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Membrane transport of non-transferrin-bound iron by reticulocytes

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The transport of non-transferrin-bound iron into rabbit reticulocytes was investigated by incubating the cells in 0.27 M sucrose with iron labelled with ^{59}Fe. In most experiments the iron was maintained in the reduced state, Fe(II), with mercaptoethanol. The iron was taken up by cytosolic, haem and stromal fractions of the cells in greater amounts than transferrin-iron. The uptake was saturable, with a K_m value of approx. 0.2 μM and was competitively inhibited by Co^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}. It ceased when the reticulocytes matured into erythrocytes. The uptake was pH and temperature sensitive, the pH optimum being 6.5 and the activation energy for iron transport into the cytosol being approx. 80 kJ/mol. Ferric iron and Fe(II) prepared in the absence of reducing agents could also be transported into the cytosol. Sodium chloride inhibited Fe(II) uptake in a non-competitive manner. Similar degrees of inhibition was found with other salts, suggesting that this effect was due to the ionic strength of the solution. Iron chelators inhibited Fe(II) uptake by the reticulocytes, but varied in their ability to release ^{59}Fe from the cells after it had been taken up. Several lines of evidence showed that the uptake of Fe(II) was not being mediated by transferrin. It is concluded that the reticulocyte can transport non-transferrin-bound iron into the cytosol by a carrier-mediated process and the question is raised whether the same carrier is utilized by transferrin-iron after its release from the protein.

Introduction

Most cells of vertebrate animals obtain iron from the plasma iron transport protein, transferrin [1]. During this process the iron is dissociated from transferrin and passes into the cytosol. The mechanism by which it crosses cellular membranes into the cytosol is very poorly understood. Investigation of this problem has been largely confined to intestinal microvillar membrane vesicles [2–8] and liver cells [9–13]. In general, these studies have involved the use of Fe(III) complexed by citrate, nitrilotriacetate or tricine (*N*-tris(hydroxymethyl)glycine), or Fe(II) complexed by ascorbate. Although there has not been complete agreement, the majority of the studies demonstrated saturable iron binding to and/or transport across the cellular membranes, suggesting the presence of carrier-mediated processes. The microvillar surface of intestinal cells normally takes up iron in a transferrin-free form. Also, liver cells efficiently clear non-transferrin-bound iron from the blood plasma [14–15]. Possibly this oc-

Abbreviations: PIH, pyridoxal isonicotinoyl hydrazone; Pipes, 1,4-piperazinediethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tf-Fe, transferrin-bound iron.

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curs normally during periods of rapid absorption of iron from the intestine. Hence, the membrane of enterocytes and hepatocytes may have different iron transport properties from those of other cells which obtain all their iron requirements from transferrin. So little information is available on membrane transport of iron in eucaryotic cells that this question cannot be answered at the present time and there is need to develop methods for its investigation. This paper describes an attempt to do this.

Immature erythroid cells, such as reticulocytes, show very active uptake of transferrin-bound iron. They were chosen for the present investigation because of this property plus the fact that the transmembrane passage and cytosolic destination of radioactive iron taken up by the cells can be readily determined by measurement of its incorporation into haem. The initial experiments were aimed at developing a procedure for studying the uptake of transferrin-free iron which was independent of the presence of any transferrin which was retained by the cells. Subsequent studies defined many of the properties of this iron transport process. Evidence for the presence of a facilitated mechanism was obtained.

Materials and Methods

Materials. Iron-59 ($^{59}FeCl_3$), ^{125}I ($Na^{125}I$) and [U-^{14}C]sucrose were purchased from Amersham International, Amersham, U.K. Pronase was obtained from Boehringer Mannheim, Mannheim, F.R.G. and the other biochemicals were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Rabbit transferrin was isolated, rendered iron-free and labelled with ^{125}I and ^{59}Fe as previously described [16]. Pyridoxal isonicotinoyl hydrazone (PIH) was prepared as described by Ponka et al. [17].

Cells. Reticulocyte-rich blood was obtained from rabbits with phenylhydrazine-induced haemolytic anaemia [16]. The blood was collected from a marginal ear vein 3–5 days after the last dose of phenylhydrazine. The cells were washed four times with ice-cold 0.155 M NaCl, the buffy coat being removed at each wash. They were then centrifuged at 2000 × *g* for 30 min at 4°C and the top one-quarter of the cell layer was removed to obtain a reticulocyte-enriched cell suspension. The reticulocyte count varied from 40 to 90% but, for brevity, the cells will be referred to as 'reticulocytes'. Mature erythrocytes were obtained from non-anaemic rabbits. They were washed in the same way as the reticulocytes except that the bottom one-quarter of the cells after the last centrifugation was used for the experiments. The reticulocyte count of these cells was less than 2%.

Radioactive iron solution. The iron solution used in all experiments, except where specified otherwise, was prepared by mixing $^{59}FeCl_3$ (1.4 mM in 0.1 M HCl) with $^{56}FeSO_4$ (2 mM in 0.1 M HCl) in a molar ratio of 1 : 10, followed by a 50-fold molar excess of 2-mercaptoethanol and 0.27 M sucrose to give an iron concentration of 62.5 μM. Varying amounts of this solution were then added to the cell suspensions to give iron concentrations in the incubation mixture of 0.05 to 4.0 μM. Except where indicated to the contrary a concentration of 1.0 μM was used. No attempt was made to eliminate oxygen from the solutions. However, the iron appeared to be in the ferrous state since it reacted immediately and completely with 2,2′-bipyridine and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine with the production of the coloured products characteristic of their reaction with ferrous iron. The iron in the above solution will be called Fe(II) throughout this paper.

The radioactive iron solution was prepared immediately before use, and was used used within 5 min. However, it has been found that the Fe(II) solution is quite stable and gives the same rate of iron uptake by reticulocytes after standing at room temperature for at least one week.

Measurement of iron uptake. The following standard procedure was used for the measurement of the uptake of non-transferrin-bound iron in all experiments except where indicated to the contrary. The washed cells were suspended in 0.27 M sucrose to give a packed cell volume (PCV) of 10–15%. A 100 μl aliquot of the cells was then added to 4.9 ml incubation solution which consisted of 0.27 M sucrose buffered to pH 6.4–6.5 with 4 mM Pipes. The radioactive iron solution was then added and the cells incubated in a shaking water bath at 37°C for the desired period of time, after which the cells were centrifuged at 1000 × *g* for 10 min at 4°C and washed three times in 5 ml ice-cold 0.155 M NaCl. During the

third wash the cells were transferred to fresh test tubes. After washing they were haemolysed with 1.0 ml of 20 mM Hepes (pH 7.4), one half was used for the extraction of haem and the other half was centrifuged at 10000 × *g* for 20 min at 4°C to separate cytosolic and stromal fractions of the cells. The haem, cytosolic and stromal fractions were counted separately for radioactive iron. In preliminary experiments it was found that increasing the final centrifugation conditions from 10000 × *g* to 50000 × *g* for 20 min did not alter the relative distribution of the ^{59}Fe between the cytosol and the stroma.

The uptake of transferrin-bound iron (Tf-Fe) was determined in many of the experiments for comparison with the uptake of transferrin-free iron. The same procedure was used except that diferric transferrin labelled with ^{59}Fe and ^{125}I replaced the Fe(II) and the standard incubation solution was 0.155 M NaCl buffered at pH 7.4 with 4.0 mM Hepes.

Analytical methods. The reticulocyte count was determined by staining with new methylene blue and the packed cell volume by the microhaematocrit method. Heme was extracted by the method of Thunell [18]. The pH was determined with a Radiometer model PHM 83 pH meter. Osmolality was measured with an Osmometer Model 3DII (Advanced Instruments Inc., Needham Heights, MA, U.S.A.). Radioactivity of ^{59}Fe and ^{125}I was measured in a three-channel γ-scintillation counter (Packard model 5320) and of ^{14}C in a liquid scintillation counter (Beckman model LS3800).

Results

Time course of Fe(II) uptake

The uptake of Fe(II) by reticulocytes was optimal when incubation was performed under the standard conditions (isotonic sucrose solution, pH 6.4–6.5, 1 μM Fe) and occurred in a linear manner for at least 30 min (Fig. 1A). The incorporation into haem was approx. 50% of that into the cytosol at each time point, while that into cytosol and stroma were approximately the same. Since iron uptake is linear with respect to time the rate of iron uptake could be determined by taking a single 20 min time point and calculating the rate of uptake in terms of nmole iron per min. Also, iron

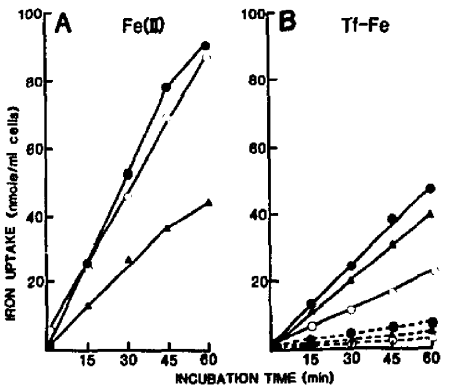

Fig. 1. Iron uptake by rabbit reticulocytes from Fe(II) solution (A) or from diferric transferrin (B) into cytosolic (●), stromal (○) or haem (▲) fractions of the cells. The uptake of Fe(II) was from 0.27 M sucrose (pH 6.5), that of Tf-Fe from 0.155 M NaCl (pH 7.4) (———) or 0.27 M sucrose (pH 6.5) (- - - - -).

uptake is directly correlated with the reticulocyte count (below). Hence, the results could be corrected for variations in reticulocyte count between experiments by calculating them in terms of nmole Fe/min per ml reticulocytes. This was the practice in all of the experiments described below except in Figs. 1 and 2. There are approx. 10^{10} cells per ml packed rabbit reticulocytes.

The validity of the haem extraction method was demonstrated in several ways. In addition to failure to detect significant ^{59}Fe in the haem fraction after zero incubation time, or after incubation of Fe(II) with mature erythrocytes at 37°C (see below), it was found that $^{59}Fe(II)$ added to lysed reticulocytes was not extracted into the haem fraction. Also, although Fe(II), was taken up by reticulocytes incubated at 37°C in the presence of 10 mM isoniazid (an inhibitor of haem synthesis) iron incorporation into haem was reduced to 5–10% of that found in the absence of isoniazid.

Iron uptake from Tf-Fe by reticulocytes in the sucrose medium at pH 6.5 was much lower than that from Fe(II) (Fig. 1B). Incubation in NaCl (pH 7.4) greatly increased iron uptake from transferrin, but it was still less than from Fe(II), even the incorporation into haem. However, a feature of the uptake of Tf-Fe was the relatively high proportion of total iron uptake which was recovered in the haem fraction and the lower proportion in the stromal fraction. The mean values

for rate of iron incorporation into haem, cytosol and stroma when reticulocytes were incubated with 1 μM Fe were 1.2 ± 0.09, 2.3 ± 0.19 and 2.5 ± 0.23 nmol/ml per min, respectively for Fe(II) in sucrose (pH 6.5) and 0.88 ± 0.05, 0.95 ± 0.08 and 0.62 ± 0.04 nmol/ml per min, respectively, for Tf-Fe in NaCl (pH 7.4) (means ± S.E. of eight measurements).

Relationship of iron uptake to reticulocyte count

The effect of reticulocyte count on iron uptake was investigated by using cell populations prepared by mixing reticulocytes and mature erythrocytes in varying proportions. Iron uptake from Fe(II) or Tf-Fe into the three cellular fractions was linearly related to the reticulocyte count, but was greater with Fe(II) than with Tf-Fe (Fig. 2). Moreover, the regression lines for iron uptake into the haem and cytosolic fractions passed very close to the origin, indicating that iron uptake was confined to the reticulocytes present in the cell mixture. Hence, maturation of reticulocytes to mature erythrocytes is accompanied by loss of the ability to transport Fe(II) into the cytosol, as well as the ability to take up Tf-Fe, as previously demonstrated [19,20]. Although the uptake of Fe(II) into the stromal fraction showed a direct linear correlation with the reticulocyte count, the regression line crossed the ordinate at a value greater than zero. This shows that the membranes

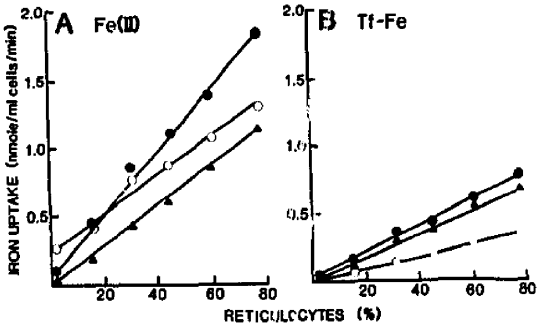

Fig. 2. Effect of reticulocyte count on the rate of uptake of Fe from Fe(II) in 0.27 M sucrose (pH 6.5) (A) and from Tf-Fe in 0.155 M NaCl (pH 7.4) (B) into cytosolic (●), stromal (○) and haem (▲) fractions of the cells. The red cell suspensions of varying reticulocyte percentage were obtained by mixing differing proportions of reticulocyte-rich red cells (78% reticulocytes) with reticulocyte-poor cells (2% reticulocytes).

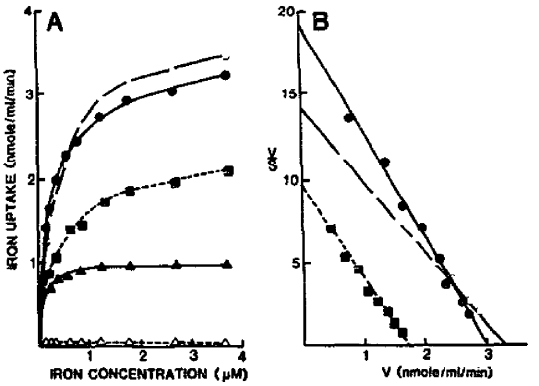

Fig. 3. (A) Effect of varying the Fe(II) concentration in the incubation medium on the rate of iron uptake into cytosolic (●———●), stromal (○———○) and haem fractions (▲———▲) of reticulocytes incubated under control conditions (0.27 M sucrose, pH 6.5) and into cytosol (■- - - - -■) and haem (△- - - - -△) of cells incubated in the same solution containing 10 mM isoniazid. (B) Eadie-Hofstee plots of the specific uptake of Fe(II) into cytosol (●———●) and stroma (○———○) of control cells, and into the cytosol of cells incubated in the presence of 10 mM isoniazid (■- - - - -■). The data are from Fig. 3A.

of mature erythrocytes can take up or bind some Fe(II) but the capacity for this is much smaller than in reticulocytes.

Effect of iron concentration

The rate of uptake of Fe(II) into the cytosolic and stromal fractions of reticulocytes showed evidence of saturation as the iron concentration of the incubation medium was raised to about 1 μM but continued to increase in a linear manner with further increase in concentration (Fig. 3A). Hence, there was evidence of both a saturable or specific uptake process and a non-saturable or non-specific process. The saturable process was analysed by the Eadie-Hofstee method to determine the maximum rate of iron uptake, V_{max}, and the Michaelis constant, K_m, after first subtracting the non-specific part of the uptake, as determined by the slope of the linear portion of the uptake curve (Fig. 3B). Ten measurements of the type shown in Fig. 3 were performed. The mean K_m values were 0.20 and 0.23 μM for uptake into the cytosolic and stromal fractions, respectively. The corresponding V_{max} values were 3.0 and 2.8 nmol/ml reticulocytes per min (Table I).

TABLE I

V_{max} AND K_m VALUES FOR SPECIFIC Fe(II) UPTAKE

The results show the maximal rates (V_{max}) and Michaelis constants (K_m) for the specific (saturable) component of Fe(II) uptake into haem, cytosolic and stromal fractions of reticulocytes. K_m values were not calculated for the haem fraction. The results for control incubations are the means ± S.E. of 10 estimations, those for incubations performed in the presence of 10 mM isoniazid or with Fe(II) prepared without addition of 2-mercaptoethanol are mean values of two estimations.

Conditions	V_{max} (nmol/ml reticulocytes per min)			K_m (μM)	
				cytosol	stroma
	heme	cytosol	stroma		
Control, mean ± S.E.	1.41 ±0.07	3.00 ±0.13	2.84 ±0.14	0.20 ±0.01	0.23 ±0.00
10 mM isoniazid	–	1.95	2.65	0.22	0.23
No mercapto-ethanol	1.42	2.92	2.76	0.22	0.25

Iron uptake into haem also showed evidence of saturation, but at a lower concentration than into the other two fractions. Also, iron uptake into haem reached a true plateau with very little or no non-saturable uptake. A relatively greater proportion of the iron uptake to the cytosolic fractions was incorporated into haem at lower iron concentrations than at higher concentrations (Fig. 3A). The V_{max} values for incorporation into haem were calculated from the plateau levels of uptake rate. The mean was approx. 50% of that for the cytosol (Table I). K_m values were not calculated for iron uptake to haem because of the doubtful significance of any such values due to the limited capacity of the cells for haem synthesis.

In two experiments measurements were made in the presence of 10 mM isoniazid, to block iron incorporation into haem. As shown in Fig. 3A, iron uptake to the cytosol was still saturable, but was reduced compared with that in the absence of isoniazid. The K_m values for iron uptake into the cytosolic and stromal fractions were similar but the V_{max} values lower than in the control incubations (Table I).

Effect of pH

The uptake of Fe(II) into the three cellular fractions were similarly influenced by the pH of the incubation medium, optimum uptake occurring at pH 6.4–6.5. These results contrast markedly with the effects of pH on Tf-Fe uptake which is maximal at about pH 8.0 (Fig. 4).

When the effect of iron concentration of the uptake of Fe(II) at pH 6.5 and 7.4 were compared it was found that the higher pH was associated with much more non-specific (unsaturable) uptake and less specific uptake. Iron uptake into haem was also less at pH 7.4, especially at the lower iron concentrations indicating that there was a less efficient transport of extracellular Fe(II) to the haem synthesis pathway at this pH than at 6.5.

Effect of salt concentration in the incubation medium

Early in the course of these experiments it was observed that the uptake of Fe(II) from NaCl solutions was much less than from sucrose solutions. Hence, the effect of adding varying quantities of NaCl to the standard incubation medium, by replacing part of the sucrose solution with 0.155 M NaCl, was investigated. As the NaCl concentration was raised the uptake of Fe(II) declined, reaching 20% of the control value for uptake to heme and cytosolic fractions and 40% of that into the stroma at an NaCl concentration of 50 mmol/l. Further increases in NaCl concentration reduced iron uptake more slowly to about 10 and 30% of the control values at 155 mM NaCl

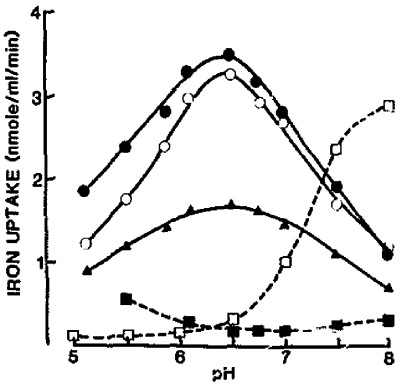

Fig. 4. Effect of pH of the incubation medium on the rate of Fe(II) uptake into the cytosolic (●——●), stromal (○——○) and haem (▲——▲) fractions of reticulocytes incubated in 0.27 M sucrose. Also shown are iron uptake from Fe(II) (■- - - - -■) and Tf-Fe (□- - - - -□) into the cytosol of reticulocytes incubated in 0.155 M NaCl.

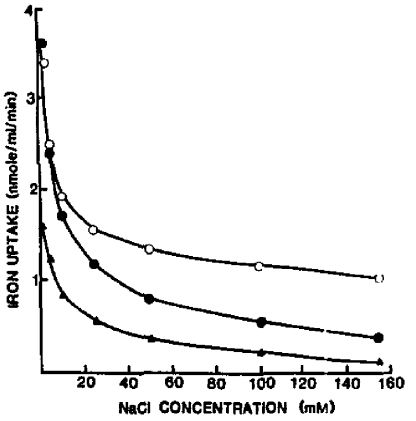

Fig. 5. Effect of NaCl concentration in the incubation medium on the rate of iron uptake into cytosolic (●), stromal (○) and haem (▲) fractions of reticulocytes. The incubation media were prepared by mixing varying proportions of 0.155 M NaCl and 0.27 M sucrose, at pH 6.5, to obtain the desired final NaCl concentrations.

(Fig. 5). When the effect of NaCl on iron uptake at various concentrations of Fe(II) was investigated results characteristic of non-competitive inhibition were obtained, with reduction in V_{max} but little change in K_m values. The rate of Fe(II) uptake from media containing 0.155 M NaCl was relatively insensitive to the pH of the incubation medium, little change occurring as the pH was lowered from 7.5 to 6.0 (Fig. 4).

The effects of NaCl on Fe(II) uptake did not appear to be specific for either Na^+ or Cl^-. Addition of KCl, NaI, Na_2SO_4 or sodium phosphate to the incubation medium produced similar degrees of inhibition of Fe(II) uptake as NaCl, while $CaCl_2$ and $MgCl_2$ gave somewhat greater inhibition.

Kinetics of iron uptake

The rate of Fe(II) uptake into all three cellular fractions increased as the incubation temperature was raised from 21°C to 38°C. Arrhenius plots of these results showed linear relationships for the uptake into each fraction, allowing calculation of the activation energy. The values so obtained were 88.2 ± 1.05, 76.7 ± 2.56 and 41.6 ± 4.65 kJ/mol for Fe(II) incorporation into haem, cytosol and stroma, respectively (means ± S.E. of four experiments).

Inhibition of iron uptake by other metals

Several divalent metal ions were tested for their ability to inhibit Fe(II) uptake by reticulocytes. When tested at 25-times the molar concentration of iron (25 μM in the presence of 1 μM Fe(II)) the degree of inhibition of iron uptake into the cytosol was 0, 10, 12, 15 and 23%, respectively, for Mg^{2+}, Ca^{2+}, Cu^{2+}, Sr^{2+} and Ba^{2+}. However, Zn^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+} produced 75–85% inhibition and were, therefore, examined in more detail. As shown in Fig. 6 the degree of inhibition was dependent on concentration and was similar for the four metals, but was less than that produced by the addition of non-radioactive Fe(II). Manganese, Ni^{2+} and Co^{2+} had relatively little effect on iron uptake from transferrin but Zn^{2+} did produce considerable inhibition. Non-radioactive Tf-Fe, as expected, produced marked inhibition of labelled Tf-Fe uptake. It also inhibited the uptake of Fe(II), but much less than did Mn^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+}. The uptake of Fe(II) into the haem and stromal fractions was also inhibited by these metals. Manganese produced the same degree of inhibition into haem as into cytosol, Zn^{2+} and Ni^{2+} slightly less and Co^{2+} more, at each concentration of the metals. Inhibition of Fe(II) incorporation into the stromal fractions was less than into the other fractions so that at 64 μM concentration of the metals the degree of inhibition varied from 65 to 80%, compared with 80 to 93% for incorporation into the cytosol.

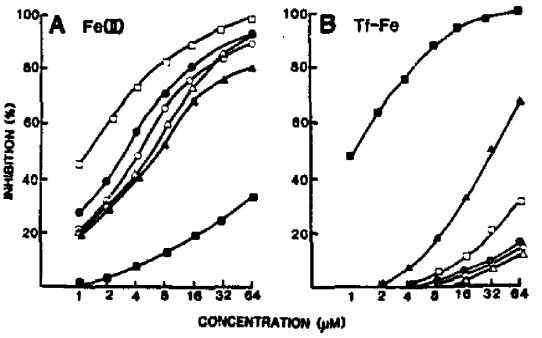

Fig. 6. Inhibition of iron uptake from radioactively labelled 1 μM Fe(II) (A) or Tf-Fe (B) by varying concentrations of non-radioactive Mn^{2+} (●), Ni^{2+} (○), Co^{2+} (△), Zn^{2+} (▲), Fe^{2+} (□) or Tf-Fe (■).

The effects of Co^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} on Fe(II) uptake at varying concentrations of Fe(II) were determined to see whether or not the inhibition was of a competitive type. The metals had similar effects, all producing reduction in the K_m values but no change in V_{max}. Hence, these results provide evidence that the inhibition is of a competitive type.

Effect of chelators on iron uptake and release

Several chelators of ferrous and ferric iron were tested for their ability to affect Fe(II) and Tf-Fe uptake by reticulocytes. All of them, except citrate, markedly inhibited Fe(II) uptake to the cytosol (Table II). They produced similar degrees of inhibition of Fe(II) uptake to the stroma as to the cytosol but less inhibition of iron incorporation into haem. That is, a greater proportion of the iron taken up by the cells was incorporated into haem in the presence of the chelators than in their absence. Citrate inhibited iron uptake when used at higher concentrations than in Table II, e.g. 65% inhibition of uptake to the cytosol at 1 mM concentration.

The ionophore A23187 which has been shown to transport Fe(II) into erythrocytes [21] was used in a similar way as the chelators. It was found to increase Fe(II) uptake (Table II). As with the chelators the effect on Fe(II) uptake to stroma was similar to that to cytosol, but there was relatively less increase in incorporation into heme, only 12% increase being observed at an A23187 concentration of 1 μM.

TABLE II

EFFECTS OF CHELATORS ON Fe(II) UPTAKE

The reticulocytes were incubated with 1 μM Fe(II) in the absence (control) or in the presence of 1, 3 and 10 μM concentrations of the chelators and the ionophore A23187. The table shows rates of iron uptake into the cytosol expressed as percentages of the values for control cells. They are the means of two measurements.

Chelator	Iron uptake (% control)		
Chelator concn. (μM):	1	3	10
Desferrioxamine	19	1	0.2
EDTA	74	1	0.1
PIH	74	3	1
2,2′-Bipyridine	68	51	25
Citrate	100	96	90
Nitrilotriacetate	64	34	21
A23187	195	152	131

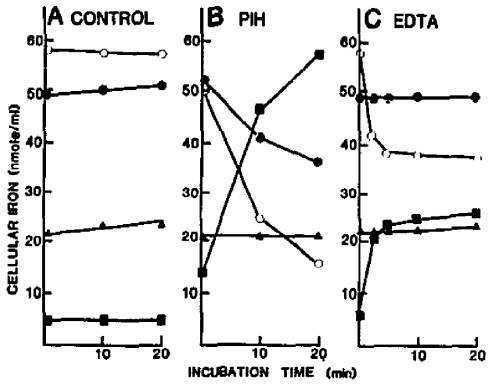

Fig. 7. Release of iron from reticulocytes into 0.155 M NaCl (pH 7.4) (A), or into the same solution containing 1 mM PIH (B) or 1 mM EDTA (C). The cells were labelled with ^{59}Fe by incubation in 0.27 M sucrose (pH 6.5) with 1 μM $^{59}Fe(II)$ for 20 min at 37°C. They were then washed three times with ice-cold 0.155 M NaCl and reincubated at 37°C with the solutions given above. The figure shows the iron released into the reincubation medium (■) and that remaining in the cytosolic (●), stromal (○) and haem (▲) fractions of the cells after 0, 10 and 20 min reincubation.

The reversibility of iron uptake by reticulocytes was investigated by incubating the cells for 20 min at 37°C with $^{59}Fe(II)$ followed by three washes in ice-cold NaCl and reincubation in NaCl buffered at pH 7.4 with Hepes (10 mM). Chelators were added to the reincubation solution in some cases. In the absence of chelators little iron was released from the cells and the distribution of ^{59}Fe between the three cellular fractions remained relatively constant, a small rise occurring in haem and cytosolic ^{59}Fe and a fall in stromal ^{59}Fe (Fig. 7A). However, when chelators were added to the reincubation solution much more of the radiolabelled iron was released. With PIH (Fig. 7B) this release occurred from both the membrane and cytosolic fractions but there was no change in the heme fraction, while with EDTA the release was entirely from the membrane fraction, again with no loss from heme (Fig. 7C). Two other chelators were tested, 2,2′-bipyridine which produced a similar pattern of changes, but only about one-third as much iron release as PIH, and desferrioxamine which released about 50% more iron than EDTA

but, like EDTA, only from the membrane fraction.

Effect of reducing agents

In the preliminary experiments in this investigation sodium ascorbate, dithiothreitol and 2-mercaptoethanol were tested as reducing agents in the Fe(II)-sucrose solution. No significant difference in iron uptake was observed with the three agents. Therefore 2-mercaptoethanol was chosen because of its weaker iron chelating ability [4]. It was used in all of the experiments described above. At the concentrations used in these experiments it had no effect on iron uptake from transferrin.

Additional experiments were performed using Fe(II)-sucrose to which no reducing agent was added and Fe(III)-sucrose, prepared by replacing the $FeSO_4$ in the iron solution with $FeCl_3$. The uptake of Fe(II) in the absence of mercaptoethanol showed no significant difference with respect to rate of uptake or effect of varying the iron concentrations (Table I). In the case of Fe(III)-sucrose the iron was used for heme synthesis almost as readily as with Fe(II). It was also taken up into the cytosolic and stromal fractions, but with much greater non-saturable uptake components of the saturation curves than found with Fe(II).

Evidence that Fe(II) uptake is not mediated by transferrin

Several lines of evidence indicate that the Fe(II) uptake by reticulocytes which was described above was not mediated by transferrin. These include the effects of pH (Fig. 4), salt concentration (Fig. 5) and competing metal ions and diferric transferrin (Fig. 6), all of which affected the uptake of Tf-Fe in a markedly different way to that of Fe(II). Three other experiments also provide evidence on this point. In one the reticulocytes were incubated with pronase (1 mg/ml) for 30 min at 37°C in order to inactivate the transferrin receptors [22], and were then washed and reincubated with Fe(II) or Tf-Fe. This treatment virtually eliminated iron uptake from transferrin but caused only about 20% reduction in Fe(II) uptake (Table III). In the second experiment reticulocytes were depleted of transferrin by incubation for 15 min at 37°C in three changes of NaCl buffered at pH 7.4 with Hepes (10 mM). By prior labelling the cells with ^{125}I-transferrin it was shown that this procedure reduced cell-bound transferrin to 10% of its original value so that the cell samples used in the subsequent measurement of Fe(II) uptake contained only 0.15 μg transferrin, compared with about 1.5 μg in the cells washed by the standard procedure. This reduction in cellular transferrin did produce a small fall in the uptake of Fe(II), especially into the heme fraction, but this decrease was less than that observed when transferrin was used as the source of iron (Table III).

In the final experiment apotransferrin was added at concentrations of 0.1, 1, 2 and 5 μM to the Fe(II)-sucrose incubation solution. The lowest concentration had no effect, but the higher con-

TABLE III

IRON UPTAKE BY PRONASE-TREATED, TRANSFERRIN-DEPLETED AND TRANSFERRIN-SUPPLEMENTED RETICULOCYTES

Reticulocytes were incubated with pronase (1 mg/ml) for 30 min at 37°C, then washed three times, depleted of endogenous transferrin by three 15 min incubations at 37°C in NaCl, or washed in the standard manner before the addition of apotransferrin to the incubation medium, and then incubated with 1 μM ^{59}Fe(II). See text for details. The results (mean ± S.E., $n = 4$) show the values for the rates of iron uptake as percentages of the uptake into control cells.

Treatment	Fe	Iron uptake (% control)		
		heme	cytosol	stroma
Pronase	Fe(II)	90.2 ± 8.8	81 ± 9.8	72 ± 3.9
	Tf-Fe	4.0 ± 1.1	1.8 ± 1.58	7.2 ± 1.35
Tf-depleted	Fe(II)	75 ± 5.0	94 ± 6.8	132 ± 4.2
	Tf-Fe	54 ± 4.4	53 ± 4.3	76 ± 10.1
Apotransferrin 1 μM	Fe(II)	98 ± 4.8	82 ± 7.4	87 ± 5.6
2 μM	Fe(II)	80 ± 3.7	69 ± 4.9	70 ± 5.3
5 μM	Fe(II)	52 ± 4.0	48 ± 2.7	36 ± 4.8

centrations did produce a concentration-dependent reduction in iron uptake (Table III). Similar results to those in Table III were obtained in one experiment in which the cells had been depleted of transferrin as described above before incubation with ^{59}Fe(II) with and without the addition of apotransferrin.

Sucrose uptake

In order to determine whether the uptake of Fe(II) by reticulocytes could be accounted for by transport of the Fe-sucrose complex into the cells, measurements were made of the uptake of ^{14}C-labelled sucrose. Duplicate samples of reticulocytes were incubated at 37°C in the sucrose-pH 6.5 medium containing 1 μM ^{56}Fe(II) to which tracer amounts of [U^{14}C]sucrose had been added or in the standard medium containing 1 μM ^{59}Fe(II). The fractional uptake of sucrose was less than 1% of that of Fe(II). Similar results for sucrose uptake were obtained with mature erythrocytes as with reticulocytes, or when the incubation with [^{14}C]sucrose was performed in NaCl-Hepes (pH 7.4) instead of sucrose.

Discussion

The results of these experiments show that Fe(II) is transported into rabbit reticulocytes and utilized for haem synthesis at a greater rate than is iron bound to transferrin, the normal source of reticulocyte iron. That the iron is not simply absorbed to the cell surface but passes through the membrane into the cytosol is demonstrated by its incorporation into haem and by the fact that ^{59}Fe in the cytosolic fraction of the cells is not available to chelators which do not penetrate the erythroid cell membrane (e.g. EDTA, desferrioxamine). Considering the relatively low centrifugal force which was used to separate the cytosol from the stroma the former fraction of the cells was probably not pure and may have contained ribosomes and some low density intracellular vesicles. However, it was probably free of plasma membranes or iron-binding organelles since no change in the related distribution of ^{59}Fe between cytosolic and stromal fractions was observed when the centrifugal force was increased from 10000 × g to 50000 × g. Some of the ^{59}Fe in the stromal fraction is available to EDTA and desferrioxamine (Fig. 7). The fact that only about one-third of the stromal ^{59}Fe was released by these chelators is probably due to the heterogeneous nature of the stroma of reticulocytes which consists of intracellular organelles (mitochondria, endocytotic vesicles, lysosomal remnants) as well as the outer cell membrane. Probably part of the iron which was transported through the cell membrane passed into these organelles, making it unavailable to the chelators and also leading to the linear rate of iron uptake and other features of iron uptake into the stroma which resembled those of the uptake to the cytosol. By contrast, lipid-soluble iron chelators (PIH, 2,2′-bipyridine) were able to mobilize ^{59}Fe from the cytosolic and stromal fractions, but not from that portion of cytosolic iron which was in the form of heme.

The Fe(II) uptake process has features of facilitated or carrier-mediated transport accompanied by some non-specific (non-saturable) uptake. Thus, it displayed saturation kinetics at iron concentrations up to about 2 μM, was competitively inhibited by other divalent transition metal ions and had an activation energy comparable with that found with other facilitated transport process (higher than expected for simple diffusion) [23]. Although complete saturation of iron uptake into the stromal and cytosolic fractions did not occur, probably due to adsorptive and diffusional processes, that into the haem fraction was saturable at relatively low Fe(II) concentrations. This is likely to be a consequence of the limited haem synthesizing capacity of the cells which was fully supplied with iron when the extracellular Fe(II) concentration was well below that required to saturate the carrier-mediated uptake pathway. The observation that inhibition of haem synthesis by the action of isoniazid did not change the saturable nature of the Fe(II) uptake process indicates that the saturation kinetics are not a consequence of the presence in the cells of limited capacity 'sink' in the form of the haem synthesis mechanism. The reason for the reduction in Fe(II) uptake as a result of inhibition of haem synthesis is uncertain but may relate to the mechanism of iron release from the putative carrier on the inner side of the cell membrane.

Another reason for believing that Fe(II) uptake occurs by a carrier-mediated process is the close correlation found between the reticulocyte count and the rate of iron uptake. Fe(II) uptake ceases as reticulocytes change into mature erythrocytes, possible due to loss of a membrane carrier for iron. The results of the haem synthesis inhibition experiments show that this loss of iron uptake was not due to the loss of haem synthesis capacity, a normal consequence of reticulocyte maturation. The similarity in the relationship between reticulocyte count and the uptake of Fe(II) or Tf-Fe suggests that the transport of both forms of iron involves the same carrier, but further work is required to substantiate this possibility. Another possibility is that the Fe(II) used in the present experiments was bound by the residual transferrin present in the reticulocytes and was taken up by the Tf-Fe uptake mechanism. However, this is ruled out by the many lines of evidence which are summarized in Results.

It is clear from the studies on the uptake of Fe(II) in the absence of reducing agents and of [^{14}C]sucrose uptake that the form of the iron transported into the cells is not an Fe-reducing agent or Fe-sucrose complex. It may be ionic iron, probably Fe(II) since this was the form of iron used in most experiments and one which was efficiently transported, and because all of the iron chelators tested inhibited iron uptake. There is some evidence that the iron of the Tf-Fe complex is reduced to the ferrous state before being transported into the reticulocyte cytosol [24]. Hence the presence of an Fe(II) carrier in these cells would not be unexpected. However, Fe(III) was also found to be transported into reticulocytes and utilized for haem synthesis, although with more non-specific uptake than with Fe(II). At least two explanations of these observations must be considered, that both Fe(II) and Fe(III) can be transported by the carrier mechanism, or that the Fe(III) is reduced to Fe(II) at the exterior surface of the cell by a transmembrane redox system capable of reducing Fe(III), as has been demonstrated in many types of cells [25]. The greater non-specific features of Fe(III) uptake may be consequences of the lower solubility and greater tendency for hydrolysis of Fe(III) than Fe(II).

Two aspects of FE(II) uptake which are of particular interest are the effects of ionic strength and pH. Iron uptake was much greater from sucrose than from NaCl solutions, and with sucrose was optimal at pH 6.5. Sucrose was chosen for these experiments because of its ability to form weak complexes with iron which are stable up to pH values above neutrality [26] plus its inability to pass through cell membranes. However, the effect of sucrose is not simply due to its ability to solubilize iron because relatively low concentrations of NaCl and other salts were able to inhibit Fe(II) uptake from sucrose solutions. This effect, which was shown to be 'non-competitive' in nature for the case of NaCl, appears to be due to the ionic strength of the solution rather than being specific for NaCl, since several other salts produced similar degrees of inhibition. Calcium and Mg produced greater inhibition than Na or K salts, possibly due to their divalent nature or to specific interactions with cell surface components. However, these two divalent cations were not nearly as effective inhibitors as Co, Mn, Ni and Zn which probably compete with Fe(II) for the putative iron carrier.

Suspension of reticulocytes in media of low ionic strength would be expected to produce many effects on the cells, including changes in the charge within or on the cell membrane [27], membrane potential [28,29], transmembrane ion fluxes [30,31], intracellular pH [32] and the conformation of membrane components [33]. Changes in extracellular pH could also affect these properties. In our present state of knowledge it is not possible to determine which are responsible for the effects observed in the present investigation. The pH effect may be due to the involvement of charged groups on the cell membrane with pK values around 6.5, one type of which needs to be protonated and another to be unprotonated for efficient transport of Fe(II).

As mentioned in the Introduction several groups of researchers have reported evidence for carrier-mediated binding or transport of Fe(II) and Fe(III) by intestinal membrane vesicles [2,4–8] and of Fe(III) by liver cells [11–3]. However, the relationship between these investigations and the present one is difficult to assess because iron bound to much stronger chelators than sucrose was used in all of the other experiments and because, in the

case of membrane vesicles, it is difficult to assess whether the iron is bound to or is transported through the vesicular membrane. In virtually all reported studies the K_m value for iron transport was found to be 40–1000-times higher than that in the present work. This may mean that the iron transport properties of reticulocytes differ from those of the intestinal mucosa and hepatic cells, or that the differences are methodological in origin, possibly resulting from the use of different iron chelators.

Reticulocytes have been previously reported to take up non-transferrin-bound iron but only when the iron was bound by another carrying agent such as A23187 and other carboxylate ionophores [21] or PIH and its analogues [34–36]. In the present work A23187 was found to enhance the uptake of Fe(II), thus confirming the earlier study, but since the ionophore acts as an artificial carrier of iron the results do not aid in understanding the normal transport process. The reported ability of PIH to transport iron into reticulocytes appears to be in conflict with the marked inhibition of Fe(II) uptake which was observed when PIH was added to the incubation medium in the present work. However, in the studies with PIH-Fe [34] very high concentrations of the complex (160 μM) were found to be necessary to give iron uptake rates comparable to those found with Tf-Fe. Moreover, the addition of excess PIH or desferrioxamine or 2,2′-bipyridine reduced iron uptake from its complex with PIH, while treatment of the reticulocytes with pronase led to a similar degree of reduction of PIH-Fe uptake as reported in Table II for Fe(II) uptake. These results raise the possibility that the iron of PIH-Fe is transported by the same carrier as postulated for Fe(II). Since no PIH was used in the present investigation of Fe(II) transport the carrier cannot be one for PIH. Instead, iron may be transported into reticulocytes from PIH-Fe in its ionic form after dissociation from its complex with the chelator.

In conclusion, this investigation has provided evidence for the presence of carrier-mediated transport of Fe(II), or Fe(II) and Fe(III), into rabbit reticulocytes. Rat reticulocytes also contain a similar transport process (unpublished observations). Future work will be directed at determining the nature of the carrier and whether it is responsible for the entry of iron derived from transferrin into the cytosol of reticulocytes and other types of cells.

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