Biochimica et Biophysica Acta, 373 (1974) 84–99

BBA 76785

THE MECHANISM OF IRON EXCHANGE BETWEEN SYNTHETIC IRON CHELATORS AND RABBIT RETICULOCYTES

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(Received June 4th, 1974)

SUMMARY

The aim of this investigation was to examine the hypothesis that the mechanism of iron uptake by reticulocytes from synthetic iron chelators is dependent on an initial exchange of iron from the chelators to transferrin present in the cells. The experiments were performed with rabbit reticulocytes and transferrin, several iron chelators, and ^{59}Fe to trace the movement of iron. The following results were obtained:

1. The amount of transferrin present in rabbit reticulocytes after washing with ice-cold saline was found to be approx. 140 μg/ml cells.

2. The relative rates of iron uptake by reticulocytes from chelators was shown to be directly related to rates at which iron exchanged between the chelators and transferrin.

3. Depletion of cellular transferrin caused a marked reduction in the rate of iron uptake by reticulocyte from synthetic chelators but had much less effect on iron uptake from transferrin.

4. Radioactive iron extracted from reticulocyte stroma after incubation of the cells with ^{59}Fe labelled chelator solutions had the properties of transferrin-bound iron when studied by dialysis, immunoprecipitation, disc electrophoresis and gel filtration.

The results of these experiments confirm the above hypothesis.

INTRODUCTION

Most or all of the iron acquired by immature erythroid cells and used for haemoglobin synthesis is derived from plasma transferrin. Experiments in vitro have shown that iron bound to transferrin [1] is utilized by reticulocytes for haemoglobin synthesis more efficiently than is inorganic iron [2] or iron bound to many small molecular weight iron complexing agents [3] (such agents will subsequently be called "chelators"). However, with certain chelators (e.g. citrate, nitrilotriacetic acid, *N*-β-hydroxyethyliminodiacetic acid (HEDA)) the iron is utilized almost as well as is transferrin-bound iron [3, 4]. The varying availability of chelator-bound iron for reticulocytes cannot be explained by the known chemical or physical properties of the chelators or

* Abbreviation: HEDA, *N*-β-hydroxyethyliminodiacetic acid.

their affinity constants for ferric or ferrous iron. The utilization of chelator-bound iron does, however, have certain features in common with the utilization of transferrin-bound iron. Thus, both processes are highly temperature dependent and are blocked by inhibitors of oxidative metabolism and by sulphydryl reagents, which have been shown to inhibit transferrin uptake by reticulocytes [4]. In addition, both processes are inhibited by vinblastine which interferes with the function of cellular microtubules [5]. Another relationship between transferrin and other iron chelators is suggested by the observation that certain chelators, such as citrate and nitrilotriacetic acid which readily donate iron to reticulocytes also exchange it quickly with transferrin, while EDTA and desferrioxamine, which are poor sources of iron for reticulocytes, exchange iron with transferrin at a very slow rate [6, 7].

The above observations suggest the possibility that an initial step in the process of reticulocyte iron uptake and utilization from chelators involves iron exchange between the chelators and transferrin which is present in the cells even though they have been washed free of plasma prior to incubation with the chelator solutions. The iron would then be transported and metabolized in the same way as transferrin-bound iron. The aim of the present investigation was to examine this hypothesis using rabbit transferrin and rabbit reticulocytes and several chelators with varying ability to donate iron to reticulocytes. The investigation was performed in four parts. Firstly, the effects of washing of reticulocytes on their content of transferrin, and the amount of transferrin bound by reticulocytes in vivo were determined. Secondly, the relationship between the rates of iron exchange between different chelators and reticulocytes, and between chelators and transferrin, was investigated. The effect of depleting reticulocytes of transferrin on their capacity to take up iron from chelators was studied in the third part of the work. Finally, attempts were made to determine whether radioactive iron in the stroma or reticulocytes after uptake from chelator was bound by transferrin.

MATERIALS AND METHODS

Chemicals

Sodium citrate, nitrilotriacetic acid, HEDA and EDTA were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., catechol (*O*-dihydroxybenzene) from B.D.H. Chemicals Ltd, Poole, England; Tiron (disodium-1,2-dihydroxybenzene-3,5-disulfonate monohydrate) from G. Frederick Smith Chemical Co., Columbus, Ohio, U.S.A., and Sephadex G-200 from Pharmacia Fine Chemicals A.B., Uppsala, Sweden. Desferrioxamine was provided by CIBA Ltd, Basle, Switzerland. ^{59}Fe ($FeCl_3$, 10–20 Ci/g), ^{125}I and ^{131}I (NaI, carrier free) were purchased from the Radiochemical Centre, Amersham, England.

Rabbits and rabbit reticulocytes

Young adult hybrid rabbits of both sexes were used. They were housed in individual cages and were fed pelleted rabbit diet plus fresh vegetables. In the in vivo experiments the drinking water contained 0.1 g NaI/l. Haemolytic anaemia was induced by the daily subcutaneous injection of neutralized phenylhydrazine hydrochloride in 0.15 M NaCl in a dose of 6 mg/kg body weight. Haemorrhagic anaemia was produced by bleeding from a marginal ear vein. Reticulocytes were obtained from

animals with either type of anaemia by bleeding from a marginal ear vein and using heparin as an anticoagulant. Except where indicated, the blood cells were washed three times with 10–15 vol. of ice-cold 0.15 M NaCl and were then suspended in Hanks and Wallace balanced salt solution [8] before use. No attempt was made to separate reticulocytes from the other blood cells, but for brevity, the cells of reticulocyte-rich blood will be referred to as reticulocytes.

Transferrin and albumin purification and labelling

Transferrin and albumin were prepared from rabbit serum as previously described [9, 10]. The transferrin was recrystallized three times before use. Both proteins were considered to be at least 99 % pure when examined by polyacrylamide gel electrophoresis. They were labelled with radioiodine by the iodine monochloride method to a level of 0.5–1.0 iodine atom per protein molecule [11]. Free radioactive iodine was removed by ion-exchange chromatography using IRA410 followed by dialysis against 0.15 M NaCl. Iron was removed from transferrin by dialysis against 0.01 M EDTA–acetate buffer, pH 5.5, until colourless, followed by dialysis in turn against water, 0.1 M $NaClO_4$, 0.02 M $NaHCO_3$ and water. The apoprotein was then freeze dried. It was labelled with ^{59}Fe by dissolving in 0.15 M NaCl–0.01 M $NaHCO_3$ and adding the iron as its nitrilotriacetic acid complex in an amount which would not exceed 30–50 % of the iron-binding capacity of the protein. The ^{59}Fe–nitrilotriacetic acid complex was prepared immediately before use by adding 1 mM nitrilotriacetic acid to the ^{59}Fe in a molar ratio of nitrilotriacetic acid to Fe of 3 : 1 and then adjusting the pH to 7 with 0.1 M $NaHCO_3$.

Antiserum to rabbit transferrin was produced in sheep by giving three intramuscular injections of 10 mg transferrin mixed with Freund's complete adjuvant at 2 week intervals. The sheep were bled from the jugular vein two weeks after the last transferrin injection. The antiserum was shown to be specific for transferrin by immunoelectrophoresis against whole rabbit serum.

Chelator solution

Solutions of the iron chelators were prepared and labelled with ^{59}Fe as previously described [4].

In vivo experiments

Haemolytic anaemia was produced in two rabbits by the daily injection of phenylhydrazine for 6 days. 2 days later they were given a single intravenous injection of 10 mg of ^{125}I-labelled transferrin. Blood samples were taken after 10 min and then at different time intervals over 40 days. Haematocrit, reticulocyte count, plasma transferrin concentration, plasma radioactivity and cell-bound radioactivity after washing the cells four times with ice-cold 0.15 M NaCl were measured on each blood sample. Cell samples obtained 24, 48 and 72 h after injecting the labelled transferrin were also used for some of the in vitro experiments. The volume of blood taken from the rabbits was 2–2.5 ml for most samples, but at 24, 48 and 72 h, samples of 4–5 ml were collected. A second series of six injections of phenylhydrazine were given on the 13–18th day after injecting the transferrin.

In vitro experiments

The procedures used for incubating reticulocytes with labelled chelators or

transferrin (and labelled albumin in one experiment), for washing the cells and for counting radioactivity were the same as in earlier work from this laboratory [4, 12].

Preparation and extraction of reticulocyte ghosts

Washed reticulocytes were haemolysed with 20 vol. ice-cold 0.02 M sodium phosphate buffer, pH 7.4. The ghosts were separated from the haemolysate by centrifugation at $10\,000 \times g_{av}$ for 20 min at 4 °C and were then washed twice with the same phosphate buffer as used for haemolysis. The washed ghosts were extracted in the cold with 10 vol. 1 % sodium deoxycholate or 1 % Triton X-100. After mixing with the detergents they were allowed to stand in an ice bath for 30 min and were then centrifuged at $40\,000 \times g_{av}$ for 60 min to separate residue and solubilized extract.

Deoxycholate was removed from the extract by dialysis in turn against 0.02 M $NaHCO_3$, water and 0.02 M Tris, pH 6.5. Deoxycholate extraction, followed by removal of the deoxycholate, was used for the experiments in which the iron-binding components of the ghost extract were examined by polyacrylamide gel electrophoresis and by immunoprecipitation with antiserum against rabbit transferrin. Triton X-100 was used where the extract was fractionated by gel filtration, using a column of Sephadex G-200 (2.5 cm × 60 cm) equilibrated with 1 % Triton X-100–0.15 M NaCl and eluting with the same solution.

Analytical methods

The haematocrit was measured using a Hawksley microhaematocrit centrifuge (Hawksley Co., London, England). Reticulocytes were counted on dry smears after staining with new methylene blue. Protein was measured by a biuret procedure [13]. Plasma transferrin was determined by radioimmunodiffusion [14]. Immunoelectrophoresis was performed by the method of Scheidegger [15]. Polyacrylamide gel electrophoresis was performed as described by Davis [16], using 7.5 % gels and a Hoffer apparatus, model EF301 (Hoffer Scientific Instruments, San Francisco, Calif., U.S.A.). Red cell size distribution curves and red cell volumes were obtained using a Coulter Counter (Coulter Electronics Ltd, Dunstable, Beds., England). Radioactivity was counted in a Packard Autogamma 3-channel well-type scintillation counter (Packard Instrument Co., Downers Grove, Ill., U.S.A.). Incorporation of ^{59}Fe into haem was determined by extracting haem from washed reticulocytes by the method of Thunell [17]. Immunoprecipitations from aqueous solution were performed by incubating transferrin solutions and dialysed deoxycholate extracts of reticulocyte ghosts with an excess of anti-transferrin serum for 1 h at 37 °C followed by standing at 4 °C for 16 h. The resultant precipitates were washed three times with ice-cold 0.15 M NaCl and then dissolved in 1 M NaOH for counting of radioactivity.

RESULTS

Effect of washing on elution of transferrin from reticulocytes

Reticulocytes from a rabbit with haemolytic anaemia and mature erythrocytes from an untreated rabbit were incubated with a mixture of ^{125}I-labelled transferrin and ^{131}I-labelled albumin for 1 min at 4 °C and for 15 min at 37 °C.The cells were then washed eight times with 17.5 vol. ice-cold 0.15 M NaCl. Radioactivity was measured in each wash solution and in the cells after the last wash. The pattern of change

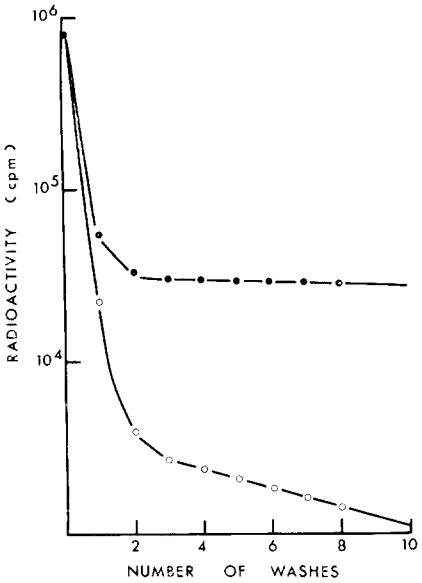

Fig. 1. Changes in cellular ^{125}I-labelled transferrin (● – ●) and ^{131}I-labelled albumin (○ – ○) during washing of rabbit reticulocytes. A sample of 0.17 ml blood cells (75 % reticulocytes) from a rabbit with haemolytic anaemia was incubated at 37 °C for 15 min with 0.2 ml of a solution containing 0.6 mg ^{125}I-labelled transferrin (spec. act. $1.3 \cdot 10^6$ cpm/mg) and 5.7 mg ^{131}I-labelled albumin (spec. act. $1.4 \cdot 10^5$ cpm/mg). The cells were then washed eight times with 3.0 ml ice-cold 0.15 M NaCl. The figure shows the amount of radioactivity present in the cell fraction after each wash.

in the radioactivity of the cell fraction with successive washes was similar for all aliquots of cells. Both labelled proteins were lost from the cell fraction rapidly during the first two washes, presumably due to removal of incubation medium, but with subsequent washes the loss of radioactivity slowed and could be represented by a single exponential function. The results obtained with reticulocytes which had been incubated at 37 °C are shown in Fig. 1. The rates of loss of transferrin and albumin from the cell fraction during washing after the second wash varied between the two

TABLE I

ELUTION OF TRANSFERRIN AND ALBUMIN FROM RETICULOCYTES AND MATURE ERYTHROCYTES DURING WASHING

The cells were incubated with ^{125}I-labelled transferrin and ^{131}I-labelled albumin for 15 min at 4 or 37 °C prior to washing eight times with 17.5 vol. ice-cold 0.15 M NaCl. The elution of the proteins was calculated from the rate of loss of radioactivity from the cells during the 3–8th washes.

Incubation temperature	Rate of protein loss from cells (percent cell radioactivity per wash)			
	Reticulocytes		Mature erythrocytes	
	Transferrin	Albumin	Transferrin	Albumin
4 °C	9.9	17.8	10.8	17.8
37 °C	1.7	12.8	5.2	18.7

proteins and between the different aliquots of cells (Table I). It was greater for albumin than for transferrin and, in addition, much less albumin than transferrin remained in the cell fraction after the second wash. The rate of elution of transferrin from the cells was greater with mature cells than with reticulocytes and was greater after incubation at 4 than at 37 °C. The rates of elution of albumin were little affected by the original incubation temperature or degree of maturity of the cells.

Transferrin binding by reticulocytes in vivo

The changes in plasma ^{125}I-labelled transferrin concentration in the two rabbits with haemolytic anaemia which were injected with the labelled protein are shown in Fig. 2. Plasma radioactivity fell rapidly during the first 2 days after injection and then more slowly at rates equivalent to single exponential curves throughout the remainder of the experiment except for the second period of anaemia when plasma radioactivity temporarily fell below the lines of the exponentials. The half-times of the exponentials for the two rabbits were 4.6 and 5.0 day, respectively.

The results shown in Fig. 3 illustrate the changes which occurred during and after the two periods of anaemia. The figure shows the reticulocyte responses which accompanied the fall in haematocrit. It may also be seen that the plasma transferrin concentration was higher during the periods of anaemia and fell after recovery of the haematocrit to normal levels. Cell-bound transferrin was calculated from the specific activity of the plasma transferrin and the amount of radioactivity remaining on the cells after washing four times. It correlated closely with the reticulocyte count. Reticulocyte-bound transferrin was also calculated for the two periods of haemolytic anaemia. The calculation was made on the assumption that transferrin was only bound by reticulocytes, not by mature erythrocytes, and by using the reticulocyte counts and the mean cell volumes of the reticulocytes and mature erythrocytes found

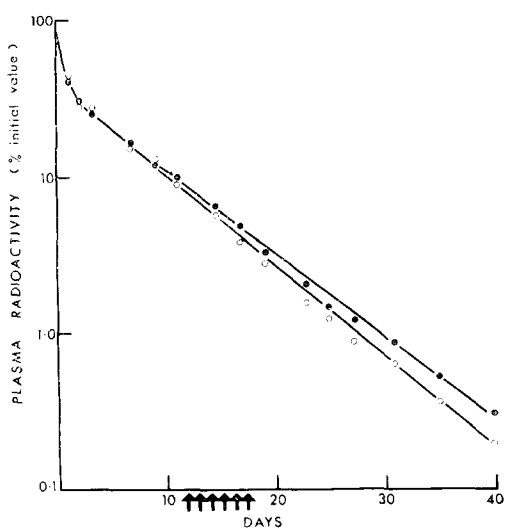

Fig. 2. Changes in plasma radioactivity in two rabbits after injection with ^{125}I-labelled transferrin. Phenylhydrazine, 6 mg/kg, was given at the times indicated by the arrows, and on days 7 to 2 prior to the injection of the labelled transferrin.

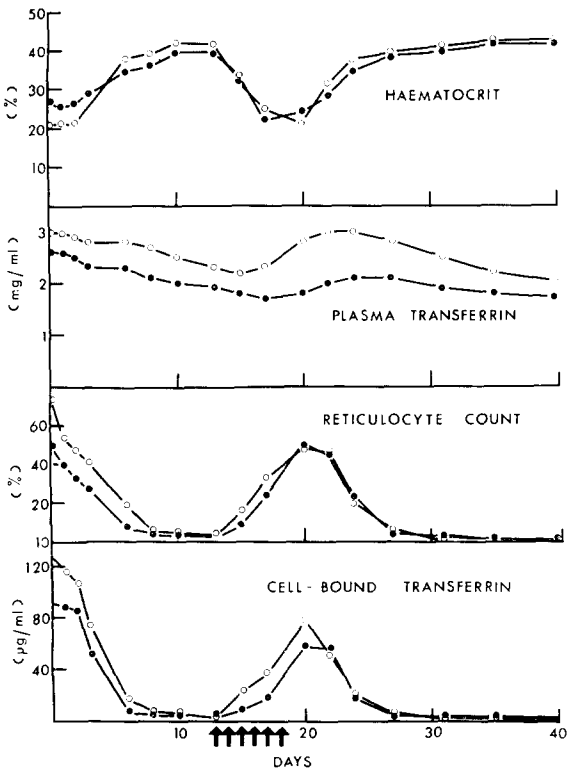

Fig. 3. Changes in haematocrit, plasma transferrin concentration, reticulocyte count and cell-bound transferrin in two rabbits treated with phenylhydrazine on days 7 to 2 prior to commencing the measurements and at the times indicated by the arrows.

during the anaemic periods (110 and 70 μCi, respectively). Mean values of 133±32 (mean±S.D.) and 140±21 μg transferrin per ml reticulocytes were found for rabbits 1 and 2, respectively.

It was shown by precipitation of plasma samples and haemolysates of washed reticulocytes with 10% trichloroacetic acid that more than 98% of the ^{125}I in the plasma and cells of the two rabbits used in the in vivo experiment was protein bound.

Rates of iron transfer from chelators to reticulocytes and to transferrin

The rates of ^{59}Fe uptake by reticulocytes from transferrin and several different chelators were measured by taking aliquots of the cells at different times during incubation at 37 °C and determining the rate of increase of cell-bound radioactivity with respect to time of incubation. The iron concentration of all solutions was 1.5 μg/ml. Transferrin and the chelators, except citrate, were used at concentrations with iron-binding capacities of three times this level. Sodium citrate was used at 20 times the molar concentration of iron. The rates of iron exchange between transferrin and most of the chelators were determined by measuring the change in absorbance at 465 nm which occurred at 22 °C when apotransferrin was added to solutions of iron bound to the chelators. However, because of the high absorbance at 465 nm of the iron com-

TABLE II

RATE OF ^{59}Fe UPTAKE AND PERCENT INCORPORATION INTO HAEM BY RETICULOCYTES FROM CHELATORS AND RATE OF IRON EXCHANGE BETWEEN CHELATORS AND TRANSFERRIN

Reticulocytes were incubated with ^{59}Fe bound to transferrin and other iron chelators. The rate of ^{59}Fe uptake was measured over a 30-min period. Cellular uptake of ^{59}Fe is expressed as a percentage of the value obtained with transferrin-bound ^{59}Fe. The percentage of the ^{59}Fe in the cells which was present in haem was measured at the end of 30 min incubation. The rate of iron exchange between chelators and transferrin was measured as described in the text. The results are given as the time required to obtain 50 % iron exchange between chelator and transferrin.

Chelator	Rate of ^{59}Fe uptake (percent transferrin value)	Incorporation of ^{59}Fe into haem (%)	Time for 50 % iron exchange, chelator to transferrin
Transferrin	100	78	–
Citrate	67	67	<0.5 min
HEDA	70	63	<0.5 min
Nitrilotriacetic acid	57	71	<0.5 min
Catechol	16	18	2 min
Tiron	10	42	15 min
EDTA	1	0	26 h
Desferrioxamine	0	0	>45 h

plexes of catechol and Tiron, iron exchange from these two chelators to transferrin was measured by immunoprecipitation of transferrin at different time intervals after mixing apotransferrin with the chelator–iron solutions labelled with ^{59}Fe and measuring the rate of disappearance of ^{59}Fe from the supernatant solution obtained after centrifugation. The results are summarized in Table II. The rates of iron uptake by reticulocytes from citrate, nitrilotriacetic acid and HEDA was 57–70 % as great as that from transferrin, while the rates from catechol and Tiron were lower, and those from EDTA and desferrioxamine were only 1 and 0 % of the transferrin value, respectively. In the case of desferrioxamine a small amount of ^{59}Fe was bound by the cells immediately on mixing but the ^{59}Fe in the cells did not increase with time of incubation at 37 °C. The incorporation of ^{59}Fe into haem with the first three chelators was almost as efficient as with transferrin. The rate of iron exchange between chelator and transferrin was greatest with the chelators which donated their iron to the cells most rapidly and was least with EDTA and desferrioxamine.

Requirement of cellular transferrin for iron uptake from chelators

Two experiments were performed to evaluate the need for cell-bound transferrin in the uptake of ^{59}Fe by reticulocytes from synthetic chelators. In the first experiment (Fig. 4) reticulocytes obtained from the rabbits used in the in vivo experiment 48 h after injecting the labelled transferrin was treated in two ways. All of the cells were first washed three times with ice-cold 0.15 M NaCl, then half was washed a further four times with cold 0.005 M glucose–0.001 M sodium phosphate–0.15 M NaCl (pH 7.4). The other half was washed 4 times with 20 vol. of the same solution but was incubated at 37 °C for 15 min with each wash solution. As shown in Fig. 4A the

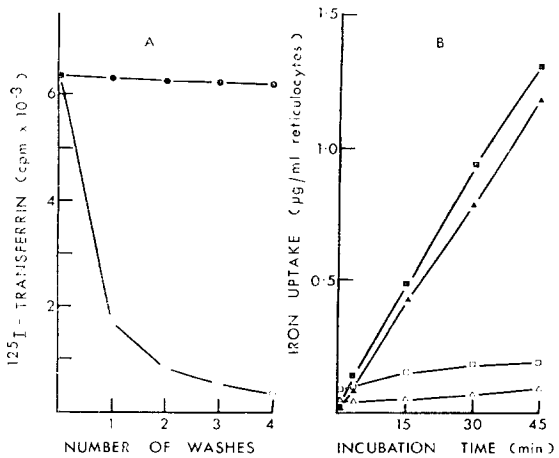

Fig. 4. (A) Changes in cell-bound ^{125}I-labelled transferrin in reticulocytes washed with ice-cold glucose–phosphate–saline (● — ●) or with the same solution but incubated for 15 min at 37 °C with the solution at each wash (○ — ○). The cells were obtained from the two rabbits used in the in vivo experiment 48 h after injecting them with ^{125}I-labelled transferrin. The cell samples consisted of 0.25 ml cells (reticulocyte count 42%). The mean specific activity of the plasma transferrin of the two rabbits at this time was $2.6 \cdot 10^5$ cpm/mg. (B) Rate of uptake of iron bound to citrate (■ — ■, □ — □) or to nitrilotriacetic acid (▲ — ▲, △ — △) by reticulocytes previously washed in the cold (■ — ■, ▲ — ▲) or at 37 °C (□ — □, △ — △) as described in A. The results have been corrected for the reticulocyte count, assuming that only reticulocytes take up significant amounts of iron [2, 4].

latter procedure depleted the cells of ^{125}I-labelled transferrin acquired in vivo, but washing with cold glucose–phosphate–saline produced little reduction in cell-bound transferrin, agreeing with the effects of washing cells labelled in vitro (Fig. 1). The two samples of cells were then incubated with ^{59}Fe bound to citrate and to nitrilotriacetic acid. Iron uptake was rapid and linear for at least 45 min with cells washed in the cold, but was reduced to very low levels in the cells depleted of transferrin (Fig. 4B).

In the second experiment reticulocytes from a rabbit with haemorrhagic anaemia were washed three times with cold 0.15 M NaCl, then incubated for 15 min with ^{125}I-labelled transferrin and re-washed with cold NaCl, in order to label cellular transferrin. The cells were then divided into four aliquots and each was treated in a different way, viz. (i) washed four times with ice-cold glucose–phosphate–saline, remaining at 4 °C in each wash solution for 15 min, (ii) washed four times with glucose–phosphate–saline but incubated at 37 °C for 15 min with each wash solution, (iii) washed in the same manner as (ii) but after the last wash incubated for 3 min with a solution of transferrin in glucose–phosphate–saline, concentration 2.5 mg/ml, (iv) washed in the same manner as (ii) but transferrin (2.0 mg/ml) was included in each washing solution. All of the cells were then washed three more times in ice-cold 0.15 M NaCl. At the end of these washing procedures the amounts of ^{125}I-labelled transferrin present in cells (ii), (iii) and (iv) were 6.9, 6.1 and 3.9%, respectively, of the ^{125}I-labelled transferrin present in cells (i). Aliquots of the four types of cells were then incubated with ^{59}Fe bound to transferrin, citrate, HEDA and nitrilotriacetic acid. The results with transferrin and citrate are shown in Fig. 5. The results with HEDA and nitrilotriacetic acid were almost identical with those of citrate. Cells (ii), (iii) and

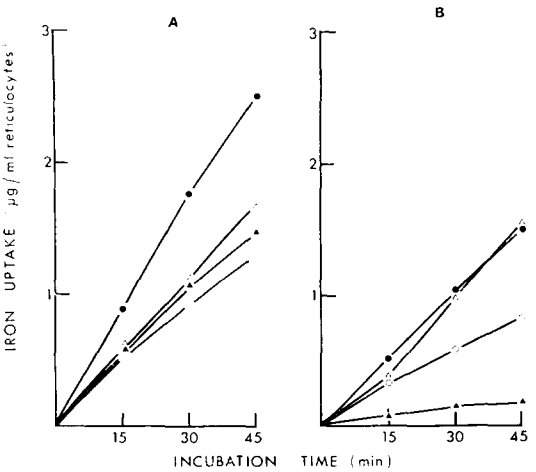

Fig. 5. Rate of uptake of iron bound to transferrin (A) or to HEDA (B) by reticulocytes which had been washed in the following ways: (i) ● — ●, washed four times in ice-cold glucose–phosphate–saline; (ii) ▲ — ▲, washed four times with glucose–phosphate–saline but incubated at 37 °C, for 15 min with each wash solution; (iii) ○ — ○, washed four times at 37 °C for 15 min with glucose–phosphate–saline, then incubated for 3 min at 37 °C with a solution of 2.5 mg/ml transferrin; (iv) △ — △, washed four times at 37 °C for 15 min with glucose–phosphate–saline containing 2.0 mg/ml transferrin. The cells were then washed a further three times with ice-cold 0.15 M NaCl before iron uptake was measured. The results have been corrected for the reticulocyte count.

(iv) showed a 30–40 % reduction in the rate of iron uptake from transferrin when compared with cells (i), but the rate for cells (ii) was similar to the rates for cells (iii) and (iv). With the ^{59}Fe-labelled chelator solutions, however, the rate of iron uptake was greatly reduced with cells of type (ii), but was increased markedly above this level when the cells were provided with additional transferrin ((iii) and (iv)), especially with cells (iv) in which iron uptake was as great as with cells (i).

Investigation of cellular substances binding Fe after uptake from chelators

The chemical nature of the ^{59}Fe present in reticulocytes following incubation with ^{59}Fe bound to transferrin, and the chelators was investigated in several experiments using a variety of techniques. In these experiments the cells were first labelled with ^{125}I-labelled transferrin, either in vivo or by incubation in vitro, in order to have labelled transferrin as a marker substance. The cells were then washed and incubated with the ^{59}Fe solutions.

In the first experiment 0.2 ml reticulocytes were incubated with ^{59}Fe bound to transferrin and other chelators for 1 and 5 min and were then washed three times, haemolysed with 0.5 ml water and the haemolysate was dialysed in turn against water (20 ml), water (100 ml) and 20 mM EDTA–sodium acetate, pH 5.4 (20 ml), each for 12 h. Control experiments using ^{59}Fe-labelled transferrin and rabbit haemoglobin showed that none of the radioactivity could be dialysed into water, but ^{59}Fe of transferrin was completely dialysed into the EDTA solution while no ^{59}Fe-labelled haemoglobin dialysed under these conditions. ^{59}Fe bound to the other chelators all passed through the dialysis membrane when water or EDTA solution was used. After incubation with transferrin, citrate, nitrilotriacetic acid and HEDA little of the cell ^{59}Fe

TABLE III

DIALYSIS OF RETICULOCYTE HAEMOLYSATE AFTER INCUBATION OF THE CELLS WITH ^{59}Fe-LABELLED CHELATORS

Reticulocytes were incubated with the labelled chelators for 1 and 5 min, then haemolysed and dialysed in turn against water and 20 mM EDTA, pH 5.4. Radioactivity was measured in the cells before dialysis (total ^{59}Fe,) in each dialysis solution and in the cell stroma after dialysis. The results for dialysis solutions and cell stroma are expressed as a percentage of the total ^{59}Fe.

Chelator	Incubation time (min)	Total ^{59}Fe (cpm)	Total ^{59}Fe (%)		
			Water dialysate	EDTA dialysate	Stroma
Transferrin	1	17 400	2	81	17
Citrate		12 800	2	68	30
HEDA		10 800	0	74	26
Nitrilotriacetic acid		6 200	4	68	28
Tiron		3 300	15	76	9
EDTA		140	100	0	0
Desferrioxamine		850	67	23	10
Transferrin	5	55 600	1	42	57
Citrate		36 800	2	54	44
Nitrilotriacetic acid		23 800	2	55	43

could be dialysed into water but a large proportion dialysed into the EDTA solution (Table III). This proportion was less after 5 min incubation than after 1 min incubation. With ^{59}Fe-labelled EDTA and ^{59}Fe-labelled desferrioxamine a large proportion of the cellular ^{59}Fe dialysed into the water used in the first two dialyses.

In subsequent experiments reticulocytes were incubated with ^{59}Fe bound to chelators or $^{59}FeSO_4$, washed, haemolysed and the washed ghosts extracted with sodium deoxycholate or Triton X-100. As shown in Table IV, 60–82 % of the ^{59}Fe

TABLE IV

PROPORTION OF RETICULOCYTE STROMAL ^{59}Fe IN TRANSFERRIN-CONTAINING FRACTIONS

Reticulocytes were incubated with chelator-bound ^{59}Fe and $^{59}FeSO_4$ for 2 or 15 min, washed, haemolysed, the stroma extracted with 1 % sodium deoxycholate or 1 % Triton X-100 and the extract fractionated as described in the text. The results are given as the percentage of the total ^{59}Fe of the stromal extracts which was found in the transferrin-containing fraction.

Extraction solution	Fractionation method	Incubation time (min)	Stromal ^{59}Fe in transferrin fraction (%)			
			Incubation solution			
			Citrate	Nitrilotri-acetic acid	HEDA	$FeSO_4$
Sodium deoxycholate	Immunoprecipitation	2	60	74	66	67
		15	60	75	68	82
Sodium deoxycholate	Polyacrylamide gel electrophoresis	2			54	76
		15			57	56
Triton X-100	Gel filtration	2	80	71	75	

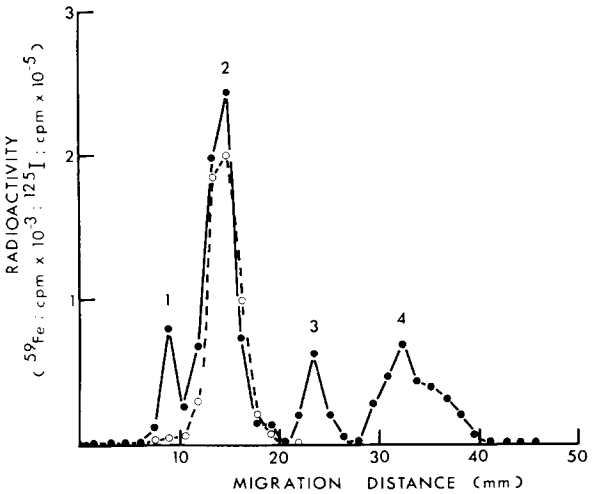

Fig. 6. Fractionation of reticulocyte stromal ^{59}Fe and ^{125}I by polyacrylamide gel electrophoresis after incubation of the cells labelled with ^{125}I-labelled transferrin with ^{59}Fe bound to HEDA for 2 min. Following incubation with ^{59}Fe-labelled HEDA an extract was prepared from the washed stroma as described in the text using sodium deoxycholate and was fractionated by polyacrylamide gel electrophoresis followed by slicing of the gel into 1.5-mm sections and counting for radioactivity. ● – ●, ^{59}Fe; ○ – ○, ^{125}I.

in extracts obtained with deoxycholate precipitated when incubated with antiserum against rabbit transferrin. Some of these extracts were also fractionated by polyacrylamide gel electrophoresis. The gels were run in duplicate, one for staining and the other was sliced into 1.5-mm sections which were counted for radioactivity. Radioactive iron appeared as 4 peaks (numbered 1 to 4 in Fig. 6). Peaks 1 and 2 corresponded to haemoglobin and transferrin, as indicated by the positions of these two proteins when run as single species on other gels. Peaks 3 and 4 migrated faster than transferrin but did not correspond to protein-staining bands. The ^{125}I-labelled transferrin present in all the samples migrated to the position of the transferrin band and was used to identify this region in the unstained gels. The proportion of the total ^{59}Fe of the samples which was found in the transferrin-containing band varied from 54 to 76 % (Table IV).

The reticulocyte ghost extracts obtained by the use of Triton X-100 were fractionated by gel filtration on Sephadex G-200. The ^{59}Fe was eluted as three peaks, a small one at the void volume of the column, a large one corresponding to the elution volume of transferrin and a third small peak at a greater elution volume. The proportion of the total ^{59}Fe which was eluted in the large transferrin-containing peak was 71–80 % (Table IV).

DISCUSSION

It has been known for many years that the initial step in the process of iron exchange between transferrin and reticulocytes involves uptake of the protein by the cells [18, 19] and that transferrin can be demonstrated on reticulocytes [20]. The pres-

ent experiments allow measurements to be made of the amount of transferrin carried by rabbit reticulocytes in vivo and suggest that cell-bound transferrin is required for the utilization by reticulocytes of iron bound to various chelators. The results also suggest that cellular transferrin acts as an intermediary carrier of iron in its transfer from the chelators or inorganic iron to the site of haemoglobin synthesis.

The mean amount of reticulocyte-bound transferrin found in the two rabbits studied in vivo was approx. 140 μg/ml reticulocytes (0.0025 μmole/ml). This is very similar to the amount of labelled transferrin taken up by rabbit reticulocytes when they have been incubated for 20–30 min in solutions of transferrin at concentrations corresponding to those in plasma [19]. By this time of incubation equilibrium has been established, the original cellular transferrin having been replaced by labelled transferrin, and transferrin molecules are entering and leaving the cells at equivalent rates.

The evidence that cellular transferrin is required for the utilization of iron bound to chelators but not for iron bound to transferrin resides in the demonstration that depletion of cellular transferrin caused a marked reduction in the rate of iron uptake from chelators (Figs. 4 and 5B). The presence of transferrin in the solution used to wash the cells at 37 °C would have prevented depletion of cellular transferrin. Iron uptake by these cells (cells (ii), Fig. 5B) from chelators was not diminished relative to control cells (cells (i), Fig. 5B). Replacement of transferrin in depleted cells by incubation at 37 °C with a transferrin solution for 3 min restored the rate of iron uptake from chelators to about 55 % of the control value (cells (iii), Fig. 5B). Probably this short period of incubation was not sufficient to completely replenish cellular transferrin, since 10–15 min incubation is usually required for reticulocytes to achieve maximal transferrin uptake [18, 19]. This may be why the rate of iron uptake did not return completely to the control level. However, the short incubation time was chosen so that the treatment of cells (iii) would differ as little as possible from that of cells (ii) and (iv) in Fig. 5B.

Further evidence that cellular transferrin is involved in the utilization of chelator-bound iron and inorganic iron is provided by the results of the dialysis, gel filtration, disc electrophoresis and immunoprecipitation investigations which showed that much of the reticulocyte stromal ^{59}Fe which had been taken up from chelators or $FeSO_4$ appeared in the transferrin-containing fractions (Table IV). The immunoprecipitation and polyacrylamide gel electrophoresis results strongly suggest that thus ^{59}Fe was bound to transferrin. The gel filtration results support such a conclusion but this procedure does not separate transferrin and haemoglobin.

The demonstration that reticulocyte transferrin is required for the uptake of iron from chelators allows a rational explanation to be made for the varying ability of chelators to donate iron to the cells. A major factor determining the capacity of chelator-bound iron to be utilized by reticulocytes would be the rate at which iron can exchange between the chelator and transferrin. This conclusion is supported by the finding of a direct relationship between the relative rates of iron transfer from the chelators to reticulocytes on one hand to transferrin on the other (Table II). In the case of inorganic iron the efficiency of uptake and utilization probably depends on how effectively the iron is maintained in an ionized form which can react with transferrin, since at neutral pH iron ions are rapidly hydrolysed to form polynuclear iron–hydroxide complexes [21] which may adsorb to cell surfaces but react

only at extremely slow rates with the iron-binding sites of transferrin.

If cellular transferrin is required for the uptake of chelator-bound iron or inorganic iron it is not necessary to postulate that such iron enters the cell by a pathway different from that taken by transferrin-bound iron. It also provides an acceptable explanation for the effects of metabolic inhibitors and other chemical agents on the uptake of iron from the chelators. Presumably sulphydryl reagents and vinblastine block the uptake of citrate-bound ^{59}Fe not because they stop transferrin uptake by the cell but because they stop the movement of transferrin in the cell. Whether this movement occurs in the cell membrane only, or whether it occurs inside the cell membrane is not certain, although some experimental results support the latter suggestion. Thus, there is evidence from autoradiography [22] and electron microscopic studies of reticulocytes incubated with transferrin conjugated to ferritin that transferrin can enter reticulocytes, probably by endocytosis [23]. If this is the case it is likely that the sulphydryl reagents and vinblastine act by inhibiting endocytosis. Possibly endocytosis is necessary for the movement of cellular transferrin between the cell membrane, where it can accept iron from chelators, and some intracellular site where the iron is donated to another cellular carrier or else directly to the site of haem synthesis.

The cell washing experiment (Fig. 1) illustrates that three washes with ice-cold saline which are usually employed in reticulocyte incubation experiments are sufficient to remove all of the suspending medium. In the case of cells incubated with transferrin at 37 °C the small amount of transferrin eluted from the cells during washing would be insufficient to produce significant errors in measurement of cell-bound protein. However, with transferrin and low incubation temperature or with albumin (and possibly other proteins) at any temperature elution during washing would produce a considerable reduction in cell-bound protein so that results obtained would underestimate the amount of protein present on the cells prior to washing. The results also show that little transferrin should be eluted from reticulocytes during washing in the cold prior to incubation experiments. This was demonstrated by determining the amount of transferrin eluted during washing cells from the rabbits used in the in vivo experiment (Fig. 4). The washing experiments demonstrate the higher affinity of reticulocytes than of mature erythrocytes for transferrin which has been observed previously [18, 24]. This is generally considered to be due to the presence of receptors for transferrin on the reticulocyte cell membrane and that such receptors disappear as the cell matures. The greater affinity of reticulocytes for transferrin after incubation at 37 °C than at 4 °C and the lack of effect of incubation temperature on affinity of the cells for albumin indicate a basic difference in the mechanisms of binding of the two proteins. This confirms the conclusions drawn from other experiments [25].

The results of the in vivo experiments illustrate several interesting aspects of transferrin metabolism in the rabbit. Firstly, there was no evidence that the rate of catabolism of transferrin was greater during the periods of haemolytic anaemia and increased erythropoiesis than it was when the animals had recovered from the anaemia. The fall in the plasma radioactivity values below the lines of the exponentials shown in Fig. 2 during the second period of anaemia was probably due to expansion of the plasma volume in compensation for the reduction in red cell volume. The radioactivity would not have returned to the levels of the exponentials if the fall during the anaemic period had been due to increased catabolism. Shepp and co-workers [25]

have recently published other evidence that transferrin catabolism is not increased in haemolytic anaemia in rabbits, whereas data from humans suggests that some increase may occur in comparable conditions [26]. Secondly, the plasma concentration of transferrin increased during the periods of haemolytic anaemia and fell upon recovery. The changes in plasma concentration were probably due to corresponding alterations in rate of synthesis, since there was no evidence of changed rates of catabolism and any alterations in plasma volume would have been in the opposite direction to those required to produce the observed alterations in plasma transferrin level. The present results confirm earlier observations that plasma iron-binding capacity increases during haemolytic anaemia in the rabbit [27]. This change contrasts with the fall which is usually found in humans under such conditions [28].

A third interesting aspect of the in vivo experiment is that it allows an estimation to be made of the total amount of cell-bound transferrin in the circulating blood volume of the rabbits. It may be calculated from the values for cell-bound transferrin, haematocrit and plasma volume that approx. 4.1 and 4.9 mg transferrin were bound during the peaks of haemolytic anaemia and 0.23 and 0.21 mg after recovery from the anaemia in the two rabbits. The two higher values represent only about 1.3 % of total circulating transferrin in each animal. Presumably a further quantity of transferrin is bound by the bone marrow but, on the basis of data obtained in vitro [29], it is unlikely that more than a further 1–2 % is bound to this tissue. Now, the amount of transferrin in the circulating blood plasma is only about half of the total extracellular transferrin [30]. Hence, the amount of transferrin bound by reticulocytes and bone marrow is probably less than 1 % of the total amount of transferrin in extracellular fluid in the normal rabbit and less than 2 % in animals with enhanced erythropoiesis. Variations in the amount of transferrin bound by immature erythroid cells are therefore unlikely to have a significant effect on the plasma transferrin concentration.

ACKNOWLEDGEMENTS

The writers are indebted to Miss A. de Melker for skilled technical assistance. This work was supported by grants from the Australian Research Grants Committee and the University of Western Australia.

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