The Behavior of Transferrin Iron in the Rat

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The plasma protein transferrin mediates iron exchange between body tissues. However, the exact manner in which this protein functions has not been clearly defined. In vitro differences in chemical and spectroscopic behavior of the two iron binding sites have been shown. It has also been suggested that there is a heterogeneous behavior of iron donated from transferrin to reticulocytes based on differences in behavior of the two iron binding sites, although this has also been denied. Also controversial has been the in vivo behavior of transferrin as it might be affected by the location of iron on the two iron binding sites. Some of the differences encountered may relate to differences in transferrin behavior in various animal species, however, even in the rat there has been disagreement concerning exchange of iron between transferrin and various body tissues.

The rat has been employed in these studies because of our familiarity with methods of isolation and purification of its transferrin and because of detailed information concerning iron exchange in this species. A difficulty was imposed by the presence of two species of transferrin in the rat referred to as transferrin fast and transferrin slow. At the same time, the use of purified fractions of these two species permitted observations concerning iron exchange that would not otherwise have been possible.

MATERIALS AND METHODS

Tagging of Plasma With Radioiron

In vitro labeling was accomplished by slowly adding 0.5 ml of a radioiron solution of ferrous sulfate (10 μCi in 1 μg iron) in 0.01 normal HCl saline (pH 2) to 4 ml of normal rat plasma over a period of 2 min. During the addition, the plasma was continuously agitated in a Vortex shaker. In some studies, a ferrous ammonium sulfate (FAS) solution in 0.01 normal HCl was added to provide carrier iron for the radioiron sulfate. Fe nitrilotriacetate (NTA) was prepared according to the recommendations of Graham and Bates except that varying ratios of Fe to NTA were employed as specified under Results. In vivo labeling was accomplished by the intravenous injection of the tracer solution of radioiron sulfate. Loading of transferrin iron in vivo was also accomplished by placing the radioiron sulfate solution in a gut loop as described elsewhere and drawing blood after 30 min to obtain the tagged plasma. Monoferric transferrin on both slow and fast transferrin was prepared by trace labeling of iron deficient plasma with FeSO₄ as described above. In some studies, Fe was used as well as Fe in order to permit simultaneous studies of two different transferrin iron preparations.

Purified Transferrin Preparations

Purified rat transferrins labeled with radioiron were prepared by first saturating plasma with radioiron, removing excess iron by passage through a Sephacryl-200 superfine or Sepharose 6B (Pharmacia) column followed by ion exchange chromatography on Sephadex A50. The latter purification step resulted in separation of transferrin fast and transferrin slow. The purity of these proteins in the diferric form was confirmed by an absorption ratio A465: A280 of 0.046 and by isoelectric focusing; monoferric transferrin was prepared by column chromatography on Sephadex G50 at pH 5.1. Double labeling of the purified transferrin was achieved by first labeling all sites with Fe, removing the iron from the acid labile site by column chromatography at pH 5.1, and then by saturating the open sites with FeSO₄. Labeling was carried out
on purified diferric transferrin according to the procedure of McFarlane, followed by extensive dialysis against buffered saline.

**Polyacrylamide Gel Electrophoresis**

Aliquots of 0.3 ml of the tagged plasma or of pure protein fractions were diluted with 0.3 ml of the saturated aqueous sucrose solution. This mixture was applied on the cathodal top of a 7.5% polyacrylamide gel in the column of 1.5 x 15 cm. Electrophoresis was carried out in an electrophoretic cell (Biorad, Model 155) at 4°C during 2.5-16 hr at 10 mA per gel that required a voltage change from 80-200 V. Thereafter the gel was cut into slices of 2 mm thickness. The 59Fe content of these slices was measured either directly in an autogamma scintillation spectrometer (Packard, Model 5330) or after addition of 10 ml of aquasol (containing 10 mm HC104) to each slice and extraction of radioiron using a shaking water bath in a liquid scintillation counter (Packard, Model 2405). The latter technique permitted measurement of 59Fe and 55Fe simultaneously.

**Isoelectric Focusing in Polyacrylamide Gel**

This procedure was carried out using 7.5% polyacrylamide in a column of 1.4 x 18 cm. The gel contained 2% ampholine (pH range 5-8) LKB, Western Instruments, Pleasant Hill, Calif.). A mixture of 0.3 ml of the radioiron tagged sample with 0.3 ml of a saturated aqueous glucose solution was applied on the cathodal top of the gel column, and isoelectric focusing was carried out in an electrophoretic cell (Biorad, Model 155) for 16 hr at 600 V and 4°C. Thereafter the gel was cut into slices and radioiron activity was measured as described above. In some experiments, the 59Fe containing protein fractions were eluted from the gel using 3 ml of a 0.3 M Tris/HCl buffer, pH 8.3, for each slice. Complete elution was achieved within 20 hr on a shaking water bath. Iron determination of these fractions was done as described elsewhere.

**Animals**

Male Sprague-Dawley rats for the most part 8-12 wk of age weighing 250 ± 50 g were employed. They were on a standard Purina rat chow diet containing approximately 350 mg of iron/kg diet. In order to produce iron deficiency, rats were either placed on an iron deficient diet at the age of 4 wk and studied at the age of 8 wk as described elsewhere or were placed on the iron deficient diet at the age of 6 wk and periodically bled so as to accelerate iron depletion. Iron overload was produced by placing animals on a high iron diet containing 1%-2% of iron as ferrous citrate. Within about 2 wk these animals usually had a transferrin saturation of over 70%.

In studies of transferrin iron exchange, the radioiron solution was injected intravenously into a tail or leg vein (0.5 ml over a period of 2 min) and samples of heparinized blood of 0.1 ml were drawn at intervals from the tail vein, the sampling time adjusted to the anticipated turnover rate. In these studies the animal was lightly anesthetized with ether. At the termination of the study, animals were sacrificed under anesthesia by exsanguination with perfusion of an amount of saline equal to 4x their blood volume. Various tissues were removed and their activity determined in a gamma counter. Plasma samples containing 2 isotopes of iron were prepared.

![Graph](image_url)

Fig. 1. Acrylamide gel electrophoresis of plasma from iron deficient animals to which increasing amounts of FeSO4 were added in vitro. Open circles represent the total 59Fe activity in the transferrin peak, closed triangles represent the transferrin fast fraction, and open triangles, the transferrin slow fraction. The total binding capacity derived from these measurements (closed arrow) is compared with that derived from a standard technique (open arrow). The relative amounts of radioiron taken up by slow and fast transferrin were proportionate to the amount of those proteins known to be present in iron deficient plasma. The electrophoretic contour of the transferrin radioiron peaks in normal plasma at 150 min and 16 hr are shown in the upper and lower figures on the right, indicating separation between slow and fast transferrin at the latter time.
for counting by first adding 1 ml of 0.1 normal perchloric acid and then centrifuged. Eight ml of aquasol were then added to 1 ml of the supernatant and the samples counted in a Tricarb scintillation spectrometer (Packard, Model #2405). The perchlorate precipitation removed more than 98% of the radioactive hemoglobin from the supernatant.

RESULTS

Iron Binding to Transferrin

The suitability of electrophoresis for distinguishing between specific and nonspecific binding of iron to transferrin is illustrated in Fig. 1. Iron tagged with $^{59}$Fe was added to aliquots of iron deficient plasma up to twice its iron binding capacity. When subjected to gel electrophoresis, virtually all added radioiron was localized in the transferrin peak up to the point of saturation of iron binding sites and was distributed proportionate to the amount of slow and fast transferrin protein as determined by electrophoresis. Once the point of saturation had been passed, all further radioiron was excluded from the transferrin peak.

In order to examine the general characteristics of iron binding, $^{59}$Fe-tagged ferrous and ferric iron salts as well as iron chelates were used to produce near saturation of plasma transferrin (Table 1). After incubation for 30 min at 37°C, the plasma aliquots were subjected to electrophoretic separation. Complete recovery of $^{59}$Fe added as ferrous ammonium sulfate was found in the transferrin fraction. Considerably lower recovery (71.4%–76.2%) was observed when ferric iron was added as the chloride, nitrate or ammonium sulfate, even though a pH of less than 2 in the iron solutions was used. Recovery values ranging from 93.0%–98.3% were obtained when citrate was added to a ferric salt and 94.8%–98.3% when nitrilotriacetic acid was employed. Consistently lower recoveries were observed when the ratio of chelate to iron was increased.

The completeness of $^{59}$FeSO$_4$ binding to transferrin is further illustrated in Table 2. Transferrin was labeled in vitro by the addition of $^{59}$FeSO$_4$ to plasma and in vivo through absorption from the gut or by slow intravenous injection of $^{59}$FeSO$_4$. Virtually all radioactivity in the plasma sample was found localized in the transferrin peak after polyacrylamide gel electrophoresis. Only in a sample with a transferrin saturation of 89% before the addition of radioiron (not included in Table 2) was binding reduced, and then only 6% of the added isotope was lost. Since injected radioiron could have been lost from the plasma to tissues instead of being bound to transferrin, the recovery of injected radioiron was also determined. This was accomplished by first injecting a known amount of $^{125}$I iodine-labeled transferrin and 2 min later injecting a known amount of radioiron as ferrous sulfate (pH 2). The ratio between radiiodine and radioiron in circulating blood was compared with the ratio of the two isotopes administered. Ratios of 0.96 in normal animals and 1.0 in iron loaded animals verified the efficiency of this binding technique.

In Vitro Stability of Iron Bound to Transferrin

Purified dfferic transferrin labeled with $^{59}$Fe was added in vitro in small amounts to iron deficient plasma. The initial radioactive profile of this plasma subjected to isoelectric focusing showed only 3% of the activity to the right of the two dfferic peaks where the two monoferric peaks would be found (Fig. 2). By 200-min incubation in vitro at 37°C, there was 7% of
the activity in these monoferric areas and by 24 hr this had increased to 23%.

In a second study, plasma was labeled by the in vivo absorption of radioiron from a gut loop in an iron deficient animal. In view of the transferrin saturation of 6% and the pattern obtained by isoelectric focusing (Fig. 2D), this may be assumed to be predominantly monoferric transferrin. A small amount of this plasma was added to plasma of an hypertransfused rat whose transferrin was 89% saturated with iron. After incubation of this plasma for 200 min at 37°C, there was no significant change in the isoelectric focusing profile (Fig. 2E). On the basis of these observations, it is assumed that no significant redistribution of iron between transferrin binding sites would be expected over the period of a few hours in rat plasma in vitro.

Is Iron Loading of Transferrin Random?

The relative proportions of the proteins transferrin fast and transferrin slow have been shown by DEAE cellulose chromatography to be 80% and 20% in the normal and 70% and 30% in the iron deficient rat. If iron is distributed uniformly on all binding sites, the distribution of added radioactivity should be similar. The plasma of 6 normal rats after the in vitro addition of a tracer amount of radioiron was subjected to electrophoresis by polyacrylamide gel. The average activity of 6 animals was 74% ± 1% in the fast transferrin peak and 26% ± 1% in the slow transferrin peak. Similar observations are recorded in Fig. 1 where the addition of increasing amounts of iron added to iron deficient plasma gave a ratio of 67% fast and 33% slow. The precision of these estimates is limited by the incomplete separation of the two peaks (see Fig. 1) but agree with the expected amounts of isotransferrins present.

Another way of examining randomness of loading was by the determination of the specific activity of the radioiron in each peak. Normal plasma with a transferrin saturation of 43% was labeled in vitro by the addition of a tracer amount of $^{59}$FeSO$_4$, and the four peaks obtained by isoelectric focusing were identified (Fig. 3). The single fraction with the highest radioactivity in each was selected for iron analyses. The two
Fig. 3. The radioiron activity of four transferrin peaks obtained by isoelectric focusing. **Fe as ferrous sulfate was added to normal plasma with an iron saturation of 43%. The transferrin of this plasma was prepurified by DEAE cellulose chromatography and concentrated by ultrafiltration as described elsewhere except omitting gel filtration. The radioactive profile obtained by subsequent isoelectric focusing is displayed. The percent of radioactivity in the 4 peaks of 13.3%, 37.7%, 11.5%, and 37.5% and the distribution between the transferrin slow and fast of 25% and 75% are about as expected from random loading of normal plasma at that saturation.

Fig. 4. Loading patterns of radioiron on transferrin. The figure on the left is the profile of **Fe entering the plasma from the gut as determined by isoelectric focusing. The figure on the right is the radioactive pattern of **Fe added in vitro to that same plasma. The distribution is generally the same although there has been a shift of 9% in activity from the two monoferric peaks on the right to the mono-diferric peaks on the left.

Plasma Disappearance Kinetics of Transferrin Bound Radioiron

Purified **Fe tagged diferric transferrin fast and **Fe-tagged transferrin slow were injected intravenously. Their simultaneous clearance rates were virtually identical (Table 3). To further examine any difference in removal of iron from individual sites, doubly labeled transferrin was employed with 100% of **Fe on the acid stable site and 78% **Fe on the acid labile site. In 3 normal animals, the mean ratio from the two isotope clearance rates from the plasma (**/**Fe) was 1.02 ± 0.01.

Previous studies have shown a difference in the disappearance rate of diferric as compared to monoferric transferrin in the rat. These were extended by examining the effect of iron status of the recipient animal (Table 4). In iron loaded animals, the ratio was close to 1, while in iron deficient animals ratios of approximately 2 were obtained. In normal animals, intermediate ratios were observed. A plot of the relationship between diferric and monoferric iron turnover as a function of transferrin saturation in individual animals is shown in Fig. 5. The linear regression of these data further substantiates the change in ratio from 1 to 2 as transferrin saturation decreased. In the various groups of animals studied, it appeared to make no difference whether a purified transferrin fast was employed in which iron was entirely located on the acid stable site or whether trace labeled iron deficient plasma was used where the label was uniformly distributed between both sites.

Additional studies were carried out at the two extremes of saturation in an attempt to further explain the changing clearance ratios between monoferric and diferric. In the iron loaded animal, monoferric transferrin was injected and subsequent plasma samples
were drawn to examine its in vivo behavior (Fig. 6). Within 15 min, virtually all activity was shown to be in the form of diferric transferrin. In the iron deficient animal, after the injection of diferric transferrin fast (Fig. 7), there was a rapid appearance of monoferric peaks of both transferrin fast and slow. Such monoferric transferrin did not appear over the same interval in the normal animal (Fig. 7). Clearance curves had been observed to show a curvature after the injection of monoferric transferrin in the iron deficient animal, not present in the normal animal (Fig. 8). On the other hand, plasma drawn from an iron deficient animal after about 80% clearance of the initial activity (injected as $^{59}$Fe diferric transferrin fast), when injected in two iron deficient animals, showed an initial $t_\frac{1}{2}$ clearance of 21 and 14 min, even longer than the simultaneous clearance of $^{56}$Fe-trace labeled iron deficient plasma of 17 and 12 min.

**Tissue Distribution**

Our previous studies have shown no difference in in vitro iron release and in internal distribution of radioiron from labeled diferric transferrin fast and slow or from iron in the monoferric form bound to the acid stable sites of transferrin fast or slow. Additional studies were carried out here to determine whether there was any difference between the labeled purified proteins previously studied and the trace labeling of plasma. In normal animals injected with trace labeled iron deficient plasma, the distribution between erythron and liver was similar to that of normal animals injected with purified monoferric transferrin labeled at the acid stable site (Table 5). Both of these had a distribution similar to that of diferric transferrin. A similarity in distribution between randomly labeled and one-site oriented

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**Table 4. Rate of Plasma Clearance of Monoferric and Diferric Transferrin Iron**

<table>
<thead>
<tr>
<th>Recipient Animal</th>
<th>Type of Tagging</th>
<th>No. of Rats</th>
<th>$%$ Saturation (%)</th>
<th>$t\frac{1}{2}$ Clearance (min)</th>
<th>Di/Mono</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficient</td>
<td>Purified proteins*</td>
<td>8</td>
<td>4 ± 1</td>
<td>9.7 ± 2.2</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Normal</td>
<td>In vitro tagging†</td>
<td>3</td>
<td>7 ± 1</td>
<td>18.0 ± 1.4</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Iron loaded</td>
<td>Purified proteins*</td>
<td>5</td>
<td>30 ± 6</td>
<td>32.6 ± 10.5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>In vitro tagging†</td>
<td>4</td>
<td>58 ± 3</td>
<td>42.7 ± 15.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Purified proteins*</td>
<td>6</td>
<td>81 ± 13</td>
<td>91.5 ± 18.0</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>In vitro tagging†</td>
<td>7</td>
<td>81 ± 8</td>
<td>80.8 ± 11.6</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

* Purified transferrin was saturated with $^{56}$FeSO$_4$ (FAS) to produce the diferric form. The monoferric form was prepared by removing iron from the acid labile site of purified diferric transferrin labeled with $^{56}$Fe.†

† Iron deficient plasma was trace labeled in vitro with $^{56}$FeSO$_4$ so as to produce the monoferric form of both transferrin slow and fast or saturated with $^{56}$FeSO$_4$ (FAS) to 95% to produce the diferric forms.32

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**Fig. 5.** The effect of transferrin saturation on clearance of diferric and monoferric transferrin. On the ordinate is shown the ratio of clearance of diferric as compared to monoferric transferrin. On the abscissa is shown the transferrin saturations of the individual animals studied that varied between 4% and 96%. The regression line is drawn with an extrapolated value at 0% saturation of 2 and 100% saturation of 1 ($r = 0.80$). The data plotted in this figure are the same as those grouped in Table 4.

**Fig. 6.** In vivo changes in radioiron distribution in an iron loaded animal. Trace labeled ($^{56}$Fe) normal plasma was injected into an iron loaded animal with a transferrin saturation of 89%. The profile of the injected plasma is shown on the left and the profile of a sample drawn 15 min later on the right. Since redistribution of iron between transferrin molecules did not occur in iron overload plasma (Fig. 2) this change may be presumed to represent the rapid saturation of monoferric transferrin by cold iron from body tissues.
Fig. 7. In vivo behavior of radioiron bound to transferrin. The top figures indicate the pattern of diferric transferrin fast (solid line) and of diferric transferrin slow (dotted line) over 60 min after their injection into a normal animal. The isoelectric focusing profile of the material injected seen on the left and the pattern to its right were made from plasma drawn 2, 31, and 60 min after its intravenous injection. Despite the progressive decrease in activity of the diferric peaks, there was no evidence of monoferric activity nor was there evidence of exchange of radioiron between transferrin slow and transferrin fast. In addition, the area under the two isotransferrin peaks remains constant excluding the possibility that one or the other might preferentially deliver iron. In the lower sequence is shown the profiles resulting from the injection of diferric transferrin fast (profile on left) into an iron deficient animal. Samples were drawn at 2 mm and 25 min. The pattern at 2 min (second from left) is similar to that of the injected transferrin fast, but the pattern at 25 min shows the appearance of all four transferrin peaks including those of transferrin slow as well as transferrin fast.

monoferric transferrin iron was also found in iron loaded rats. Trace labeled monoferric and diferric transferrin iron injected into iron deficient animals also showed similar distribution.

There were differences in the amount of plasma activity relating to the time of sacrifice. Differences also occurred as the iron status of the animal changed (Table 5). Iron deficient animals had a proportionately larger radioactive iron uptake in circulating red cells than did normal animals. Both iron deficient and iron loaded animals had relatively larger iron uptake in the liver as compared to the erythron. In these various studies, therefore, the differences observed related to differences in iron metabolism of the animals studied and not the way in which transferrin iron was presented.

**DISCUSSION**

Internal iron exchange was initially examined by tagging the plasma compartment with radioiron. Measurements of plasma iron turnover and of radio-

iron localization in the circulating red cell mass were originally translated directly into total tissue iron uptake and erythron iron turnover, respectively. It was soon realized that reflux occurred from extravascular fluids and from iron taken up by body tissues. In addition, an effect of plasma iron concentration on plasma iron turnover was identified and related to the difference in clearance between mono- and diferric transferrin. Adjustments for these phenomena made it possible to determine with some accuracy the level of erythroid marrow activity. However, a number of reports in recent years have raised questions concerning the validity of these kinetic measurements. There has been concern about the adequacy of (radio)-iron binding to the specific sites of transferrin and more important from a theoretical standpoint has been the proposal that the two sites of transferrin behave differently in respect to iron release (see discussion by Aisen and Brown). In the rat, the additional possibility has been raised that there may be differences in tissue distribution of iron delivered from transferrin fast and
transferrin slow. This study was undertaken to evaluate the nature of transferrin iron exchange in the rat with these issues in mind.

The first question addressed was the completeness and specificity of radioiron binding to transferrin. Binding of ferrous ammonium sulfate to plasma transferrin carried out in vitro as described in Materials and Methods was found to be complete as long as there were unsaturated binding sites. Least satisfactory were ferric salts where binding over a period of 30-min was incomplete, presumably a reflection of their limited solubility. The iron of citrate or nitrilotriacetate complexes showed a binding only 3%–6% less than that of ferrous sulfate, the difference being more pronounced when larger amounts of chelate were used. However, there are other reasons to avoid the use of such ligands. When present in millimolar concentrations in plasma, these chelates may permit intramolecular transferrin iron exchange and in addition will compete for the anion binding site of the transferrin which is normally occupied by bicarbonate. To counteract this, the amount of bicarbonate in the medium must be increased. Further objection to the use of nitrilotriacetate or citrate is the delivery of iron preferentially to the acid stable binding site of transferrin whereas simple ferrous salts of iron show no preferential binding.

A more complex question relates to the behavior of the two sites of transferrin. In 1968, Fletcher and Huehns published a provocative discussion of the function of transferrin based primarily on differences observed in a reticulocyte incubation model. One

![Graphs](From www.bloodjournal.org by guest on October 30, 2017. For personal use only.)
Table 5. Tissue Distribution of Transferrin Iron Complexes

<table>
<thead>
<tr>
<th>Type of Tagging</th>
<th>Animals Studied</th>
<th>TF Saturation (%)</th>
<th>Sacrifice Time (min)</th>
<th>Radioiron Content as a Percentage of Injection Dose</th>
<th>Activity Ratio Erythron/Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Wt (gm)</td>
<td>Hgt (g/dl)</td>
<td>PI</td>
<td>6.3 ± 3.1</td>
</tr>
<tr>
<td>Normal animals</td>
<td>6</td>
<td>211 ± 9</td>
<td>39 ± 1</td>
<td>147 ± 40</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>Monoferric (purified transferrin, acid stable site)</td>
<td>6</td>
<td>187 ± 3.1</td>
<td>42 ± 1</td>
<td>168 ± 93</td>
<td>38 ± 23</td>
</tr>
<tr>
<td>Diferric (trace labeled &amp; satur.)</td>
<td>6</td>
<td>222 ± 4.3</td>
<td>42 ± 2</td>
<td>138 ± 15</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>Iron loaded animals</td>
<td>Monoferric (trace labeled iron deficient plasma)</td>
<td>6</td>
<td>302 ± 21</td>
<td>45 ± 1</td>
<td>370 ± 34</td>
</tr>
<tr>
<td>Monoferric (purified transferrin, acid stable site)</td>
<td>6</td>
<td>313 ± 14</td>
<td>47 ± 1</td>
<td>381 ± 29</td>
<td>82 ± 13</td>
</tr>
<tr>
<td>Iron deficient animals</td>
<td>Monoferric (trace labeled iron deficient plasma)</td>
<td>8</td>
<td>226 ± 30</td>
<td>18 ± 1</td>
<td>43 ± 13</td>
</tr>
<tr>
<td>Diferric (purified transferrin, acid labile site)</td>
<td>3</td>
<td>269 ± 42</td>
<td>22 ± 6</td>
<td>90 ± 27</td>
<td>9 ± 4</td>
</tr>
</tbody>
</table>
portion of transferrin iron was observed to be removed more rapidly by reticulocytes than another. It was proposed that iron from one iron binding site might be preferentially delivered to the erythroid marrow, while the iron of the other binding site might be preferentially delivered to the liver. The results of Fletcher and Huehus seemed to be substantiated by a number of other investigators not only in the reticulocyte model but also in in vivo studies. As this hypothesis has been examined, various possibilities have been raised, including not only a differential unloading of iron from one site to its favorite tissues, but also that differential loading of the individual sites in the process of intestinal absorption may occur.

The feasibility of measuring specific binding by gel acrylamide electrophoresis, of separating slow and fast transferrin by both gel electrophoresis and isoelectric focusing, and the ability to isolate slow and fast diferric transferrin as well as monoferic transferrin peaks, have provided new opportunities to study specific site behavior. With such techniques, it was possible to explore iron loading on transferrin both in vitro and in vivo and to follow the behavior of specifically labeled fractions in the plasma and in their tissue distribution. Loading was shown to be random in respect to slow and fast transferrin and in respect to the two sites of transferrin. Little difference was observed in the pattern of transferrin loading obtained in vitro from that obtained by intravenous injection of iron salt, or that obtained through absorption from the intestine. In all instances, the profile of distribution of radioiron between the two transferrins and between the two sites of transferrin is considered consistent with a random process as formulated by Wenn and Williams. Slight differences observed may well be explained by methodological limitations and slight changes in transferrin saturation introduced by the iron content of the isotope.

The rate of unloading of transferrin iron in vivo was shown to be greater for the diferric than the monoferric form, consistent with previous in vivo observations. However, there was no difference between monoferric transferrin on the acid labile site as compared to monoferric transferrin on both sites. The removal of radioiron from diferric transferrin in the plasma in normal animals was not associated with the appearance of monoferric transferrin, indicating that both iron atoms were removed at the same time. Thus the previous demonstration of the “all-or-none” exchange of iron from the transferrin iron complex to reticulocytes was also true of the in vivo disposal of plasma iron. Finally, the disposal of the radioiron from the fast and slow transferrin was uniform in pattern, presumably showing a random release whenever the transferrin iron complex interacted with a tissue membrane receptor.

Most previous statements concerning a heterogeneous iron release from transferrin have been derived from studies of iron exchange between transferrin and reticulocytes. These studies appear simple but are actually difficult to control. Problems identified include inadequate or abnormal iron complexing with transferrin, alterations in transferrin saturation affecting the rate of exchange, and the presence of radioactively labeled iron derived from hemolysis. Because of these alternate explanations and the indirectness of the observations, it would seem inappropriate to interpret differences observed to site behavior. In the present study, the distribution of radioiron on the transferrin molecule has been examined under conditions where there is little exchange of iron between transferrin binding sites in vitro. Evidence is provided of a random loading and unloading behavior of the different sites of the transferrin molecule in the rat.

An aspect of plasma iron turnover that has emerged during these studies is the effect of the iron status of the experimental animal on radioiron kinetics. In the iron deficient animal, diferric transferrin was cleared at twice the rate of monoferric transferrin. However in the animal with a high transferrin saturation, this difference disappeared. Observation on the state of the labeled transferrin in the plasma explained this. At high saturations, trace labeled monoferric iron was rapidly converted to the diferric form in vivo so that the disappearance curve was actually that of the diferric form. In the iron deficient animal, the difference in iron delivery from monoferric and diferric transferrin was evident. In addition, there was an early reflux back into the plasma of iron, this time as monoferric transferrin. This rapid reflux that was seen only in the iron deficient animal explained the curvilinear disappearance curve in iron deficiency.

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The behavior of transferrin iron in the rat

H Huebers, W Bauer, E Huebers, E Csiba and C Finch