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Magnetic Measurements on Crystallized Fe-Transferrin Isolated from the Blood Plasma of Swine

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The paramagnetic susceptibility of the iron in Fe-transferrin is determined to be 15 700 ($\pm$ 500) · 10^{-6} cgs emu at 20° C, which corresponds to an effective magnetic moment of 6.08 ($\pm$ 0.10) Bohr magnetons. The iron is thus trivalent and bound with essentially ionic bonds. Some magnetic interaction between the two iron atoms in each protein molecule is possible. After reduction and decolorisation with hydrosulfite the iron is present as free or bound divalent ions with an effective moment of 5.1 Bohr magnetons. The diamagnetic gram susceptibility of transferrin was determined to be -0.586 ($\pm$ 0.007) · 10^{-6} cgs emu.

The biochemical and physico-chemical properties of the serum protein, transferrin (siderophilin), and its role as an iron transporting factor in blood have recently been reviewed [1]. The molecular weight of transferrin is about 90 000 [2,3], and it is capable of reversible combination [2,4,5] with two atoms of iron per molecule of protein [2,3,6]. The compound between iron and transferrin, Fe-transferrin, is optically characterized by a relatively broad absorption band with its maximum at 470 mμ. The formation of Fe-transferrin when di- or trivalent ionic iron is added to a solution of transferrin can thus be followed colorimetrically. Such experiments disclose the interesting fact that in plasma under aerobic conditions the Fe-transferrin is formed more rapidly with ferrous than with ferric ions [5], while in solutions of purified transferrin the ferric ions react faster than the ferrous ions do [4,6,7]. Colorimetrically judged the formed compound is identical in all these cases. The effect might be due to different rates of autoxidation of ferrous iron [6].

Since most chemical evidence points in the direction that the iron of Fe-transferrin is trivalent, this is commonly believed to be the case. No direct proof of this hypothesis has yet been obtained, and the mode of binding of iron in Fe-transferrin is still obscure. Michaelis, however, made magnetic measurements on Fe-transferrin, but his results are only published as a personal communication to Koechlin [6] and no experimental data are given. Koechlin simply states that Michaelis had found the iron to be trivalent in the red complex, and that he also had found "that hydrosulfite reduced the ferric iron and dis-

charged the red color in the complex at reactions where dissociation of the complex would not normally have occurred".

For further discussion of the role of transferrin in the iron transporting mechanism of the blood it is important to know as much as possible about the state of iron in the compound, Fe-transferrin. As the above statements are only qualitative in nature, we have reinvestigated the magnetic properties of Fe-transferrin in a highly purified form.

MATERIAL AND METHODS

Iron-saturated Fe-transferrin was prepared from swine plasma according to Laurell [8] and recrystallized four to six times. During the steps of the preparation, which were performed at a pH below 6, a small amount of ferrous ammonium sulfate was added to prevent dissociation of the Fe-transferrin compound. The excess of iron was eliminated during the later preparative steps. Since it was difficult to remove all of the non-specifically bound iron in this way, one sample (No. 2) was run through a column containing the ion exchange resin, IRA 120, pretreated with $M/15$ phosphate buffer of pH 7.8. In this way the iron content was brought to 0.125 %. An iron content of 0.124 % is to be expected from a molecular weight of 90 000 and two atoms of iron per molecule. The other samples contained 0.127 to 0.134 % iron (See Table 1). The recrystallizations were repeated until no more hemi-muco-β-globulin could be spectrophotometrically detected [8].

To get iron-free transferrin for the determination of its diamagnetism, one sample of Fe-transferrin was dialysed at pH 3.5 in the presence of cysteine and *o*-phenanthroline. After twenty-four hours all the iron was in the form of the ferrous *o*-phenanthroline complex, which was dialysed away against glass-distilled water.

All samples were analysed for dry weight (at 110° C) and for iron. For the iron determinations two colorimetric methods were used, with sulfo-salicylic acid or with *o*-phenanthroline as complexing agents. They gave closely agreeing values in all cases.

The extinction values were determined in a Beckman spectrophotometer, Model DU. The magnetic measurements were carried out with the apparatus constructed by Theorell and Ehrenberg and a solution of nickel chloride was used as calibration agent [9]. All measurements were made in the temperature range 20 to 22° C.

RESULTS

Four samples of Fe-transferrin from different preparations were investigated for the paramagnetism of the iron by measurements on their salt-free solutions. The results are given in Table 1 together with the characteristics of the samples. The extinction at 470 mμ is proportional to the amount of specifically bound iron. Sample *2* contains practically all of its iron as Fe-transferrin compound. Samples *3* and *4* are saturated with specifically bound iron and contain above that level respectively 2.9 and 9.0 % of iron non-specifically bound to the protein. Sample *1* may be impure or partly denatured since, according to the extinction value, 9 % of its specific sites are unoccupied by iron in spite of an iron content slightly above the theoretical saturation level.

The susceptibility values are calculated per gram atom of iron and reduced to 293° K with the assumption that Curie's law is valid. The spreading of the separate determinations on every sample gives a standard deviation of the mean of about $\pm 150 \cdot 10^{-6}$ cgs emu. Another error of $\pm 350 \cdot 10^{-6}$ cgs emu is due to uncertainties in the diamagnetic correction and has the same sign in all determinations. As is seen, the different and small amounts of non-specifically bound iron do not influence the average susceptibility to any detect-

ible extent. The non-specifically bound iron has thus much the same susceptibility as the specifically bound. As a mean value of the susceptibility of the iron in Fe-transferrin we accept 15 700 ($\pm$ 500) $\cdot$ 10^{-6} cgs emu at 293° K. This corresponds to an effective magnetic moment, μ_{eff} = 6.08 ($\pm$ 0.10) Bohr magnetons. Preliminary measurements on three samples, containing slightly less iron than those described in the table, also indicated a μ_{eff} of about 6.1. No foreign heavy metal ions that could contribute to the paramagnetism of the solution could be detected by common chemical methods.

One part of sample No. *4* was diluted with phosphate buffer of pH 6.5 to final concentrations of 3.19 % protein and *M*/30 phosphate. When hydrosulfite, 5 mg per ml solution, was added, the solution started to decolorize rather slowly. The extinction values in the wavelength region, 420—600 mμ, decreased in proportion. The process could not be followed to the end as turbidity occurred. When the extinction at 470 mμ had diminished to 50 %, the solution, which was still clear, was measured in the magnet. The result showed that the decolorized iron had a susceptibility of 11 000 $\cdot$ 10^{-6} cgs emu corresponding to μ_{eff} = 5.1 Bohr magnetons.

Table 1.

Sample No.	Protein conc. mg/ml	Fe conc. γ/ml	Fe/protein ‰	$\beta_{470m\mu} \times 10^{-7}$ cm²/g AtFe	Number of magnetic determ.	$\chi_{Fe,\ 293°\ K}$ 10^{-6} cgs emu
1	27.3	34.7	1.27	0.486	4	15 900
2	22.7	28.4	1.25	0.556	4	15 500
3	44.5	56.4	1.27	0.540	4	15 800
4	63.8	85.2	1.34	0.506	3	15 600

The diamagnetism of the protein was determined by a series of five measurements on an "iron free" sample with 21.4 mg protein per ml and 2.0 γ iron per ml. The mean difference reading on the micrometer could be used immediately for diamagnetic correction of the readings in the experiments already described, if the iron contents were properly reduced because of the small but not negligible iron content of the "iron free" sample. To evaluate the true diamagnetism of the protein we have corrected for the paramagnetic contribution of this iron. For the partial specific volume of transferrin we take the figure 0.725, determined by Oncley, Scatchard and Brown [10] on a 70 % pure material. If we assume that Wiedemann's additivity law is applicable and that the gram susceptibility of water is equal to —0.7200 $\cdot$ 10^{-6} cgs emu we find the gram susceptibility of transferrin to be —0.586 ($\pm$ 0.007) $\cdot$ 10^{-6} cgs emu at 293° K. A small correction has been made, due to the physically dissolved oxygen, but it does not amount to more than one unit in the third decimal.

DISCUSSION

As already mentioned the transferrin molecule is capable of combining with two atoms of iron. There is some doubt whether this reaction takes place in one step or two, or, whether the active sites of the molecule are close enough

to each other to bring about an interaction between them [11]. Different possible structures have to be proposed and their theoretical magnetic moment has to be compared with the value $\mu_{eff} = 6.08$ ($\pm$ 0.10) B. m. (Bohr magnetons) obtained in the present investigation.

We first assume that *no interaction* exists between the two iron atoms bound to the same protein molecule. In this case the possibility of divalent iron can be ruled out at once, as it would exhibit a maximal effective moment of 4.90 B. m. or slightly higher, due to incomplete quenching of the orbital moment. All experimental values of μ_{eff} of ionic ferrous iron in magnetically dilute solids or solutions lie between 5.1 and 5.6 B. m., which is definitely lower than the 6.08 B. m. of Fe-transferrin. Ferric iron, however, would give $\mu_{eff} = 5.92$ B. m., if it is bound with essentially ionic bonds. Experimental values are often close to that figure, but usually lower, probably due to deviations from the Curie law. Higher values seem to be rare, if any. Because of the small difference between 6.08 ($\pm$ 0.10) B. m. and 5.92 B. m. a structure with ionic trivalent iron is rather possible in the case of Fe-transferrin.

The other extreme possibility is that *complete magnetic coupling* occurs between the two iron atoms in Fe-transferrin. This means that they can be treated as an entity and, if the electronic spins only are magnetically active, the effective magnetic moment per iron atom can be calculated according to the formula $\mu_{eff} = \sqrt{\frac{1}{2} n(n+2)}$ B. m., where n is the total number of odd electrons in the entity. Two trivalent iron atoms with five odd electrons each would thus give $\mu_{eff} = \sqrt{60} = 7.75$ B. m. The large difference between this figure and the experimental one makes the proposed structure improbable. Two trivalent iron atoms, one with five and one with three odd electrons, or two divalent iron atoms with four odd electrons each would both give $\mu_{eff} = 6.33$ B. m. Since the orbital contribution has been overlooked, the figure 6.33 is probably somewhat too low but anyhow close enough to the experimental value to require discussion. The strong magnetic coupling assumed here calls for a rather compact and stable structure, especially in the case of trivalent iron, where a kind of resonance between the iron atoms must be postulated. Such a stable structure is not easy to reconcile with the fact that the combination between iron and transferrin is an easily reversible reaction. From a chemical point of view the case of divalent iron is further improbable. On these grounds we believe that strong interaction does not occur between the iron atoms of Fe-transferrin.

The best explanation of the experimental value $\mu_{eff} = 6.08$ ($\pm$ 0.10) B. m. seems to be that the iron in Fe-transferrin is trivalent and bound with essentially ionic bonds. The fact that the observed value is higher than the expected one of 5.92 B. m. might be interpreted as due to a weak interaction between the two iron atoms in each protein molecule, which in such a case may not be situated too far from each other. To illuminate the proposed interaction further magnetic measurements over a temperature range and detailed studies of the coupling reaction between ionic iron and transferrin are highly desirable.

The value $\mu_{eff} = 5.1$ B. m. obtained for the iron after decolorisation is just what would be expected for divalent ionic iron. We can thus state that if transferrin forms a compound with ferrous iron, it is either uncolored and ionic or it is unstable in the presence of phosphate ions at pH 6.5.

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