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# EFFECT OF LEAD ON THE TRANSPORT OF TRANSFERRIN-FREE AND TRANSFERRIN-BOUND IRON INTO RABBIT RETICULOCYTES

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**Abstract**—The effects of Pb on iron transport into rabbit reticulocytes was investigated using two sources of iron, non-transferrin-bound ferrous iron, Fe(II), and transferrin-bound iron, and fractionating the cells into haem, cytosolic and stromal fractions. Uptake of Fe(II) into all three fractions was inhibited by low concentrations of Pb, 50% inhibition of uptake to the cytosol ($IC_{50}$) occurring at 1 $\mu$M Pb. Fe(II) uptake could be divided into saturable and non-saturable components. The saturable component was inhibited at lower concentrations of Pb than the non-saturable component. Pb reduced the $V_{max}$ and increased the $K_m$ values for saturable Fe(II) transport. The effects of Pb on Fe(II) transport were reversible and were observed with $PbCl_2$ and Pb $(NO_3)_2$ as well as with lead acetate. Pb also inhibited the uptake of transferrin-bound iron but at higher concentrations ($IC_{50}$, 4 $\mu$M) and the inhibition was less readily reversible. The effect was attributable to inhibition of transferrin endocytosis which resulted in a redistribution of transferrin receptors from intracellular to cell surface sites. These results show that Pb can inhibit transferrin endocytosis and iron transport across the cell membrane of reticulocytes and raise the possibility that these effects may contribute to the hypochromic anaemia associated with Pb poisoning, in addition to the previously established inhibition of enzymes of the haem synthesis pathway.

Anaemia of a mild to moderate degreee is a frequent consequence of chronic Pb poisoning in humans. The erythrocytes are usually hypochromic and may be normocytic or microcytic. The cause of the anaemia is believed to be impaired haemoglobin synthesis due to the inhibitory effects of Pb on many of the enzymes involved in haem synthesis [1]. Pb has also been shown to inhibit the uptake of transferrin-bound iron by immature erythroid cells [2–4], although the mechanism by which this occurs is unknown. Possibly the reduction in iron uptake represents a way that Pb impairs haem synthesis in addition to the inhibition of haem-synthesizing enzymes. The aim of the present work was to determine the mechanism of the inhibitory action of Pb on iron uptake by erythroid cells. The experiments were performed with rabbit reticulocytes and two sources of iron. One was iron bound to rabbit transferrin. The other was non-transferrin-bound ferrous iron, Fe(II), present in a stable form in isotonic sucrose. It has been shown that reticulocytes can readily assimilate this form of iron in the absence of transferrin and utilize it for haem synthesis [5]. The uptake process has features of carrier-mediated transport. Hence, the investigation provided the opportunity to study the effects of Pb on what appears to be a ferrous iron carrier in reticulocyte membranes.

## MATERIALS AND METHODS

*Materials.* Iron-59 ($^{59}FeCl_3$) and $^{125}I$ ($Na^{125}I$) were purchased from Amersham International (Amersham, U.K.). Pronase was from Boehringer Mannheim (Mannheim, F.R.G.) and the other biochemicals were from the Sigma Chemical Company (St Louis, MO, U.S.A.). Rabbit transferrin was isolated from plasma and labelled with $^{125}I$ and $^{59}Fe$ as previously described [6]. It was used in the diferric form.

*Cells.* Reticulocyte-rich blood was obtained from rabbits with phenylhydrazine-induced haemolytic anaemia [6] 3–6 days after the last injection of phenylhydrazine. The cells were washed four times in ice-cold 0.155 M NaCl and were then centrifuged at 2000 *g* for 30 min at 4°, the buffy coat removed and discarded and the top one-quarter of the red cell layer then taken off with a Pasteur pipette to obtain a reticulocyte-enriched cell suspension. This was diluted to a haematocrit of 10–20% with 0.27 M sucrose. The reticulocyte count varied from 45–90%. These cells will be referred to as reticulocytes since the mature cells present in the suspension take up very little Fe(II) or transferrin-bound iron when compared with reticulocytes [5].

*Measurement of iron uptake.* For the measurement of the uptake of Fe(II) 0.1 mL of the cell suspension was suspended in a solution of 0.27 M sucrose buffered to pH 6.5 with 4 mM Pipes (piperazine-*N*,*N*′-bis [2-ethanesulfonic acid]) with or without the addition of the Pb salt (usually lead acetate) in concentrations varying from 0.0625 $\mu$M to 32 $\mu$M. The cells were preincubated for 10 min at 37° before the addition of the $^{59}Fe(II)$ in 0.27 M sucrose, prepared as described previously [5], in the amounts required to give iron concentrations from 0.01 to 3 $\mu$M. The incubation was then continued at 37° for the desired time, usually 20 min, after which the cells were washed three times in ice-cold 0.155 M NaCl, transferred to new test tubes, haemolysed and fractionated into haem, cytosol and stromal fractions as

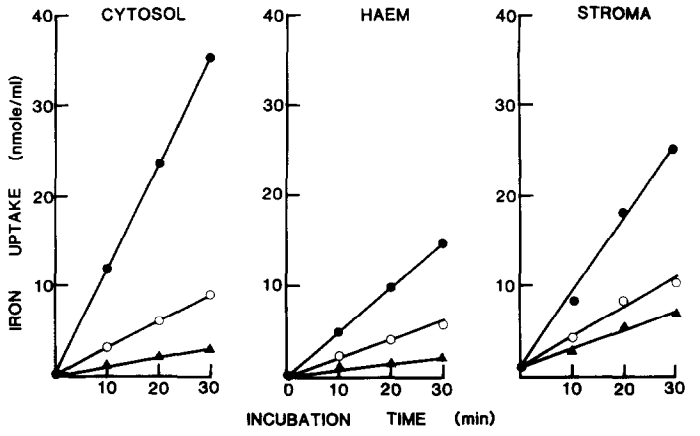


Fig. 1. Uptake of non-transferrin-bound iron into the cytosol, haem and stroma of rabbit reticulocytes. The cells were incubated with 1 μM Fe(II)-sucrose in the absence of Pb (●) or in the presence of 1 μM(○) or 4 μM (▲) lead acetate for the indicated times, then washed and fractionated as described in the text.

in earlier work [5]. Each fraction was then counted for radioactivity. The stromal fraction consists of outer cell membrane plus intracellular organelles such as endosomes and mitochondria.

The uptake of transferrin-bound iron was measured in a similar way except that the incubation medium was 0.155 M NaCl buffered by pH 7.4 with a 4 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and the source of iron was diferric transferrin labelled with $^{125}I$ and $^{59}Fe$. After the incubation the cells were washed and fractionated as for Fe(II) uptake.

In certain experiments the rate of transferrin endocytosis and intracellular accumulation of iron were measured by using a procedure which involves incubation of the radiolabelled and washed cells with Pronase (1.0 mg/mL) for 30 min at 4° as described previously [7]. This leads to the release of receptor-bound transferrin on the outer cell membrane and allows the separation of membrane-bound and intracellular radioactive transferrin and iron. This fractionation procedure is different from that described above which is based on the centrifugal separation of cytosol and stroma of osmotically haemolysed cells. In the Pronase procedure the intracellular fraction contains intracellular organelles such as endosomes and mitochondria as well as the cytosol. In the haemolysis method these intracellular organelles are included with the plasma membrane in the stromal fraction and are separated from the cytosol fraction is devoid of intracellular organelles.

*Analytical methods.* The reticulocyte count was determined by staining with new Methylene Blue and the packed cell volume by the microhaematocrit method. Haem was extracted by the method of Thunell [8]. Radioactivity was counted in a three-channel γ-scintillation counter (LKB-Wallac 1282 Compu-gamma).

## RESULTS

### *Uptake of non-transferrin-bound iron*

Lead acetate inhibited the incorporation of ferrous iron into the cytosol, haem and stromal fractions of the cells. Iron uptake into each of these fractions was linear with respect to incubation time up to at least 30 min (Fig. 1). Hence, it was possible to determine the rate of iron uptake by measuring the amount of $^{59}Fe$ incorporated into cell fractions after incubation for a single time period, 20 min. This procedure was used to determine the effects of Pb concentration on iron uptake (Fig. 2). The transport of iron into the cytosol was inhibited to a greater degree than that into haem at each Pb concentration examined up to 8 μM. Beyond that the inhibition into both fractions was approximately the same and was more than 90% complete. Fifty per cent inhibition of iron uptake to the cytosol and to haem occurred at Pb concentrations of approximately 1 and 2 μM, respectively.

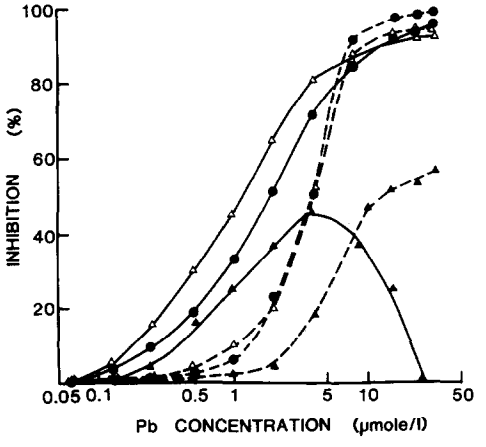


Fig. 2. Effect of Pb concentration on the uptake of non-transferrin-bound iron (——) or transferrin-bound iron (– – –) into the cytosol (△), haem (●) or stromal fraction (▲) of reticulocytes. The cells were incubated with 1 μM Fe(II) or 1 μM transferrin-bound iron for 20 min in the presence of varying concentrations of lead acetate. Each point is the mean from four experiments.

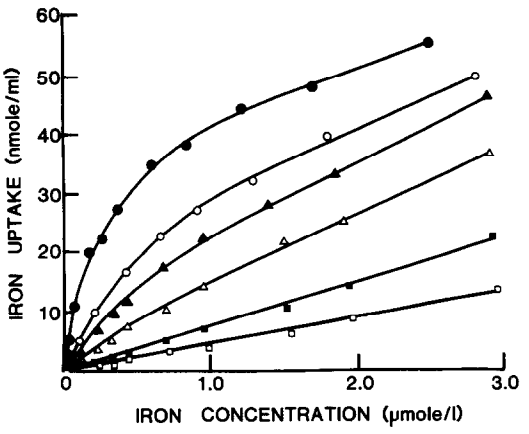


Fig. 3. Uptake of iron into the cytosol of reticulocytes incubated in varying concentrations of Fe(II)-sucrose in the absence of (●) or the presence of 0.5 (○), 1.0 (▲), 2.0 (△), 4.0 (■) or 8.0 (□) μM lead acetate. The incubations with Fe(II) were performed for 20 min. Four experiments of this type were performed, with similar results.

Incorporation of iron into the stromal fraction was also inhibited as the Pb concentration was raised from 0.25 to 4 μM when it reached almost 50% inhibition. However at higher Pb concentrations the inhibition diminished and by 25 μM Pb iron uptake to the stromal fraction had returned to the control level.

The effects of $PbCl_2$ and $Pb(NO_3)_2$ were compared with those of lead acetate. No appreciable differences were observed between the effects of the three lead salts when used at concentrations of 0.5, 2.0 and 8.0 μM.

As was found previously [5], when the reticulocytes were incubated with increasing concentrations of non-transferrin-bound iron the uptake into the cell cytosol showed evidence of saturation at about 1.0 μM Fe(II) (Fig. 3). At higher iron concentrations iron uptake continued to increase in a linear manner. Hence, there is evidence for a saturable, probably carrier-mediated, uptake process and a non-saturable process [5]. In the presence of increasing concentrations of Pb the saturable process was progressively inhibited so that it was no longer detectable above 2 μM Pb (Fig. 3). The non-saturable process was little affected up to this Pb concentration but at higher concentrations it also started to diminish, as indicated by the slopes of the linear parts of the curves in Fig. 3.

Analysis of the results of four experiments of the type illustrated in Fig. 3 using a Newton–Raphson nonlinear curve fitting programme to determine the maximum rate of iron uptake, $V_{max}$, and the Michaelis constant, $K_m$, of the saturable components of the uptake curves showed that Pb reduced the $V_{max}$ values but elevated those for $K_m$ (Table 1). When comparable studies were performed using reticulocytes which were incubated with 0.1 mM succinylacetone (an inhibitor of haem synthesis) a similar effect of Pb on the $V_{max}$ and $K_m$ values was observed although the $V_{max}$ values in the control presence of succinylacetone were less at each Pb concentration than it is absence (Table 1). In control experiments it was shown that 0.1 mM succinylacetone caused approximately 95% inhibition of $^{59}Fe$ incorporation into haem.

As shown in Fig. 4A the inhibitory effect of lead on Fe(II) transport to the cytosol could be reversed by washing the cells with 0.15 M NaCl after incubation with lead acetate. A lesser degree of reversibility was observed with respect of Fe(II) incorporation into haem. Three experiments of the type shown in Fig. 4 were performed, with similar results.

*Uptake of transferrin-bound iron*

The uptake of iron from transferrin was inhibited by Pb, although a higher concentration was required to produce the same degree of inhibition as that observed with ferrous iron; 4 μM Pb produced about 50% inhibition (Fig. 5A). The inhibition of iron uptake was accompanied by a decrease in the rate and amount of transferrin endocytosis, and a redistribution of transferrin receptors, as indicated by the distribution of transferrin taken up by the cells, so that a relatively higher proportion of the total cellular receptors was at the cell surface and less at intracellular sites (Fig. 5B).

A more detailed examination of the effects of Pb concentration on the rate of iron uptake showed that the process of iron uptake from transferrin is less sensitive to Pb than that from ferrous iron. Fifty per cent inhibition of iron transport into the cytosol did

Table 1. Effect of lead acetate on the $V_{max}$ and $K_m$ values for the transport of Fe(II) into the cytosolic fraction of rabbit reticulocytes

| Pb Concentration (μmol/L) | $V_{max}$ (nmol/mL reticulocytes/min) | | $K_m$ (μmol/L) | |
|---|---|---|---|---|
| | −SA | +SA | −SA | +SA |
| 0 | 39.7 ± 5.48 | 25.1 ± 6.81 | 0.246 ± 0.101 | 0.203 ± 0.109 |
| 0.5 | 30.3 ± 4.16 | 18.7 ± 5.52 | 0.302 ± 0.074 | 0.260 ± 0.120 |
| 1.0 | 22.3 ± 3.16 | 12.3 ± 4.06 | 0.313 ± 0.075 | 0.293 ± 0.144 |

The incubations were performed with $^{59}Fe(II)$ in the presence of the indicated concentrations of lead acetate, without (−SA) or with (+SA) the addition of 0.1 mM succinylacetone. The values are the means ±SE of six (−SA) or three (+SA) determinations on the saturable component of the iron uptake process.

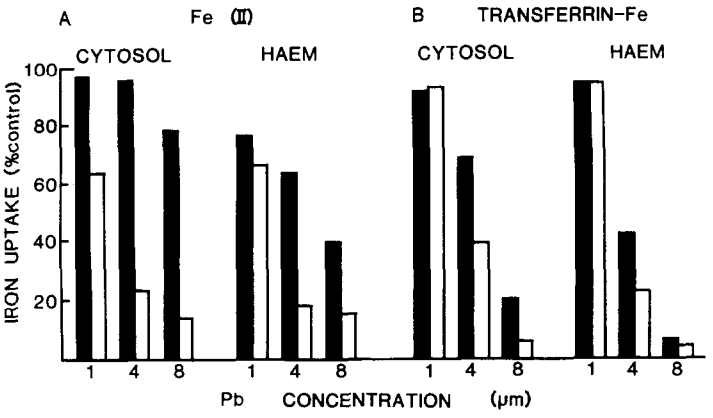


Fig. 4. Reversibility of the effect of Pb on iron uptake by reticulocytes. Triplicate samples of reticulocytes were incubated at 37° for 10 min with lead acetate at the indicated concentrations. Two of each three samples were washed three times with 0.15M NaCl. Pb at the given concentration was then added to one of each of these two samples, and all three samples were incubated with (A)Fe(II) or (B) Fe-transferrin for 20 min at 37°. The figure shows the iron uptake into cytosol and haem of the washed samples to which Pb was not restored (closed columns) and the unwashed samples (open columns) expressed as percentages of the values obtained with control cell samples treated in the same manner but in the absence of lead acetate. Very similar results to those shown by the open columns were obtained with the washed cells to which Pb was restored before incubation with the iron compounds. Three additional experiments of this type were performed with comparable results to those in the figure.

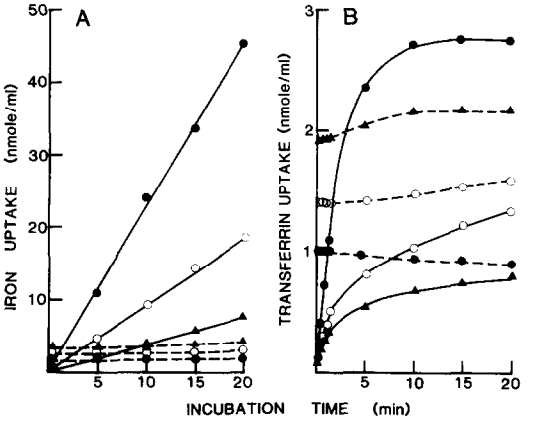


Fig. 5. Uptake of iron (A) and transferrin (B) by rabbit reticulocytes incubated with transferrin-bound iron (1 μM Fe) in the absence of (●) or the presence of 4 (○) or 8 (▲) μM lead acetate. After incubation with diferric [$^{59}$Fe-$^{125}$I]-transferrin for the indicated periods of time the cells were washed and treated with Pronase at 4° as described in the text in order to separate membrane-bound (- - -) and intracellular (——) iron and transferrin. The figure illustrates one out of four experiments, all of which had similar results.

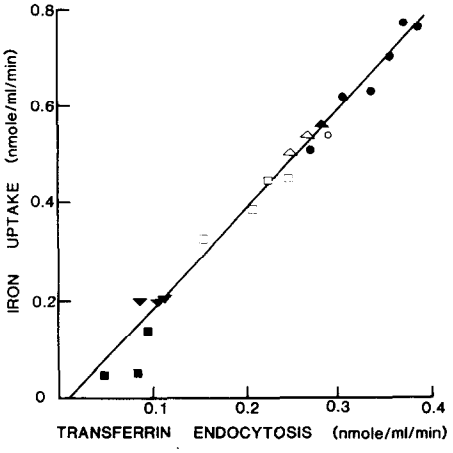


Fig. 6. Relationship between the rates of iron uptake and transferrin endocytosis by rabbit reticulocytes in the presence of varying concentrations of Pb. The cells were incubated with diferric [$^{59}$Fe-$^{125}$I]transferrin in the absence of (●) or in the presence of 0.25 (○), 0.5 (▲), 1.0 (△), 4.0 (□), 8.0 (▼) or 10.0 (■) μM lead acetate. The equation for the regression line is $y = 0.48x + 0.015$ and the correlation coefficient, $r$, is 0.991.

not occur until the Pb concentration was 4 μM (Fig. 2). Other differences are that the degree of inhibition of iron incorporation into haem was almost the same as that into the cytosol at all Pb concentrations, inhibition of iron incorporation into the cell stroma continued to increase as the Pb concentration was raised above 4 μM (Fig. 2) and the inhibition of iron uptake was less readily reversed by washing the cells after incubation with Pb than was the case with Fe(II) uptake (Fig. 4B).

The above results suggested that the reduction in iron uptake produced by Pb could be the result of inhibition of transferrin endocytosis. Hence, the effects of different Pb concentrations on the rates of transferrin endocytosis and cellular accumulation of iron were determined by taking serial cell samples during 15 min incubation with labelled transferrin and calculating the rate of iron uptake and the initial rate of transferrin endocytosis as previously described [7]. A very close correlation ($r = 0.991$) was observed (Fig. 6). The slope of the regression

line indicated that approximately two iron atoms were accumulated by the cell for each molecule of transferrin which was endocytosed, as expected if iron uptake from diferric transferrin is dependent on transferrin endocytosis and the endocytic cycle leads to the donation of both iron atoms for each transferrin molecule taken up even in the presence of Pb.

## DISCUSSION

The results of these experiments demonstrate that Pb inhibits the uptake of both transferrin-free and transferrin-bound iron by rabbit reticulocytes. In neither case was there any evidence that haem synthesis, as indicated by $^{59}Fe$ incorporation into haem, was more sensitive to inhibition by Pb than was iron uptake by the cells. Hence, there is no evidence that the inhibition of iron uptake was secondary to prior inhibition of haem synthesis. Indeed, when haem synthesis was inhibited independently of Pb by the use of succinylacetone it was found that Pb still inhibited the uptake of Fe(II) by the reticulocytes. It is generally believed that the hypochromia of the erythrocytes found in the anaemia of Pb poisoning is primarily due to inhibition of one or more of the enzymes involved in haem synthesis [1]. The present work raises the possibility that inhibition of uptake of iron by erythropoietic cells may also contribute to the lack of haemoglobinization of the cells.

The mechanisms by which Pb inhibits the uptake of Fe(II) and Fe-transferrin by reticulocyte are different. In the case of Fe(II) the inhibitory effects were exerted on both the saturable and non-saturable components of the uptake process. The former has features of carrier-mediated transport [5] and was sensitive to low concentrations of Pb. The reduction in $^{59}Fe$ uptake into the cytosol produced by varying concentrations of Pb was similar to that previously reported as resulting from the addition of the same concentrations of non-radioactive Fe(II) to the incubation medium and was greater than that of several divalent metals at similar concentrations [5]. However, unlike the other metals which act as competitive inhibitors of Fe(II) uptake ($Co^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Zn^{2+}$) the action of Pb appeared to be of mixed type, as evidenced by the reduction of $V_{max}$ as well as elevation of apparent $K_m$ values for the saturable component of the Fe(II) uptake process.

The non-saturable component of Fe(II) uptake was also inhibited by Pb but at higher concentrations, at about the same concentrations at which the inhibitory effects of Pb on Fe(II) incorporation into the stromal fraction of the cells began to diminish (4–8 $\mu$M Pb). The stromal fraction of reticulocytes is a multi-component fraction of the cells, containing intracellular organelles (e.g. mitochondria, endosomes) as well as external membrane. It seems likely that Pb exerts at least two effects on the cell membrane which affect Fe(II) uptake, namely, (1) inhibition of transport through the membrane by a carrier mechanism or channel and (2) effects on the cell membrane which lead to increased adsorption of Fe(II) without transport to the cytosol, the first being more sensitive to low concentrations of Pb. At low concentrations Fe(II) transport into the cytosol and into mitochondria which are included in the stromal fraction would be inhibited, leading to the reduction of Fe(II) uptake into the stromal as well as cytosolic fraction of the cells. At higher levels of Pb the second effect would lead to an increase, rather than a reduction in Fe(II) incorporation into the stromal fraction. The mechanism responsible for this effect is unknown.

These experiments confirm that Pb inhibits the uptake of transferrin-bound iron by reticulocytes [2–4] and they also provide evidence for the mechanism involved. There was a reduction in transferrin endocytosis as well as iron uptake, and when the rates of the two processes which occurred at varying concentrations of Pb were compared a highly significant correlation was observed. That is, at all concentrations of Pb, and in the absence of Pb, the rate of transferrin endocytosis was approximately equivalent to the rate of iron uptake, taking into account the diferric state of the transferrin used in these experiments. Hence, the inhibitory effects of Pb on iron uptake can be attributed to an inhibition of transferrin endocytosis. This did not appear to be due to a reduction in the affinity of the transferrin receptors for transferrin since there was increased, rather than decreased, binding of [$^{125}I$]transferrin to the outer cell membrane in the presence of Pb when compared with its absence. Overall, the observed results may be explained by an inhibitory effect of Pb on the endocytotic process, with less effect on exocytosis, so that there is a redistribution of transferrin receptors from intracellular sites (endosomes) to the outer cell membrane.

An important question in cellular iron metabolism is the mechanism by which iron released from transferrin within endosomes passes through the membrane lining these organelles into the cytosol. Does it occur by the same carrier mechanism as is responsible for Fe(II) uptake? The results of the present investigation appear to oppose this possibility, since the uptake of Fe(II) was inhibited by Pb concentrations which had little effect on the uptake of transferrin-bound iron, and the primary inhibitory effect on the latter process was on endocytosis which is the rate-limiting step in iron uptake from transferrin under most conditions [9]. However, the conditions used for the measurement of Fe(II) and Fe-transferrin uptake were quite different with respect to pH, ionic strength and salt composition of the incubation media. Hence, it would be inappropriate to draw any conclusions from the apparent different sensitivities of the two processes to Pb. It is necessary to use the pH 6.5 sucrose-containing low ionic strength medium for measurement of Fe(II) uptake because Fe(II) transport is inhibited by NaCl or other salts and by higher pH levels [5].

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## REFERENCES

1. Waldron HA and Stofen D, *Sub-clinical Lead Poisoning*. Academic Press, New York, 1974.
2. Jandl JH, Inman JK, Simmons RL and Allen DW,

Transfer of iron from serum iron-binding protein to human reticulocytes. *J Clin Invest* **38**: 161–185, 1959.
3. Allen DW and Jandl JH, Kinetics of intracellular iron in rabbit reticulocytes. *Blood* **15**: 71–81, 1960.
4. Lothe K and Falbe-Hansen I, Incorporation of $^{59}Fe$ into erythrocyte nonhaem iron and haemoglobin in anaemic and lead-poisoned rabbits. *Clin Sci* **24**: 47–54, 1963.
5. Morgan EH, Membrane transport of non-transferrin-bound iron by reticulocytes. *Biochim Biophys Acta* **943**: 428–439, 1988.
6. Hemmaplardh D and Morgan EH, The mechanism of iron exchange between synthetic iron chelators and rabbit reticulocytes. *Biochim Biophys Acta* **373**: 84–89, 1974.
7. Iacopetta BJ and Morgan EH, The kinetics of transferrin endocytosis and iron uptake from transferrin by rabbit reticulocytes. *J Biol Chem* **258**: 9108–9115, 1983
8. Thunell S, Determination of the incorporation of $^{59}Fe$ in hemin in peripheral red cells and red cells in bone marrow cultures. *Clin Chim Acta* **11**: 321–333, 1965.
9. Morgan EH, Transferrin, biochemistry, physiology and clinical significance. *Mol Aspects Med* **4**: 1–123, 1981.