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FACTORS INVOLVED IN THE HANDLING OF IRON BY THE RETICULO-ENDOTHELIAL SYSTEM

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A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MEDICINE OF THE UNIVERSITY OF THE WITWATERSRAND

JOHANNESBURG, 1972.

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TO MY WIFE

This is to certify that this thesis is my own work and has not been presented at any other university.



D.A. Lipschitz.

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CHAPTER I

INTRODUCTION

INTRODUCTION

For centuries iron was regarded as a source of health and vigour, and it has been known for at least 200 years that it is a component of blood and effective in the treatment of chlorosis. However, its metabolism remained largely a mystery until the advent of radioactive iron isotopes in 1938. Since then most of the major pathways of this metal into, through and out of the body have been elucidated. In addition the functions which it subserves have been partially characterized.

Iron performs its most critical function in the body in combination with protoporphyrin to form haem. Haem plus various protein moieties form compounds which bind oxygen reversibly. Haemoglobin in erythrocytes transports oxygen from the lungs to the periphery, while myoglobin enables muscle cells to store oxygen. In the tissues haem enzymes such as the cytochromes are concerned with oxidative metabolism. Iron compounds are thus pivotally involved in the basic energy-exchange reactions of cellular metabolism.

The human body shows great economy in its handling of iron and only small amounts are lost each day. Iron is thus rigidly conserved by the body (McCance and Widdowson, 1938) and this serves to emphasize its metabolic importance. The total iron content of an average adult varies between 2 and 5g, values being higher for the male than the female (Pritchard and Mason, 1964; Weinfeld, 1965). The major proportion of body iron is present in circulating haemoglobin (approximately 66%); 3% is in myoglobin and less than 1% is bound to transferrin or in the haem enzymes (Bothwell and Finch, 1962). The remainder comprises the body store of iron, which does not appear to participate in active metabolic processes. It represents a reserve from which iron can be drawn if demands for it increase. This has been demonstrated following phlebotomy (Haskins et al, 1952), and in iron deficiency these stores become depleted (Rath and Finch, 1948).

The metabolically active iron-containing compounds in the body are continually being renewed, the iron being used again and again as fresh proteins

and porphyrins are synthesised. In normal individuals about 30mg iron passes through the plasma pool each day, most of it en route to the erythroid marrow where it is incorporated into the haemoglobin of developing red cells (Huff et al, 1950). The major source of this iron is the reticulo-endothelial cells of the liver and spleen, which engulf aged erythrocytes and liberate the haemoglobin iron for re-utilization (Noyes et al, 1960). Only relatively small contributions are made by iron newly absorbed from the gastro-intestinal tract and by the body iron stores, which are located principally in reticulo-endothelial cells; but the supply from these sources is increased if the requirements for erythropoiesis are enhanced (Haskins et al, 1952; Hahn et al, 1943). At any moment in time only about 4 mg iron is present in the plasma, and there is a rapid turnover with a half-time of approximately 90 minutes.

Iron transport through the plasma is mediated by a specific binding protein known as transferrin. It is a beta 1 globulin with a molecular weight of 74,000 (Roberts et al, 1966). This protein is a

true transport vehicle and is not destroyed in the process of acquiring or relinquishing its iron (Laurell, 1951). Each molecule of transferrin is capable of binding two atoms of ferric iron. There is good evidence that transferrin cannot give up its iron to reticulo-endothelial cells *in vivo* (Finch *et al*, 1965), or acquire iron from red cell precursors. The unidirectional transfer is presumably due to the nature of the exchange mechanisms at the different cell membranes (Finch *et al*, 1970). Normally transferrin is only $\frac{1}{3}$ saturated with iron, the concentration representing a dynamic equilibrium between the amount of iron entering and the amount leaving the plasma at any one time. If input exceeds the marrow demand, the plasma iron concentration will rise; if demand exceeds supply, the level will fall. In normal individuals there is a distinct diurnal variation in the plasma iron, values being highest in the morning and lowest in the late evening (Laurell, 1953). This is reversed in normal subjects who work at night (Bowie *et al*, 1963) and is absent in many disease states (Bothwell and Finch, 1962). These daily fluctuations probably represent changes

in reticulo-endothelial iron release (Funk, 1970), but the mechanism is unknown.

The percent saturation of transferrin with iron appears to influence its destination. At normal levels of saturation and with normal marrow demands, 80% of the iron on transferrin is taken up by red cell precursors and incorporated into haem within one hour (Noyes et al, 1964). If, however, the plasma iron level rises to near saturation, either due to decreased marrow demand (aplastic anaemia), or increased input (haemochromatosis), more is deposited in liver parenchymal stores (Hosain and Finch, 1966). Recently Fletcher and Huehns (1967, 1968) have suggested that functional differences between the two binding sites for iron on transferrin account for those observations. They believe that the one site preferentially gives up its iron to the erythroid marrow and placenta, while the other is more involved with transport to stores. This suggests an active role for transferrin in internal iron exchange. The basis for this hypothesis is derived from observations on the uptake of transferrin iron by reticulocytes

in vitro. In vivo studies, however, have thus far not provided support for this attractive hypothesis (McChernelch and Brown, 1970, Lane and Finch, 1970).

Although most of the iron entering the plasma has been newly liberated from haemoglobin in reticulo-endothelial cells, there is also a contribution from storage compounds in these cells and in other tissues such as the liver parenchyma. There are two storage compounds; haemosiderin, an insoluble compound which is visible histologically as green granules when stained with prussian blue, and the soluble ferritin. Ferritin consists of a central core of six electron dense, closely associated iron clusters (Kerr and Muir, 1960; Bessis and Breton-Gorius, 1960), surrounded by a protein envelope. The protein shell is made up of about 20 identical polypeptide chain sub-units and has a molecular weight of 460,000 (Harrison, 1963). Ferritin from different species is immunologically specific (Mazur and Shorr, 1948); in addition there are electrophoretic differences between ferritins derived from liver, spleen and bone marrow (Alfrey

et al, 1967; Gabuzda and Gardner, 1967). By various techniques all the iron can be removed from ferritin without demonstrably damaging the protein shell. Lacking iron the protein is called apoferritin.

The iron is present for the most part as ferric hydroxide-ferric phosphate micelles and accounts for 17 to 23% of the dry weight (Granick, 1942). A small proportion of the iron is on the surface of the molecule, in the ferrous form, linked to sulfhydryl groups (Mazur et al, 1955). This iron is available for transfer to endogenous or exogenous chelates, and is in equilibrium with the larger centrally situated ferric pool. When the sulfhydryl groups are oxidized to disulphide linkages, the ferrous ions are also oxidized to the ferric form and become attached to the internally situated iron micelles. Sulfhydryl groups are essential for the binding of iron to ferritin. As iron-poor ferritin has more sulfhydryl groups than iron-rich ferritin, relatively more of the metal binds to the former than the latter (Mazur et al, 1960).

The mechanisms involved in the incorporation of plasma iron into ferritin have been studied in

vitro, using liver slices (Mazur et al, 1960), and in vivo, in the experimental animal (Mazur et al, 1961). The postulated mechanism involves interactions between adenosine triphosphate (ATP), ascorbic acid and a molecule of iron on transferrin. ATP stimulates the oxidation of ascorbic acid in the presence of iron. During this reaction the iron is reduced to the ferrous form, which dissociates from its binding site on transferrin, and is transferred to acceptor sulfhydryl groups on the ferritin surface. This process is energy dependant.

The entry of iron into a cell results in the stimulation of ferritin protein synthesis and in addition, stabilizes pre-existing ferritin and decreases its rate of degradation (Drysdale and Munro, 1966). Smith et al (1968) have shown that iron given orally or parenterally in a single dose stimulates ferritin synthesis in the rat intestinal mucosa. In iron-loaded rats, rates of ferritin synthesis remain persistently higher than in normal or iron deficient rats (Cumming et al, 1970). Furthermore, rates of ferritin synthesis appear to vary proportionally to the amount of iron

given as an oral iron load (Bernier et al, 1970). Studies of iron-induced ferritin synthesis have shown remarkably similar results in the liver parenchyma (Drysdale and Munro, 1966) and in the intestinal mucosa (Millar et al, 1970). Following a single parenteral dose of iron, the rate of incorporation of ^{14}C-labelled leucine into ferritin rapidly increases to a peak at 5 hours and falls to normal levels by 8 hours.

Mazur and his associates have also investigated some of the factors involved in the release of iron from ferritin. On the basis of animal (Mazur et al, 1958) and in vitro tissue studies (Green and Mazur, 1957), the enzyme xanthine oxidase may play a part. Induced hypoxia results in an increase of xanthine and hypoxanthine in the liver. In the presence of ferritin and under anaerobic conditions, hypoxanthine and xanthine are oxidized to uric acid by the dehydrogenase activity of xanthine oxidase. Ferric ferritin acts as an electron acceptor, thereby oxidizing xanthine oxidase, and the iron is reduced to the ferrous state when it becomes available for transfer out of

the cell. Confirmatory evidence for this hypothesis comes from the following in vivo observations:-
1) Haemorrhagic or traumatic shock increases both uric acid and plasma iron concentrations. 2) Administration of xanthine, hypoxanthine or inosine leads to increased release of iron and uric acid from the liver. 3) Total hepatic iron stores appear to be inversely related to xanthine oxidase levels (Mazur and Sackler, 1967). 4) Co-existant haemochromatosis and xanthinuria, a disease associated with a congenital absence of xanthine oxidase, has been reported (Ayvazian, 1964).
5) Finally, immature rats have increased storage iron concentrations and low levels of xanthine oxidase. As the concentration of the enzyme rises to that found in adult rats, the storage iron concentration falls (Mazur and Carleton, 1965).
While the above observations strongly suggest a central role for xanthine oxidase in the release of iron from ferritin, there is other evidence to the contrary. For example, a poor correlation between changes in body demands for iron and changes in measured xanthine oxidase activity has been reported

(Awai and Brown, 1969), and patients with xanthinuria do not necessarily show changes in iron metabolism (Seegmiller et al, 1964). In vitro other reducing agents, including ascorbic acid, cysteine and glutathione, can release iron from ferritin (Bothwell and Finch, 1962). The effects of allopurinol, a potent inhibitor of xanthine oxidase, on iron metabolism are controversial. Some studies indicate that it has no effect (Boyett et al, 1968; Green et al, 1968; Awai and Brown, 1969), but in others changes in iron metabolism have been reported. These include an increase in the rate of hepatic iron accumulation (Powell and Emmerson, 1966), and a decrease in the amount of desferrioxamine-chelatable iron (Hedenberg, 1969).

The second of the two storage forms of iron, haemosiderin, differs from ferritin in that it is insoluble and visible under the light microscope. Originally haemosiderin was erroneously assumed to result from excessive and rapid destruction of red cells (Cook, 1929), but there is now a good deal of evidence that it is derived partially or wholly from ferritin. Studies with the electron-microscope

reveal that the iron granules of haemosiderin have an appearance identical to those found in the ferritin molecule (Richter, 1958). Furthermore, in vitro oxidation and denaturation of ferritin results in granules with the characteristics of haemosiderin (Matioli and Baker, 1963). Unlike ferritin, however, haemosiderin is not homogeneous, and its iron, nitrogen and protein contents vary (Richter, 1960). It may contain porphyrins, other pigments and lipids (Shoden and Sturgeon, 1960). Matioli and Baker (1963) have postulated the following sequence of events in haemosiderin formation. Ferritin remains freely distributed in the cell cytoplasm until it is denatured by oxidizing agents. High concentrations of ferritin in the liver have been shown to inhibit the enzyme catalase. As a result, hydrogen peroxide, derived from the auto-oxidation of flavoproteins, accumulates. Hydrogen peroxide is a powerful oxidizing agent and may well lead to the conversion of ferritin to haemosiderin. The denatured molecules are sequestered in vacuoles and aggregate. Until the protein component of the molecule is destroyed by tryptic digestion, the granule will maintain a ferritinic

substructure. Removal of the protein envelope favours the polymerization and precipitation of the ferric micelles. This gives rise to the large amorphous iron complexes which characterize the non-ferritin forms of haemosiderin.

Normally the storage iron in a tissue is composed of equal amounts of ferritin and haemosiderin. When animals are loaded with iron, there is initially an increase in the ferritin. Later, however, haemosiderin is deposited while the ferritin stabilizes at a constant level (Shoden et al, 1953). Haemosiderin iron appears to be less easily mobilized from stores than ferritin iron (Shoden and Sturgeon, 1968).

The size of the body iron stores may be investigated during life by several means, but some are not suitable for routine clinical practice. These include estimates of the total iron available for new haemoglobin synthesis following vigorous phlebotomy (Haskins et al, 1952; Pritchard and Mason, 1964), determination of the miscible iron stores by radioisotope dilution techniques (Finch, 1959) and histological and chemical evaluation of

iron stores from liver biopsy specimens (Bothwell and Bradlow, 1960; Frey et al, 1968). Furthermore, while stainable marrow iron shows a good correlation with total iron stores (Gale et al, 1963), marrow aspiration has some limitations at a clinical level. The only two non-traumatic procedures which can be used on a wide scale are estimates of the plasma iron concentration and total iron binding capacity, and measurement of chelatable iron using the iron chelate desferrioxamine.

The total iron binding capacity correlates well with iron deficiency but cannot be used to assess iron overload (Weinfeld, 1970). The plasma iron concentration is not a good index of iron stores. It remains normal until all the storage iron has been mobilized (Bothwell and Finch, 1962), and although it is often elevated if the stores are excessive, it provides no clue to the severity of the siderosis. Furthermore, in the presence of ascorbic acid deficiency, the plasma iron may be only minimally elevated despite massive iron overload (Wapnick et al, 1970), and some degree of ascorbic acid deficiency is

frequently associated with severe siderosis (Seftel et al, 1966). In recent years desferrioxamine has assumed an important place in the assessment of iron stores. Studies on urinary iron excretion following a standard dose of desferrioxamine indicate that an accurate measure of the size of the iron stores may be obtained in this way (Hallberg, et al, 1966). This approach is discussed in greater detail in chapter 4.

The immediate source of the iron in the major storage depots of the body differs. The liver parenchymal cell acquires iron from transferrin. There are species variations in the percentage of the daily plasma iron turnover which enter the liver. In rats it is about 15% (Cheney et al, 1967), but the figure is considerably less in normal humans (Hosain and Finch, 1966). The proportion is, however, increased in conditions associated with a high plasma iron and low unsaturated iron binding capacity (Hosain and Finch, 1966). Little is known of the day to day exchange between transferrin and hepatic parenchymal cells, but a mechanism for mobilization of hepatic storage iron exists, and

these stores have been shown to be readily available for haemopoiesis after bleeding (Finch and Finch, 1955).

The cells of the reticulo-endothelial system obtain most of their iron from catabolized haemoglobin, either from senile erythrocytes or from wastage during erythropoiesis. Some haemoglobin iron leaves the developing red cell with extrusion of its nucleus, and some structurally abnormal cells with a short life span are phagocytosed (Cook et al, 1970). A small amount of haemoglobin bound to haptoglobin is cleared daily by reticulo-endothelial cells, but this accounts for only a minute fraction of recircuited iron (Garby and Noyes, 1959a). There is in addition a small amount of free haem in the plasma. This is complexed either to albumin, or to a specific haem binding protein, haemopexin (Muller-Eberhard, 1970). In contrast to haemoglobin, haem is cleared by the liver parenchymal cells and may be involved in the regulation of haem synthesis in these cells.

The spleen is the major site for sequestration of circulating red cells (Crosby, 1959). Non-viable

erythrocytes are cleared exponentially from the circulation, and there is some evidence that the most severely damaged cells are preferentially removed (Noyes et al, 1960). Splenectomy in rats results in a slowed rate of clearance of acetyl-phenylhydrazine damaged cells (Azen and Schilling, 1963). The survival of the normal red cell depends on its ability to deform (Weed, 1970). This property enables cells with a diameter usually greater than 8 μ to pass through splenic channels with diameters less than 3 μ. With age, an alteration in the red cell membrane occurs; it becomes more rigid and thus the cell is trapped in the splenic sinusoids. Effete red cells have been shown to adhere readily to hepatic, splenic and alveolar macrophages (Lee and Cooper, 1966), but the mechanism whereby the phagocytes recognize and clear altered red cells is poorly understood. The initial phase of phagocytosis is thought to be the adhesion of the red cell to a receptor site on a macrophage (Rabinovitch, 1964). Contact between the two cells results in excitation of the macrophage membrane, and phagocytosis follows.

Kornfeld et al. (1969) have followed the intracellular pathway of sequestered haemoglobin in rat livers. Initially the haemoglobin is found predominantly in endocytic vacuoles known as phagosomes. These particles then merge with lysosomes and thus acquire lytic enzymes capable of degrading haemoglobin. The iron is released from haem and enters the cytoplasm. The nature of this in transit iron is unknown. It may then either leave the cell or be incorporated into ferritin. Very little is known of the factors which determine how much iron is released into the plasma and how much is diverted into the stores: this topic is examined in greater detail in chapter 2. There is no doubt, however, that considerable variation does occur, and that supply matches erythropoietic demand reasonably closely under most circumstances.

While transferrin is undoubtedly the principal acceptor of iron from the reticulo-endothelial system, there is some electron-microscopic evidence that reticulo-endothelial cells in the marrow can deliver iron in the form of ferritin directly to erythroblasts. Bessis and Breton-Gorius (1962) have shown that

developing red cell precursors tend to group around a reticulum cell, the "nurse-cell", and ferritin can be seen in the cytoplasm of both cells and in invaginations of the membrane (ropheocytosis). However, it is possible that the direction of ferritin movement is from the red cell precursor to the reticulum cell, and that excess iron is being excreted in this way. Some support for this interpretation is provided by the evidence that splenic reticulum cells can extract ferritin from siderocytes (Crosby, 1957). Even if iron does enter erythrocyte precursors in this way, quantitative considerations make it clear that it can constitute only a tiny fraction of haemoglobin iron.

The iron bound to transferrin is in the ferric form, whereas there is suggestive evidence that iron released from ferritin is in the ferrous form. If this is so, it must be oxidised before or during binding to the transport protein. Caeruloplasmin, which is present in the plasma, accelerates the in vitro oxidation of iron from the ferrous to the ferric form (Osaki et al, 1966; Osaki, 1966), and Cartwright and co-workers have provided elegant

evidence for the role of this enzyme in iron metabolism. In copper deficient swine the iron in intestinal mucosal cells, reticulo-endothelial cells and liver parenchymal cells is not given up to transferrin. In contrast, the ability of these cells to take up iron and to incorporate the metal into ferritin is not impaired (Lee et al, 1968). The defect in iron metabolism is corrected immediately by the injection of caeruloplasmin, but not by administration of equivalent amounts of copper (Ragan et al, 1969). This suggests that the enzyme itself (and not the copper) is responsible for iron release to transferrin. Measurement of plasma caeruloplasmin levels indicate that it must fall to below 1% of normal values before abnormalities of iron metabolism are noted (Roeser et al, 1970). This suggests that the enzyme subserves a permissive function, and may well not be involved in the day to day regulation of reticulo-endothelial iron release. It must be noted, however, that in pathological states such as haemolytic anaemias, the amount of caeruloplasmin required to maintain the elevated plasma iron turnover increases markedly.

Although oxidation of the iron is thus necessary before it can be taken up by transferrin, the preceding step seems to be a reduction, since ferritin iron is predominantly in the ferric form. There is some evidence that ascorbic acid may be involved at this stage. In siderotic Bantu of South Africa who develop scurvy, plasma iron levels are reduced. Administration of ascorbic acid to such subjects results in a prompt rise in plasma iron without an associated reduction in plasma iron turnover (Bothwell et al, 1964). The interrelationships between ascorbic acid and iron metabolism will be discussed further in Chapter 3.

It is apparent from this review of the current state of knowledge of iron metabolism in the reticulo-endothelial system that there is a fair degree of insight into certain aspects, but that much remains to be elucidated. The experiments described in this thesis were designed to gain further information about three particular areas of uncertainty, namely, the mechanisms involved in the variable release of liberated haemoglobin iron into the plasma, the source of iron chelated by desferrioxamine and the role of ascorbic acid in storage iron metabolism.

CHAPTER II

SOME FACTORS AFFECTING THE RELEASE OF IRON FROM RETICULO-ENDOTHELIAL CELLS

INTRODUCTION

As already indicated in Chapter 1, the major source of the iron entering the plasma pool is the haemoglobin from senile erythrocytes which have been engulfed by reticulo-endothelial cells, and its major destination is the erythroid marrow where it re-enters the red cell cycle. From the limited data available it would appear that under physiological conditions, little of the iron from catabolized haemoglobin exchanges with storage iron (Garby and Noyes, 1959b), and most of it is released. If, however, a larger load of non-viable erythrocytes is injected, more than half the iron remains within the reticulo-endothelial cells (Noyes *et al*, 1960).

The rate of entry of iron into the plasma varies under the influence of a number of factors. One of the most important of these is the rate of erythropoiesis. Venesection has been shown to increase iron release, so that even when large quantities of non-viable red cells are injected, almost all the iron from catabolized haemoglobin enters the circulation (Noyes *et al*, 1960). The supply of iron is thus adjusted to meet the requirements of the erythroid marrow, but the response of the

reticulo-endothelial system is not immediate. An increase in the rate of erythropoiesis following ascent to high altitudes is at first followed by a fall in the plasma iron concentration; within a few days, however, the rate of delivery of iron to plasma is accelerated, and the concentration returns to normal even though the plasma iron turnover is increased (Reynefarje et al, 1959). Furthermore, experiments with rats using an altitude chamber have shown that the plasma iron is considerably elevated 7 days after returning to normal atmospheric pressure (Weintraub et al, 1965). In patients with aplastic anaemia, a marked fall in plasma iron turnover is noted, which indicates that the reticulo-endothelial system can conserve iron not required for erythropoiesis (Hosain and Finch, 1966). The reticulo-endothelial system cannot, however, retain all the iron from catabolized haemoglobin. Even in aplastic anaemia half of it is released to the circulation, and a marked rise in plasma iron concentration results (Finch et al, 1970).

In pernicious anaemia, kinetic data reveal a plasma iron turnover which is 3 - 4 times normal.

Plasma iron levels are usually elevated (Finch et al, 1956), indicating that the reticulo-endothelial system supplies iron in amounts adequate to meet the demands of the hyperplastic marrow. Most of the new red cells are, however, destroyed before entering the circulation. With treatment, ineffective erythropoiesis ceases, and the major source of iron supply to the marrow is eliminated. This is reflected in a rapid fall both in plasma iron and plasma iron turnover in spite of the reticulocyte crisis. Thalassaemia affords an even more impressive illustration of the capacity of the reticulo-endothelial system to process haemoglobin iron (Finch et al, 1970). While the plasma iron turnover may be 10 times normal in this condition, plasma iron concentrations are usually elevated. From these data it would seem that the amount of iron released by the reticulo-endothelial system depends on the amount of iron entering the system. This has been confirmed experimentally. When increased loads of non-viable erythrocytes are administered to normal subjects, a proportional increase of reticulo-endothelial iron release is noted (Noyes et al, 1960).

Little is known about the factors controlling the entry of reticulo-endothelial iron into the

plasma. Following venesection, the increased marrow demand results in a fall in plasma iron, with a corresponding rise in percent transferrin saturation. Pollycove (1966) has proposed that it is this that stimulates increased iron release from stores until supply meets demand. As the anaemia becomes less severe, the rate of iron removal by the marrow decreases, the plasma iron rises and thus transfer from stores diminishes. This hypothesis has, however, as yet no experimental evidence to support it. Furthermore, saturation of circulating transferrin with ionic iron does not decrease the re-utilization of a load of non-viable erythrocytes given two hours later (Noyes et al, 1960).

There is little evidence that any other factors are involved. Most hormones probably do not directly alter iron handling by the reticulo-endothelial system. Adreno-corticotrophic hormone or cortisone do not alter plasma iron concentrations (Paterson et al, 1952). Plasma iron turnover is reduced in myxoedema and increased in thyrotoxicosis (Finch et al, 1970), but this may merely reflect the effect of thyroxin on erythropoiesis. Red cell production is reduced in myxoedema and increased in thyrotoxicosis, but

the rate of erythropoiesis is under the direct control of erythropoietin. The possibility that erythropoietin enhances reticulo-endothelial iron supply has been considered, but there is some evidence that it does not directly stimulate reticulo-endothelial iron release. In response to altitude induced hypoxia, the increase in plasma iron turnover lags well behind the rise in circulating erythropoietin levels (Faura et al, 1969). Furthermore, while plasma erythropoietin levels are usually elevated in aplastic anaemia (Lange et al, 1961), reticulo-endothelial iron release is markedly reduced.

A number of disease processes are accompanied by inefficient entry of iron into the plasma, as evidenced by a modest anaemia and a low plasma iron concentration in the face of normal or increased reticulo-endothelial iron stores (Cartwright and Wintrobe, 1952). Ferrokinetic data in chronic infections, malignancy and rheumatoid arthritis, are remarkably similar (Bush et al, 1956; Chodos et al, 1956; Freireich et al, 1957a). Evidence of impaired reticulo-endothelial iron release has

been obtained, both in animals (Freireich *et al*, 1957b) and in man (Hourani *et al*, 1963; Hourani *et al*, 1965). While restricted iron supply may well be the prime defect in these anaemias, other abnormalities have been noted. These include a shortened red cell survival due to an extra-corpuscular factor (Freireich *et al*, 1957a), and an inadequate erythropoietin response (Gutnisky and Van Dyke, 1963). Cartwright (1966) believes that relative erythropoietin lack results in a failure of the marrow to compensate for the shortened red cell survival. He reasons that once the anaemia has reached a steady state, reticulo-endothelial iron release is again appropriate, in that output equals the input from a reduced red cell mass. He believes that the experimental results indicating impaired iron release may be explained on the basis of dilution of the radioactive label in the enlarged intra-cellular iron pool. It must be noted however, that direct estimates of erythropoietin levels have not been made in these disorders, and that all the features of the anaemia may be explained on the basis of relative iron deficiency due to impaired reticulo-endothelial release.

While there is thus some doubt that iron release is impaired in these chronic diseases, it is clear that in acute infections the reticulo-endothelial iron supply is markedly diminished. In man, hypoferraemia develops early in the course of febrile illnesses (Cartwright and Wintrobe, 1952). Administration of small doses of endotoxin to rats causes a rapid fall in both plasma iron and plasma iron turnover (Kampschmidt and Arrendondo, 1960). Soon after turpentine abscess formation, rats show impaired re-utilization of catabolized haemoglobin iron. In addition, the rate of sequestration of non-viable erythrocytes by the reticulo-endothelial system is increased (Quastel and Ross, 1966). The reasons for these alterations in reticulo-endothelial function are, however, not understood.

Because of the paucity of information about the factors involved in the regulation of iron supply from the reticulo-endothelial system to the erythroid marrow via the plasma pool, the studies described in this chapter were undertaken. The experiments were performed with rats, and reticulo-endothelial function was assessed by examining the release of ^{59}Fe from the liver and spleen after injecting heat-denatured erythrocytes containing ^{59}Fe-haemoglobin.

MATERIALS AND METHODS

Adult male rats of the Sprague-Dawley strain, weighing between 200 and 300g were used. In each study rats were matched for weight, the maximal difference being 25g. They were housed in cages containing a maximum of 6 animals, and received a diet of rat production pellets (Delmas Milling Company, Randfontein, South Africa) and tap water.

In some studies erythropoiesis was inhibited by hypertransfusion or stimulated by venesection. Rats were hypertransfused by intravenous injections of 4ml packed erythrocytes on the eighth and seventh day prior to starting the experiment. In other animals erythropoiesis was stimulated by removing 4ml blood from the retro-orbital plexus of veins on the sixth and fifth day before the start of the experiment. To ensure that significant changes in erythropoiesis had occurred, incorporation of transferrin-bound radioiron into circulating erythrocytes was measured in 5 hypertransfused, 5 normal and 5 venesected rats.

Radioiron bound to transferrin was prepared by incubating buffered plasma with tracer amounts of

$^{59}FeCl_3$ for 30 minutes at 37°C. The amount of $^{59}FeCl_3$ added was always less than the binding capacity of the plasma present. After 44 hours the animals were weighed and bled to death via the aorta. The packed cell volume and the radioactivity present in a 2ml sample of washed erythrocytes were determined. The mean (±SD mean) percentage red cell utilization of radioiron (calculated on the assumption that the blood volume was 50ml/kg body weight in normal and venesected animals and 60ml/kg body weight in hypertransfused animals) was 9(±4)%, 75(±8)% and 95(±3)% in hypertransfused, normal and venesected animals respectively. The mean (±SD mean) packed cell volumes were 63(±3)%, 51(±2)% and 33(±2)%.

Erythrocytes containing (^{59}Fe)-haemoglobin were obtained from a rat given weekly injections of 50 µc ^{59}Fe intraperitoneally in the form of ferrous ascorbate. The radioactive solution was prepared by adding the $^{59}FeCl_3$ to 1ml sterile isotonic saline containing 100mg ascorbic acid. Immediately prior to the start of the experiment blood was obtained in heparin from the retro-orbital

plexus of veins. After centrifugation and aspiration of plasma the erythrocytes were washed in sterile isotonic saline and then suspended in four times their original volume of a 1:1 mixture of isotonic saline and ACD (5g citric acid, 13.89g sodium citrate and 13.9g dextrose per litre of solution). They were denatured by heating for 25 minutes at 40°C.

Once the erythrocytes had been damaged, the haemoglobin concentration was determined so that the amount of iron could be calculated. The cell suspension was then diluted in isotonic saline, and each experimental animal received in 1ml the required amount of haemoglobin iron. Cells damaged in this way were taken up approximately equally by the liver and spleen. The temperature influenced the site of sequestration of the damaged erythrocytes; when cells were heated to 49°C a much greater percentage was cleared by the liver. At this temperature varying degrees of haemolysis were noted and a significant percentage of radioactivity was lost in the urine. Even when a standard procedure was followed, however, the relative proportions taken up by the two organs varied. In some experiments

more radioactivity was cleared by the spleen, in others more by the liver. The reasons for this variation were not elucidated.

In some studies injections of denatured unlabelled erythrocytes were given to rats before the cells containing ^{59}Fe. The unlabelled cells were treated in the same way as the radioactive erythrocytes. In other studies iron was injected intravenously in the form of iron-nitrilotriacetic acid (NTA-iron). Nitrilotriacetic acid was dissolved in isotonic saline adjusted to pH 9.0 with solid sodium bicarbonate, and the pH was then lowered to 3.0 with concentrated hydrochloric acid. Iron was added as $FeCl_3$ in a molar ratio of iron to NTA of 1:2, and the pH finally raised to 7.0 with sodium bicarbonate. The concentration of iron was adjusted to 100 µg/ml.

Rats received intravenous injections via the tail veins. A Packard whole-body Armac counter was used to measure the total amount of radioactivity in the animals. At timed intervals groups of animals were killed by bleeding from the aorta under ether anaesthesia. Their livers and spleens were removed

and cooled rapidly in crushed dry ice. The radioactivity in the organs was determined and expressed as a percentage of ^{59}Fe injected. They were stored at - 20°C until fractionation was performed.

The stored liver and spleen from each animal were thawed and homogenized together in four times their weight of distilled water. To ensure minimal geometric errors all samples were made up to the same volume, and the radioactivity was assessed by means of a well-type scintillation counter. Total radioactivity present in the combined liver and spleen was calculated from a 2ml aliquot of the homogenate.

^{59}Fe in ferritin was determined by heating a 4ml aliquot of the homogenate to 80°C for 10 minutes (Drysdale and Munro, 1965). When cool, the sample was centrifuged at 1,500 X g and an equal volume of saturated ammonium sulphate was added to the supernate. After standing at 0°C for two hours to allow complete precipitation, it was centrifuged and the radioactivity in the precipitate was determined.

The method was checked by determining the recovery of labelled ferritin which had been added to a non-radioactive rat liver homogenate. The (^{59}Fe)-ferritin was prepared by the above method from the liver of a rat killed one hour after 30 µg iron as ferrous sulphate labelled with 10 µc $^{59}FeCl_3$ had been injected intravenously. The mean (±SD mean) percentage recovery in 5 experiments was 91(±3)%. The possibility that (^{59}Fe)-haemoglobin might contaminate this fraction was examined by adding (^{59}Fe)-haemoglobin to a non-radioactive liver homogenate. (^{59}Fe)-haemoglobin solution was obtained by lysing in ice-cold distilled water washed erythrocytes labelled in vivo, and removing the stroma by centrifugation. In 5 experiments a mean (±SD mean) of only 0.75(±0.2)% of the radioactivity was found in the ferritin fraction.

As a final validation, the method was compared with the immunological isolation of ferritin. An antiserum to crystalline horse spleen ferritin was prepared in rabbits by the method of Mazur and Shorr (1948). This antiserum cross-reacts with rat liver ferritin (Mazur et al, 1960), and 0.5ml

was found to precipitate 190 ug ferritin iron. Two rats were killed 24 hours after an intravenous injection of transferrin-bound radioiron. The livers were removed and 4ml aliquots of the homogenate from each organ were transferred to 10 test tubes. The heat supernates were obtained as described above, and the radioactivities determined. An equal volume of saturated ammonium sulphate was added to half the tubes and 1ml of ferritin antiserum was added to the remainder. The former were kept at $0^{o}C$ for 2 hours and the latter incubated at $37^{o}C$ for one hour. After centrifuging the radioactivities of the precipitates were measured. By the ammonium sulphate method the mean ($\pm$SD mean)% labelled ferritin in the livers of the two rats was 54($\pm$4)% and 63($\pm$6)%, respectively, while by the antiserum technique the figures were 52($\pm$3)% and 65($\pm$4)%. It was concluded that the ammonium sulphate method gave very similar results to the specific anti-serum. Furthermore, while other heat stable proteins exist in rat liver homogenates, these must contain minimal, if any, radioiron and could be ignored.

Organ radioactivity in the form of haemoglobin was determined by the method of Gale et al (1963).

5ml saturated sodium pyrophosphate and 5ml 20% trichloracetic acid were added to a 1ml aliquot of the original homogenate. After heating to 80°C for 10 minutes and cooling, radioactivity in the washed precipitate was determined.

The method was checked by determining the recovery of (^{59}Fe)-haemoglobin added to non-radioactive liver homogenate. Mean ($\pm$SD mean) percentage recovery from 5 samples was 95($\pm$2)%.

In order to discover whether this fraction was significantly contaminated with ferritin, the above procedure was repeated, but labelled ferritin was substituted for (^{59}Fe)-haemoglobin. A mean ($\pm$SD mean) of only 0.4($\pm$0.1)% of ferritin radioiron was found in the haem precipitate.

Serum iron concentrations were measured by the method of Bothwell and Mallet (1955) and unsaturated iron binding capacities by that of Herbert et al (1967).

The rate of clearance of heat damaged erythrocytes labelled with (^{59}Fe)-haemoglobin or of transferrin-bound radioiron was assessed in some experiments.

When transferrin-bound radioiron was injected, serum rather than plasma was used as it was found that heparin tended to cause excessive blood loss. 0.2ml blood was removed from the retro-orbital plexus of veins exactly 10, 20 and 30 minutes after injection. The animal was anaesthetized with ether and the blood removed by gently inserting a capillary tube behind the eye. Once the required amount of blood had been obtained, the tube was removed and excessive bleeding prevented by applying firm digital pressure to the orbit. At 60 minutes the animal was bled to death, and the radioactivity present in plasma and in washed erythrocytes determined. All samples were made up to 2ml with isotonic saline prior to counting. The half time of clearance of injected radioactivity was obtained by plotting the results semi-logarithmically.

RESULTS

UPTAKE OF DENATURED ERYTHROCYTES AND RELEASE OF IRON IN NORMAL RATS.

Heat-denatured ^{59}Fe-labelled erythrocytes containing 50 µg iron as haemoglobin were injected intravenously into five rats, and the rate of disappearance was followed. The mean half-disappearance time (± SD mean) was 18(± 1) minutes. The subsequent fate of the ^{59}Fe was followed in 40 further rats (Table I). One hour after injection a mean (± SD mean) of 87(± 4)% of the injected radioactivity was in the liver and spleen. The ^{59}Fe was equally distributed between the two organs, mean figures (± SD mean) being 43(± 8)% in the liver and 44(± 9)% in the spleen. At this time 78(± 7)% was still in the form of haemoglobin, while 5(± 2)% had already been converted into ferritin (Fig 1). Four hours after injecting the denatured erythrocytes, the mean percentage (± SD mean) of the ^{59}Fe still present in the liver and spleen was 50(± 7)%, with 24(± 5)% in the liver and 26(± 4)% in the

TABLE I

UPTAKE, RELEASE AND INTRA-CELLULAR DISTRIBUTION OF ^{59}Fe FOLLOWING THE INJECTION OF DENATURED ERYTHROCYTES CONTAINING 50 µg HAEMOGLOBIN IRON LABELLED WITH ^{59}Fe

Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)					µg/100ml	
	Liver and spleen	Ferritin	Haemoglobin	Liver	Spleen	Serum Iron	UIBC
1	87(±4)	5(±2)	78(±7)	43(±8)	44(±9)	182(±28)	262(±32)
4	50(±7)	25(±6)	14(±4)	24(±5)	26(±4)	272(±98)	160(±55)
24	41(±4)	26(±5)	11(±4)	19(±2)	22(±4)	150(±28)	306(±21)

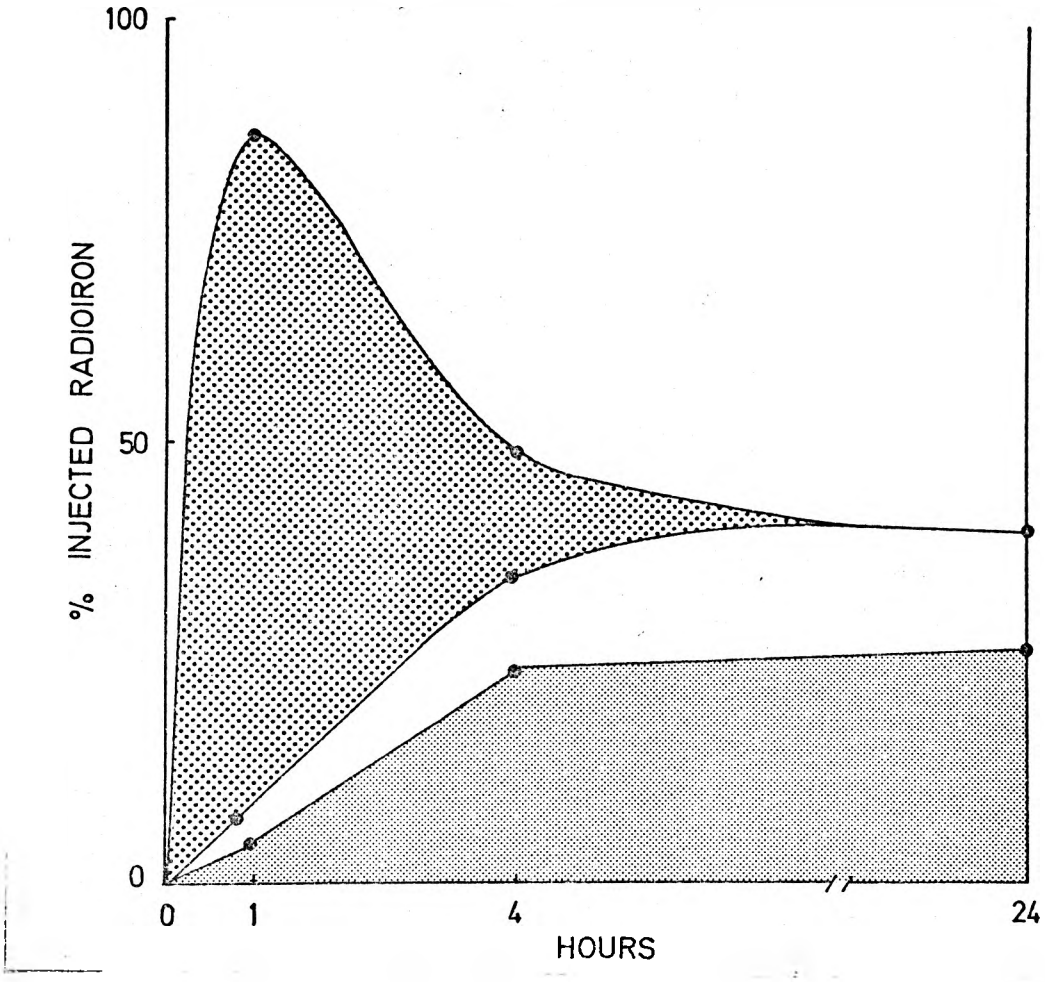

Figure 1. Distribution of ^{59}Fe in combined livers and spleens at various times after injecting denatured erythrocytes containing (^{59}Fe)-haemoglobin: Stippled, haemoglobin; shaded, ferritin.

spleen. Only 14(± 4)% was still in the form of haemoglobin, while 25(± 6)% was in ferritin. Approximately 37% had therefore been released between one and four hours. The mean serum iron concentration had risen to 272(± 98) µg/100 ml from 182(± 28) µg/100 ml at one hour. The rate of release of ^{59}Fe during the succeeding 20 hours was considerably slower, since 24 hours after injecting the labelled red cells the mean radioactivity (± SD mean) present in the two organs was 41(± 4)%. In subsequent studies attention was therefore focussed on the early phase of major release of labelled iron between one and four hours.

The effect of varying the load of denatured erythrocytes was then examined in groups of eight rats each (Table II). The pattern was remarkably similar whether the amount of labelled iron in the haemoglobin was 10 µg, 25 µg, 50 µg or 100 µg (Fig 2). Although the percentage of injected ^{59}Fe present in the two organs after one hour was slightly less with 10 µg than with 100 µg the means (± SD mean) being 83(± 4)% and 94(± 4)%

TABLE II

EFFECT OF VARYING THE LOAD OF DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN ON THE DISTRIBUTION OF INJECTED RADIOIRON

Quantity of haemoglobin iron in labelled cells	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)					µg/100ml	
		Liver & spleen	Ferritin	Haemoglobin	Liver	Spleen	Serum Iron	UIBC
10 µg	1	83(±4)	14(±4)	71(±12)	47(±4)	36(±5)	127(±10)	348(±41)
	4	47(±4)	35(±5)	22(±1)	22(±7)	25(±6)	152(±19)	313(±52)
	Released	36			25	11		
25 µg	1	89(±6)	8(±2)	83(±4)	40(±9)	49(±11)	170(±20)	293(±71)
	4	51(±5)	36(±6)	13(±7)	20(±4)	31(±4)	192(±42)	259(±50)
	Released	38			20	18		
50 µg	1	87(±2)	10(±4)	80(±9)	44(±3)	43(±4)	176(±25)	230(±32)
	4	50(±2)	24(±6)	12(±3)	30(±3)	20(±6)	261(±32)	172(±21)
	Released	37			14	23		
100 µg	1	94(±4)	7(±2)	74(±8)	46(±9)	48(±9)	207(±13)	236(±43)
	4	60(±6)	25(±3)	22(±9)	28(±5)	32(±4)	364(±9)	81(±59)
	Released	34			18	16		

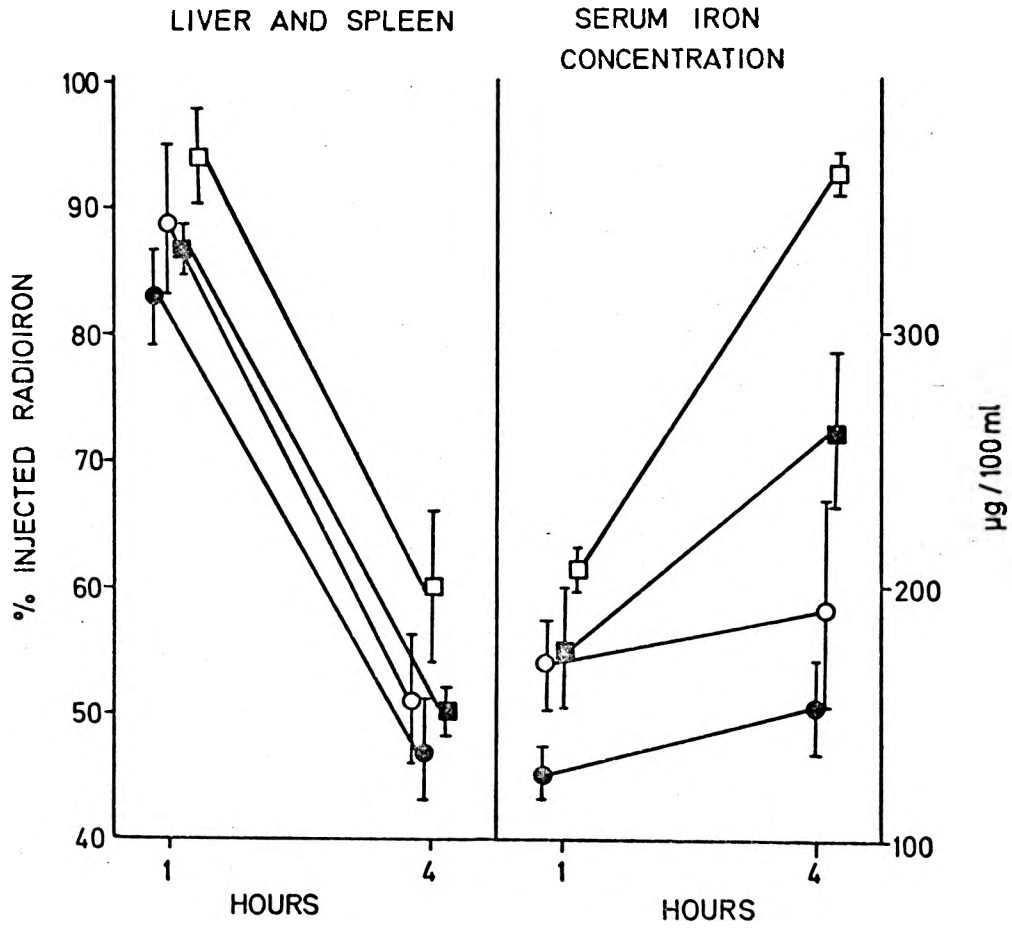

Figure 2. Effect of varying the load of denatured labelled erythrocytes upon the release of iron from hepatic and splenic reticulo-endothelial cells. Mean values (± SD mean) in rats given denatured erythrocytes containing 10/ug (closed circles), 25/ug (open circles), 50/ug (closed squares) and 100/ug (open squares) ^{59}Fe.

respectively, the rates of release of ^{59}Fe between one and four hours was virtually identical. At four hours the mean percentages of radioactivity ($\pm$ SD mean) still present in the organs were 47($\pm$ 4)% with the 10 µg dose and 60($\pm$ 6)% with 100 µg, indicating that 36% and 34% respectively had been released. Although the amounts of radioactivity released were similar, the quantity of iron was naturally ten times as great with the bigger dose. This was reflected by the serum iron concentrations (Fig 2). One hour after injecting the cells containing 10 µg iron, the mean serum iron concentration ($\pm$ SD mean) was 127($\pm$ 10) µg/100 ml, and at four hours 152($\pm$ 19) µg/100 ml. The comparable figures after injecting 100 µg iron were 207($\pm$ 13) µg/100 ml and 364($\pm$ 9) µg/100 ml. Intermediate values were obtained with the 25 µg and 50 µg doses. No significant differences were noted in the rates of breakdown of labelled haemoglobin or in the percentages of ^{59}Fe in ferritin with the different loads of labelled erythrocytes. After four hours between 12% and 22% was still present as haemoglobin, while 24 - 35% was in ferritin.

EFFECTS OF HYPERTRANSFUSION AND OF VENESECTION.

Uptake and release were compared in 18 hyper-transfused, 18 venesected and 24 control rats (Fig 3). A third of the animals in each group were killed one hour after injecting the labelled denatured erythrocytes. At this time the mean percentages of injected radioactivity present in the combined livers and spleens were virtually identical in each group, but more was in the spleens and less in the livers of both the venesected and the polycythaemic rats than in the normal animals (Table III). Half the remaining rats from each group were killed at four hours. In all the animals the amount of ^{59}Fe in the spleens had decreased, but the fall was greater in the venesected animals than in the other two groups, which were very similar. Radioiron had also been released from the livers of the normal and venesected rats, but the percentage in the livers of the hypertransfused animals had increased by 16% which matched the fall in splenic activity almost exactly. The mean percentages of injected radioactivity present

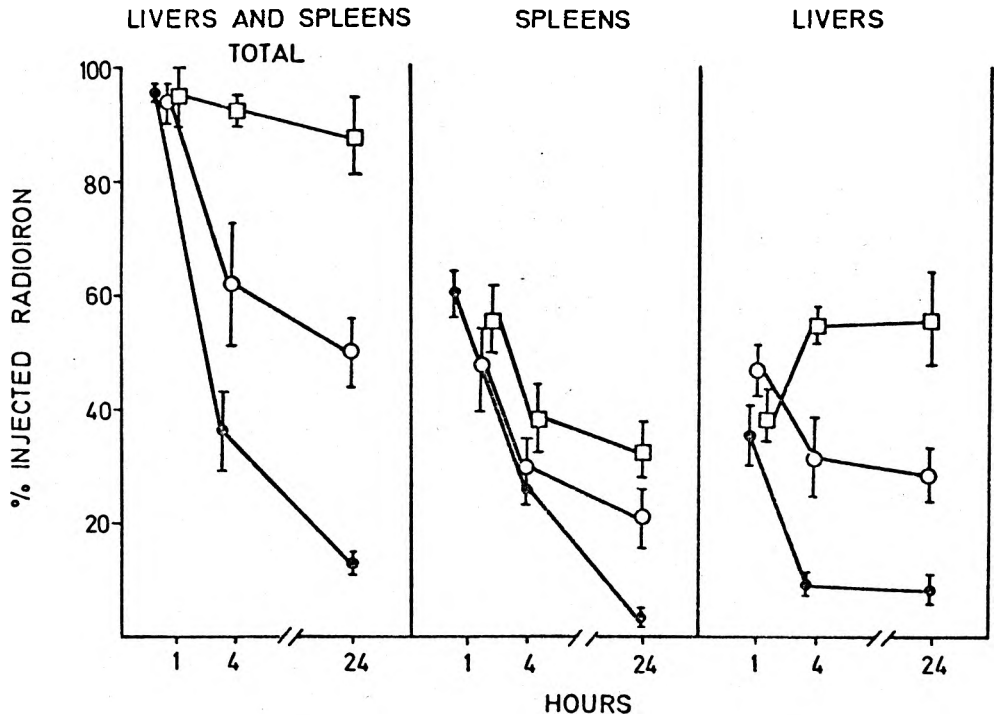

Figure 3. Effects of venesection and hypertransfusion upon the release of iron from hepatic and splenic reticulo-endothelial cells. Mean values (± SD mean) in normal (open circles), venesected (closed circles) and hypertransfused (open squares) rats.

TABLE III

EFFECTS OF VENESECTION AND HYPERTRANSFUSION UPON DISTRIBUTION OF ^{59}Fe AFTER INJECTING DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)					µg/100ml	
		Liver & spleen	Ferritin	Haemoglobin	Liver	Spleen	Serum Iron	UIBC
NORMAL	1	94(±3)	8(±2)	81(±9)	47(±4)	47(±7)	205(±48)	232(±34)
	4	62(±11)	35(±8)	12(±4)	32(±7)	30(±5)	203(±62)	136(±51)
	24	50(±6)	30(±5)	6(±2)	29(±4)	21(±5)	97(±21)	281(±20)
VENESECTED	1	96(±1)	3(±1)	90(±8)	36(±5)	60(±4)	80(±21)	291(±17)
	4	36(±7)	5(±1)	26(±8)	10(±2)	26(±3)	97(±18)	291(±25)
	24	13(±2)	1(±0.5)	8(±3)	9(±2)	4(±1)	46(±7)	315(±29)
HYPERTRANSFUSED	1	95(±5)	10(±2)	82(±5)	39(±5)	56(±6)	231(±40)	51(±13)
	4	93(±2)	62(±2)	10(±4)	55(±3)	38(±4)	236(±20)	34(±5)
	24	88(±7)	65(±4)	4(±2)	56(±8)	33(±5)	231(±69)	56(±10)

in the combined livers and spleens in the form of ferritin at four hours were markedly different in each group. Only a mean of 5% was in this form in the venesected rats, compared with 62% in the hypertransfused rats and 35% in the controls. In the venesected animals a mean of 26% of the injected radioactivity was still present as haemoglobin, compared with only 10% in the hypertransfused and 12% in the control animals. After 24 hours these figures were all below 10%, so that the differences between the four hour figures could not be ascribed to the presence of labelled reticulocytes in the liver and spleen.

After 24 hours all the remaining animals were killed. At this time there had been virtually no further changes in the amounts of ^{59}Fe present in the livers of all three groups. In the spleens, on the other hand, there had been a considerable further decrease (22%) in the case of the venesected animals, and a smaller fall in the controls (9%) and in the hypertransfused animals (5%). Almost none of the ^{59}Fe in

the combined livers and spleens was in the form of ferritin at this time in the venesected animals, compared with a mean of 65% in the hypertransfused rats and 30% in the controls.

EFFECTS OF PRIOR LOADING WITH IRON IN VARIOUS FORMS.

Denatured erythrocytes.

Heat-denatured erythrocytes containing 100 µg unlabelled iron as haemoglobin were injected intravenously into six groups of 12 animals each at various times up to 24 hours before the ^{59}Fe-labelled cells. Half the animals in each group were killed after one hour and the remainder after four hours. The percentage of injected radioactivity present in the combined livers and spleens at one hour was not significantly altered by injecting the unlabelled cells beforehand (Table IV), but the distribution between spleen and liver varied in the different groups. In the control group a mean of 30% was in the spleen and 56% in the liver (Fig 4). The maximum deviation from this pattern was in the group given the unlabelled erythrocytes three hours

TABLE IV

EFFECT OF UNLABELLED DENATURED ERYTHROCYTES INJECTED AT VARIOUS TIMES BEFORE DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Timing of first injection before labelled cells injected	Hours after injecting labelled cells	Mean % Injected Radioiron (± SD)					µg/100ml	
		Liver & Spleen	Ferritin	Haemo-Globin	Liver	Spleen	Serum Iron	UIBC
Control	1	86±5	9±2	76±10	56±3	30±6	217±32	224±19
	4	50±6	28±9	14±3	32±5	18±4	286±32	191±14
	Released	36			24	12		
2	1	97±3	13±3	89±10	65±5	32±5	357±28	46±3
	4	62±5	35±4	14±6	39±2	23±5	298±9	88±9
	Released	35			26	9		
3	1	93±6	9±3	80±12	70±5	23±8	318±34	65±20
	4	61±3	37±5	20±7	40±4	21±3	327±30	129±62
	Released	32			30	2		
6 X	1	94±4	9±2	85±5	39±3	55±7	229±42	183±22
	4	72±3	47±4	14±3	38±2	34±4	234±27	190±16
	Released	22			1	21		
9	1	87±4	16±6	66±6	56±8	31±12	134±40	260±22
	4	65±4	43±5	11±4	42±5	23±3	143±14	253±34
	Released	22			14	8		
12	1	89±9	16±3	69±6	54±4	35±3	148±19	309±30
	4	61±3	39±2	5±2	38±6	23±4	225±32	260±16
	Released	28			16	12		
24	1	96±3	13±4	77±10	66±4	30±2	152±36	315±41
	4	56±6	35±8	6±4	35±8	21±6	194±34	239±43
	Released	40			31	9		

X The labelled erythrocytes in this group were obtained from a different rat.

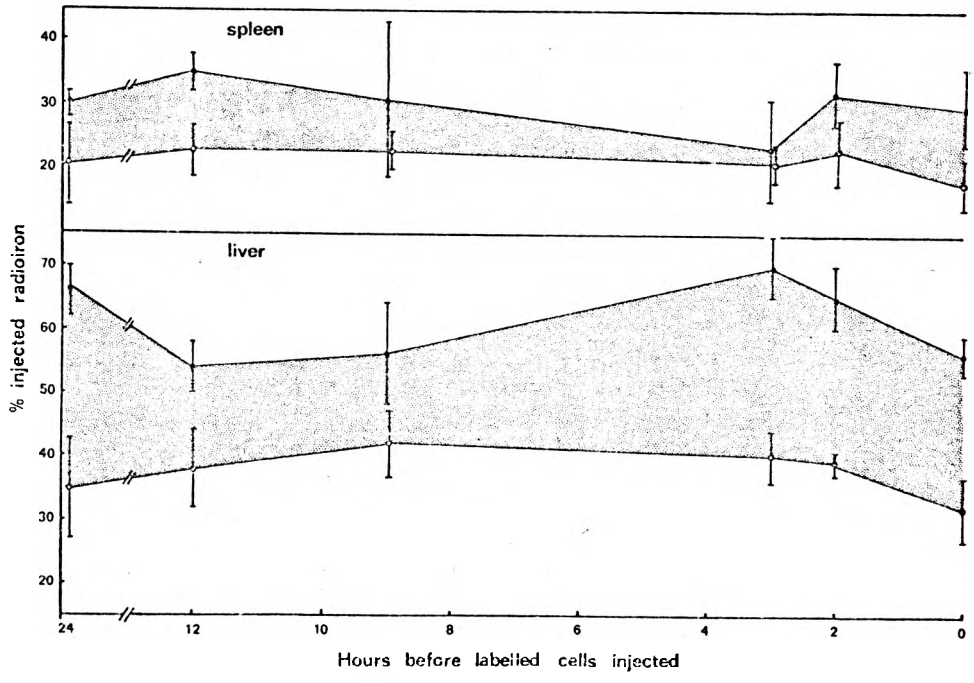

Figure 4. Effect of injecting unlabelled heat damaged erythrocytes at various times before the labelled erythrocytes. Mean (± SD mean) percentages of injected ^{59}Fe in the individual livers and spleens one hour (closed circles) and four hours (open circles) after injecting the denatured erythrocytes containing (^{59}Fe)-haemoglobin. The shaded areas represent the release of radioiron from the two organs between one and four hours.

beforehand, splenic uptake being decreased to 23% and hepatic uptake increased to 70%. However, in the groups given the unlabelled cells 9, 12 and 24 hours beforehand, the pattern was approximately the same as in the controls. The mean serum iron concentration in the two-hour and three-hour groups were considerably higher than in the control animals, but in the 9-hour, 12-hour and 24-hour groups they were lower than in the controls.

In contrast to the situation at one hour, there were marked differences between the percentages of injected ^{59}Fe present in the combined livers and spleens of the different groups at four hours. The maximum effect was seen in the groups given the unlabelled denatured erythrocytes six and nine hours beforehand; means of 72% and 65% respectively were still present in the combined organs of these groups compared with 50% in the control group. Between one and four hours approximately 36% had therefore been released in the controls, compared with only 22% in the groups given the unlabelled erythrocytes six and

nine hours beforehand. The inhibition of release was not as marked if the interval between the unlabelled and the labelled cells was 12 hours, while in the 24-hour group the amount released was similar to that in the control group. The maximum inhibition of release from the liver and spleen individually did not occur in the same groups. Approximately 12% was released from the spleens of the control group, and this figure was only 2% in the group given the unlabelled cells three hours before the labelled cells. In this group hepatic release was approximately 30%, compared with 24% in the control group. Total release in the three-hour group was thus hardly altered. In the nine-hour group, on the other hand, hepatic release was 14% and splenic 8% (Fig 4). In the six-hour group, ^{59}Fe-labelled erythrocytes from a different donor had to be used because not enough blood could be obtained from a single donor animal; although total release in this group appeared to be in line with the findings in the other groups, the distribution of labelled erythrocytes between liver and spleen was quite different, and it was

interesting to note that inhibition of release was much more marked in the liver than in the spleen. This suggested that the degree to which release was inhibited was influenced by the size of the load of denatured labelled cells in each organ.

The percentages of injected radioactivity present as ferritin in the organs of the animals killed after four hours followed an inverse pattern to that of total release. In the six- and nine-hour groups, mean percentages of 47% and 43% respectively were in this form, compared with 28% in the control rats. The mean percentage still present as haemoglobin in the animals killed after 4 hours was lower in the 12-hour and 24-hour rats than in the control animals.

A second experiment was performed to discover whether there was any relationship between the extent to which the release of iron was inhibited and the load of denatured labelled erythrocytes. Two groups of 24 rats each were studied, the first being given cells containing 15 μg ^{59}Fe as haemoglobin and the second 100 μg.

Six hours beforehand, half the animals in each group were given unlabelled denatured erythrocytes containing 100 µg iron, while the remaining rats received isotonic saline. Half the rats in each of the four groups obtained in this way were killed after one hour. The percentage of injected radioactivity present in the combined livers and spleens was lower in the animals given the 15 µg dose of denatured labelled cells than in those given 100 µg (Table V). When the remaining animals were killed after four hours, however, it was apparent that release between one and four hours was remarkably similar in the two control groups. Release was inhibited in both groups which had been given the unlabelled cells six hours before the labelled cells; however, the inhibition was greater in the rats given the 15 µg dose of labelled cells (23%) than in those given the 100 µg dose (13%). The percentage in ferritin was also greater, and in both groups was greater than in the respective control groups. Splenic release was inhibited to a greater extent than hepatic release.

TABLE V

INHIBITION OF ^{59}Fe RELEASE BY UNLABELLED DENATURED ERYTHROCYTES INJECTED 6 HOURS BEFORE DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN.

EFFECT OF VARYING THE LOAD OF LABELLED ERYTHROCYTES.

Quantity of haemoglobin iron in labelled cells	Nature of injection 6 hours prior to labelled cells	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)			
			Liver & spleen	Ferritin	Liver	Spleen
15 µg	Saline	1	80(±3)	12(±4)	41(±6)	39(±2)
		4	39(±5)	20(±4)	22(±3)	17(±4)
		Released	41		19	22
	Unlabelled denatured cells	1	84(±2)	13(±2)	54(±3)	30(±3)
		4	66(±4)	44(±3)	42(±3)	24(±2)
		Released	18		12	6
100 µg	Saline	1	98(±3)	5(±1)	46(±4)	52(±3)
		4	57(±6)	20(±2)	28(±5)	29(±3)
		Released	41		18	23
	Unlabelled denatured cells	1	96(±3)	9(±1)	45(±5)	51(±3)
		4	68(±5)	34(±7)	29(±5)	39(±5)
		Released	28		16	12

A final experiment was undertaken to determine whether the enhanced iron release in venesected rats could be inhibited by the injection of unlabelled denatured erythrocytes six hours previously. Twelve venesected and 12 normal rats were used (Table VI). Approximately 34% was released in the normal rats compared with 53% in the venesected animals. In the animals given the unlabelled denatured erythrocytes 6 hours beforehand, release was reduced to 9% and 26% respectively. Splenic and hepatic release were inhibited to approximately the same extent.

NTA-Iron

Unlabelled NTA-iron in a dosage of 100 µg was injected intravenously into groups of eight rats at the same times as previously, and the same procedure was followed. The mean percentage of injected radioactivity present in the combined livers and spleens again varied only slightly, but the distribution of the labelled erythrocytes between the two organs was altered, on this occasion in the opposite direction to the previous experiment (Table VII). In the control group

TABLE VI

INHIBITION OF ^{59}Fe RELEASE BY UNLABELLED DENATURED ERYTHROCYTES INJECTED 6 HOURS BEFORE DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN:

EFFECT ON NORMAL AND VENESECTED RATS

	Nature of injection 6 hours prior to labelled cells	Hours after injecting labelled cells	Mean(range)% Injected Radioiron		
			Liver & spleen	Liver	Spleen
Venesected	saline	1	86(85-89)	36(29-44)	50(42-58)
		4	33(30-38)	10(9-12)	23(21-27)
		Released	53	26	37
	Unlabelled Denatured cells	1	72(69-78)	27(20-35)	45(30-49)
		4	46(45-49)	18(17-19)	28(26-30)
		Released	26	9	17
Normal	saline	1	78(65-85)	41(35-48)	37(29-49)
		4	44(38-48)	21(13-26)	23(22-25)
		Released	34	20	14
	Unlabelled Denatured cells	1	80(77-88)	35(24-40)	45(40-48)
		4	71(67-76)	28(26-32)	43(35-49)
		Released	9	7	2

TABLE VII

TABLE VII

EFFECT OF 100 µg NTA-IRON INJECTED AT VARIOUS TIMES BEFORE DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Timing of first injection - hours before labelled cells injected	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)					µg/100ml	
		Liver & spleen	Ferritin	Haemoglobin	Liver	Spleen	Serum Iron	UIBC
CONTROL	1	88(±3)	6(±2)	80(±9)	42(±9)	46(±7)	181(±29)	290(±60)
	4	52(±5)	27(±2)	13(±4)	27(±4)	25(±3)	250(±80)	165(±30)
	Released	36			15	21		
2	1	87(±6)	16(±3)	67(±7)	23(±4)	63(±7)	300(±49)	159(±136)
	4	67(±6)	40(±2)	10(±2)	22(±3)	44(±7)	227(±6)	193(±31)
	Released	20			1	19		
3	1	95(±3)	16(±2)	69(±3)	39(±4)	56(±1)	299(±30)	121(±20)
	4	84(±4)	49(±3)	11(±3)	33(±3)	51(±4)	219(±32)	218(±21)
	Released	11			6	5		
6	1	94(±6)	21(±4)	67(±14)	37(±6)	57(±4)	144(±21)	260(±32)
	4	81(±2)	48(±2)	11(±3)	34(±2)	47(±3)	149(±26)	271(±42)
	Released	13			3	10		
9	1	92(±1)	19(±3)	71(±2)	57(±7)	35(±7)	124(±20)	305(±16)
	4	70(±6)	43(±5)	14(±2)	44(±5)	27(±9)	182(±32)	272(±47)
	Released	22			13	8		
12	1	98(±3)	16(±2)	74(±7)	46(±3)	49(±5)	150(±16)	263(±23)
	4	71(±7)	36(±5)	13(±5)	35(±8)	36(±8)	193(±23)	233(±29)
	Released	27			11	13		
24	1	94(±3)	14(±7)	66(±13)	50(±5)	44(±4)	165(±18)	267(±19)
	4	55(±5)	27(±3)	11(±2)	29(±3)	26(±5)	244(±29)	209(±29)
	Released	39			21	18		

the radioactivity was distributed approximately equally between the two organs, whereas in the previous experiment there was almost twice as much in the liver as in the spleen. The serum iron concentrations were once again markedly elevated in the two-hour and three-hour groups, and depressed in the six-, nine- and twelve-hour groups.

At four hours there was more radioiron still present in the combined livers and spleens of all the groups given NTA-iron beforehand than in the control group, the only exception being the 24-hour group. The maximum inhibition of release was in the three-hour and six-hour groups (Fig 5). Hepatic release was virtually zero in the two-hour group, splenic release being unaltered at this time. In the other groups, however, release was inhibited to an approximately equal extent in each organ (Fig 6).

As in the previous experiment, the mean percentage of injected radioactivity present as ferritin in the animals killed after four hours

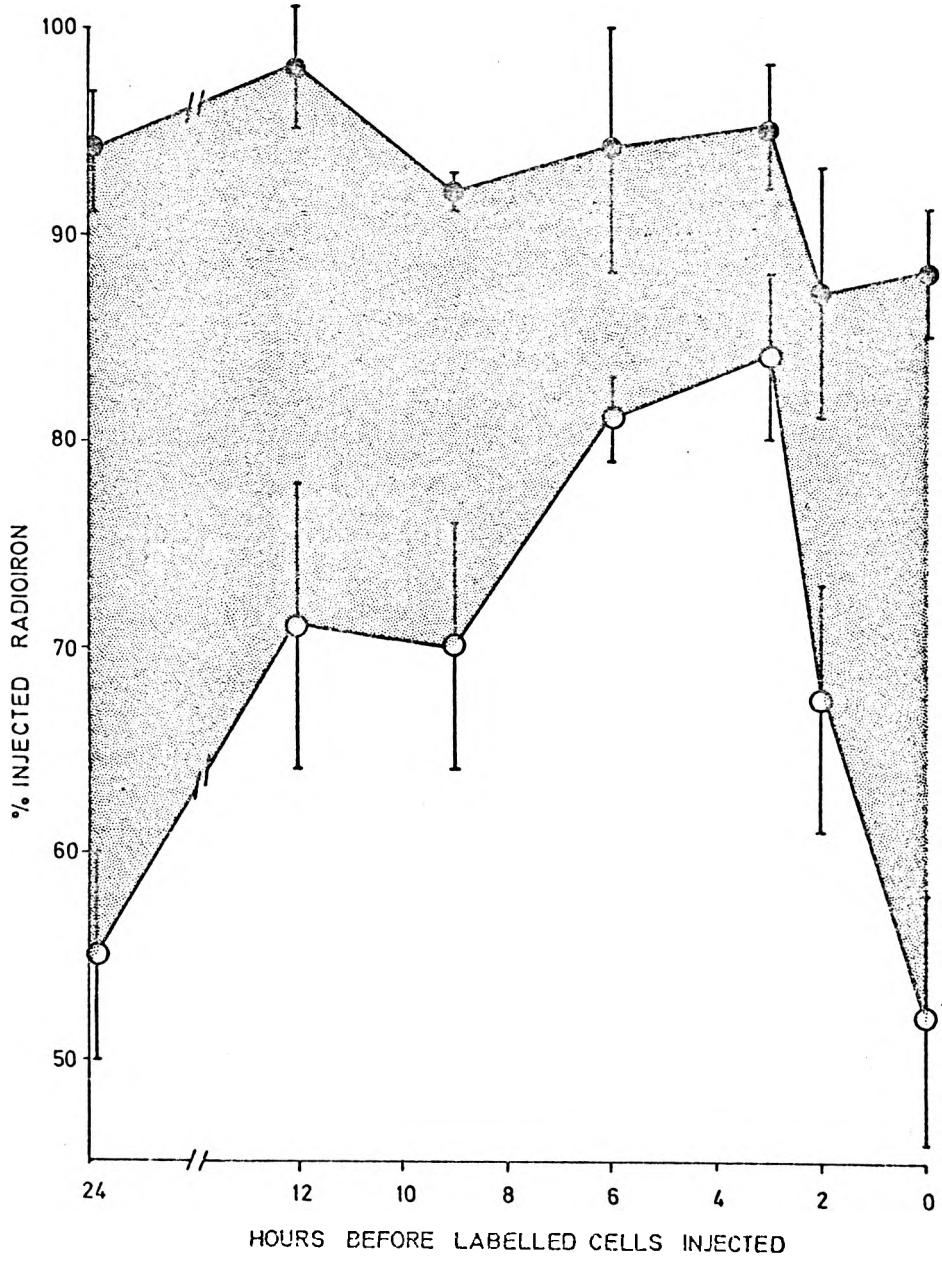

Figure 5. Effect of injecting 100 ug NTA-iron at various times before the denatured labelled erythrocytes. Mean (± SD mean) percentages of injected ^{59}Fe in the combined livers and spleens one hour (closed circles) and four hours (open circles) after injecting the denatured erythrocytes containing (^{59}Fe)-haemoglobin. The shaded area represents the release of radioiron between one and four hours.

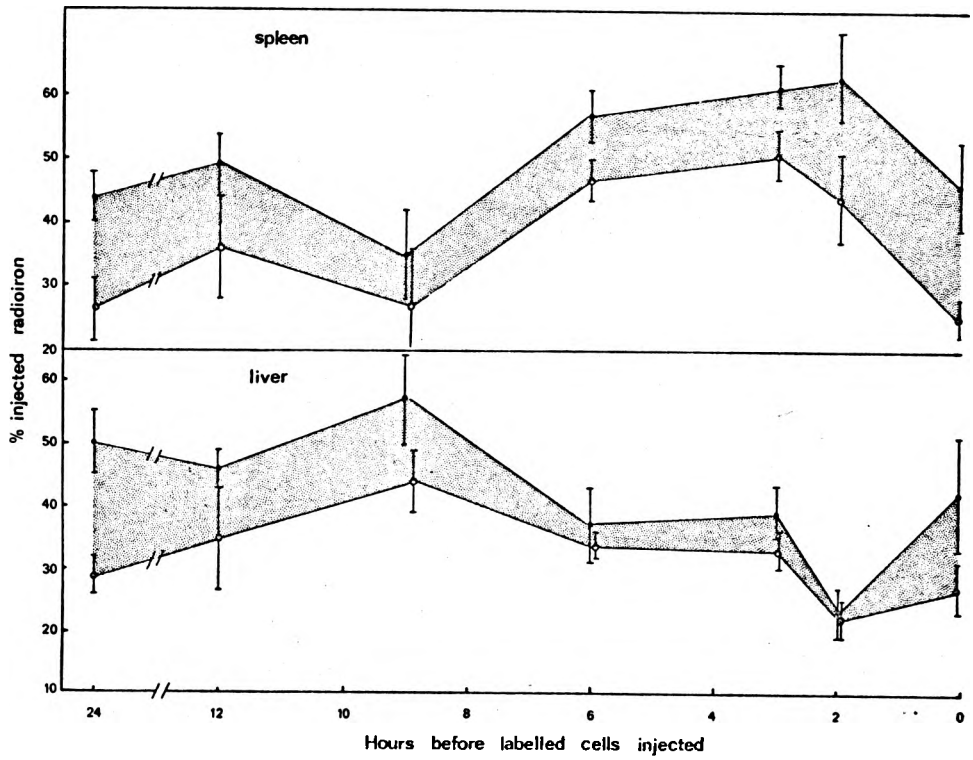

Figure 6. Effect of injecting 100 µg NTA-iron at various times before the labelled erythrocytes. Mean (±SD mean) percentages of injected ^{59}Fe in the individual livers and spleens one hour (closed circles) and four hours (open circles) after injecting the denatured erythrocytes containing (^{59}Fe)-haemoglobin. The shaded areas represent the release of radioiron from the two organs between one and four hours.

was greatest in those groups in which total release was maximally depressed (Fig 7). In this experiment, however, the percentage present as haemoglobin in the animals killed after four hours did not vary significantly.

A further experiment was then performed in which the amount of NTA-iron was reduced, so that most or all of it would be taken up by unsaturated transferrin. Initial experiments using rats weighing 300 g established that 35 µg iron as NTA-iron increased the serum iron concentration to a mean of 346 µg/100 ml (UIBC 59 µg/100 ml); one hour later the serum iron had fallen to a mean of 278 µg/100 ml, and a second injection of 12 µg iron as NTA-iron at this time raised it to a mean of 366 µg/100 ml. After a further hour this figure was 304 µg/100 ml. By labelling with ^{59}Fe, it was established that the distribution of the 35 µg NTA-iron in the blood and organs was the same as that of tracer amounts of radioiron incubated with rat plasma before injection, suggesting that all the injected NTA-iron had been bound by transferrin. In contrast, when 100 µg

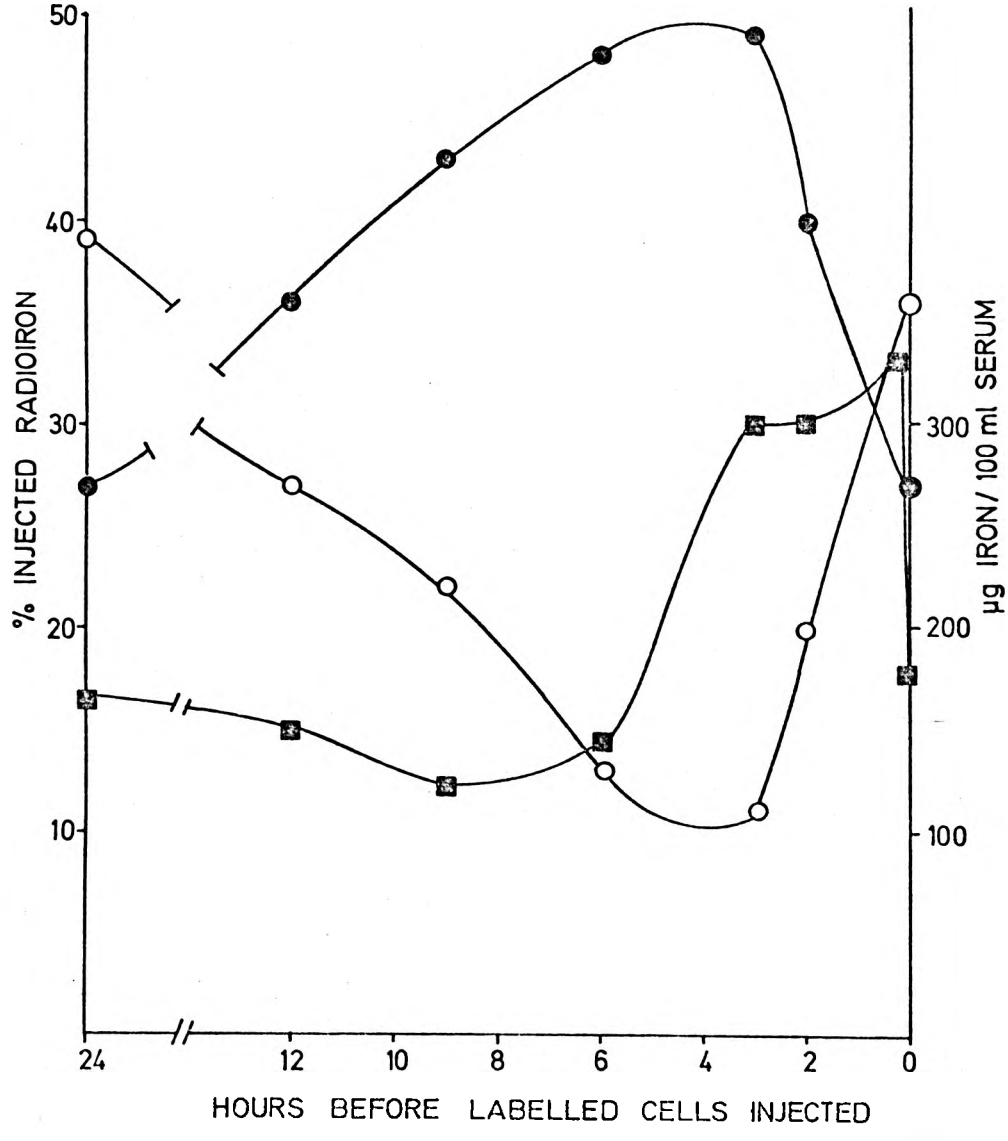

Figure 7. Effect of injecting 100 µg NTA-iron at various times before the denatured labelled erythrocytes on the percentage of injected ^{59}Fe released from reticulo-endothelial cells (open circles) and present in ferritin (closed circles) four hours after injecting the denatured labelled erythrocytes. Closed squares, serum iron concentrations one hour after injecting the denatured labelled erythrocytes.

TABLE VIII

THE DISTRIBUTION OF RADIOIRON AFTER INJECTING ^{59}Fe BOUND TO TRANSFERRIN AND TO VARYING AMOUNTS OF NTA ^{59}Fe

	Mean(range)% Injected Radioactivity		
Organ	100 µg NTA ^{59}Fe	35 µg NTA ^{59}Fe	^{59}Fe Transferrin
Liver	31.0(26.3-34.1)[X]	6.7(6.2-7.1)	9.0(8.1-10.3)
Spleen	1.1(0.9-1.3)	0.4(0.35-0.45)	0.4(0.3-0.5)
G.I.T.	9.1(7.4-10.5)	6.5(5.4-7.6)	3.8(3.6-4.9)
Kidney	4.8(4.5-5.1)	3.8(3.6-4.0)	1.9(1.6-2.3)
Heart	1.3(0.7-2.3)	1.2(1.0-1.5)	1.3(0.5-1.5)
Lung	1.9(1.4-2.7)	2.1(1.9-2.4)	2.9(2.0-4.1)
Blood	12.8(6.6-16.8)	42.5(41.0-44.3)	46.0(43.2-49.1)
Carcass	52.6(48.4-54.9)	29.0(27.0-31.8)	22.0(21.2-22.7)
Serum Iron (µg/100ml)	361(340-391)	346(321-363)	180(170-181)

[X] 3 animals/group, killed 15 minutes after the radioiron injection.

NTA-iron labelled with ^{59}Fe was injected, an increased percentage of radioactivity was located in the liver (Table VIII).

Sixteen rats were given injections of 35 µg and 12 µg NTA-iron six and five hours respectively before the denatured ^{59}Fe-labelled erythrocytes, while a further 12 received isotonic saline. Half the animals in each group were killed at one hour and the remainder at four hours (Table IX). At one hour the mean percentages ($\pm$ SD mean) of injected radioactivity present in the livers and spleens were 98($\pm$ 2)% and 96($\pm$ 3)% in the NTA-iron and control groups respectively. At four hours significantly less radioiron had been released from the NTA-iron group, the mean percentage ($\pm$ SD mean) still present in the two organs being 68($\pm$ 6)% compared with 59($\pm$ 7)% in the control rats (t_{12}, 2.69; $P < .02$).

A striking feature of the results of experiments in which denatured erythrocytes or NTA-iron were given prior to the labelled denatured red cells, was the association between impaired release of labelled cells and an increased percentage of

TABLE IX

INHIBITION OF ^{59}Fe RELEASE BY NTA-IRON: 35 µg INJECTED 6 HOURS AND 12 µg INJECTED 5 HOURS BEFORE DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Nature of injections 6 and 5 hours prior to labelled cells	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)[X]				µg/100ml	
		Liver & spleen	Ferritin	Liver	Spleen	S.I.	UIBC
35 µg NTA-Iron-6 hours. 12 µg NTA-Iron-5 hours	1	98(±2)	10(±3)	42(±8)	56(±8)	189(±29)	252(±74)
	4	68(±6)	41(±2)	30(±2)	38(±6)	268(±48)	157(±67)
	Released	30		12	18		
Saline - 6 hours	1	96(±3)	7(±3)	40(±7)	56(±8)	219(±25)	209(±64)
Saline - 5 hours	4	59(±7)	32(±6)	26(±3)	33(±6)	277(±30)	188(±68)
	Released	37		14	23		

[X] 6 animals/group - controls; 8 animals/group - experimental

labelled ferritin in the liver and spleen. It was therefore decided to examine the effects of inhibiting ferritin synthesis on reticulo-endothelial release. Such an inhibition has previously been achieved using cycloheximide (Chu & Fineberg, 1969), and preliminary studies were therefore carried out to establish the best time for administering it. It was found that cycloheximide in a dose of 200 mg intramuscularly inhibited the uptake of denatured labelled erythrocytes by the liver and spleen, haemoglobin catabolism and the release of ^{59}Fe, unless it was injected at least 15 hours before the erythrocytes (Table X). This time interval was therefore selected for an experiment to determine whether inhibiting ferritin synthesis would abolish the effect of a previous injection of iron.

Twelve rats were given cycloheximide and 24 isotonic saline (Table XI). Nine hours later the cycloheximide group and half the saline group were given 100 µg NTA-iron intravenously, while the remaining rats were given a second injection

TABLE X

EFFECT OF 200 µg CYCLOHEXIMIDE INJECTED INTRAMUSCULARLY AT VARIOUS TIMES BEFORE DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Hours before labelled cells	Injection before labelled cells	Mean % Injected Radioiron				
		Liver & Spleen			Ferritin	Haemoglobin
		1 hour	4 hours	Rel.	4 hours	4 hours
1[X]	cyclohexamide	43 (35-50)	73 (70-79)	-	20 (17-21)	35 (30-41)
	saline	91 (87-94)	62 (55-69)	29	23 (18-25)	16 (12-18)
6[X]	cyclohexamide	79 (74-83)	72 (65-78)	7	38 (32-42)	25 (14-35)
	saline	90 (85-92)	51 (47-55)	39	27 (23-30)	14 (10-18)
10[+]	cyclohexamide	85 (±8)	65 (±8)	20	-	-
	saline	92 (±4)	54 (±4)	38	-	-
15[+]	cyclohexamide	97 (±2)	54 (±6)	43	-	-
	saline	94 (±4)	46 (±3)	48	-	-
24[+]	cyclohexamide	90 (±6)	50 (±3)	40	-	-
	saline	94 (5)	50 (6)	44	-	-

X = 4 animals/group - range in parenthesis; + 6 animals/group - SD in parenthesis.

rel = released.

TABLE XI

INHIBITION OF ^{59}Fe RELEASE BY 100 µg NTA-IRON INJECTED 6 HOURS BEFORE DENATURED CELLS CONTAINING (^{59}Fe)-HAEMOGLOBIN:

EFFECT OF 200 µg CYCLOHEXIMIDE INJECTED INTRAMUSCULARLY 9HOURS BEFORE THE NTA-IRON

Nature of injection 15 hours before labelled cells	Nature of injection 9 hours before labelled cells	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)				
			Liver & spleen	Ferritin	Haemo-globin	Liver	Spleen
200 µg cyclohex-imide i.m.	100 µg NTA-Iron	1	92(±8)	8(±2)	64(±6)	38(±6)	53(±7)
		4	68(±6)	28(±2)	18(±3)	28(±3)	40(±7)
		Released	24			10	13
saline i.m.	100 µg NTA-Iron	1	91(±3)	8(±3)	66(±6)	39(±2)	52(±4)
		4	64(±5)	29(±4)	17(±5)	27(±6)	37(±4)
		Released	27			12	15
saline i.m.	saline	1	93(±4)	5(±2)	82(±5)	39(±6)	54(±7)
		4	59(±4)	29(±3)	16(±7)	23(±4)	36(±3)
		Released	34			16	18

of isotonic saline. After a further six hours all the animals received denatured labelled erythrocytes. Release was inhibited to the same extent in the rats given NTA-iron alone (7%) and NTA-iron plus cycloheximide (10%), and the percentages of injected radioiron present in ferritin were also virtually identical (28% and 29% respectively).

Ferritin

Preliminary experiments with ^{59}Fe-labelled ferritin revealed that it was rapidly and completely taken up by the liver after intravenous injection (See chapter 4). Rat liver ferritin containing 100 µg iron was injected intravenously into 12 normal rats, while a further 12 received injections of isotonic saline. Six hours later heat-denatured isotopically labelled rat erythrocytes were injected, and after a further hour half the animals in each group were killed (Table XII). The mean percentages of injected radioactivity (±SD mean) present in the combined livers and spleens were 94 (± 4)% in the control rats and 93 (± 2)% in those given ferritin. The remaining rats were killed at 3 hours, when a

TABLE XII

EFFECT ON THE DISTRIBUTION OF RADIOIRON OF 100 µg FERRITIN INJECTED 6 HOURS BEFORE DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Nature of injection 6 hours prior to labelled cells	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)[X]					µg/100ml	
		Liver & spleen	Ferritin	Haemo-globin	Liver	Spleen	S.I.	UIBC
100 µg Ferritin	1	93(±2)	4(±1)	86(±2)	39(±5)	54(±5)	156(±19)	334(±50)
	3	56(±6)	28(±4)	15(±2)	24(±3)	32(±4)	220(±52)	250(±60)
	Released	37			15	22		
Saline	1	94(±4)	4(±2)	86(±7)	40(±4)	54(±5)	197(±18)	216(±24)
	3	52(±3)	23(±7)	16(±5)	24(±5)	28(±3)	271(±44)	288(±42)
	Released	42			16	26		

[X] 6 animals/group.

mean (± SD mean) of 52(± 3)% was in the livers and spleens of the control group, 23(± 7)% as ferritin, compared with 56(± 6)% and 28(± 4)% in the animals given ferritin.

EFFECT OF GIVING NTA-IRON AFTER THE LABELLED NON-VIABLE RED CELLS.

In two further experiments the effects of giving NTA-iron after the labelled red cells were studied. In the first, 11 rats were given 100 µg NTA-iron intravenously 30 minutes after the ^{59}Fe-labelled denatured erythrocytes, while 11 control rats received isotonic saline (Table XIII). Four animals from each group were killed one hour after the injection of the labelled cells, at which time the mean(range) percentages in the livers and spleens were 92(90-94)% and 87(84-93)% respectively. After 2½ hours four further rats from each group were killed, when the mean figures (range) were 82(79-86)% and 59(56-64)%. Release from the spleens was inhibited to approximately the same extent as the livers. The remaining animals were killed at four hours, the mean percentages at this time were 67(58-73)%

TABLE XIII

EFFECT OF 100 µg NTA-IRON INJECTED 30 MINUTES AFTER DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Nature of injection after labelled cells	Hours after injection of labelled cells	Mean(range)% Injected Radioiron				µg/100ml
		Liver & spleen	Ferritin	Liver	Spleen	UIBC
100 µg NTA-Iron	1	92(90-94)	7(4-11)	42(35-50)	50(44-55)	30(23-44)
	2½	82(79-86)	24(12-38)	38(37-39)	44(41-47)	108(48-148)
	Released	10		6	4	
	4	67(58-73)	39(32-45)	35(32-40)	32(26-36)	132(123-138)
	Released 2½-4	15		3	12	
Saline	1	87(84-93)	8(6-10)	41(32-49)	46(35-51)	234(230-238)
	2½	59(56-64)	15(11-21)	26(22-31)	33(28-41)	187(176-192)
	Released	28		15	13	
	4	53(50-56)	27(22-32)	26(23-29)	27(21-35)	200(181-212)
	Released 2½-4	6		0	6	

and 53(50-56)%.

In a second experiment 20 rats were given denatured labelled erythrocytes containing 100/ug iron and 10/ug iron (Table XIV). Release was inhibited by NTA-iron in the group receiving the larger load of red cells (approximately 15% compared with 25% in the controls), but not in those given the smaller amount (24% and 21% respectively).

EFFECT OF INJECTING HUMAN TRANSFERRIN.

Initial studies were performed to test the efficacy of using iron free human transferrin (Behringwerke AG, Marburg-Lahn, Germany) in rats. The rate of clearance of ^{59}Fe bound to human transferrin and to rat serum was determined in groups of 3 rats. The mean(range) half-disappearance time was similar in the two groups, values being 90(87-93) minutes and 92(86-94) minutes, respectively. Studies were then performed to investigate the effect of increased available transferrin on reticulo-endothelial radioiron release in normal, venesected and hyper-transfused animals.

TABLE XIV

INHIBITION OF ^{59}Fe RELEASE BY 100 µg NTA-IRON INJECTED 30 MINUTES AFTER DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN:

EFFECT OF VARYING THE LOAD OF LABELLED ERYTHROCYTES

Quantity of haemoglobin iron in labelled cells	I.v. injection given 30 minutes after the labelled cells	Hours after injecting labelled cells	Mean % Injected Radioiron (±SD)					µg/100ml	
			Liver & spleen	Ferritin	Haemo-globin	Liver	Spleen	S.I.	UIBC
10 µg	NTA-Iron	1	81(±3)	19(±6)	59(±10)	58(±4)	23(±4)	556(±35)	25(±2)
		4	57(±9)	50(±14)	14(±4)	30(±2)	27(±9)	351(±33)	199(±64)
		Released	24			28	-		
	saline	1	80(±4)	14(±1)	66(±12)	47(±3)	33(±3)	161(±20)	337(±35)
		4	59(±4)	37(±4)	7(±3)	32(±4)	27(±3)	243(±32)	288(±58)
		Released	21			15	6		
100 µg	NTA-Iron	1	97(±6)	5(±1)	95(±3)	45(±7)	52(±8)	470(±41)	39(±8)
		4	82(±5)	44(±11)	27(±4)	40(±5)	42(±4)	516(±81)	44(±19)
		Released	15			5	10		
	saline	1	93(±3)	7(±2)	84(±8)	49(±10)	44(±12)	314(±72)	247(±86)
		4	68(±3)	35(±4)	30(±2)	31(±3)	37(±4)	408(±31)	31(±14)
		Released	25			18	7		

In the first experiment groups of 10 rats were killed 1 and 4 hours after an injection of denatured erythrocytes containing (^{59}Fe)-haemoglobin. Half the animals received 50mg human transferrin intravenously 30 minutes after the erythrocytes, while the remainder received saline (Table XV). The release of radioiron was similar in the animals who received transferrin and in the controls (40% and 44%, respectively). The plasma iron concentrations were, however, higher at 4 hours in the rats given human transferrin than in the control animals. This suggested that additional iron had been taken up from a source other than reticulo-endothelial cells.

Studies were then performed on venesected rats. The dose of transferrin and the load of labelled cells injected was varied, but no effect on reticulo-endothelial radioiron release was noted (Table XVI). In all the studies, the plasma iron concentrations at 4 hours were higher in the animals who received transferrin.

In a final study 100mg human transferrin in divided doses administered to 6 hypertransfused rats, the first injection being together with and the second 30 minutes

TABLE XV

EFFECT OF 50mg HUMAN TRANSFERRIN INJECTED 30 MINUTES AFTER DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Nature of injection 30 minutes after labelled cells	Hours after labelled cells	Mean % Injected Radioiron (±SD)					µg/100ml	
		Liver & spleen	Ferritin	Haemo-globin	Liver	Spleen	Serum Iron	UIBC
50mg Transferrin	1	90(±1)	4(±1)	88(±4)	36(±3)	54(±5)	195(±16)	425(±36)
	4	50(±4)	23(±5)	14(±3)	22(±1)	28(±4)	334(±11)	357(±22)
	Released	40			14	26		
Saline	1	91(±5)	4(±1)	83(±5)	46(±1)	45(±5)	159(±21)	296(±31)
	4	47(±6)	18(±6)	16(±2)	21(±3)	26(±5)	236(±30)	189(±33)
	Released	44			25	19		

TABLE XVI

HUMAN TRANSFERRIN INJECTED INTO VENESECTED RATS:

EFFECT OF VARYING THE LOAD OF TRANSFERRIN AND OF DENATURED ERYTHROCYTES CONTAINING (^{59}Fe)-HAEMOGLOBIN

Quantity of haemoglobin iron in labelled cells	Nature of Injection 30 minutes after labelled cells	Hours after labelled cells	Mean% Injected Radioiron(±SD)[X]			µg/100ml	
			Liver & spleen	Liver	Spleen	Serum Iron	UIBC
100µg	50mg transferrin	1	95(±3)	38(±5)	57(±2)	208(±16)	427(±37)
		4	46(±6)	21(±4)	25(±6)	540(±74)	286(±51)
		Released	49	17	32		
	saline	1	95(±1)	46(±1)	49(±5)	258(±50)	220(±69)
		4	46(±7)	17(±3)	29(±5)	460(±38)	85(±24)
		Released	49	19	30		
10µg	50mg transferrin	1	83(±3)	48(±11)	35(±11)	241(±86)	447(±44)
	saline	1	84(±6)	49(±9)	35(±8)	226(±72)	250(±81)
150µg	100mg transferrin	1	79(±9)	24(±9)	55(±3)	199(±56)	780(±133)
		4	79(±7)	23(±12)	56(±7)	584(±67)	195(±111)
	saline	1	61(±10)	21(±7)	40(±7)	51(±6)	317(±42)
		4	84(±12)	30(±4)	54(±12)	354(±46)	71(±30)

[X] 5 animals/group

after the injection of denatured erythrocytes containing (^{59}Fe)-haemoglobin. A further 6 animals received saline instead of transferrin. At one hour a mean (range) of 97(96-98)% of injected radioiron was in the livers and spleens of the animals that received transferrin compared with 94(91-96)% in the controls. At 4 hours the values were 89(85-94)% and 91(86-97)% respectively. Once again enhancement of release was not noted.

EFFECT OF A TURPENTINE ABSCESS.

Twelve rats were given intramuscular injections of 0.2 ml mineral turpentine 20 hours before the denatured labelled erythrocytes, while twelve control rats received injections of isotonic saline. At one hour a mean ($\pm$ SD mean) of 94($\pm$ 5)% of the injected radioiron was in the livers and spleens of the control animals, compared with 99($\pm$ 5)% in the turpentine group. At four hours these figures were 53($\pm$ 5)% and 65($\pm$ 8)% respectively (t_{10}, 3.49; $P<0.01$), indicating that approximately 41% and 34% had been released. The inhibition of release in the turpentine group was reflected in the mean serum iron

concentrations; at one hour the figure (± SD mean) was 104(± 7) µg/100 ml compared with 244(± 33) µg/100 ml in the control rats, and at four hours 131(± 26) µg/100 ml compared with 298(± 12) µg/100 ml. The radioiron which had not been released was present as ferritin, since a mean (± SD mean) of 42(± 3)% was in this form in the turpentine group at 4 hours compared with 30(± 3)% in the controls. The percentages still present as haemoglobin were very similar, namely 10(± 1)% and 11(± 2)%.

DISCUSSION

The initial experiments using normal rats confirmed the observations of Noyes et al (1960) that not all the radioiron liberated from catabolized haemoglobin enters the plasma when the load of denatured erythrocytes is unphysiological. The maximum release occurred between one and four hours after injecting the denatured cells, and the superfluous radioiron was stored. It was noteworthy that similar percentages of erythrocyte radioiron were released in the face of a tenfold variation in the load of denatured cells. At least under these experimental conditions, therefore, a tenfold increase in the rate of red cell breakdown resulted in a proportional increase in the rate of delivery of iron to the plasma even though the requirement for erythropoiesis was unaltered, and this was reflected by a considerable rise in the serum iron concentration.

If the load of denatured cells was kept constant, the amount of labelled haemoglobin iron released into the plasma could be altered

by manipulating the rate of erythropoiesis. Release was enhanced if the marrow had been stimulated by previous venesections, but the situation in the animals in which erythropoiesis had been inhibited by hypertransfusion was less clear. When the liver and spleen were considered together, there appeared to have been marked inhibition of release, but in fact radioiron had been released from the spleen to approximately the same extent as in the normal animals, and this had been balanced by a reciprocal increase in the amount of radioiron in the liver. The rise in hepatic radioactivity may have been partly due to the uptake of radioiron from the plasma by liver parenchymal cells, since this would be facilitated by saturation or near saturation of the transferrin entering the liver from the splenic vein. However, the fact that hepatic uptake was rapid and complete suggests that the radioiron released from the spleen was present in some other form. Whatever the explanation, it appeared that there was a difference in the behaviour of hepatic and splenic reticulo-endothelial cells.

In further experiments it was possible to show that certain other manoeuvres inhibited the release of radioiron from both liver and spleen. Inhibition was produced by transfusing unlabelled erythrocytes which had been denatured by heating in the same way as the labelled cells. An intravenous injection of NTA-iron had a similar effect, but in this case the maximum depression was observed with a somewhat shorter time interval between the two injections. Iron chelated by nitrilo-triacetic acid is very rapidly given up to transferrin (Bates et al, 1967). Since release was inhibited when the amount of NTA-iron was small enough not to exceed the binding capacity of the plasma, it seems reasonable to suppose that the rise in serum iron concentration (or the fall in unsaturated iron binding capacity) was the effective stimulus. This could also have been the trigger in the case of the unlabelled denatured erythrocytes, since the quantity of haemoglobin iron in the cells was sufficient to produce a significant elevation in the serum iron concentration (Table IV). The time necessary for liberation of the iron from haemoglobin before its

release into the plasma might explain the slightly longer interval between the two injections needed for maximum inhibition. Injecting ferritin intravenously did not alter either the serum iron concentration or the pattern of release.

If an elevation in the serum iron concentration does indeed directly alter the behaviour of reticulo-endothelial cells, so that after a few hours a smaller proportion of haemoglobin iron is released into the plasma, a fall in the serum iron concentration might have the opposite effect. There is a temporary decrease in the serum iron concentration when erythropoiesis is stimulated by venesection or by a reduction in atmospheric pressure, and this could be the stimulus to increased iron release. However, it was not found possible to devise manoeuvres which reduced the serum iron concentration without at the same time altering the rate of erythropoiesis. Injecting iron-free human transferrin increased the unsaturated iron binding capacity, but the release of radioiron was not enhanced. In all these experiments the

serum iron concentration rose following the injection of the transferrin, suggesting that there had been an increase in the release of unlabelled iron; the source of this iron was not, however, elucidated. While this suggested that the human transferrin was exerting a physiological effect, it still remains possible that it was not acting as effectively as rat transferrin would have done.

Consideration must be given not only to the stimulus which initiates the change in the pattern of release, but also to the mechanism by which it is brought about. The iron which is freed from catabolized haemoglobin follows one of two possible pathways. Either it is released within a few hours into the plasma, or else it is incorporated into storage compounds in the reticulo-endothelial system. Nothing is known about the chemical form of the iron after it has been freed from porphyrin; it does, however, seem reasonable to postulate that there is a common pool from which the iron enters either the plasma or the storage complexes. If entry

into the plasma is delayed because the supply of transferrin binding sites for iron is reduced, this pool would tend to enlarge. Evidence that increasing the percentage saturation of plasma transferrin does delay the release of iron into the plasma was provided by the experiments in which NTA-iron was injected 30 minutes after the denatured labelled erythrocytes. The fact that the release of iron from a small load of labelled erythrocytes was not inhibited by this manoeuvre is compatible with this hypothesis, since a shortage of free transferrin binding sites would be expected to have a more marked effect when the amount of iron awaiting release was large. An increase in the size of the common pool would make more iron available for incorporation into storage compounds. It has been shown that iron stimulates ferritin synthesis, and it is of some interest that the maximum effect occurs 5 hours after injecting the iron (Drysdale and Munro, 1966), the time interval found to be necessary to produce maximum depression of release by NTA-iron. It is therefore suggested that induction of ferritin synthesis by enlargement

of the "pre-release" iron pool is the mechanism which diverts iron into the stores. Some support for this hypothesis is provided by the demonstration that more radioiron was present in ferritin in all the experimental situations in which release was inhibited. Furthermore, the fact that the inhibition following the injection of unlabelled cells was more marked when the load of labelled erythrocytes was small (Table V) is consistent with an increased capacity of the ferritin synthetic pathway. An attempt was made to test this hypothesis directly by using cycloheximide to inhibit ferritin synthesis (Chu & Fineberg, 1969), but this was inconclusive since neither the inhibition of release nor the incorporation of radioiron into ferritin was altered. Presumably the radioiron was taken up by the preformed ferritin since ferritin synthesis is known to be almost totally impaired under these experimental conditions (Goldberg, 1970).

While stimulation of ferritin synthesis by the accumulation of iron seems the strongest possibility at the present time, other mechanisms

by which alterations in release might be effected must also be considered. When release was stimulated by venesection, the rate of catabolism of haemoglobin was slowed (Table III), and it seemed possible that if iron were entering the "pre-release pool" more slowly, the pool might be smaller, and this might be the reason why less iron was deviated into stores. The finding that the liberation of radioiron from haemoglobin was possibly faster at the time of maximum depression of release following the transfusion of unlabelled denatured cells (Table IV) was consistent with this idea. However, no change in the rate of haemoglobin catabolism was detected in the experiments with NTA-iron (Table VII), although the inhibition of release was more marked, and it was also not apparent in the turpentine experiment. It therefore appears that while the rate of breakdown of haemoglobin probably does vary, this is unlikely to exert a direct influence on the proportion of radioiron released.

Several of the manoeuvres producing a change

in the amount of haemoglobin iron released into the plasma also altered the distribution of the denatured labelled erythrocytes between the liver and spleen. These included not only venesection, hypertransfusion and the transfusion of unlabelled denatured erythrocytes, but also the injection of NTA-iron. No consistent pattern of altered organ uptake could be identified, except that the most marked inhibition of release seemed to occur in the organ in which uptake was less.

CHAPTER III

THE ROLE OF ASCORBIC ACID IN THE METABOLISM OF STORAGE IRON

INTRODUCTION

There is reason to believe that the metabolism of ascorbic acid and iron are interrelated. For example, scurvy is common in the Bantu people of Southern Africa, but it is almost never seen in individuals who are not heavily siderotic (Seftel *et al*, 1966). Evidence has been obtained that irreversible oxidation of a proportion of the dietary ascorbic acid occurs, probably mediated by the massive tissue deposits of ferric iron (Lynch *et al*, 1967). The severity of the resultant ascorbic acid deficiency depends upon the amount of ascorbic acid in the diet, and consequently the incidence of clinical scurvy varies with the seasons.

Other observations, both clinical and experimental, suggest that deficiency of ascorbic acid may, in its turn, affect the metabolism of iron. In Bantu individuals with severe siderosis the plasma iron concentration is typically elevated, but if the tissue levels of ascorbic acid are depleted, the plasma iron is usually low (Bothwell *et al*, 1964). The administration of ascorbic acid to such subjects results in the prompt return

of the plasma iron concentration to elevated levels. This suggests that the release of iron to the plasma may be inhibited by deficiency of the vitamin. Finally, there are certain experimental observations which strengthen the suspicion that the metabolism of storage iron is abnormal in the presence of ascorbic acid deficiency. It has been reported that deposits of haemosiderin are greater than normal in the tissues of scorbutic guinea-pigs, while the soluble fraction of liver iron is reduced (Banerjee and Chakrabarty, 1965).

These various findings are not altogether surprising since the results of studies in vitro suggest that ascorbic acid may be necessary, not only for the incorporation of transferrin-bound iron into hepatic ferritin (Mazur et al, 1960), but also for the release of iron from ferritin (Mazur et al, 1955). The present investigation was undertaken to obtain more information on the role of ascorbic acid in the uptake and release of iron from stores.

MATERIALS AND METHODS

Adult male guinea-pigs of the Onderstepoort strain were used. The diet consisted of ascorbic acid-free guinea-pig/rabbit production pellets (Delmas Milling Company, Randfontein, South Africa), hay which had been heated to 80°C for 1 hour, and tap water. This diet produced frank scurvy in 28-32 days. Control animals were fed the same diet except that ascorbic acid was added to the water (140 mg/l). The experiments were performed after 21 days, at which time the mean liver ascorbic acid concentrations (±SD mean) by the method of Roe (1954) were 0.6(±0.5)mg/100g in the scorbutic group and 24(±5)mg/100g in the control group. The mean haemoglobin concentrations (±SD mean) were 13.4(±2.1)g/100ml and 14.1(±2.2)g/100ml respectively, and the reticulocyte counts were normal in all animals studied. The mean weights (±SD mean) of the scorbutic and control groups were 589(±157)g and 629(±123)g respectively.

In kinetic studies radioiron was given to the experimental animals in one of three ways. Radioiron bound to transferrin was prepared by

incubating guinea-pig plasma with tracer amounts of $^{59}FeCl_3$ for 30 minutes at 37°C. In another experiment 1000 µg iron as ferrous sulphate labelled with $^{59}FeCl_3$ was injected intravenously. No immediate effects were observed, and within 15 minutes 70-80% of the radioactivity was present in the liver. Finally, erythrocytes containing haemoglobin labelled with ^{59}Fe were prepared by incubating guinea-pig reticulocytes in vitro with ^{59}Fe of high specific activity, bound to transferrin. (Fractionation of such cells by the method of Gale et al (1963) revealed that more than 70% of the radioactivity was in the haemoglobin residue). Prior to injection the cells were rendered non-viable in the following way. After washing, the cells were suspended in eight times their volume of a 1:1 mixture of 0.9% NaCl solution and ACD (5 g citric acid, 13.8g sodium citrate, 13.9g dextrose per litre of solution) and heated in a water bath at 49°C for 4 minutes. When erythrocytes which had been treated in this way were injected intravenously into normal guinea-pigs, almost all the radioactivity was present in the liver and spleen after 45 minutes.

In all studies the animals were killed by bleeding from the aorta under ether anaesthesia. The portal vein was cannulated and 30 ml 0.9% NaCl solution was perfused through the liver to wash out the blood. The liver and spleen were removed, cooled rapidly on ice, and were then stored at - 20°C prior to processing. This was done by thawing and homogenizing the organs in four times their weight of distilled water. In the isotopic experiments total body radioactivity was measured in an Armac Counter. This was also used for measuring the localization of radioiron in the liver and spleen. Radioactivity in ferritin and haemoglobin was determined as described in Chapter II, but in the present studies insoluble radioiron was also estimated. A 2 ml aliquot of the homogenate was heated to 80°C and the radioactivity in the washed precipitate determined. Since the radioactivity present in the haemoglobin was already known, a crude estimate of insoluble radioiron could be obtained by subtraction. In studies in which ^{59}Fe-haemoglobin was not present, the total heat precipitate was taken to represent insoluble radioiron. Ferritin iron was measured

by the method of Drysdale and Munro (1965) and total non-haem iron by the method of Torrance and Bothwell (1968). "Haemosiderin", or insoluble non-haem iron was then obtained by difference.

RESULTS

NON-HAEM IRON AND FERRITIN IRON CONCENTRATIONS IN LIVERS AND SPLEENS OF NORMAL AND SCORBUTIC GUINEA-PIGS

The total non-haem iron and ferritin iron concentrations were measured in the livers of 18 scorbutic guinea-pigs, 18 control animals, and 11 animals which had been maintained on the ascorbic acid-free diet, but which had received an intraperitoneal injection of 40 mg ascorbic acid 24 hours before being killed. Similar determinations were performed on the spleens of 10 animals from each group.

The effects of ascorbic acid deprivation were different in the two organs (Fig 1). In the liver, the total non-haem iron concentration was significantly lower in the scorbutic animals than in the controls, mean figures ($\pm$SD mean) being 90($\pm$30) μg/g and 130($\pm$36) μg/g respectively (t_{34}, 3.67; $P < 0.001$). In the spleens, on the other hand, the concentration of non-haem iron was significantly higher in the scorbutic animals, the mean figure ($\pm$SD mean) being 833($\pm$195) μg/g

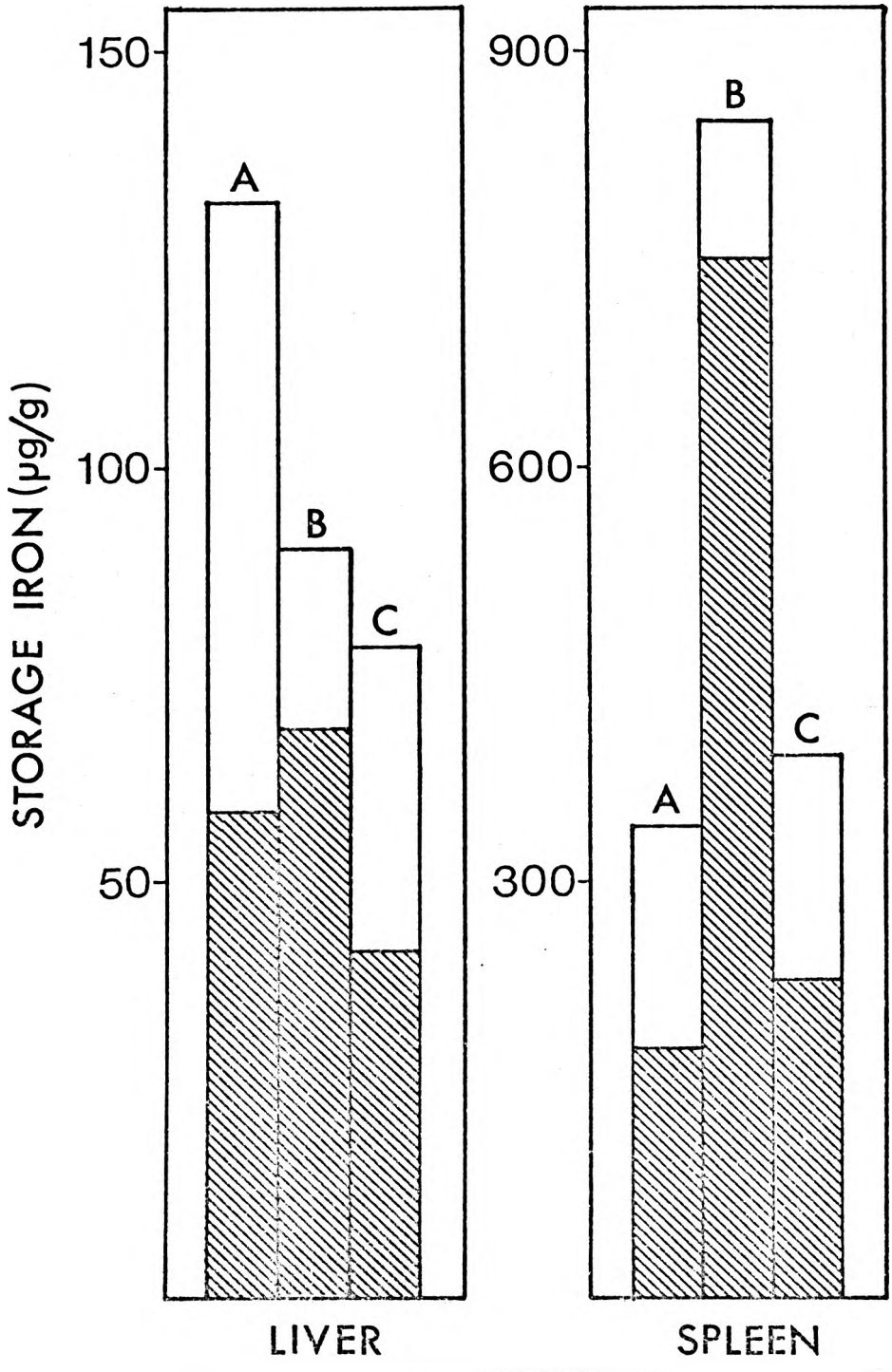

Figure 1. Total non-haem iron concentrations in livers and spleens of control animals (A), scorbutic animals (B) and scorbutic animals given ascorbic acid 24 hours before analysis (C). Unhatched=ferritin.

compared with only 339($\pm$90)/ug/g in the control group (t_{18}, 7.28; $P<0.001$). In both livers and spleens the proportion of non-haem iron present as ferritin was much lower in the scorbutic than in the control animals. In the livers, a mean ($\pm$SD mean) of 23($\pm$11)% was present as ferritin in the scorbutic guinea-pigs compared with 55($\pm$8)% in the normal animals (t_{34}, 10.0; $P<0.001$). In the group of scorbutic animals given an injection of ascorbic acid 24 hours before death, the mean ($\pm$SD mean) non-haem iron concentration in the livers was similar to that in the scorbutic animals (78 $\pm$23/ug/g), but the percentage of ferritin had risen to a mean ($\pm$SD mean) of 51($\pm$2)%. In the spleens of the scorbutic animals, ferritin formed a mean ($\pm$SD mean) of only 9($\pm$2)% of the total non-haem iron, compared with a mean ($\pm$SD mean) of 47($\pm$5)% in the normal animals. In the scorbutic guinea-pigs given the injection of ascorbic acid 24 hours before death, the splenic non-haem iron concentration was much lower than that in the untreated scorbutic group (mean 383/ug/g, SD mean $\pm$162) (t_{18}, 5.62; $P<0.001$), and the percentage present as ferritin had risen to a mean ($\pm$SD mean) of 42($\pm$16)%.

UPTAKE AND RELEASE OF HEAT-DAMAGED ERYTHROCYTES CONTAINING ^{59}Fe-LABELLED HAEMOGLOBIN

Twelve control and 12 scorbutic animals received intravenous injections of heat-damaged erythrocytes containing 75 µg iron as haemoglobin. Three guinea-pigs from each group were killed respectively at 45 minutes and 3, 24 and 48 hours. The spleen and liver of each animal were homogenized together and the total radioactivity as well as the distribution of the ^{59}Fe between the various fractions determined (Fig 2).

In the control animals a mean (range) of 96(90-99)% of the injected radioactivity was present in the liver and spleen at 45 minutes, but by 3 hours the figure had fallen to a mean of 48 (47-49)% and by 24 hours to 36(35-40)%. In the scorbutic animals, less of the injected ^{59}Fe was located in the livers and spleens at 45 minutes (mean and range, 70(69-83)%), but the major difference from the control group was that there was little change in this figure during the course of the experiment. The subsequent means

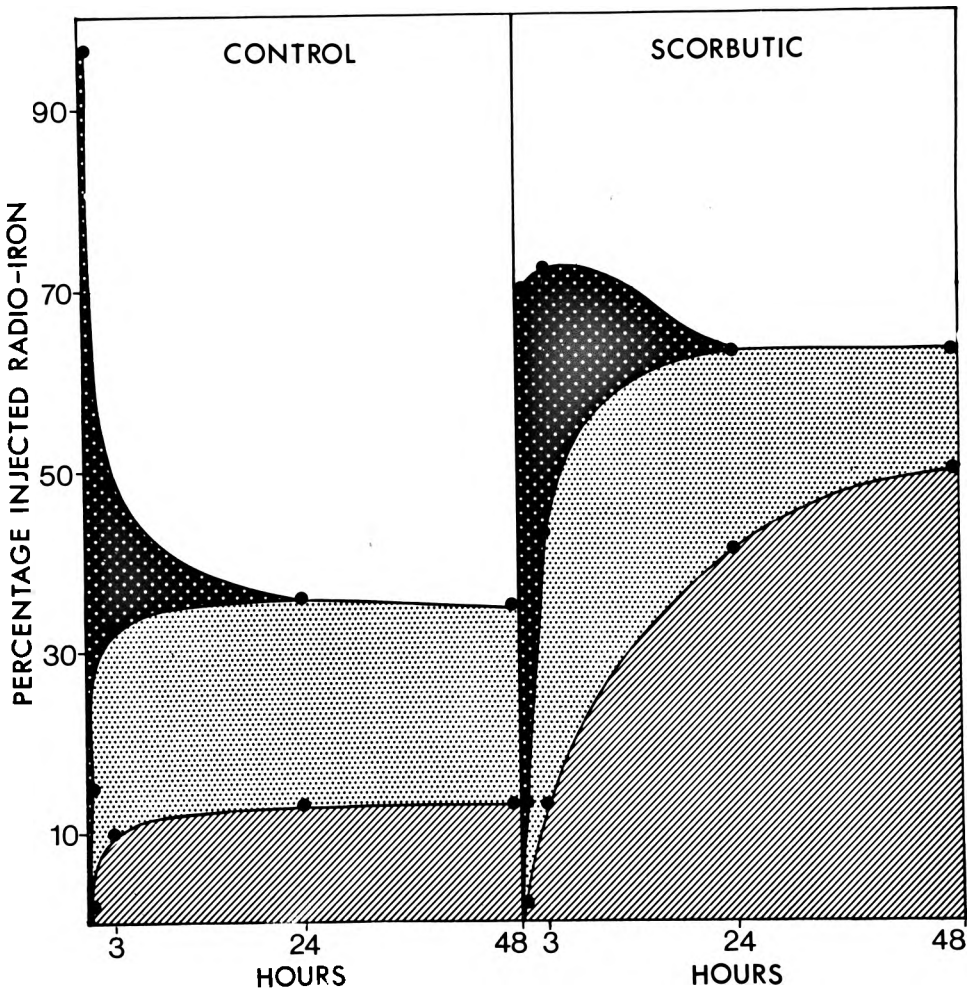

Figure 2. Distribution of ^{59}Fe in combined livers and spleens of control and scorbutic animals at various times after injecting denatured red cells containing ^{59}Fe-haemoglobin. Heavy stipple, haemoglobin; light stipple, ferritin; hatched, insoluble (haemosiderin).

and ranges were: at 3 hours, 73(70-79)%; at 24 hours, 63(61-65)%; and at 48 hours, 64(62-65)%.

The rate of disappearance of ^{59}Fe-labelled haemoglobin was somewhat more rapid in the control animals. Although the initial uptake of denatured cells was slightly greater in the control group, by 3 hours only a mean (range) of 17(15-18)% of the injected radioactivity was still in the form of haemoglobin, compared with 31(30-35)% in the scorbutic guinea-pigs. By 24 hours, however, it had all been processed in both groups. The mean percentage (range) of injected radioactivity present as ferritin increased slightly in the control animals from 13(11-15)% at 45 minutes to 24(19-27)% at 3 hours, after which there was little change. The means and ranges in the scorbutic guinea-pigs were not very different; at 45 minutes 11(9-12)% was in ferritin; at 3 hours 29(27-31)%, at 24 hours 27(22-30)% and 48 hours 13(12-15)%.

The major difference between the two groups was in the fraction of ^{59}Fe present in the heat precipitate after subtracting haemoglobin iron ('insoluble iron'). In the control guinea-pigs

the mean figure (range) rose from 2(1-3)% at 45 minutes to 10(9-11)% at 3 hours, and thereafter was 13(10-15)% at both 24 and 48 hours. In the scorbutic animals, on the other hand, the amount of radioactivity in this fraction continued to increase; at 24 hours a mean (range) of 41(37-44)% was in this form, and at 48 hours the figure had risen to 50(47-53)%.

UPTAKE AND RELEASE OF TRANSFERRIN-BOUND ^{59}Fe

Nine scorbutic and nine control animals were given intravenous injections of 1 ml guinea-pig plasma labelled with ^{59}Fe. Three animals from each group were killed at 3, 6 and 24 hours respectively, and total hepatic radioactivity as well as the percentage present in ferritin and heat-insoluble fractions were determined.

The patterns were significantly different in the two groups (Fig 3). In the control animals, the percentage of injected radioactivity present in the liver rose from a mean (range) of 12(10-15)% at 3 hours to 13(11-15)% at 6 hours and 19(16-22)% at 24 hours. In the scorbutic animals there was also an

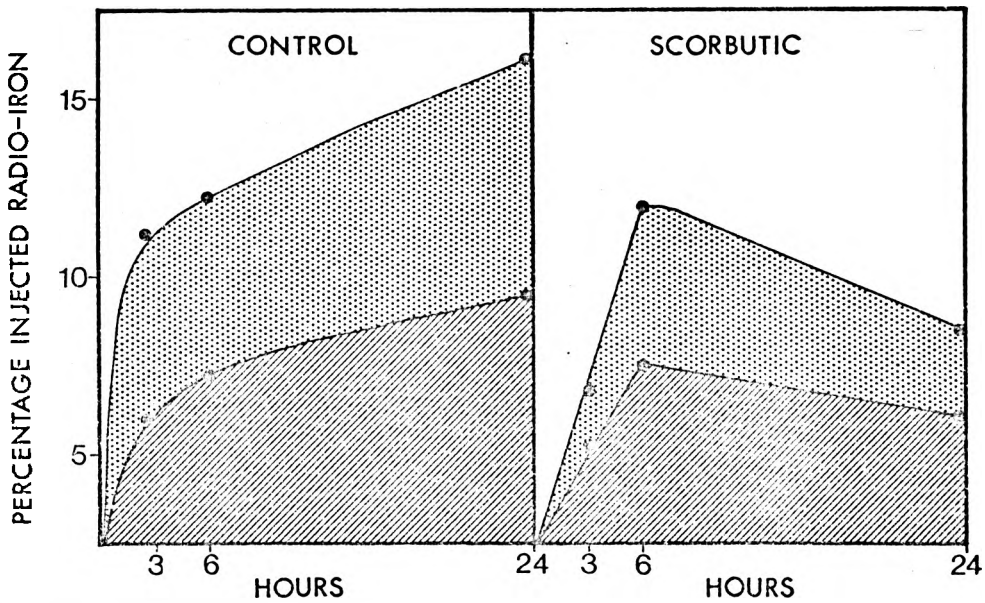

Figure 3. Distribution of ^{59}Fe in the livers of control and scorbutic animals at various times after injecting transferrin-bound ^{59}Fe. Stippled, ferritin; hatched, insoluble (haemosiderin).

initial increase in hepatic radioactivity to a mean (range) of 13(11-15)% at 6 hours, but at 24 hours the figure had declined to 8(7-10)%. The distribution of ^{59}Fe between the ferritin and heat-insoluble fractions was not very different in the two groups, the proportion which was insoluble being slightly more in the scorbutic animals.

This experiment was repeated using six control and six scorbutic guinea-pigs, all of which had been hypertransfused with homologous packed red cells 7 and 6 days previously in order to inhibit erythropoiesis. Haemoglobin values ranged between 24 and 26 g/100 ml. Three animals from each group were killed at 3 hours, and the remaining three at 6 hours. The mean percentage (range) of injected radioactivity in the livers of the control animals was 15(13-16)% at 3 hours and 20(18-23)% at 6 hours, while in the scorbutic group the corresponding figures were 20(15-22)% and 30(23-36)%. The mean percentages of injected radioactivity present in the form of ferritin at these times were similar in the two groups, but greater amounts were present as insoluble iron in the scorbutic

animals. The mean figures (range) at 6 hours were 7(6-9)% and 15(12-19)% in the control and scorbutic groups respectively.

UPTAKE AND RELEASE OF $^{59}FeSO_4$

Twelve scorbutic and 12 control animals were given intravenous injections of 1000 µg iron as $^{59}FeSO_4$. Three animals from each group were killed at 12 hours, and 2, 4 and 6 days thereafter, and the total hepatic radioactivity together with the fractions present as ferritin and as insoluble iron determined.

The results resembled those obtained with plasma-bound ^{59}Fe. Initially similar percentages of injected radioactivity were present in the livers of each group, the mean figures (range) at 12 hours being 76(74-77)% and 68(65-71)% in scorbutic and control animals respectively. Thereafter there was a steady decline in radioactivity in the scorbutic group, whereas in the control animals the figures remained high. At 6 days the mean figures (range) were 39(38-41)% and 72(70-73)% respectively.

DISCUSSION

In the present study ascorbic acid deficiency was found to produce changes in the distribution of storage iron in guinea-pigs. The total non-haem iron concentration was lower in the livers and considerably higher in the spleens of the scorbutic animals than it was in the controls (Fig 1). Not only was there a redistribution of storage iron between the two organs, but the proportion present in each of the two iron storage compounds was also different, much less being present as ferritin and much more as haemosiderin. These results confirm and extend those of Banerjee and Chakrabarty (1965), who reported a higher total iron concentration in the spleens of scorbutic guinea-pigs compared with pair-fed control animals; in the livers the total iron concentration was not diminished, but in the scorbutic group the soluble iron fraction was smaller and the insoluble fraction larger. In the present study it was possible to demonstrate the role of ascorbic acid deficiency in the production of these changes. After a single intraperitoneal injection of the vitamin 24 hours

before death, there was a drop in mean total non-haem iron concentration in the spleens of the treated animals to less than half that in the untreated group. This indicated that a considerable amount of storage iron had been released. In addition, there was a very considerable decrease in haemosiderin and an absolute increase in the amount of ferritin, which now formed more than half the total as compared with less than 10% in the scorbutic animals. In the livers the relative proportions of the two storage compounds also returned to normal on injecting ascorbic acid, but the total non-haem iron concentration did not change significantly. These observations suggest that the balance between ferritin and haemosiderin is shifted towards the insoluble form in the presence of ascorbic acid deficiency, and that the balance is rapidly restored when the deficiency is corrected. In this connection, the observations in vitro of Matioli and Baker (1963) may be relevant. These workers have obtained evidence suggesting that haemosiderin is formed as a result of the oxidative denaturation of ferritin. It therefore seems

possible that the proportion of storage iron present in each form might be influenced by the cellular concentration of a reducing agent such as ascorbic acid.

The increase in total storage-iron concentration in the spleens of the scorbutic animals together with the decrease in the livers suggested that ascorbic acid deficiency might have different effects on the release of iron from stores in different types of cell. This possibility was strengthened by the marked effect on splenic-iron release when ascorbic acid was administered to scorbutic guinea-pigs, and the absence of such an effect in the liver. Hepatocytes might conceivably be capable of different metabolic reactions from reticulo-endothelial cells. However, since hepatic storage-iron is located not only in hepatocytes but also in cells of the reticulo-endothelial system, further studies were necessary in order to test this hypothesis. Iron within each cell type was separately labelled with ^{59}Fe by injecting the isotope in different forms. It is generally agreed that damaged red cells are not engulfed by

hepatocytes, so that ^{59}Fe in the form of haemoglobin within heat-denatured red cells was located in reticulo-endothelial cells, in the liver as well as in the spleen (Hosain et al, 1962). Iron bound to transferrin, on the other hand, does not enter reticulo-endothelial cells (Hosain et al, 1962). Most of it is taken up by the erythroid bone marrow and incorporated into developing red cells, but a fraction goes to the liver and enters hepatocytes. Furthermore, free iron injected intravenously is predominantly taken up by the parenchymal cells of the liver (Finch et al, 1950). The effect of ascorbic acid deficiency upon the uptake, storage and release of ^{59}Fe injected in these three forms was therefore studied.

Significant differences between scorbutic and control guinea-pigs were observed (Fig 2). In normal animals, iron was rapidly freed from haemoglobin and released into the plasma, whereas in scorbutic animals very little ^{59}Fe left the reticulo-endothelial cells. There was, in addition, some suggestion that a smaller percentage of the damaged erythrocytes had been taken up by the

scorbutic animals, and that the rate of liberation of iron from haemoglobin was slower. Much more striking, however, was the progressive build-up of radioactivity in haemosiderin (heat-insoluble fraction) in these animals, whereas in the control animals there was little change after the first 3 hours. These observations therefore provide support for the suggestion that the effect of ascorbic acid deficiency is to inhibit the release of iron from reticulo-endothelial cells, and to promote the formation of haemosiderin at the expense of ferritin.

The behaviour of hepatocytes in terms of the handling of storage iron was found to be different to that of reticulo-endothelial cells (Fig 3). When ^{59}Fe-labelled plasma was injected intravenously there was a steady build-up in hepatic radioactivity between 6 and 24 hours in the control animals. However, in the scorbutic animals the radioiron levels diminished over the same period, thus indicating a release of ^{59}Fe into the plasma. These conclusions were confirmed by the results of the experiment with $^{59}FeSO_4$.

Mazur et al (1960) have suggested that ascorbic acid is necessary for the incorporation of plasma iron into hepatic ferritin. However, no evidence to support this contention was obtained in the present study. In the experiment in which erythropoiesis was inhibited by hypertransfusion, similar quantities of labelled ferritin were formed from plasma-bound ^{59}Fe in both control and scorbutic animals, while the total percentage incorporated into storage complexes (ferritin + haemosiderin) was actually greater in the scorbutic group.

The observations made in the present study may well have relevance to the cellular and organ distribution of storage iron in Bantu subjects with iron overload. The siderosis in these individuals is a consequence of the excessive amounts of available iron in the diet, and over the years there is a steady accumulation of iron in storage depots (Walker and Arvidsson, 1953; Bothwell and Bradlow, 1960). In the majority of affected subjects the most striking deposits are found in reticulo-endothelial cells throughout the body. This

distribution differs from that found in idiopathic haemochromatosis, in which reticulo-endothelial deposits are much less prominent than those in parenchymal cells. The differing hepatic localization of iron in the two conditions has, in fact, been shown to be distinctive enough to allow for differentiation on histological appearances alone (Bothwell et al, 1965). Several possible reasons for the difference in iron distribution have been advanced, and it has even been suspected that it may hold a clue to the aetiology of the idiopathic condition. The results of the present study, however, suggest that the answer may lie in the low ascorbic acid content of the Bantu diet (Lynch et al, 1967) compared with that of most subjects with idiopathic haemochromatosis. Significant deficiency of ascorbic acid in the Bantu might inhibit the release of iron from reticulo-endothelial sites sufficiently to allow for the slow accumulation of storage iron in these cells. This notion is supported by the low serum-iron concentrations in such subjects when frank scurvy is present, and the sharp increase in serum-iron

concentration on administering ascorbic acid (Bothwell *et al*, 1964). Although this suggests that the release of iron from stores is inhibited, it should be noted that the plasma-iron turnover in scorbutic Bantu is usually between two and three times normal, and that it does not change when ascorbic acid is first administered (Bothwell *et al*, 1964). It is obvious, therefore, that a considerable amount of iron does enter the plasma in scorbutic individuals; the present study suggests that most of it comes from hepatic parenchymal cells rather than reticulo-endothelial cells. Unanswered questions relate to the factors responsible for the increased release of iron from hepatocytes under these circumstances, and the relationship (if any) between the inhibition of iron release from reticulo-endothelial cells and the enhanced conversion of ferritin to haemosiderin.

CHAPTER IV

THE SITE OF ACTION OF DESFERRIOXAMINE

INTRODUCTION

Desferrioxamine methanesulphonate (DFA) is an iron chelate of great avidity and selectivity. The DFA-induced urinary iron excretion is a valuable diagnostic test of iron overload (Balcerzak et al, 1968; Ploem et al, 1966), and provides a good assessment of normal and deficient iron stores (Dagg et al, 1966; Hallberg and Hedenberg, 1967). In spite of investigation by a number of workers, the source of the iron chelated by DFA remains uncertain. There is, however, agreement on the sources which are not available to the chelate. Iron bound to transferrin in the plasma is not removed in vivo (Hallberg and Hedenberg, 1965a; Balcerzak et al, 1966), and the incorporation of plasma-bound radioiron into the haemoglobin of new red cells is unchanged when DFA is injected (Hallberg and Hedenberg, 1965a). Haemoglobin iron is inviolate (Keberle, 1964) and there is nothing to implicate myoglobin and other functional iron compounds. While iron in process of absorption from the intestine can be bound, it does not represent an important chelatable pool (Brown et al, 1967; Hallberg and Hedenberg, 1965b; Hedenberg, 1969).

On balance it would seem that the storage compounds ferritin and haemosiderin represent the major source, since the quantity of iron chelated is roughly proportional to the quantity of storage iron in the body (Fielding, 1965; Ploem et al, 1966; Hallberg et al, 1966; Balcerzak et al, 1968). Furthermore, DFA has been shown to remove some iron from ferritin in vitro (Wöhler, 1964), and also from liver homogenates, the amount being proportional to the non-haem iron concentration (Hedenberg, 1969). Certain observations, however, suggest that at least some of the iron chelated in vivo has a different immediate origin. For example, DFA-induced urinary iron excretion is often high in patients with pernicious anaemia (Fielding, 1965; Karabus and Fielding, 1967), and shortly after starting treatment there is a rapid drop unrelated to any significant change in the amount of storage iron (Hallberg, 1964; Karabus and Fielding, 1967; Balcerzak et al, 1968). Additional evidence suggesting that DFA is able to chelate iron which is not in fixed storage compounds is provided by the effect of the transfusion of non-viable erythrocytes. DFA-induced urinary iron

excretion doubles between 4 and 8 hours after such a transfusion before returning to normal at 20 hours (Hedenberg, 1969). Such observations suggest that iron in transit both to and from the stores can be chelated, but it remains uncertain whether this represents a major or a minor source of the iron bound by desferrioxamine. These observations are, in addition, of special relevance to this thesis, for they suggest that alterations in the amount of iron processed by reticulo-endothelial cells results in alterations in the quantity of iron excreted after injecting the chelate.

Another area of uncertainty relates to the cellular origin of the chelated iron. The major physiological pathway for iron involves the break-down of senescent red cells in reticulo-endothelial cells, with subsequent delivery of the released iron into the plasma. Much of the storage iron of the body is in these cells, but there is also some in parenchymal cells, particularly those of the liver. In states of iron overload the deposits of storage iron are increased in all tissues, but not necessarily to the same extent. Harker et al

(1968) have produced evidence to suggest that the iron chelated by DFA may originate largely or entirely from parenchymal cells. They found that the amount of iron excreted after the injection of DFA was more closely correlated with the quantity of histologically visible iron in hepatic parenchymal cells than with the amount in reticulo-endothelial cells.

The present study was done in an attempt to shed light on some of these problems. The cellular origin of the chelated iron was studied by labelling the storage iron in the two cell types separately. In addition, the rate of release of storage iron into the plasma was manipulated in various ways in an attempt to define more closely the chemical source or sources of the chelated iron.

MATERIALS AND METHODS

Adult male rats of the Sprague-Dawley strain weighing between 200 and 300 g were used. In some experiments erythropoiesis was inhibited by hypertransfusion or stimulated by venesection as described in Chapter II.

Transferrin-bound radioiron was prepared by incubating buffered rat plasma with a tracer amount of $^{59}Fe\ Cl_3$. In order to prepare radioactive ferritin, 30 µg iron as ferrous sulphate labelled with 10 µc $^{59}FeCl_3$ was injected intravenously into a rat. One hour later the rat was killed, and ferritin was prepared from the homogenized liver by precipitation with ammonium sulphate after heating (Drysdale and Munro, 1965). After precipitation it was redissolved in isotonic saline. Erythrocytes containing haemoglobin labelled with ^{59}Fe were obtained as described in Chapter II.

Desferrioxamine methanesulphonate (Desferal, Ciba) was supplied in sterile vials containing 500 mg crystallized powder, which was dissolved in

isotonic saline immediately prior to use. Control animals received injections of isotonic saline.

A Packard whole-body Armac counter was used to measure the radioactivity present in the total animal and in individual organs. Excretion of ^{59}Fe was assessed by determining the decrease in whole-body counts after allowing for decay. The methods for determining ^{59}Fe in ferritin and in haemoglobin have been described above (Chapter II). Radioactivity present in circulating erythrocytes was determined by counting an aliquot of blood. The percentage red-cell utilization of radioiron was calculated on the assumption that the blood volume was 50 ml/kg body weight in normal and bled animals and 60 ml/kg in polycythaemic animals. The total non-haem iron content of the livers and spleens was estimated chemically by the method of Torrance and Bothwell (1968).

RESULTS

EFFECT OF ERYTHROID MARROW ACTIVITY UPON QUANTITIES OF CHEMICAL IRON CHELATED BY DESFERRIOXAMINE

Forty rats were given intramuscular injections of 5 mg iron as iron sorbitol citrate (Jectofer, Astra) on the eighth and seventh days prior to commencing the study. They were then randomly divided into two groups, one of which was hypertransfused on the same two days and also on the first day of the study. The mean packed cell volume at this time was 69%. Each group was subdivided again to yield four groups of 10 rats each; 10 hypertransfused and 10 normal rats then received intramuscular injections of 10 mg DFA twice daily for 14 days, while the remaining animals were given injections of isotonic saline.

On the fifteenth day all the animals were killed and the non-haem iron contents of their livers and spleens were determined. The figures were closely similar in the two hypertransfused groups. The mean total liver non-haem iron ($\pm$ SD mean) was 4,614($\pm$958) /ug in the hypertransfused

animals given DFA and 4,390(±1,041)/ug in those given isotonic saline, while the splenic non-haem iron contents were 636(±5)/ug and 634(±6)/ug respectively. In contrast, the concentrations in the normal rats given DFA were significantly lower than those in the animals given isotonic saline. Mean hepatic values (± SD mean) were 1,493(±84)/ug and 2,328(±272)/ug respectively (t_{10}, 9.3; $P<0.001$), while the comparable splenic figures were 184(±52)/ug and 260(±80)/ug (t_{10}, 2.5; $0.05>P<0.02$). Approximately 900/ug iron had thus presumably been removed by DFA from the two organs.

EFFECT OF ERYTHROID MARROW ACTIVITY UPON QUANTITIES OF RADIOIRON CHELATED BY DESFERRIOXAMINE

Transferrin-bound radioiron

Tracer quantities of ^{59}Fe bound to plasma were injected intravenously into 22 rats. Preliminary experiments in normal rats revealed that the ^{59}Fe was cleared exponentially from the plasma with a mean half time of 66 minutes. Ten animals received injections of 10 mg DFA immediately

following the injection of radioiron, and thereafter intramuscularly each hour for 3 hours. The remaining 12 animals acted as controls. After 4 hours, three rats from the DFA group and four control animals were killed. Further injections of DFA were given at 12, 24 and 36 hours. Half the remaining animals in each group were killed at 24 hours and the remainder at 48 hours.

A mean of 16.8% of the injected radioactivity was in the livers of the control animals at 4 hours and this amount changed very little during the course of the experiment (Table I). By 48 hours a mean of 7.5% had been excreted by this group, and virtually all the remaining radioiron could be accounted for in circulating erythrocytes. The administration of DFA did not significantly affect the amount of radioiron incorporated into red cell haemoglobin at 48 hours, but more ^{59}Fe had been excreted (mean 14.2%) and less was in the liver (mean 5.8%) than in the control group. The difference between the two groups in terms of hepatic radioactivity was apparent as early as 4 hours after injecting the ^{59}Fe.

TABLE I

EFFECT OF DESFERRIOXAMINE (DFA) ON THE DISTRIBUTION OF RADIOIRON IN NORMAL RATS AT VARIOUS TIMES AFTER THE INJECTION OF ^{59}Fe-LABELLED PLASMA

	Time after injection					
	4 hr		24 hr		48 hr	
	DFA	Control	DFA	Control	DFA	Control
No. of rats	3	4	3	4	4	4
Mean % in liver	7.4	16.8	8.4	16.2	5.8	13.6
	(6.7-8.3)	(16.2-17.6)	(6.1-10.2)	(14.4-17.5)	(5.4-6.2)	(11.4-15.6)
Mean % in hepatic ferritin	4.1	11.3	5.2	11.6	3.0	9.5
	(3.2-4.5)	(10.6-12.6)	(3.4-6.8)	(9.5-13.5)	(2.6-3.5)	(8.8-11.0)
Mean % in circulating red cells	-	-	17.8	24.7	58.0	61.1
			(9.3-31.8)	(16.9-29.4)	(49.0-70.9)	(49.7-68.0)
Mean % excreted	-	-	18.8	6.8	14.2	7.5
			(11.2-19.7)	(4.9-7.4)	(10.8-16.2)	(5.1-10.5)

The range of results is given in parentheses.

The experiment was repeated using 14 hypertransfused animals, but observations were made only at 4 and 24 hours. After 24 hours less than 3% had been incorporated into circulating erythrocytes, thus reflecting the successful inhibition of erythropoiesis. In the control animals the amount of ^{59}Fe in the liver increased from a mean(range) of 13.7(11.4-15.2)% at 4 hours to 29.6(28.2-32.1)% at 24 hours whereas in the previous experiment there had been no significant change after 4 hours. The mean(range) corresponding figures in the hypertransfused rats given DFA were 5.5(4.7-5.8)% and 15.7(7.6-20.5)% respectively. The mean(range) percentages excreted after 24 hours were virtually identical to those in the previous experiment, namely 5.2(2.5-7.0)% in the control rats and 19.1(17.4-20.5)% in those given DFA.

Ferritin labelled with ^{59}Fe.

The distribution of intravenously injected ferritin was first examined. (^{59}Fe)-ferritin containing 50 µg iron was injected into eight normal rats, half of which were killed after 2 hours and the remainder after 24 hours. At both times

all the radioactivity was present in the livers, and approximately 75% could still be identified as ferritin. The same amount of labelled ferritin was then injected into 10 normal rats. After 24 hours half the rats were given intramuscular injections of 10 mg DFA twice daily for 16 days, while the remainder received isotonic saline injections. In the control animals at this time only a mean (range) of 50.2(33.3-66.4)% of the injected radioactivity remained in the liver, 32.8(21.3-47.8)% being ferritin. A mean(range) of 22.6(17.5-30.4)% was in circulating erythrocytes and 16.6(11.0-19.6)% had been excreted. In the animals given DFA a similar amount had been incorporated into red cells (mean 26.3, range 21.0-34.2)% but a considerably greater quantity had been excreted (mean 57.7, range 54.5-63.2)%, and this was reflected in the lower figure for hepatic radioactivity (mean 21.5, range 9.3-30.2)%; 10.1 (range 2.9-13.5)% was in ferritin.

A similar experiment was performed on nine hypertransfused animals, but on this occasion only 30 µg iron as (^{59}Fe)-ferritin was injected.

Two animals were killed at 3 hours, when once again all the injected radioactivity was present in the liver. A mean of 72.3% was identifiable as ferritin. Three of the remaining rats received intramuscular injections of 10 mg DFA twice daily for 8 days, while four animals acted as controls. Further transfusions of 3 ml packed homologous erythrocytes were given on the first, third and sixth days. On the eighth day the animals were killed, at which time there was no radioiron in circulating erythrocytes. The control animals had excreted a mean(range) of 5.0(4.1-7.2)% of the ^{59}Fe, while the animals given DFA had excreted a mean(range) of 55.7(54.5-63.2)%. All the remaining radioactivity in both groups of animals was in the livers. A mean(range) of 73.2(68.3-77.5)% of injected radioactivity was still present as ferritin in the control animals, while the corresponding figure in the DFA group was 36.0 (30.1-39.6)%.

The third experiment using (^{59}Fe)-ferritin was performed on 12 animals in which erythropoiesis had been stimulated by venesections. On this

occasion only a mean(range) of 82.2(80.8-84.7)% of the 30 µg injected ferritin iron was in the liver at 2 hours, and this had diminished to 16.0(12.1-24.1)% in the five control animals killed after 6 days. A mean(range) of 14.2(12.6-16.4)% had been excreted at this time, while the remainder (mean 56.0, range 49.3-59.0)% was in circulating erythrocytes. The five rats given DFA injections in the same way as before had excreted a mean(range) of 45.8(40.0-50.3)% of the radioiron. It was noteworthy that in this experiment the amount of radioactivity in circulating haemoglobin was a good deal less in the DFA animals than in the controls (mean 37.0, range 32.3-40.2)%. Only a mean(range) of 8.1(6.2-11.3)% was still in the liver, with 3.7(1.6-6.1)% as ferritin.

Heat-damaged erythrocytes.

Damaged red cells containing 50 µg iron as (^{59}Fe)-haemoglobin were injected into 21 normal animals. After 45 minutes three of the animals were killed, and the remaining rats were divided into equal groups. An intravenous injection of

10 mg DFA was given to the one group at this time, and this was followed by an intramuscular injection of the same amount every hour for 3 hours. Three animals from each group were killed at 4, 24 and 48 hours respectively.

At 45 minutes virtually all the injected radioactivity was in the livers and spleens, but by 4 hours the figure had diminished to almost half (Table II). Thereafter it continued to decline, but at a slower rate. In the animals given DFA the figures were somewhat lower at each time, but the differences were small. Excretion of radioiron after 48 hours was less than 1% in the control group, but in the animals given DFA it rose from a mean of 6.6% at 4 hours to 15.1% at 48 hours. Almost identical amounts of ^{59}Fe were incorporated into circulating erythrocytes in both the control and the desferrioxamine groups.

In a final study, nine hypertransfused animals were given the same amount of labelled haemoglobin iron in denatured erythrocytes as in the previous experiment. On this occasion, however, no procedures were carried out before 24 hours, at which time

TABLE II

EFFECT OF DESFERRIOXAMINE (DFA) ON THE DISTRIBUTION OF RADIOIRON IN RATS AT VARIOUS TIMES AFTER THE INJECTION OF DENATURED ERYTHROCYTES CONTAINING 50 µg ^{59}Fe IN HAEMOGLOBIN

	Time after injection						
	45 min		4 hr		24 hr		48 hr
	Control	DFA	Control	DFA	Control	DFA	Control
No. of rats	3	3	3	3	3	3	3
Mean % in liver and spleen	99.0 (98.4-99.9)	51.7 (48.7-55.5)	54.8 (47.1-62.9)	32.2 (25.7-36.4)	46.0 (37.6-54.7)	30.0 (23.9-37.1)	37.5 (35.0-41.8)
Mean % in hepatic and splenic ferritin	8.9 (8.7-9.2)	24.2 (22.8-25.9)	30.3 (18.1-36.6)	14.2 (11.8-15.5)	21.7 (17.8-29.5)	14.1 (9.7-19.2)	20.8 (19.9-21.9)
Mean % in hepatic and splenic haemoglobin	83.9 (81.5-86.2)	12.8 (11.1-14.8)	13.7 (12.9-14.8)	2.1 (1.2-3.3)	4.2 (3.5-5.1)	0.4 (0.0-0.6)	0.3 (0.1-0.5)
Mean % in circulating erythrocytes	-	-	-	23.2 (14.3-33.8)	31.5 (28.6-34.2)	48.2 (45.0-54.0)	47.9 (43.4-49.2)
Mean % excreted	-	6.6 (5.1-9.5)	0.6 (0.0-2.9)	11.6 (9.5-15.4)	-	15.1 (11.7-20.0)	1.0 (0.0-1.3)

The range of results is given in parentheses.

three animals were killed. Virtually all the radioactivity was present in the livers and spleens. Three of the remaining animals were given intramuscular injections of 10 mg DFA twice daily for 8 days, the control rats receiving injections of isotonic saline, and further transfusions of 3 ml packed homologous erythrocytes were given at 24 hours, 3 days and 6 days. When the animals were killed after 8 days, the controls had excreted a mean(range) of 4.8(0.3-11.2)% compared with 47.4(41.0-49.8)% in the DFA animals. Virtually all the remaining radioiron was in the livers and spleens in both groups, a mean(range) of 59.7 (54.5-63.3)% being present as ferritin in the control rats and 23.0(21.9-24.3)% in the DFA rats. Less than 2% had been incorporated into circulating erythrocytes in either group.

DISCUSSION

Under most circumstances the quantity of iron excreted after an injection of DFA is at least roughly proportional to the amount of storage iron in the body. This suggests that the iron storage compounds ferritin and haemosiderin must be the ultimate source of the chelated iron. Support for this concept was obtained in the present study. Ferritin labelled with ^{59}Fe was injected intravenously and was rapidly taken up by the liver. This iron appeared to enter the physiological circuit, since a large proportion of the ^{59}Fe was incorporated into haemoglobin during the succeeding days. In the animals given DFA some of the ^{59}Fe was chelated and excreted, but without affecting the supply of isotope to the bone marrow. When the marrow demand for iron was stimulated by prior venesections, however, there was not enough ^{59}Fe to supply both erythropoiesis and DFA; in this experiment a mean of only 37% of the injected radioactivity was present in circulating erythrocytes in the DFA group compared with a mean of

56% in the control animals. It is therefore apparent that the chelate was binding iron destined for incorporation into haemoglobin. However, this must have occurred prior to the entry of the iron into the plasma, since the results of several previous investigations (Hallberg and Hedenberg, 1965a; Balcerzak et al, 1966) indicate that DFA does not chelate transferrin-bound iron in vivo.

While these findings supported the concept that ferritin is the ultimate source of the iron chelated by DFA, they did not define whether iron is removed directly from ferritin by the chelate, or whether some other iron compound or compounds in equilibrium with storage iron is the proximate source. The same holds for studies by other workers, in which the ferritin concentrations in the livers and spleens of rabbits and rats were shown to be significantly reduced by the administration of DFA (Wöhler, 1964; Cumming et al, 1969). Moreover, in the present study it was not possible to estimate the actual quantities of iron excreted by the animals in the experiments using

^{59}Fe without making assumptions concerning the sizes of the pools labelled by the isotope. These difficulties were, however, overcome in the experiment in which the storage iron contents of the livers and spleens were estimated chemically. The results obtained indicate that the iron storage compounds are not the major immediate source of the chelated iron. There was approximately 900 µg less non-haem iron in the livers and spleens of the rats given DFA injections than in the control animals, and this iron had presumably been chelated and excreted. In contrast, there was no significant differences between the storage iron concentrations in the organs of the hypertransfused rats given DFA and those given saline. By inhibiting erythropoiesis, hypertransfusion reduced greatly the amount of iron released from the stores into the plasma. If ferritin or haemosiderin were the immediate source of the chelated iron, this would not be expected to decrease the amount of iron bound by DFA in the hypertransfused animals. On the contrary, more should have been chelated, since the stores were larger in these animals. The fact that the DFA-induced iron excretion was

reduced by hypertransfusion suggests strongly that the chelate obtains iron predominantly from some compound or compounds on the pathway between ferritin and plasma transferrin.

Experiments with radioiron demonstrated that chelation of iron from the liver and spleen was not totally abolished by hypertransfusion. In whatever form the ^{59}Fe was introduced, the radioactivity present in the organs diminished significantly when DFA was injected. This was noted not only in normal rats but also in animals in which erythropoiesis had been suppressed by hypertransfusion. It should, however, be noted, that there is still some exchange between stores and plasma under such circumstances. On this basis the apparent discrepancies between the chemical and isotopic data suggest that it is only the most recently deposited iron which participates in this exchange.

The nature of the iron compound (or compounds) vulnerable to the chelate remains a matter for speculation. Little is known about the processes involved in the liberation of iron from haemoglobin

and its subsequent re-entry into the plasma or incorporation into storage compounds. It is, however, known that there is only limited exchange between pre-existing storage iron and iron released into the plasma from catabolized haemoglobin (Garby and Noyes, 1959b). It seems reasonable to postulate that after seperation from the porphyrin the iron enters a pool from which it may be taken up by transferrin or be incorporated into ferritin. Karabus and Fielding (1967) suggest that iron released from stores passes through the same pool before entering the plasma. There is some indirect evidence as to the form of iron passing through cells en route to transferrin. In the previous chapter evidence was presented that the reducing agent ascorbic acid is necessary for the release of catabolized haemoglobin iron to transferrin. In addition the vitamin is necessary for the release of iron from ferritin in reticulo-endothelial cells, while xanthine oxidase has been shown to play a part in the liver (Mazur et al, 1958). These findings suggest that iron from both ferritin and haemoglobin must be converted to the ferrous form at some stage prior

to its release to plasma. Since both transferrin and DFA bind only ferric iron, the re-oxidation of the released iron must presumably occur before it is chelated by them. Experiments with copper-deficient swine suggests that this may be brought about by caeruloplasmin (Lee et al, 1968; Ragan et al, 1969). This enzyme acts intravascularly (Roeser et al, 1970). It seems reasonable, therefore, to postulate that iron is delivered to the cell membrane in the ferrous form where it is oxidized and thus becomes available for chelation by either transferrin or DFA.

The findings of this investigation indicate that the amount of iron chelated by DFA depends, not only on the quantity of storage iron but also on the amount of iron delivered to the cell membrane for release to transferrin. Since under most circumstances DFA-induced urinary iron excretion correlates well with the amount of storage iron, an equilibrium must exist between the quantity of iron in ferritin and haemosiderin and the quantity at the cell membrane at any one time. When marrow demands for iron are suppressed, this equilibrium

presumably shifts so that less reaches the cell membrane and less is chelated by DFA. The results of previous investigations support this hypothesis. Administration of ascorbic acid to scorbutic iron loaded Bantu results in a prompt rise both of the plasma iron (Bothwell et al, 1964; Wapnick et al, 1970) and of the quantity of iron excreted after injecting DFA (Wapnick et al, 1969). Furthermore, administration of non-viable erythrocytes similarly leads to an increase in both the amount of iron delivered to the plasma (Noyes et al, 1960) and the DFA-induced urinary iron excretion (Hedenberg, 1969). Finally at the commencement of therapy of pernicious anaemia with vitamin B_{12} a prompt fall in both DFA-induced urinary iron excretion (Karabus and Fielding, 1967) and the plasma iron concentration and plasma iron turnover is noted (Finch et al, 1956). Once again, therefore, less iron is being released to transferrin and less iron is consequently chelated by DFA.

Information was also obtained concerning the question of the cellular origin of the iron chelated by DFA. Although it has previously been

shown (and confirmed in the present study) that iron stores are diminished in both spleen and liver by the administration of DFA (Wöhler, 1964; Cumming et al, 1969), it is not possible to deduce from this that iron in both organs was directly chelated. The possibility that only one of the organs was supplying iron to the chelate, so that a greater proportion of the iron for erythropoiesis was provided by the other, cannot be excluded. The experiments with radioiron in the present study have, however, provided evidence that iron within hepatic parenchymal cells and hepatic and splenic reticulo-endothelial cells is chelated by DFA. Labelled iron was introduced into the different cell types by injecting heat-denatured erythrocytes in one set of experiments and transferrin-bound iron in the other. Damaged erythrocytes are engulfed by reticulo-endothelial cells, and not by the cells of the liver parenchyma, whereas the fraction of transferrin iron which is not taken up by the red cell precursors enter parenchymal cells, and not reticulo-endothelial cells (Finch et al, 1965). Radioiron was introduced in both forms, and as early as 4 hours after the start

of the experiments considerable differences were observed between the DFA and control rats with regard to the amounts of radioactivity present in the organs. Little re-distribution could have occurred at this stage, so that it seems reasonable to conclude that the chelate obtained ^{59}Fe from both cell types. The quantitative importance of each of these sources could not, however, be calculated, as this would involve assumptions concerning the size of the pools in which the radioiron was diluted.

SUMMARY

The factors modifying the release of iron from reticulo-endothelial cells were studied in rats by injecting heat-denatured erythrocytes containing (^{59}Fe)-haemoglobin. The cells were rapidly taken up by the liver and spleen, and a proportion of the ^{59}Fe was released into the plasma, the maximum rate being between one and four hours after injection. The remaining ^{59}Fe was incorporated into storage compounds. A ten-fold variation in the load of denatured erythrocytes produced a proportional change in the amount of iron released, the percentage remaining constant. Percentage release of ^{59}Fe was enhanced in venesected rats and diminished in hypertransfused rats. Release was inhibited by injecting either unlabelled denatured erythrocytes or NTA-iron before the ^{59}Fe-labelled cells, the maximum effect being obtained if the interval between the two injections was 3-9 hours. Release was also inhibited by injecting NTA-iron 30 minutes after the denatured labelled erythrocytes. Inhibition was always preceded by a rise in the serum iron concentration, and was associated with an increase in the percentage of ^{59}Fe incorporated into ferritin. It is postulated that the shortage of free binding

sites for iron on transferrin delays the entry of liberated haemoglobin iron into the plasma, and consequently there is enlargement of a 'pre-release' iron pool. Iron has been shown by other workers to induce the synthesis of ferritin, and the presence of a stimulated mechanism for ferritin synthesis within the reticulo-endothelial cells would result in the diversion of an increased percentage of erythrocyte iron into storage compounds.

The role of ascorbic acid in the metabolism of storage iron was investigated in guinea-pigs. Ascorbic acid deprivation increased the total non-haem iron concentration in the spleen and reduced it in the liver, and in both organs ferritin was diminished and haemosiderin increased. Replacing the ascorbic acid restored the normal distribution of iron between the two storage compounds, and in the spleen the total storage iron concentration returned to control levels within 24 hours. Evidence was obtained in experiments with ^{59}Fe that the accumulation of iron in the spleen was due to a diminished release from reticulo-endothelial cells. When ^{59}Fe-labelled haemoglobin in denatured red

cells was injected, release of the isotope was inhibited in scorbutic animals. In contrast, ^{59}Fe in the liver parenchymal cells after injecting labelled transferrin was released to a greater extent than in normals. These observations suggest that ascorbic acid is necessary for the release of iron from reticulo-endothelial cells but not from liver parenchymal cells. The vitamin also appears necessary for the prevention of excessive formation of haemosiderin from ferritin. These results, in addition, may explain certain ferrokinetic peculiarities in patients with scurvy, and possibly also the predominantly reticulo-endothelial localization of the iron in Bantu subjects with siderosis.

Finally, the site of action of desferrioxamine was investigated in rats. Repeated injections of desferrioxamine significantly depleted the hepatic and splenic iron stores in normal rats, but not in rats which had been hypertransfused so as to reduce the rate of release of iron into the plasma. These observations indicate that the storage compounds ferritin and haemosiderin are not important direct donors of iron to desferrioxamine. Since plasma

iron is not bound by the chelate, some compound or compounds on the pathway between the stores and the plasma is probably the major immediate source. It is postulated that the chelate acquires iron at the cell membrane after it has been oxidized by caeruloplasmin. By differentially labelling the iron in hepatic parenchymal cells and reticulo-endothelial cells with ^{59}Fe, evidence was obtained that desferrioxamine chelates iron in both cell types.

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