

AN IN VITRO AND IN VIVO STUDY OF THE
MECHANISMS OF UPTAKE AND UTILIZATION
OF IRON BY RED CELL PRECURSORS.

by

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T O M Y W I F E

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INTRODUCTION AND SCOPE OF THE PRESENT STUDY.

Iron is absorbed from the bowel and passes into the blood where it attaches to a specific iron-binding β_1 globulin called transferrin. Attached to this protein, iron is distributed to most tissues in the body, but the bulk of the iron is transported to the bone marrow and liver. Iron transported to the liver is incorporated mainly into storage forms of iron such as ferritin and haemosiderin, whereas in the erythroid marrow iron is utilized mainly in haemoglobin synthesis.

The process whereby iron is released from transferrin to the red cell precursors and is incorporated into haemoglobin, is incompletely understood. It has been postulated that a labile pool of iron exists in the red cell precursor membrane from which iron can feed back into the plasma or enter the cell where it is utilized in haemoglobin synthesis. Several investigators have suggested that once iron has entered the cell it passes through a non-haemoglobin protein intermediate before it is finally incorporated into haemoglobin. The nature of this intermediate is as yet

undetermined. Another non-haemoglobin iron-containing protein, ferritin, can be seen in the cytoplasm of red cell precursors on electron microscopy, but its role in the incorporation of iron into haemoglobin in these cells has not yet been satisfactorily elucidated.

This thesis has been divided into two parts. The first part is a review of what is at present known of the mechanisms of transfer of iron from transferrin to the red cell precursors in the bone marrow, and the intracellular pathways whereby it is subsequently incorporated into haemoglobin. It was felt necessary in this section to review in some detail the process whereby iron is taken up by the immature red cell membrane, in order to have a more complete background to the overall movement of iron into and out of the erythroid cell. This has necessitated a review of

- a) the properties of transferrin,
- b) in vivo studies of iron uptake by red cell precursors which have indicated the existence of a 'labile pool' of iron in the erythroid cell membrane,
- c) electron microscopic studies in which iron is believed to be assimilated by red cell precursors by a process

akin to pinocytosis,

d) in vitro studies, which have yielded most information on the intimate mechanisms of iron uptake by red cell precursors.

In the second part of this thesis, experimental studies of the transfer of iron from transferrin to the red cell precursors in human and rat bone marrow, and its subsequent incorporation into haemoglobin, have been presented. Both in vitro and in vivo techniques have been used. Emphasis has been placed on the pathways of iron within these cells. In this context particular attention has been paid to the role of ferritin as until recently no information about the movement of iron into and out of ferritin in the erythroid cell was available.

PART I

REVIEW OF THE LITERATURE

CHAPTER I

THE MECHANISMS OF IRON UPTAKE BY RED CELL PRECURSORS

I. The transport of iron.

Once iron has crossed the intestinal mucosa, it is picked up in the blood by a specific transport protein, which has been variously called transferrin (Laurell, 1947), iron-binding protein (Rath and Finch, 1949), siderophilin (Schade et al., 1949) and metal-combining globulin (Surgenor et al., 1949). The specific iron-binding fraction of human plasma was almost simultaneously discovered by Holmberg and Laurell (1945) and by Schade and Caroline (1946). It was subsequently concentrated and purified in Cohn's fraction IV (Surgenor et al., 1949), and crystallized in 1952 (Koechlin, 1952).

Transferrin has the electrophoretic mobility of a β_1 globulin (Wallenius, 1952; Neale, 1955). Its molecular weight has been estimated to be approximately 90,000, each molecule of transferrin being able to bind two atoms of iron (Laurell and Ingleman, 1947; Surgenor et al., 1949; Koechlin, 1952). Recent work indicates that the molecular weights of human, rat and monkey transferrins are closer to 68,000

(Charlwood, 1963). When the protein is fully saturated with iron, about 0.13 per cent of the total molecule consists of iron, (Surgenor et al., 1949). It should be noted, however, that as far as is known 'serum contains only iron-free siderophilin molecules and/or those with two atoms of iron chelated to the protein' (Schade, 1964). Iron-transferrin complexes containing only one atom of iron have thus far not been isolated in a stable form (Schade, 1964).

Transferrin is routinely measured in terms of the amount of iron it is capable of binding (i.e. the iron-binding capacity of the serum), and not in terms of the absolute weight of the protein. Normally about 30 per cent of the protein is saturated with iron, the total binding capacity having an average value between 300 - 340 ug. per 100 ml. of plasma in normal adults (Bothwell and Finch, 1962 a).

Transferrin is a glycoprotein, the protein moiety of which is thought to consist of a single polypeptide chain (Bearn and Parker, 1964). The carbohydrate portion of the molecule

consists of four chains, each containing two residues of hexosamine, two of galactose and one each of mannose and sialic acid (Schultze et al., 1958; Jamieson, 1963). The iron attached to transferrin is in the ferric form (Surgenor et al., 1949), and is extremely tightly bound to it under physiological conditions, the affinity constant of the iron-transferrin complex being between 10^{26} and 10^{30} (Saltman and Charley, 1960). At physiologic pH values, the iron cannot be removed from transferrin by chelates which have a very strong affinity for ferric iron (Rubin et al., 1960). Maximal binding of iron to transferrin occurs when the pH is above 7.0, and dissociation occurs when the pH falls below 6.5. The dissociation is reversible as the pH is raised to 7.0 (Surgenor et al., 1949). The mode of binding of iron to transferrin has been thought to involve phenolic groups of the protein, and bicarbonate groups from the plasma (Schade et al., 1949; Fiala and Burk 1949). Recent work has suggested that the iron is bound to tyrosine and histidine molecules in transferrin (Hazen, 1963).

Up until 1961, electrophoretic studies had revealed

that there were at least eight genetically controlled variants of human transferrin, differing in their electrophoretic mobility (Smithies and Hillier, 1959; Giblett et al., 1959; Turnbull and Giblett, 1961). The transferrins were thought to represent allelomorphic genes at a single locus and were named B₀, B₁, B₂, C, D₀, D₁, D₂, D₃ in order of decreasing electrophoretic mobility. Most individuals have the single transferrin C. The recent detection of several new transferrin variants has revealed that altogether there are fifteen species of transferrin that can be distinguished electrophoretically, seven migrating faster than transferrin C and seven migrating slower (Bearn and Parker, 1964). The transferrin variants do not appear to differ in their ability to carry iron and deliver it to the tissues (Turnbull and Giblett, 1961).

II. In vivo studies of iron uptake by immature red cells.

The concept of the 'labile erythropoietic iron pool'.

Although in vivo tracer studies have not been of much value in contributing to the intimate molecular mechanisms

of iron uptake by the red cell precursors, they have nevertheless been invaluable in obtaining an insight into the quantitative aspects of the movement of iron from the plasma to red cell precursors in the marrow and reticuloendothelial cells and parenchymal cells of various organs.

Several studies have been performed in which tracer amounts of radioiron bound to transferrin of plasma have been administered intravenously, and the subsequent fate of the iron followed by appropriate sampling of the blood, and by detection of radio-activity in various organs by external scintillation counters (Huff et al., 1950; Huff et al., 1951; Bothwell et al., 1956; Bothwell et al., 1957; Bohannon et al., 1961; Pollycove, 1959; Pollycove and Mortimer, 1961). The techniques were initially described by Huff and his co-workers (Huff et al., 1950; Huff et al., 1951). After injection of radioiron into a normal human subject, all the iron is initially in the plasma; seven to ten days later almost all the iron can be found in circulating red cells. The intermediate stages of this overall reaction have revealed much interesting information.

Virtually all the radioiron moves rapidly from the plasma to the bone marrow. Initial counting following the injection of the tracer is a measure of the radioiron in the circulating plasma. As the iron leaves the plasma and accumulates in the marrow, as evidenced by an increased count over the sacrum, the counts over the spleen and liver decrease. The radioiron remains in the marrow for approximately 24 hours, and during the next 5 - 6 days the radioiron leaves the marrow and can be detected in the haemoglobin of circulating red cells. Once iron is incorporated into haemoglobin in the red cell precursor, it remains part of the molecule until the erythrocyte is destroyed.

Although in early studies it appeared that radioiron disappeared from the plasma in a simple exponential fashion, with more refined techniques it soon was shown that this was true only for times up to eight hours. The slope of the curve thereafter became progressively less steep, i.e. at a time when the level of radioactivity was about 20 per cent of its initial value (Sharney et al., 1954; Huff and Judd, 1956; Pollycove, 1959). This finding implied that a portion of the iron initially

removed from the plasma was not irreversibly fixed at the receptor sites for iron, but that there was a significant interchange with, or feedback from, these sites to the plasma. Further evidence against irreversible fixation of radioiron in developing red cells was the fact that the maturation time of human red cell precursors was estimated at from 2 to 4 days (Osgood 1954; Astaldi, 1960) and therefore the appearance of radioiron in circulating red cells after intravenous injection should be complete in approximately 4 days. As maximal iron incorporation does not occur until 7 - 10 days, irreversible fixation of iron in red cell precursors would appear unlikely.

On the basis of their analysis of the kinetic curves of iron turnover, Pollycove and Mortimer (1961) proposed the existence of a 'labile erythropoietic iron pool' which is intermediate between the plasma iron and the iron that is irreversibly fixed for incorporation into haemoglobin in red cell precursors. This pool was located at, or in the membrane of the developing erythrocyte. Supporting evidence for this concept was the finding that eight hours after intravenous injection of a tracer amount of labelled iron in dogs, about

half of the iron taken up by the marrow was non-haemoglobin iron associated with the red cell precursor membrane. It was also shown that when labelled human and rabbit reticulocytes were reincubated in unlabelled plasma, up to 17 per cent of the radioiron could be transferred back to plasma. Similar findings in dogs and man were also reported in a subsequent communication (Pollycove, 1961). Pollycove and Mortimer (1961) could also find no evidence that iron in plasma exchanged with body stores to any appreciable extent except in states of iron overload such as haemochromatosis.

Pollycove and Mortimer (1961) have estimated that the feedback of iron is approximately 11 mg. per day, and that the labile erythropoietic pool is 84 mg. in size. As 32 mg. of iron leave the plasma per day, and the feedback is 11 mg. per day, 21 mg. per day must be used for haemoglobin synthesis. As 1 gram of haemoglobin contains 3.4 mg. of iron, this would allow for a daily synthesis of haemoglobin of 6.3 grams. This figure is compatible with a red cell lifespan of about 120 days.

The Pollycove concept of an erythropoietic labile pool

of iron is however at variance with studies by Noyes and Finch (1961) who showed that 20 minutes after intravenous injection of radioiron in rabbits, about 80 per cent of the iron taken up by the marrow was in the form of haem. Similar findings with reference to the distribution of radioiron in red cell precursors in in vivo studies have been reported in dogs and man (Hosain et al., 1962) and more recently in humans (Gale, 1964; Myhre, 1964 b). The findings of Noyes and Finch (1961) indicate that there is a relatively rapid fixing of transferrin iron into haem in red cell precursors, and would appear to be incompatible with the presence of a significantly large labile pool of iron in the marrow as postulated by Pollycove. On the basis of these findings it has been stated that 'the anatomic reality of the Pollycove model must be regarded as a matter of contention' (Bothwell and Finch, 1962 b). In view of these differences, an attempt has been made to follow the movement of iron into and out of red cell precursors in the present study.

III. Electron microscopic studies of iron uptake by red cell precursors. The uptake of iron by ropheocytosis.

The presence of ferritin, a known non-haem iron protein complex, was demonstrated in erythroid marrow cells on electron microscopy in a series of elegant studies by Bessis and his co-workers (Bessis and Breton-Gorius, 1957; Bessis, 1958; Bessis, 1959; Bessis and Breton-Gorius, 1959). On the basis of these morphological studies, a dynamic picture of the role of ferritin in the erythroid cells has been evolved by Bessis and his co-workers and this will be discussed in some detail.

Ferritin was first isolated from horse spleen by Laufberger in 1937 (Laufberger, 1937). It is a water soluble, crystalline protein, stable to heating to 80°C. The molecule contains 17 - 23% of its dry weight as iron (Granick, 1942), and consists of ferric-hydroxide phosphate micelles which are attached to a protein moiety called apoferritin. The molecular weight of horse spleen apoferritin has been determined as 465,000 (Rothen, 1944) and as 480,000 (Harrison, 1964). Farrant (1954) showed by electron microscopic studies that the iron micelles are located within the protein molecules, and appear to be gathered in the four corners of a square whose sides measure 55 Å.

More recent work indicates that the iron within the ferritin core is arranged at the vertices of an octahedron, and that the different appearances of ferritin as seen with the electron microscope are due to images of the octahedron when viewed at different angles (Kerr and Muir, 1960; Muir, 1960). On the basis of X-ray diffraction evidence, it has been proposed that the quaternary structure of apoferritin consists of twenty subunits, each consisting of a single peptide chain and situated at the twenty vertices of a pentagonal dodecahedron (Harrison, 1964). The structure of the iron micelles in ferritin is specific. In addition to being easily detected in the erythroblast, ferritin can be also seen in the reticuloendothelial cells of marrow and liver, and also in parenchymal liver cells and intestinal epithelium on electron microscopy. Ferritin cannot be seen on light microscopy in contrast to haemosiderin which is a closely related iron protein complex. Haemosiderin is an ill-defined entity containing variable amounts of ferritin in addition to other substances such as lipids, porphyrins, sugars, copper and calcium.

pictures of bone marrow constitutes one of the two currently held ideas on the mechanism of uptake of iron by red cell precursors (Bessis, 1962). He has suggested that iron enters the developing red cell mainly in the form of ferritin. This hypothesis is based on the demonstration of erythroblastic islands in the bone marrow, consisting of red cell precursors surrounding a central reticuloendothelial 'nurse' cell which contains ferritin. This cell appears to feed ferritin into the surrounding red cell precursors by a process called rhopheocytosis. The molecules of ferritin adhere to the erythroid cell membrane and are then invaginated into the cytoplasm to form a vacuole. The vacuole then loses its membrane and the ferritin is incorporated into the cytoplasm of the red cell precursor. The ferritin molecules are then believed to enter the mitochondria of the cell, where they lose their characteristic appearance and take on a more dispersed form and are called 'ferruginous micelles'. It is postulated that this iron is utilized for haem synthesis. The ferritin in the reticular 'nurse' cells of the marrow is believed by Bessis to be derived from the plasma transferrin (Bessis, 1962).

The alternative concept that iron uptake by red cell precursors is due to direct transfer of iron from plasma transferrin will be discussed in detail in the following section, the evidence for this being that several workers have shown that in vitro suspensions of immature cells (reticulocytes), free of reticular 'nurse' cells, are able to take up iron from transferrin and utilize it for haemoglobin synthesis. The fact that radio-iron appears rapidly in haem in marrow cells after intravenous administration of plasma-bound radioiron (Noyes and Finch, 1961) also supports the concept of a direct transfer of transferrin-iron to immature red cell precursors. Bessis has recently stated (Bessis, 1962) that probably both mechanisms co-exist, i.e. that transferrin imparts iron to both the reticular cell and the erythroblast. Ferritin is formed in the reticular cells and then transferred to the erythroblast by ropheocytosis. He maintains that the possibility 'that ferritin is manufactured within erythroblasts seems unlikely, although the ability to synthesize ferritin is widespread in other tissues' (Bessis, 1962).

There are several serious criticisms of Bessis' hypothesis. Firstly, the direction of movement of ferritin between

the red cell precursors and the reticuloendothelial cells in the marrow has been questioned. Several workers have suggested that the ferritin could well be moving from the erythroid to the reticular 'nurse' cells, and not vice versa (Lajtha, 1959; Jandl, 1960; Bothwell and Finch, 1962 c). This may represent phagocytosis of effete or normal red cell precursors in the marrow. Secondly, it appears highly unlikely that the reticuloendothelial cell is able to synthesize significant quantities of ferritin when the iron is derived from transferrin. Autoradiographic studies of human bone marrow cultured with ^{59}Fe -labelled plasma have revealed that it is only the erythroid series of cells which assimilate iron from transferrin (Lajtha and Suit, 1955; Austoni, 1956; Nyhre, 1964 a). Also Hosain et al. (1962) have recently shown that in groups of hypertransfused rats (i.e. having depressed marrow activity) the amount of radioiron from plasma that localized in the spleen was negligible. As the spleen contains only reticuloendothelial cells and no erythroid elements, it was concluded that reticuloendothelial cells cannot assimilate iron from transferrin.

An attempt has thus been made in the present study to

try and elucidate some of the problems relating to the movement of ferritin in the erythroid marrow.

IV. In vitro studies of iron uptake from transferrin by red cell precursors.

In vitro studies have contributed materially to an understanding of the mechanisms whereby transferrin-bound iron is taken up by red cell precursors. In almost all these studies the iron of the iron-transferrin complex has been labelled with radioiron, but in more recent studies transferrin itself has been tagged with ^{131}I in an attempt to determine its fate in relation to the uptake of iron. It must be emphasized at the outset that the detailed molecular mechanisms of iron uptake by immature red cells have not yet been completely elucidated.

(i) The specific uptake of transferrin-bound iron by red cell precursors.

In mammals and birds, iron bound to transferrin is taken up by immature red cells, and not by adult

red cells. This finding has been amply confirmed by several workers in in vitro studies in which iron uptake by reticulo-cyte suspensions has been compared to uptake by adult red cells (Walsh et al., 1949; Jensen et al., 1953; Jandl et al., 1959; Morgan and Laurell, 1963). Autoradiographic studies of bone marrow cultures incubated with transferrin-bound iron have also confirmed the inability of adult red cells to take up iron, and have established that non-erythroid cells do not assimilate iron (Lajtha and Suit, 1955; Austoni, 1956; Myhre, 1964 a). Adult red cells can however take up ferric iron in a saline suspension, but almost all this iron is confined to the membranes of the cell (Jandl et al., 1959).

In man, iron uptake starts in the earliest recognizable nucleated red cell, the pronormoblast (Lajtha and Suit, 1955). In vitro studies have shown that there is a gradient of iron uptake by erythroid cells of man, less iron being assimilated as the cell matures (Lajtha and Suit, 1955; Austoni, 1956; Myhre, 1964 a). A similar gradient exists in dog marrow cells (Alpen and Cranmore, 1959), but was not observed in rat marrow (Austoni, 1954), where

maximal uptake occurs in the polychromatic and orthochromatic stages of erythroblast maturation. Quantitative estimates of the amounts of iron taken up by human erythroid cell cultures show fairly good agreement between some workers. Mean erythroblast uptakes of 1.7×10^{-9} and 1.8×10^{-9} ug./hour/cell were obtained by Vas et al. (1962) and Myhre (1964 a) respectively. A higher value was reported by Thorup et al. (1958) who found a mean erythroblast iron uptake of 2.75×10^{-9} ug./hour/cell. Myhre (1964 a) estimated that the approximate uptake of iron per cell per hour was 3.4×10^{-9} ug. in the early human erythroblast, 1.8×10^{-9} ug. in the polychromatic erythroblast, 0.6×10^{-9} ug. in the orthochromatic erythroblast, 9×10^{-11} ug. in early reticulocytes and 2×10^{-12} ug. in late reticulocytes. In rabbit bone marrow and reticulocyte suspensions, nucleated red cells were calculated to take up 2.01×10^{-9} ug./hour/cell, and mature and young reticulocytes were estimated to assimilate a mean of 0.16×10^{-9} and 1.26×10^{-9} ug./hour/cell, respectively (Erslev and Hughes, 1960). Belcher and Courtenay (1959) found that rat reticulocytes took up 0.25×10^{-9} ug. of iron/cell/hour, the uptake diminishing as the cells matured in the circulation. Species differences probably explain the differences in iron uptake

by the reticulocytes of the various species studied.

(ii) The kinetics of uptake of transferrin and transferrin-bound iron by red cell precursors.

Early studies of the uptake of transferrin-bound iron by red cell precursors in vitro resulted in an incomplete understanding of the process as the uptake of the transferrin molecule itself, in relation to the iron, was not elucidated.

The rate of uptake of iron by the reticulated nucleated red cells of ducks was found to be rapid over the first few hours of incubation, and then diminished markedly after approximately six hours (Sharpe et al., 1952; Jensen et al., 1953). The uptake of iron by pigeon erythrocytes was found to be linear over the first six hours, but progressed at a slower rate up to 24 hours (Clark and Walsh, 1960). In studies on human reticulocytes a slowly diminishing rate of uptake of transferrin-bound iron over four hours incubation was demonstrated (Jandl et al., 1959). Erslev and Hughes (1960) found that the rate of uptake of iron by rabbit reticulocytes decreased gradually after four

hours incubation, but only after ten to twelve hours in rabbit bone marrow suspensions. Very little further uptake of iron occurred in suspensions incubated for 24 - 48 hours. With rat reticulocytes, the maximum rate of iron uptake was found to occur within the first half hour of incubation (Clark and Walsh, 1960). In a recent study Myhre (1964 a) found that the rate of iron uptake by human reticulocytes was relatively rapid over the first two hours of incubation, and then slowed down, so that after ten hours further uptake was minimal. However, with human marrow cells, the rate of iron uptake was constant during the initial ten hours of incubation and then gradually slowed down, findings which are similar to those of Erslev and Hughes (1960) using rabbit cells.

By comparing the disappearance rate of iron and transferrin in vivo after intravenous injection of doubly-labelled transferrin (i.e. transferrin labelled with ^{131}I and ^{59}Fe), it appeared that transferrin itself was not utilized in the process of unloading its iron (Katz, 1961). Also, it had been shown that in vitro, transferrin is not destroyed during this process (Paoletti and Durand, 1958). The kinetics of transferrin

uptake by immature red cells in relation to iron uptake have been recently studied in vitro by two groups of workers (Jandl and Katz, 1961; Jandl and Katz, 1963; Morgan and Laurell, 1963; Morgan, 1964).

Using ^{131}I - ^{59}Fe - transferrin, Jandl and Katz (1961, 1963) found that negligible quantities of transferrin were taken up by adult human red cells. Reticulocytes, however, rapidly took up transferrin within the first few minutes of incubation, approximately half of the two hour uptake occurring in the first two minutes. ^{59}Fe was taken up at a more steady rate, with the eventual accumulation of many more moles of iron than transferrin after two hours incubation. Almost all the transferrin was attached to the cell membranes, while over half of the iron taken up had entered the soluble cell contents. When ^{131}I -labelled reticulocytes were reincubated in unlabelled transferrin solutions the rate of detachment of labelled transferrin was found to be equal to the rate of its attachment. Half the transferrin on the cell exchanged with transferrin in solution in approximately one minute. Transferrin detachment was found to be dependent of the concentration of transferrin

in the incubation medium. On the other hand, iron taken up by the cell could not be eluted subsequently by transferrin solutions or by native plasma (Jandl et al., 1959). These findings led to the following concept (Jandl and Katz, 1963) of iron transfer to reticulocytes: 'A molecule of plasma transferrin containing iron attaches to a receptor on the immature red cell surface. There a mechanism exists for actively removing the iron from the protein. The protein then is displaced from the cell by another molecule of iron laden transferrin. Thereby iron is delivered to the cell in an orderly fashion'.

In experiments in which ^{131}I -transferrin and ^{59}Fe -transferrin were incubated with rabbit reticulocytes, Morgan and Laurell (1963) confirmed the finding that transferrin molecules bound to the cell surface exchanged with the transferrin in the incubating medium. The rate of release of transferrin from the reticulocytes was related to the amount of transferrin bound, and was not due to competition for the binding site by unbound transferrin. Small amounts of iron taken up by reticulocytes could, however, be eluted when the cells were reincubated

in unlabelled transferrin solutions. This finding was at variance with that of Jandl et al. (1959) but in agreement with the findings of Pollycove and Maqsood (1962). In the latter experiments the reticulocytes were, however, labelled in vivo and then reincubated in vitro. In a more recent study Morgan (1964) has extended his observations on the interaction between transferrin and the reticulocytes of rats, rabbits and humans in in vitro experiments. The initial reaction between transferrin and the reticulocyte surface appeared to be one of physical absorption as evidenced by the relatively large zero uptake of transferrin, and its insensitivity to metabolic inhibitors and to reduction in incubation temperatures. This union was not a firm one, since considerable amounts of transferrin could be eluted by washing with cold saline. Subsequently transferrin became more firmly bound to the reticulocyte surface and iron was transferred into the reticulocyte. Maximum binding of transferrin occurred in 2 - 5 minutes in rabbits, but took 15 - 30 minutes in rats and in man. Transferrin release (elution) was found to be independent of the concentration of transferrin in the incubating medium, a finding at variance with that of Jandl and Katz (1963).

The pattern of release of transferrin and iron from the reticulocyte surface at various times of incubation indicated that this process consisted of two parts. Initially, absorbed transferrin molecules exchanged with those in the media, this phase occurring before the iron had been transferred from transferrin to the cell, and also probably accounting for any iron that could be eluted from the reticulocyte. Thereafter more transferrin was released once its iron had been given up to the cell.

(iii) The effect of valency of iron on iron uptake
by red cell precursors.

Conflicting data exists in the literature as to whether valency of iron is of importance in determining the uptake of iron by red cell precursors. Walsh et al. (1949) found that iron bound to transferrin was assimilated less rapidly than free ferric iron, which in turn was taken up less rapidly than ferrous iron by human reticulocytes. These data do not however, indicate the valency of transferrin-bound iron at the moment it is transferred to reticulocytes. Sharpe et al. (1952)

found that equal amounts of ferric and ferrous iron were taken up when added to duck blood, the ferrous iron probably being oxidised immediately to ferric iron. Prior incubation of rat reticulocytes with ferric or ferrous iron (Clark and Walsh, 1960) resulted in an equal depression of ^{59}Fe uptake from transferrin indicating that valency was not of importance in determining the uptake of iron by the cell. At the same time it is possible that the added ferrous iron could have been oxidised to ferric iron in these experiments. However, prior incubation of the rat reticulocytes with ethylenediamine-tetra-acetic acid (E.D.T.A.) (a predominantly ferric iron chelator) and α,α' -dipyridyl or orthophenanthroline (ferrous iron chelators) resulted in a more consistent depression of subsequent ^{59}Fe uptake in those samples initially incubated with the ferrous iron chelators. Further evidence that transferrin-bound iron is in the ferrous form on transfer to reticulocytes was cited by Cleton et al. (1963) who noted that ferric iron chelators had no effect on the uptake of transferrin-bound iron by rabbit reticulocytes, a finding also reported by Jandl et al. (1959) in human reticulocytes.

(iv) Evidence for a receptor for iron in the
immature red cell membrane.

The presence of a receptor for iron in the human reticulocyte membrane was shown in a series of ingenious experiments by Jandl et al. (1959). The uptake of iron from transferrin was found to be depressed by prior incubation of reticulocytes with small concentrations of trypsin which did not affect glycolysis or respiration i.e. the effect appeared to be mediated on a receptor on the surface of the cell. A much weaker effect on iron uptake was observed with 'receptor destroying enzyme' obtained from vibrio cholera filtrates. Although both trypsin and 'receptor destroying enzyme' inactivate the neuraminic acid group of substances present in red cell membranes which are believed to be the receptors on red cells which react with several viruses (Howe et al., 1957), the relatively weak effect of 'receptor destroying enzyme' on iron uptake indicated that the iron receptors of reticulocytes were not identical to these substances. The possibility that certain ketose amino acids might act as iron binding receptors in the membranes of reticulocytes was also investigated by Jandl et al.

(1959). These studies were based on the previous demonstration that ketose amino acids were able to render iron available for bacterial utilization (Neillands, 1957), while Kruh and Borsook (1956) had shown that ketose amino acids could increase the incorporation of amino acids into the proteins of reticulocytes when added in the presence of iron. However, Jandl and his co-workers found that several ketose-amino acids had no effect on iron uptake by reticulocytes.

Further information on the nature of the receptor for iron in the immature red cell membrane has been obtained by studying the effect of adenosine triphosphate (A.T.P.) and ascorbic acid on iron uptake by rabbit reticulocytes (Morgan and Laurell, 1963) and rat marrow and reticulocytes (Mazur and Carleton, 1963).

Mazur et al. (1960, 1961) had previously found that A.T.P. and ascorbic acid could facilitate the incorporation of iron from transferrin into ferritin in rat liver. It was suggested that A.T.P. stimulated the oxidation of ascorbic acid in the presence of iron bound to transferrin, resulting in the reduction of the ferric iron to ferrous iron. In this way the iron was released from the

protein and incorporated into ferritin. Morgan and Laurell (1963) found that A.T.P. and ascorbic acid had no effect on iron uptake by whole reticulocytes (as these cells are impermeable to A.T.P.), or on the incorporation of iron into haem in a haemolysate of reticulocytes. However, the uptake of iron by reticulocyte ghosts was stimulated by A.T.P. and ascorbic acid, and it was postulated on this basis that transferrin, having bound to a specific receptor site on the reticulocyte membrane, might release its iron after chelation by ascorbate and A.T.P. and be transferred to a non-haem compound, which was postulated to be ferritin, in the membrane. Concurrent studies by Mazur and Carleton (1963) utilizing rat reticulocytes and marrow, have resulted in very similar conclusions concerning the mechanism of iron transfer. It was shown that lysis of reticulocytes or marrow cells resulted in a marked decrease in ^{59}Fe incorporation into both haem and ferritin within these cells, but that on addition of both ascorbic acid and A.T.P. to the lysate, the inhibition of radioiron incorporation into ferritin could be reversed. A reversal of inhibition of $[2\text{-}^{14}\text{C}]$ glycine incorporation into ferritin under the same conditions could not be demonstrated, indicating that preformed ferritin was available for combination

with iron once it had been liberated from transferrin by ascorbate and A.T.P.

(v) The effect of chelating agents on iron transfer to red cell precursors.

Experiments utilizing chelating agents have revealed much information on the mechanism of transfer of iron to immature red cells.

The inability of ethylenediaminetetraacetic acid (E.D.T.A.), a strong chelate of iron, to modify iron uptake by human retic locytes from transferrin until the molar ratio of E.D.T.A. to Fe exceeded 10, was strong evidence that iron does not exist in the free ionic form in its transfer from the iron-binding protein to the cell (Jandl et al., 1959). The transfer of iron to rabbit reticulocytes by synthetic chelating agents as compared to transferrin has been studied in some detail by Princiotto et al. (1964), and to a lesser extent by Clifton et al. (1963). Hydrophilic, aliphatic chelates related to E.D.T.A. were unable to transfer iron to those sites on the reticulocyte

membrane from where it could be utilized for haem synthesis (Princiotto et al., 1964). This was thought to be due to these negatively charged chelates being repelled by negatively charged acid groups on the red cell membrane. Neutral lipophilic iron chelates such as N, N' - Ethylenebis [2 - (O-hydroxyphenyl) glycine] (E.D.D.H.A.) were found to mimic transferrin in releasing iron to reticulocytes for haem synthesis. A similar finding had also been reported previously by Cleton et al. (1963). The more lipophilic the compound, the greater was the utilization of iron for haemoglobin production, provided the compound was neutral (Princiotto et al., 1964). The mode of uptake of iron from some of the chelates was however different to transferrin as previous studies (Aserkoff et al., 1963) had indicated that the processes were not competitive, nor did they respond in the same way to metabolic inhibitors. The inability of iron chelated to E.D.D.H.A. to compete with transferrin - iron in giving up its iron to reticulocytes was also noted by Cleton et al. (1963). Princiotto et al. (1964) interpreted their results to indicate that the effect of binding of iron to transferrin was to 'change the charge and environment of the metal in that it may approach areas on the reticulocyte membrane surface where active metabolic intervention may then further the process

of iron utilization'.

- (vi) The effect of varying iron transferrin ratios on iron uptake by immature red cells.

In vitro studies in which the pattern of iron uptake by red cell precursors has been compared at various iron transferrin ratios have been of great value in arriving at a more dynamic understanding of the transfer of iron from transferrin to the receptors on the immature red cell membrane. These studies are also of interest with respect to the patterns of iron uptake by red cell precursors and other tissues in conditions where the percentage saturation of transferrin is low, e.g. in iron deficiency anaemia, or high, e.g. in idiopathic haemochromatosis or Bantu nutritional siderosis.

In early studies Jensen et al. (1953) found that when increasing amounts of labelled iron were added to suspensions of duck erythrocytes, the amount of iron taken up by the cells increased progressively. When insufficiency of iron was not the limiting factor the amount of iron incorporated

into haem soon reached a plateau, while the incorporation into a non-haem fraction increased progressively. The non-haem iron fraction was taken as the difference between total cellular uptake and haem activity. No attempt was made to determine the nature of the non-haem iron in this study. Unfortunately, the percentage saturations of the plasma in each aliquot could not be ascertained at that time because of technical difficulties (the iron binding protein of ducks is peculiar in that it does not give colour reactions characteristic of animal transferrins). The possibility therefore exists that the increasing amounts of iron added to the duck blood could have oversaturated the iron-binding protein, and uptake of free iron could have been measured. In a recent study, Naïean (1962) found essentially similar results with human reticulocyte suspensions, when he showed that comparable aliquots of cells which had taken up increasing quantities of iron, incorporated the same amount of iron into haem.

Thorup et al. (1958) showed that in human marrow suspensions the rate of removal of iron from plasma decreased progressively as the concentration of iron available to the cells decreased below a level of $175 - 200 \times 10^{-9}$ ug. per cell per 24

hours. Concentrations of iron above this level did not result in any further increase in iron uptake. The percentage saturations of transferrin with iron were however, not reported.

Jandl et al. (1959) determined the effect of presenting human reticulocytes with increasing quantities of iron, the iron being bound to transferrin. In this study the percentage saturation of the transferrin was determined, and the results were expressed relative to this variable. In experiments in which the total iron binding capacity of purified transferrin was kept constant, and the amount of iron was varied, it was shown that relatively small amounts of iron were taken up below saturations of 20%, and that between saturations of 20 and 70% the uptake tended to plateau. Above saturations of 70% there appeared to be a 'nonspecific off-loading' of iron onto the cells. It was also shown that if the amount of iron was kept constant and the amount of transferrin varied, increasing amounts of iron were taken up at higher saturations of transferrin i.e. that the uptake of iron appeared to be dependent on transferrin saturation. From these findings Jandl and co-workers made the

interesting speculation that the homeostatic limits of 20 to 70% saturation of transferrin are maintained in vivo because below a saturation of 20% the iron-binding protein competes successfully with the marrow for iron, while above 70% saturation there appears to be a nonspecific off-loading of iron onto immature cells. In a subsequent paper (Jandl and Katz, 1963), using doubly-labelled transferrin (^{131}I - ^{59}Fe - transferrin), it was shown also that the higher the degree of saturation of transferrin with iron, the more ^{131}I -transferrin was taken up by the cells. The avidity of iron-saturated transferrin for the reticulocyte exceeded that of unsaturated transferrin by about five fold. The conclusion was thus drawn that iron might be involved in the chemical attachment of transferrin to the cell surface, or that in some way, iron affects the physico-chemical properties of transferrin which allow it to be more easily taken up by immature cells.

In a recent paper Morgan and Laurell (1963), using separately labelled ^{131}I - transferrin and ^{59}Fe - transferrin incubated with rabbit reticulocytes, obtained results which differed in several respects to those of Jandl et al. (1959)

and Jandl and Katz (1963). Morgan and Laurell found that iron uptake was independent of transferrin concentration and suggested that this difference might be due to the fact that the purified transferrin used by Jandl and his co-workers was in some way subtly altered as a result of a harsher fractionation procedure. They also showed that the amount of transferrin bound by reticulocytes was independent of iron concentration, a finding at variance with that of Jandl and Katz (1963).

In a more recent paper Katz and Jandl (1964) repeated some of their earlier experiments, but used ^{59}Fe -labelled plasma instead of purified iron binding protein. They also incubated the radioiron with the plasma for approximately 18 hours before use, instead of approximately 1 hour as they had previously done. They then confirmed Morgan and Laurell's observation that iron uptake was independent of transferrin concentration, and also could no longer demonstrate the marked increase in iron uptake at percentage saturations above 70%. The latter finding was shown to be due to the more complete binding of iron to transferrin as a consequence of allowing

the radioiron and transferrin to react for 18 hours instead of one hour.

In a stimulating and critical paper Schade (1964) has reported his observations on the uptake of iron by immature red cells. He obtained essentially similar results with human reticulocytes, rabbit reticulocytes and marrow cells. The total cellular uptake of iron was found to be independent of the amount of iron present in serum in which the total iron binding capacity was kept constant. This finding is in direct contrast to those of Jandl and his co-workers (Jandl et al., 1959; Katz and Jandl, 1964) and of Morgan and Laurell (1963). It should be noted however, that Schade's conclusions were true only for incubation times up to 45 minutes, after which time the rate of iron uptake (and total cellular uptake) began to fall off with low saturations of iron. Schade however explains this fall off as 'reflecting a decrease in the absolute concentration of the iron siderophilin complex to levels below that required to saturate the iron absorbing capacity of the reticulocytes present in the suspension'. It is difficult to explain the differences between the results of Schade (1964),

and Katz and Jandl (1964) as the latter workers also measured total cellular uptakes after 45 minutes incubation. In experiments in which the amount of iron in the serum was kept constant but the total amount of transferrin varied Schade (1964) found that the cellular uptake of iron was independent of the amount of transferrin present (i.e. independent of the percentage saturation of transferrin). This finding is in agreement with that of Katz and Jandl (1964) and Morgan and Laurell (1963).

Schade (1964) also demonstrated that the mechanism of iron uptake by red cell precursors was different from that of iron uptake by liver. Iron uptake by liver tissue was approximately 3.8 times greater at 90% saturation of transferrin than at 50% saturation, this uptake obeying the theoretical equilibrium condition in which the dissociation of the two transferrin-bound atoms of iron were dependent on each other. As mentioned above, these findings were not observed with immature red cells, and were regarded by Schade as good evidence for the presence of specific complementary sites on mature red cell membranes 'peculiar to the physico-chemical properties of the

iron-siderophilin molecule'.

Schade (1964) also pointed out the danger of using purified iron binding proteins in studies of this type, because of the difficulty of obtaining a siderophilin that was not subtly altered in some way. The use of E.D.T.A. in the preparation of isolated siderophilins was also deprecated, as small traces of E.D.T.A. could cause significant errors in estimating the percentage saturation of a particular specimen.

The in vitro studies cited above all indicate that it is not the percentage saturation of transferrin that determines the uptake of iron by red cell precursors, but rather the concentration of iron-transferrin complex present in the incubation medium. These findings are of some interest when compared to the recent studies of Bainton and Finch (1964) who showed in a large series of patients with various haematological disorders that there was a better correlation between the sideroblast count and the percentage saturation of transferrin, than between the sideroblast count and the plasma iron level. These studies indicate that in vivo the percentage saturation of transferrin

may determine the amount of iron taken up by the immature red cell, and are difficult to reconcile with the in vitro findings.

- (vii) The effect of temperature on iron and transferrin uptake by red cell precursors.

The in vitro uptake of transferrin-bound iron by red cell precursors has been shown to be temperature dependent, optimal uptake of iron occurring at the physiological temperature of the species investigated (Walsh et al., 1949; Sharpe et al., 1952; Jensen et al., 1953; Jandl et al., 1959; Morgan, 1964). In these studies wide variations of temperature from the physiological value were tested. Erslev and Hughes (1960), however, found that minor variations in temperature of incubation of rabbit marrow (35°C as against 40°C) had little effect on iron uptake. Incorporation of iron into haem was also diminished at low and high temperatures (Jensen et al., 1953; Jandl et al., 1959). The uptake of iron not bound to transferrin (i.e. ferric iron in saline suspensions) was also shown to be temperature dependent, but less so than iron bound to transferrin (Jandl et al., 1959). Transferrin uptake (as ^{131}I)

was also found to be depressed at low temperatures, but less so than iron uptake (Jandl and Katz, 1963; Morgan, 1964). The rate of release (elution) of ^{131}I - transferrin from reticulocytes when reincubated in unlabelled transferrin solutions was also reduced at low temperatures (Morgan, 1964). The obvious temperature dependence of iron and transferrin uptake suggests that these processes are dependent on cellular metabolic energy. Transferrin release may also be dependent on metabolic energy (Morgan, 1964).

(viii) The effect of pH, CO_2 tension and O_2 tension on iron uptake by red cell precursors.

Maximal iron uptake from transferrin by human reticulocytes was found to occur between pH 7.2 and pH 7.6, with sharp reductions outside this range (Jandl et al., 1959). In rabbit bone marrow suspensions, optimal iron uptake occurred at pH 7.4 (Erslev and Hughes, 1960). Depression of iron uptake was found to be more marked at pH values on the alkaline side, as compared to values on the acid side of pH 7.4. Proliferative activity of the marrow, as determined in suspensions treated with

colchicine, did not change significantly between pH 7.2 and 7.7 (Erslev and Hughes, 1960). These findings are of interest in view of the demonstration by Thomas (1955) that oxygen consumption and glycine incorporation into haem in rabbit bone marrow suspension cultures were maximal at pH 8.0 to 8.5, at which values Erslev and Hughes (1960) found negligible cellular iron uptake. The effect of pH on transferrin uptake has not been documented yet.

Erslev and Hughes (1960) found that the proliferative activity of rabbit erythroblasts was suppressed at low CO₂ tensions and low bicarbonate content of the suspending medium. Iron uptake was also suppressed, but to a lesser extent, by low CO₂ tensions in the incubation medium.

In the intact animal, reduced tissue oxygen tension has been shown to cause the production of a humoral factor (erythropoietin) which stimulates the proliferative activity of the red cell precursors (Gordon, 1959). Further evidence for the existence of a humoral factor is the finding that rabbit bone marrow suspensions do not show an increase in proliferative

activity at low oxygen tensions, nor is the uptake of transferrin-bound iron increased (Erslev and Hughes, 1960). On the contrary, proliferative activity and iron uptake are somewhat depressed. Reduced oxygen tensions have also been shown to slightly depress iron uptake by human reticulocytes (Jandl et al., 1959). These findings indicate that the process of iron uptake is to some extent dependent on an intact oxidative metabolism in red cell precursors. The effect of reduced oxygen tension on transferrin uptake has not been reported.

(ix) Effect of metabolic stimulators and inhibitors
on iron and transferrin uptake by red cell
precursors.

A variety of metabolic inhibitors have been shown to depress iron uptake by the immature red cell, and also its subsequent incorporation into haemoglobin, indicating that these processes are dependent on cellular energy. In early studies on duck erythrocytes (Sharpe et al., 1952; Jensen et al., 1953) cyanide was found to markedly inhibit cellular iron uptake, but the inability of carbon monoxide to depress iron uptake

indicated that the effect of cyanide was not mediated through inhibition of cytochrome oxidase (Sharpe et al., 1952). The possibility of formation of a non-transferable complex between iron and cyanide could not be excluded in the latter experiments, although it appeared unlikely. Evidence for the dependence of iron uptake on an intact energy metabolism in human marrow suspensions was forthcoming in the studies of Thorup et al. (1958), who found that concentrations of sodium fluoride above $10^{-3}M$ progressively suppressed iron uptake. In studies on human reticulocyte suspensions, Jandl et al. (1959) came to similar conclusions when it was found that $10^{-2}M$ cyanide, $10^{-2}M$ fluoride, $10^{-2}M$ azide, $10^{-3}M$ arsenate, $10^{-3}M$ iodoacetate and $10^{-4}M$ 2:4 dinitrophenol all markedly inhibited cellular iron uptake from transferrin. Fluoroacetate in high concentration had no inhibitory effect, however. An interesting finding (Jandl et al., 1959) was that although $10^{-4}M$ lead ions markedly inhibited iron incorporation into haemoglobin, cellular uptake of iron was not depressed. This indicated that the membrane uptake of iron by the cell was not dependent on an intact haemoglobin synthetic mechanism.

Iron uptake and incorporation into haem in human marrow suspensions has been found to be inhibited by chloramphenicol, but only in concentrations 10 - 15 times normal therapeutic levels (Erslev and Iossifides, 1962). The effect on cellular uptake was thought to be mediated through a direct action on the membrane receptors for iron, rather than on energy yielding processes in the cell. The intracellular effect on iron incorporation into haemoglobin was explained on its known inhibitory effect on protein synthesis. Vas et al. (1962) were, however, unable to show an effect on cellular iron uptake in human marrow suspensions with chloramphenicol, but found an inhibitory effect on iron incorporation into haem.

In studies on rat reticulocytes (Clark and Walsh, 1960), it was found that $10^{-2}M$ cyanide had no effect on cellular uptake of iron, a finding in contrast to that of Jandl et al. (1959), but that the percentage of cellular iron incorporated into haem was reduced. On the other hand, $10^{-3}M$ mercuric chloride inhibited the cellular uptake of iron but had no effect on the proportion of iron incorporated into haem. $10^{-2}M$ iodoacetate and $10^{-3}M$ fluoride had no effect on either iron uptake or iron incorporation

into haem, findings at variance with those of Thorup et al. (1958) and Jandl et al. (1959) using human red cell precursors. Because of the differential effect of cyanide and mercuric chloride, Clark and Walsh (1960) postulated that in the intact cell, iron incorporation into haem consists of a phase of membrane iron uptake, and subsequent incorporation into haem. An inability to depress iron uptake by rat reticulocytes with cyanide and fluoride was also observed by Mazur and Carleton (1963). Cyanide again depressed iron incorporation into haem, while fluoride had no effect. Lower concentrations of inhibitors were used compared to the studies of Clark and Walsh (1960). However Mazur and Carleton (1963) found that $5 \times 10^{-4}M$ cyanide markedly inhibited iron uptake by rat marrow suspensions, and also its incorporation into haem. $5 \times 10^{-4}M$ dinitrophenol inhibited rat reticulocyte iron uptake, but not iron incorporation into haem. No effect was found on rat marrow with this inhibitor. $5 \times 10^{-3}M$ azide depressed both iron uptake and incorporation into haem in rat reticulocytes and marrow, and similar effects were noted with $5 \times 10^{-4}M$ cobalt ions and $5 \times 10^{-4}M$ α,α' -dipyridyl.

Transferrin uptake by human reticulocytes was also shown

to be dependent on cellular energy, but less so than iron uptake (Jandl and Katz, 1963). More detailed studies of the effect of metabolic inhibitors on transferrin and iron uptake using doubly-labelled transferrin have been reported by Morgan (1964). The immediate reaction between transferrin and the reticulocyte membrane, which was one of loose physical absorption, was found to be insensitive to cyanide, fluoride, azide, iodoacetamide, 2:4 dinitrophenol and arsenite in concentrations of 10^{-3} M. However, as more transferrin and iron became bound to the reticulocyte surface with time, cellular energy was required for these processes. It was found that at this stage, arsenite completely inhibited both transferrin and iron uptake, while 2:4 dinitrophenol had a much more marked inhibitory effect on iron uptake than on transferrin uptake. Little effect on either process was noted with the other inhibitors used. During this phase iron uptake was also found to be more sensitive to incubation at low temperatures than transferrin uptake. It was thus postulated that iron transfer was distinct from the process of progressive transferrin uptake. It was further postulated that the energy obtained from cellular metabolism either brought the transferrin molecule into a specific spatial arrangement with the

cellular receptor for iron, or that it caused a change in the tertiary structure of transferrin necessary for the release of the iron. The rate of release of transferrin from the reticulo-
cyte surface into the medium was also found to be energy dependent, probably as a consequence of the energy dependence of transferrin and iron uptake.

Various metabolic stimulants with the exception of glucose have been shown to have no effect on iron uptake by human reticulocyte suspensions (Jandl et al., 1959). The substances tested were inosine, glycine, succinic acid, methylene blue and citrate. Two $\times 10^{-2}$ M glucose resulted in moderate stimulation of iron uptake. In earlier studies (Walsh et al., 1949) it was found that absence of glucose from the incubating medium suppressed iron uptake.

(x) The effect of erythropoietin on iron uptake by
red cell precursors.

The failure to observe a stimulating effect of reduced levels of oxygen tension on iron uptake and proliferative

activity in rabbit marrow suspensions indicated that anoxia was not a direct stimulant to the bone marrow, and was regarded as indirect evidence for a humoral regulator (erythropoietin) of bone marrow activity (Erslev and Hughes, 1960). Several workers using starved or polycythaemic rats as the assay animal have shown increased erythropoietic activity in the serum obtained from subjects suffering from several types of anaemia (Gordon, 1959). Erslev and Hughes (1960) were, however, unable to demonstrate any increase in ^{59}Fe uptake or in proliferative activity when rabbit marrow was suspended in anaemic as against normal serum. However, Erslev (1962 a) has subsequently shown that purified sheep erythropoietin stimulates the cellular uptake of ^{59}Fe and also its incorporation into haem in marrow suspension cultures, the effect being most marked when using cultures of low cellularity and incubating for 45 hours. The effect of erythropoietin was thought to be due either to prevention of autolysis of erythroid cells after 45 hours incubation, or to 'promote the creation or metabolism of immature erythroid cells capable of in vitro multiplication'. In a subsequent paper Erslev (1962 b) showed that erythropoietin had no stimulatory effect on ^{59}Fe uptake by reticulocyte suspensions. When the proportion of stem

cells in a marrow culture was increased by methods which made the more mature erythroid cells non viable (e.g. by freeze-thawing), erythropoietin was however found to have no stimulatory effect on iron uptake by the marrow cells.

The effect of erythropoietin and anaemic plasma on ^{59}Fe uptake by short term marrow cultures obtained from rat, sheep and human sources was also investigated by Korst et al. (1962). The most striking results occurred with rat marrow in 20 hour cultures. These workers found that anaemic plasma from phenylhydrazine treated rats significantly stimulated both marrow uptake of iron and iron incorporation into haem in normal and 'anaemic' marrows, as compared to the controls. Possible artefacts due to differences in the specific activity of the iron-transferrin complex used were ruled out in appropriately controlled experiments. The effect of erythropoietin in increasing iron uptake was thought to be due to increased cell proliferation rather than increased haemoglobin synthesis without an increase in cells. These findings are of some interest in view of the recent demonstration that erythropoietin markedly stimulated ^{59}Fe incorporation into haem in an in vitro rat marrow culture

system (Krantz and Goldwasser, 1963). It was also recently shown that erythropoietin caused an increased synthesis of messenger ribonucleic acid in immature red cells in vitro (Krantz and Goldwasser, 1964). This work indicates that erythropoietin has a direct effect on haemoglobin synthesis, in addition to the postulated effect on proliferation of stem cells in the marrow.

V. Summary of current concepts of iron uptake by
immature red cells.

Two methods of providing iron for haemoglobin synthesis in red cell precursors have been described. The predominant source of iron appears to be that which is transferred directly from transferrin to red cell precursors. The other method is that described by Bessis and his co-workers in which a central reticular 'nurse' cell imparts its iron to surrounding erythroblasts by the process of ropheocytosis. The belief that this latter method is a major source of the iron taken up by immature red cells has been questioned by several workers. It has been postulated that a labile pool of iron exists in the immature

red cell membrane which can feed iron into the cell or back into the plasma, but others have expressed doubts as to its existence.

The uptake of iron directly from transferrin is dependent on specific receptor sites for both iron and transferrin on the red cell precursor membrane, and is an energy dependent process. Transferrin is more rapidly bound to the precursor membrane than is iron, but in contrast to the process of iron uptake, the binding of transferrin is readily reversible. Once the transferrin has released its iron in the cell membrane it can then be displaced by another molecule of iron-laden transferrin, and the process is then repeated. A difference of opinion exists as to whether iron-rich transferrin is preferentially bound as compared to iron-poor transferrin.

It has been postulated that transferrin having bound to a specific receptor on the immature red cell membrane releases its iron in the presence of ascorbate and adenosine triphosphate and is transferred to a non-haem compound in the membrane, which is thought to be ferritin.

CHAPTER II

INTRACELLULAR PATHWAYS OF IRON

IN RED CELL PRECURSORS

I. Unidentified non-haemoglobin protein intermediates
for iron incorporation into haemoglobin.

Once iron has been taken up by the immature red cell membrane, it enters the interior of the cell and is eventually incorporated into haemoglobin. The pathways the iron takes before final incorporation into haemoglobin are however, still controversial. Although the ultimate incorporation of iron into haemoglobin must involve protoporphyrin and globin, several workers have investigated the intracellular pathways which iron takes before this step.

Basic contributions to the understanding of haemoglobin synthesis in erythroid cells were made by Thorell (1947) when the relationship of haemoglobin synthesis to normoblast maturation was determined. On the basis of microspectrophotometric studies of rat, human and rabbit erythroblasts, Thorell showed that appreciable haemoglobin synthesis occurred only after the basophilic normoblast stage. However, autoradiographic studies of rat bone marrow (Austoni, 1954) and human bone marrow suspensions cultured with ⁵⁹Fe-labelled plasma

(Lajtha and Suit 1955; Austoni, 1956) showed that erythroid cells in an earlier stage of maturation than the basophilic normoblast took up appreciable quantities of the label. As these cells do not make haemoglobin, the iron taken up must be present as a non-haem substance. That this assumption was basically correct was demonstrated several years later by Sondhaus and Thorell (1960) when non-haem iron was determined spectrophotometrically in the erythroid cells of *Salamandra maculosa*. The highest concentration of non-haem iron was present in the early erythroblast, and decreased progressively as the cells matured.

It is only in recent years that definitive knowledge has been obtained of the pathways which iron takes in red cell precursors before it is finally incorporated into haemoglobin. Allen and Jandl (1960) recently investigated some aspects of the kinetics of intracellular iron in in vitro rabbit reticulocyte suspensions. In these experiments, reticulocyte suspensions were incubated for 4 to 5 minutes with plasma-bound radioiron, and further labelling of the cells was then stopped by adding an excess of non-active iron in plasma to the incubation medium.

In this way a 'phalanx' of radioiron was followed through the cells by taking samples at intervals thereafter. By differential centrifugation of the haemolysate of the cells, it was shown that iron rapidly passed into the stroma, reaching a maximum in 4 minutes, and was then released into the soluble cell contents in 6 - 8 minutes. The activity of the mitochondria and microsomes reached a maximum simultaneously with the stroma. The ^{59}Fe present in the microsomal fraction was shown not to be incorporated into the ribonucleoproteins of the microsomes, and was also not due to absorption of the supernatant haemoglobin which may have occurred in preparation of the microsomes. These findings are of interest when compared to the earlier work of Rabinowitz and Olsen (1956) who showed that labelled amino acids incorporated into haemoglobin in in vitro reticulocyte preparations had initially passed through the microsomal ribonucleoprotein.

Allen and Jandl (1960) showed that the ^{59}Fe activity of the stroma was not in the form of haemin, nor did it have the solubility of ferritin. The possibility was raised however, that the ^{59}Fe activity in the various sub-cellular fractions

could have been present in a single substance, such as ferritin, but with different solubilities in the various fractions. The nature of the ^{59}Fe in the soluble cell contents was characterized more completely by absorbing the supernate (soluble cell contents) onto I.R.C. - 50 resin in equilibrium with phosphate buffer at pH 6.5. The pale yellow non-absorbed material in solution was called non-haemoglobin protein, and the haemoglobin fraction was eluted from the resin with 1 M Cyanide. The non-haemoglobin protein had a kinetic curve intermediate between the cell particles and haemoglobin or another iron compound more rapidly synthesized and then degraded. A similar kinetic pattern was obtained when the non-haemoglobin protein was prepared by differential 'salting out' with ammonium sulphate. The non-haemoglobin protein was shown to contain approximately 35 - 37% of its ^{59}Fe activity in the form of haem. When the reticulocyte suspension was poisoned with lead, incorporation of iron into this non-haemoglobin protein was blocked.

The non-haemoglobin protein intermediate isolated by Allen and Jandl differs in several important respects from that isolated by Greenough et al. (1962) from in vitro suspensions

of human and dog marrow cells incubated with transferrin-bound radioiron, and also with [^{14}C]L - leucine. Greenough and his co-workers studied the movement of ^{59}Fe and [^{14}C]L - leucine through a protein fraction (Fraction I) moving with the solvent front when aliquots of supernate from marrow haemolysates were subjected to column chromatography on Amberlite I.R.C. - 50 resin. The starting buffer was 0.0067 M sodium phosphate with 0.0033 M KCN at pH 7.0. A similar fraction had been isolated earlier by Allen et al. (1958) on Amberlite I.R.C. - 50, and by Huisman and Meyering (1960) on carboxymethyl-cellulose chromatography of adult haemoglobin solutions. Huisman and Meyering called this fraction, V_1 , and found that its spectrum was distinctly different to haemoglobin, although the presence of a Soret peak at $4100 \text{ \AA}$ and of some smaller peaks between $5000 \text{ \AA}$ and $6500 \text{ \AA}$ indicated that it was a haem protein. Amongst other proteins not identified, this fraction was shown to have T.P.N.H. - activated methaemoglobin reductase activity.

Greenough et al. (1962) found that less than 1 per cent of the ^{59}Fe activity of Fraction I was in the form of haem,

and the iron was firmly bound to the protein. Solutions of Fraction I were not stable after standing for several hours, but were stable to heating to 70°C. Although Fraction I shared the property of heat resistance with ferritin, it was distinguished from ferritin on the basis of cellulose-acetate electrophoresis and immunochemical studies using the Ouchterlony agar gel technique. It should be noted however, that in a preliminary report of their work (Greenough and Thomas, 1961) it was stated that Fraction I had similar N - terminal residues to ferritin.

When human or dog marrow suspensions were incubated with ^{59}Fe bound to transferrin, Greenough et al. (1962) found that significant amounts of ^{59}Fe appeared in haemoglobin only after a delay of 7 - 10 minutes, and thereafter haemoglobin synthesis occurred at a linear rate for 4 - 6 hours. The haemoglobin fraction consisted of all components eluted from the column with the limiting buffer. There was however no delay in the appearance of radioactivity in Fraction I and its activity rose steeply to peak values in 10 - 30 minutes. [^{14}C] L - leucine showed a similar pattern of incorporation into Fraction I

and haemoglobin. When further ^{59}Fe or $[^{14}\text{C}]$ L - leucine labelling of the marrow was stopped after 10 minutes by re-incubating in unlabelled substrate for a further 4 hours, the radioactivity in Fraction I fell, while that in haemoglobin rose reciprocally. However, the fall in ^{59}Fe activity did not account for all the activity appearing in haemoglobin. This was explained by postulating a large stromal pool of ^{59}Fe which was continually feeding into Fraction I. A further possibility however, appears to be that another intermediate for iron incorporation into haemoglobin is present and is not being measured. On the other hand, the fall in $[^{14}\text{C}]$ L - leucine activity of Fraction I was greater than that which appeared in haemoglobin. This was thought to be due to an active turnover of proteins not on the pathway of haemoglobin synthesis. Lead ions did not block the incorporation of iron into Fraction I in contrast to the finding obtained with the fraction isolated by Allen and Jandl (1960). Because of the marked similarity in the kinetics of ^{59}Fe and $[^{14}\text{C}]$ L - leucine incorporation into Fraction I and haemoglobin, Greenough and his co-workers suggested that the protein they had isolated might be an intermediate for globin synthesis as well as for

iron incorporation into haemoglobin. It should be noted that [^{14}C] L - leucine is not a precursor for the haem portion of haemoglobin. Furthermore 'if the active portion of Fraction I is incorporated in-toto into haemoglobin, it is necessary that the leucine-containing material be closely related to, or identical with part of the globin molecule'. Although the nature of Fraction I has not been determined exactly, Greenough and his co-workers have postulated that iron, once it has entered the cell, combines with globin, or a part of it, to form a complex called sideroglobin. Sideroglobin then combines with protoporphyrin to form haemoglobin. This hypothesis has accentuated the controversy over the sequence of combination of iron, protoporphyrin and globin.

A non-haemoglobin iron containing protein has also been isolated in rabbit bone marrow homogenates by Salera and his co-workers (Salera et al., 1961; Salera, 1963) which appears to be similar to the fraction isolated by Greenough et al. (1962). This fraction was isolated by means of paper, starch block and agar electrophoresis, and appeared to be an intermediate for iron incorporation into haemoglobin in in vitro and in vivo

studies in normal rabbits. It could be distinguished electrophoretically from ferritin, which was also demonstrated in the marrow supernate. Lead ions, while blocking iron incorporation into haemoglobin, did not block its entry into the iron-protein intermediate, a finding similar to that of Greenough et al. (1962).

Labelling of a non-haem iron fraction with ^{59}Fe has also been found in in vitro studies of iron uptake by human marrow cells (Myhre, 1964 b). The non-haem fraction was taken as the difference between total cellular uptake of iron and iron incorporation into haem. No further identification of the non-haem fraction was attempted. In cultures in which radio-iron was taken up continuously up to 24 hours, radioactivity increased both in the haem and the non-haem fraction. The same percentage utilization of iron for haem synthesis was found irrespective of the total cellular iron uptake. These findings were interpreted to mean that one fraction of iron was rapidly incorporated into haem, while another was incorporated into a non-haem iron pool which was only slowly utilized for haem synthesis. As an active non-haemoglobin protein intermediate

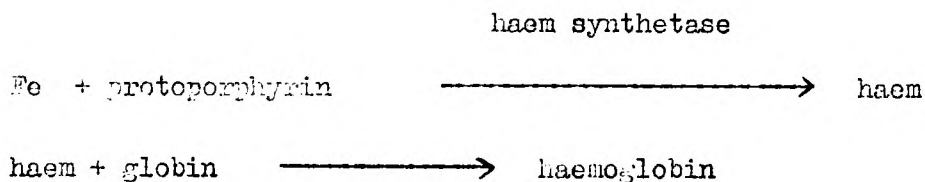
(Fraction I) for iron incorporation into haemoglobin had been demonstrated by Greenough et al. (1962) in marrow cultures, it must be assumed on kinetic grounds that the non-haem fraction 'isolated' by Nyhre contains both Fraction I and another iron containing compound which is not utilized rapidly for haem synthesis.

II. The 'iron incorporating enzyme'.

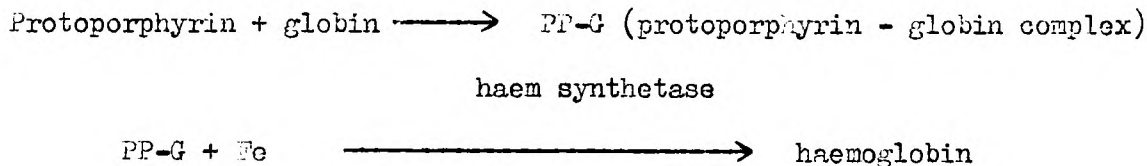
A large volume of work exists on the enzymic formation of haem from protoporphyrin and iron in extracts of both avian red cells and mammalian red cell precursors and other tissues, e.g. liver (Granick, 1954; Goldberg et al., 1956; Krueger et al., 1956; Kagawa et al., 1959; Nishida and Labbe, 1959; Goldberg, 1959; Lochhead and Goldberg, 1961; Schwartz et al., 1961). The enzyme has been named haem synthetase (Schwartz et al., 1961) and iron-incorporating enzyme (Goldberg, 1959). Active sulphydryl groups are required for its activity (Schwartz et al., 1961) and its activity is augmented by reducing substances such as reduced glutathione, cysteine and ascorbic acid.

Of great interest was the finding that the activity of the 'haem synthetase' system was augmented by the presence of globin in the reaction mixture (Schwartz et al., 1961).

In the latter study, the enzyme was obtained from an extract of chicken red cell stroma, and was incubated in the presence of stroma-free supernate (Fraction II), protoporphyrin and radioiron. The end product of the reaction was shown to be haemoglobin. Haemoglobin formation was minimal if either the enzyme or Fraction II was omitted. However, optimal haemoglobin synthesis occurred if globin was substituted for Fraction II. The optimal ratio of globin concentration to protoporphyrin concentration was 1.2:4, which was in good agreement with the expected 1:4 ratio i.e. four haem groups per molecule of haemoglobin. These findings are of interest in relation to the work of Greenough et al. (1962) as the active protein in Schwartz's Fraction II and in Greenough's Fraction I appears to be globin. As pointed out by Schwartz et al. (1961), their studies do not elucidate the exact mechanism of haemoglobin formation from iron, protoporphyrin and globin. One possibility considered by them was the following sequence of reactions:



Another possibility is that based on the work of Eriksen (1955), who believes that protoporphyrin combines with globin to form a protoporphyrin - globin complex (PP-G), and iron is subsequently enzymically incorporated to form haemoglobin, viz:



Yoshikawa and Yonehama (1964) have recently presented evidence that the iron-incorporating enzyme in duck erythrocytes is a lipoprotein, the phospholipid moiety of which may act as the binding site of protoporphyrin for the incorporation of iron. This synthesis occurs mainly in the mitochondria, and it is postulated that the haem so formed is transferred to globin, which has been synthesized by the microsomes. Yoshikawa and his co-workers were unable to find any stimulation of haemoglobin formation when

globin was added to their system, in contrast to the previously mentioned findings of Schwartz et al. (1961). However, the possibility of a subtle denaturation of the globin used could not be excluded.

III. Ferritin as an intermediate for iron incorporation into haemoglobin.

A non-haem intermediate for iron incorporation into haemoglobin in rabbit red cells has been studied by Falbe-Hansen and Lothe (1962). This intermediate was shown to be present in haemoglobin - free stroma obtained from peripheral red cells of rabbits at intervals after intravenous injection of radioiron. Starch block electrophoresis of homogenates of the stromal fraction showed that there were two fractions in which iron could be detected chemically, one with a migration velocity similar to ferritin, and the other at the point of application, neither of which were radioactive. However, a radioactive fraction (with no chemically detectable iron) was found on electrophoresis of homogenized stroma obtained from rabbits 2 hours after intravenous

injection of ^{59}Fe . As this fraction was not present in the stroma at 5 hours the conclusion was drawn that it represented a short lived intermediate in iron incorporation into haemoglobin.

In subsequent work Lothe and Falbe-Hansen (1963) attempted to increase the specific activity of the non-haem iron of the stroma by using anaemic (bled) rabbits. Starch block electrophoresis of homogenized stromal ghosts obtained from anaemic rabbits 15 minutes, 4 hours, 27 hours and 29 days after intravenous injection of radioiron, showed a radioactive fraction at the point of application and a radioactive fast and slow anodic fraction. The high concentration of activity at the origin was in contrast to the findings in normal rabbits. When sodium desoxycholate was added to the homogenized red cell ghosts in the 15 minute and 27 hour samples, there was a marked increase in the activity of the slow anodic compound in the 15 minute sample, and also in the fast anodic compound in the 27 hour sample. The slow anodic compound had a migration velocity similar to ferritin. Lothe and Falbe-Hansen suggested that their experiments show that iron is first incorporated into the

particles, presumably into ferritin of the mitochondria, and then passes onto the fast moving fraction'. These findings were compared to the electron microscopic studies of Bessis in normal erythroblasts, in which ferritin inside the mitochondria was shown to be changed to a more dispersed form called ferruginous micelles (Bessis, 1962). It was postulated that iron in this form was used for haemoglobin synthesis.

Lothe and Falbe-Hansen (1963) also performed starch block electrophoresis of homogenized red cell ghosts obtained from lead poisoned rabbits after intravenous injection of radio-iron. A highly active fraction sticking to the point of application was found in samples obtained at 20 minutes, 2 hours and 24 hours. Addition of sodium desoxycholate to the homogenized ghosts resulted in the appearance of a radioactive fraction moving to the anode with the same migration velocity as ferritin. However, no trace of the fast anodic fraction found in red cell ghosts from normal and anaemic rabbits could be observed in the samples obtained from lead poisoned rabbits. It thus appeared that there was a block in the transfer of iron from ferritin to the fast anodic fraction in lead poisoned

immature red cells. The fast moving anodic fraction was thought to be similar to the non-haemoglobin protein isolated by Allen and Jandl in rabbit reticulocytes (1960), as lead also blocked the incorporation of iron into this fraction. It should be noted however, that lead did not block the incorporation of iron into the non-haemoglobin protein isolated by Greenough et al. (1962) and Salera (1963). A serious drawback to the studies of Falbe-Hansen and Lothe is that the identification of ferritin by starch block electrophoresis alone cannot be regarded as definitive.

After intravenous injection of radioiron into rabbits, Salera and his co-workers (Salera et al., 1961; Salera, 1963) isolated labelled ferritin from the supernatant of bone marrow homogenates subjected to paper, starch block and agar electrophoresis. The ferritin was identified in the agar electrophoretic strip by antiferritin antiserum. In normal rabbits ferritin was not labelled until at least 3 hours after injection of radioiron, maximum labelling occurring at 24 hours. In iron-loaded rabbits, labelled ferritin could be detected earlier, while in bled rabbits, maximal labelling of ferritin occurred

after half-an-hour. However, no conclusions were drawn in these studies as to whether or not ferritin acted as an intermediate for iron incorporation into haemoglobin.

On the basis of his electron microscopic studies, Bessis (1962) maintains that the ferritin observed in red cell precursors represents a temporary excess of iron which is utilized for haemoglobin synthesis. In support of this argument he has shown that as the red cell matures, and haemoglobin synthesis increases, the amount of ferritin observed decreases progressively. It is possible however, that this could be due to extrusion of the ferritin into surrounding reticuloendothelial cells in the marrow. As mentioned previously, the direction of movement of ferritin from reticuloendothelial cell to erythroblast has been questioned by other workers. Crosby (1957) has also suggested that the 'siderin granules' seen in the erythroblast indicate that more iron is present in the cell than is required for haemoglobin synthesis. On the basis of his studies, in which he showed that transfused siderocytes disappeared rapidly from the circulation of normal subjects, but persisted in

splenectomized subjects, Crosby suggested that the spleen removes the excess iron not utilizable for haemoglobin synthesis.

Mazur and Carleton (1963) have recently reported the results of their studies on the relation of ferritin iron to haem synthesis. The kinetics of radioiron incorporation into ferritin and haem from ^{59}Fe -labelled plasma were investigated in rat reticulocytes and marrow in vitro and in vivo. Studies were also done using $[^{14}\text{C}]$ glycine. Ferritin was isolated by quantitative precipitation from the marrow lysate with anti-ferritin serum and carrier ferritin. On the basis of their kinetic data Mazur and Carleton maintain that in immature red cells iron is rapidly incorporated into ferritin, which then 'appears to function as an active iron storage protein and is intermediate between serum-bound iron and that which is incorporated in haem'. The interpretation of their experimental findings is however open to criticism and will be discussed in detail in relation to the results obtained in the present study. (see page 136).

Matioli and Eylar (1964) recently reported that

[14] DL - leucine was incorporated maximally into apoferritin at 30 minutes in in vitro suspensions of reticulocytes obtained from normal, lead-poisoned and iron-overloaded rabbits. The apoferritin was isolated immunochemically. By 2 hours, the radioactivity in apoferritin had decreased markedly. This fall in activity was interpreted to be due to conversion of ferritin to haemosiderin in these cells. Increased apoferritin synthesis was found in reticulocytes obtained from lead-poisoned rabbits, and rabbits with turpentine abscesses. It thus appeared that the ferritin of erythroblasts arose predominantly from intracellular protein synthesis and that significant transfer of ferritin from reticuloendothelial cells to erythroblasts as postulated by Bessis (1962) was unlikely. In discussion of their results, Mاتيoli and Eylar (1964) made the interesting postulate that apoferritin synthesis could represent a mechanism to regulate haemoglobin synthesis in the erythroblast. Based on the scheme of Jacob and Monod (1961) for the genetic regulation of protein synthesis, Mاتيoli and Eylar (1964) postulated that 'in the erythroblasts the regulator for apoferritin synthesis responds to high levels of iron by formation of an inactive complex which thereby derepresses

the apoferritin gene. As apoferritin is made, iron is required in large amounts to give ferritin. The level of free iron in the cell drops sharply to a level commensurate with the synthesis of haemoglobin. Thus the concentration of iron within the cell is kept at a convenient level by the presence of apoferritin. As the cell matures, less iron is absorbed. The repressor for apoferritin synthesis would progressively increase in concentration and lead to an inhibition of the apoferritin gene. In this way iron would be preferentially utilized for stimulation and production of haemoglobin. Consequently, by the time that the cell loses its nucleus, the reticulocyte will contain a large amount of messenger R.N.A. for haemoglobin and only a little messenger R.N.A. for apoferritin'. This hypothesis implies that iron, once it has combined with apoferritin to form ferritin, is not actively released for incorporation into haemoglobin. Mantioli and Eylar (1964) also raise the interesting question as to whether the large amounts of ferritin seen in thalassaemic erythroblasts in the face of depressed haemoglobin synthesis (Bessis, 1962) is due to a genetically determined aberration in haemoglobin production, or whether 'the ferritin

production is in competition with the haemoglobin synthesis as a result of a disturbance of specific repressors that control the genes for the two proteins'.

Yonehama et al. (1963) recently showed that iron bound to ferritin was utilizable to a much lesser extent than free inorganic iron for incorporation into haem in vitro. The incubation mixture consisted of protoporphyrin, a source of iron-incorporating enzyme (obtained from duck erythrocytes), cysteine and the source of radioiron. In this system it was also found that transferrin-bound iron was not released for incorporation into haem. Similar studies were done by Lochhead and Goldberg (1963), the source of the iron-incorporating enzyme being rat liver. They showed that iron bound to rat liver proteins or to transferrin was only minimally utilized in haem formation, but that on the addition of reducing substances such as ascorbic acid, cysteine, reduced diphosphopyridine nucleotide and reduced glutathione in physiological concentrations, a marked potentiation of iron incorporation into haem occurred. Adenosine triphosphate (A.T.P.) had no potentiating effect on iron bound to rat liver protein. A.T.P. was

found to potentiate the transfer of transferrin-iron to haem and also increase the potentiation produced by the reducing agents. The significance of these experiments is difficult to assess, but it would appear that under the conditions of the experiments, both ferritin iron and transferrin iron can be incorporated into haem, and that 'iron in a state of transfer must be in the ferrous or reduced form'.

The concept that ferritin iron may be an active storage protein and precursor for haem iron in the erythroblast would appear to be open to doubt. The studies described in this thesis have attempted to elucidate some of the problems relating to iron incorporation into ferritin and haemoglobin in red cell precursors.

IV. Summary of current concepts of intracellular pathways of iron in red cell precursors.

Once iron is released from transferrin onto the red cell precursor membrane, it passes rapidly into the

interior of the cell. Several studies indicate that it is then taken up by a non-haemoglobin protein before being incorporated into haemoglobin. The nature of the precursor complex is, however still not clear. It appears to be distinct from ferritin, and one group of workers have found that it has properties in common with globin. They therefore suggested that the iron combines with a protein, which may be part of the globin molecule, and that this complex (sideroglobin) then unites with protoporphyrin to form haemoglobin. The more generally accepted view is that iron combines with protoporphyrin to form haem, which then combines with globin to form haemoglobin.

Significant quantities of ferritin can be seen in erythroid precursors on electron microscopy, and evidence has recently been presented that a proportion of the iron entering the cell is temporarily stored in ferritin before being used for haemoglobin synthesis. Alternatively, the ferritin iron may represent a surplus that is excreted when the cell has matured.

The relationship between ferritin and the non-haemoglobin protein intermediates isolated by various workers has not been elucidated as yet.

P A R T I I

EXPERIMENTAL STUDIES

CHAPTER III

MATERIALS AND METHODS

I. In vitro studies.

The basic method used in the in vitro study of the uptake and utilization of iron by red cell precursors was to incubate a suspension of bone marrow cells under varying conditions with autologous plasma labelled with ^{59}Fe . The total cellular uptake and the incorporation of ^{59}Fe into various fractions in the cell was then determined.

(i) Technique of marrow culture.

Marrow was obtained either from the ribs of human subjects undergoing thoracotomy, or by aspiration from the posterior iliac crest. All the marrows were obtained from haematologically normal subjects, except for two specimens which were obtained from patients with proven iron-deficiency anaemia. Venous blood containing approximately 20% reticulocytes was obtained from two patients with pernicious anaemia in early remission, and from one subject with idiopathic autoimmune haemolytic anaemia. Reticulocyte counts were performed as described by Dacie (1956). Reticulocyte-rich blood was

also obtained from rats of the Wistar strain 3 to 4 days after the parenteral administration of an ethanolic solution of phenylhydrazine (30 mg. per kg.). All these samples were treated the same as marrow.

Specimens of marrow were collected at 0°C in Balanced Salt Solution (B.S.S.) (Hanks and Wallace, 1947) containing 200 units penicillin, 100 ug. streptomycin and 100 ug. neomycin per ml., and were processed and used within one hour after removal from the patient. Marrow obtained from ribs was shaken vigorously and then syringed repeatedly through a 20 - gauge Luer lock needle until adequate dispersion of the cells was obtained. The marrow was washed once in cold B.S.S. to remove the supernatant fat, and was then resuspended in B.S.S. containing 10 to 30 per cent autologous plasma.

Spleens were removed from rats of the Wistar strain under ether anaesthesia and were placed in B.S.S. at 0°C. A suspension of splenic cells was made by syringing the spleen through the nozzle of a Luer lock syringe and then syringing repeatedly through a 20 - gauge Luer lock needle until adequate

dispersion of cells was obtained. Suspensions of these cells were subsequently treated the same as marrow.

$^{59}\text{Fe Cl}_3$ (specific activity 20-30 $\mu\text{c.}/\mu\text{g.}$) was incubated with autologous plasma at 37°C for approximately 1 hour and was then added to the marrow suspension at the start of incubation. The amount of $^{59}\text{Fe Cl}_3$ added was always less than the binding capacity of the plasma present. Plasma iron levels were estimated by a modification of the method of Bothwell, Jacobs and Kamener (1959). In some experiments, metabolic stimulators or inhibitors were added to the marrow suspension just prior to the addition of ^{59}Fe - labelled plasma. The inhibitors or stimulators were initially made up in B.S.S. and the pH adjusted to 7.4. Equal aliquots of marrow suspension were incubated at 37°C in air in 25 ml. Erlenmeyer flasks placed on top of a slowly revolving drum inclined at 10° to the horizontal. Aliquots of marrow were removed at varying times and the reaction was stopped by rapid cooling to 0°C and the addition of 1 ml. of 0.5% potassium cyanide in B.S.S. adjusted to pH 7.4. In some experiments further ^{59}Fe -labelling of the marrow was stopped after 5-10 minutes incubation by cooling the marrow

rapidly to 4°C, washing it free of external radioactivity, and reincubating it in unlabelled incubation medium.

After incubation the marrow cells were washed three times with saline and the radioactivity of the cells was determined by counting in a well-type scintillation counter. The cells were then lysed by the addition of 4 volumes of distilled water. The samples were shaken vigorously and kept at 4°C for 30 minutes. The lysate was then centrifuged for 20 minutes at 1,500 x G. The resulting clear red supernatant solution was siphoned off and kept. The precipitate ('stromal fraction') was washed twice in 10 volumes of cold distilled water and centrifuged at 1,500 x G for 20 minutes. The ^{59}Fe activity of the 'stromal fraction' was determined.

(ii) Isolation of components in the supernatant solution.

a) Isolation of Fraction I and Main

Haemoglobin. An aliquot of supernate was chromatographed on the carboxylic acid ion-exchange resin, Amberlite CG-50 Type II,*

*Manufactured by the Rohm and Haas Co., Philadelphia.

prepared according to the method of Hirs, Moore and Stein (1953) as modified by Greenough et al. (1962).

The resin was prepared as follows: to 1.5 kilos of the resin, 3.5 litres of water were added and the suspension was stirred for 20 minutes. After a settling period of 30 minutes, foam on the surface was removed, and the supernatant suspension was removed. The settling process was repeated four or five times with 2 litre portions of water, until the supernatant fluid was clear after about 15 minutes of settling. The resin was then air dried on a Buchner funnel, and the filter cake was added to 4 litres of acetone and stirred for 3 hours. The resin was filtered and washed with acetone (about 8 litres) until the filtrate was clear. The air dried resin was suspended in water, stored until all bubbles were eliminated, and all traces of acetone were removed by washing on the filter with 24 litres of water. The resin was then cycled once through the sodium form before use. To the resin in 5 litres of water, 560 grams of NaOH (as a 40% solution) were added over a period of 30 minutes. The pH of the suspension rose to about 11. Stirring was continued until the evolution of heat had subsided

after about 3 hours, and the sodium salt of the resin was then washed by decantation with five 2 litre portions of water. The resin was then washed on a filter with water until the filtrate had a pH of about 10. The resin was converted to the acid form by passing 10 litres of 3 N HCl through the filter over a 4 hour period, and was then washed with approximately 6 litres of water. The resin was then titrated from the acid side to near pH 7.0 with NaOH, and washed with approximately 100 volumes of water. The resin was then equilibrated with the starting buffer until the pH was regulated to within 0.05 pH U of pH 7.0. According to Greenough et al. (1962) haemoglobin binding varies greatly with very small pH changes between pH 6.8 and 7.2, and accurate equilibration of a batch of resin is critical. Columns (1 x 10 cm) were packed by gravity approximately 30-60 minutes before use. The starting buffer was 0.0067 M sodium phosphate with 0.0033 M KCN at pH 7.0. The limiting buffer was 0.5 M NaCl dissolved in the starting buffer.

The yellow coloured fraction eluting with the solvent front, termed Fraction I by Greenough et al. (1962) was separated

with the starting buffer, and its activity determined. All other components, termed Main Haemoglobin by Greenough et al. (1962), were eluted from the column by changing directly to the limiting buffer. The activity of the Main Haemoglobin fraction was determined. Recoveries of radioactivity were between 85 - 95%.

b) Isolation of haemin. In order to ascertain whether the Main Haemoglobin fraction represented an accurate measure of haem synthesis, the percentages of counts of ^{59}Fe incorporated into Main Haemoglobin and into haemin in aliquots of supernate were compared. Also, in several experiments in which aliquots of supernate were not chromatographed on I.R.C. CG - 50 resin, haemin was isolated by crystallization and its activity determined.

Haemin was crystallized by the method of Labbe and Nishida (1957). In order to correct for the incomplete recoveries obtained with this technique an aliquot of a solution of rabbit haemoglobin labelled with ^{55}Fe was added

to the ^{59}Fe -labelled marrow supernatant solution at the beginning of the procedure, and the washed haemin crystals were digested, electroplated onto copper discs, and counted for ^{55}Fe and ^{59}Fe as described below. Identical aliquots of ^{55}Fe rabbit haemoglobin were taken as standards of the number of counts of ^{55}Fe haemoglobin added to the ^{59}Fe supernatant and were digested and electroplated onto copper discs and counted for ^{55}Fe . The ^{55}Fe -labelled rabbit haemoglobin solution was prepared by injecting $100\text{ }\mu\text{C}$ $^{55}\text{Fe Cl}_3$ into an ear vein of a rabbit and collecting venous blood one week later. The cells were washed 3 times in saline, haemolysed with 4 volumes of distilled water, and the supernatant haemoglobin solution was obtained after centrifuging the lysate at 2,000 x G for 30 minutes.

The method of haemin crystallization, digestion, electroplating and counting was performed as follows. Twelve volumes of extraction solvent were added to one volume of the mixture of ^{55}Fe haemoglobin and ^{59}Fe supernatant solution with stirring. (The extraction solvent consisted of one part of stock solution of 2% strontium chloride hexahydrate in glacial

acetic acid plus three parts of acetone, and was prepared immediately before use to prevent crystallization of the strontium chloride). The mixture was allowed to stand for 20-30 minutes with occasional stirring, and was briefly heated to the boiling point during this period. The mixture was then centrifuged at 2,000 x G for 20 minutes and the supernatant solution was decanted into a beaker. The residue was washed twice with one volume of extraction solvent, and the supernatant solutions were pooled. The pooled supernatant solution was heated to 100°C in a water bath. Crystallization of haemin began as the solution became concentrated, and was complete when the solution was allowed to cool to room temperature. The haemin crystals were centrifuged at 2,000 x G for 10 minutes and washed twice with 50% acetic acid and once with ethanol and once with ethyl ether.

The haemin crystals were then digested, electroplated onto copper discs and counted for ^{55}Fe and ^{59}Fe by a modification of the methods of Peacock et al. (1946) and Halberg and Brise (1960). The haemin crystals were transferred to 100 ml. Kjeldahl flasks and 2 ml. of concentrated sulphuric and 1 ml. of concentrated nitric acid were added to each flask. A glass bead was

added to each flask to prevent bumping and the flask was heated over a flame until the sample became oily in appearance. The flask was removed from the burner and allowed to cool. Approximately 3 ml. of H_2O_2 were carefully added to each flask and the flask was heated again for 10 minutes. Further addition of H_2O_2 was usually required before the contents became clear. When the contents of the flask had become colourless, boiling was continued for an additional ten minutes to ensure that all the H_2O_2 had been driven off. When the sample was cool it was transferred to a pyrex centrifuge tube with three washings of 0.6 N sulphuric acid. Carrier iron was added to make up a total quantity of 7.5 mg. in the sample. (It was assumed that there are 34 mg. of iron in each 10 grams of haemoglobin). The iron was then precipitated out of solution. Three to four drops of Brom-cresyl purple indicator (0.1% in 0.01 N NaOH) were added to each tube. While the contents of the tube were stirred vigorously, concentrated ammonium hydroxide was added drop by drop until the iron was completely precipitated, the solution turning purple. The tube was then centrifuged at 2,000 x G for 10 minutes, and the clear supernatant discarded.

The ferric hydroxide precipitate was dissolved by adding 6 drops of 6 N sulphuric acid and left to stand for 20 minutes. The sample was then electroplated onto a clean copper planchet (initially washed in 30% nitric acid and rinsed in distilled water) in a water tight electrolysis cell. The anode consisted of a platinum spiral with a diameter of 2 cm. melted into a Pyrex glass tube filled with mercury. The sample, dissolved in 6 N sulphuric acid was washed into the electrolysis cell with 35 ml. of an 8% ammonium oxalate solution heated to about 60°C. Electrolysis was started immediately and continued for 5 hours, an amperage of 0.4 being maintained through each cell. The planchets were then removed, immediately rinsed in distilled water, dipped in acetone and dried with a fan. They were then covered with a few drops of a solution containing 1 ml. of 3 - in - 1 oil made up to 100 ml. with benzene.

The emissions of the ^{59}Fe were then measured by counting the planchet with a helium-filled Geiger-Müller tube, and the emissions of the ^{55}Fe were measured by counting the same planchet with an argon-filled Geiger-Müller tube covered by a beryllium filter. ^{59}Fe and ^{55}Fe standards were counted

on both tubes to make appropriate corrections for cross-counting. The divergence was usually not more than 3% in either direction.

In this way it was possible to calculate the number of counts of ^{59}Fe -labelled haemin in the original supernatant solution from the following formula:

$$\begin{array}{l} \text{No. of cts. of } ^{59}\text{Fe} \\ \text{haemin in original} \\ \text{supernate} \end{array} = \frac{\begin{array}{l} \text{No. of cts. of } ^{55}\text{Fe in haemoglobin standard} \\ \times \text{No. of cts. of } ^{59}\text{Fe haemin recovered} \end{array}}{\text{No. of cts. of } ^{55}\text{Fe haemin recovered.}}$$

The percentage of counts of ^{59}Fe in haem in the original supernatant was then calculated. Using this approach it was found that the mean percentage of ^{59}Fe incorporated into haemin in six different samples was 83.4% (range 77% - 90%) of the value for Main Haemoglobin.

c) Isolation of ferritin. Human liver

ferritin was prepared from siderotic Bantu livers by the method of Mazur and Shorr (1948) as follows. One kilogram of liver tissue was minced in a meat grinder and to this 1.5 litres of water were added. The tissue was then homogenized in a large Waring Blender and heated over 5 minutes in a water bath with stirring to 80°C. The hot suspension was run through coarse cheesecloth and the filtrate was run through fluted filter paper. After cooling, the filtrate was adjusted to pH 4.6 with 50% acetic acid and was allowed to stand overnight at 4°C. The solution was then centrifuged and the precipitate discarded. Twenty-five ml. of 20% cadmium sulphate were added to each 100 ml. of solution, and the solution was left for 2 days at room temperature. The solution was then centrifuged, the crystals kept and the supernatant solution discarded. The crystalline ferritin was then dissolved in the smallest possible quantity of 2% ammonium sulphate and dialysed against tap water overnight to remove most of the cadmium. One volume of saturated ammonium sulphate was then added to one volume of the solution and the precipitate was recovered by centrifugation. The precipitate was dissolved in water and reprecipitated three times with saturated ammonium sulphate to further reduce the cadmium content. The

ammonium sulphate was then removed by dialysis against tap water for 2 days. The concentrated ferritin was kept at 4°C with toluene as preservative.

An antiserum to human liver ferritin was then prepared by bleeding rabbits immunized with aliquots of alum-precipitated human ferritin which was prepared by a modification of the method of Proom (1943). The alum-precipitated ferritin was prepared as follows. Twenty-five ml. of freshly prepared ferritin solution were pipetted into 80 ml. of distilled water and well mixed. Ninety ml. of 10% aluminium potassium sulphate were then added with stirring. The pH of the solution was carefully adjusted to 6.5 with 5 N NaOH with constant stirring. The precipitate was recovered by centrifugation and the supernatant discarded. The deposit was washed twice with 200 ml. of isotonic saline and finally resuspended in 100 ml. isotonic saline. Five ml. of the final suspension were injected intramuscularly into each buttock of a rabbit. The procedure was repeated 14 days later. Booster injections of aqueous ferritin were given intraperitoneally 10 days before each bleed. The rabbits were bled periodically from an ear vein, 20 - 30 ml. being collected in a heparinized tube. The

plasma was siphoned off after centrifugation and was pooled.

Five ml. aliquots were stored at 0°C.

The titre of each pool of antiserum was determined in the following way. Increasing quantities of antiserum from 0.1 ml. to 2.0 ml. were added to a constant volume of ferritin solution of known iron content e.g. 0.2 ml. ferritin containing 125 ug. of iron. The aliquots were incubated at 37°C for 1 hour and left in the refrigerator overnight. The precipitates were washed twice with saline. The amount of iron in the ferritin-antiferritin complex was then determined. To each tube 1 ml. of concentrated HCl, 2 drops of thioglycollic acid and 2 ml. of ammonium hydroxide were added. The volume was made up to 50 ml. in a measuring cylinder and the pink colour developed was measured at 540 m μ and read against a standard curve. The amount of antiserum in each tube was plotted against the amount of iron in the precipitate in each tube and a curve obtained (Figure 1). The minimum volume of antiserum that precipitated all the ferritin was read off from the curve, and

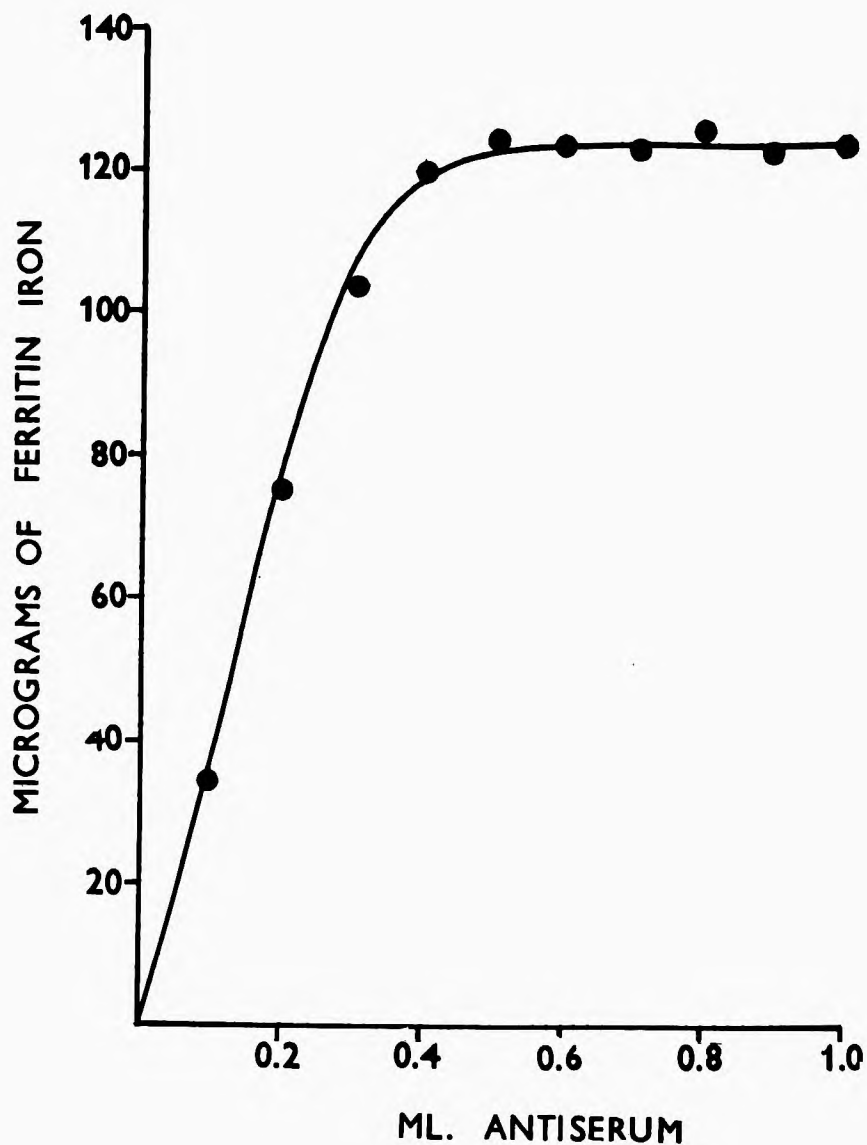


Fig. 1: Determination of the titre of ferritin antiserum.

The minimum volume of antiserum that could precipitate all the ferritin (as ferritin iron) in the aliquots was read off from the curve.

the amount of ferritin that could be precipitated by 1 ml. of antiserum was calculated. One ml. of pooled antiserum was able to precipitate approximately 250 ug. of ferritin iron, i.e. equivalent to approximately 1 mg. of ferritin protein.

In studies on rats, an antiserum to purified horse spleen ferritin was prepared in identical fashion to that described for human liver ferritin. A cross immunity has been demonstrated between horse spleen and rat liver ferritin (Mazur et al., 1960).

The method for isolation of ferritin in rat marrow was identical to that for human marrow. To one volume of ⁵⁹Fe-labelled marrow supernatant solution was added an equal volume of 0.2 M disodium ethylenediaminetetra-acetic acid (E.D.T.A.) buffered to pH 7.0. One ml. of antiserum and 0.2 ml. of carrier ferritin solution containing approximately 500 ug. ferritin were then added and the mixture incubated for one hour at 37°C. The ferritin-antiferritin complex, which appeared as a visible precipitate, was washed three times with distilled water after centrifuging at 1,500 x G for 10 minutes.

The ^{59}Fe activity of the precipitate was determined. In this method, carrier ferritin was added to the solution for two reasons. Firstly, precipitation could be verified by chemical methods and in the second place it allowed for the measurement of minute amounts of labelled ferritin. As it was theoretically possible that free ^{59}Fe or some other labelled small molecule in the supernatant solution might attach to carrier ferritin and be precipitated with the ferritin - antiferritin complex, E.D.T.A. was added to the solution since it was found that E.D.T.A. prevented the non-specific attachment of free ^{59}Fe to carrier ferritin. In order to prove that E.D.T.A. would not remove ^{59}Fe from physiologically labelled ferritin, antiserum was added to a marrow haemolysate containing 0.1 M E.D.T.A. and ferritin labelled physiologically with ^{59}Fe . Under these circumstances 96% of the radioactivity was precipitated. Physiologically labelled ferritin was prepared as follows. Rats were loaded with iron by giving 1 mg. of iron-sorbitol-citrate intramuscularly to each rat on alternate days for 3 doses. Each rat was then given 50 $\mu\text{c.}$ of $^{59}\text{Fe Cl}_3$ intravenously and the following day the animals were sacrificed and the labelled ferritin was isolated from their livers by the

method of Mazur and Shorr (1948) described above.

The specificity of the antiserum was confirmed by the absence of any radioactivity in the precipitate when the antiserum was incubated with ^{59}Fe -labelled haemoglobin solutions or with ^{59}Fe -labelled plasma transferrin. In addition, negative results were also obtained with ^{59}Fe -labelled Main Haemoglobin, obtained after chromatography of marrow supernates.

Ferritin was also identified in some studies using agar gel diffusion plates. These were set up as described by Bodman (1960). Antiferritin serum was placed in the centre well, while human liver ferritin, labelled in vitro with ^{59}Fe , and marrow supernatant solution and Fraction 1, were placed in the peripheral wells. After the bands of precipitation had formed, the gels were washed for 3 days with frequent changes of distilled water. They were dried until paper thin and were then placed in apposition with X-ray films for several days in order to obtain autoradiographs.

II. In vivo studies.

The incorporation of ^{59}Fe bound to plasma transferrin into red cell precursors in vivo was determined in a group of 24 normal laboratory rats of the Wistar strain. Twelve rats from each group were initially given 30 mg./kg. of an ethanolic solution of phenylhydrazine intraperitoneally to stimulate erythropoiesis. Reticulocyte counts on blood obtained from the tail vein of each rat were done as described by Dacie (1956). Three days after administration of the phenylhydrazine, both the normal and phenylhydrazine-treated rats were given intravenous injections of 2 $\mu\text{c.}$ $^{59}\text{Fe Cl}_3$ bound to 1 ml. of rat plasma. Three rats from each group were then sacrificed at 1, 5, 12 and 23 hours after administration of the ^{59}Fe . The femurs from each rat were obtained and the marrow syringed out into Hanks B.S.S. The marrow aliquots were washed once in Hanks B.S.S. and the marrows from each group of three rats were pooled and their wet weights and ^{59}Fe activity determined. The incorporation of ^{59}Fe ferritin and Main Haemoglobin in each group of three pooled marrows was determined as described under in vitro methods (anti-horse spleen ferritin was used in the isolation of ferritin).

The incorporation of ^{59}Fe into ferritin in the peripheral red cells of a group of 12 splenectomized and 12 control rats was also determined at 4 hours, 24 hours, 48 hours and 4 days after intravenous administration to each rat of 2 $\mu\text{c. } ^{59}\text{Fe Cl}_3$ bound to 1 ml. rat plasma. Packed red cells were obtained after three washings with isotonic saline, and the cells were then haemolysed in 4 volumes of saline by freeze-thawing twice. (The red cells were not haemolysed with water as the rat haemoglobin tended to crystallize out readily under these conditions). The haemolysate was centrifuged at 1,500 x G for 20 minutes, and the supernatant collected. The number of counts of ^{59}Fe present in ferritin in the supernate was determined as described under in vitro methods. In addition, at 4 hours and 24 hours the incorporation of ^{59}Fe into ferritin was determined in the pooled marrows obtained from 3 rats of the control and splenectomized groups. Ten ml. autologous plasma labelled with 25 $\mu\text{c. } ^{59}\text{Fe}$ were also given intravenously to one haematologically normal splenectomized human subject. Venous blood was obtained at intervals over 7 days from the subject, and the packed red cells washed once in saline. The cells were haemolysed with four volumes of water, and the

supernatant was obtained after centrifuging at 1,500 x G for 30 minutes. The number of counts of ^{59}Fe incorporated into ferritin in the supernatant was determined as described under in vitro methods.

III. A note on the use of ^{59}Fe as a measure of the haem synthetic capacity of red cell precursors.

A factor of considerable importance with reference to the incorporation of radioiron into haem or haemoglobin is whether in fact this is an accurate measure of haem synthesis, particularly in in vitro whole cell systems. Bannerman et al. (1959), in investigating haem synthesis in thalassaemic blood with ^{59}Fe and $[2\text{-}^{14}\text{C}]$ glycine found that haem synthesis was approximately six times less when measured by ^{59}Fe incorporation as compared to $[2\text{-}^{14}\text{C}]$ glycine incorporation into haem. This finding was thought to be due to dilution of the ^{59}Fe in the non-haem iron pool in the cell. Similar conclusions were drawn by Lochhead et al. (1963) when they found that ^{59}Fe incorporation into haem in liver homo-

genates from iron loaded rabbits, was much reduced when compared to the uptake by normal liver homogenates and liver homogenates from iron deficient rabbits. The findings of Bannerman et al. (1959), however, are difficult to interpret, as the comparison of ^{59}Fe and $[2\text{-}^{14}\text{C}]$ glycine uptake was made in experiments in which ^{59}Fe was added to saline suspensions of washed cells, thus excluding the physiological method of uptake of iron from transferrin. On the basis of in vitro studies on rabbit red cell precursors, Erslev and Hughes (1960) pointed out that the total amount of iron needed for the formation of haem in one mature red cell could be incorporated in approximately 70 hours, if the value which was obtained for the in vitro uptake of iron/hour/cell was used in the calculation. As 70 hours is approximately the life span of the immature red cells of the rabbit and as 70 per cent of cellular ^{59}Fe uptake could be found in haemoglobin in in vitro cultures, it seemed justified to use ^{59}Fe incorporation as a measure of the haem synthetic capacity of immature red cells.

A comparison of the utilization of ^{59}Fe for haem synthesis in vitro and in vivo reveals discrepant results in

different species. Clark (1962) found that ^{59}Fe incorporation into haem in rat reticulocytes was similar in vivo and in vitro. On the other hand, the proportion of cellular ^{59}Fe incorporated into haem in in vitro suspensions of human marrow was only about half of that incorporated in vivo (Myhre, 1964 b). Marked species variations also exist in the proportion of cellular ^{59}Fe incorporated into haem in in vitro studies. In duck erythrocytes the utilization of cellular ^{59}Fe for haem synthesis has been reported as approximately 85% (Jensen et al., 1953); in pigeon erythrocytes approximately 40% (Clark and Walsh, 1960); in rat reticulocytes about 80% (Belcher and Courtenay, 1959) and 70% (Clark and Walsh, 1960; Mazur and Carleton, 1963); in rat erythroblasts approximately 25% (Mazur and Carleton, 1963); in rabbit erythroblasts about 70% (Erslev and Hughes, 1960); in human reticulocytes 30 to 80% (Jandl et al., 1959) and 50 to 60% (Erslev and Iossofides, 1962) and approximately 50% in human erythroblasts (Myhre, 1964 b).

In the present studies, iron incorporation into haem was used as a measure of the haem synthetic capacity of the red cell precursor, bearing in mind the limitations mentioned above.

In determining the kinetics of iron incorporation into various fractions in the cell and their relationship to iron incorporation into haem, the use of ^{59}Fe as a measure of haem synthesis is of course mandatory.

CHAPTER IV

STUDIES ON THE UPTAKE AND UTILIZATION OF IRON BY RED CELL PRECURSORS

I. Demonstration of physiologically labelled ferritin in
marrow suspensions at various times of incubation.

The incorporation of ^{59}Fe into the 'stromal fraction', Fraction 1, ferritin, and Main Haemoglobin was followed over several hours in six experiments. Since the major changes in the pattern of incorporation of ^{59}Fe into the various fractions occurred in the first hour of incubation, most samples were analysed during this period. To permit comparison between results obtained in the different experiments, the amounts of radioactivity present in the various fractions at different times were expressed as percentages of the total cellular uptake at 1 hour. Figures 2 and 3 illustrate the pattern of changes obtained in two of the experiments.

Radioiron was rapidly taken up by the marrow suspension. After 5 minutes between 23 and 37% was already present in cells, and this rose to between 71 and 79% by 30 minutes. Approximately half of this uptake was present in the 'stromal fraction'. Between 5 and 30 minutes the mean percentage present in Fraction 1 rose from 5.5 (range 3.7 to 9.9%) to

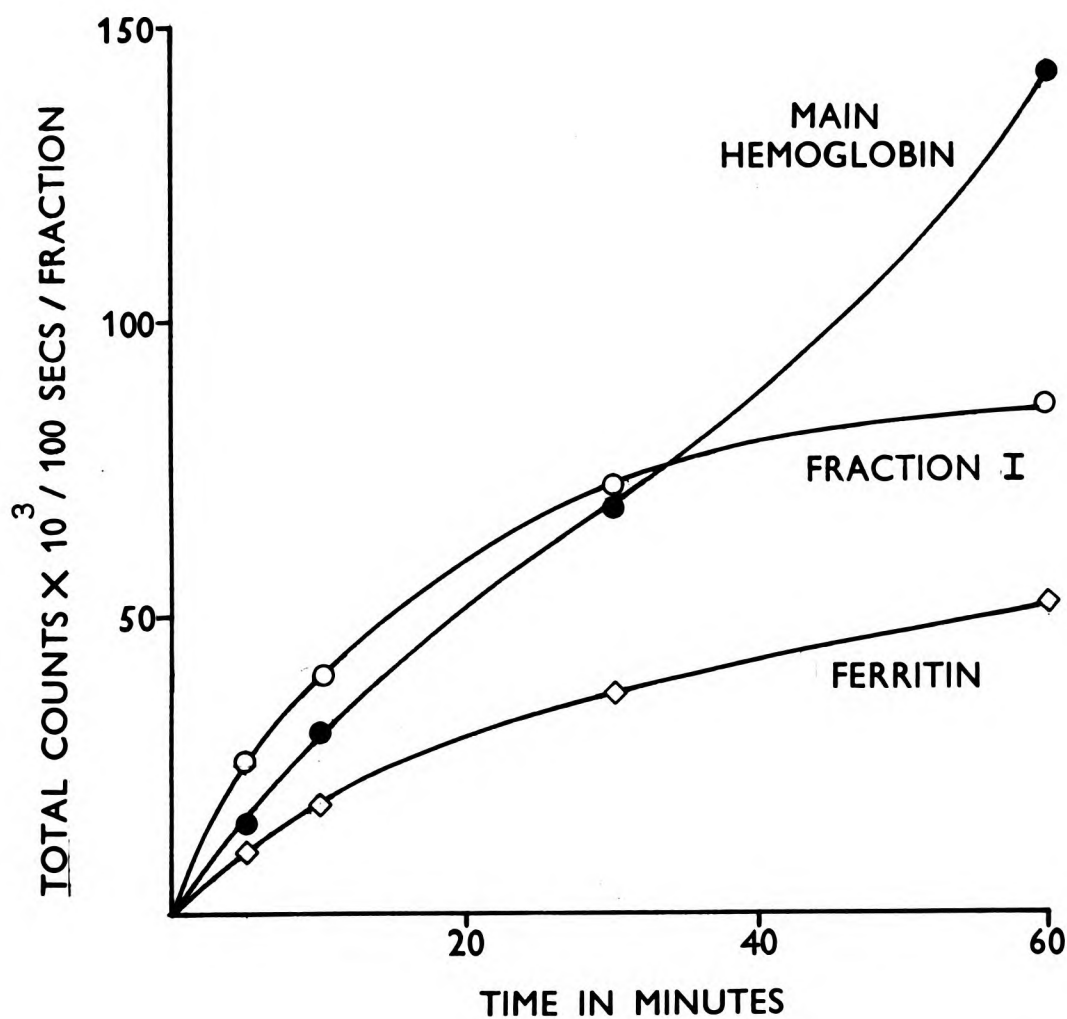


Fig. 2: Incorporation of ^{59}Fe into Fraction I.
Main Haemoglobin and ferritin.

Analysis of samples of human rib marrow culture
 at various times up to 1 hour.

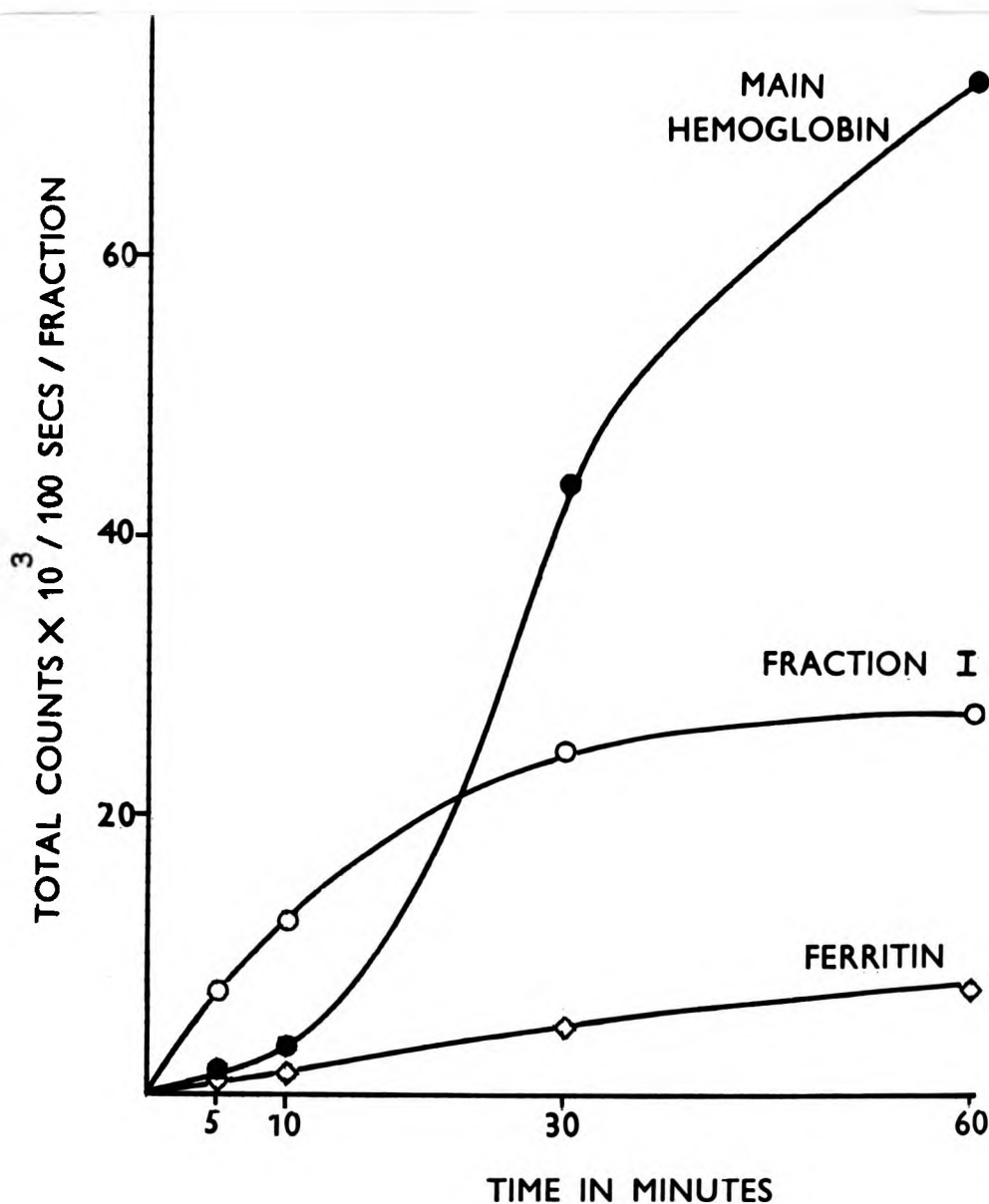


Fig. 3: Incorporation of ^{59}Fe into Fraction I,
Main Haemoglobin and ferritin.

Analysis of samples of human rib marrow culture
 at various times up to 1 hour.

16.8 (range 12.7 to 22.0%). The rate of increase over the next 30 minutes was much slower, the mean figure at 1 hour being 18.5% (range 15 to 28%). Between 2.0 and 4.6% of the ^{59}Fe was incorporated into ferritin by 10 minutes, and thereafter the radioactivity in ferritin increased in linear fashion up to 1 hour, when between 8.3 and 16.4% was present in this fraction. It was noteworthy that the amount of radioactivity in ferritin was always less than in Fraction 1. Only small amounts of ^{59}Fe (between 2.0 and 5.9%) were incorporated into Main Haemoglobin in the first 10 minutes, but by 60 minutes between 19.0 and 36% was present in this compound.

Between 1 and 12 hours there were no marked changes in the distribution of ^{59}Fe between the various fractions. The rate of uptake of radioactivity by the cells was not as rapid as over the first hour, the figures after 4 hours ranging between $1\frac{1}{2}$ and 3 times the 1-hour uptakes. At this stage between 17.6 and 32.5% of the activity taken up was in Fraction 1, from 10.2 to 23.0% in ferritin, and between 22 and 46% in Main Haemoglobin.

II. Site of formation of ferritin.

As marrow is a heterogeneous tissue it was necessary to determine whether the ferritin isolated in the marrow supernatant solution had been labelled in red cell precursors or in reticuloendothelial cells. Since preliminary studies indicated that bone marrow suspensions obtained from the femurs of rats metabolized iron in similar fashion to human bone marrow suspensions, three in vitro studies were done in which the uptake of ^{59}Fe from labelled autologous plasma by marrow suspensions was compared with that of splenic suspensions prepared in the same way (see Methods). After 3 hours' incubation the samples were washed 3 times with normal saline and were then counted for radioactivity. The total uptakes by the splenic suspensions were less than 10% of those obtained with equivalent wet weights of marrow. After homogenization with 4 volumes of water, samples of the supernatant solution from the splenic suspensions were set up with anti-ferritin serum. It was found that 0, 2 and 14% of the activity taken up by splenic cells was precipitated as ferritin. The comparable figures obtained with the marrow suspensions were 10,

23 and 16% respectively. When these figures were related to the total uptakes of the two tissues, the spleen's capacity to incorporate ^{59}Fe into ferritin was between 0 and 10% of a comparable weight of marrow. Although it is possible that reticuloendothelial cells in the marrow may behave differently from those in the spleen, this seems unlikely, and the results obtained therefore suggest that the labelling of ferritin observed in marrow cultures was occurring predominantly in red cell precursors and not in reticuloendothelial cells.

III. Demonstration of physiologically labelled
 ferritin in Fraction 1.

Addition of antiferritin serum to Fraction 1 resulted in the precipitation of a significant number of counts as ferritin. When a solution of Fraction 1 was placed opposite antiferritin serum on an agar gel diffusion plate no visible band resulted, but a radioautograph of the washed plate showed a line of radioactivity opposite Fraction 1 that coalesced with the lines of radioactivity corresponding to the bands of labelled

pure ferritin (Figure 4). A similar band was noted when a sample of the whole supernatant solution was treated in the same way. These results, therefore confirmed the presence of ferritin in both the supernatant solution and Fraction 1.

When the percentage of counts incorporated into ferritin at various time intervals was measured directly on the supernatant solution, the results were in close agreement with those obtained when the same estimation was carried out on Fraction 1 (Table I). These findings indicate that all the ferritin present in the marrow supernatant solution was in Fraction 1. In addition, the percentage of radioactive ferritin present in Fraction 1 increased with time (Table II).

In the experiments of Greenough et al. (1962), the marrow lysate was centrifuged at $1,500 \times G$, and the resulting supernatant solution was then subjected to further centrifugation at $100,000 \times G$ before chromatography on I.R.C. - 50 resin. The possibility that the ferritin content of the marrow supernate might be reduced by this procedure was investigated. It was found that approximately 50% of the activity precipitable as ferritin in the marrow supernatant solution was removed by prior centrifugation of the supernate at $100,000 \times G$.

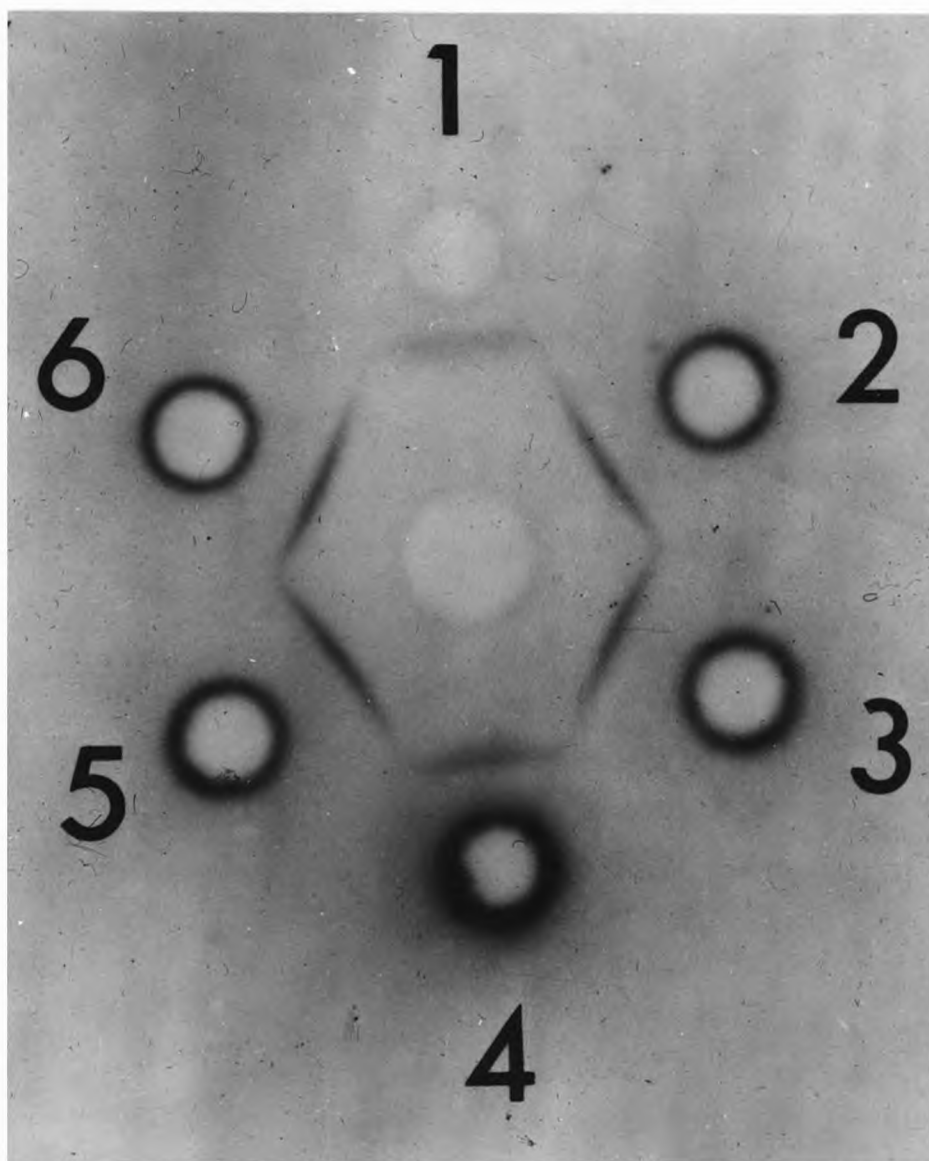


Fig. 4: Radioautograph of agar-gel diffusion plate.

Centre well, human liver ferritin antiserum; 1, ^{59}Fe -labelled Fraction 1; 4, ^{59}Fe -labelled marrow supernatant solution; 2, 3, 5 and 6, ^{59}Fe -labelled human liver ferritin. Although no visible precipitate could be seen opposite the wells containing Fraction 1 and the marrow supernatant solution, bands of radioactivity coalescing with the liver ferritin bands are demonstrated.

Exp't. No.		Minutes			Hours				
		5	10	30	1	2	3	4	6
I.	Supernatant solution	9.7	7.0	8.3		7.9		16.8	
	Fraction 1	7.4	8.0	8.9		13.5		15.3	
II.	Supernatant solution	9.9	8.4	9.8	9.4		15.1		
	Fraction 1	8.4	9.2	9.9	10.0		16.1		
III.	Supernatant solution	7.2	9.9	10.1	10.3	11.0			
	Fraction 1	4.2	6.6	8.3	8.3	10.2			
IV.	Supernatant solution		10.5	11.4	11.5	11.8		11.3	
	Fraction 1		8.5	10.5	8.4	10.5		10.7	
V.	Supernatant solution	11.1		9.2	11.2			11.2	8.9
	Fraction 1	10.0		9.6	10.2			8.1	8.9

Table I: A comparison of radioactivity in ferritin (expressed as a percentage of total marrow uptake) determined in marrow supernatant solution and in Fraction 1, after various periods of incubation in vitro.

Exp't. No.	Minutes			Hours				
	5	10	30	1	2	3	4	6
I.	43	51	51	59	69		74	
II.	41	46	51	61		67		
III.	30	43	49	50	58			
IV.	31	33	55	58		71		78

Table II: The percentage radioactivity precipitable as ferritin in Fraction 1 after various periods of incubation of marrow in vitro (labelling with ⁵⁹Fe not stopped).

IV. Effects of stopping labelling on different fractions.

Four experiments were performed in which the marrow was incubated with ^{59}Fe -labelled plasma for a period of 5 to 10 minutes, and further labelling of the marrow with ^{59}Fe was then stopped. In this way it was possible to follow the fate of a 'cohort' of radioiron at various time intervals. Figure 5 illustrates the pattern obtained in one experiment. The radioactivity in Fraction 1 and the 'stromal fraction' fell with time up to 6 hours, whereas the activity of haemoglobin rose rapidly at first, and then reached a plateau. In contrast, the radioactivity in ferritin remained the same throughout the time of incubation. When the counts present in Fraction 1 were corrected for the ferritin present, the non-ferritin fraction fell rapidly to a very low level. During this period there was a reciprocal increase in the activity present in Main Haemoglobin. In the three other experiments the results were very similar. The percentages of radioactivity precipitable as ferritin when labelling was stopped were 9.9; 5.1; and 6.5 as compared with figures of 9.9; 6.5; and 8.9, respectively, at 6 hours. During this period the non-ferritin activity in Fraction 1 fell from 9.6

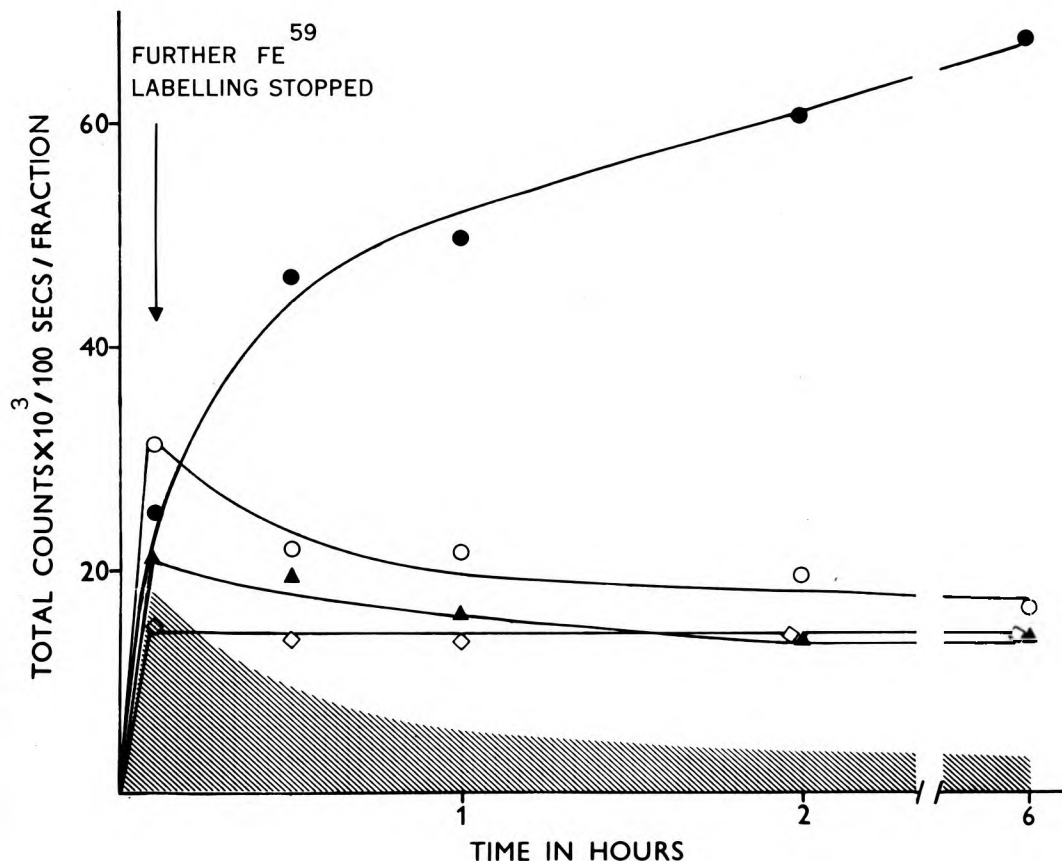


Fig. 5: Incorporation of ⁵⁹Fe into 'stromal fraction',
Fraction 1, Main Haemoglobin, and ferritin.

Analysis of samples of human rib marrow culture at various times up to 6 hours. Further labelling with ⁵⁹Fe was stopped after 5 minutes by washing the cells and re-suspending them in unlabelled medium. Closed triangles, 'stromal fraction'; open circles, Fraction 1; closed circles, Main Haemoglobin; open diamonds, ferritin; hatched area, haemoglobin precursor, i.e. Fraction 1 minus ferritin.

to 2.7%, from 11.1 to 2.2% and from 11.3 to 6.1%. The increase in Main Haemoglobin was from 26.3 to 64.6%, from 19.8 to 44.3%, and from 37.6 to 67.3% in the three experiments. It thus appears that the portion of Fraction 1 not precipitable by antiferritin serum in the active intermediate for iron incorporation into haemoglobin (haemoglobin precursor). Since in control experiments in which labelling was not stopped the activity in haemoglobin continued to rise to between 12 and 25 times the figure at 10 minutes, these data also suggest that ferritin does not act as an active intermediate for the incorporation of iron into haemoglobin in marrow cultures over 6 hours.

V. Effect of antiferritin serum on ^{59}Fe incorporation into haemoglobin in a marrow lysate system.

Two human marrow lysates were prepared by lysing equal aliquots of washed iliac crest marrow cells with four volumes of water. The 'stroma' from one lysate was removed by centrifuging at 1,500 x G for 20 minutes, and both lysates were then incubated with antiferritin serum for 10 minutes at 37°C

to inactivate any ferritin in the lysate. The lysates were then incubated with ^{59}Fe citrate for 2 hours at 37°C . The transferrin of the antiferritin serum had initially been saturated by adding excess nonactive iron to it, and then dialysed overnight against iron free 0.08 M Tris buffer pH 8.4 to remove the excess iron. This prevented the ^{59}Fe citrate attaching to the transferrin of the antiserum. A control of normal rabbit plasma containing no antibodies to ferritin and which was treated in the same way was set up. A further control using B.S.S. instead of rabbit serum was also employed. At the end of the incubation, the lysate containing stroma was centrifuged at $1,500 \times G$ for 20 minutes and the supernates from both lysates were then dialysed overnight against running iron free water to remove any free iron. The supernatant solutions were then chromatographed on I.R.C. 50 resin and the percentage of the added activity in Main Haemoglobin was determined. It can be seen in Table III that antiferritin serum had no effect on ^{59}Fe incorporation into haemoglobin as compared to the controls, indicating that under the conditions of the experiment, ferritin does not appear to be a necessary intermediate for iron incorporation into haemoglobin in vitro.

	Total Haemolysate				1,500 x G Supernate		
	Haemolysate plus anti-ferritin serum	Haemolysate plus normal rabbit serum	Haemolysate plus B.S.S.	Supernate plus anti-ferritin serum	Supernate plus normal rabbit serum	Supernate plus B.S.S.	
% of added activity in Main Haemoglobin	7.5	7.0	8.9	10.7	6.5	10.3	

Table III. Effect of antiferritin serum on iron incorporation
into haemoglobin in a marrow lysate system.

The test lysates were incubated with antiferritin serum for 10 minutes prior to incubation with ^{59}Fe citrate. The antiferritin serum had no effect on ^{59}Fe incorporation into Main Haemoglobin.

VI. Kinetics of ^{59}Fe incorporation into ferritin
in marrow cells *in vivo*.

Since it was not possible to show any drop in ferritin activity during the incubation of marrow *in vitro*, further studies were carried out *in vivo* in rats to find out whether labelled ferritin was still present in the reticulo-cytes entering the circulation. Since it seemed possible that the ferritin might be removed by the spleen, studies were done in both normal and splenectomized animals. Twelve splenectomized rats and 12 controls were given an intravenous injection of ^{59}Fe -labelled plasma, and batches were killed at intervals thereafter (see Methods, page 98). Four hours after injection the pooled marrows from three splenectomized and three control rats contained 9.6 and 12.0% respectively, of the cellular radioactivity in the ferritin fraction. At 24 hours the corresponding figures were 1.8 and 2.2%. However, it was not possible to show radioactive ferritin in the circulating red cells of the remaining animals at any time up to 4 days after the administration of the radioiron. In an extension of the study, 10 ml. of autologous plasma labelled with 25 $\mu\text{c.}$ of

$^{59}\text{Fe Cl}_3$ was injected into a normal splenectomized human subject, and blood samples were collected at daily intervals for 7 days thereafter. At no time was labelled ferritin demonstrated. These findings suggest that the ferritin present in red cell precursors is either ultimately used for haemoglobin synthesis or is extruded before the cells leave the marrow.

The incorporation of ^{59}Fe into ferritin and Main Haemoglobin in marrow was also determined in a group of 12 control and 12 phenylhydrazine treated rats over a period of 23 hours after intravenous injections of ^{59}Fe -labelled plasma (see Methods, page 97). Groups of 3 rats were sacrificed at 1, 5, 12, and 23 hours, and the marrow obtained from each group of 3 rats was pooled. The pattern of incorporation of ^{59}Fe into ferritin and Main Haemoglobin in the marrow can be seen in Figure 6. In the control group the radioactivity in ferritin decreased between 5 and 12 hours, while that in haemoglobin rose. In the phenylhydrazine treated rats, radioactivity in ferritin rose and fell in parallel with the activity in haemoglobin.

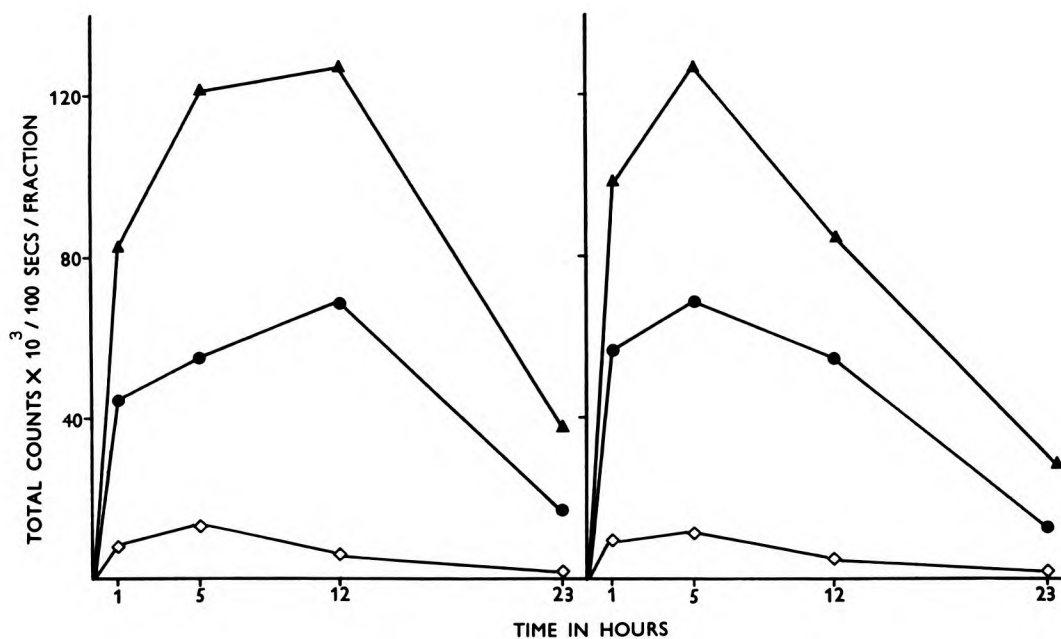


Fig. 6: Incorporation of ^{59}Fe into rat marrow in normal and phenylhydrazine-stimulated rats after intravenous injection of ^{59}Fe -labelled plasma.

Analysis of pooled samples of marrow from groups of 3 rats at various times up to 23 hours. Left hand side of figure - normal rats. Right hand side of figure - ^{59}Fe rats given phenylhydrazine. Closed triangles, total ^{59}Fe uptake per gram wet weight of marrow; closed circles, Main Haemoglobin; open diamonds, ferritin.

VII. Iron uptake by reticulocyte suspensions.

Reticulocyte suspensions, obtained from the blood of two patients with pernicious anaemia in early remission, were incubated with labelled plasma for 4 hours. In both cases haemoglobin and Fraction 1 formation was similar to that observed in normal marrow. No label was detectable in ferritin in one experiment, but small and possibly significant amounts of ^{59}Fe were incorporated into ferritin in the other. In a third experiment, a reticulocyte suspension from a patient with idiopathic autoimmune haemolytic anaemia was incubated for 1 hour in labelled plasma. At this time 10.3% of the activity taken up by the reticulocytes was in ferritin. Several samples of the suspension were then re-incubated in unlabelled medium after the cells had been washed 4 times in cold saline. After a further 3 hours incubation 8.5% of the radioactivity was still present in the ferritin fraction.

Two similar experiments were carried out on reticulocyte suspensions obtained from rats that had been given phenylhydrazine 3 to 4 days previously. After 1 hour's incubation

2.0 and 0.9% respectively, of the ^{59}Fe were present in ferritin. After 3 further hours of incubation in unlabelled substrate the comparable figures were 3.2 and 0.6%.

VIII. Iron uptake by iron-deficient marrows.

Marrow obtained from a patient with iron deficiency anaemia was incubated with labelled plasma, and samples were taken at progressive times of incubation up to 4 hours. The incorporation of ^{59}Fe into haemoglobin and Fraction 1 was similar to that observed in normal marrows, but no labelling of ferritin could be detected.

In an experiment using another severely iron-deficient marrow in which further labelling with ^{59}Fe was stopped after 8 minutes incubation (Figure 7), the activity in Fraction 1 fell to much lower levels relative to the total uptake than in normal marrows. This can be ascribed to the fact that it contained only the haemoglobin precursor, since no labelled ferritin was demonstrable at any time.

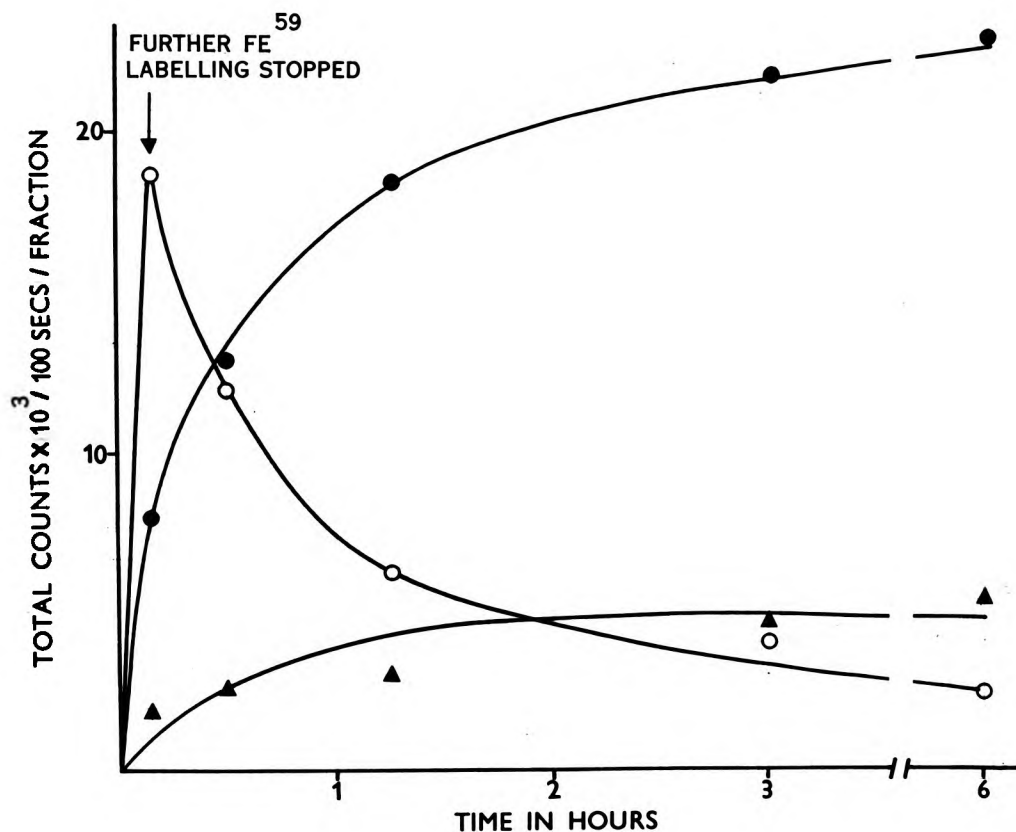


Fig. 7: Iron-deficient human iliac crest marrow culture.
Incorporation of ⁵⁹Fe into 'stromal fraction',
Fraction 1, and Main Haemoglobin.

Analysis of samples at various times up to 6 hours. Further labelling was stopped after 8 minutes by washing the cells and resuspending them in unlabelled medium. Closed triangles, 'stromal fraction'; open circles, Fraction 1; closed circles, Main Haemoglobin. No ferritin could be identified at any stage.

IX. Effect of lead on the incorporation of ^{59}Fe into
'stromal fraction', ferritin, Fraction 1, and
Main Haemoglobin.

In five experiments 10^{-4} M lead acetate was added to the marrow suspension at the start of incubation. There was little effect upon total iron uptake, but radioactivity in the 'stromal fraction' increased by a mean of 95% (range 40 to 154%) compared with the control tubes. Both Fraction 1 and ferritin activity were greater by mean percentages of 38 (range 12 to 97) and 40 (range 12 to 90), respectively. The effect upon ^{59}Fe in the haemoglobin precursor (i.e. Fraction 1 minus ferritin) was variable, ranging from a decrease of 46% to increases of 2, 34, 36, and 125%. Incorporation of radioactivity into Main Haemoglobin was inhibited by from 38 to 80% (mean 55%). The results in one experiment are illustrated in Figure 8.

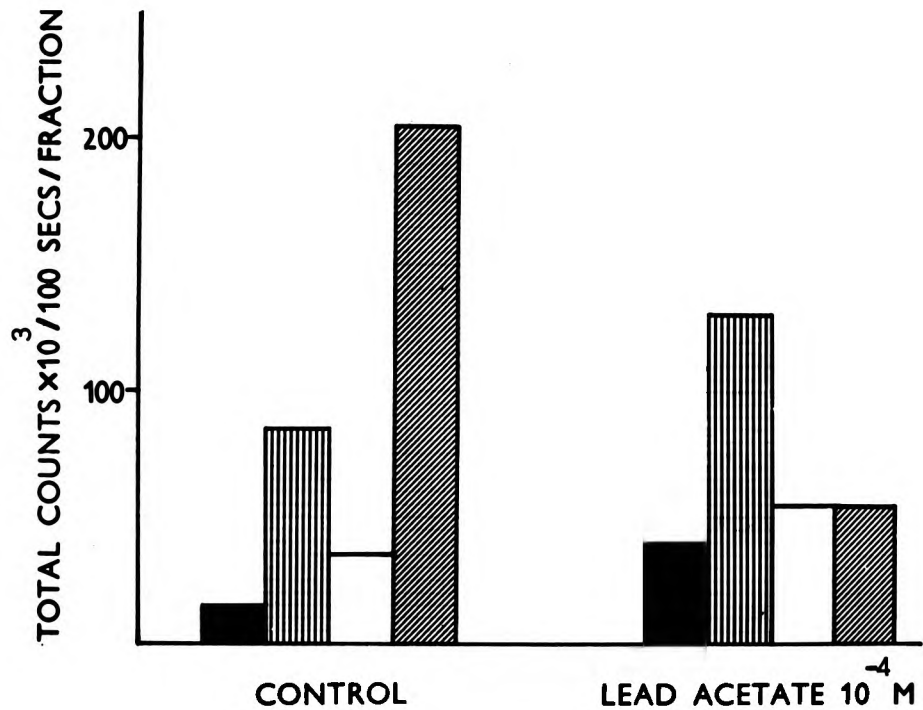


Fig. 8: Effect of 10^{-4} M lead acetate on human rib marrow culture.
Incorporation of ^{59}Fe into 'stromal fraction', haemoglobin
precursor, Main Haemoglobin, and ferritin after 4 hours.

Black, 'stromal fraction'; vertical lines, ferritin; unshaded, haemoglobin precursor, i.e. Fraction 1 minus ferritin; diagonal lines, Main Haemoglobin. There is diminished radioactivity in haemoglobin and increases in 'stromal fraction', ferritin, and haemoglobin precursor in the lead-poisoned culture as compared with the control.

X. Effect of metabolic inhibitors on ^{59}Fe uptake
and incorporation into 'stromal fraction',
ferritin and haem.

The effect of 10^{-2} M sodium cyanide, 10^{-2} M sodium fluoride, 10^{-2} M sodium arsenite, 10^{-3} M iodoacetamide and 10^{-4} M 2:4 dinitrophenol on ^{59}Fe uptake and incorporation into 'stromal fraction', ferritin and haem in an iliac crest marrow cultured for 3 hours was determined. All the inhibitors were made up in B.S.S. and buffered to pH 7.4. The results are depicted in Table IV. As these inhibitors (with the exception of 2:4 dinitrophenol) resulted in a marked depression of total cellular uptake, the ^{59}Fe activity in each of the fractions in this experiment was expressed as a percentage of the total cellular uptake. In this way one could detect any effect of the inhibitor on the intracellular distribution of ^{59}Fe .

An increase in the percentage of ^{59}Fe incorporated into the 'stromal fraction' was noted with all the inhibitors, the effect being most marked with sodium arsenite and sodium fluoride. Sodium cyanide and iodoacetamide caused a

	Total uptake cts./100 secs.	% Uptake of control uptake.	% Counts in 'stromal fraction'.	% Counts in ferritin.	% Counts in haem.
Control	48,000	100	5.6	25.0	48.5
Sodium cyanide $10^{-2}M$	20,535	41.9	9.3	33.1	2.1
Sodium fluoride $10^{-2}M$	28,815	58.8	19.3	24.1	23.6
Sodium arsenite $10^{-2}M$	6,633	13.5	43.5	16.0	1.8
2:4 Dinitro- phenol $10^{-4}M$	46,280	96.5	7.2	19.0	48.7
Iodoacetamide $10^{-3}M$	29,880	61.0	7.1	30.0	22.3

TABLE IV. The effect of metabolic inhibitors on ^{59}Fe uptake and utilization by red cell precursors.

Human iliac crest marrow aliquots were incubated for 3 hours. Haem was crystallized by the method of Labbe and Nishida (1957).

32% and 20% increase respectively in the proportion of counts incorporated into ferritin, in the face of a marked inhibition of ^{59}Fe incorporation into haem. Sodium fluoride had no effect on ^{59}Fe incorporation into ferritin, while depressing iron incorporation into haem. Sodium arsenite caused a 36% depression of ^{59}Fe incorporation into ferritin in the presence of an almost complete block of iron incorporation into haem. On the other hand, 2:4 dinitrophenol caused a 24% depression of ^{59}Fe incorporation into ferritin while not affecting haem synthesis at all.

XI. Effect of anoxia on ^{59}Fe uptake and incorporation into 'stromal fraction', ferritin and haem.

In two experiments identical aliquots of human rib marrow were incubated in duplicate with ^{59}Fe -labelled plasma in an atmosphere of 94% oxygen and 6% CO_2 , and in an atmosphere of 94% nitrogen and 6% CO_2 , respectively for 2 hours. As total cellular uptake of ^{59}Fe was markedly depressed in the anoxic marrows (Table V), the radioactivity in each of the fractions was again expressed as a percentage of the cellular uptake. In experiment I (Table V) anoxia had no effect on the proportion of ^{59}Fe incorporated into the 'stromal fraction'.

Incubation atmosphere	Total uptake ^{59}Fe cts./100 secs.	% Counts in 'stromal fraction'.	% Counts in ferritin.	% Counts in haem.
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Expt. I.

94% O_2 6% CO_2	184,300	54.7	10.2	9.8
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94% N_2 6% CO_2	59,170	54.2	12.2	5.0
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Expt. II.

94% O_2 6% CO_2	56,634	43.7	4.3	20.8
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94% N_2 6% CO_2	24,890	35.4	7.0	14.7
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TABLE V. The effect of anoxia on ^{59}Fe uptake and utilization by red cell precursors.

Human rib marrow aliquots were incubated for 2 hours. The results are expressed as the mean of duplicate analysis in each experiment. Haem was isolated by the method of Labbe and Nishida (1957).

There was an increase of 19.6% in the percentage of counts incorporated into ferritin while ^{59}Fe incorporation into haem was depressed by almost 50% in the anoxic marrow. In experiment II (Table V), ^{59}Fe incorporation into the 'stromal fraction' and haem were depressed by 19% and 29.3% respectively in the anoxic marrow, while the proportion of counts incorporated into ferritin was increased by 62.8%.

XII. Effect of metabolic stimulators on ^{59}Fe uptake and incorporation into 'stromal fraction', ferritin and haem.

The effect of 10^{-2}M citrate, $5 \times 10^{-2}\text{M}$ succinate and $5 \times 10^{-3}\text{M}$ succinate on ^{59}Fe uptake and incorporation into 'stromal fraction', ferritin and haem was determined in an iliac crest marrow cultured for 3 hours. All the stimulators were made up in B.S.S. and buffered to pH 7.4. Neither citrate nor succinate in these molarities had any effect on the cellular uptake and incorporation of ^{59}Fe into 'stromal fraction', ferritin or haem when compared to the control values.

XIII. Effect of varying saturations of transferrin
on ferritin and Main Haemoglobin synthesis.

A comparison was made between the uptake of iron into ferritin and Main Haemoglobin, respectively, at various saturations of transferrin. After the addition of 10 μ c. ^{59}Fe to 20 ml. plasma, unlabelled iron as ferrous ammonium sulphate was added to equal samples of plasma in amounts calculated to produce the desired saturation of the transferrin. The samples were then dialysed against running iron-free water for 16 hours before use. Equal marrow aliquots were incubated for 4 hours with the plasma samples. At high saturations the amount of iron incorporated into Main Haemoglobin did not increase, whereas incorporation into ferritin and into 'stromal fraction' continued to rise progressively. The results in one experiment are shown in Figure 9. In five other experiments (Figure 10) the general pattern was confirmed, with a progressive drop in the ratio of the ^{59}Fe Main Haemoglobin to ^{59}Fe ferritin occurring with increasing saturation of transferrin.

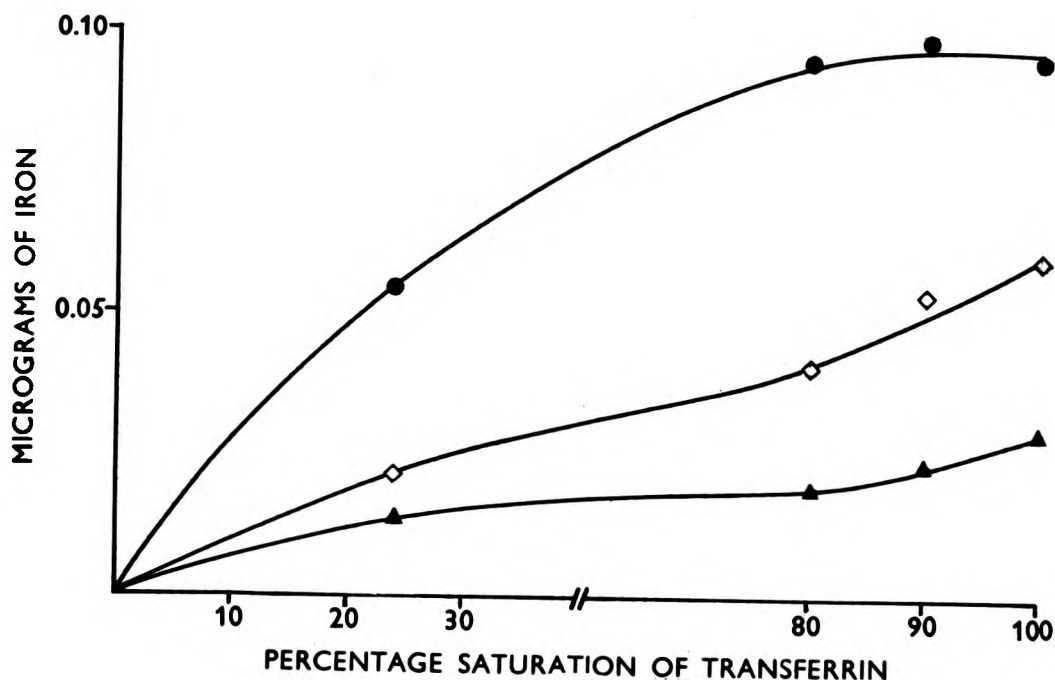


Fig. 9: Relationship between transferrin saturation and the incorporation of iron into 'stromal fraction', ferritin, and Main Haemoglobin in human marrow culture.

The marrow aliquots were incubated for 4 hours. Closed triangles, 'stromal fraction'; open diamonds, ferritin; closed circles, Main Haemoglobin. At high saturations of transferrin more iron was incorporated into ferritin.

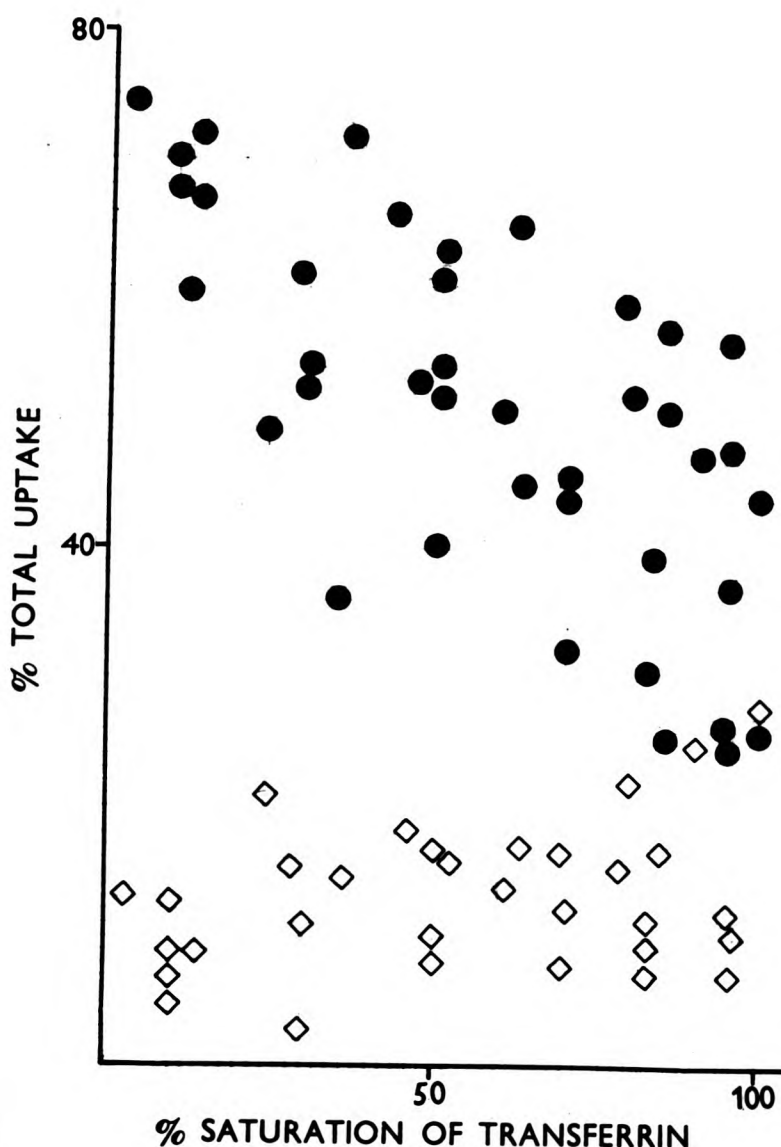


Fig. 10: Relationship between transferrin saturation and the incorporation of iron into ferritin and Main Haemoglobin in human marrow cultures.

The results of five experiments are depicted. The incubation time was 4 hours. Closed circles, Main Haemoglobin; open diamonds, ferritin. There was a progressive fall in the ratio of ^{59}Fe Main Haemoglobin to ^{59}Fe ferritin with increasing saturations of transferrin. The radioactivity of Main Haemoglobin and ferritin are expressed as percentages of the total cellular uptake.

Discussion.

Greenough and his collaborators have shown that iron in red cell precursors passes through a non-haemoglobin iron-protein complex (Fraction 1) before being incorporated into haemoglobin (Greenough et al., 1962). Although these workers were not able to identify radioactive ferritin in Fraction 1, the present studies indicate that it contains an appreciable amount and that the proportion present rises progressively with time. There are two possible reasons for these discrepant findings. In the first place experiments done in the current study revealed that approximately 50% of the radioactive ferritin in Fraction 1 is removed by the high-speed centrifugation (100,000 x G for 30 minutes) to which Greenough and co-workers subjected the marrow lysate before chromatography, and secondly there were differences in the immunochemical techniques used in the two studies. As a result, the kinetic patterns for Fraction 1 reported by Greenough and co-workers correspond more closely to those obtained for the haemoglobin precursor (Fraction 1 minus ferritin) in the present investigation. The current findings lend further support to the suggestion (Greenough et al., 1962) that this

substance is an important precursor of haemoglobin. In experiments where labelling was allowed to continue for several hours radioactivity appeared in this fraction at an earlier stage than in haemoglobin and then levelled off. In addition, in experiments where further uptake of ^{59}Fe was stopped after 5 minutes incubation, the radioactivity present in the fraction dropped while that in haemoglobin rose reciprocally. The fact that the fraction only fell to a limited extent is compatible with the suggestion that it was being fed from a large stromal pool, or alternatively, it is possible that some other active precursor substance was present.

Significant amounts of iron taken up by marrow cultures were rapidly incorporated into ferritin. The results obtained with metabolic inhibitors and incubation under anoxic conditions indicated that the incorporation of radioiron into ferritin was far less sensitive to curtailment of available metabolic energy than iron incorporation into haem. Similar findings have been reported by Mazur and Carleton (1963). The absence of any inhibitory effect of anoxia or cyanide on iron incorporation into ferritin indicates that this process is not dependent on

oxidative metabolism. However, an inhibitory effect on iron incorporation into ferritin was noted with sodium arsenite and 2:4 dinitrophenol, both of which are uncouplers of oxidative phosphorylation. The latter findings are compatible with the thesis that adenosine triphosphate (A.T.P.) is necessary for iron incorporation into ferritin (Mazur and Carleton, 1963; Morgan and Laurell, 1963). The possibility thus exists that in the cyanide poisoned and anoxic marrows, A.T.P. derived from sources other than oxidative phosphorylation (e.g. from glycolysis) may have been used in mediating the incorporation of iron into ferritin. The reason for the absence of any effect of succinate on marrow iron uptake and utilization is not clear, although the absence of any effect with citrate may be due to its inability to penetrate the red cell membrane (Garby and Thoren, 1962).

Since ferritin is visible on electron microscopy both in red cell precursors and in reticuloendothelial cells of the marrow (Bessis and Breton-Gorius, 1957) it was important to establish the cellular location of the labelled ferritin in the various experiments. In an attempt to resolve this problem the uptake of plasma-bound radioiron and its incorporation into

ferritin were compared in bone marrow and splenic suspensions. Since only negligible amounts of radioiron were incorporated into ferritin in the splenic suspensions, it seemed likely that the reticuloendothelial cells of the bone marrow were not responsible for the degree of ferritin labelling observed in marrow cultures. These findings are in agreement with the results of other workers (Lajtha and Suit, 1955; Austoni, 1956; Hosain et al., 1962), who have shown in several different ways that reticuloendothelial cells have an extremely limited capacity to take up iron bound to transferrin. Although it therefore seems probable that the incorporation of radioiron into ferritin observed in the present study was taking place predominantly in developing red cell precursors, there is still some doubt as to its significance. Recently Mazur and Carleton (1963) have suggested on the basis of both in vivo and in vitro studies that most of the iron derived from plasma is incorporated into ferritin before its incorporation into haem. They based this conclusion on several observations. When rats were given ^{59}Fe intravenously, the activity in marrow ferritin dropped between 4 and 12 hours while the activity in haemoglobin was still rising. This relationship was not shown in animals in which

radioiron uptake had been stimulated by phenylhydrazine or depressed by endotoxin. Under these circumstances the rise and fall of radioactivity in ferritin and haemoglobin fractions ran parallel. Similar findings have also been obtained in the present study in rats given phenylhydrazine. In further experiments Mazur and Carleton incubated reticulocytes in vitro with ^{59}Fe -labelled plasma. Further labelling was then stopped after 20 minutes. At this time the specific activity of the ferritin iron was much lower than that of the iron in haem. However, since ferritin contains approximately 2,400 atoms of iron per molecule as compared with only 4 in haem, they calculated that each ferritin molecule had accumulated many more atoms of iron than each haem molecule. They therefore interpreted these observations as indicating a precursor relationship between ferritin iron and haem iron. At the same time these authors pointed out that when incubation of reticulocytes was continued for 120 minutes after further labelling had been stopped, the calculated loss of ^{59}Fe per micromole of ferritin was many times greater than the gain by haemoglobin. These results suggested that the major portion of the labelled iron in ferritin might eventually leave the cell. These interpretations depend on the

assumption that ^{59}Fe taken up by ferritin is uniformly dispersed in a large iron pool. It is, however, doubtful whether such an assumption is justifiable, since it has been shown that plasma-bound ^{59}Fe is incorporated to a greater extent into the low density, iron-poor molecules of hepatic ferritin than into the high density ferritin molecules of high iron content (Mazur et al., 1960). This lack of homogeneity of hepatic ferritin indicates the difficulties associated with any quantitative interpretation of the uptake and release of ^{59}Fe by ferritin.

In the present study an attempt was made to elucidate the significance of the ferritin formed in red cell precursors by studying the incorporation of ^{59}Fe into this fraction under various experimental conditions. At high saturations of transferrin more iron was taken up by red cell precursors, but this additional uptake was not incorporated into haemoglobin. These results are in agreement with those obtained by Najean (1962). In the present investigation the bulk of the extra iron was found to be incorporated into ferritin. A similar build-up in ferritin activity was noted when haemoglobin synthesis was depressed by lead. In contrast, when marrow from an iron-deficient subject

was incubated in radioactive plasma, there was a rapid movement of iron through the haemoglobin precursor into haemoglobin, but no ferritin was formed at any time. These observations suggest that iron is only incorporated into ferritin when more iron enters the cell than can be immediately utilized for haemoglobin synthesis. Since this storage form of iron could not be identified in circulating red cells, it is either utilized for the synthesis of haem at some later stage, or is removed from the red cell precursors. Although it seems entirely possible that ferritin iron is available for haemoglobin formation if required, no evidence in support of this thesis was obtained in the present investigation. In the experiments in which isotope labelling was stopped, the activity in ferritin rapidly reached a plateau, and there was no significant fall in activity for several hours afterwards. In contrast, there was a sharp decline in the activity present in the haemoglobin precursor while the activity in the haemoglobin rose. In addition, in studies where labelling was allowed to continue for as long as 12 hours there was a steady build-up in ferritin over the whole period. Antiferritin serum was also unable to depress ^{59}Fe incorporation into haemoglobin in a marrow lysate system. Although these results in no way

exclude the possibility that iron taken up and stored in ferritin is slowly released for haemoglobin synthesis, they afford no positive evidence that this occurs.

If ferritin is not utilized for haem synthesis, it must leave the red cell precursors, since labelled ferritin could not be identified in circulating erythrocytes. This could theoretically occur either within the bone marrow or after the reticulocyte enters the circulation. Since it has been established that the spleen has the capacity to remove haemosiderin granules from young red cells (Crosby, 1957), in vivo experiments were carried out in splenectomized rats to find out whether labelled ferritin could be identified in circulation at any time after the intravenous administration of radio-iron. Although appreciable quantities of radioactive ferritin were present in the marrows of these animals, none was found in the circulation. Although these observations appear incompatible with the demonstration that reticulocytes incubated in vitro can incorporate radioiron into ferritin, the explanation for this apparent discrepancy may lie in the limited capacity of the reticulocyte to take up iron from the plasma under normal circum-

stances (Lajtha and Suit, 1955). If ^{59}Fe incorporated into ferritin within red cell precursors is not utilized for haem synthesis, then it seems probable that it leaves the cell before it enters the circulation. This possibility would fit in well with previous electron microscopic findings in which it has been demonstrated that ferritin can move freely between the red cell precursors and the reticuloendothelial cells of the marrow (Bessis and Breton-Gorius, 1957). Although it was originally suggested by Bessis that the movement was in the opposite direction, several workers have questioned the validity of this interpretation (Bothwell and Finch, 1962 c; Lajtha, 1959; Jandl, 1960; Mاتيoli and Eylar, 1964). If the present proposal is a valid one it might explain, in part at least, ferrokinetic data which indicate that some of the iron delivered to the marrow enters a labile pool which may feed it back into the plasma again (Pollycove and Mortimer, 1961). Although evidence has been produced to show that this pool is located on the stroma of red cell precursors, the present findings are compatible with another short circuit involving the movement of iron from plasma into ferritin in red cell precursors, and thence to reticuloendothelial cells that release the iron back into the plasma.

CHAPTER V

SUMMARY OF THE EXPERIMENTAL STUDIES

In vitro and in vivo studies were carried out in human subjects and in rats on the uptake and utilization of transferrin-bound iron by red cell precursors. In in vitro human marrow cultures iron rapidly appeared in a non-haemoglobin protein fraction which was isolated on column chromatography on I.R.C. -50 resin. The iron subsequently was incorporated into haemoglobin. The non-haemoglobin fraction was shown to consist of ferritin, and an active haemoglobin precursor which is identical to the non-haemoglobin protein intermediate isolated by Greenough and his co-workers. Experiments were then carried out to elucidate the significance of the ferritin component and to determine the metabolic energy requirements for iron incorporation into ferritin.

The cellular location of the labelled ferritin was shown to be in the red cell precursors and not in reticulo-endothelial cells, as only negligible amounts of ^{59}Fe were incorporated into ferritin in splenic suspensions. Iron incorporation into ferritin was shown to be far less sensitive to curtailment of available metabolic energy than iron incorporation into haem in red cell precursors. Several metabolic

inhibitors, while markedly inhibiting iron incorporation into haem had little effect on iron incorporation into ferritin once iron had been taken up by the cell.

With increasing percentage saturation of transferrin more iron was incorporated into ferritin relative to the amount incorporated into haemoglobin. Also no labelling of ferritin with ^{59}Fe was detected when marrow from an iron deficient subject was cultured, the iron rapidly moving through the active haemoglobin precursor into haemoglobin. A build-up in ferritin activity was also observed when haemoglobin synthesis was depressed by lead. The observations suggest that the iron not immediately required for haemoglobin synthesis in red cell precursors is incorporated into ferritin. No evidence of the later utilization of this ferritin iron for the formation of haemoglobin was obtained, although this possibly could not be definitely excluded. These results are at variance with those of Mazur and Carleton who maintain that ferritin is an active intermediate for iron incorporation into haemoglobin in red cell precursors.

Since labelled ferritin could not be identified in circulating erythrocytes after intravenous injection of ^{59}Fe -labelled plasma, ferritin iron not utilized for haemoglobin synthesis must leave the red cell precursors. This could either occur within the bone marrow or after the reticulocyte enters the circulation. Since it has been shown that the spleen has the capacity to remove haemosiderin granules from reticulocytes, the possibility that the spleen might remove ferritin from reticulocytes was investigated in a group of control and splenectomized rats. Although labelled ferritin was demonstrated in the marrow of these animals after the intravenous injections of ^{59}Fe at no stage could it be detected in circulating red cells. Labelled ferritin could also not be detected in circulating red cells of a normal splenectomized human subject after intravenous injection of ^{59}Fe -labelled plasma. If ^{59}Fe incorporated into ferritin within red cell precursors is not utilized for haem synthesis, it appears probable that it leaves the cell before it enters the circulation and that this removal is effected by reticulo-endothelial cells in the marrow. This concept would fit in

well with the electron microscopic findings in which it has been shown that ferritin moves freely between red cell precursors and the reticuloendothelial cells of the marrow. This interpretation might also explain at least in part the ferrokinetic data of Pollycove and his co-workers, which indicate that some of the iron taken up by the marrow enters a labile pool which may then feed back into the plasma again. The present findings are compatible with a short circuit in which iron moves from plasma into ferritin in red cell precursors, and then to reticuloendothelial cells that release the iron back into the plasma.

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