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Use of Inhibitors of Ion Transport to Differentiate Iron Transporters in Erythroid Cells

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ABSTRACT. Iron uptake by rabbit reticulocytes and mature erythrocytes was investigated using 4 incubation systems: 1. Fe-transferrin in NaCl at pH 7.4, 2. Fe-transferrin in sucrose at pH 5.9, 3. Fe(II)-sucrose in sucrose at pH 6.5, and 4. Fe(II)-sucrose in KCl at pH 7.0. These systems were compared with respect to their magnitude and response to many membrane transport inhibitors and modifying agents. Iron uptake *via* the first 3 systems had many similar features that were quite distinct from those of iron uptake in the fourth system. On the basis of these results, it is concluded that erythroid cells contain two iron transport mechanisms, one with a high affinity and relatively low capacity for iron transport, which can be studied using incubation systems 1–3, and the other of low affinity but high capacity (incubation system 4). High-affinity transport is present only in immature erythroid cells, is relatively sensitive to inhibition by N-ethylmaleimide (NEM), N,N^1-dicyclohexylcarbodiimide (DCCD), and 7-chloro-4-nitrobenz-2-oxa-1,3 diazole (NBD), and is probably the mechanism by which iron, released from transferrin within endosomes, is transported across the endosomal membrane into the cytosol. DCCD and NBD are also inhibitors of the endosomal H^+-ATPase, which is in keeping with the hypothesis that this ATPase functions as the iron transporter in endosomal membranes. However, the more specific inhibitor of this enzyme, bafilomycin A_1, inhibited iron uptake only in incubation system 1, where its action can be attributed to inhibition of endosomal acidification. Hence, it is unlikely that the ATPase also functions as the iron transporter. The low-affinity uptake mechanism is sensitive to inhibition by amiloride, valinomycin, quinidine, imipramine, quercetin, and diethylstilbestrol (to all of which high-affinity transport is relatively resistant), and is present in mature erythrocytes as well as reticulocytes. BIOCHEM PHARMACOL 52;2: 371–377, 1996.

KEY WORDS. iron; membrane transport; transport inhibitors; reticulocytes; erythrocytes; H^+-ATPase

Immature erythroid cells acquire the iron required for metabolic functions and haemoglobin synthesis from plasma transferrin by receptor-mediated endocytosis [1, 2]. During this process, the iron is released from transferrin in the acidic environment of the endosome and, then, crosses the endosomal membrane into the cytosol. The mechanism by which the iron is transported across this, and other cell membranes, is obscure. In the case of erythroid cells, it has been investigated by incubating the cells with transferrin-bound and transferrin-free iron under a variety of conditions. These include 1. Fe-transferrin in NaCl at pH 7.4 [3, 4], 2. Fe-transferrin in sucrose at pH 5.9 [5], 3. Fe(II)-sucrose in sucrose at pH 6.5 [6], 4. Fe(II)-sucrose in KCl at pH 6.5 [7], and 5. Fe(II)-ascorbate in NaCl or KCl at pH 7.4 [8]. The results obtained by the use of all of these experimental systems have provided evidence for carrier-mediated transport of iron across the cell membranes. However, it is not clear whether they demonstrate different transport mechanisms or the same one studied under different conditions.

The primary aim of the present investigation was to answer this question through the use of a variety of reagents that have been shown to affect different types of membrane transport. Only the first 4 of the systems listed above were studied because there is good evidence that systems 4 and 5 represent 2 ways of investigating the same process [7]. The results lead to the conclusion that there are two distinct mechanisms of iron transport into erythroid cells that can be distinguished by the use of inhibitors of ion transport.

MATERIALS AND METHODS

Materials

Iodine^{125}I ($Na^{125}I$) and ^{59}Fe ($^{59}FeCl_3$) were purchased from Dupont, Australia (Sydney, Australia). The biochemical reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Transferrin was isolated from rabbit plasma and labelled with ^{59}Fe and ^{125}I, as previously described [9].

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† *Abbreviations:* NEM, N-ethylmaleimide; DCCD, N, N^1-dicyclohexylcarbodiimide; NBD, 7-chloro-4-nitrobenz-2-oxa-1, 3 diazole.

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Cells

Blood with a phenylhydrazine-induced reticulocytosis was obtained from rabbits 4–6 days after the last injection of phenylhydrazine. Blood with a low reticulocyte count was from untreated rabbits. The blood cells were washed 3 times in 0.15 M NaCl by centrifugation at 1800 × *g* for 10 min. The buffy coat was removed after each centrifugation. The cells were then centrifuged a fourth time, at 1800 × *g* for 30 min. The top quarter of the red cell layer was removed from reticulocyte-rich blood and the bottom quarter from normal blood to give cell suspensions with reticulocyte counts of 45–70% and 1–2%, respectively. These cells will be referred to as reticulocytes and erythrocytes. The cells were washed once in the medium to be used in the subsequent incubation, and suspended in that medium at an haematocrit of approximately 30%. For most experiments these media were 1. 0.15 M NaCl, 4 mM Tris-Hepes, pH 7.4, 2. 0.27 M sucrose, 4 mM Tris-Hepes, pH 5.9, 3. 0.27 M sucrose, 4 mM Tris-Hepes, pH 6.5, and 4. 0.15 M KCl, 4 mM Tris-Hepes, pH 7.0. The osmolality of all of the incubation solutions was 285–295 mOsM/kg.

Iron Solutions

The radioactive iron solutions were either ^{125}I-^{59}Fe-labelled diferric rabbit transferrin or Fe-sucrose, prepared by mixing $^{59}FeCl_3$ and $^{56}FeSO_4$ in a ratio of approximately 1:100, and diluting with 0.27 M sucrose to the desired concentrations. These stock solutions were diluted a further 20 times in the incubation media to give the final incubation solutions.

The Fe-sucrose solution was prepared just before commencing the cell incubations and was used between 5 and 15 min after preparation. Due to the very rapid rate of self exchange of Fe (II) and Fe (III) that occurs in aqueous solutions [10], all of the iron would be uniformly labelled with ^{59}Fe by the time the incubations were commenced. The iron remains predominantly in the ferrous state and is taken up by the cells in this form, as shown by its reaction with α,α′-bipyridine and the effects of reducing agents on iron uptake. The Fe-sucrose solution, whether diluted with sucrose or KCl, reacts immediately with α,α′-bipyridine to produce the pink colour typical of ferrous iron. The amount of immediate colour formation falls at approximately 15% per hour during incubation at 37°C. Hence, it appeared that more than 90% of the iron present during the 15-min incubations with the cells would be in the ferrous form. It was also found that ferric iron in sucrose or KCl solutions, prepared in the same way as the Fe-sucrose used in the experiments but with substitution of $FeCl_3$ for $FeSO_4$, produces no pink colour when mixed with α,α′-bipyridine, and that the typical Fe(II)-bipyridine color appears only slowly over a period of hours. Thus, it is unlikely that the results obtained with Fe(II)-sucrose and bipyridine are due an effect of the chelator on the equilibrium ratio of Fe(III) to Fe(II) toward the ferrous side within the time-scale of the experiments. Moreover, it has been shown that the addition of reducing agents, such as ascorbic acid or mercaptoethanol, to incubations containing Fe(II) in sucrose or KCl does not increase iron uptake by reticulocytes [6, 7], and iron uptake from Fe(III) is less than from Fe(II) ([6] and unpublished observations). Therefore, it may be concluded that the results for cellular uptake of iron from the transferrin-free forms of iron described below relate to the uptake of Fe(II) by the cells from Fe(II) present in the incubation solutions.

Measurement of Iron Uptake

The cells were incubated with the ^{59}Fe-labelled solutions in the media described above at 37°C in an oscillating water bath and were, then, washed once with ice-cold 0.15 M NaCl containing 5 mM EDTA, pH 7.4, and twice with cold 0.15 M NaCl, as described previously [5]. Usually, an 0.08 mL cell suspension was incubated with 2.0 mL incubation medium for 15 min. This time was chosen because it has been shown that, with all of the incubation procedures, iron uptake occurs linearly with respect to time for at least 30 min [5, 6, 7]. After the incubation and washing, the cells were haemolysed with 15 mOsM/kg Hepes pH 7.4, separated by centrifugation into cytosolic and stromal fractions, and radioactivity counted in each fraction, as in earlier work [6]. Iron uptake was calculated in terms of nmole Fe per mL cells.

Analytical Methods

The reticulocyte count was made on a dried smear after staining with new Methylene Blue, and the haematocrit by the microhaematocrit method. Measurements of pH were made with a PHM82 pH meter (Radiometer, Copenhagen, Denmark), osmolality with a Fiske One-Ten Osmometer (Fiske, Needham Heights, MA, U.S.A.) and radioactivity with a LKB-Wallac 1282 Compugamma scintillation counter (LKB, Stockholm, Sweden).

RESULTS

Incubation Conditions

In previous investigations, we showed that the uptake of transferrin-bound iron by reticulocytes suspended in NaCl, pH 7.4 or sucrose, pH 5.9, and of sucrose-Fe by cells suspended in sucrose, pH 6.5, saturates when the iron concentration is about 0.5–1 μM [5, 6, 11]. Also, iron uptake by erythroid cells is confined to reticulocytes, and does not occur with mature erythrocytes [5, 12]. Hence, the present studies using these incubation conditions were performed only with reticulocytes, and the Fe concentration in the incubation solutions was 0.5 μM. However, iron uptake by cells suspended in isotonic KCl solutions saturates at a much higher concentration (40–50 μM), and occurs with

erythrocytes as well as reticulocytes [7]. Therefore, cells were incubated in KCl with 20 μM Fe, and many of the experiments were performed using erythrocytes as well as reticulocytes.

In the earlier experiments on sucrose-Fe uptake by cells suspended in sucrose, the maximal rate of iron uptake was found to occur at pH 6.5 [6], and this pH was used for measurement of Fe uptake by cells suspended in KCl [5]. It was decided to determine whether or not this is the optimum pH for such incubation conditions. The pH optimum for reticulocytes was found to be 7.0 and that for erythrocytes, 6.75. However, with erythrocytes, the difference between the values obtained at pH 6.75 and 7.0 was small. Hence, in the experiments described below, incubations with reticulocytes and erythrocytes in KCl were performed at pH 7.0.

The mean values for iron uptake into the cytosol and stromal fractions of cells incubated for 15 min under the standard incubation conditions are presented in Table 1. The amount of uptake from the solutions containing 0.5 μM Fe were similar, with approximately the same relative distribution between cytosol and stromal fractions. The uptake from 20 μM Fe in KCl was much higher, and occurred with erythrocytes as well as reticulocytes, although it was greater in reticulocytes than in mature cells. With reticulocytes, the stroma consists of plasma membrane plus intracellular organelles (e.g. mitochondria, endocytotic vesicles) and iron uptake by the stroma includes iron taken up by all of these components. This probably accounts for the relatively high amount of iron uptake to the stroma. With the short incubation time (15 min), much of the iron taken up would be in mitochondria in transit into haem, as well as in endosomes and plasma membrane. However, mature erythrocytes have no internal organelles, so that stromal uptake is a measure of the iron taken up only by the plasma membrane.

TABLE 1. Iron uptake by reticulocytes and erythrocytes in 4 incubation systems

Incubation system	n	Reticulocyte iron uptake (nmole/mL cells)	
		cytosol	stroma
Tf-NaCl	28	17.9 ± 1.93	8.8 ± 1.22
Tf-sucrose	19	12.6 ± 1.27	9.4 ± 1.11
FeII-sucrose	19	16.1 ± 1.68	11.7 ± 0.95
FeII-KCl	30	197 ± 10.9	86.9 ± 3.83
FeII-KCl	17	107 ± 7.80 E	41.6 ± 2.82 E

The results show iron uptake into the cytosolic and stromal fractions of reticulocytes and erythrocytes (E) after 15-min incubation at 37°C with ^{59}Felabelled iron in 4 different incubation systems. These systems were: transferrin-bound iron in NaCl pH 7.4 (Tf-NaCl), transferrin-bound iron in sucrose pH 5.9 (Tf-sucrose, Fe(II) in sucrose pH 6.5 (FeII-sucrose) and Fe(II) in KCl pH 7.0 (FeII-KCl). The iron concentration in the first 3 systems was 0.5 μM and in FeII-KCl was 20 μM. For details, see text. The results are expressed as nmole Fe per mL cells and are the means (± SEM) of 17–30 estimations. The measurements with erythrocytes were performed only in the FeII-KCl system because these cells take up negligible amounts of iron in the other systems.

Inhibitors of Iron Uptake

Several biochemical reagents, and also Ca^{2+} and Mg^{2+} were found to inhibit iron uptake (Table 2). In general, the biochemical reagents produced quite different degrees of inhibition of iron uptake from the KCl solution than with the other incubation conditions. With the exception of valinomycin, bafilomycin A_1, and NBD,† the effects of the inhibitors on iron uptake from the 3 solutions containing 0.5 μM iron were all very similar. Valinomycin partially inhibited iron uptake from transferrin in NaCl solution, but stimulated the uptake from transferrin or Fe(II) in sucrose solution. Bafilomycin A_1 and NBD inhibited uptake from transferrin in NaCl solution to a greater degree than from transferrin or Fe(II) in sucrose solution. DCCD, NBD, and NEM inhibited iron uptake from these solutions to a much greater degree than uptake from KCl, but the other inhibitors had a greater effect on uptake from KCl.

One mechanism of inhibition of iron uptake from transferrin during incubation of cells in NaCl at pH 7.4 is through inhibition of the rate of endocytosis. This has been shown to be the case with NEM [12] and the possibility was examined in the present work with respect to bafilomycin A_1, DCCD, and NBD (Fig. 1). Bafilomycin was without any effect on endocytosis of transferrin, although it markedly inhibited iron uptake, but the other two reagents inhibited endocytosis as well as iron uptake. NBD almost completely blocked both iron uptake and endocytosis. The effects of these inhibitors on transferrin recycling and efflux were also examined, as described previously [11]. Bafilomycin and DCCD were without effect, and NBD produced a reduction in the rate of transferrin release from the cells (Fig. 2).

The effects of valinomycin and diethylstilbestrol were particularly interesting. Valinomycin markedly inhibited iron uptake from the KCl solution and produced a lesser degree of inhibition with transferrin-iron in NaCl, and it stimulated iron uptake from the sucrose solutions. Other studies will be undertaken to investigate these effects. At a concentration of 10 μM, diethylstilbestrol produced 90% inhibition of iron uptake from the KCl solution. Further studies were performed using concentrations from 0.1 to 50 μM. Iron uptake from KCl solution by reticulocytes and erythrocytes was equally sensitive to the reagent, 50% inhibition (IC_{50}) occurring at 1.1 and 1.2 μM for reticulocytes and erythrocytes, respectively. The diethylstilbestrol analogues, hexestrol and dienestrol, were also inhibitors of the process, with IC_{50} values for reticulocytes and erythrocytes of 0.78 μM and 0.72 μM, respectively, with hexestrol, and 1.15 μM and 1.40 μM with dienestrol.

$CaCl_2$ and $MgCl_2$ at 100 μM concentration had moderate inhibitory effects on iron uptake from the sucrose solutions, the cells being slightly more susceptible to Ca^{2+} than Mg^{2+}. However, with cells incubated in KCl, iron uptake by both reticulocytes and erythrocytes was inhibited to a considerably greater degree by Mg^{2+} than Ca^{2+}. The effects of varying concentrations of the two ions on iron uptake from

TABLE 2. Effects of reagents that inhibit iron uptake by reticulocytes and erythrocytes

Reagent	(μM)	Reticulocyte iron uptake (% control value)				Erythrocyte iron uptake
		Tf-NaCl	Tf-sucrose	FeII-sucrose	FeII-KCl	FeII-KCl
Amiloride	500	91 ± 4.1	88 ± 6.6	94 ± 3.8	12.5 ± 1.0	11.7 ± 2.7
Valinomycin	0.5	45 ± 4.7	214 ± 38	217 ± 38	7.2 ± 1.6	9.4 ± 1.6
Oligomycin	1.0	40 ± 2.3	38 ± 1.6	36 ± 2.3	24 ± 1.5	28 ± 3.5
NEM	100	10.3 ± 1.7	27 ± 2.8	28 ± 5.4	96 ± 1.9	101 ± 3.5
Quinidine	100	89 ± 7.8	68 ± 2.6	70 ± 1.2	31 ± 3.3	36 ± 6.7
Imipramine	10	88 ± 4.2	97 ± 9.8	94 ± 11.3	33 ± 3.6	44 ± 5.0
Quercetin	20	86 ± 3.7	102 ± 12.3	128 ± 14.6	13 ± 3.0	20 ± 3.2
Diethylstilbestrol	10	93 ± 4.5	114 ± 4.7	109 ± 10.7	9.7 ± 0.3	11.0 ± 1.2
Bafilomycin A_1	0.01	10 ± 3.1	88 ± 2.1	81 ± 5.6	101 ± 3.9	99 ± 0.84
DCCD	50	14 ± 1.6	11 ± 1.8	14 ± 2.4	75 ± 6.6	51 ± 3.4
NBD	50	1.5 ± 0.5	26 ± 7.1	26 ± 3.6	81 ± 6.8	108 ± 0.44
$CaCl_2$	100	100 ± 8.7	69 ± 8.3	65 ± 5.6	90 ± 2.8	93 ± 4.0
$MgCl_2$	100	96 ± 5.6	78 ± 8.9	80 ± 2.5	52 ± 1.4	46 ± 2.0

The results show iron uptake into the cytosolic fraction of reticulocytes and erythrocytes that had been preincubated with the reagents for 10 min, before incubation for 15 min with ^{59}Fe-labelled iron, in 4 different incubation systems, as in Table 1. The results are expressed as percent of control values obtained in the absence of the reagents, and are means (± SEM) of 3–4 measurements.

KCl solution were, then, examined. The IC_{50} values for Mg^{2+} were found to be 102 and 90 μM for reticulocytes and erythrocytes, respectively, and 750 and 980 μM, respectively, for Ca^{2+} (means of 2 determinations).

DISCUSSION

Two Iron Transporters

Several lines of evidence show that there is a clear difference between the uptake of Fe(II) from KCl solution and iron uptake from the solutions used in the 3 other systems examined in this investigation. The first arises from the effects of the inhibitors listed in Table 2. Several of the inhibitors (amiloride, quercetin, quinidine, imipramine, diethylstilbestrol, Mg^{2+}) inhibited iron uptake from KCl, but had little or no effect on uptake by the other systems. Others (NEM, DCCD, NBD) inhibited the latter systems, but had much less effect on uptake from KCl. Second, the uptake from KCl occurs in both erythrocytes and reticulo-

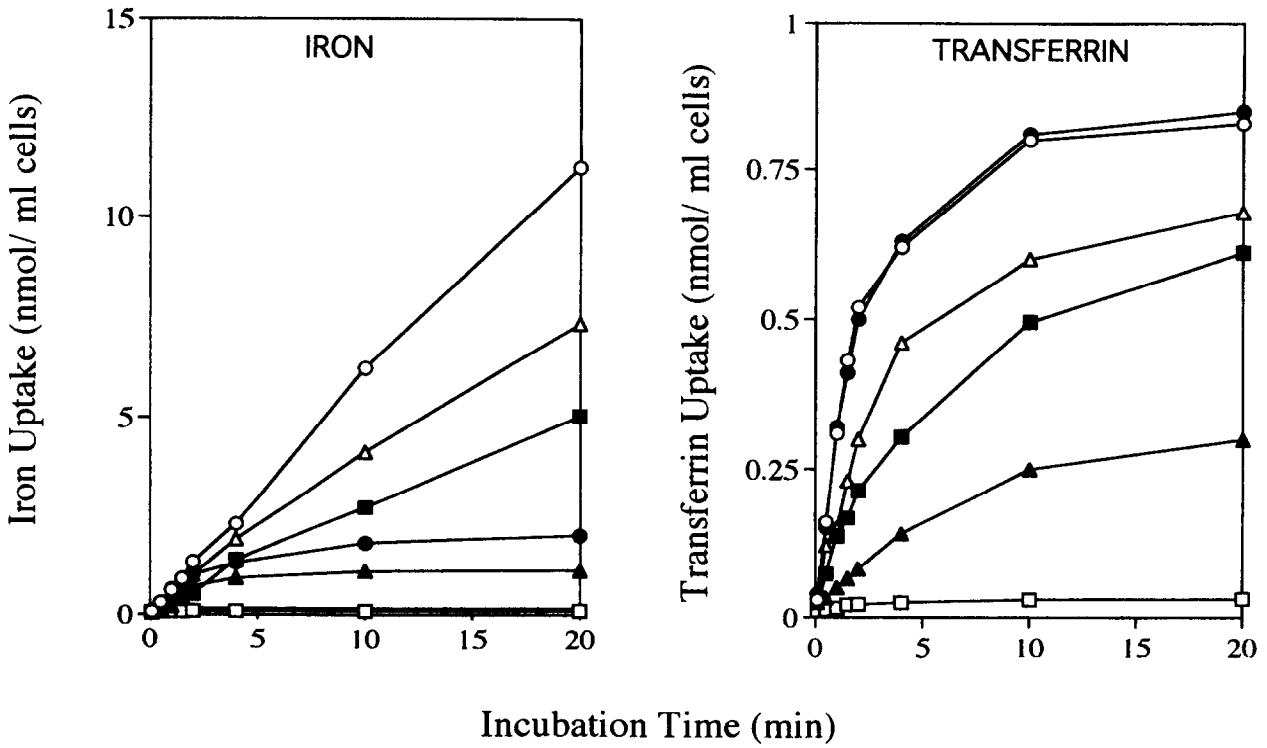

FIG. 1. Iron uptake and transferrin endocytosis by rabbit reticulocytes incubated with 1 μM ^{59}Fe-^{125}I-labelled diferric transferrin at 37°C in 0.15 M NaCl, pH 7.4. The cells were preincubated for 10 min and then incubated with the labelled transferrin in the presence of the following inhibitors: nil (○), 0.5 μM valinomycin (■), 0.01 μM bafilomycin A_1 (●),50 μM DCCD (▲), 50 μM NBD (□).

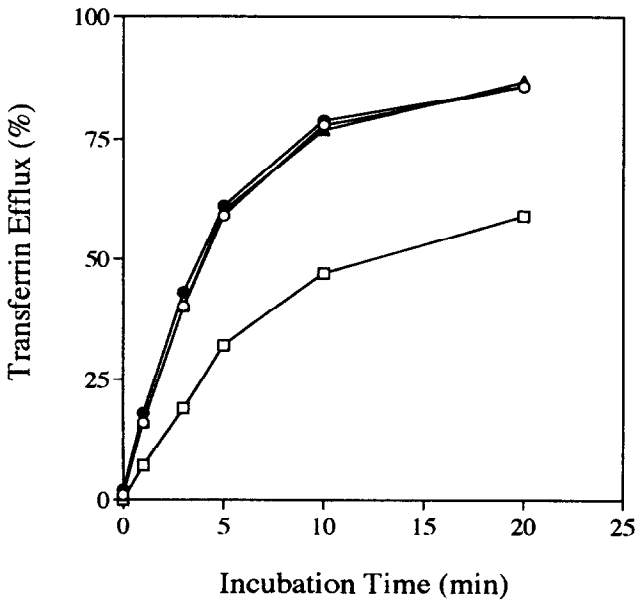

FIG. 2. Effect of bafilomycin A_1, DCCD, and NBD on transferrin efflux from rabbit reticulocytes. The cells were incubated for 15 min with 1 μM ^{59}Fe-^{125}I-labelled diferric transferrin at 37°C in 0.15 M NaCl, pH 7.4. They were, then, washed 3 times with ice-cold NaCl and reincubated in 0.15 M NaCl, pH 7.4 at 37°C with no added reagent (○), 0.01 μM bafilomycin (●), 50 μM DCCD (▲), or 50 μM NBD (□). At the times of reincubation indicated in the figure, 0.2-mL samples of the cells were transferred to 2.0 mL ice-cold NaCl, mixed, centrifuged, and radioactivity counted in the cell and supernatant fractions. The results are expressed as the percentage of the labelled transferrin that was released from the cells at each time-point.

cytes, but occurs only with reticulocytes in the other 3 systems [5, 6]. Third, the rate of iron uptake from KCl is far greater than with the other systems, all of which have similar rates (Table 1). The much higher rate of iron uptake from KCl than from the other solutions is not a simple consequence of the higher iron concentration that was used because, with both erythrocytes and reticulocytes incubated with 20 μM Fe(II), uptake from KCl is much greater than from sucrose [7]. The apparent K_m values are also much greater for uptake from KCl (approximately 17 μM) than from the other 3 solutions which, again, are very similar (0.07–0.2 μM) [5, 12].

Overall, the above evidence points to the close similarity between iron uptake by erythroid cells from transferrin, when incubated in NaCl at pH 7.4 or sucrose at pH 5.9, and from Fe(II) in sucrose at pH 6.5, and a distinct difference between these systems and iron uptake from Fe(II) dissolved in KCl solution. The similarity among the first 3 systems is so close that it strongly suggests that iron transport into the cells occurs by the same transport mechanism, one that has been shown to be saturable, to disappear when reticulocytes mature into erythrocytes, and which is dependent on active cell metabolism [5, 6, 7]. Hence, it probably occurs by the same active transport process. This view is further supported by studies with cells from the Belgrade rat. Homozygous Belgrade rats have a hypochromic, microcytic anaemia that is inherited as an autosomal recessive trait [13]. This abnormality is due to impaired transport of iron across the membranes of immature erythroid cells [14, 15]. Iron transport into the cells from transferrin and from Fe(II) in sucrose was found to be equally impaired [16], but transport of Fe(II) from KCl solution is unaffected by the genetic defect [7].

The most reasonable conclusion from all of the above evidence is that erythroid cells possess 2 distinct iron transport mechanisms, one present only in immature cells that has a low K_m value, and the other present in mature erythrocytes as well as immature cells, that has a higher K_m. For brevity, these 2 processes will be referred to as the high-affinity and the low-affinity mechanisms.

Iron uptake from transferrin in NaCl solution occurs by receptor-mediated endocytosis of the transferrin-iron complex, release of iron from transferrin within the acidic endosome, and transport across the endosomal membrane [1, 2]. However, incubation of reticulocytes in isotonic sucrose blocks endocytosis [17]. Hence, iron transport into cells suspended in sucrose must occur through the outer cell membrane and, if the above conclusion is correct, the transport sites must cycle from the outer membrane through the endosomes during the endocytosis-exocytosis cycle, as does the transferrin receptor. The effects of a low-ionic-strength extracellular medium, such as sucrose, on iron uptake by reticulocytes were studied earlier [5, 18]. It was concluded that the major effect that was responsible for efficient iron uptake was the development of a high negative surface potential on the cell membrane. Other effects of the sucrose medium, such as changes in membrane permeability, transmembrane potential difference, and cell size had little influence on iron transport. The high negative surface potential would aid the approach of Fe(II) to the iron transporter. In the case of iron uptake by endocytosis of transferrin, the interaction of transferrin with its receptor may achieve the same purpose by localizing the transferrin and its iron close to the carrier, and away from cations present in the extracellular or endosomal environment that could compete with iron for binding to the carrier.

The results obtained with some of the inhibitors (valinomycin, bafilomycin, NBD) may, at first sight, appear to conflict with the above conclusions. Valinomycin inhibited iron uptake from transferrin in NaCl, but accelerated it when the uptake was studied using transferrin or Fe(II) in sucrose solution, and bafilomycin markedly inhibited uptake in the first of these systems, but had little effect with the other systems. In the case of valinomycin, the effects can be explained by inhibition of transferrin endocytosis in cells incubated in NaCl (Fig. 1) and an alteration in membrane potential in cells incubated in sucrose. Valinomycin increases the membrane conductance of K^+ and changes the membrane potential from a high positive (inside) value dependent on the $[Cl_i]/[Cl_o]$ distribution ratio, to a lower value dependent on K^+ [18]. This change would be ex-

pected to aid the transport of positively-charged Fe(II) ions into the cell. Bafilomycin is a relatively specific inhibitor of vacuolar H^+-transporting ATPases [19, 20] and, undoubtedly, inhibits iron uptake from transferrin in NaCl solution by inhibiting acidification of the endosome without inhibiting endocytosis (Fig. 1). The high degree of inhibition of iron uptake from transferrin in NaCl solution produced by NBD was shown to be the result of inhibition of endocytosis (Fig. 1).

Mechanism of Iron Transport

The results obtained with the inhibitors used in these experiments give some insight into the mechanisms involved in the two iron transport processes. The inhibitors fall into 3 general types: 1. amiloride, an inhibitor of Na^+-associated cation transport systems such as Na^+/Na^+ and Na^+/H^+ exchange [21], 2. valinomycin, an ionophore with high selectivity for K^+, Rb^+, and Cs^+ [22], and 3. the other inhibitors, which have been shown to block ion-motive ATPases responsible for membrane transport of a variety of mono- and divalent cations, including H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} [23–27]. The sensitivity of both the high- and low-affinity iron transport processes to the latter group of inhibitors suggests that they both involve ion-motive ATPases.

Ion-motive ATPases are considered to be of at least 3 types, "P" or phosphorylated, "F", of the F_oF_1 type found in mitochondria, and "V" or vacuolar because they are often found in membrane-bound organelles other than mitochondria, but may also occur in the plasma membrane [24, 25]. It is unlikely that iron transport involves P- and F-type ATPases. P-type ATPases are resistant to oligomycin and NBD, inhibitors of one or both of the iron transport mechanisms, and are sensitive to vanadate, which does not inhibit low-affinity iron transport [7]. In animal cells, the F-type ATPases are confined to mitochondria, which rules them out as mediators of iron transport across endosomal and plasma membranes.

Different ion transport processes that are mediated by the V-type ATPases are sensitive to one or more of the reagents that inhibited iron transport. This suggests that these ATPases are involved in iron transport, either as an additional function of a known ATPase or through the function of a previously undescribed enzyme. Indeed, the former possibility has been proposed for iron transport across the endosomal membrane, the high-affinity transport process as defined in this paper. The evidence for this comes from experiments in which the H^+-ATPase from reticulocyte endosomes, after isolation and reconstitution into liposomes, was found to be capable of mediating iron transport that was inhibited by DCCD and NBD [27]. The results obtained with bafilomycin, DCCD, and NBD in the present work appear to be in support of this idea because these reagents are known inhibitors of the endosomal proton pump. However, DCCD and NBD are not specific for this process, as shown by their ability to inhibit endocytosis and their actions on other types of ATPases [24, 25]. Although bafilomycin, which is believed to be a more specific inhibitor of the proton pump [19, 20], did inhibit iron uptake from transferrin in NaCl solution, it had little effect on iron uptake using the other 2 high-affinity iron transport systems. It is likely that its inhibitory action on iron uptake from transferrin is dependent on inhibition of endosomal acidification, which has been shown using other techniques to block iron uptake from transferrin effectively [28]. Hence, either bafilomycin acts on the endosomal H^+-ATPase in a manner that blocks its proton but not its iron transporting function, or the proton pump and iron transporter are two different entities.

Inhibition of the low-affinity pathway by amiloride raises the possibility that iron transport is linked with Na^+ transport, possibly through the action of a Na^+/Fe^{2+} countertransport system [29]. In addition, the high degree of sensitivity of iron transport to a number of inhibitors of various transport ATPases suggests that an ATPase is also involved. Particularly interesting is the inhibitory effect of diethylstilbestrol, which had an IC_{50} value of approximately 1 μM, 10 or more times lower than corresponding values for other transport systems [30–32]. Another pertinent observation is the high sensitivity of iron transport to inhibition by Mg^{2+} compared with Ca^{2+}. This suggests that the transport mechanism involves Mg^{2+} as well as Na^+. There is evidence for the presence in erythroid cells of a Mg^{2+}/Na^+ antiport that is inhibited by amiloride, quinidine, and imipramine at concentrations similar to those that inhibit iron uptake [26, 33]. Hence, the question arises as to whether or not this transporter can also mediate iron transport. Evidence in support of this hypothesis has recently been obtained in our laboratory (unpublished observations).

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