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Competitive advantage of diferric transferrin in delivering iron to reticulocytes

(monoferric transferrin/behavior of transferrin receptors)

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ABSTRACT **Radioiron- and radioiodine-labeled forms of human diferric and monoferric transferrin and apotransferrin, isolated by preparative isoelectric focusing, were used to define transferrin-iron uptake by human reticulocytes. In mixtures of human diferric and monoferric transferrin, the diferric molecule had a constant 7-fold advantage in delivering iron to reticulocytes, as compared with the 2-fold advantage when single solutions of mono- and diferric transferrins were compared. This was shown to be due to competitive interaction in iron delivery, probably at a common membrane-receptor binding site for transferrin. Apotransferrin did not interfere with the iron-donating process and its limited cellular uptake was inhibited in noncompetitive fashion by diferric transferrin.**

Transferrin mediates iron exchange between body tissues. There has been considerable discussion concerning the behavior of this protein in view of the chemical differences between its two iron binding sites (1). Our recent studies, however, have shown that iron loading of the binding sites is essentially a random process and the release of iron on incubation with erythroid cells is an "all-or-none" phenomenon (2–5). In addition, it was shown that iron delivery from the two monoferric transferrins is identical when they are incubated *in vitro* with reticulocytes or bone marrow cells (4, 6) or injected *in vivo* into rabbits (7). In all these experiments, diferric transferrin proved to be a better donor than the single-iron-loaded molecule (2–5, 8), in variation with an earlier report (9). While part of this advantage relates to the simultaneous release of both iron atoms, an additional preference for the diferric form has been suggested but not quantitated (10). In this report, the relationship of iron supplied to human reticulocytes from human mono- and diferric transferrin is examined at physiological levels of plasma iron. In addition, the effect of apotransferrin on the iron delivery process was studied.

MATERIALS AND METHODS

Transferrin Preparations. Purification and radioiodination of human diferric transferrin by using ^{125}I and ^{131}I were done as described (3, 4). Conversion of the transferrin into apotransferrin was accomplished by using desferrioxamine (4).

For the preparation of di[^{55}Fe]ferric transferrin, 90 μCi of ^{55}Fe (10 μCi/μg as $FeSO_4$; 1 Ci = 37 GBq; New England Nuclear) was added to 200 mg of ^{131}I-labeled apotransferrin (100 μCi of ^{131}I dissolved in 5 ml of 0.05 M Tris·HCl, pH 8.0/0.01 M $NaHCO_3$) and this was followed by the addition of sufficient iron as ferrous ammonium sulfate (39 μg of iron/ml in 0.01 M HCl) to exactly saturate the free iron binding sites present in the transferrin solution.* This goal was achieved by spectrophotometric titration techniques in which the increase in color development at λ = 465 nm was observed (11). At the point of saturation, the A_{465}/A_{280} ratio of the transferrin solution was 0.045, which is characteristic of the diferric state of the transferrin molecule (2, 3). Transferrin solutions were prepared at iron saturations varying from 5% to 100% by adding ferrous ammonium sulfate (273.4 μg of iron/ml, pH 2) to apotransferrin under spectrophotometric control as described above.

Monoferric transferrin was prepared from a solution of 200 mg of apotransferrin in 5 ml of 0.2 M NaOAc, pH 5.9/0.01 M $NaHCO_3$ (4). To this solution, 140 μg of iron containing 30 μCi ^{59}Fe from a freshly prepared ^{59}Fe–nitriloacetate complex [radioiron and carrier ($FeCl_3$, pH 2) was treated with sodium nitriloacetate at a molar ratio of 1:8 and the pH was adjusted to 5.9 with 1 M $NaHCO_3$ (5)] was added. The amount of iron added was calculated to result in a 50% iron saturation of the transferrin solution. After incubation (20 min at 25°C), 1 M $NaHCO_3$ was added to increase the pH to pH 7.6. This solution was concentrated to 3 ml on a Centriflo PM30 cone (Amicon), the cone was washed with two 3-ml portions of water and the combined solution was concentrated to 2 ml. After dilution with an equal volume of saturated aqueous sucrose, 1.0-ml aliquots were layered on the cathodal top of a 7.5% polyacrylamide column [1.4 × 18 cm; 2% (wt/vol) Ampholine, pH 5–8] and isoelectric focusing was started (4). The procedures to elute the pink band of the acid-stable human monoferric transferrin from the gel are described elsewhere (3, 4). In some studies, the apotransferrin used had been previously labeled with ^{125}I so that uptake of the protein as well as its iron content could be followed.

Before incubation with reticulocytes, 5-ml aliquots of the solutions containing the individually labeled transferrin species were buffer exchanged against Hanks' buffer (pH 7.4) (GIBCO) on a Sephadex G-50 column (1.5 × 25 cm). Mixtures of radioiron-labeled transferrin were stable and did not exchange their iron on *in vitro* incubation, as reported for similarly labeled rat transferrins (5).

Reticulocyte Assay. Reticulocyte-rich blood was obtained from a patient with sickle cell anemia who was subjected to weekly venesections. Reticulocyte counts were carried out using brilliant cresyl blue for staining, and the number of reticulocytes in 1,000 erythrocytes was determined. The cells were washed with Hanks' balanced salt solution and incubated in the same medium with the labeled transferrin preparations. After incubation, aliquots of media were analyzed for the amounts of apo-, mono-, and diferric transferrin present. These separations were carried out by isoelectric focusing. Similar measurements of the purified transferrin preparations originally prepared and the various mixtures and amounts of mono- and diferric trans-

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* In control studies, the radioiron was mixed with carrier ferrous ammonium sulfate before it was added to the apotransferrin or, alternatively, the sequence of the labeling procedures was switched. These modifications altered neither the total amount of iron nor the amount of radioactive iron delivered to reticulocytes.

ferrin used in the incubation studies were also carried out. The methods of incubation—washing, wet ashing, and simultaneous measurement of radioactivity of ^{59}Fe and ^{55}Fe together with ^{131}I and ^{125}I in media and in erythrocytes—have been described (2–4, 8, 12, 13). Additional competitive studies of iron uptake from diferric and monoferric transferrin mixtures were conducted and evaluated as described by Dixon (14). This technique has shown to be applicable because the rates of iron and protein uptake show Michaelis–Menten type kinetics (15–17).

Plasma iron and transferrin saturation were determined by standard methods (13, 18). Hematocrits were determined by the microhematocrit technique.

Variations in results in multiple determinations are expressed throughout as mean ± 1 SD.

RESULTS

Iron-Donating Efficiencies of Diferric and Monoferric Transferrin. Mixtures of ^{131}I-labeled di[^{55}Fe]ferric transferrin and ^{125}I-labeled mono[^{59}Fe]ferric transferrin at equal iron concentrations (118 μg/dl) were incubated with human reticulocytes (Fig. 1). Whereas the amount of iron delivered from the pure diferric species was twice that of the pure monoferric species, the uptake of iron from the various mixtures showed a diferric/monoferric iron ratio of 3.6 ± 0.3 (Fig. 1*A* and Table 1, Exp. 1).

To determine changes in this uptake ratio that might occur as a function of the iron concentration in the medium, solutions containing di[^{55}Fe]ferric and mono[^{59}Fe]ferric transferrin at equal iron concentrations were serially diluted with Hanks' solution and then incubated with identical populations of human reticulocytes (Table 1, Exp. 2). The respective iron uptakes as well as the total uptake showed little change in amount over a range from 40 to 240 μg iron/dl. Below this, iron uptake decreased rapidly. Relative uptake of diferric over monoferric transferrin, which was relatively constant at a ratio of about 3.5, progressively decreased low iron concentrations until it approached a ratio of 1.

The results of additional studies carried out to further characterize the interaction between di- and monoferric transferrin for cellular iron uptake are shown as a Dixon plot in Fig. 2. The intersection of the two lines at the ordinate constitutes evidence of a competitive type of inhibition ($K_i = 1.2 \pm 0.3 \times 10^{-6}$ M). The same conclusion can be reached from the data summarized in Table 1, where the preferential utilization of iron from the diferric over the monoferric form was relatively constant at a ratio of 3.3–3.6.

Reticulocyte Uptake of Transferrin. To determine membrane binding of mono- and diferric transferrin, radioiodine-labeled compounds were incubated at 4°C for 10 min with reticulocytes. Very small amounts of monoferric transferrin remained

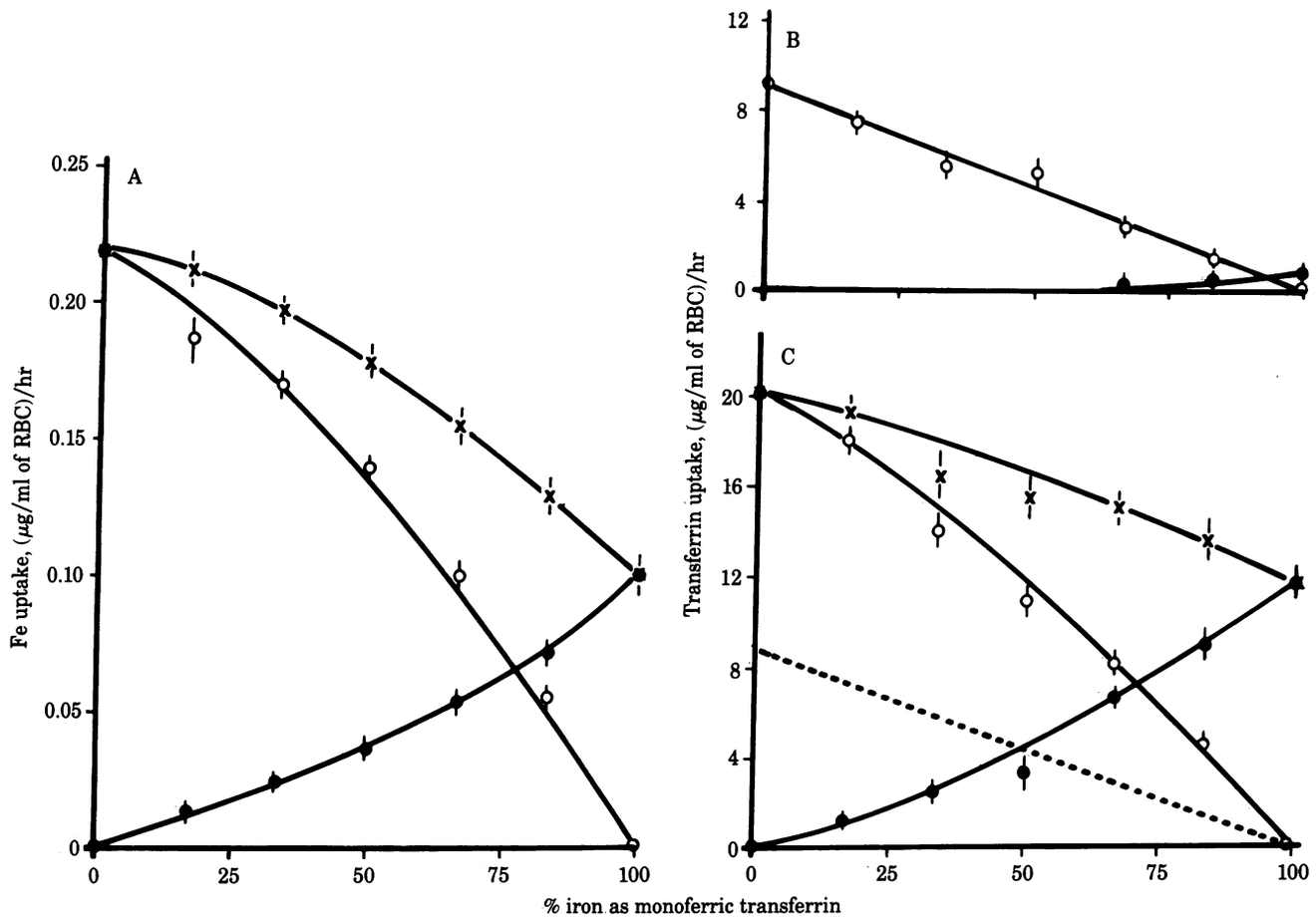

FIG. 1. Iron uptake from mixtures of di[^{55}Fe]ferric and mono[^{59}Fe]ferric transferrin. The two transferrin solutions at equal iron concentrations (118 μg/dl) were combined in different proportions and then incubated for 2 hr at 37°C with washed human reticulocytes (hematocrit, 18 ± 1; reticulocyte count, 18 ± 2%). Over this time period, uptake was linear (3). Results are mean ± SD of triplicate values. (*A*) Total iron uptake (x) and individual iron uptakes from diferric (○) and monoferric (●) transferrin. (*B*) ^{131}I-Labeled diferric (○) and ^{125}I-labeled monoferric (●) transferrin binding after 10 min of incubation at 4°C. (*C*) ^{131}I-Labeled diferric (○) and ^{125}I-labeled monoferric (●) transferrin after 60 min of incubation binding and total iron uptake (x) at 37°C. ---, Membrane binding of diferric transferrin as shown in *B*. RBC, erythrocytes.

Table 1. Evaluation of the advantage of diferric transferrin in delivering iron to reticulocytes

Exp.	Concentration: Iron, μg/dl	Concentration: TIBC, μg/dl	Concentration: Transferrin: Total, mg/ml	Concentration: Transferrin: Monoferric, %	Concentration: Transferrin: Diferric, %	Diferric/monoferric in medium	Relative uptake advantage*
1	118.0	118.0	0.84	16.6	83.3	5.01	3.00
	118.0	147.0	1.05	33.3	66.6	2.01	3.60
	118.0	177.0	1.26	50.0	50.0	1.00	3.78
	118.0	206.0	1.47	66.6	33.3	0.50	3.78
	118.0	236.0	1.68	83.3	16.6	0.20	3.81
							3.6 ± 0.3
2	2.3	3.4	0.016	50.0	50.0	1.00	1.00†
	4.7	7.0	0.036	50.0	50.0	1.00	1.10†
	9.3	14.0	0.066	50.0	50.0	1.00	1.43†
	18.6	28.0	0.133	50.0	50.0	1.00	2.60†
	37.5	56.0	0.267	50.0	50.0	1.00	2.87
	75.0	112.0	0.530	50.0	50.0	1.00	3.85
	152.0	228.0	1.080	50.0	50.0	1.00	3.67
	231.0	346.0	1.650	50.0	50.0	1.00	3.56
							3.5 ± 0.4
3	10.0	200.0	1.43	95.0	5.0	0.05	3.27
	30.0	200.0	1.43	85.0	15.0	0.18	3.17
	50.0	200.0	1.43	75.0	25.0	0.33	3.40
	74.0	200.0	1.43	63.0	37.0	0.59	3.40
	114.0	200.0	1.43	43.0	57.0	1.39	3.39
	166.0	200.0	1.43	17.0	83.0	4.90	3.60
	200.0	200.0	1.43	0	100.0	—	—
							3.4 ± 0.1
4	60.4	98.0	0.70	62.6	37.4	0.60	3.50
	65.4	103.0	0.74	57.8	42.2	0.73	3.76
	69.4	107.0	0.77	54.5	45.5	0.84	3.41
	72.4	110.0	0.79	52.2	47.8	0.92	3.26
	80.4	118.0	0.84	47.0	53.0	1.13	3.09
	102.4	140.0	1.00	36.9	63.1	1.71	3.74
	145.4	182.0	1.31	26.0	74.0	2.85	3.43
	270.4	308.0	2.20	14.0	86.0	6.15	3.12
	476.4	539.0	3.85	7.9	92.1	11.60	2.62
							3.3 ± 0.3

TIBC, total iron binding capacity.
* Ratio of diferric/monoferric transferrin iron uptake by reticulocytes divided by ratio of diferric/monoferric transferrin iron in medium.
† Insufficient transferrin iron complexes to saturate receptors.

on the membrane after washing, whereas there was significant binding of diferric transferrin (Fig. 1*B*). Temperature-dependent uptake studies over 1 to 2 hr were then carried out at 37°C. Cell-related uptake from a solution containing only diferric transferrin was approximately 1.7 times that from a monoferric transferrin solution having the same protein concentration (Fig. 1*C*). If allowance was made for the difference in membrane transferrin (uptake at 4°C), intracellular uptake of the two complexes appeared to be similar. However, when mixtures were used, the uptake of diferric transferrin was 1.8 ± 0.2 times that of monoferric transferrin.

To further characterize the relationship between transferrin complexes and receptors, experiments in which the concentration of apotransferrin was varied were carried out. The Lineweaver–Burk plot of the data provided evidence that diferric transferrin acts as a strong noncompetitive inhibitor in the temperature-dependent uptake of apotransferrin into reticulocytes (Fig. 3*A*). The K_m calculated for apotransferrin was 0.5×10^{-6} M. The effect of diferric transferrin on temperature-dependent uptake of ^{125}I-labeled apotransferrin by reticulocytes was further examined in a Dixon plot (Fig. 3*B*). The straight lines obtained for the two different apotransferrin concentrations at increasing concentrations of diferric transferrin met on the abscissa, once again indicating noncompetitive inhibition (14). The K_i was calculated to be 0.9×10^{-6} M. The simultaneous measurement of radioiron uptake from the labeled diferric transferrin preparation allowed approximation of the K_m as a function of iron concentration. In two experiments, the K_m values found for diferric transferrin were close to 0.5×10^{-6} M (Fig. 4).

DISCUSSION

We have examined the uptake of iron from mixtures of mono- and diferric transferrin in detail. Studies were conducted in which the diferric/monoferric transferrin ratio was varied but the total amount of iron remained constant, in which the diferric/monoferric transferrin ratio was held constant but the amount of iron was varied, and in which both the ratio and the amount were varied. At total iron levels in excess of about 40 μg/100 ml, all mixtures showed a constant iron uptake advantage ratio of about 3.5 for the diferric form. Because only half

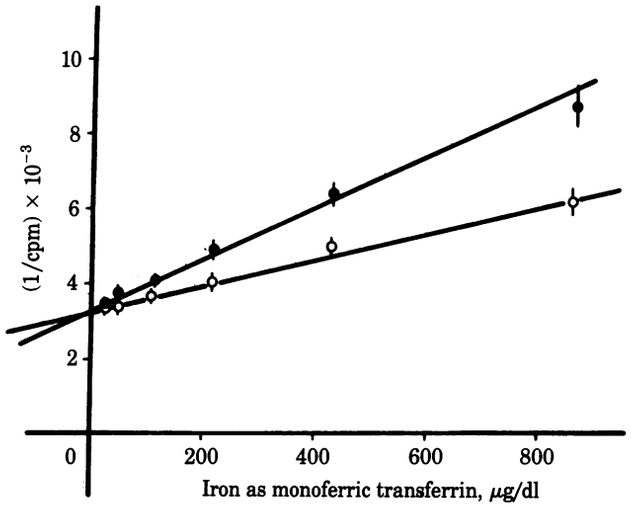

FIG. 2. Dixon plot for the evaluation of uptake of diferric and monoferric transferrin iron by reticulocytes. Diferric transferrin was added to mono[^{59}Fe]ferric transferrin at 34 μg/dl (●) or 68 μg/dl (○). The mixtures were incubated with reticulocytes (20%; hematocrit, 25%) for 1 hr at 37°C. Radioiron uptake was quantitated. The straight lines obtained for the two different diferric transferrin concentrations intersect at a point left of the ordinate, indicating competitive inhibition (14).

as many molecules of diferric transferrin are required to supply the same amount of iron as a given amount of monoferric transferrin, the molecular advantage of diferric transferrin in donating iron to reticulocytes is 7 times that of monoferric transferrin. A Dixon plot, along with a variety of other studies, indicated

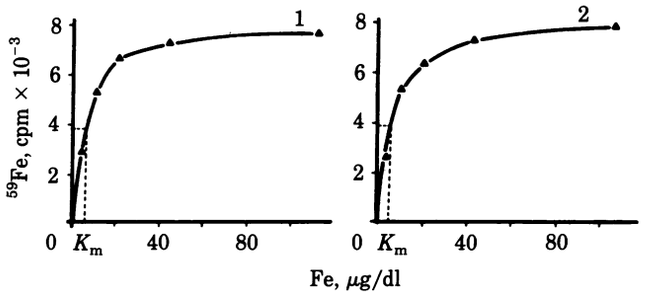

FIG. 4. Uptake of ^{59}Fe by reticulocytes at two concentrations of apotransferrin (Fig. 3*B*) as a function of diferric transferrin iron concentration. Iron uptake at the two apotransferrin concentrations was identical and showed Michaelis–Menten type kinetics. K_m values are indicated.

a competitive relationship between the two as long as the receptor sites were saturated by transferrin iron complexes. Below saturation, the ratio of receptor interaction with di- and monoferric transferrin progressively decreased until the chances that diferric and monoferric transferrin molecules would donate iron were equal. Such a loss in competitive advantage of the diferric molecule would be expected when the total number of molecules became insufficient to saturate membrane receptors. The amount of apotransferrin present did not affect the uptake of transferrin iron complexes or iron delivery.

The reaction between transferrin and cell membrane receptors was evaluated by incubating reticulocytes at 4°C. There was firm binding of diferric transferrin iron whereas mono- and apotransferrin were easily washed from the cell surface. Substantial data have been presented by others to indicate that the

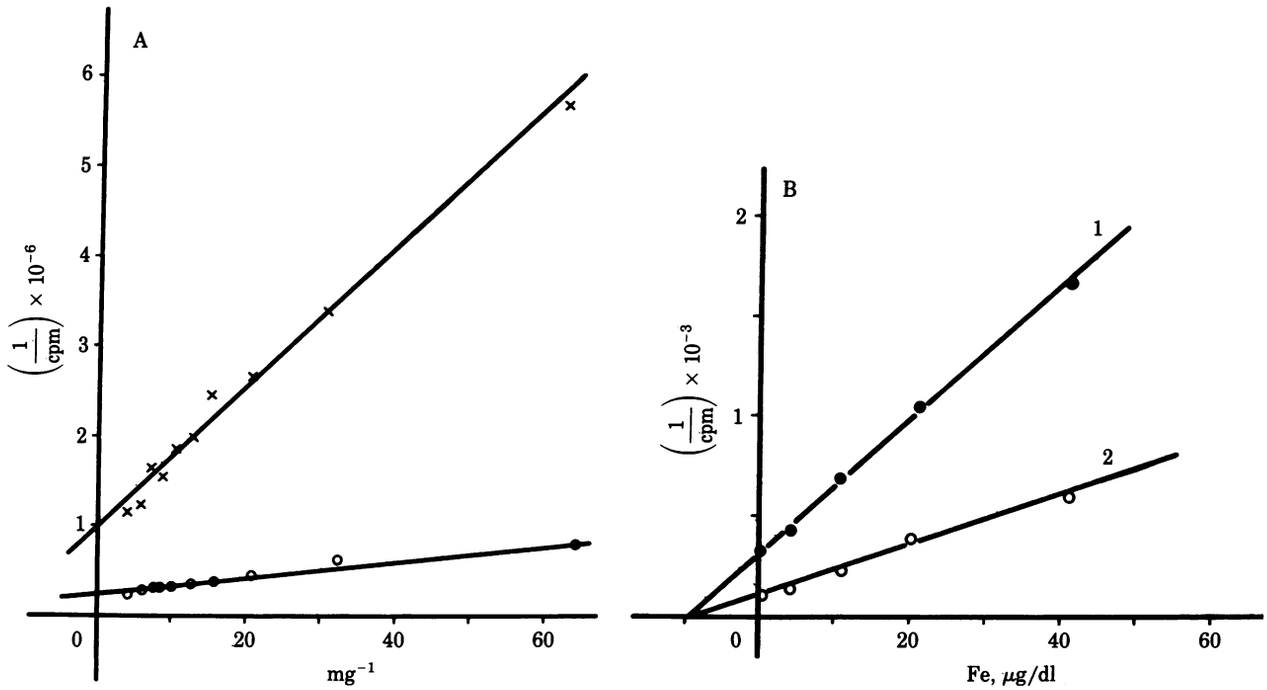

FIG. 3. Evaluation of the mode of interaction between apotransferrin and diferric transferrin for uptake by reticulocytes. (*A*) Lineweaver–Burk plot. The concentration of ^{125}I-labeled apotransferrin was varied while the concentration of diferric transferrin was kept constant (49 μg of iron/dl). The lines representing the reciprocal of ^{125}I uptake in the presence (x) and absence (○) of diferric transferrin cross the ordinate at different points, indicating noncompetitive inhibition. (*B*) Dixon plot. Various amounts of di[^{59}Fe]ferric transferrin were added to solutions of ^{125}I-labeled apotransferrin while keeping the apotransferrin concentration constant. The mixtures were incubated with reticulocytes as described in Fig. 1. For two apotransferrin concentrations [0.1 mg/ml (curve 1) and 0.6 mg/ml (curve 2)], straight lines were obtained when the reciprocal of the ^{125}I uptake by reticulocytes was plotted against the concentration of diferric transferrin. The lines meet on the abscissa, indicating noncompetitive inhibition.

process of iron delivery to the reticulocyte also involves internalization of the membrane receptor–transferrin iron complex (17, 19). Incubation at 37°C for 1 hr provided a means of examining all cell-related transferrin. Significant amounts of apo-, mono-, and diferric transferrin were taken up when pure solutions of each were used separately. While the amounts of apo- and monoferric transferrin were less than that of diferric transferrin, it is reasonable to assume that they represented only internalized transferrin whereas cell-released diferric transferrin included membrane-adherent transferrin as well. Allowing for this, the amount of cell-related monoferric transferrin would seem proportional to the delivery of iron from this moiety, suggesting that the process of iron delivery may be identical once access to the cell has been achieved. Apotransferrin, while it can be taken up in the absence of iron-bearing transferrin, is noncompetitive. It thus appears that the amount of apotransferrin is unimportant and that diferric transferrin is the dominant form of transport iron.

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1. Aisen, P. (1980) in *Iron in Biochemistry and Medicine II*, eds. Jacobs, A. & Worwood, M. (Academic, London), pp. 87–129.
2. Huebers, H., Huebers, E., Csiba, E. & Finch, C. A. (1978) *J. Clin. Invest.* **62**, 944–951.
3. Huebers, H., Csiba, E., Josephson, B., Huebers, E. & Finch, C. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 621–625.
4. Huebers, H., Josephson, B., Huebers, E., Csiba, E. & Finch, C. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2572–2576.
5. Huebers, H., Bauer, W., Huebers, E., Csiba, E. & Finch, C. A. (1981) *Blood* **57**, 218–228.
6. Van der Heul, C., Kroos, M. J., Van Hoort, W. L. & Van Eijk, H. G. (1981) *Clin. Sci.* **60**, 185–190.
7. Huebers, H. A., Celada, C. & Finch, C. A. (1982) in *Biochemistry and Physiology of Iron*, Proceedings of the Fifth International Conference on Proteins of Iron Storage and Transport, eds. Saltman, P. & Hegenauer, J. (American Elsevier, New York), pp. 127–131.
8. Christensen, A. C., Huebers, H. & Finch, C. A. (1978) *Am. J. Physiol.* **235**, 18–22.
9. Harris, D. C. & Aisen, P. (1975) *Biochemistry* **14**, 262–268.
10. Zapolski, E. J. & Princiotto, J. V. (1977) *Biochem. J.* **166**, 175–179.
11. Graham, G. & Bates, G. W. (1976) *J. Lab. Clin. Med.* **88**, 477–486.
12. Eakins, J. E. & Brown, D. A. (1966) *Int. J. Appl. Radiat. Isot.* **17**, 391–397.
13. The International Committee for Standardization in Hematology (1978) *Br. J. Haematol.* **38**, 291–294.
14. Dixon, M. (1953) *Biochem. J.* **55**, 170–171.
15. Kornfeld, S. (1969) *Biochim. Biophys. Acta* **194**, 25–33.
16. Neame, K. D. & Richards, T. G. (1972) in *Elementary Kinetics of Membrane Carrier Transport* (Blackwell, Oxford), pp. 41–55.
17. Jacopetta, B. J., Morgan, E. H. & Yeoh, G. C. T. (1982) *Biochem. Biophys. Acta* **687**, 204–210.
18. Cook, J. D. (1970) *J. Lab. Clin. Med.* **76**, 497–506.
19. Hemmaplardh, D. & Morgan, E. H. (1977) *Br. J. Haematol.* **36**, 85–96.