PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **179**, 222–226 (1985)

Molecular Advantage of Diferric Transferrin in Delivering Iron to Reticulocytes: A Comparative Study (42090)

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Abstract. The delivery of transferrin iron from four animal species and man to homologous reticulocytes was measured at different transferrin saturations. Total iron uptake in the *in vitro* reticulocyte incubation model employed followed a hyperbolic curve, increasing as the transferrin saturation increased but at a progressively slower rate. In all species, there was a much greater iron delivery from diferric as compared to monoferric transferrin, the molecular advantage varying from 8:1 to 14:1. The majority of iron was delivered from diferric transferrin when transferrin saturations exceeded 13–19% depending on the species. Thus a general similarity exists in the transferrin–iron receptor interactions in these mammalian species. Formuli have been provided whereby the iron utilization curve may be calculated when uptake has been determined at any one transferrin saturation.

Iron supply of body tissues is mediated by the plasma protein, transferrin (1). This protein has two binding sites for iron, so that it may exist in the apo-, mono-, and diferric form. The pattern of tissue distribution from the two monoferric species is the same (2). The only difference observed is the preference of membrane transferrin receptors for the diferric form (3, 4). In this study, employing an *in vitro* homologous reticulocyte model, the receptor preference is measured in four species of animals and in man. The effect of the varying degrees of preference in these different species for di- over monoferric transferrin on the total iron uptake by reticulocytes is determined, and formuli are presented by which this effect may be calculated.

Materials and Methods. *Materials.* The radioiron isotopes were purchased from New England Nuclear (^{59}Fe and ^{55}Fe, as ferrous sulfate, sp act 13–22 μCi/μg of iron, dissolved at 0.5 *M* HCl (1 μCi = 3.7×10^{10} Bq). Ferrous ammonium sulfate and other chemical reagents used were of analytical grade. Hanks' buffered salt solution was obtained from Gibco (Grand Island, N.Y.).

Preparation and labeling of transferrin. ^{59}Fe-tagged diferric rat, rabbit, dog, and baboon transferrin were isolated from plasma by methods described elsewhere (2, 3, 5), after each plasma had been brought to the point of iron saturation under spectrophotometric control (6, 7). The resulting ^{59}Fe-tagged diferric preparations had an absorption ratio of A465/A280 of 0.046 and were found to be free of hemopexin. Apotransferrin was prepared using desferrioxamine (7). ^{59}Fe-tagged diferric transferrin of high specific activity was prepared by adding 30 μCi of carrier-free ^{59}Fe sulfate to 1 mg of purified apotransferrin (dissolved in 0.5 ml of 0.1 *M* Tris–HCl, pH 8.3, containing 0.01 *M* sodium bicarbonate). This was followed by addition of ferrous ammonium sulfate (42.2 μg iron/ml, pH 2.0) in an amount sufficient to saturate the transferrin iron binding capacity (5, 6). This preparation was extensively dialyzed against Hanks' buffer (Amicon 8MC, PM 10 membrane) and analyzed for its degree of iron saturation. An absorption ratio of A465/A280 = 0.046 confirmed the diferric state of the various transferrin preparations (5).

^{55}Fe-tagged monoferric transferrin was prepared by adding 30 μCi of carrier-free ^{55}Fe ferrous sulfate (Amersham & Buchler, Arlington Heights, Ill.) to 20 mg of apotransferrin, employing the same conditions as described above for diferric transferrin. Because of random loading of the two binding sites, this monoferric preparation usually contained more than 98% of the activity bound to monoferric transferrin and less than 2% to diferric transferrin. This distribution could be verified in the rat and the rabbit by isoelectric focusing (2).

Iron-free plasma from the individual spe-

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cies was prepared using desferrioxamine followed by gel chromatographic separation on a Sephadex G-50 column equilibrated with Hanks' buffer (7).

Measurement of total iron uptake and determination of molecular advantage. Total iron uptake may be determined by uniform labeling of plasma at increasing transferrin saturations. Under spectrophotometric control, ^{59}Fe-tagged ferrous ammonium sulfate was added in increasing amounts to iron-free plasma to produce a series of solutions of increasing transferrin saturation.

The molecular advantage of diferric transferrin was determined by employing Eq. (I) to the results of the total iron uptake (Method a). The molecular advantage could also be determined by individual trace labeling of plasmas at different saturations with ^{59}Fe monoferric and ^{55}Fe diferric transferrin (Method b). The uptake of these two isotopes during incubation at 37°C was then determined (9, 10). Parallel mixtures were prepared in this instance with the same degree of saturation of transferrin but labeled with a ^{59}Fe radioiron solution as described under Method a.

Reticulocyte assay system. In vitro uptake of iron by reticulocytes was studied in the homologous systems of the rat, rabbit, dog, baboon, and man. A patient with sickle cell anemia provided human reticulocytes. The only difference between sickle cell reticulocytes and those of other patients with hemolytic anemia was the higher absolute uptake of iron in the former, presumably due to their greater immaturity. Reticulocytosis was induced by phlebotomy in the rabbit, and by a combination of phlebotomy and a low iron diet in the rat (2, 3). Phenylhydrazine treatment was used in the dog and baboon to produce a hemolytic anemia. At the time reticulocyte counts reached 25–30%, heparinized blood samples (30–50 ml) were collected and centrifuged at 1000*g* for 1 hr. The plasma was removed and the sediment washed twice with 10 vol of ice-cold Hanks' buffer. This procedure was repeated for a third time after 15-min incubation period at 37°C to deplete the cells of any prebound transferrin (1). The preparation was then kept at 4°C and divided into 10 aliquots and centrifuged again. The supernatant was removed by aspiration and the tagged plasma samples with transferrin saturation ranging 5–100% were layered on top of the sediment. This step was carried out at 0°C. After mixing, the suspension was incubated at 0°C for 10 min and 0.5-ml aliquots were taken. The total volume of an individual incubation study was 3.1 ml with a hematocrit of 20 ± 2% and reticulocyte counts of 28 ± 4%. Incubation at 37°C was started and samples were taken after 15, 30, and 60 min. Methods of washing the cells and determination of radioiron uptake have been reported previously (3, 6, 7). Because of the large size of the transferrin iron pool, there was virtually no change in the concentration of plasma iron during the incubation time. Thus, the system could be regarded as in a stable state.

Mathematical analysis. Calculations were predicted on the basis of a random distribution of iron among the transferrin binding sites, identical iron donating properties of the two monoferric species and absence of effect of apotransferrin on iron delivery. The validity of these assumptions has been established (3, 9). This being so, the relative amounts of di- and monoferric transferrin could be calculated from the transferrin saturation (s)*. The total iron uptake (U) as determined at any given saturation depended on the molecular advantage (a) and the relative amounts of di- and monoferric transferrin. Total uptake as percentage of maximum was calculated from

$$U_{\text{total (at } s)} = \frac{a \cdot s^2}{[s(100 - s)]/50 + (a \cdot s^2)/100}. \quad \text{(I)}$$

In this formula, the molecular advantage (a) is expressed in terms of iron donating capacity. Since twice as much iron is donated from diferric transferrin, the molecular advantage will represent the transferrin preference × 2. The formula can be rearranged to solve for the molecular advantage as

$$a = \frac{200U_{\text{total}} - 2U_{\text{total}} \cdot s}{100s - U_{\text{total}} \cdot s}. \quad \text{(II)}$$

* The respective proportions of diferric and monoferric transferrin iron at any given saturation (s) are expressed by the formula $S^2/100$ and $S(100 - S)/50$, respectively (7).

The iron uptake (U) from diferric transferrin at any saturation (s) expressed as a percentage of maximum uptake is then determined by

$$\text{di}U\ (\text{at } s) = \left(\frac{U_{\text{total}} \text{ at } s}{10}\right)^2. \qquad \text{(III)}$$

The U_{mono} at s is determined subtracting the U di at s from the U_{total} at s. The U_{mono} at any saturation may then be calculated as a percentage of maximum uptake according to

$$\text{mono}U\ (\text{at } s) = \frac{200as\ (100 - s)}{[s(a - 2) + 200]^2}. \qquad \text{(IV)}$$

Equal iron contributions from the diferric and monoferric pools will occur when total uptake is 50%. At this point, the saturation will be:

$$s = \frac{200}{2 + a}. \qquad \text{(V)}$$

The molecular advantage may also be determined as previously described from relating the individual uptake of di- and monoferric iron labeled with different isotopes by reticulocyte as compared to their ratio in the media (10):

$$a = \frac{\text{diferric radioiron/monoferric radioiron (reticulocytes)}}{\text{diferric radioiron/monoferric radioiron (media)}}. \qquad \text{(VI)}$$

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

Results. Total iron uptake by reticulocytes of four animal species and man as a function of transferrin saturation is shown in Fig. 1. There was a steep increase in uptake at low transferrin saturation, while at high transferrin saturation incremental uptake became progressively less. Uptake at 100% transferrin saturation varied with different animal species. In rats the uptake was 3.9 μg, in the rabbit 3.4 μg iron, in the dog 2.4 μg iron, in the baboon 0.65 μg, and in man 0.3 μg of iron/ml reticulocytes/hour. It is recognized, however, that this uptake is influenced by the relative maturity of the reticulocyte population present and that this may vary depending upon the degree of marrow stimulation and the nature of marrow release in the different species.

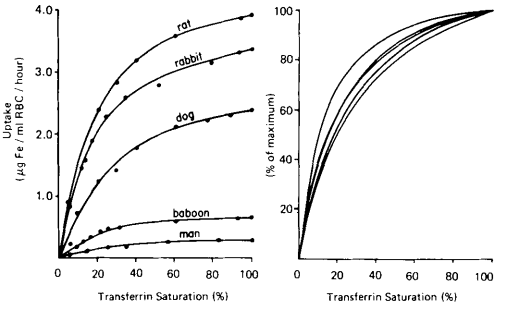

FIG. 1. Iron uptake by reticulocytes of five different species is shown on the left. On the right, where uptake is expressed as percentage of maximum, the general similarity in the relative uptake curves as a function of transferrin saturation is evident. In this plot the baboon shows the greatest advantage, rat and rabbit intermediate, and man and dog the least.

Analysis of the individual uptake curves was carried out in order to determine the molecular advantage of the diferric over monoferric transferrin molecule in delivering iron to reticulocytes. By using the Eq. (II) (see Materials and Methods) the advantage of di/monoferric transferrin was determined to be $a = 11.6 \pm 0.7$ ($n = 6$) for the rat, 11.0 ± 2.0 ($n = 6$) for the rabbit, 8.3 ± 0.6 ($n = 6$) for the dog, 13.9 ± 1.9 ($n = 6$) for the baboon, and 8.4 for man.

A second independent method by which molecular advantage of diferric transferrin may be determined is by the use of two different isotopes, one labeling diferric and the other monoferric transferrin as shown in Eq. (VI) (Materials and Methods). The results of this approach are summarized in Table I. These values were slightly lower than those determined by the first method, i.e., 8.4 ± 0.4, 7.4 ± 0.4, 7.4 ± 0.3, and 12.0 ± 1.0 for the rat, rabbit, dog, and baboon, respectively. The advantage in man by this method had been previously reported to the 7.0 (9).

From the data given in Fig. 1 (Method a) and Table I (Method b), the individual contribution of iron uptake from the diferric and monoferric pools could be calculated for any given transferrin saturation (8). An example of this evaluation for the rat system is given

TABLE I. EVALUATION OF THE ADVANTAGE OF DIFERRIC TRANSFERRIN IN DELIVERING IRON TO RETICULOCYTES OF FOUR ANIMAL SPECIES

Tf. sat	$^{55}Fe_2$ Tf	^{59}Fe, Tf	Ratio 55/59	Reticulocyte uptake 55/59				Molecular Advantage			
				Rat	Rabbit	Dog	Baboon	Rat	Rabbit	Dog	Baboon
5	0.25	9.5	0.026	0.234	0.189	0.182	0.263	8.9	7.2	6.9	10.0
10	1.00	18	0.056	0.455	0.422	0.383	0.611	8.2	7.6	6.9	11.0
15	2.25	25.5	0.088	0.696	0.706	0.644	1.085	7.9	8.0	7.3	12.3
20	4.00	32	0.125	1.05	0.950	0.963	1.600	8.4	7.6	7.7	12.8
25	6.25	37.5	0.167	1.33	1.166	1.233	2.099	8.0	7.0	7.4	12.6
30	9.00	42	0.214	1.75	1.457	1.607	2.785	8.2	6.8	7.5	13.0
40	16	48	0.333	2.96	2.466	2.533	4.133	8.9	7.4	7.6	12.4
50	25	50	0.500	4.2	3.900	3.750	6.000	8.4	7.8	7.5	12.0
75	56.25	37.5	1.500	13.0	11.100	11.700	17.85	8.7	7.4	7.6	11.9
100	100	—	∞	∞	∞	∞	∞				
							Mean	8.4	7.4	7.4	12.0
							SD	±0.4	±0.4	±0.3	±1.0

in Fig. 2. Theoretical values for uptake from mono- and diferric transferrin at different transferrin saturations are shown in Fig. 2a, while experimental data by method b is shown in Fig. 2b. At low transferrin saturation, monoferric transferrin provides most of the iron. Equal amounts from mono- and diferric transferrin [Eq. (V)] in rat, rabbit, dog, baboon, and human were provided at saturation points of 14.7, 15.4, 19.4, 12.6, and 19.2% saturation, respectively.

Discussion. Transferrin iron exchange has been shown to involve a random loading of iron on open iron binding sites, one iron atom at a time (9). This indicates that the proportion of apo-, mono-, and diferric molecules can be calculated if the transferrin saturation is known. On the other hand, the uptake of the transferrin iron complex by receptors on cell membranes is not random. In all animal species studied, there is a marked preference of receptors for diferric over monoferric transferrin, varying from 3.5 to 1 in man to as much as 7 to 1 in the baboon. Since all of the iron is delivered from each transferrin molecule which binds to a membrane receptor, regardless of whether there are one or two molecules of iron bound (3), the ratio of iron delivery from di- and monoferric transferrin in these same species varies from 7 to 1 to 14 to 1. Since apotransferrin has been shown not to compete for receptors, it plays no part in iron delivery.

Two different methods were employed to evaluate the molecular advantage. Results

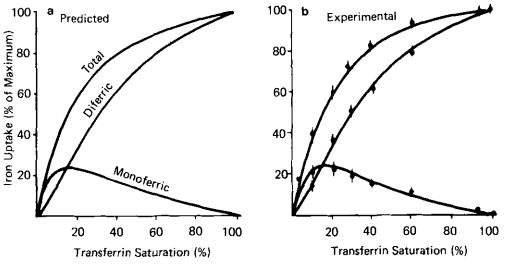

FIG. 2. Effect of transferrin saturation on iron uptake by rat reticulocytes is shown. On the left the uptake from mono- and diferric and total uptake are calculated, based on a receptor preference for diferric transferrin 11.6 to 1. On the right, the uptake of each of the three parameters was determined experimentally as described under Materials and Methods.

were similar but not identical, and Method a appeared to be the most accurate. The slightly lower values in Method b (Table I) can be assumed to be due to small amounts of diferric transferrin contaminating the monoferric preparation (see Materials and Methods). The receptor preference for diferric transferrin results in a hyperbolic curve of iron uptake by the cell with increasing transferrin saturation. In general, the normal range of transferrin saturation is on a steep part of the curve, suggesting that physiologic or pathologic changes in plasma iron will have a substantial effect on iron supply to tissues.

The amount of iron taken up from each form of transferrin depends on the number of each molecular species present and the molecular advantage. Because of the preference for diferric transferrin, its overall contribution dominates the iron supply. In the various species studied, the point at which diferric transferrin exceeded monoferric transferrin varied from 12.6 to 19.2% saturation. At low transferrin saturations, where there are not enough iron loaded transferrin molecules to saturate receptors, there will be no difference between monoferric and diferric transferrin in their interaction with receptors (5). This occurred in the *in vitro* reticulocyte incubation system employed in these studies when the saturation was less than about 10%. Above that level, the molecular advantage between mono- and diferric transferrin remained constant to 100% saturation. The formuli provided make it possible to construct an iron utilization curve, given any single measurement of radioiron uptake and the transferrin saturation at which this uptake occurred as long as there is receptor saturation. This makes it possible to characterize transferrin-receptor function independent of the mono/diferric effect.

Two quite different biologic effects can result from the described relationship between transferrin saturation and plasma iron turnover. The plasma iron concentration will be stabilized, since, as it rises, removal will be increased, and when it falls, removal will be decreased. In addition a means is provided whereby the supply of iron to tissues may be regulated other than by having the plasma iron level fall so low that the membrane receptors for transferrin can no longer be saturated.

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Received November 16, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted February 28, 1985.