Role of Transferrin in Determining Internal Iron Distribution

By Pensri Pootrakul, Alan Christensen, Betty Josephson, and Clement A. Finch

The behavior in vivo of transferrin in loading and unloading iron from its two sites was examined in rats. Radioiron entering the plasma from the gastrointestinal tract in iron-deficient, normal, and iron-loaded rats did not differ in its subsequent tissue distribution between erythroid marrow and liver of normal recipients from a second isotope added to the same plasma in vitro. Loading studies in vitro were then carried out employing a reticulocyte incubation model designed to place one isotope predominantly on one site of transferrin, more available to the erythron, and the second isotope on the other site, more available to the liver. In 15 groups of animals in which 3 different iron salts were employed to load transferrin with iron, the mean isotope ratio in the erythron was $1.03 (\pm 0.06 \text{ SD})$ and the mean liver ratio was $0.75 (\pm 0.21 \text{ SD})$. It was found that the incubation of plasma with reticulocytes resulted in contamination of the plasma by radioactive hemoglobin. After allowance was made for hepatic uptake of radiohemoglobin in the 15 groups in which proper correction could be made, the isotope ratio in the liver became $0.97 (\pm 0.17 \text{ SD})$. It is concluded that iron atoms from the two sites of transferrin have similar tissue distributions in vivo in the experimental situations examined.

The plasma protein transferrin mediates the exchange of iron between various body tissues in accordance with their needs. It has been generally assumed that transferrin iron acts as a single pool from a kinetic standpoint. However, the possibility has been raised that iron on each of the two binding sites present on the transferrin molecule behaves differently: iron from site A is delivered principally to the erythroid marrow, and iron from site B is preferentially released to the hepatocyte. Manipulations designed to provide tagged fractions of transferrin iron especially oriented to one or the other site have provided evidence both in vitro and in vivo to support this hypothesis.

We have employed a similar reticulocyte incubation model and have studied the internal distribution of tagged fractions of iron in rats. Our studies indicate a similar behavior of the two sites, and at the same time explanations have been found for some of the divergent results which have been previously reported.

MATERIALS AND METHODS

Iron binding to transferrin in vitro was accomplished by adding various iron compounds to iron-deficient rat plasma. The iron-deficient plasma was obtained from rats 2-4 mo of age who had been on an iron-deficient diet since the first month of life. It was usually used immediately but was...
sometimes stored up to 1 wk at 4°C. The average plasma iron was 40 μg/dl and the average binding capacity was 800 μg/dl, so that the transferrin saturation was about 5%.

Radioactive iron was obtained as 59FeCl3 in 0.5 N HCl with a specific activity of 12-36 mCi/mg and as 55FeCl3 in 0.5 N HCl with a specific activity of 20-34 mCi/mg. Ferrous ammonium sulfate was prepared by mixing a tracer dose of 59FeCl3 (< 10% of total iron) with Fe(NH4)2(SO4)2·6H2O at a pH of <2. The iron salt was then added to plasma under a CO2 atmosphere, 0.1 ml of iron solution per milliliter of plasma. Additional studies were done with iron citrate, which was prepared by adding radioactive FeCl3 and/or carrier FeCl3, as indicated, to sodium citrate in a molar ratio of 1:30. The third salt examined was iron nitrilotriacetate (Fe-NTA), which was prepared by mixing equal volumes of a solution of FeCl3 in 0.1 N HCl with NTA freshly prepared in 0.05 N NaOH at a pH of >8 in a molar ratio of 1:5. The pH of the citrate and NTA complexes was adjusted to 5 with solid sodium bicarbonate and to 7.4 with solid Tris base. They were then added at once to iron-deficient plasma.

In the first series of studies iron loading of transferrin was carried out in vivo. Radioiron (10-100 μCi of radioiron ascorbate) was given by stomach tube to normal animals, to iron-deficient animals, and to animals who had been iron loaded by the administration of 30 mg of iron dextran intraperitoneally 1 mo before the study. Blood was drawn 30 min later in normal animals, 20 min later in iron-deficient animals, and 40 min later in iron-loaded animals and the plasma separated at once. The second isotope as a tracer dose of ferrous ammonium sulfate was added in vitro to each of these plasmas, and the combined, doubly labeled plasma was injected intravenously. At 30 min the animals were sacrificed (see below) and tissue localization of the two isotopes was determined.

A second series of studies was carried out in which tracer amounts of radioactive iron were added to plasmas from animals with different iron levels and the proportion of activity localized in the liver as a function of the plasma iron level was determined. Twenty-nine normal animals were fasted to lower their plasma iron concentration, or injected with ferrous ammonium sulfate to raise it. 59Fe citrate in tracer amounts was added to iron-deficient plasma and injected intravenously. Recipient animals were sacrificed at 30 min; radioiron in liver and femora was determined and the plasma iron at the time of sacrifice was determined. From the data the following regression line was obtained (correlation coefficient 0.82):

$$\frac{\text{liver}}{\text{liver + marrow}} = 3.32 \times 10^{-4} \times \text{plasma iron (μg/dl)} + 0.0973. \quad (1)$$

Selective binding of the two sites of transferrin was attempted by reticulocyte incubation as previously described. Radioiron with carrier (59Fe) was first added to iron-deficient plasma in vitro to produce a transferrin saturation of 50%, 90%, and was incubated for 30 min. This plasma was then incubated with reticulocytes at 37°C for 60 min. The number of reticulocytes employed was adjusted so as to result in a decrease in plasma iron of approximately 50% in 60 min. The actual decrease was determined in each study by periodic measurements of plasma 59Fe activity. A second isotope of iron along with carrier iron (55Fe) was then added to return the transferrin saturation to its original level and the plasma was incubated for 30 min. The amount of the two isotopes was adjusted to provide a ratio of 1:3 between 59Fe and 55Fe, since this provided for accurate differential counting. In studies employing citrate and NTA complexed iron, the tagged plasma was passed through a Sephadex column to remove excess NTA or citrate less than 5 mm after addition of that iron complex. The doubly tagged plasma was given to a group of 2-4 animals as displayed in Table 3.

Reticulocytes used in these incubation studies were prepared either by phenylhydrazine administration or by bleeding. We were unable to detect differences in results relating to the type of reticulocytes employed. Phenylhydrazine, 100 mg/kg, was injected intraperitoneally and resulted in about 65% reticulocytes in blood drawn 5-6 days later. Reticulocytes were also obtained by bleeding animals 5 ml/day on 3 successive days, and on the fifth day drawn blood contained 50% reticulocytes. These young cells were washed once in saline immediately before their use in most of the studies, but it was later found that hemolysis could be reduced if the saline wash was omitted.

The plasma incubated with reticulocytes always had some free hemoglobin, especially when iron saturation was very high, necessitating an increased number of reticulocytes or an increased time...
of incubation to reduce the iron content to half of the original level. Direct measurements of hemoglobin concentration in plasma were made by the pyridine hemochromogen method. A portion of this hemoglobin was radioactive, but there was no assurance that it would be a constant proportion of the plasma hemoglobin. Accordingly, a dialysis method was developed to distinguish between transferrin and hemoglobin activity. In this procedure, 0.2-0.3 ml of plasma was placed in a cellophane bag and was dialyzed against 4 liters of 0.01 M EDTA and 0.1 M sodium acetate buffer at pH 5. At this pH, iron was dissociated from transferrin, became bound to EDTA, and moved into the surrounding solution. In order to establish the efficiency of this separation, 59Fe-labeled hemoglobin was added to plasma which also contained 55Fe bound to transferrin. The mean results of five 7-day dialyses carried out at 4°C are shown in Table 1. After 2 days 1% of activity from transferrin remained, while 89.9% of hemoglobin activity remained. Thus the activity in the bag was

\[ P = 0.01T + 0.899H \]  

(2)

where \( T \) = activity from transferrin-bound iron, \( H \) = activity from hemoglobin iron, and \( P \) = total activity in the dialysis bag. Considering that the original activity was \( (100 = T + H) \), the formula could be rewritten:

\[ H = \frac{P - 1}{0.889} \]  

(3)

On the basis of prior measurements, it was assumed that all hemoglobin would be cleared from the plasma within 30 min and that 50% would be localized in the liver.

Male rats of the Sprague-Dawley strain weighing 240-450 g were used as recipients in the in vivo studies. Nonfasting animals were anesthetized with ether at the time of an intravenous injection of tagged plasma, and again 30 min later at the time of their sacrifice by bleeding. During the 30-min period, 0.1-ml blood samples were drawn at 5-min intervals from the tail of some animals to determine the rate of disappearance of radioactivity from the circulation. At sacrifice, approximately 4 ml of blood/100 g body weight was removed from the heparinized animal through the abdominal aorta for determination of plasma iron, total iron-binding capacity, and radioactivity. Then, sequentially, 10-ml aliquots of saline were introduced into the aorta with subsequent removal of an equivalent amount of circulating blood to a total of 40 ml, at which time the animal was bled out. Since only 3% of injected radioactivity was found in the liver in most studies, it seemed important that activity from residual blood be minimized. Saline perfusion as described reduced contamination due to plasma to 2.3 ± 0.5%, and red cells to 0.6 ± 0.18% of that present in the circulating blood at the time of sacrifice (mean ±SD of six animals). After the death of the animal, tissues were removed for determination of residual tissue activity. A more detailed perfusion was carried out in some animals to avoid contamination of tissues by residual blood radioactivity. Two days before, a cannula was placed in the superior venae cava of these animals. Thirty minutes after the intravenous injection of radioactive transferrin, the animal was exchange transfused with 60 ml of stroma-free hemoglobin solution* to a hematocrit of 5%. At the end of this, the animal was further exchanged with saline as previously described. This double perfusion reduced plasma contamination to 1.6 ± 0.65%, and red cell contamination to 0.35 ± 0.28%.

At the time of sacrifice, the liver was cut into small pieces and 1 g of tissue was weighed out

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*Kindly supplied by the Warner Lambert Company.
in duplicate for wet ashing. The femora and duplicate 1-ml samples of whole blood were also digested. Samples were prepared for counting by a modification of the method of Eakins and Brown.\textsuperscript{14} \(^{59}\)Fe and \(^{55}\)Fe counts were determined in a Model 2405 Packard Tricarb liquid scintillation spectrometer with proper allowance for cross counting. The amount of each isotope in the liver was calculated from the counts in the sample analyzed and the proportion of the liver it represented. Marrow radioactivity was estimated by multiplying the activity found in one femur by 13.\textsuperscript{12} Activity in the circulating blood was determined from the activity in plasma and washed red cells, and from an assumed blood volume of 60 ml/kg.\textsuperscript{15} Erythron activity was calculated from the combined activity of marrow and circulating red cells. The radioactivity in the blood, liver, marrow, kidneys, and spleen was compared to the total activity injected to determine total recovery.

The plasma iron and total iron-binding capacity were determined according to the recommendations of the Standardization Committee of the International Society of Hematology,\textsuperscript{16} except that twice the amount of iron was required in the total iron-binding capacity measurement due to the high transferrin concentrations found in iron-deficient rat plasma. Sephadex filtration of plasma according to the method of Cavill\textsuperscript{17} was carried out in some studies to remove excess citrate and NTA. In this procedure Sephadex G-25 medium was swollen with 0.155 M Tris buffer at pH 7.4 overnight and then packed into a column (1.5 x 10 cm). The labeled plasma was applied to this column and was then eluted with 0.155 M Tris buffer at pH 7.4, thereby isolating that portion of the eluate containing a high concentration of protein. A decrease of approximately 5% in both the plasma iron concentration and the total iron-binding capacity resulted from this procedure due to dilution by the aqueous phase of the column. There was no evidence in these studies for removal of a measurable fraction of radioiron which had not been protein bound, nor was there evidence of contamination of the plasma by iron from the column.

RESULTS

Studies on Iron Loading of Transferrin

In order to determine whether loading in vitro was similar to that which occurred in vivo, a comparison of tissue distribution after both types of binding was carried out (Table 2). Studies utilizing radioiron absorbed by iron-deficient, normal, and iron-loaded animals showed no difference in distribution between marrow and liver as compared to plasma labeled in vitro by a second radioiron isotope. Thus the mean ratio in iron-deficient animals in marrow and liver was 0.91 and 0.90, in normal animals 0.99 and 0.99, and in iron-overload animals 1.01 and 1.02. The lower ratios in both marrow and liver with iron-deficient plasma are unexplained.

Release of Iron From Reticulocyte-incubated Plasma In Vivo

In order to examine differences in iron release between the two sites of transferrin, \(^{59}\)Fe \((\text{NH}_4)_2\text{(SO}_4\text{)}_2 \cdot 6\text{H}_2\text{O} \) was loaded onto transferrin in vitro to

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*Iron added as ferrous ammonium sulfate.
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<th>Transferrin Saturation of Injected Plasma</th>
<th>Post-incubation Final Isotope</th>
<th>Tissue Radioisotope Uptake (Per Cent of Injected)</th>
<th>Liver Uptake Corrected for Hemolysis (Per Cent of Injected)</th>
<th>Plasma Radioisotope Uptake (Per Cent of Injected)</th>
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*Phenylhydrazine: rats were fed phenylhydrazine.*
*Values in parentheses indicate range in animals of the group.*
*These high ratios due to incomplete clearance of radiolabeled plasma.*
*Labelled plasma passed through the Sephadex G 25 column.*
*Hemoglobin solution was used to permit a more complete exchange perfusion.*
a saturation of 36%–100%. This tagged transferrin was then exposed to reticulocytes as described under Materials and Methods to remove half of the activity; then the plasma transferrin was reconstituted to the original iron saturation by the addition of $^{55}$Fe $(\text{NH}_4)_2\text{(SO}_4)_2 \cdot 6\text{H}_2\text{O}$. The localization of the two isotopes in vivo at 30 min in seven groups of animals is shown in Table 3, part A. The half-time clearance of the two isotopes in two animals was similar. The mean uptake by the marrow of the first isotope was 20.69% and of the second isotope 20.31% with an isotope ratio (mean of individual ratios) of 1.04. Mean liver uptake was 6.5% and 4.9% for the two isotopes, with a mean ratio of 0.8.

In the second series of studies ferric citrate was used as the donor salt (Table 3, part B). Column elution was carried out before labeled plasma was exposed to reticulocytes and within 5 min of the addition of the second isotope to remove excess citrate, thereby minimizing any redistribution of iron between binding sites that might be citrate mediated. In the studies in vivo no differences were observed in the plasma-iron disappearance time of the two isotopes, and the total liver activity was essentially that expected. However, despite normal marrow ratios, substantial differences between the four groups of animals were found in hepatic isotope ratios with a range of 0.49–1.05.

In a third series of studies (Table 3, part C), NTA was employed to load iron on transferrin. The first four groups studied showed marrow ratios of 0.96–1.08 and liver ratios of 0.52–0.98. In two animals exchange-transfused with hemoglobin to reduce tissue contamination by blood radioiron, the same ratio of isotopes in tissues was found, and the amount of hepatic activity was as expected. The last two groups studied, however, showed erythron ratios of 2.0 and 1.3 and hepatic ratios of 0.13 and 0.90. A noteworthy finding was the very high hepatic uptake of $^{59}$Fe in one study and of both isotopes in the other study. These results, although reasonably consistent for each animal of the group, differed from all of the other studies with the three iron salts employed.

To obtain further insight into the amount of hepatic uptake to be expected, studies were carried out in which the plasma iron of experimental animals was altered (see Materials and Methods) and plasma labeled in vitro was injected. In animals with a plasma iron of 150 and 300 µg/dl, liver activity at 30 min as derived from the linear regression formula would amount to 4% and 5.7% of the injected dose, or about 20% of the mean uptake of 23.2% by the erythroid marrow.

**Effect of Hemolysis on Organ Distribution of Radioactive Iron**

Hemolysis was observed in some of the plasmas after reticulocyte incubation. It was appreciated that such hemoglobin would contain radioactive iron, about 50%, of which would be cleared by the hepatocyte. Accordingly, a determination was made of the tissue localization of radioactive hemoglobin added in known amounts to plasma. (The transferrin of this plasma had also been tagged in vitro with tracer amounts of the same isotope.) In these studies varying proportions of $^{59}$Fe activity were added in the form of hemoglobin, the remainder as transferrin-bound radioiron. $^{55}$Fe was all bound to transferrin. The presence of radioactive hemoglobin increased liver $^{59}$Fe uptake and lowered the isotope ratio in proportion to the amount of hemoglobin present. When
Values for two animals were averaged in each study. Formula (1) in the Materials and Methods section was employed to make this calculation.

Fig. 1. Relationship between radioactive hemoglobin and excess hepatic radioiron (calculated by subtracting the hepatic activity of the second isotope from that of the first isotope). Symbols refer to prepared mixtures of radiohemoglobin and transferrin radioiron (○), and to reticulocyte incubation studies when radioiron was loaded with NTA (●), citrate (●), and ammonium sulfate (●).

The excess liver uptake of the reticulocyte-incubated isotope (59Fe) was related to the amount of hemolysis present (Fig. 1), a correlation coefficient of 0.92 was observed in the first 15 studies summarized in Table 3 (the last two NTA studies excluded). Large amounts of hemolysis, however, were not properly corrected for by assuming a 50% uptake by the liver because much of the activity still remained in the plasma. For example, the group with 15.8% hemolysis showed a clearance of only 38% of hemoglobin activity from the plasma. Excluding the two groups with hemolysis of over 10%, a linear regression line for hemolysis versus excessive liver uptake was obtained which was almost identical to that obtained with direct measurements shown in Table 4.

DISCUSSION

The transferrin molecule has two binding sites. The general impression from studies of the chemistry of binding has been that these sites have a similar affinity for iron. However, not all of the reports are in agreement with this thesis and physical-chemical differences between sites have been observed. Because of its importance to an understanding of iron transport, we have restudied this question.

Considerations of the behavior of the two sites observed in these studies can be divided into observations of loading and of unloading. While iron loading of transferrin has been generally assumed to occur at random, this concept has been questioned by Brown. He has described studies in which tagged plasma

<table>
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<tr>
<th>Study*</th>
<th>Plasma Iron (μg/dl)</th>
<th>Radiohemoglobin (per cent of total 59Fe)</th>
<th>Liver/Carcass (%) Predicted From Transferrin Iron</th>
<th>59Fe Liver/Carcass (%)</th>
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*Values for two animals were averaged in each study.

†Formula (1) in the Materials and Methods section was employed to make this calculation.
was obtained by perfusing the portal system of an animal given radioiron by mouth, and the distribution of this isotope in a recipient animal was compared with that of a second isotope of iron added as the NTA salt to the same plasma. We have carried out similar studies but have obtained the plasma tagged in vivo by sampling the circulating blood and have added the second isotope as ferrous ammonium sulfate.

Because of the slow rate of uptake of transferrin iron by the liver, normally about $0.2\%$ per min, and presumably an even slower subsequent release from that organ, it was thought that the plasma obtained within 20-40 min of absorption would be for the most part the complex entering the circulation and would more likely represent physiologic labeling from the gut than would that obtained through venous cannulation and perfusion of isologous blood through the portal system. In the studies reported here little difference has been observed in the distribution among body tissues of the loaded isotopes in vivo and in vitro. Furthermore, the distribution of radioiron absorbed into the plasma in iron-deficient and iron-loaded animals was similar to that of a second isotope added in vitro. Thus there was no evidence that a selective unloading of transferrin as a result of selective loading occurred even in those animals most likely to show such a phenomenon.

The approach used to evaluate the release of iron from the two binding sites of transferrin has been the reticulocyte incubation model originally described by Fletcher and Huehns\textsuperscript{35} as modified to include two isotopes of iron.\textsuperscript{9,10} Reticulocyte incubation studies employing ferrous ammonium sulfate for iron loading of transferrin showed a mean erythron iron uptake of the two isotopes of 1.04 and with citrate of 1.01. Four of six studies with NTA showed an erythron uptake ratio of 1.03 (the two outlyers will be discussed below). Thus there was no evidence to support a differential uptake by the marrow.

Liver ratios appeared disproportionately reduced regardless of the loading salt employed, with one-third of all values at 0.6 or below. Assuming erythron measurements to be correct, the low hepatic ratio could only be due to a small fraction of $^{59}$Fe which localized selectively in the liver. Radioactively tagged hemoglobin, resulting from hemolysis during incubation of reticulocytes with plasma tagged by the first isotope, was shown to be present in the incubated plasma. Because such hemoglobin would be selectively taken up by the liver,\textsuperscript{12} it had a marked effect on the liver ratio.* Corrected liver ratios for all three salts, with the exclusion of the last two groups of NTA (for which total liver uptake was highly abnormal), was $0.99 \pm 0.18$ SD (Table 3).

These results are at variance with those reported by Awai et al.\textsuperscript{9,10} They have observed isotope ratios at 30 min of 2.5-6.3 in the marrow and 0.10-0.12 in the liver. It seems likely that their data are comparable to the last group of animals reported in Table 3C, since they describe a hepatic uptake of $30\%$ at 30 min, compared to a marrow uptake in the same animal of $2.1\%$. Such a rapid

*Thus, if transferrin iron uptake with each isotope was $4\%$, and there was $2\%$, in one study and $4\%$, in a second study of the first isotope as radioactive hemoglobin, of which $50\%$ would localize in the liver at 30 min, the calculated ratios would be 0.8 and 0.67, respectively. This is further illustrated by data in Table 4.
uptake of iron by the liver appears inconsistent with ferrokinetic data, for ablation of the erythroid marrow results in a long half-time disappearance of transferrin-bound radioiron. In normal animals and man radioiron uptake by the erythroid marrow is approximately five times greater than that by the liver. Even if it is assumed that the labeled iron is on a liver-oriented site of transferrin, half of the radioiron of a plasma whose transferrin is saturated with iron in vitro should be on this site, in which case a hepatic clearance of at least half of the manipulated plasma should be observed. In our studies of the distribution of radioiron in animals whose transferrin was saturated in vitro, the mean hepatic uptake was less than one-fourth of that found in the marrow. Having also observed excessive uptakes on occasion with NTA loading of iron without reticulocyte incubation, we feel that the most likely explanation is an inadequate loading of iron on the iron-binding sites of transferrin on some occasions when NTA was employed. It is quite possible that the pH conditions were not optimal as carried out and probably caused the unphysiologic distribution observed in two experiments by us and by Awai et al. Nevertheless, in studies designed to examine the difference between iron-binding sites, the use of chelates, such as citrate and NTA, which can mediate iron exchange between transferrin-binding sites, is undesirable.

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Role of transferrin in determining internal iron distribution

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