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# CHELATOR-MEDIATED IRON EFFLUX FROM RETICULOCYTES

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**The mechanism by which bipyridine and phenanthroline types of iron chelator inhibit iron uptake from transferrin and iron efflux mediated by pyridoxal isonicotinoyl hydrazone was investigated using rabbit reticulocytes with the aim of providing more information on the normal process of iron uptake by developing erythroid cells. It was shown that the chelators block cellular uptake by chelating the iron immediately after release from transferrin while it is still in the membrane fraction of the cells. The iron-chelator is then released from the cells by a process which is very similar to that of transferrin release with respect to kinetics and sensitivity to incubation temperature and the effects of metabolic inhibitors and other chemical reagents. These results are compatible with the conclusion that both transferrin and the iron-chelators in the cells are mainly present in endocytotic vesicles and are released from the cells by exocytosis. The chelators were also shown to block the pyridoxal isonicotinoyl hydrazone-mediated efflux of iron from cells which had taken up iron in the presence of isoniazid, an inhibitor of haem synthesis, by chelating the iron in the cytosol and the mitochondria. In this case, the iron-chelator complexes were not released from the cells. Measurement of the diethyl ether/water partition coefficients of bipyridine and 1,10-phenanthroline and their iron complexes gave much higher values for the free chelators, supporting the concept that the chelators trap the iron intracellularly because of differences in the lipid solubility and, hence, membrane permeability to the free chelators and their iron complexes.**

## Introduction

Iron uptake by developing erythroid cells involves binding of the iron-transport protein, transferrin, to receptors on the cell membrane [1]. There is considerable evidence that the transferrin molecules are then taken into the cell by endocytosis [2–4]. Iron release from the transferrin probably occurs within the endocytotic vesicles [5,6] followed by its uptake by the cell. The apotransferrin is then released into the extracellular fluid [1].

In earlier experiments from this laboratory, it was shown that iron uptake from transferrin by reticulocytes could be inhibited by the iron chelators 2,2′-bipyridine and 1,10-phenanthroline and that during the inhibition the iron appeared in the medium bound to the chelator [7]. It was concluded, but not shown, that the two chelators inhibit iron uptake by entering the cells and chelating the iron during its transit between the sites of release from transferrin and of incorporation into haem, followed by diffusion of the chelated iron from the cells. However, recent observations by Ponka and his associates with the newly discovered iron chelator, pyridoxal isonicotinoyl hydrazone, have thrown doubt on these conclusions. They showed that radioactive iron

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

taken up by reticulocytes in the presence of isoniazid, an inhibitor of haem synthesis, was released from the cells when they were subsequently reincubated with pyridoxal isonitotinoyl hydrazone or related substances [8–10]. This release was found to be inhibited by the presence of bipyridine in the reincubation solution. Hence, it was concluded that bipyridine could enter reticulocytes and bind iron but the iron-bipyridine complex could not be released from the cells [9]. Why, then, is iron-bipyridine released from reticulocytes when the chelator is present during the incubation with iron-transferrin but not when the chelator is added to cells which have already taken up the iron from transferrin? Possibly the theory that endocytosis of iron-transferrin is necessary for iron uptake is incorrect and iron release from transferrin occurs while it is bound to receptors on the outer cell membrane. The iron could then be chelated by bipyridine without the necessity of the chelator entering the cells and becoming trapped as its complex with iron.

The aim of the present investigation was to resolve the above problem and to help determine whether iron release from transferrin occurs at the outer cell membrane or within intracellular vesicles. This is an important question which relates to the functions of cell membranes and of the endocytotic process in the general sense as well as to the specific problem of the mechanism of iron uptake by immature erythroid cells. It was also hoped that the experiments would shed light on the ways in which chelators can interfere with iron metabolism.

## Materials and Methods

*Chemicals.* The iron chelators 2,2′-bipyridine (hereafter termed bipyridine), 1,10-phenanthroline, bathophenanthroline and sulphonated bathophenanthroline were obtained from the G. Frederick Smith Chemical Co., Columbus, OH, U.S.A. The other biochemical compounds were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Iron-59 ($FeCl_3$, 10–20 $\mu Ci/\mu g$) and $^{125}I$ (NaI, carrier free) were from Amersham International, U.K. Pyridoxal isonicotinoyl hydrazone and solutions of this reagent were prepared as described by Ponka et al. [9].

*Purification and labelling of transferrin.* Transferrin was isolated from rabbit plasma by ion-exchange chromatography, gel filtration and crystallization, and was labelled with $^{59}Fe$ and $^{125}I$ as described previously [11]. It was used in the diferric form for all of the experiments described in this paper.

*Reticulocytes.* Reticulocytosis was induced in rabbits by injection of phenylhydrazine [11]. Blood was collected 4–7 days after the last injection of phenylhydrazine using heparin as an anticoagulant. The cells were washed three times with ice-cold 0.15 M NaCl and were suspended in approx. 4 vol. Hanks & Wallace balanced salt solution [12]. The reticulocyte count of the different cell preparations varied between 40 and 65%.

*Incubation procedure.* Transferrin and iron uptake by reticulocytes were measured by incubating the cells with ($^{59}Fe$,$^{125}I$)-labelled transferrin in Hanks & Wallace solution in a shaking water-bath at 37°C unless specified otherwise. After varying times of incubation, cell samples were washed three times with ice-cold 0.15 M NaCl, transferred to counting tubes and haemolysed with water and, the radioactivity was counted in a three-channel gamma scintillation counter. When chelators, inhibitors or other chemical reagents were used the cells were preincubated with the agents for 10 min at 37°C before the addition of the radiolabelled transferrin. Solutions of these reagents were adjusted to pH 7.4 and 300 mosM before they were added to the cells.

Two basic procedures were used to measure iron or transferrin release (efflux) from reticulocytes. The first was based on the method described by Ponka and Neuwirt [13] for labelling the non-haem iron pool with $^{59}Fe$, followed by reincubation in the presence of pyridoxal isonicotinoyl hydrazone or other chelators. The reticulocytes, suspended in Hanks & Wallace solution, were pre-incubated at 37°C for 10 min with 10 mM isoniazid, then labelled transferrin was added at a concentration of 5 $\mu$M and the incubation was continued for a further 60 min. The cells were then washed three times with ice-cold 0.15 M NaCl and suspended in ice-cold Hanks & Wallace solution. Pyridoxal isonicotinoyl hydrazone and/or other chelators, metabolic inhibitors or chemical reagents were then added as required and the cell

suspension was reincubated. At the end of the reincubation period the cell suspension was centrifuged at 4°C for 10 min at 1000 × *g* and the supernatant was transferred to a separate counting tube for the measurement of $^{59}$Fe and $^{125}$I-labelled transferrin efflux. Radioactivity was also counted in the cells or in subcellular fractions separated as described below in order to measure the distribution of the isotopes between the efflux solution and the cells or the fractions of the cells. Unless specified to the contrary, the concentration of pyridoxal isonicotinoyl hydrazone used in these experiments was 1.0 mM, the reincubation time was 30 min and the reincubation temperature was 37°C.

For the second type of efflux experiment, the reticulocytes were labelled by incubation with ($^{59}$Fe,$^{125}$I)-labelled transferrin in the presence of 1 mM bipyridine or 1 mM 1,10-phenanthroline. The cells were suspended in Hanks' solution, preincubated for 10 min at 37°C with the chelator, labelled transferrin was then added (5 μM), and incubation was continued at 37°C for a further 30 min. The cells were then washed and reincubated as above in Hanks & Wallace solution, inhibitors and other reagents being included as required. No bipyridine or 1,10-phenanthroline was added at this time. At the end of the reincubation period, cells and efflux solution were separated by centrifugation and, in most experiments, the cells were fractionated into membranes and cytosol as described below before counting for radioactivity.

*Subcellular fractionation of reticulocytes.* Two methods of fractionation of reticulocytes were used. In the first, the cells were haemolysed with ice-cold 20 mM Tris-HCl buffer (pH 7.8), and then sufficient 3 M KCl/30 mM $MgCl_2$ was added to give final concentrations of 150 mM and 1.5 mM for the two salts. The haemolysed cells were centrifuged at 4°C for 30 min at 30000 × *g* in order to separate the membrane fraction (ghosts) and cytosol fraction of the cells.

In the second method the reticulocytes were suspended in ice-cold 0.15 M NaCl and fragmented by the nitrogen cavitation method using a pressure of 800 lb/inch$^2$ [14]. The suspension was separated by differential centrifugation according to De Duve et al. [15] into five fractions (1, 6000 *g* · min; 2, 33000 *g* · min; 3, 250000 *g* · min; 4, 3000000 *g* · min; 5, supernatant). Radioactivity measurements and protein and enzyme assays were performed on each fraction.

*Analytical methods.* Reticulocytes were counted in dry smears after staining with New methylene blue. Ultrafiltration was performed using an Amicon apparatus and PM-10 filters (nominal molecular mass cut-off, 10000 daltons). Separation of protein-bound $^{59}$Fe from chelator-bound $^{59}$Fe was achieved by precipitation of the proteins with 85–90% ice-cold ethanol followed by centrifugation at 4°C for 10 min at 1000 × *g* as described previously [16]. This procedure precipitates $^{59}$Fe bound to transferrin, haemoglobin or ferritin while $^{59}$Fe bound to pyridoxal isonicotinoyl hydrazone, bipyridine or 1,10-phenanthroline remains in the supernatant solution. Protein was measured by a modification of the method of Lowry et al. [17] using bovine serum albumin as a standard. Published methods were used for the assay of the enzymes, cytochrome oxidase [18] and acid phosphatase [19].

## Results

*Iron efflux with pyridoxal isonicotinoyl hydrazone*

The effects of pyridoxal isonicotinoyl hydrazone on iron and transferrin efflux from reticulocytes which had been incubated with ($^{59}$Fe,$^{125}$I)-labelled transferrin in the presence of isoniazid are summarized in Table I. The results confirm the observations of Ponka and his colleagues [8–10] that the hydrazone greatly increases the release of $^{59}$Fe from the cells and that this release can be inhibited by bipyridine and by reagents which block cellular metabolism (NaCN, 2,4-dinitrophenol, antimycin A, CCCP). Another iron chelator, 1,10-phenanthroline and substances which react with sulphydryl groups (*N*-ethylmaleimide, diamide) or amino groups (dinitrofluorobenzene, phenylglyoxal) were also found to block iron efflux. The major source of the $^{59}$Fe lost from the cells during incubation with pyridoxal isonicotinoyl hydrazone was the membrane fraction of the cells. All of the reagents which inhibited iron efflux produced an increase in the amount of $^{59}$Fe in the cytosolic fraction of the cells (Table I). The presence of the $^{125}$I label on the transferrin enabled measurements to be made

TABLE I

DISTRIBUTION OF $^{59}Fe$ AND EFFLUX OF $^{125}I$-LABELLED TRANSFERRIN AFTER 30 MIN REINCUBATION OF RABBIT RETICULOCYTES WHICH HAD BEEN INCUBATED WITH ($^{59}Fe$,$^{125}I$)-LABELLED TRANSFERRIN FOR 60 MIN IN THE PRESENCE OF ISONIAZID

The reincubation conditions were 37°C in Hanks' solution (control) or Hanks' solution containing 1 mM pyridoxal isonicotinoyl hydrazone (PIH) with or without the addition of 1 mM bipyridine of 1,10-phenanthroline, metabolic inhibitors (25 mM NaCN, 2.5 mM dinitrophenol or 50 $\mu$M antimycin A), -SH reagents (2.5 mM *N*-ethylmaleimide or 5 mM diamide) or $-NH_2$ reagents (5 mM dinitrofluorobenzene or 5 mM phenylglyoxal). The results are given as the mean ± 1 S.E.

| Reincubation conditions | *n* | $^{59}Fe$ (% total) | | | Transferrin efflux (% total) |
|---|---|---|---|---|---|
| | | Efflux | Cytosol | Membranes | |
| Control | 5 | 1.7 ± 0.2 | 27 ± 2.4 | 72 ± 2.5 | 85 ± 4.0 |
| PIH | 10 | 52 ± 1.2 | 30 ± 1.3 | 18 ± 0.8 | 84 ± 2.0 |
| PIH and bipyridine | 5 | 9.8 ± 1.7 | 48 ± 4.6 | 42 ± 5.9 | 89 ± 2.6 |
| PIH and 1,10-phenanthroline | 6 | 7.5 ± 1.8 | 30 ± 7.5 | 63 ± 8.8 | 83 ± 2.9 |
| PIH and metabolic inhibitors | 14 | 25 ± 4.6 | 56 ± 2.6 | 19 ± 4.7 | 73 ± 2.3 |
| PIH and -SH or $-NH_2$ reagents | 15 | 17 ± 3.1 | 54 ± 6.0 | 29 ± 4.9 | 17 ± 2.1 |

TABLE II

FRACTIONAL DISTRIBUTION OF ($^{59}Fe$,$^{125}I$)-LABELLED TRANSFERRIN, CYTOCHROME OXIDASE AND ACID PHOSPHATASE IN SUBCELLULAR FRACTIONS OF RABBIT RETICULOCYTES

The cells were incubated at 37°C for 60 min with ($^{59}Fe$,$^{125}I$)-labelled transferrin in the absence (control) or presence of 10 mM isoniazid, or for 30 min in the presence of 1 mM bipyridine. The cells were then disintegrated by nitrogen cavitation and four particulate fractions were obtained by centrifugation for 1600 *g*·min (1), 33000 *g*·min (2), 250000 *g*·min (3) and 3000000 *g*·min (4), plus a cytosol fraction (5).

| Fraction | Activity (% total) | | | |
|---|---|---|---|---|
| | $^{59}Fe$ | $^{125}I$-labelled transferrin | Cytochrome oxidase | Acid phosphatase |
| Control | | | | |
| 1 | 4 | 3 | 3 | 3 |
| 2 | 4 | 5 | 32 | 8 |
| 3 | 5 | 27 | 61 | 44 |
| 4 | 4 | 48 | 4 | 25 |
| 5 | 83 | 15 | 1 | 3 |
| Isoniazid | | | | |
| 1 | 11 | 5 | 7 | 5 |
| 2 | 21 | 5 | 21 | 15 |
| 3 | 29 | 34 | 60 | 36 |
| 4 | 4 | 45 | 7 | 37 |
| 5 | 34 | 11 | 3 | 7 |
| Bipyridine | | | | |
| 1 | 3 | 2 | 2 | 4 |
| 2 | 7 | 6 | 32 | 7 |
| 3 | 28 | 33 | 56 | 49 |
| 4 | 34 | 45 | 4 | 36 |
| 5 | 28 | 14 | 5 | 5 |

of transferrin efflux as well as that of iron. Neither pyridoxal isonicotinoyl hydrazone nor the other iron chelators affected this efflux; the metabolic inhibitors reduced it significantly ($P > 0.05$) but only by about 15%, while the sulphydryl and amino reagents caused approx. 80% reduction.

*Subcellular distribution of $^{59}Fe$ and $^{125}I$-labelled transferrin*

The subcellular distributions of $^{59}Fe$ and $^{125}I$-labelled transferrin in reticulocytes after incubation with the labelled protein and with or without isoniazid for 60 min are shown in Table II. In the control cells approx. 80% of the $^{59}Fe$ and 15% of the $^{125}I$-labelled transferrin was found in the cytosol fraction (fraction 5). When isoniazid was present during the initial incubation, more than 60% of the $^{59}Fe$ taken up by the reticulocytes was in the mitochondrion-rich fractions, fractions 2 and 3, as indicated by the distribution of cytochrome oxidase. Reincubation of these cells with pyridoxal isonicotinoyl hydrazone led to iron efflux but this occurred proportionately from these fractions so that the relative distribution of $^{59}Fe$ between them showed no change (results not shown). The distribution of $^{125}I$-labelled transferrin and $^{59}Fe$ between the subcellular fractions of control cells differed in that a much greater proportion of the $^{125}I$ label was found in the fractions which were enriched with acid phosphatase (fractions 3 and 4). The transferrin was lost from these fractions dur-

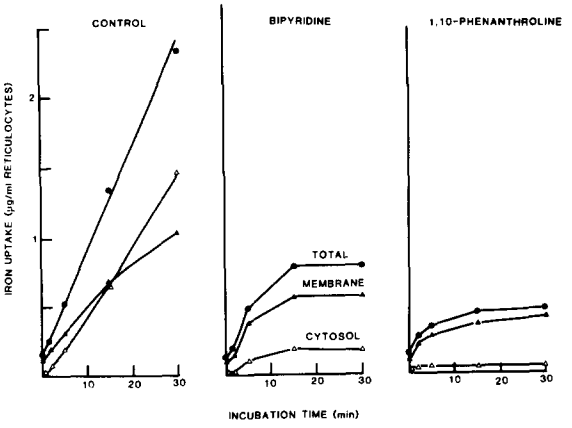


Fig. 1. Effect of 2,2'-bipyridine (1 mM) and 1,10-phenanthroline (1 mM) on the uptake of transferrin-bound iron by reticulocytes. The figure shows the total iron uptake (●———●) and its distribution between the membrane (▲———▲) and cytosol (△———△) fractions of the cells.

ing the reincubation. Also shown in Table II are the results obtained using reticulocytes which had been incubated for 30 min with the labelled transferrin in the presence of 1 mM bipyridine. This treatment did not alter the relative distribution of transferrin or the enzymes between the subcellular fractions but led to a reduction of $^{59}$Fe in the cytosol fraction and a marked increase in fractions 3 and 4.

The results of the above experiments support earlier conclusions that inhibition of haem synthesis with isoniazid leads to the accumulation of iron in mitochondria [20] and that pyridoxal isonicotinoyl hydrazone mobilizes this iron. Bipyridine and 1,10-phenanthroline inhibited iron efflux from the cells, apparently by trapping the iron in the membrane fraction of the cells (mainly in mitochondria) and in the cytosol. The metabolic inhibitors and reagents which react with sulphydryl and amino groups also blocked efflux. Several experiments were then undertaken in an attempt to explain these results and also the earlier observations that bipyridine and 1,10-phenanthroline can inhibit iron uptake from transferrin by reticulocytes [7,21,22].

*Uptake and efflux of iron in presence of bipyridine and 1,10-phenanthroline*

Reticulocytes were incubated with $^{59}$Fe-labelled transferrin for varying periods of time, either in the absence or presence of bipyridine or 1,10-phenanthroline, and the distribution of $^{59}$Fe between the membrane and cytosol fractions of the cells was determined. Radioactive iron uptake by the control cells was linear throughout the 30 min period of incubation; initially more of the $^{59}$Fe was present in the membrane fraction but subsequently the radioactivity was transferred to the cytosolic fraction (Fig. 1). By contrast, in the presence of the chelators, the rate of $^{59}$Fe uptake by the cells began to diminish after about 5 min incubation and reached a plateau after 15 min at a much lower level than in the control cells. Additionally, more than 75% of the $^{59}$Fe taken up by the cells remained in the membrane fraction throughout the entire period of incubation. The rate of accumulation of $^{59}$Fe in this fraction during the first 2 min of incubation was similar to that in the controls but thereafter lagged behind that of the controls.

In a subsequent experiment, reticulocytes were incubated with $^{59}$Fe-labelled transferrin with and without bipyridine for 30 min, washed and haemolysed, and the membrane fraction was solubilized with the non-ionic detergent, Teric 12A9 (I.C.I., Australia). The incubation solutions, cytosols and the extracts of the membranes were studied by ultrafiltration and by precipitation with ethanol. As shown in Table III, very little of the $^{59}$Fe in incubation solution, cytosol or membrane extract of the control cells was ultrafilterable and soluble in ethanol. Incubation of reticulocytes in the presence of bipyridine led to release of iron from transferrin at the same rate as in control cells, but with bipyridine most of the $^{59}$Fe was found in the incubation solution and membrane fraction of the cells in a low-molecular-weight, ethanol-soluble form, presumably bound to the chelator.

The lability of $^{59}$Fe taken up from transferrin by reticulocytes in the presence of bipyridine or 1,10-phenanthroline was examined by washing the cells and reincubating them in Hanks' solution. When the incubation was performed at 37°C the $^{59}$Fe was rapidly released from the cells, entirely from the membrane fraction. Similar results were obtained using 1,10-phenanthroline (Fig. 2) or bipyridine as the chelator. Transferrin $^{125}$I was re-

TABLE III

ULTRAFILTRATION AND ETHANOL PRECIPITATION

Ultrafiltration and ethanol precipitation of incubation solution, cytosol and detergent (Teric 12A9) extract of membrane fraction of reticulocytes after incubation with $^{59}$Fe-labelled transferrin for 30 min at 37°C in the absence (control) or presence of 1 mM bipyridine. The reticulocyte count was 53% and the transferrin concentration 4 μM. Ultrafiltration was performed through an Amicon PM-10 membrane and ethanol precipitation using 85% ice-cold ethanol.

| | $^{59}$Fe | | |
|---|---|---|---|
| | cpm | % ultra-filterable | % ethanol soluble |
| Control | | | |
| Incubation solution | 7245 | 1.2 | 1.8 |
| Cytosol | 5860 | 0.1 | 0.5 |
| Membranes | 3635 | – | – |
| Detergent extract of membranes | 2845 | 1.1 | 7.7 |
| Bipyridine | | | |
| Incubation solution | 12125 | 42 | 46 |
| Cytosol | 1255 | 38 | 43 |
| Membranes | 3070 | – | – |
| Detergent extract of membranes | 2460 | 79 | 82 |

leased from the cells, also from the membrane fraction, at the same rate as $^{59}$Fe. However, approx. 85% of the $^{59}$Fe in the efflux solution was soluble in ethanol which precipitates transferrin-bound iron [16]. Hence, the $^{59}$Fe was released as its complex with the chelator, not bound to transferrin. When the reincubation was performed at 50°C, the initial rate of efflux was similar to that observed at 37°C, but after about 2 min efflux of both $^{59}$Fe and transferrin slowed and was virtually completely stopped after 5 min at 50°C (Fig. 2). Incubation of the cells at temperatures below 37°C slowed the rate of efflux of $^{59}$Fe and transferrin to the same degree, and by lowering the temperature to 4°C efflux was almost completely inhibited (Fig. 3). In this experiment, samples of the labelled cells were haemolysed with 20 mM Tris-HCl buffer (pH 7.8) and centrifuged, and the membrane fraction of the cells was reincubated in parallel with the intact cells. Haemolysis caused the release of 20–25% of the $^{59}$Fe and 15–20% of the $^{125}$I-labelled transferrin present in the cells. During reincubation of the ghosts there was virtually no progres-

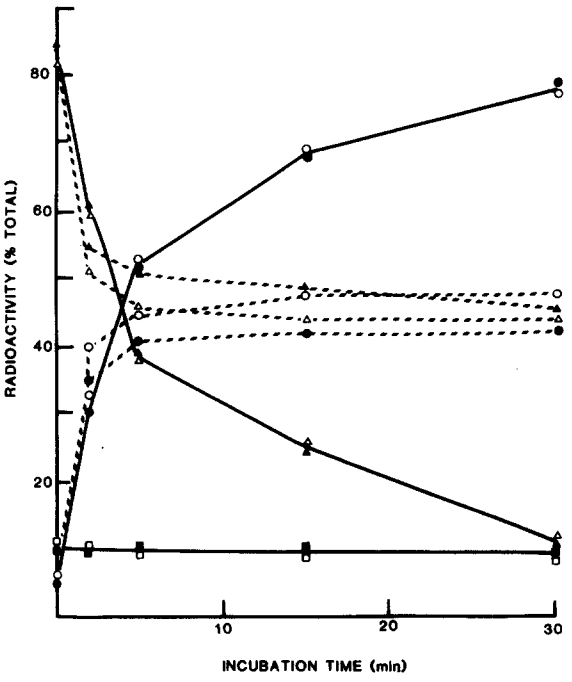


Fig. 2. Efflux of $^{59}$Fe (●) and $^{125}$I-labelled transferrin (○) and the distribution of cellular radioactivity between cytosol ($^{59}$Fe, ■; $^{125}$I, □) and membrane ($^{59}$Fe, ▲; $^{125}$I, △) fractions of reticulocytes during incubation at 37°C (———) or 50°C (— — —). The cells were labelled by incubation at 37°C for 30 min with ($^{59}$Fe,$^{125}$I)-labelled transferrin in the presence of 1 mM 1,10-phenanthroline before being washed and reincubated in Hanks & Wallace solution.

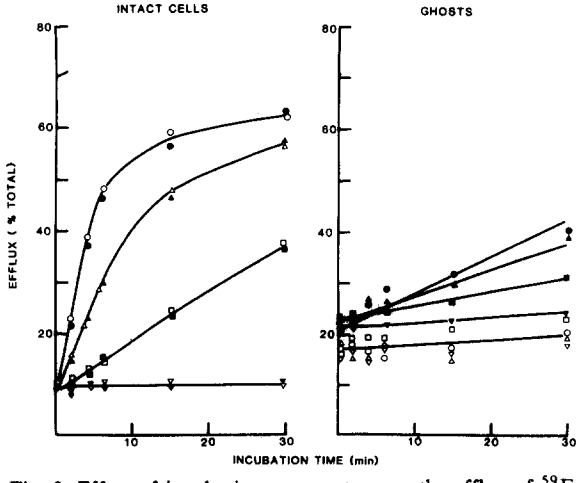


Fig. 3. Effect of incubation temperature on the efflux of $^{59}$Fe (filled symbols) and $^{125}$I-labelled transferrin (open symbols) from reticulocytes or their ghosts after prior incubation of intact cells with ($^{59}$Fe,$^{125}$I)-labelled transferrin at 37°C for 30 min in presence of 1 mM 1,10-phenanthroline. 37°C, ●, ○; 30°C, ▲, △; 21°C, ■, □; 4°C, ▼, ▽.

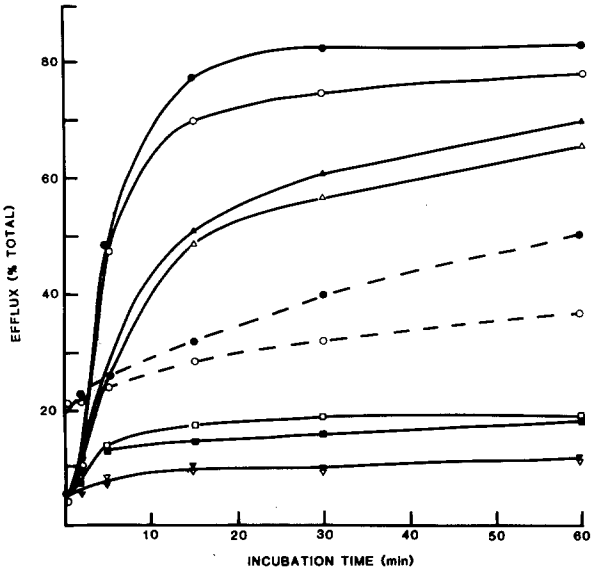


Fig. 4. Effect of 2.5 mM dinitrophenol (▲, △), 5 mM dinitrofluorobenzene (■, □) and 2.5 mM *N*-ethylmaleimide (▼, ▽) on $^{59}$Fe (filled symbols) and $^{125}$I-labelled transferrin (open symbols) efflux from reticulocytes which had been incubated for 30 min with ($^{59}$Fe,$^{125}$I)-labelled transferrin at 37°C for 30 min in the presence of 1 mM 1,10-phenanthroline. Also shown is the efflux from control cells (●———●, ○———○) or ghosts prepared from such cells after the 30 min labelling period (●------●, ○------○).

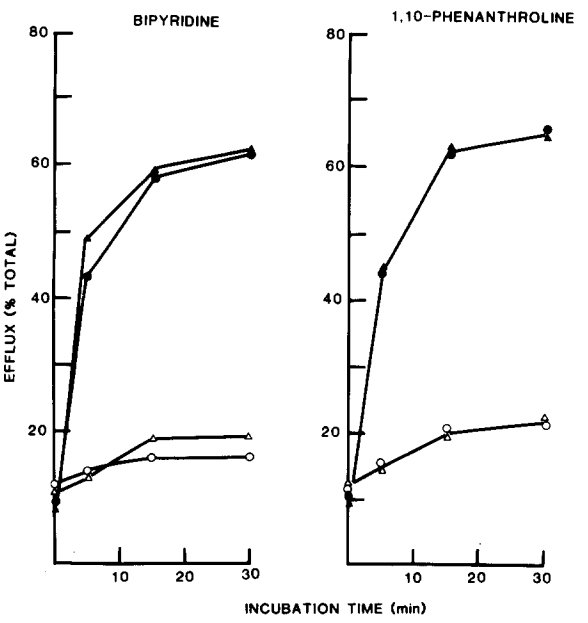


Fig. 5. Effect of 0.25 mM vinblastine (open symbols) on efflux of $^{59}$Fe (●, ○) and $^{125}$I-labelled transferrin (▲, △) from reticulocytes. The cells were labelled by incubation with ($^{59}$Fe,$^{125}$I)-labelled transferrin at 37°C for 30 min in the presence of 1 mM bipyridine or 1 mM 1,10-phenanthroline. Filled symbols: control.

sive release of transferrin, and only a slow release of $^{59}$Fe which was much lower than with intact cells and was less affected by incubation temperature (Fig. 3).

Investigations were then performed to determine the effects of metabolic inhibitors and chemical agents on the efflux of $^{59}$Fe and $^{125}$I-transferrin taken up in the presence of 1,10-phenanthroline. As shown in Fig. 4, 2,4-dinitrophenol caused a moderate degree of inhibition and dinitrofluorobenzene and *N*-ethylmaleimide caused marked inhibition of efflux of both $^{59}$Fe and transferrin. Pyridoxal isonicotinoyl hydrazone (1 mM) had no effect on efflux (results not shown). As was observed in the earlier experiment, the rate of efflux of $^{59}$Fe and transferrin from ghosts was much less than from intact reticulocytes (Fig. 4). This efflux was not inhibited by the above reagents (results not shown).

Another reagent tested in this series of experiments was the microtubule inhibitor, vinblastine (Fig. 5). This was found to inhibit both $^{59}$Fe and $^{125}$I-labelled transferrin efflux from reticulocytes which had been incubated with doubly labelled transferrin in the presence of bipyridine or 1,10-phenanthroline. Once again there was a striking correspondence between the efflux of both isotopes. As in the earlier experiments, the $^{125}$I was precipitated with ethanol, but the $^{59}$Fe was not, indicating that it was bound to the chelators, not to transferrin.

*Effect of chelator concentration on iron efflux*

The mechanism of pyridoxal isonicotinoyl hydrazone-mediated iron efflux from reticulocytes which had been labelled with $^{59}$Fe-labelled transferrin in the presence of isoniazid was investigated by studying the effects of varying concentrations of iron chelators and pyridoxal isonicotinoyl hydrazone and of incubation temperature on this process. Four iron chelators were used, 1,10-phenanthroline, bipyridine, bathophenanthroline

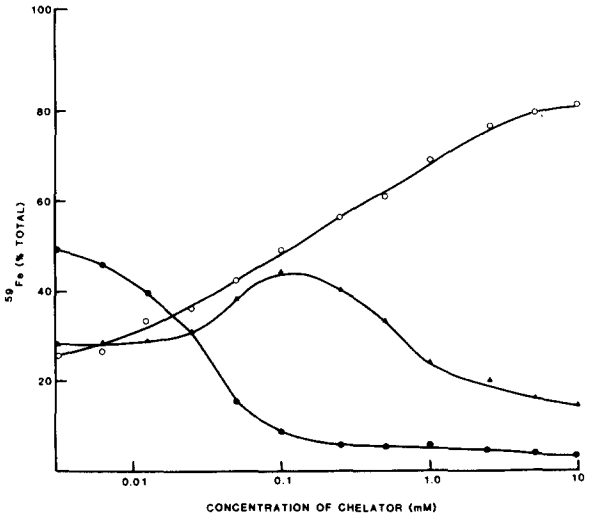


Fig. 6. Effect of concentration of ferrous iron chelators on efflux of $^{59}Fe$ (●) and distribution of cellular $^{59}Fe$ between membranes (○) and cytosol (▲) during incubation with pyridoxal isonicotinoyl hydrazone. Reticulocytes were labelled by incubation with ($^{59}Fe$,$^{125}I$)-labelled transferrin at 37°C for 60 min in the presence of 10 mM isoniazid. They were then washed and reincubated at 37°C for 30 min in the presence of 1 mM pyridoxal isonicotinoyl hydrazone and varying concentrations of 1,10-phenanthroline, bipyridine, bathophenanthroline and sulphonated bathophenanthroline. The figure shows the results obtained with 1,10-phenanthroline. Comparable results were obtained with the other chelators (see text).

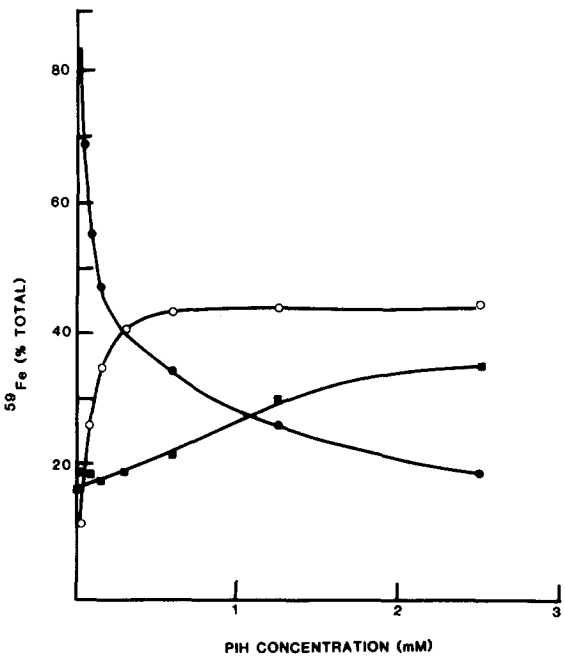


Fig. 7. Effect of the concentration of pyridoxal isonicotinoyl hydrazone (PIH) on the efflux of $^{59}Fe$ from reticulocytes (○———○) and the distribution of cellular $^{59}Fe$ between membrane (●———●) and cytosol (■———■) fractions. The cells were labelled by incubation with ($^{59}Fe$,$^{125}I$)-labelled transferrin at 37°C for 60 min in the presence of 10 mM isoniazid.

and sulphonated bathophenanthroline. Fig. 6 shows the results obtained with 1,10-phenanthroline. The degree of inhibition of $^{59}Fe$ efflux and the amount of $^{59}Fe$ retained in the membrane fraction increased as the concentration of chelator was increased. By contrast, the $^{59}Fe$ in the cytosol increased to a maximum at the minimal concentration of 1,10-phenanthroline which produced a high degree of inhibition of $^{59}Fe$ efflux, and then diminished as the chelator concentration was increased above this level. A similar pattern of results was obtained with the other three chelators but they varied in their potency. The millimolar concentrations of chelators which caused 50% inhibition of $^{59}Fe$ efflux were 0.008, 0.035, 0.20 and 2.5 for bathophenanthroline, 1,10-phenanthroline, bipyridine and sulphonated bathophenanthroline, respectively.

The efflux of $^{59}Fe$ from reticulocytes also varied with the concentration pyridoxal isonicotinoyl hydrazone (Fig. 7). However, in this case, increasing the concentration of the reagent produced a rise in the efflux, until a maximum was reached at about 0.5 mM. Increases in the concentration above 0.5 mM resulted in movement of the isotope from the membrane fraction of the cells into the cytosol, even though no increase in release from the cells occurred.

*Effect of incubation temperature on pyridoxal isonicotinoyl hydrazone-induced efflux*

Iron efflux from reticulocytes increased as the incubation temperature was raised from 14°C to 37°C. This was associated with a fall in the amount of $^{59}Fe$ in the membrane fraction of the cells, and with a varying effect on the $^{59}Fe$ in the cytosol (Fig. 8). The changes in incubation temperature had a much greater effect on $^{59}Fe$ efflux from the cells than it did on the movement of $^{59}Fe$ from the membranes. At 37°C, the $^{59}Fe$ content of the

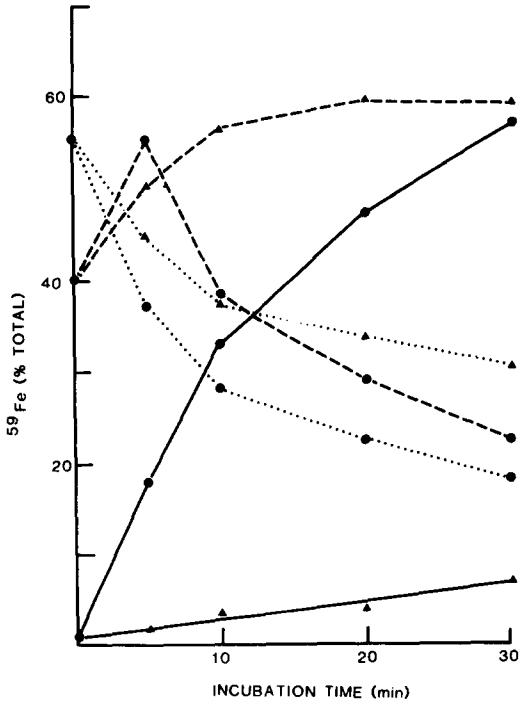


Fig. 8. Effect of incubation temperature on $^{59}$Fe efflux from reticulocytes (———) and distribution of cellular $^{59}$Fe between membrane (·····) and cytosol (— — —) fractions of the cells. The cells were labelled by incubation with ($^{59}$Fe,$^{125}$I)-labelled transferrin for 60 min at 37°C in the presence of 10 mM isoniazid, washed and then reincubated in the presence of 1 mM pyridoxal isonicotinoyl hydrazone at 37°C (●) or 14°C (▲).

cytosol rose quickly during the first 5 min of incubation, and then fell as $^{59}$Fe left the cells. At 14°C the rate of rise in cytosol $^{59}$Fe was slower and there was relatively little fall even after 30 min incubation, in association with the very slow efflux of $^{59}$Fe from the cells. The results obtained at the intervening temperatures used, 23°C and 31°C, were between those observed at 37°C and 14°C (results not shown).

*Diethyl ether / water partition coefficients*

Measurements were made of the diethyl ether/water partition coefficients of bipyridine, 1,10-phenanthroline and their iron complexes by equilibration of 1 mM solutions of the chelators in 0.15 M NaCl/0.01 M Tris-HCl (pH 7.4) with diethyl ether. The values so obtained were 2.77 and 0.23 for bipyridine and Fe(bipyridine)$_3$, respectively and 0.66 and 0.016 for 1,10-phenanthroline and Fe(phenanthroline)$_3$, respectively (means of four estimations).

## Discussion

These experiments confirm that bipyridine and 1,10-phenanthroline can inhibit iron uptake from transferrin [7] and that bipyridine and inhibitors of cellular metabolism can reduce pyridoxal isonicotinoyl hydrazone-mediated efflux of iron from reticulocytes which have been pre-incubated with $^{59}$Fe-labelled transferrin and isoniazid [8,9]. Other phenanthroline-type iron chelators and reagents which interact with sulphydryl groups (*N*-ethylmaleimide, diamide) or amino groups (dinitrofluorobenzene, phenylglyoxal) also inhibited this efflux (Table I). The cell fractionation studies (Table II) confirmed that the major site of $^{59}$Fe accumulation of reticulocytes during incubation in the presence of isoniazid is the mitochondria and that this is the primary source of the $^{59}$Fe released with pyridoxal isonicotinoyl hydrazone [8,9]. However, the present results indicate that the earlier conclusion that bipyridine and metabolic inhibitors block the release of $^{59}$Fe from mitochondria [9] is not necessarily correct. The site of action of the chelators was dependent on concentration, higher concentrations trapping more of the $^{59}$Fe in the membrane fraction of the cells, but with lower concentrations much of the $^{59}$Fe was released from the membrane fraction and was trapped in the cytosol (Fig. 6). Since the membrane fraction of the reticulocytes included the mitochondria, it is likely that the $^{59}$Fe in this fraction was mainly in the mitochondria. In the case of metabolic inhibitors and sulphydryl- and amino-reactive agents, the main site of the inhibition was at the stage of iron exchange between the cytosol and the extracellular medium.

Several experiments were performed in order to determine the mechanism by which the iron chelators blocked iron uptake by reticulocytes. Fig. 1 illustrates this effect and demonstrates that the chelators inhibit the movement of $^{59}$Fe from the membrane fraction of the cells into the cytosol, so that most of the $^{59}$Fe accumulated by the cells in the presence of the chelators remains in the membrane. However, the amount of $^{59}$Fe in this frac-

tion did not reach the level found in control cells and the kinetics of $^{59}Fe$ uptake into membranes and cytosol of control and treated cells are such as to suggest that the chelators blocked the movement of $^{59}Fe$ from cell membranes to mitochondria where it normally incorporated into haem before it appears in the cytosol as haemoglobin. Instead, as shown by the efflux experiments, in the cells treated with the chelators the $^{59}Fe$ which was released from transferrin in the membrane fraction returned to the medium bound to the chelators. In this form it was ultrafiltrable and soluble in ethanol, in contradistinction to transferrin-bound $^{59}Fe$. Also the $^{59}Fe$ solubilized by detergent from the membranes of chelator-treated cells was also largely ultrafilterable and ethanol-soluble (Table III), indicating that most of the $^{59}Fe$ in this fraction was bound to the chelators, not to transferrin or membrane iron-binding proteins. By contrast, nearly all of the $^{59}Fe$ in control cells, in membranes or cytosol, or in the incubation medium appeared to be protein-bound, since it was not ultrafilterable and was precipitated by ethanol, along with transferrin and, presumably, membrane proteins.

The $^{59}Fe$ taken up by reticulocytes in the presence of bipyridine or 1,10-phenanthroline was rapidly released from the cells when they were reincubated in isotope-free media (Figs. 2–5). The efflux proceeded directly from the membrane fraction of the cells to the extracellular medium, not via the cytosol (Fig. 2). The efflux was temperature-dependent, increasing as the temperature was raised from 4°C to 37°C (Fig. 3) but was inhibited within 2–5 min by incubation at 50°C (Fig. 2). It was also inhibited by sulphydryl- and amino-reactive agents, by vinblastine, which interferes with the function of microtubules, and to a lesser extent by inhibitors of energy metabolism (Figs. 4 and 5). A striking feature of all of these efflux experiments was the marked similarity in the patterns of efflux of $^{59}Fe$ and $^{125}I$-labelled transferrin, even though the $^{59}Fe$ was not bound to transferrin. These results suggest that the $^{59}Fe$ was derived from the same cellular site and was released from the cells by the same mechanism as was the transferrin.

A considerable body of evidence indicates that transferrin is taken up by reticulocytes by the process of endocytosis and leaves by exocytosis [23]. Within the cells it is present mainly or entirely within intracellular vesicles [23] which sediment with the membrane fraction of the cells when they are fractionated by the methods used in the present work. The results of the experiments illustrated in Figs. 2–5 are consistent with the conclusion that transferrin efflux from the cells was the result of exocytosis. Earlier work has shown that endocytosis and exocytosis of transferrin by reticulocytes are temperature-dependent processes, and are inhibited by incubation at 46°C and by metabolic inhibitors, sulphydryl reagents and inhibitors of microtubular function [4,24]. In addition, phenylglyoxal has been shown to block endocytosis in other cell systems [25,26]. Since, in the presence of bipyridine and 1,10-phenanthroline, the $^{59}Fe$ appears to leave reticulocytes in the same manner as transferrin, it appears likely that the $^{59}Fe$ which accumulated in the cells in the presence of chelators was located in intracellular vesicles complexed to the chelators, and that these complexes were released from the cells during exocytosis.

An alternative explanation for the results shown in Figs. 4–7 is that the $^{59}Fe$-chelator complex is bound to or dissolved in the cell plasma membrane. Under these circumstances $^{59}Fe$ efflux would occur by diffusion from the membrane into the extracellular medium. Such a diffusion process would show kinetics different from those of transferrin release which occurs by exocytosis, and would not be expected to be inhibited by metabolic or microtubule inhibitors. Moreover, heating to 50°C should increase the rate of diffusion of $^{59}Fe$ from the cells rather than inhibit it. The results for $^{59}Fe$ efflux from reticulocyte ghosts (Figs. 3 and 4) are also against this alternative explanation. Haemolysis resulted in a diminished rate of efflux of $^{59}Fe$ and transferrin and a lesser effect of incubation temperature and metabolic inhibitors. This can readily be explained by the disruption of cell integrity leading to inhibition of exocytosis, as indicated by the reduced efflux of transferrin, but it would not be expected to reduce the rate of diffusion of $^{59}Fe$-chelator or transferrin from external cell membranes. The slow rate of loss of $^{59}Fe$ from ghosts which was observed may have resulted from an increase in permeability of vesicle and/or outer cell membrane as a conse-

quence of the haemolytic process.

The iron complexes of bipyridine and 1,10-phenanthroline consist of three molecules of chelator and one iron atom [27]. Presumably the iron-free forms of the chelators can diffuse through cell, vesicle and mitochondrial membranes but passage of the much larger and charged iron-chelator complexes occurs much more slowly. A similar explanation was presented by Ponka et al. [9] to explain the inhibitory effect of bipyridine on pyridoxal isonicotinoyl hydrazone-mediated iron efflux from reticulocytes. It is supported by the results of water/diethyl ether partition measurements which showed that the iron complexes were less soluble in the organic phase than were the free chelators. Also, differences in lipid solubility may explain the differing sensitivity of pyridoxal isonicotinoyl hydrazone-mediated efflux to the chelators, bathophenanthroline, 1,10-phenanthroline, bipyridine and sulphonated bathophenanthroline.

After incubation of reticulocytes with $^{59}$Fe-labelled transferrin in the presence of isoniazid the largest proportion of the $^{59}$Fe taken up by the cells was in the mitochondria (Table II and Ref. 20) and this was the major source of the $^{59}$Fe which is released by the action of pyridoxal isonicotinoyl hydrazone. The results for the effect of incubation temperature (Fig. 8) showed that the $^{59}$Fe passed through the cytosol during its exit from the cell and that this efflux was much more dependent on incubation temperature than was movement from the membrane fraction of the cells to the cytosol. Also, as the concentration of pyridoxal isonicotinoyl hydrazone was increased $^{59}$Fe efflux reached a maximum at a concentration of 0.5 mM, while movement from membranes to cytosol did not show this effect (Fig. 7). These results raise the possibility that efflux occurs by mediated transport, possibly, as suggested by Ponka et al. [9,10], via a carrier for pyridoxal.

In conclusion, the results of the present experiments support the concept that iron release from transferrin in reticulocytes occurs within endocytotic vesicles and show that bipyridine and phenanthroline-type iron chelators inhibit iron uptake by complexing the iron within these vesicles. In contrast to the mechanism of their effects on iron uptake from transferrin, the inhibition of pyridoxal isonicotinoyl hydrazone-induced iron efflux produced by these iron chelators results from complexation of iron either within the mitochondria or cytosol.

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## References

1 Jandl, J.H. and Katz, J. (1963) J. Clin. Invest. 42, 314–336
2 Appleton, T.C., Morgan, E.H. and Baker, E. (1971) in The Regulation of Erythropoiesis and Hemoglobin Synthesis (Travnicek, T. and Neuwirt, J., eds.), pp. 310–315, Universita Karlova, Prague
3 Sullivan, A.L., Grasso, J.A. and Weintraub, L. (1976) Blood 47, 133–143
4 Hemmaplardh, D. and Morgan, E.H. (1977) Br. J. Haematol. 36, 85–96
5 Morgan, E.H. (1981) Biochim. Biophys. Acta 642, 119–134
6 Armstrong, N.J. and Morgan, E.H. (1982) in The Biochemistry and Physiology of Iron (Saltman, P. and Hegenauer, J., eds.), pp. 149–157, Elsevier, New York
7 Morgan, E.H. (1971) Biochim. Biophys. Acta 244, 103–116
8 Ponka, P., Borová, J., Neuwirt, J. and Fuchs, O. (1979) FEBS Lett. 97, 317–321
9 Ponka, P., Borová, J., Neuwirt, J., Fuchs, O. and Necas, E. (1979) Biochim. Biophys. Acta 586, 278–297
10 Ponka, P. and Wilczynska, A. (1982) in The Biochemistry and Physiology of Iron (Saltman, P. and Hegenauer, J., eds.), pp. 275–277, Elsevier, New York
11 Hemmaplardh, D. and Morgan, E.H. (1974) Biochim. Biophys. Acta 373, 84–99
12 Hanks, J.H. and Wallace, R.E. (1949) Proc. Soc. Exp. Biol. Med. 71, 196–200
13 Ponka, P. and Neuwirt, J. (1970) Br. J. Haematol. 19, 593–604
14 Wallach, D.F.H. and Schmidt-Ullbrick, R. (1973) Methods Cell Biol. 15, 235–276
15 De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Applemans, F. (1975) Biochem. J. 60, 604–617
16 Morgan, E.H. (1976) Biochem. J. 158, 489–491
17 Maddy, A.H. and Spooner, R.O. (1970) Vox Sang 18, 34–41
18 Yonetani, T. (1967) Methods Enzymol. 10, 332–335
19 Widnell, C.C., Schneider, Y.-J., Pierre, B., Baudhin, P. and Trouet, A. (1982) Cell 28, 61–70
20 Borová, J., Ponka, P. and Neuwirt, J. (1973) Biochim. Biophys. Acta 320, 143–156

21 Mazur, A. and Carleton, A. (1963) J. Biol. Chem. 238, 1817–1824
22 Primosigh, J.V. and Thomas, E.D. (1968) J. Clin. Invest. 47, 1473–1482
23 Morgan, E.H. (1981) Mol. Aspects Med. 4, 1–123
24 Light, A. and Morgan, E.H. (1982) Scand. J. Haematol. 28, 205–214
25 Oliver, J.M., Yin, H.H. and Berlin, R.D. (1976) in Leukocyte Membrane Determinants Regulating Immune Reactivity (Eijsvoogel, V.P., Roos, D. and Zeijlemaker, W.P., eds.), pp. 3–14, Academic Press, New York
26 Kaplan, J. and Neilsen, M.L. (1979) J. Biol. Chem. 254, 7329–7335
23 Smith, G.F. and Richter, F.P. (1944) Phenanthroline and Substituted Phenanthroline Indicators, G. Frederick Smith Chemical Co., Columbus, OH