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EARLY EVENTS IN GUINEA PIG RETICULOCYTE IRON UPTAKE

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Summary

Hemolysates, prepared from guinea pig reticulocytes incubated with ^{59}Fe-labelled serum, can be resolved into five peaks utilizing molecular sieve chromatography: ferritin, transferrin, hemoglobin, an M_r 17 000 fraction, and a low molecular weight fraction. The hemoglobin peak also contains a nonhemoglobin component (III-X), demonstrated by heme extraction and by isoelectric focusing. Transferrin, the III-X component and the low molecular weight fraction are the first to accumulate radioactive iron during the reticulocyte incubation. The ^{59}Fe in each of these also chases. Therefore, a role for these components as precursors to iron incorporation into heme is suggested.

Introduction

The ligands which provide iron to protoporphyrin for the synthesis of heme and to apoferritin for the production of ferritin have not been identified. In vitro, transferrin can donate iron directly to mitochondria for heme synthesis [1], but the process is relatively inefficient [2] and it is not clear that in vivo iron transfer follows this route. A labile intracellular iron pool has been postulated to be the intermediary of intracellular iron exchange [3]. The hypothesis rationalizes a variety of observations but its experimental support is limited to the finding that a substantial portion of iron which has newly entered the cell can be chelated. Iron bound to transferrin, ferritin or heme would not be readily released to a chelate and these molecules are probably not the source of readily chelatable iron.

We have attempted to identify intracellular iron ligands in the reticulocyte and to assess their role in iron transport. Hemolysates, obtained from guinea

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

pig reticulocytes incubated with ^{59}Fe-labelled serum for short time periods, were subjected to Ultrogel filtration. Transferrin, a nonheme ^{59}Fe-containing peak eluting in almost the same position as hemoglobin, and a low molecular weight ^{59}Fe-containing peak accumulated counts earliest. The counts in these peaks chased in pulse-chase experiments. A role for these ligands in intracellular iron transport is suggested.

Materials and Methods

Materials. Reticulocytes were prepared by repeated bleeding of guinea pigs (approximately 10 ml per bleed, 3—5 bleeds). Guinea pig transferrin was purified from guinea pig plasma by ammonium sulfate precipitation, Sephadex filtration and DEAE-Sephadex chromatography [4]. Ferritin was prepared from guinea pig liver by heat coagulation, ammonium sulfate precipitation, G200 Sephadex filtration and Sepharose 6B filtration [5]. Antisera to these proteins were prepared as previously described [5].

The iron-binding protein found in guinea pig liver (called GIBP) [6] was prepared [7]. Antibody to purified iron binding protein was raised in male New Zealand rabbits by giving 50 μg iron-binding protein emulsified in complete Freund's adjuvant, in multiple intracutaneous injections. The rabbits were also given single injections of pertussis vaccine [8]. Antisera gave single precipitin lines of identity when tested by immunodiffusion against iron-binding protein and liver homogenate. Immunoprecipitation with antisera employed a double antibody technique [5].

Incubation of reticulocytes. Reticulocyte-rich blood was collected in heparinized tubes and washed three times in ice-cold 0.1 M NaCl/0.02 M Hepes (pH 7.0) (buffer). In some experiments cells were incubated in the buffer at 37°C for 20 min prior to the last wash to deplete the cells of endogenous transferrin [9] ('pre-incubated'), in other experiments this warming step was omitted. After the last wash, the blood was mixed with an equal volume of ice-cold ^{59}Fe-labelled serum and placed in a 37°C water bath. Samples were removed at designated intervals, plunged into ice-cold buffer and washed three times. In chase experiments the labelled, washed reticulocytes were resuspended in iron-saturated, unlabelled serum and incubated at 37°C for designated times and then washed a further three times. When the chase was to be prolonged beyond 10 min, the serum contained 1000 units/ml penicillin and 0.5 mg/ml glucose.

Cells were hemolyzed in 3 vol. deionized water and centrifuged at 40 000 $\times g$ for 30 min at 4°C. The supernatant was frozen in aliquots and stored in liquid nitrogen. When pellets were to be counted they were washed twice by suspension in buffer and collected after each washing by centrifugation (40 000 $\times g$ for 30 min). (A third wash removed less than 4% of counts remaining after the second wash.)

Filtration through Ultrogel. Hemolysate was thawed, centrifuged at 169 000 $\times g$ for 70 min and the supernatant was applied to Ultrogel columns (1.6 cm $\times$ 131—135 cm) and eluted with 0.05 M NaCl/0.02 M Hepes (pH 7.0) [5].

Heme extraction. Samples were extracted with methylethylketone and chromatographed [5].

Isoelectric focusing. 4% polyacrylamide gels, containing 2% ampholyte (pH 5—7, Biorad) were used. Samples were concentrated on Amicon UM 10 membranes. In one experiment an LKB 8102 electrofocusing column was used to separate whole hemolysate. The hemolysate, mixed with 1/10 vol. 50% sucrose, was layered onto the column at approximately the midpoint in its filling with the ampholyte/sucrose mixture.

Results

In the first set of experiments, reticulocyte-rich blood (39% reticulocytes) was washed in ice-cold buffer mixed with ice-cold ^{59}Fe-labelled serum, placed in a 37°C water bath and sampled at timed intervals. Iron uptake was 71 ng Fe/ml packed cell volume at 5 min and 85 ng Fe/*ml* packed cell colume at 10 min. In the second set of experiments, reticulocyte-rich blood (40% reticulocytes) was incubated at 37°C for 20 min prior to the third wash in order to deplete the reticulocytes of endogenous transferrin [9]. The red cells, rechilled by the third wash, were mixed with ice-cold ^{59}Fe-labelled serum and placed in the 37°C water bath. Uptake was 129 ng Fe/ml packed cell volume at 5 min and 170 ng Fe/ml packed cell volume at 10 min. In both sets of experiments aliquots of the 10-min incubations were subjected to cold chases. Hemolysates from these incubations were filtered through Ultrogel columns and this resulted in five count peaks. Representative single experiments are shown in Fig. 1. The average of repeated experiments is shown in Table I.

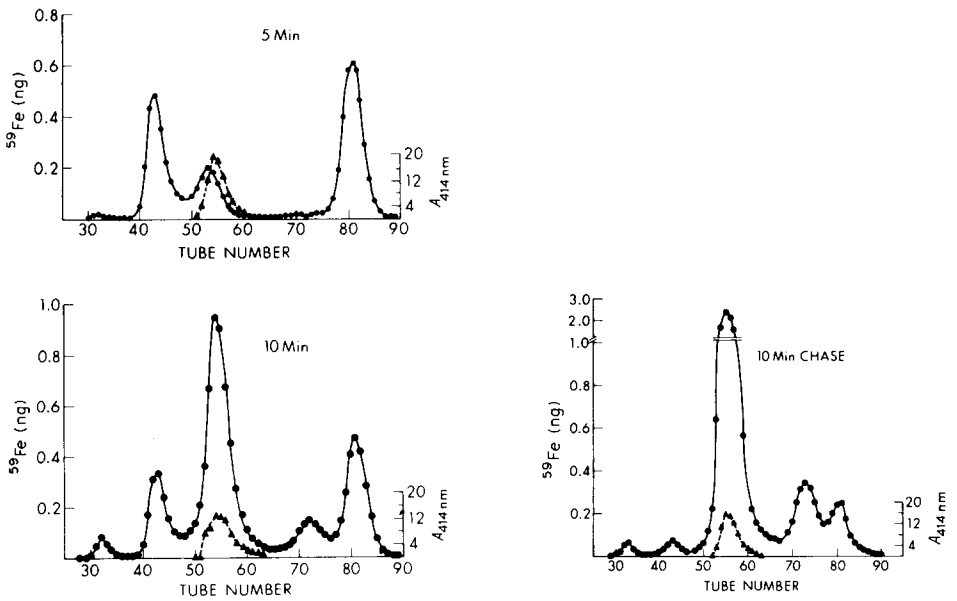

Fig. 1. Reticulocytes (depleted of endogenous transferrin by 'pre-incubation') were incubated for 5 or 10 min in ^{59}Fe-labelled serum and then processed directly to prepare a hemolysate, or were washed and incubated for an additional 10 min in unlabelled serum and then processed to prepare a hemolysate. 0.8 ml samples of these hemolysates were applied to Ultrogel AcA 44 columns (131—135 cm) and eluted with upward flow at 12—13 ml/h. 15 min fractions were collected. ●, ng ^{59}Fe; ▲, A_{414nm}.

TABLE I

IRON CONTENT OF ULTROGEL PEAKS

Guinea pig reticulocytes (prepared either with or without pre-incubation to deplete endogenous transferrin) were incubated with ^{59}Fe-labelled guinea pig serum. In the chase experiments reticulocytes were washed after 10 min and resuspended in iron-saturated, unlabelled serum for 10 min. Hemolysates, prepared as described in the text, were filtered through Ultrogel AcA 44 columns. Peak III was extracted with methylethylketone to assess the proportion of counts in heme. Calculation of iron content was based on specific activity of the labelled serum. Count recovery in the nonpreincubated reticulocyte experiments averaged 83%, and in the pre-incubated reticulocyte experiments, 90%. The values represent ng ^{59}Fe and are the mean of several determinations, the number of which is given in parentheses.

	Peak I	Peak II	Peak III	Peak IV	Peak V
Reticulocytes (non-preincubated)					
5 min (2)	0.005	0.83	0.17 (16%) *	0.02	0.29
10 min (3)	0.005	0.65	1.17 (88%)	0.13	0.70
Chase (2)	0.17	0.19	4.7 (95%)	0.46	1.18
Reticulocytes (preincubated)					
5 min (2)	0.086	2.08	1.24 (33%)	0.13	2.09
10 min (4)	0.23	1.59	5.18 (83%)	0.97	1.79
Chase (3)	0.34	0.29	11.3 (96%)	2.45	1.60

* % counts extractable with methylethylketone.

Peak I. Peak I was the first peak to elute from the Ultrogel column. The counts in this peak were predominantly in ferritin: 60% of the counts were precipitated by rabbit antiferritin serum in the 10-min samples. The ferritin peak radioactivity was barely detectable in the 5-min sample, more easily seen in the 10-min samples, and increased further after the 10 min chase (Table I).

Peak II. Peak II eluted in the same position as ^{59}Fe-labelled serum. 90% of the counts, in the 10-min samples, precipitated with rabbit antitransferrin serum. More transferrin was present in the 5-min hemolysates than in the 10-min hemolysates. This surprising result, which is probably due to the lower temperature at 5 min, is similar to that observed by Martinez-Medellin et al. [10]. 70—80% of the counts in peak II were chased at 10 min (Table I).

Peak III. Peak III contained the visible hemoglobin. The counts in this peak increased with time. Peak III was extracted with methylethylketone to assess the proportion of counts in heme. 70—80% of counts did not extract in 5-min samples; 10—20% of counts did not extract in 10-min samples; 4—5% of counts did not extract in 10-min chase samples (Table I).

The heme extraction data suggested the possibility of a second, nonheme iron-binding component in peak III which equilibrated with ^{59}Fe early and then remained at a plateau as hemoglobin continued to accumulate counts (the second, nonheme component, thus, would have a decreasing fraction of the total counts in peak III as time elapsed). In some columns counts and 414 nm absorbance were nonconcordant (Fig. 1, 5-min) but discrepancies were small and not reproducible.

Isoelectric focusing electrophoresis, using polyacrylamide gel as a supporting medium, showed a nonhemoglobin count component in peak III with a p*I* of 5.7—5.8 (Fig. 2).

Whole hemolysate was also subjected to isoelectric focusing in an LKB apparatus using a sucrose gradient as the supporting medium. A p*I* 5.7 component

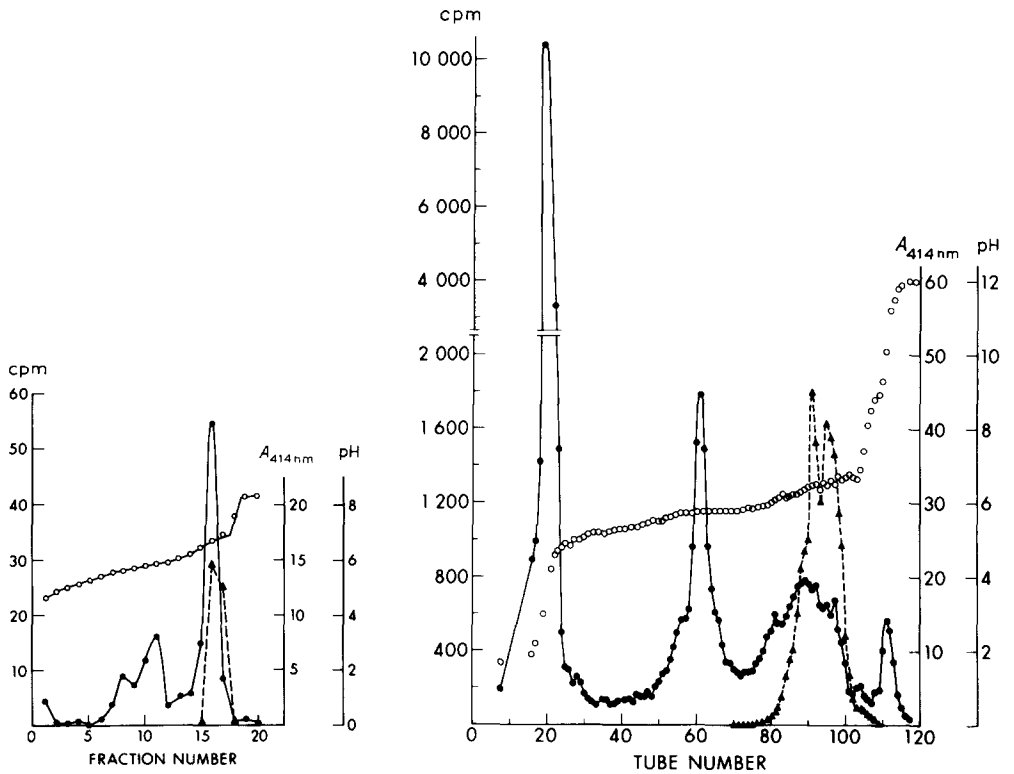

Fig. 2. Peak III, (from a 5-min-incubated reticulocyte hemolysate) was concentrated and subjected to isoelectric focusing in a polyacrylamide gel. The gel was cut into 4-mm segments. Gel segments were eluted and hemoglobin quantified by measuring absorbance at 414 nm (▲) pH of the eluant was measured (○). The nonhemoglobin component of peak III is seen as a count (cpm, ●) peak at pH 5.7—5.8, with a secondary peak at pH 5.6.

Fig. 3. Hemolysates obtained from 5-min incubated reticulocytes (total volume 10 ml) were subjected to isoelectric focusing on an LKB electrofocusing column (column volume 440 ml) in 1% ampholyte (pH 5—7) (3-ml fractions). ●, cpm; ▲, A_{414nm}; ○, pH.

was again observed (Fig. 3). The p*I* 5.7 fraction apparently did not contain the transferrin ^{59}Fe of the hemolysate because: (1) antitransferrin did not precipitate the p*I* 5.7 counts, and (2) counts in labelled guinea pig serum focused at p*I* 6.2. The p*I* 5.7 component apparently contained none of the peak V counts because peak V, obtained from an Ultrogel column and tested separately, did not focus. The polyacrylamide gel isoelectric focusing experiment, in which peak III was concentrated, and the sucrose gradient isoelectric focusing experiment, in which whole hemolysate was used directly, cannot be readily compared because of fractional (60—70%) recovery of peak III counts following concentration.

We considered that the nonhemoglobin component of peak III might be related to the intracellular iron binding protein isolated from guinea pig intestine and identified in guinea pig liver [6]. However, antiserum to this protein did not precipitate peak III counts and did not cross-react, on immunodiffusion plates, with hemolysates prepared from guinea pig reticulocytes.

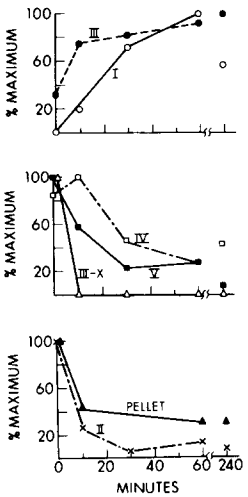

Fig. 4. Guinea pig reticulocytes (depleted of endogenous transferrin by pre-incubation) were incubated in ^{59}Fe-labelled serum for 10 min, washed and resuspended in 5 vol. unlabelled, iron-saturated guinea pig serum. Samples were removed at designated intervals, processed as described in the text and the hemolysates filtered through Ultrogel columns. Aliquots of peak III from the columns were subjected to isoelectric focusing to separate hemoglobin-bound ^{59}Fe from the nonheme ^{59}Fe component of peak III. ^{59}Fe content of each fraction is normalized to show the percent deviation from the maximum for that fraction observed during the chase experiment. 100% values have the following correspondence to ^{59}Fe content: peak I, 0.44 ng; peak II, 1.48 ng; peak III, 29 ng; nonheme component of peak III (peak III-X), 0.9 ng; peak IV, 0.61 ng; peak V, 2.03 ng; pellet, 22 ng.

Peak IV. Earlier work has shown that the counts in peak IV are in heme bound to a moiety with a molecular weight of 17 000 [5], and our speculation is that peak IV is an α chain pool. The counts in peak IV are detectable at 5 min, increase at 10 min and increase further following the 10 min chase.

Peak V. The appearance of ^{59}Fe in peak V, which has a molecular weight of less than 2000 [5], was prompt (Table I). Peak V contained ninhydrin-positive bands on paper electrophoresis, suggesting peptides, and ultraviolet-absorbing bands on paper chromatography, suggesting nucleotides and related compounds. But the radioactive iron did not comigrate with any of these species.

We followed ^{59}Fe during a prolonged chase in a third set of experiments. The reticulocyte count in this experiment was 52%; ^{59}Fe uptake was 239 ng/ml red cells at 10 min. These labelled cells were washed, resuspended in unlabelled serum and samples were removed at designated intervals (Fig. 4).

Counts in peak I, just detectable in the 10-min sample, increased during the first hour of the chase and decreased thereafter. Counts in peak II decreased more than 70% after 10 min of chase and more than 90% after 30 min of chase. The hemoglobin component of peak III showed a steady increase in counts during the progress of the chase. The counts in the nonhemoglobin component of peak III, assayed by isoelectric focusing, became undetectable after 10 min of chase. Peak IV gained counts marginally during the first 10 min of the chase and then lost counts as the chase progressed. Peak V lost 80% of its counts after 30 min of chase (Fig. 4).

Discussion

Transferrin (peak II), the nonheme iron-binding component of peak III and the low molecular weight iron fraction (peak V) are all involved in iron transit in the reticulocyte, as judged by the uptake and chase results of these experiments.

Mazur and Carleton [11] suggested that ferritin was a precusor to the iron in heme because it reached a high specific activity at 4 h in rat erythroid precursors and had a declining specific activity as the specific activity of the iron in heme increased. However, calculations were based on the assumption that the isotope in ferritin was uniformly mixed in the iron, an assumption now believed to be false [12]. Speyer and Fielding [13] found that iron was promptly incorporated into ferritin in the reticulocyte and that, with a chase, the amount of iron in ferritin dropped 50% in 90 min, while the amount of iron in hemoglobin increased progressively. They suggested that ferritin was an obligatory precursor of the iron in heme. Our data do not support this suggestion since the ^{59}Fe accumulation in nonferritin fractions preceded the accumulation of iron in ferritin and since counts appeared to chase into ferritin during the first hour of the experiment.

The evidence that transferrin is involved in intracellular iron transport may be summarized as follows: (1) iodination of transferrin bound to the reticulocyte results in labelling of only a minor fraction of transferrin, suggesting that the major portion of transferrin is inside the cell [10]; (2) electron microscopy shows transferrin inside the reticulocyte [14]; (3) transferrin is able to donate iron to mitochondria and the iron can be incorporated into heme [1]. Our experiments do not advance these observations since we do not know what proportion of the transferrin in our hemolysates may have been bound to the external surface of the red-cell membrane and released with hemolysis. There is no doubt that transferrin iron is an obligatory precursor of heme iron since iron finds its way to the reticulocyte initially bound to transferrin. But the function of transferrin in intracellular iron transport is less certain. Our data point to the existence of other intracellular iron-binding moieties which may function in this capacity.

Studies suggesting that nonhemoglobin iron-binding components and low molecular weight iron are involved in intracellular iron transport include those of Allen, Zail, Primosigh and their respective co-workers. Allen and Jandl [15] incubated rabbit reticulocytes with ^{59}Fe bound to transferrin. Nonhemoglobin, ^{59}Fe-containing protein was prepared by passing the hemolysate through a column containing IRC 50 resin. The nonhemoglobin ^{59}Fe component, in chase experiments, reached a peak 6 min after the addition of the radioactive iron, at a time when the ^{59}Fe content of hemoglobin was still rising, suggesting that it had a precursor role.

Zail et al. [16] filtered hemolysates obtained from human erythroid precursors through Amberlite CG 50 resin, removing hemoglobin from a nonhemoglobin-containing fraction. Their starting buffer contained phosphate and cyanide, thus removal of iron from ligands binding it only weakly was possible. The initial eluate from the Amberlite column, called fraction I, consisted of ferritin and nonferritin iron. When movement of iron through fraction I was

followed, with the iron content of ferritin subtracted, an iron-binding moiety was identified which contained more iron early in the uptake process, and which had less iron following a chase.

Primosigh and Thomas [17] incubated canine marrow cells with transferrin-bound ^{59}Fe. They separated the post-(34 900 × g, 20 min) supernatant into hemoglobin and nonhemoglobin fractions. The nonhemoglobin fraction was further separated into a fraction containing heme proteins; a fraction containing ferritin; a third fraction, not further characterized, and a major low molecular weight fraction which chased with unlabelled iron. The low molecular weight iron fraction depended on the addition of EDTA for its demonstration when separation was on Sephadex columns, and cyanide for its demonstration when separation was on DEAE-cellulose columns.

Our data extend these earlier studies. We have demonstrated a nonhemoglobin iron-binding component which coelutes with hemoglobin on a molecular sieve column, and a low molecular weight iron-binding component which elutes separately. The prompt accumulation of iron by these two components on incubation of reticulocytes with ^{59}Fe-labelled serum and their subsequent loss of ^{59}Fe in chase experiments is consistent with a role for these fractions as precursors to iron incorporation into heme.

Nunez and Cole [18] have recently described an "iron binding protein I" in rabbit reticulocytes which resembles our peak III-X.

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References

1 Ulvik, R., Prante, P.H., Koller, M.E. and Romslo, I. (1976) Scand. J. Clin. Lab. Invest. 36, 539—546
2 Romslo, I. (1977) in Iron Metabolism, Ciba Foundation Symposium 51 (New Series), pp. 189—190, Excerpta Medica, Amsterdam
3 Jacobs, A. (1977) Blood 50, 433—439
4 Pollack, S. and Lasky, F.D. (1976) Biochem. Biophys. Res. Comm. 70, 533—539
5 Pollack, S. and Campana, T. (1980) Blood 56, 564—566
6 Pollack, S. and Lasky, F.D. (1977) in Proteins of Iron Metabolism (Brown, E.B., Aisen, P., Fielding, J. and Crichton, R.C., eds.), pp. 393—396, Grune and Stratton, New York
7 Pollack, S. and Lasky, F.D. (1976) Biochem. Biophys. Res. Comm. 70, 533—539
8 Vaitukatis, J., Robbins, J.B., Nieschlag, E. and Ross, G.T. (1971) J. Clin. Endocrinol. 33, 988—991
9 Hemmaplardh, D. and Morgan, E.H. (1974) Biochim. Biophys. Acta 373, 84—99
10 Martinez-Medellin, J., Schulman, H.M., DeMiguel, E. and Benarides, L. (1977) in Proteins of Iron Metabolism (Brown, E.B., Aisen, P., Fielding, J. and Crichton, R.C., eds.), pp. 305—310, Grune and Stratton, New York
11 Mazur, A. and Carleton, A. (1963) J. Biol. Chem. 238, 1817—1824
12 Hoy, T.G. and Harrison, P.M. (1976) Brit. J. Haem. 33, 497—504
13 Speyer, B.E. and Fielding, J. (1979) Brit. J. Haem. 42, 255—267
14 Morgan, E.H. and Appleton, T.C. (1969) Nature 223, 1371—1372
15 Allen, D.W. and Jandl, J.H. (1960) Blood 15, 71—81
16 Zail, S.S., Carlton, R.W., Torrance, J.D. and Bothwell, T.H. (1964) J. Clin. Invest. 43, 670—680
17 Primosigh, J.V. and Thomas, E.D. (1968) J. Clin. Invest. 47, 1473—1482
18 Nunez, R.M.T. and Cole, E.S. (1980) Blood 55, 1051—1055