr-Fe_2 was negligible. Within the limits of our experimental data small amounts of Tr-Fe_2 would not affect the values reported for pK_2 .

These results confirm and extend the preliminary studies of Rubin et al. (1960). The order of magnitude of these constants is most remarkable. Not only does this protein exhibit a high degree of specificity for iron, but the affinities are such that it would easily be able to remove iron from depot cells and carry it to sites where utilized by the organism.

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