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Research Article



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# THE PLASMA–TO–CELL CYCLE OF TRANSFERRIN *

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Iron is transported from its sites of absorption, deposition, and storage through the plasma by a specific iron-binding protein, transferrin (siderophilin). Each molecule of transferrin binds up to two atoms of iron to form reddish chelates of extraordinary stability (1, 2). The stability constant of the iron transferrin complex under physiologic conditions appears to be of the order of $10^{29}$ (3).[1] It has also been found that the rate of equilibration of iron between the iron-saturated and the unsaturated form of transferrin is extremely slow (5), further indicating a very low order of dissociation.

Despite the great affinity of transferrin for iron, this metal is yielded to the immature red cells of the bone marrow rapidly and efficiently. In patients whose marrow utilization of iron is high, as in iron deficiency, half the iron in the circulating plasma may be removed in as little as 11 minutes (6, 7), almost entirely by the bone marrow (8). This corresponds to a clearance of iron from the plasma at the rate of about 6% per minute. The flow of blood to the bones in the normal man has been estimated to represent about 5% of the cardiac output (9). Thus, although bone marrow blood flow may well increase somewhat during erythroid hyperplasia, it is apparent that in individuals such as those with iron deficiency or polycythemia, close to 100% of the plasma perfusing the marrow may be cleared of iron in each passage.

From the considerations cited above it is highly improbable that iron could be transferred from the plasma to the immature red cells of the marrow by its dissociation into free ions and a simple equilibrium between intravascular and extravascular ionic iron. Two other proposed mechanisms have received study. One is that iron is taken up from the plasma by an intermediate cell in the bone marrow, the reticulum cell, and then incorporated within ferritin molecules. Granules of ferritin, in turn, are ingested by contiguous erythroblasts by a kind of microphagocytosis, as deduced from electron microscopic studies by Bessis and Breton-Gorius (10). Although this mechanism may account for a portion of the iron delivered to immature red cells, there are two lines of evidence which suggest that immature red cells acquire most of their iron by a second proposed method, whereby iron is yielded by transferrin directly to the cell: *1*) radioactive iron has been shown to be taken up directly from plasma (11) or from solutions of purified transferrin (12–14) by freely suspended immature red cells (reticulocytes), both *in vitro* (11–14) and *in vivo* (15); *2*) radioactive iron can be found in bone marrow heme within a few minutes of its intravenous injection as transferrin-bound iron (16).

In previous studies (14) it was learned that ethylenediamine tetraacetic acid (EDTA) failed to block the movement of iron from transferrin to reticulocytes unless it was added in amounts sufficient actually to remove iron from transferrin. On the other hand, enzymatic alteration of the reticulocyte surface by trypsin did block iron transfer. Accordingly, it was suggested that iron may be transferred to cell receptors without being released as a free ion (14). This raised the possibility that transferrin itself became attached to the cell surface or actually entered the red cell transiently while unloading its iron. Since it is known from comparison of the rates of turnover of iron (6–8) and of transferrin (17–19) that

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[1] Recently it has been reported (4) that the two atoms of iron are bound to transferrin with distinctive affinities. The two stability constants obtained were $10^{30.3}$ and $10^{27.7}$.

transferrin is not consumed during iron-unloading, it must be assumed that if transferrin attaches to the cell, or enters it, this is a reversible process. Recent studies (20) revealed that immature red cells of man do manifest a protein on their surfaces, and this protein has the immunologic properties of transferrin.

The presence of a transferrin-like protein on reticulocytes could be caused either by the attachment of plasma transferrin or by the presence in the cell membrane of a component having some properties of transferrin. To investigate this matter, studies were made of the interactions between immature human red cells and human transferrin that had been labeled with radioactive iodine. A preliminary report of these studies has been published (21).

## MATERIALS AND METHODS

*$I^{131}$-transferrin.* Transferrin is well suited for iodine labeling in several respects. It is rich in tyrosine, the site of iodine incorporation, and when iron-saturated, it is a very stable, highly water-soluble protein (2, 22, 23). The saturation of transferrin with iron prior to iodination is desirable not only because iron increases the stability of the protein but also because the binding of iron by transferrin may involve tyrosyl linkages (24).

The human transferrin employed in these studies was prepared and given to us by Dr. John K. Inman; the material was recrystallized three times and was of 97 to 98% purity on electrophoretic analysis (25, 26). Electrophoresis at pH 8.5 on starch gel (27) revealed a single band corresponding to the transferrin C phenotype.

The methods employed in the preparation and analysis of iodinated transferrin are reported in full detail elsewhere (19). The following criteria were used to determine the structural and functional integrity of the $I^{131}$-labeled transferrin:

1) The visible extinction coefficient (465 mμ) was unchanged.

2) There was no change in iron binding capacity as determined by direct analysis and iron specific activity.

3) The pattern on ultracentrifugation was unaltered.

4) Ouchterlony patterns (28) of iodinated transferrin were not different from those of the unlabeled proteins.

5) Quantitative precipitation (29) was unchanged.

6) On dialysis of iron-saturated, iodinated protein against serum (i.e., against unsaturated transferrin), there was a very slow exchange of iron (9% of expected equilibrium values in 48 hours); this was similar to the rate of exchange of unlabeled transferrin. There was no movement of radioactive iodine into the dialysate. The stability of the iodine complex with transferrin was further attested to by the fact that incubating $I^{131}$-transferrin with a large excess of human albumin did not result in the attachment of any of the $I^{131}$ to the albumin, as determined by subsequent electrophoretic separation of the proteins followed by autoradiography.

7) On injection into human subjects iron bound to this iodinated transferrin was cleared from the plasma at a normal rate and was utilized by red cells to a normal extent (19).

$I^{131}$-transferrin was doubly labeled in some experiments by the addition of $Fe^{59}$. In these instances the stable iron was removed from transferrin by lowering the pH to 5.5 to 5.6 with an acetate-EDTA buffer (final concentrations: 0.3 M acetate, 0.01 M EDTA, and 4 to 5% protein). Decolorization, indicating removal of the iron, was complete in 1 hour, after which the solutions were dialyzed against 0.02 M $NaHCO_3$ and 0.1 M NaCl to bring the pH to 7.5 and to remove the EDTA. $Fe^{59}$ with carrier $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was then added to the desired level of saturation [2] and allowed to stand overnight to permit complete oxidation of the iron and protein binding. Actual determinations of the iron content and "iron binding capacity" of purified transferrin, of mixtures of transferrin and albumin, and of plasma were made as described by Peters, Giovanniello, Apt, and Ross (31, 32).

*Red cell suspensions.* Suspensions of red cells rich in reticulocytes were obtained largely from the blood of patients with pernicious anemia undergoing therapeutic responses to vitamin $B_{12}$. In one instance reticulocyte-rich blood of a patient responding to iron therapy was employed, in another the blood of a patient with paroxysmal nocturnal hemoglobinuria. Red cells poor in reticulocytes were derived from the blood of normal individuals or from the bottom layer of centrifuged blood from the patients described above. All blood specimens were heparinized, the plasma and most of the buffy coats were removed after centrifugation, and the red cells were washed twice in about four volumes of physiologic saline before their suspension in saline to a concentration of about 50%. Blood cell counts and hematocrits were performed by standard methods (33) on each of these suspensions. Reticulocyte concentrations were determined by the "dry" method (33) on small samples of the washed cells that had been resuspended in 2 volumes of plasma.

*Experimental procedure.* In most experiments a given volume of the washed cell suspension was added to each of a series of siliconized tubes set up in duplicate. To these tubes were added equal volumes of a 0.5% solution of transferrin, labeled or unlabeled. The transferrin was dissolved in an isotonic buffered solution, pH 7.4 to

[2] Complete saturation was attained by the binding of 1.30 μg of iron per mg transferrin. Although this is slightly greater than previous estimates of iron-binding by transferrin (i.e., 1.25 μg per mg), the figure of 1.30 gives an estimated molecular weight for transferrin (86,000) which is more in keeping with current estimates with ultracentrifugation (30).

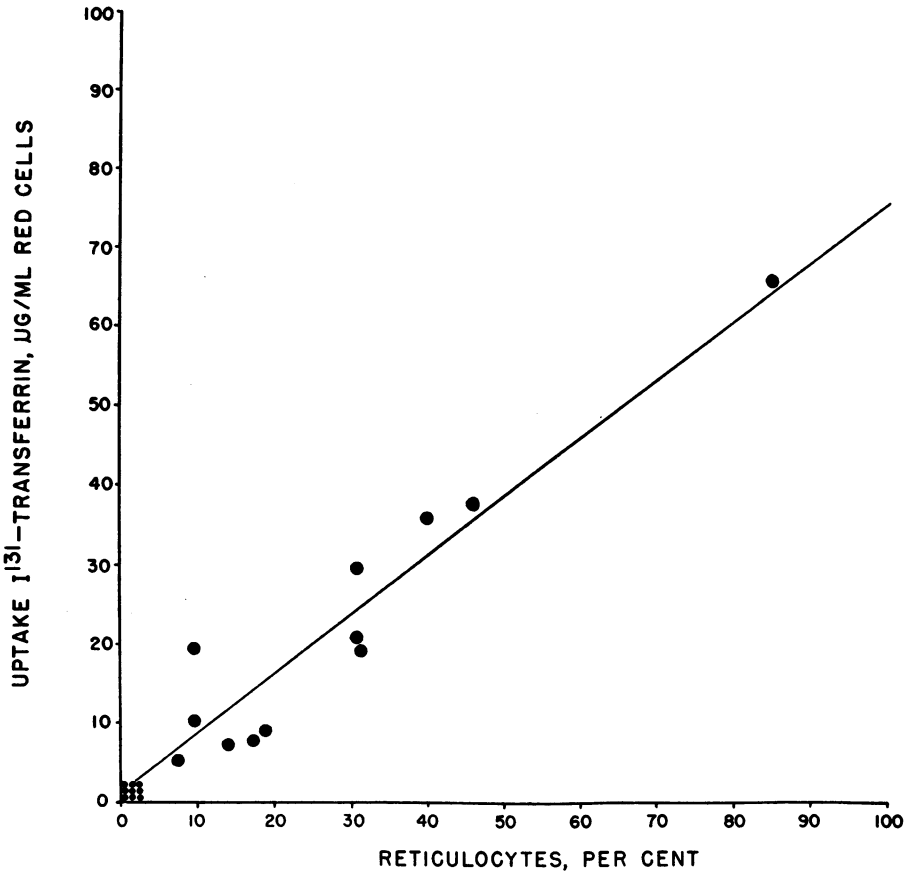


FIG. 1. THE RELATION BETWEEN THE UPTAKE OF $I^{131}$-TRANSFERRIN BY WASHED RED CELL SUSPENSIONS AND THEIR CONCENTRATION OF RETICULOCYTES.

7.5, that provided the following final concentrations of ingredients: glucose, 0.01 M; sodium bicarbonate, 0.02 M; potassium diacid phosphate, 0.01 M; and sodium chloride, 0.12 M. In most experiments this solution included human serum albumin [3] in a concentration of 4% for the purpose of preserving normal red cell morphology during incubation and of counteracting possible nonspecific absorption of transferrin on the cells and glassware. Although for these considerations albumin was routinely included in most of the experiments, duplicate studies of transferrin "uptake" (see below) by the red cell suspensions with and without albumin showed no difference. Albumin was not employed in those experiments concerned with the influence of iron saturation on transferrin uptake, since the albumin preparation itself contained some iron. The red cell suspensions and the transferrin solutions were preincubated for several minutes at 37° C before admixture. Incubation at this temperature was carried out in a Dubnoff metabolic shaker and was terminated by flushing into the tubes large (10 or more) volumes of cold saline, followed immediately by a brief, rapid centrifugation. The supernatant solution was discarded, and the cells were then "washed" 5 more times with saline as quickly as possible, and then transferred to "counting" tubes for measurement of their radioactivity.

The radioactivity of the washed cells and of appropriate standards was determined in a well-type scintillation counter. In experiments involving doubly labeled transferrin, counts were made simultaneously by the use of two scalers, set at different levels of discrimination (200 and 700 kv) and operating simultaneously (34). By knowing the counting sensitivities of the two scalers to each of the two isotopes, it is possible to employ simultaneous equations that calculate the amounts of each of the isotopes in a mixed sample.

With a knowledge of the specific activity of the transferrin and of the iron employed, the quantity of these substances taken up by the cell suspensions was calculated. This uptake was expressed in some instances as micrograms per milliliter of red cells and in other specified instances as micrograms per milliliter of reticulocytes by dividing the measured uptake by either the volume of cells or the volume of reticulocytes present in the sample.

[3] Nutritional Biochemicals Corp., Cleveland, Ohio.

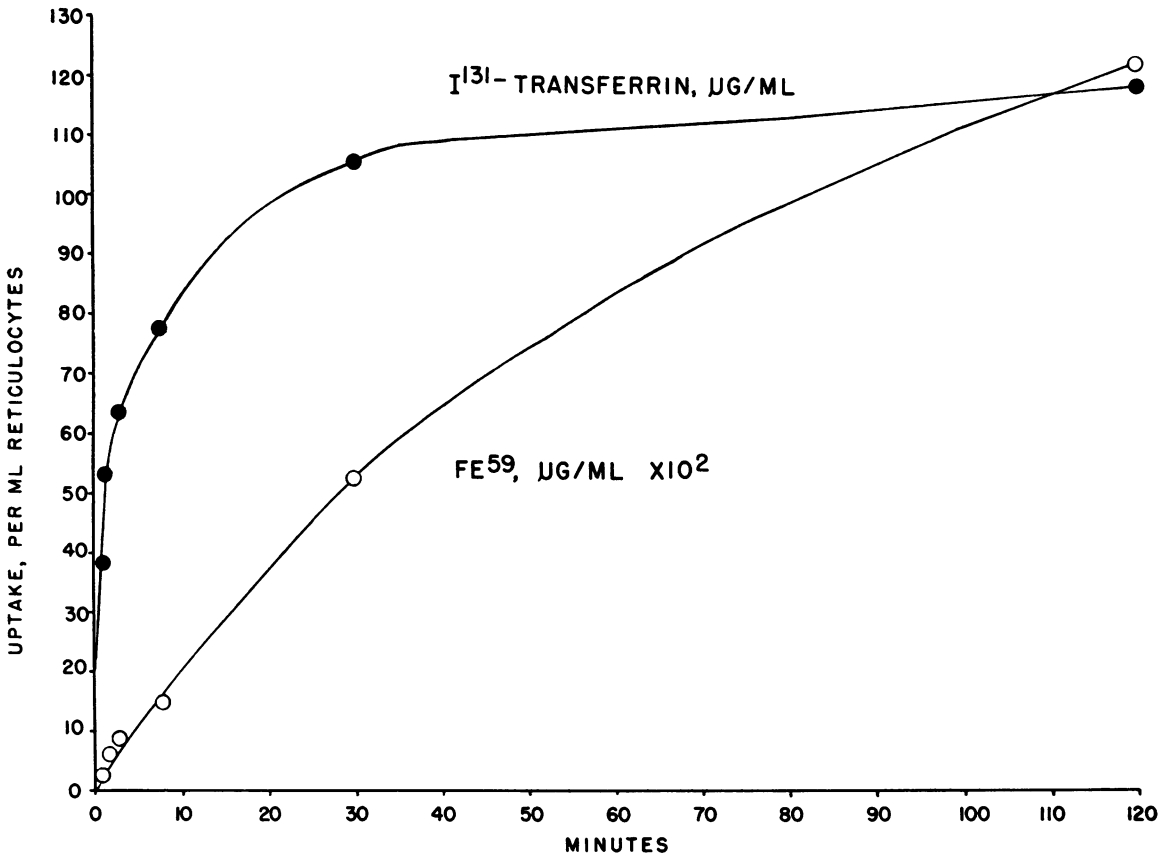


FIG. 2. COMPARISON OF THE UPTAKE BY RETICULOCYTES OF TRANSFERRIN-BOUND IRON ($Fe^{59}$) AND OF TRANSFERRIN ITSELF ($I^{131}$-TRANSFERRIN), WITH DOUBLY LABELED TRANSFERRIN.

## RESULTS

### *Uptake of $I^{131}$-transferrin by reticulocytes*

Studies were made of the uptake of $I^{131}$-transferrin by red cells from 15 individuals. Seven of these individuals had reticulocyte concentrations of less than 1.5%, the other 8 had variously elevated concentrations. The blood of two of the latter individuals was subjected to centrifugation, and the top (reticulocyte-rich) and bottom (reticulocyte-poor) layers were compared. The relationship between the reticulocyte concentration of these various cell suspensions and the uptake of $I^{131}$-transferrin is expressed in Figure 1. A line was fitted to these points by the method of least squares. If we judge from the slope of this line, the average uptake of $I^{131}$-transferrin by the immature cells was 75 $\mu$g transferrin per ml reticulocytes (the intersection of the fitted line and a vertical line representing 100% reticulocytes). Since at its other end the fitted line intersected the ordinate at 0.6, the average uptake by mature cells (0% reticulocytes) was of the order of 0.6 $\mu$g transferrin per ml red cells. Thus, reticulocytes were roughly 100 times as avid for transferrin as were mature red cells. There was no correlation between the white cell or platelet counts of these cell suspensions and their uptake of $I^{131}$-transferrin.

Albumin labeled with $I^{131}$ and incubated with red cells as was transferrin was not taken up preferentially by reticulocytes. In both reticulocyte-rich and reticulocyte-poor suspensions, the "uptake" of albumin ranged between 0.4 and 1.0 $\mu$g per ml red cells. Since this amount is similar to the quantity of transferrin remaining with mature red cells, it is possible that remnants of up to 1 $\mu$g of protein reflect the limit of the efficiency of the cell-washing method. Masouredis, Chi, and Ferguson (35, 36) have found a similar amount

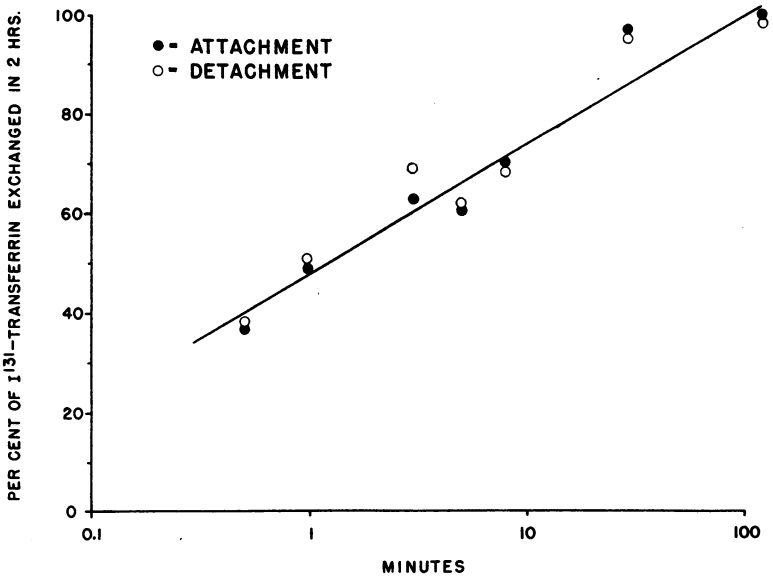


FIG. 3. THE RATE OF UPTAKE ("ATTACHMENT") OF $I^{131}$-TRANSFERRIN BY RETICULOCYTES COMPARED WITH THE RATE OF RELEASE ("DETACHMENT") OF $I^{131}$-TRANSFERRIN BY RETICULOCYTES SUSPENDED IN SOLUTIONS OF UNLABELED TRANSFERRIN. Note the use of a semilogarithmic scale. By "exchange" is meant the quantity of $I^{131}$-transferrin attached to, or detached from, the cells at a given time as a percentage of that at two hours' incubation.

of $I^{131}$-labeled anti-D antibody to attach non-specifically to D-negative red cells.

The effect of autologous serum on the uptake of $I^{131}$-transferrin by reticulocytes was compared to that of the buffered 4% human serum albumin medium. As compared to the protein-free buffer, albumin had no effect on the uptake of either $Fe^{59}$ or of $I^{131}$-transferrin by reticulocytes. However, the presence of serum, in an amount that diluted the $I^{131}$-transferrin with an equal amount of serum transferrin (iron saturation, 48%), diminished the uptake of $I^{131}$-transferrin by 32%.

The site of attachment of $I^{131}$-transferrin to reticulocytes was studied in reticulocyte-rich suspensions of cells after they had been incubated for one hour with transferrin labeled both with $I^{131}$ and with $Fe^{59}$. To each volume of washed, packed cells were added 5 volumes of ice cold water; 5 minutes later, one-half volume of 10% sodium chloride was added and part of the hemolyzed suspension was spun at 6,600 × *g* for 15 minutes and another part at 105,000 × *g* for 1 hour. The levels of radioactivity in the soluble, supernatant portion of the rapidly spun hemolyzed suspension and in the "stroma"-free (but not particle-free) supernatant portion of the slowly spun, hemolyzed suspension were compared to that of the whole, unspun preparation. Whereas 55.3% of the total cellular $Fe^{59}$ was recovered in the soluble fraction, as was expected (14), only 10.9% of the cellular $I^{131}$ was so recovered. Almost all of the remaining 90% of the $I^{131}$ was in the large particle ("stromal") fraction.

### *Studies of the rate of attachment and detachment of transferrin*

The rate at which $I^{131}$-transferrin was taken up by reticulocytes was compared to the rate of iron uptake by employing the doubly labeled protein (i.e., labeled both with $I^{131}$ and $Fe^{59}$). As shown in Figure 2, transferrin itself, as measured by $I^{131}$, was largely taken up within the first few minutes of incubation; half the 2-hour uptake occurred within 2 minutes. On the other hand, $Fe^{59}$ was taken up more steadily during the 2-hour period of incubation, with the eventual accumulation of many more moles of iron than of transferrin. At one minute approximately 2 moles of iron were taken up per mole of transferrin. By 30 minutes

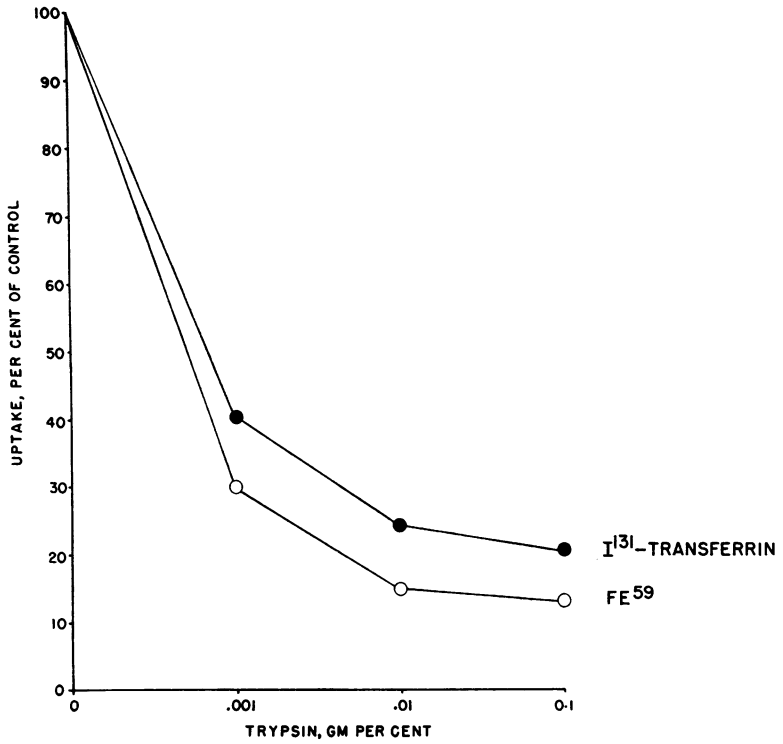


FIG. 4. THE EFFECT OF ENZYMATIC ALTERATION OF RETICULOCYTE SURFACES ON THEIR SUBSEQUENT ABILITY TO TAKE UP TRANSFERRIN-BOUND IRON ($Fe^{59}$) AND TRANSFERRIN ITSELF ($I^{131}$-TRANSFERRIN). As in a previous study (14), exposure of reticulocytes to small amounts of trypsin for 1 hour at 37° C impaired their ability to take up iron thereafter. Trypsinization of the cells blocked transferrin uptake similarly.

this ratio had increased to about 13 and by 2 hours it had reached about 17.

When reticulocytes that had been incubated with doubly labeled transferrin were washed and then were incubated further in buffered saline, there was a slight gradual loss from the cells of $I^{131}$, indicating, presumably, a slow elution of $I^{131}$-transferrin. When in the second incubation period reticulocytes were suspended in human serum albumin in various concentrations up to 4 g per 100 ml, this detachment of $I^{131}$ was somewhat inhibited. When the radioactive reticulocytes were incubated in unlabeled transferrin, however, there was a rapid detachment from the cells of $I^{131}$-transferrin. This displacing effect of newly added unlabeled transferrin upon the cell-attached labeled transferrin was evident with relatively low concentrations of the former. Thus, half the cell-attached $I^{131}$-transferrin was detached within 1 hour by the presence of 100 $\mu$g of unlabeled transferrin per ml of packed cells, and almost all of the $I^{131}$ transferrin became detached in the presence of 1 mg or more of unlabeled transferrin. This suggests a specific dynamic equilibrium between cell-attached transferrin and free transferrin for attachment to the cells.

The rate at which unlabeled transferrin caused cell-attached, $I^{131}$-labeled transferrin to detach was compared to the rate at which $I^{131}$-transferrin became attached (Figure 3). These comparative observations were carried out with the same reticulocyte suspensions and materials on the same day. To samples of washed, 50% suspensions of $I^{131}$-labeled reticulocytes were added equal volumes of 0.5% unlabeled transferrin, 34% saturated with iron. It is evident that the rate of transferrin detachment was rapid and was much the same as the rate of attachment. These data indicate that free transferrin exchanges quite rapidly with cell-attached transferrin, half this exchange occurring in approximately 1 minute.

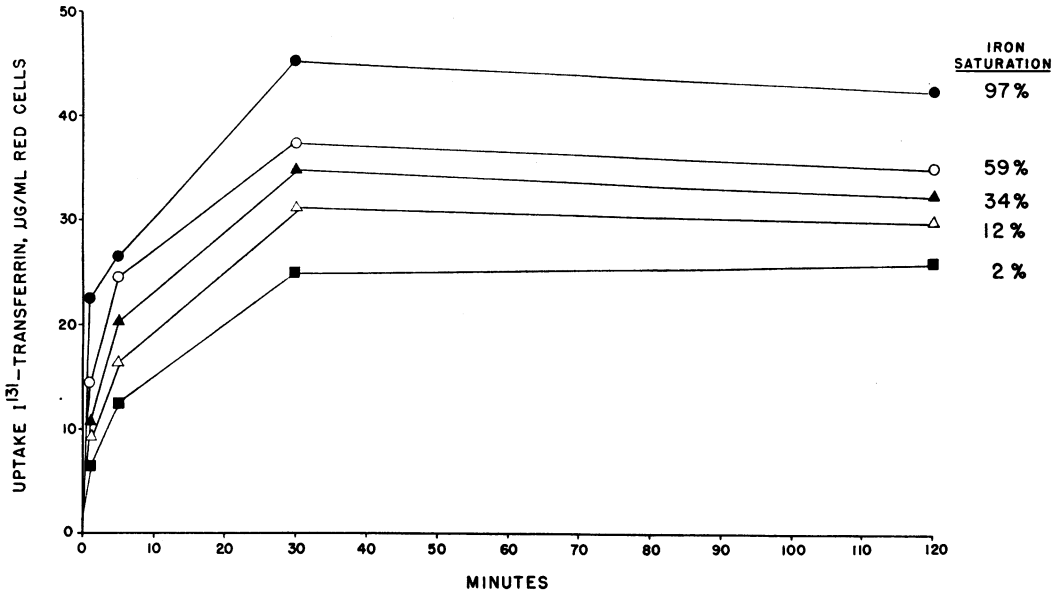


FIG. 5. INFLUENCE OF IRON SATURATION OF TRANSFERRIN ON ITS RATE AND EXTENT OF UPTAKE BY RETICULOCYTES. The uptake of transferrin was proportional to its iron saturation at every time interval studied after the admixture of reticulocyte rich red cells and $I^{131}$-labeled transferrin. The effect of iron saturation on the uptake of transferrin was most striking shortly after admixture.

*Factors influencing the rate of transferrin attachment to reticulocytes*

1. *The effect of metabolic inhibition.* With doubly labeled transferrin, it was found that cyanide ($10^{-2}$ M) and 2,4-dinitrophenylphenol ($10^{-3}$ M) strongly inhibited the uptake of $Fe^{59}$ by reticulocytes, as reported (14); thus, the uptake was reduced by cyanide to 7% of control and by dinitrophenylphenol to 8% of control. The uptake of $I^{131}$-transferrin was also inhibited by these compounds, to 27% and 32% of control values, respectively. The uptake of $Fe^{59}$ by reticulocytes at 2° C was only 9% of that at 37° C; the uptake of $I^{131}$-transferrin was also inhibited in the cold, but to a lesser extent, the uptake at 2° C being 44% of that at 37° C. Thus, in general, metabolic inhibition depressed the rate of transferrin attachment, although not so strikingly as it depressed iron accumulation.

2. *The effect of enzymatic alteration of the cell surface.* It has been reported (14) that relatively small amounts of trypsin blocked the subsequent ability of reticulocytes to take up iron, and that this defect could be partially reversed in the presence of glucose. In an experiment analogous to that cited above, samples of a washed, reticulocyte-rich cell suspension were incubated with various concentrations of crystalline trypsin[4] at 37° C, pH 7.8 for 1 hour. The cells were then washed three times before resuspension in buffered saline (pH 7.4) containing glucose, whereupon doubly labeled transferrin was added. The ability of trypsinized reticulocytes to take up both $Fe^{59}$ and $I^{131}$ transferrin was strongly suppressed, as depicted in Figure 4. As little as 0.001 g per 100 ml (10 µg per ml) trypsin had a pronounced effect on the subsequent capacity of the cell to take up transferrin and iron, despite the presence of glucose. This study suggests that cell receptors for transferrin, analogous to or identical with those for iron (14), were damaged by the enzyme.

3. *Effect of iron saturation on the avidity of transferrin for reticulocytes.* $I^{131}$-transferrin was divided into 5 batches that were then variously saturated with nonradioactive iron. Samples equal in transferrin content were then added to samples of a washed reticulocyte-rich cell suspension in the usual way. The uptake of $I^{131}$-transferrin was assessed at various time intervals thereafter (Figure 5). At every time interval studied,

[4] Trypsin, 2× cryst., Mann Research Labs., Inc., New York, N. Y.

from 1 minute to 120 minutes, the uptake of $I^{131}$-transferrin was roughly proportional to its degree of saturation with iron. The relative difference in uptake was greater shortly after admixture than it was later; thus the uptake 97% saturated transferrin/2% saturated transferrin was 3.3 at 1 minute but only 1.6 at 2 hours. This study suggested a greater avidity of reticulocytes for iron-saturated transferrin as compared to the iron-unsaturated form.

A more sensitive method for demonstrating such a difference in avidity was designed whereby the saturated and unsaturated proteins were compared within a single system, rather than by comparing duplicate systems. Thus, into a series of tubes were pipetted 0.5 ml volumes of 0.5% $I^{131}$ transferrin, either unsaturated (2% iron-saturated) or saturated (97% iron-saturated) with nonradioactive iron. Next, equal amounts of unlabeled transferrin, either unsaturated (0% iron-saturated) or saturated (96% iron-saturated), were added. To each of the four mixtures of labeled and unlabeled transferrin a given volume of washed reticulocyte-rich red cells was added, and the suspensions were incubated at 37° C for 30 minutes before washing as usual. During this period of time there should be no appreciable exchange of iron between the labeled and unlabeled transferrin (5). With this system, as recorded in Table I, it was found that $I^{131}$-transferrin was taken up to a much lesser extent in the presence of iron-saturated, unlabeled transferrin (mixtures no. 2 and 4) than when unsaturated, unlabeled protein was present (mixtures no. 1 and 3). Presumably when the mixtures consisted of labeled and unlabeled protein that had been equally saturated with iron, as in mixtures no. 1 and 4, the total uptake of transferrin was twice that of the $I^{131}$-transferrin alone; assuming this, the estimated total uptake of unsaturated transferrin was 72 μg per ml red cells and that of the saturated protein was 78 μg per ml red cells (Table I). In the unequally saturated mixtures (no. 2 and 3) the total uptake presumably was intermediate in amount, and a halfway estimate of 75 μg per ml red cells was made. By subtracting the known $I^{131}$-transferrin uptake from the estimated total uptake, the uptake of unlabeled transferrin was estimated, and the ratio of the uptake of iron-saturated transferrin to iron-unsaturated transferrin could be calculated (last column, Table I). By this means it was estimated that iron-saturated transferrin is 4 to 5 times as avid for the reticulocyte as is the iron-unsaturated form.

TABLE I

*Influence of iron saturation on the uptake of transferrin by reticulocytes*

| Mixture no. | $I^{131}$-transferrin, iron-sat. | Unlabeled transferrin, iron-sat. | Uptake $I^{131}$-transferrin | Estimated total uptake transferrin | Estimated uptake unlabeled transferrin | Ratio of uptake sat./unsat. transferrin |
|---|---|---|---|---|---|---|
| | % | % | μg/ml red cells | μg/ml red cells | μg/ml red cells | |
| 1 | 2 | 0 | 36 | 72 | 36 | |
| 2 | 2 | 96 | 12 | 75 | 63 | 5.3 |
| 3 | 97 | 0 | 60 | 75 | 15 | 4.0 |
| 4 | 97 | 96 | 39 | 78 | 39 | |

A similar, second experiment was conducted in which every tube of a series was loaded with 0.5 ml of a 0.5% solution of $I^{131}$-transferrin that was 34% saturated with iron. To these tubes were then added equal amounts of 0%, 10%, 33%, 58%, or 96% iron-saturated, unlabeled transferrin. As shown in Figure 6, the 0% saturated protein had little inhibiting effect on the uptake of $I^{131}$-transferrin as compared to saline. With increasing saturations of the unlabeled protein, however, there was progressive inhibition of the $I^{131}$-transferrin uptake. As indicated in Figure 6 the extent of inhibition was similar to that predicted if inhibition was due to competition by iron-saturated transferrin, but was dissimilar to that predicted if there was competition by transferrin regardless of its saturation with iron.

## DISCUSSION

These studies reveal that $I^{131}$-transferrin has an affinity for the surfaces of immature red cells. Since this iodinated protein does not have such an affinity for mature red cells, white cells, or platelets, and since a physically similar protein, albumin, does not have such an affinity when iodinated, the tendency of $I^{131}$-transferrin to attach to reticulocytes appears to be a specific property. That this property is not imparted to the protein by the process of iodination is indicated by the fact that attachment is competitively inhibited by uniodinated transferrin and by plasma to an extent proportional to the transferrin content. Fur-

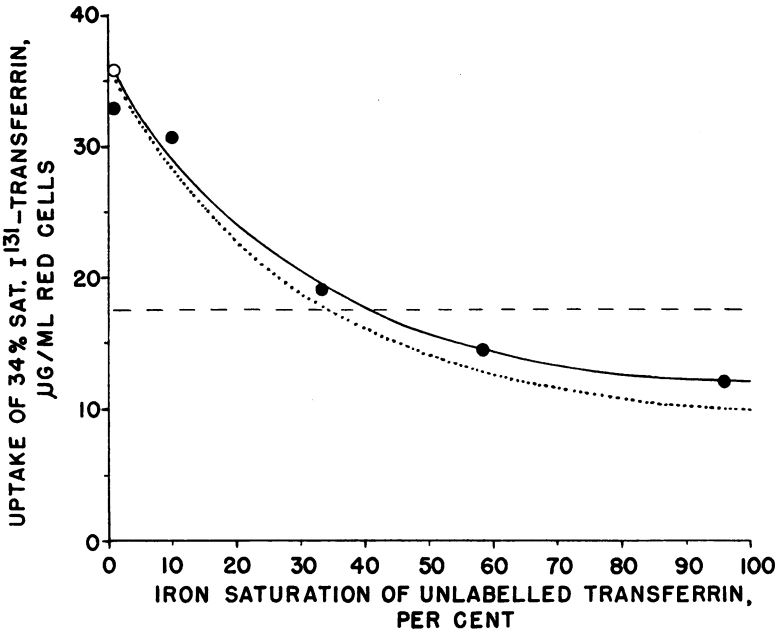


FIG. 6. INFLUENCE OF IRON SATURATION ON THE UPTAKE OF TRANSFERRIN BY RETICULOCYTES. The uptake of 34% saturated $I^{131}$-transferrin (solid line) was inhibited more by highly saturated unlabeled transferrin than by the less saturated forms. The resulting uptake of $I^{131}$-transferrin (solid line) resembled that predicted if there was competition only from iron-saturated transferrin (dotted line). As indicated by this dotted line, the uptake of labeled transferrin would have been reduced by one-half by equal amounts of 34% saturated unlabeled transferrin, reduced by two-thirds by equal amounts of 68% saturated transferrin, and so forth. If the unlabeled transferrin preparations had competed equally, regardless of iron content, the uptake of labeled transferrin would merely have been halved throughout (interrupted line). The white circle denotes the uptake of $I^{131}$-transferrin when no unlabeled transferrin had been added.

ther evidence of specificity is indicated by the fact that $I^{131}$-transferrin attached to reticulocytes is displaced by unlabeled transferrin but not by albumin. The rate of attachment and the rate of detachment of $I^{131}$-transferrin are equally rapid, half the protein on the cell exchanging with protein in solution in approximately 1 minute. Thus, transferrin in solution, as in plasma, exists in a dynamic equilibrium with transferrin attached to the surfaces of immature red cells.

Calculations indicate that roughly 50,000 transferrin molecules are attached to the surface of each reticulocyte, or approximately one molecule per 400,000 square Ångstroms. Since transferrin is believed to measure about 40 by 190 Å (37), it occupies roughly 2% of the surface of this cell.

The amount of transferrin estimated to attach to reticulocytes is very similar to the amount of albumin which, when attached to red cells by metallic cations, renders the cells agglutinable by Coombs (antihuman serum protein) serum (38). It is actually somewhat more than the amount of anti-D globulin estimated by Masouredis (35, 36) to attach to D-positive red cells. It has been reported (20, 39) that the reticulocytes of several species studied are agglutinable by Coombs serum, and that in man this agglutinating activity of Coombs serum can be blocked by its prior incubation with human transferrin (20). The present study indicates that this agglutinability of immature red cells is due, at least in part, to the attachment of plasma transferrin.

Of course, the possibility must be weighed that the adsorption of transferrin, although specific for reticulocytes, is a coincidental physicochemical phenomenon of no physiologic importance. It may

reflect, or certain features of the interaction such as the dependence on iron saturation may reflect, a subtle denaturative change that occurred during the preparation of transferrin. However, the behavior of the labeled protein in serum, the evidence by immunologic methods for the presence of transferrin on reticulocytes, and the accordant kinetic calculations strongly favor the conclusion that transferrin adsorption to immature red cells is a physiologic event.

Although care was taken in the present studies to minimize nonspecific adsorption to cells, the actual amount of adsorption of iodinated transferrin and albumin by washed mature red cells was relatively small (less than 1 $\mu$g protein per ml red cells) even when such precautions were not taken. Masouredis (35, 36), who also used the tri-iodide method of iodination and a purified protein (anti-D), has reported similar results. The reports by others (40, 41) of relatively large amounts of nonspecific adsorption in part reflect the use of unpurified protein preparations containing glycoproteins and heavy globulins, and may in part reflect the method of iodination employed. Initial studies by us of the behavior of transferrin iodinated in the presence of iodate and cysteine showed that such protein tended to adhere nonspecifically to mature red cells, to glass, and to wet filter paper, and that it had a lowered affinity for iron.

Like the uptake of iron by reticulocytes, the attachment of transferrin is blocked by metabolic inhibitors such as cyanide and 2,4-dinitrophenylphenol. As with respect to iron (14), the ability of reticulocytes to take up transferrin is lost after their enzymatic alteration by small amounts of trypsin. Such data show that the uptake both of the metabolite, iron, and of its transport protein is similarly dependent upon the metabolic function and the surface integrity of the immature red cell. The data suggest, but do not prove, the possibility that there are on the cell surface receptors for the iron-transferrin complex, and that these receptors depend upon active cellular metabolism.

The uptake of iron-saturated transferrin by reticulocytes was greater and more rapid than that of the unsaturated protein. This difference was most clearly evident in systems containing mixtures of saturated and unsaturated transferrin; in such mixtures the avidity for the cell surface of iron-saturated transferrin exceeded that of unsaturated transferrin by about fivefold. This preference for iron-laden transferrin might mean that iron is involved in the chemical attachment of transferrin to the cell surface. On the other hand, iron is known to affect markedly certain general properties of transferrin including its stability, solubility, and probably its shape (2, 22, 23), and it may be that these general features of the iron-protein complex favor interaction with the cell surface. A transient linkage of transferrin-bound iron to the surface of liver cells by complexing with adenosine triphosphate has been postulated by Mazur, Green, and Carleton (42). The present findings are compatible with the existence of such an interaction on the surfaces of immature red cells. Whatever the mechanism, the preferential attachment of iron-saturated transferrin results in the delivery of iron to the cell with relatively little competitive inhibition for cellular receptors by transferrin lacking iron. That the presence of unsaturated transferrin does inhibit iron utilization by reticulocytes to some extent, however, has been observed (14); in that study the rate of iron uptake was reduced by half when the iron-bearing transferrin was diluted with 6 times the amount of unsaturated transferrin. This would be so if the unsaturated protein had one-sixth the affinity for the cell as the saturated protein, an estimate similar to that obtained directly in the present study.

The findings described above may be interpreted as follows: A molecule of plasma transferrin containing iron attaches to a receptor on the immature red cell surface. There a mechanism exists for actively removing the iron from the protein. The protein then is displaced from the cell by another molecule of iron-laden transferrin. Thereby iron is delivered to the cell in an orderly fashion. A solution of transferrin that is partially saturated with iron, as in plasma, may, by analogy with conalbumin (43), be a mixture of transferrin containing 2 atoms of iron and of transferrin lacking iron. Accordingly, if half the reticulocyte-bound transferrin turns over with soluble transferrin in 1 minute, approximately 25,000 molecules of protein and up to 50,000 atoms of iron will arrive on each cell every minute, or about 3 $\mu$g of iron per ml reticulocytes per hour. At one-third saturation with iron, competitive

binding of iron-laden transferrin by iron-free transferrin might reduce this by about one-fourth. If, on the other hand, much of the transferrin contains only a single atom of iron, delivery of iron to the cell might be reduced by as much as one-half of this estimate. This range of iron uptake (1 to 2 $\mu$g per ml reticulocytes) is similar to that actually observed in reticulocytes incubated with $Fe^{59}$ *in vitro* (14). Since each milliliter of red cells contains about 1,000 $\mu$g of iron, this mechanism would require at least 14 days to provide the full cellular complement of iron, a period of time that exceeds the period of normal maturation by 3 or 4 times (33). It should be noted, however, that these calculations are based on studies of cells that are in the last, waning stage of maturation; nucleated red cells are known to take up iron more rapidly than reticulocytes (14, 44). This greater rate of iron uptake may reflect a more active mechanism for binding transferrin in nucleated cells or the availability of additional pathways of iron utilization such as that described by Bessis and Breton-Gorius (10). In those experiments involving doubly labeled transferrin, the accumulation of iron was actually somewhat less than that expected on the basis of the calculated turnover of $I^{131}$-transferrin. This may reflect the profitless attachment of some iron-free transferrin or the rejection of a portion of the iron delivered. Conceivably the latter possibility may contribute to the "labile erythropoietic pool" of iron described by Pollycove and Mortimer (8) on the basis of ferrokinetic studies. It should be noted, however, that *in vivo* only a very small fraction of the total body pool of transferrin would be attached to the membranes of immature red cells at any one time. Based on current estimates of the volume of erythroid marrow and on the present studies, the total quantity of cell-fixed transferrin at any given moment would be of the order of 10 or 20 mg. As compared to the total exchangeable pool of transferrin, estimated to be at least 14 g in the average adult male (19), fluctuations in the cellular phase of transferrin would have no discernible effect either on the plasma level or on the rate of degradation of transferrin.

The possibility that transferrin attachment represents the normal route of iron uptake by cells in the marrow is a reasonable one; the essentially "open" character of the marrow circulation, as described by Weiss (45) and by Pease and Zamboni (46, 47) permits extensive exposure of the maturing red cells to circulating plasma proteins. The sinusoidal "walls" of the marrow are normally discontinuous and are unsupported by a basement membrane, thus permitting a continuous and rapid flux of plasma, and even of particulate matter (47), from the vascular channels into the "parenchymal cells," i.e., the developing erythroid and myeloid cells. Indeed, the observations of Zamboni and Pease (47) reveal that the erythroid precursors of the marrow exist in an actively motile, agitated suspension within intersinusoidal spaces that are constantly being flushed by plasma penetrating through the numerous interstices of the sinusoidal walls. Thus, the frail, pervious, seemingly fugitive vascular structure of the marrow may be ideally suited to the simultaneous nourishment and retention of a free suspension of growing cells.

Although the attachment of a protein to a specific cell is often considered a unique feature of immune bodies, it is apparent that such attachments also may be physiologic. It is probable that transport proteins for the various hormones (48) and certain metabolites distribute these substances by some type of specific interaction with the target cells, possibly involving a reversible attachment to these cells, as is the case with transferrin and immature red cells. Factors influencing the attachment of such proteins to specific cell receptors may influence, or even govern, the utilization of the transported metabolites.

## SUMMARY

A study was made of the interaction between human blood cells and human transferrin labeled with $I^{131}$. Transferrin was found to attach specifically to immature red cells. Transferrin exists in a dynamic equilibrium between that in solution, as in plasma, and that attached to immature red cells. The avidity of iron-laden transferrin for these cells exceeds that of iron-free transferrin. These findings suggest the following mechanism for delivering iron to the immature red cell: *1*) Plasma transferrin containing iron selectively attaches to the surface of immature red cells; *2*) this attachment permits the active removal of iron

from transferrin by these cells; *3*) the transferrin, freed of iron, is preferentially displaced by other molecules of plasma transferrin laden with iron; *4*) the displaced transferrin then acquires more iron from storage sites and the plasma-to-cell cycle is repeated.

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