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Preferential Utilization *in vitro* of Iron Bound to Diferric Transferrin by Rabbit Reticulocytes

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^{59}Fe uptake by rabbit reticulocytes from human transferrin-bound iron was studied by using transferrin solutions (35, 50, 65, 80 and 100% saturated with iron) whose only common characteristic was their content of diferric transferrin. During the early incubation period, ^{59}Fe uptake from each preparation by reticulocytes was identical despite wide variations in amounts of total transferrin, total iron, monoferric transferrin and apotransferrin in solution. During the later phase of incubation, rate of uptake declined and was proportional to each solution's monoferric transferrin content. Uptake was also studied in a comparative experiment which used two identical, partially saturated transferrin preparations, one uniformly ^{59}Fe-labelled and the other tracer-labelled with [^{59}Fe]diferric transferrin. In both experiments, iron uptake by reticulocytes corresponded to utilization of a ferric ion from diferric transferrin before utilization of iron from monoferric transferrin.

A basic tenet of the hypothesis of Fletcher & Huehns (1968) on the function of transferrin is that immature erythrocytes will preferentially remove iron from diferric transferrin molecules. Several investigators have demonstrated that reticulocytes exhibit an increased affinity for saturated (diferric) transferrin in studies using ^{125}I-labelled transferrin preparations (Jandl & Katz, 1963; Baker & Morgan, 1969; Kornfeld, 1969), and reticulocytes bind ^{125}I-labelled diferric transferrin molecules faster and to a greater extent than monoferric transferrin molecules (Harris & Aisen, 1975). However, Phillips (1976) demonstrated that ^{125}I-labelled transferrin can bind to polypropylene tubes, mimicking cell receptors, and he has questioned the validity of investigations using such label.

Results obtained from other experiments suggest that reticulocytes preferentially react with diferric transferrin molecules (Morgan & Laurell, 1963; Fletcher & Huehns, 1967; Fletcher, 1969; Kornfeld, 1969; Lane, 1973*a*; Zapolski *et al.*, 1974). This conclusion was derived from experimental data relating variation in reticulocyte ^{59}Fe uptake with the degree of iron saturation of transferrin, and from reticulocyte iron uptake, derived from transferrin preparations that were partially iron-depleted after incubation with reticulocytes. These studies tacitly presume a preferential reticulocyte utilization of diferric transferrin-bound iron. Additional evidence has been obtained by studies *in vivo*, which demonstrated faster plasma ^{59}Fe clearance from highly saturated transferrin than from less-saturated transferrin, to support these observations *in vitro* (Hahn, 1973; Brown *et al.*, 1975; Hahn *et al.*, 1975). The present investigation was undertaken to demonstrate *in vitro* the preference by rabbit reticulocytes for human diferric transferrin-bound iron.

Experimental

The collection of rabbit reticulocytes, preparation of human ^{59}Fe-tracer-labelled transferrin and procedures for iron uptake by reticulocytes used in the present study were described previously (Zapolski & Princiotto, 1976). Reticulocyte-rich cell suspensions (2 vol. of 50% packed cells) were incubated with 1 vol. of ^{59}Fe-labelled transferrin solution, samples were removed and the percentage of ^{59}Fe taken up by cells was determined in washed cells. From these data, metal uptake was calculated from the iron content of the solution. For example, if cells took up 50% of the radioactivity from a 2μM solution of saturated transferrin, the calculated metal uptake was 2 nmol of Fe/ml of packed cells.

Results

Uptake by reticulocytes of iron from saturated transferrin

Cell iron-uptake values, measured from saturated transferrin solutions ranging in concentration from 0.5 to 10μM-transferrin, are shown in Fig. 1. When diferric transferrin concentration exceeded 2.5μM the rate of metal uptake by the cells was constant,

but below this concentration the uptake rate was proportional to the concentration. There was no evidence that any ^{59}Fe-labelled transferrin was adsorbed on glassware used in our experiments.

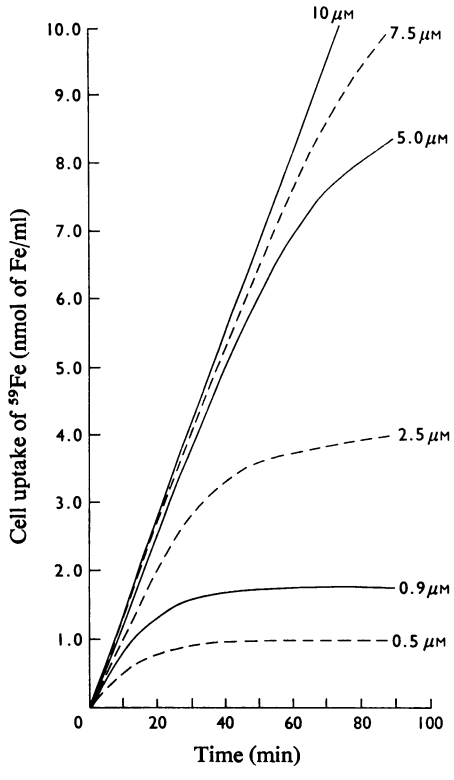

Fig. 1. *Reticulocyte iron uptake as a function of diferric transferrin concentration*
Data points have been omitted for clarity. Diferric transferrin concentration of each solution is presented next to each curve. Uptake is expressed as nmol of Fe/ml of packed cells. All data are from the same reticulocyte suspension.

Uptake by reticulocytes of iron from partially saturated transferrin solutions

The proportions of iron and transferrin used to prepare the partially saturated transferrin solutions used in this study, along with theoretical concentrations of diferric, monoferric and apotransferrin species, are listed in Table 1. Reactant concentrations were chosen to yield solutions (35–100% iron-saturated) of which each contained 0.5 μmol of diferric transferrin/litre. Reticulocyte iron-uptake

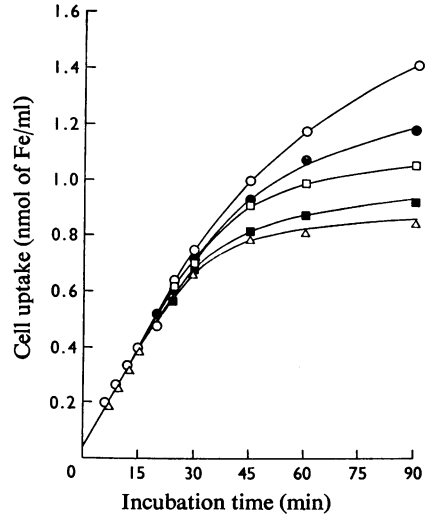

Fig. 2. *Reticulocyte iron uptake from fully and partially saturated transferrin solutions described in Table* 1
All data are from the same reticulocyte suspension. Uptake is expressed as nmol of Fe/ml of packed cells. Degrees of saturation of transferrin were: ○, 35%; ●, 50%; □, 65%; ■, 80%; △, 100%. Each solution was prepared to yield 0.5 μM-diferric transferrin concentration. Concentration of monoferric transferrin and apotransferrin are listed in Table 1.

Table 1. *Composition of transferrin solutions*
Amounts of products were computed by using the equations of Wenn & Williams (1968). For details see the Results section and Fig. 2.

% saturation with iron	Reactants (μM)		Products (μM)		
	Iron	Transferrin	Diferric transferrin	Monoferric transferrin	Apotransferrin
35	2.86	4.07	0.50	1.86	1.81
50	2.00	2.00	0.50	1.00	0.50
65	1.54	1.18	0.50	0.54	0.14
80	1.25	0.78	0.50	0.25	0.03
100	1.00	0.50	0.50	0	0

data are shown in Fig. 2. Despite wide variations in total iron, total transferrin, monoferric transferrin and apotransferrin content of these solutions, reticulocyte iron uptake (calculated as nmol of Fe/ml of cells) during the first 15–20min of incubation was identical from each solution until almost 0.5nmol of Fe was incorporated. After incorporation of this amount of iron, which corresponds to the utilization of half the diferric transferrin iron content, uptake by the reticulocytes then slowed and varied with each solution's content of monoferric transferrin or apotransferrin.

Selective uptake by reticulocytes of diferric-transferrin-bound iron

For this study, two 55%-saturated transferrin solutions (5μM-transferrin) were prepared: one was ^{59}Fe-labelled and the other was not. To 10ml of the unlabelled solution, we added 1ml of 1μM-^{59}Fe-labelled diferric transferrin (solution I); likewise to 10ml of ^{59}Fe-labelled transferrin, we added 1ml of 1μM-unlabelled diferric transferrin (solution II). These mixtures were prepared just before incubation with cells. Uptake data from these preparations' reticulocytes are shown in Fig. 3. Percentage ^{59}Fe uptake by reticulocytes was greater and more rapid from solution I. These data were used to calculate metal uptake (Fig. 3*b*), by using 5.5nmol of Fe/ml as the iron content of solution II neglecting the small contribution (3.5% of total Fe) owing to the unlabelled diferric transferrin. The theoretical concentration (Wenn & Williams, 1968) of iron bound as

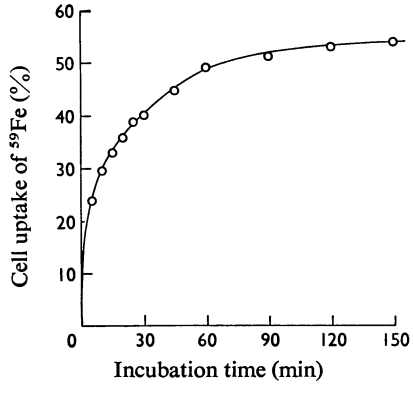

Fig. 4. *Percentage of ^{59}Fe taken up from 4%-saturated-transferrin solution by reticulocytes*
Transferrin concentration: 4μM; iron concentration: 0.32μM.

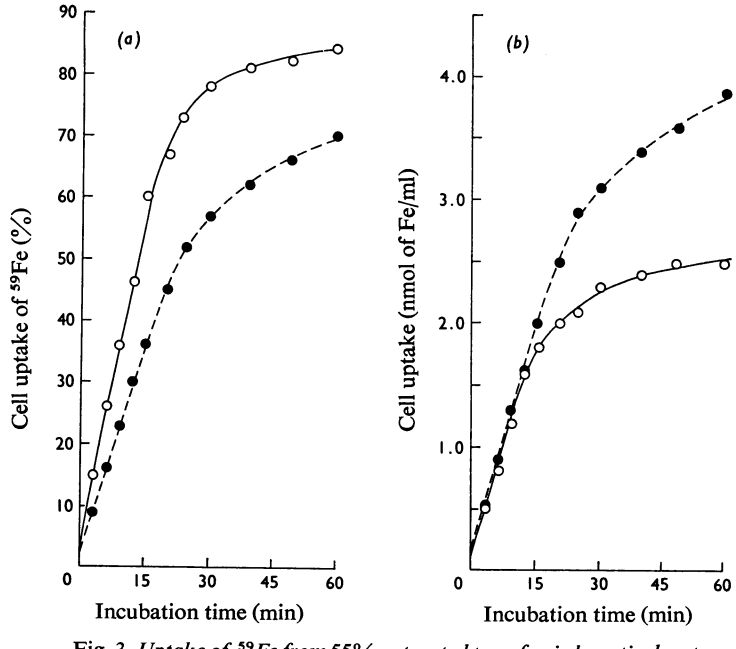

Fig. 3. *Uptake of ^{59}Fe from 55%-saturated transferrin by reticulocytes*
——, Solution I: 55%-saturated-transferrin solution, tracer labelled with [^{59}Fe]diferric transferrin. – – – –, Solution II: 55%-saturated-transferrin solution uniformly labelled with ^{59}Fe. (*a*) Percentage uptake; (*b*) calculated iron uptake by reticulocytes (see the text).

diferric transferrin in 55%-saturated transferrin (5 μM-transferrin, 5.5 μM-Fe) is 3 μM, and this value was used to calculate metal uptake from solution I [again neglecting the contribution (6%) of unlabelled diferric transferrin].

Iron uptake from solution II (broken line, Fig. 3*b*) was coincident with calculated uptake from the solution that was only labelled with diferric transferrin (solid line, Fig. 3*b*) and was 1.5 nmol of Fe/ml of cells after approx. 10 min. This value corresponds to cell uptake of half the iron bound as diferric transferrin. The rate of uptake from solution I then declined markedly compared with that from solution II, as the resultant ^{59}Fe-labelled monoferric transferrin became diluted in the large pool of unlabelled monoferric transferrin.

Uptake by reticulocytes of iron from monoferric transferrin

We have also studied uptake from a highly unsaturated transferrin solution (4%) where most of the iron will be present as monoferric transferrins. Percentage ^{59}Fe uptake by reticulocytes is shown in Fig. 4. Theoretically, 4% of the iron present is bound as diferric transferrin and 96% is bound to both monoferric transferrins. During the first hour of incubation, reticulocytes at first rapidly, and then slowly, took up nearly half the iron. During the next 2 h relatively little further iron was amassed, even though the cells at the end of the 3 h of incubation were able to incorporate further iron on reincubation with fresh transferrin solution. Thus failure of the cells to incorporate further iron from this preparation reflects an inability of the cells to utilize iron bound as one form of monoferric transferrin.

Discussion

Previous investigators have explored the relationship between iron uptake by reticulocytes with different iron or transferrin concentrations and degree of transferrin saturation (Jandl *et al.*, 1959; Morgan & Laurell, 1963; Morgan, 1964; Zapolski *et al.*, 1974). We have investigated uptake as a function of diferric transferrin concentration. The results (Fig. 1) show that some characteristic of the reticulocyte involves a saturable phenomenon, since the rate of reticulocyte iron uptake was the same at the higher concentrations. Whether or not this represents saturation of actual receptors (Jandl *et al.*, 1959), transmembrane or intracellular pathways (Hemmaplardh *et al.*, 1974) or anion-detaching enzyme (Aisen & Leibmen, 1973; Martinez-Medellin & Schulman, 1973; Egyed, 1973) remains to be elucidated.

The data that we have collected from partially saturated transferrin solutions (Fig. 2) illustrate that reported variability in iron uptake by reticulocytes as related to iron or transferrin content and degree of transferrin saturation was not a consequence of the diferric transferrin content. These solutions all contained an identical concentration of this species. The variability arises from the monoferric transferrin content. Had this study been terminated at 15–30 min, or had we used higher, near-physiological transferrin concentrations, one could conclude there were no differences in iron acquisition from these preparations by reticulocytes. At the lower concentrations that we used, it is obvious that in the face of the wide variations of transferrin, iron, monoferric transferrin and apotransferrin content of these test solutions (Table 1), whose only common characteristic was diferric transferrin content, iron uptake by reticulocytes during the early incubation period was identical. Only after cellular acquisition of half the content of diferric transferrin-bound iron did we see any variability in uptake. Since apotransferrin does not influence acquisition by reticulocytes of transferrin-bound iron (Jandl *et al.*, 1959; Zapolski *et al.*, 1974) this variability must be due to differences in monoferric transferrin content.

That this initial phase of reticulocyte iron uptake involves diferric transferrin is apparent from Fig. 3. Percentage of ^{59}Fe incorporation from each preparation was different (Fig. 3*a*). In terms of percentage, cells accumulated iron from tracer-labelled diferric transferrin (initially and throughout the course of the experiment) more efficiently and to a greater magnitude, but on the basis of metal uptake, this initial difference disappears (Fig. 3*b*). In each study (Figs. 2 and 3), initial iron uptake by reticulocytes reflects preferential utilization of iron from diferric transferrin. This involves specific incorporation of one of the two diferric transferrin-bound atoms (Fletcher & Huehns, 1967; Zapolski *et al.*, 1974; Harris & Aisen, 1975). Uptake during the later incubation period reflects uptake of monoferric transferrin-bound iron.

Our results are in accord with the postulate by Fletcher & Huehns (1968) and the observation by Jandl & Katz (1963) about the preferential affinity of reticulocytes for diferric rather than monoferric transferrin species. Lane (1973*b*) has attributed this difference to protein conformation and charge that are associated with each transferrin species. A maximum difference was observed between monoferric transferrin and apotransferrin. Lane (1975) subsequently reported that such differences extend to the two different iron-binding forms of monoferric transferrin. One monoferric transferrin species resembled diferric transferrin in its chromatographic behaviour and the other was similar in this respect to apotransferrin. Such differences might also account for the reported inability of reticulocytes to utilize iron when it is bound to a particular site as monoferric transferrin (Zapolski *et al.*, 1974).

Harris & Aisen (1975) reported that rabbit reticulocytes incorporated substantially more than

half the iron present in monoferric transferrin preparations and suggested that reticulocytes can utilize iron from monoferric transferrin bearing iron at either the A or B iron-binding site. Their observation was based on experiments using monoferric transferrin collected after isoelectric focusing which separated monoferric transferrin from diferric transferrin and apotransferrin at the isoelectric point, pI 5.3. However, at this pH transferrin only binds iron at one site (Princiotto & Zapolski, 1976) to yield haematopoietically (B-site) oriented monoferric transferrin (Zapolski *et al.*, 1974). The alternate species, A-site monoferric transferrin, was described as non-haematopoietically oriented transferrin, and it is this site from which reticulocytes preferentially abstract ferric iron when it is bound as diferric transferrin.

The data obtained from our study of iron acquisition by reticulocytes from 4% iron-saturated transferrin indicated that these cells are capable of removing iron only from one specific form of monoferric transferrin (Fig. 4). The difference between the present study and the experiment of Harris & Aisen (1975) can be attributed to random distribution of transferrin-bound iron in the present study (Lehrer, 1969; Lane, 1975; Zapolski *et al.*, 1974), which resulted in equimolar proportions of each monoferric transferrin species, whereas the Harris & Aisen (1975) study reflected uptake of iron which was only bound to the specific transferrin iron-binding site that retains an ability to bind iron at low pH (Princiotto & Zapolski, 1975; Lestas, 1976).

Whether the present findings or the Fletcher–Huehns (1968) hypothesis can be extended to the homologous human transferrin–reticulocyte system is still questionable. Harris & Aisen (1976) reported that human transferrin was functionally homogeneous towards human reticulocytes. However, their observation is in contrast with Fletcher's (1969) finding for this system. Likewise there are also conflicting reports about the functional behaviour of each rabbit transferrin iron-binding site in the homologous rabbit transferrin–reticulocyte system (Fletcher, 1969; Lane, 1973*a*; Harris & Aisen, 1975).

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