FACTORS INVOLVED IN THE REGULATION OF INTERNAL IRON EXCHANGE

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A Dissertation Submitted to the Faculty of Medicine
University of the Witwatersrand, Johannesburg
for the Degree of Masters of Science in Medicine

June 1989

DECLARATION

This thesis is my own work. No part of it has been presented at any other university. I obtained the information used in this thesis while employed by the University of the Witwatersrand and the Medical Research Council of South Africa (in the MRC Iron and Red Cell Metabolism Research Unit).

All investigations included in this thesis have been approved by the Animal Ethics Committee of the University of the Witwatersrand (certificate of approval number: 88/28.)

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ABSTRACT

The effects of various manoeuvres on the handling of ^{59}Fe labelled heat damaged red cells (^{59}Fe HDRC) by the reticuloendothelial system were studied in rats. The study demonstrated that prior saturation of transferrin with oral carbonyl iron had little effect on splenic release of ^{59}Fe but markedly inhibited hepatic release. Splenic release was, however, inhibited by the prior administration of unlabelled HDRC. The combination of carbonyl iron and unlabelled HDRC exerted a similar effect. When carbonyl iron was administered with unlabelled free haemoglobin, the pattern of ^{59}Fe distribution was the same as that observed when carbonyl iron was given alone. ^{59}Fe ferritin was identified in the serum after the administration of ^{59}Fe HDRC but the size of the fraction was not affected by prior saturation of transferrin. Sizing column analyses of tissue extracts from the spleen at various times after the administration of ^{59}Fe revealed a progressive shift of ^{59}Fe from haemoglobin to ferritin, with only small amounts of ^{59}Fe present in a small molecular weight fraction. The small molecular weight fraction was greater in hepatic extracts, with the difference being marked in animals that had received prior carbonyl iron. The increased hepatic retention of ^{59}Fe associated with saturation of transferrin was reduced by a hydrophobic ferrous chelator (2,2' bipyridine), by a hydrophilic ferric chelator (desferrioxamine) and by an extracellular hydrophilic ferric chelator (diethylene triaminepenta-acetic acid). Transmembrane iron transport did not appear to be a rate limiting factor in iron release, since no

differences in ^{59}Fe membrane fractions were noted in the different experimental settings. These findings are consistent with a model in which reticuloendothelial cells release iron from catabolized red cells at a relatively constant rate. When transferrin is saturated a significant proportion of the iron is transported from the spleen to the liver in small molecular weight complexes or in ferritin. While a saturated transferrin has no effect on the release of iron from reticuloendothelial cells, prior loading with HDRC does condition them to release less iron.

ACKNOWLEDGEMENTS

I would like to express my gratitude and sincere appreciation to Dr R.D. Baynes under whose guidance, support and inspiration the investigations in this thesis were carried out.

I would like to thank Professor T.H. Bothwell, in whose research unit these investigations were carried out, for his helpful comments and suggestions.

Thanks to Dr B Macfarlane and Mr P Sera for help with laboratory animals and to Mrs U Schmidt, Mrs G Bukofzer, Mrs A Tal, Dr R Lamparelli, Dr G Carr and Dr S Lapinsky for technical assistance. My thanks also to Sabax Ltd for performing the limulus lysate assay.

During the performance of this work I was employed by the Medical Research Council of South Africa in their Iron and Red Cell Metabolism Research Unit, and I am grateful to them for their support.

Thanks to my wife for her moral support and encouragement at all times during the completion of this thesis.

PREFACE

Internal iron exchange involves a circuit in which iron is released from cells of iron procurement (gut mucosal cells, reticuloendothelial cells, hepatic parenchymal cells and placental cells) to the transport protein transferrin and is delivered to erythroid precursors and other actively growing cells. Senescent red blood cells are taken up by reticuloendothelial cells and the iron so released is recycled (Bothwell et al, 1979; Morgan, 1981; Huebers and Finch, 1987). While the processes involved in transferrin iron delivery to erythroid and other cells have been well characterized (Morgan, 1981; Huebers and Finch, 1987) little is known of the mechanism of and factors regulating reticuloendothelial iron metabolism and release. Previous work involving the parenteral infusion of elemental iron to block transferrin binding sites for iron has suggested that cellular iron release is inhibited in the presence of a highly saturated transferrin (Lipschitz et al, 1971c; Bergamaschi et al, 1986). The current investigation aims to define the effects of various manoeuvres on internal iron exchange. These manoeuvres include an increased transferrin saturation after enteral carbonyl iron administration, prior administration of heat damaged erythrocytes, venesection, haptoglobin depletion and the administration of various classes of chelators. Through these manoeuvres it is hoped to obtain further insight into not only the regulation of internal iron exchange but also the nature of reticuloendothelial iron release. A knowledge of the mechanisms and nature of iron release may shed further light on the

pathogenesis of disorders of iron overload such as idiopathic haemochromatosis.

In chapter one, an overview of the factors involved in internal iron exchange is presented. Chapter two deals with the methods and materials utilized in this investigation. In chapter three the results of the investigation are presented. Chapter four contains a discussion of the results obtained and concludes with a model of internal iron exchange formulated from the results of this investigation.

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CHAPTER ONE

AN OVERVIEW OF THE FACTORS INVOLVED IN INTERNAL IRON EXCHANGE

1.1. INTRODUCTION

Iron is an essential element of nearly all living cells and organisms and participates in numerous metabolic pathways (Morgan, 1981; Crichton and Charloteaux-Waters, 1987). The major portion of body iron in man is in the iron porphyrin complexes haemoglobin, myoglobin and a variety of haem containing enzymes. There are also many non-haem enzymes which either contain iron or which require it as a co-factor (Bothwell et al, 1979). Haemoglobin and myoglobin function as reversible binders of molecular oxygen, with haemoglobin transporting oxygen to respiring tissues and myoglobin serving as an oxygen store in skeletal muscle. The haem and non-haem enzymes participate in reactions ranging from activation of oxygen, nitrogen, and hydrogen to the control of electron flow through numerous bio-energetic pathways (Bothwell et al, 1979).

Iron is also stored within the body as ferritin and haemosiderin which are designed for holding iron in a relatively non-reactive form. About 35 to 40 mg per kg of total body iron is present in the functional iron compounds in man and depending on the iron status of the individual, between 0 and 20 mg per kg of total body iron may be present in the storage iron compartment of man (Bothwell et al, 1979). In total the iron content of man is approximately 50 mg per kg.

Despite the fact that iron is the fourth most abundant element and the second most abundant metal in the earth's crust, in the environment

iron exists almost exclusively in the oxidised state, a factor that greatly reduces its accessibilty (Bothwell et al, 1979). Man in particular finds iron difficult to obtain and absorbs each day only 1-2% of the amount that other mammals achieve (Finch and Huebers, 1982). For this reason the body exhibits great economy in its handling of iron, and internal iron kinetics are largely concerned with the continuous redistribution of iron within the body.

Internal iron exchange involves the circuit of iron release from cells of iron procurement, namely gut mucosal cells, reticuloendothelial cells, hepatic parenchymal cells and placental cells, to the transport protein transferrin, the delivery of transferrin iron to erythroid precursors and other actively growing cells and finally the recycling of erythroid cell iron by the reticuloendothelial and hepatic parenchymal cell (Bothwell et al, 1979; Morgan, 1981; Huebers and Finch, 1987). Ferrokinetic analyses have shown that the largest fraction of plasma iron (24 mg per day) is destined for the synthesis of haemoglobin in developing erythrocytes, whilst about 5 mg per day is exchanged between transferrin and parenchymal tissues (Bothwell et al, 1979).

The factors involved in internal iron exchange are presented in this chapter. Plasma transport of iron is described in section 2. In section 3, iron delivery to erythropoietic cells, the regulation of iron uptake by erythropoietic cells and erythrocyte ageing are discussed. Section 4 deals with the iron entry into

reticuloendothelial cells, haemoglobin degradation in the reticuloendothelial cell and iron metabolism in the reticuloendothelial cell. Hepatocyte iron uptake is covered in section 5 and the proteins of iron storage are discussed in section 6. Iron release from reticuloendothelial cells and hepatocytes is discussed in section 7, and the low molecular weight iron pool is covered in section 8. Section 9 deals with chelatable iron and chelating agents.

1.2. PLASMA TRANSPORT OF IRON

1.2.1. Introduction

Iron is the fourth most abundant metal in the earth's crust (Bothwell et al, 1979). However, iron exists almost exclusively in the ferric state in the external environment. Its existence in the ferric state is a factor which greatly reduces the accessibility of iron to living organisms. This is because ferric iron is unstable in solution at neutral pH and is rapidly hydrolysed to insoluble or soluble but biologically unavailable forms of ferric hydroxide . This problem has been overcome by living organisms in their development of iron complexing agents. These agents serve to solubilize iron in the extracellular fluid and are involved in the transport and delivery of iron to cells.

In vertebrates, over 95% of plasma iron is normally bound to the transport protein apotransferrin which provides the mechanism by which the needs of the individual body tissues for iron can be satisfied (Bothwell et al, 1979). The remainder of plasma iron is known collectively as non-transferrin bound iron and is complexed to other iron compounds which are present in plasma in much smaller quantities than transferrin. Such compounds include haptoglobin, haemopexin and ferritin. Non-specific binding of iron to plasma proteins and low molecular weight complexes, as occurs in situations where the specific iron binding sites of transferrin are saturated with iron, also constitutes non-transferrin bound iron.

The following section will describe transferrin and non-transferrin-bound iron.

1.2.2. Transferrin

1.2.2.1. Chemistry

Transferrin is a glycoprotein with an ellipsoid shape (Morgan, 1981; Huebers and Finch, 1987). It consists of a single polypeptide chain that has two similar domains, each of which constitutes half the molecule and each of which contains one iron binding site (Morgan, 1981). The 2 binding sites are the acid labile N terminal and the acid stable C terminal site (Huebers and Finch, 1982). The polypeptide chain is composed of 678 amino acid residues which, together with 2 N-

linked oligosaccharide chains gives it a molecular weight of 80000 D (MacGillivray et al, 1983). The function of these two oligosaccharide chains, which are localized to the carboxy half of the transferrin molecule, is unknown (Morgan, 1981; Huebers and Finch, 1987).

The two iron binding sites of transferrin each bind iron independently (Morgan, 1981; Huebers and Finch, 1987). The transferrin pool is not homogenous and transferrin circulates in plasma and interstitial fluid in four forms depending on its degree of saturation with iron. Transferrin may exist as the diferric-, monoferric a-, monoferric b-, or apotransferrin form. These forms may exist simultaneously in plasma (Bothwell et al, 1979; Finch and Huebers, 1982; Huebers et al, 1984). There is no evidence that the demonstrable differences in in vitro behaviour of the two binding sites on transferrin have any significance insofar as physiologic iron transport is concerned (Van der Heul et al, 1981; Huebers et al, 1981; Delaney et al, 1982). Diferric transferrin does however appear to deliver iron more efficiently to tissues than does monoferric transferrin (Huebers et al, 1983a). Loading of transferrin with iron appears to occur at random so that it is possible to predict the distribution of diferric, monoferric and unloaded molecules at various saturations of transferrin with iron (Finch and Huebers, 1982).

Transferrin is similar to two other iron-binding glycoproteins, namely lactoferrin and conalbumin. All three proteins have similar molecular weights, length of polypeptide chains, carbohydrate moieties, iron-

binding sites and some shared amino acid sequences (Aisen and Leibman, 1972; Metz-Boutigue et al, 1984). However, they differ in their immunologic properties, their function and their sites of highest concentration. The maximal iron-binding capacity of these different proteins is highly pH dependent. Whereas transferrin binds iron most avidly at a neutral to alkaline pH, conalbumin binds iron at an alkaline pH and lactoferrin binds iron at an acidic pH (Montreuil et al, 1960; Weinberg, 1984). Lactoferrin cannot provide iron to reticulocytes (Brock and Esparza, 1979). When present in plasma, diferric lactoferrin is cleared by reticuloendothelial cells and hepatocytes.

1.2.2.2. Transferrin synthesis

The principal site of serum transferrin production is the hepatocyte (Morgan, 1981). The other major site of transferrin production is the lactating mammary gland (Jordan and Morgan, 1969). Regulation of transferrin production has been closely linked with the status of iron stores. Transferrin production is increased with iron store depletion and decreased with iron store repletion (Lipschitz et al, 1974).

It has not yet been elucidated exactly what molecular events regulate transferrin gene expression in iron deficiency (Idzerda et al, 1986). Studies on the livers of iron deficient chicks and rats showed them to contain elevated levels of transferrin mRNA (Mcknight et al, 1980a; Idzerda et al, 1986). In rat hepatocytes, the elevated levels of

transferrin mRNA were found to result from induction of transferrin gene transcriptional activity rather than from the regulation of translational activity (Idzerda et al, 1986).

Stimulation of transferrin synthesis also occurs in pregnancy and during the administration of oestrogens (Bothwell et al, 1979; Morgan, 1981). The effects of oestrogens also appear to be related to increased mRNA transcription (Mcknight et al, 1980b). Plasma transferrin concentration is decreased in protein depleted states, probably as a result of depletion of the amino acid protein synthesis pool (Morgan, 1981).

1.2.2.3. Iron exchange with transferrin

As mentioned above, there are two specific metal binding sites on transferrin, each of which binds one iron atom (Morgan, 1981). The binding of iron to transferrin requires the association of the transferrin molecule with carbonate or bicarbonate anions (Morgan, 1981). These anions function to secure the iron into place in the transferrin molecule by serving as a bridging ligand between iron and the protein (Huebers and Finch, 1987). The anions also appear crucial in iron release from transferrin (Egyed, 1973). While in vitro studies appear to indicate that both ferrous and ferric iron serve as the source of transferrin iron, it is still unknown whether iron in vivo is presented to the transferrin molecule in the ferric or the ferrous form (Crichton and Charloteaux-Wauters, 1987; Huebers and Finch, 1987).

Iron binding by transferrin *in vitro* is pH dependent and is maximal above pH 7 (Morgan, 1981). While iron is bound to transferrin, the diffusion properties and rate of penetration of iron through cellular membranes such as the capillary wall have the characteristic features of its carrying protein transferrin rather than the features of a small molecule or ion. Thus, when iron is bound to transferrin, iron loss from the body by passage through surface membranes, as occurs in the kidney is greatly reduced. Another major consequence of transferrin-iron binding is that the free iron concentration in body fluids is extremely low, thereby avoiding the harmful effects of adsorption of iron to cell membranes. In addition, transferrin minimizes iron losses from the body by depositing surplus iron in tissues designed for iron storage (Bothwell et al, 1979).

As a result of the high affinity of transferrin for iron, the spontaneous dissociation of iron from transferrin cannot occur at a significant rate (Aisen and Leibman, 1968). The consequence of this is that either the whole transferrin-iron complex must be broken down to supply iron to cells, or special mechanisms for the detachment of iron from transferrin must exist. The mechanism of iron delivery to cells by transferrin has been described in detail recently, and is discussed in section 1.3.1. A review of current knowledge on the subject of iron release from reticuloendothelial and hepatic cells to transferrin is presented in section 1.7. The experimental findings of this study with regard to this subject are presented in chapter 4.

1.2.2.4. The role of transferrin in intestinal iron absorption

Besides being found in serum, mammalian transferrins have been found at other sites in the body. One such site is the small intestine, where transferrin is thought to be involved in the process of inorganic iron absorption.

Transferrin has been found in the lumen of the small intestine, on the mucosal surface and inside the mucosal cells of the duodenum and jejunum (Huebers et al, 1976). The source of this transferrin is uncertain. It has been claimed that small intestinal epithelium contains an isotransferrin which is secreted into the gastrointestinal lumen in a similar manner to the secretion of siderophores by bacteria (Huebers et al, 1976; 1983b; Pollack and Lasky, 1976). The iron-transferrin complex formed in the intestinal lumen may then enter the intestinal epithelium by a process of receptor mediated endocytosis. This hypothesis is supported by the finding of a high molecular weight elutable factor in the lumina of iron deficient rats which appears to promote iron absorption (Huebers et al, 1976). A further possibilty on the source of the transferrin found intraluminally in the intestine is that it may be derived from biliary secretion of plasma transferrin.

Recent evidence against the mucosal production of an isotransferrin involved in intestinal iron absorption has been the inability to demonstrate transferrin mRNA in gastrointestinal mucosal cells (Idzerda et al, 1986).

Further doubt on the role of transferrin in intestinal iron absorption has recently been cast by the inability to demonstrate with the use of immunocytochemical tests the presence of transferrin receptors on the luminal surface of mucosal cells (Parmley et al, 1985) and on the brush border of mucosal cells (Banerjee et al, 1986). The inability to demonstrate an enhancing effect of transferrin on iron absorption in achlorhydric subjects (Bezwoda et al, 1986) may be seen as further evidence disputing the role played by mucosal transferrin in iron absorption.

While there is evidence disputing the direct role transferrin may play in intestinal iron absorption, transferrin may function in an as yet undetermined manner to increase iron absorption in iron deficiency. This hypothesis is supported by the finding of an increased concentration of intestinal transferrin in rats when iron deficiency is present (Halliday et al, 1976) and by the finding of an increased concentration of biliary transferrin in iron deficient rats (Huebers et al, 1983b).

1.2.2.5. The transferrin receptor

In its delivery of iron to cells, iron carrying transferrin binds to specific receptor sites on the cell membrane. The transferrin receptor is a transmembrane glycoprotein which is coded for on human chromosome 3q 21-25 (Yang et al, 1984; Rabin et al, 1985). It contains approximately 5% carbohydrate by weight (Seligman et al, 1979). The

receptor is a dimer with a disulphide linkage and each component monomer is capable of binding a diferric transferrin molecule (Morgan, 1981; Huebers and Finch, 1987). Each monomer has a molecular weight of approximately 90000 D (Enns and Sussman, 1981). The transferrin receptor has a small intracellular amino terminal domain and a large extracellular carboxy terminal domain consisting of 672 amino acids (Schneider et al, 1984). Like other receptor proteins, the transferrin receptor is amphipathic.

The binding process of transferrin carrying iron to the transferrin receptor is very efficient. Binding of transferrin to its receptor is calcium dependant (Hemmaplardh and Morgan, 1977), and maximal at pH 7.8 (van Bockxmeer et al, 1978).

The iron status of the transferrin molecule exerts an important effect on the affinity of the molecule for its receptor. The transferrin receptor has a very high affinity for differic transferrin (Trowbridge et al, 1984). Monoferric transferrin and apotransferrin respectively have less affinity for the transferrin receptor at physiological pH (Huebers et al, 1983a).

The development of monoclonal antibodies with specificity for the transferrin receptor has allowed for the immunological demonstration of transferrin receptors on cell surfaces (Schneider et al, 1982), and transferrin receptors have been found to be distributed on almost all human cell types evaluated. Receptor affinity for transferrin by

different tissues of an organism appears to be constant (Trowbridge et al, 1984).

Regulation of transferrin receptor expression by cells is not fully understood. Transferrin receptor expression on cell membranes appears to be regulated largely by the intracellular chelatable iron pool (Bridges and Cudkowicz, 1984; Bottomley et al, 1985). Evidence supporting this finding is that increased transferrin receptor expression occurs when the iron supply to cells is diminished by chelating agents or by growing cells in iron deficient media (Bottomley et al, 1985). It appears that the synthesis of the transferrin receptor is under transcriptional control. However recent data suggests that iron may down-regulate transferrin receptor expression by a mechanism similar to the one by which it induces increased translation of ferritin mRNA (Casey et al, 1988). What is clear is that non replicating cells appear to have a stable number of transferrin receptors. In contrast to this, cells undergoing multiplication markedly increase their number of transferrin receptors (Trowbridge and Omary, 1981). The finding of so many transferrin receptors on rapidly growing cells and on malignant cells raises the possibility that the transferrin receptor may play a role in host defence against neoplasia. The exact mechanism by which this may occur has not been fully elucidated. The increase in transferrin receptor number on multiplying cells occurs because transferrin-cell-iron interaction is important in the regulation of cellular growth and proliferation. Evidence for this has been the finding that transferrin

is essential for cultured cells to grow in serum-free conditions (Barnes and Sato, 1980). The proliferation of cultured cells is inhibited if their receptors are blocked by monoclonal antibodies specific for transferrin binding sites, as well as if the cells are deprived of transferrin. It would appear that the transferrin receptor interaction is the important component in growth regulation and that the effect may be independent of the iron content of transferrin (May and Cuatrecasas, 1985).

1.2.3. Non-transferrin-bound iron

1.2.3.1. Haptoglobin and haemopexin

Haemoglobin, present in the plasma as the result of intravascular haemolysis or the lysis of red cell precursors in the marrow, normally constitutes approximately 2% of plasma iron. This haemoglobin is complexed in the plasma by a special haemoglobin carrier called haptoglobin which is synthesized primarily in the liver (Bothwell et al, 1979). In the plasma, haemoglobin dissociates into dimers and each haptoglobin molecule binds two of these dimers (Nagel and Gibson, 1971). The binding of haemoglobin to haptoglobin is irreversible, and since haptoglobin production is not stepped up in response to an increased haemoglobin load, the plasma concentration of haptoglobin provides an indication of the amount of ongoing intravascular haemolysis (Bothwell et al, 1979). Haemoglobin bound to haptoglobin is

transported to the liver where it is taken up by hepatocytes (see section 1.5.3.1.).

When haemoglobinaemia exceeds the haptoglobin binding capacity of plasma, oxidation of the iron in the haemoglobin molecule permits the haem moiety to dissociate from its polypeptide chain and a second iron transport protein present in plasma called haemopexin binds the haem which is liberated (Bothwell et al, 1979). Haemopexin is synthesized in the liver and the haem haemopexin complexes are also removed from the circulation by hepatocytes (see section 1.5.3.1.). Since haemopexin production, like haptoglobin production, does not increase in response to an increased demand, its plasma concentration has a similar significance to the plasma concentration of haptoglobin (Muller-Eberhard et al, 1968). If the haemopexin is exhausted, haem binds to albumin until fresh haemopexin is synthesized (Bothwell et al, 1979).

1.2.3.2. Serum ferritin

Small amounts of ferritin (usually less than 1% of serum iron) are found circulating in the plasma of normal individuals (Bothwell et al, 1979). While under normal circumstances the serum ferritin concentration is a function of the size of the body iron stores (Finch et al, 1986), its full physiological significance is not yet entirely understood.

Serum ferritin is discussed more fully in section 1.6.3. and the hepatic clearance of serum ferritin is discussed in section 1.5.3.2.

1.2.3.3. Low molecular weight plasma iron complexes

While in normal individuals the plasma concentration of iron complexed with low molecular weight compounds such as ascorbate, carbonate, certain amino acids and plasma proteins such as albumin is extremely small, much higher concentrations of total iron in plasma in this form have been found circulating in patients with severe iron overload (Hershko et al, 1978b; Anuwatanakulchai et al, 1984; Gutteridge et al, 1985; Wang et al, 1986). This may occur as a result of saturation of the binding capacity of transferrin (Brissot et al, 1985). While hepatic transferrin receptors have been found to be reduced in haemochromatosis, the underlying defect remains unknown (Sciot et al, 1987). The highly efficient hepatic uptake process of this form of iron (discussed in section 1.5.3.3.), together with the high plasma levels found in haemochromatosis have led many researchers to suggest that the low molecular weight complexes not bound to transferrin may be primarily responsible for the hepatic iron loading and toxicity that characterizes haemochromatosis and other iron loading states (Hershko et al, 1978b; Brissot et al, 1985; Wright et al, 1986; 1988).

Low molecular weight iron complexes have been shown to promote the formation of free hydroxyl radicals and to accelerate the peroxidation of membrane lipids in vitro (Gutteridge et al, 1985). The availability

of this non-transferrin plasma iron for chelation by therapeutic agents such as desferrioxamine is important as it serves as a route whereby the level of iron stores can be reduced and also as a means of decreasing the amount of iron in iron-loaded cells (Jacobs, 1977; Hershko and Weatherall, 1988).

1.3. THE ERYTHROCYTE

1.3.1. Iron delivery to erythropoietic cells

1.3.1.1. Introduction

As discussed previously, the major function of transferrin is the transport of iron from wherever it enters the plasma to the developing erythroblasts of the marrow. Iron is utilized by the erythroblasts in haemoglobin synthesis (Fairbank and Beutler, 1983). On morphological grounds, a number of stages in the development of the erythrocyte have been described. The successive morphological alterations observed as the proerythroblast develops into the erythrocyte reflect the functional specialization of these cells and their maturation (Bessis et al, 1983). Studies utilizing radiolabelled iron have shown that iron is taken up from the plasma by all erythrocyte precursors in the bone marrow (Myhre, 1964; Ward et al, 1966). Maximal iron uptake is observed at the earlier stages of red cell precursor development. However, even after loss of the nucleus, the immature erythrocyte or

reticulocyte as it is known is still able to utilize plasma iron for haemoglobin synthesis (Jandl et al, 1959). The majority of investigations concerned with the mechanism of iron exchange between transferrin and erythroid cells have been performed using reticulocytes. This is due to the fact that these cells are obtained with relative ease from peripheral blood. However, most of the data presented also applies to nucleated erythroid cells and to non erythroid cells.

While initial studies suggested that transferrin iron was delivered to and released at the erythroblast membrane (Jandl and Katz, 1963), a great deal of evidence now indicates that the release of iron from transferrin occurs within the cell itself. The uptake of transferrin iron by receptors and its intracellular cycle have been described in detail recently (Dautry-Varsat et al, 1983; Klausner et al, 1983).

The steps involved in the uptake of transferrin-bound iron by reticulocytes and nucleated erythroid precursors are: (1) transferrin binding to specific receptors on the cell membrane, (2) transferrin entry into the cell, (3) iron release from transferrin and (4) release of transferrin from the cell.

1.3.1.2. Transferrin-receptor interaction

Diferric transferrin binds to specific transferrin receptors on the cell surface. The nature of these receptors has already been dealt

with (see section 1.2.6.). The binding of transferrin to the transferrin receptor is reversible and exhibits saturation kinetics (van Bockxmeer et al, 1978; van Bockxmeer and Morgan 1977; 1979). Approximately 80000 transferrin molecules can be bound by each reticulocyte. As reticulocytes mature, there is progressive disappearance of functional transferrin receptors (Pan and Johnstone, 1984).

As mentioned previously, the binding of transferrin to its receptor is dependent on pH, the presence of calcium ions and the iron content of transferrin. Evidence for this includes the findings that apotransferrin has a lower affinity for the transferrin receptor than does diferric transferrin (Morgan, 1981), and that incubation of reticulocytes with calcium chelators causes inactivation of receptors and loss of iron uptake (Hemmaplardh and Morgan, 1977). Further evidence that cellular uptake of iron from transferrin is dependent on the binding of transferrin to the transferrin receptor is that following the proteolytic digestion of reticulocytes, there is a reduction of transferrin binding to receptors and a decrease in the rate of iron uptake by the cells (Hemmaplardh and Morgan, 1976).

1.3.1.3. Diferric transferrin internalization

Once bound to the transferrin receptor on the cell surface, diferric transferrin, together with its receptor, is taken into the cell by a process of receptor mediated endocytosis (Morgan, 1981; May and

Cuatrecasas, 1985; Wileman et al 1985; Bomford and Munro, 1985; Stahl and Schwartz, 1986). Binding to the cell membrane appears to be essential for endocytosis to occur, since other proteins such as albumin and IgG for which there are no specific binding sites are not endocytosed by reticulocytes (Hemmaplardh and Morgan, 1977).

Substances other than transferrin are also taken into cells by receptor mediated endocytosis. These include cholesterol, vitamin B_{12}, epidermal growth factor, platelet-derived growth factor, insulin, gonadotrophins, toxins such as diphtheria and pseudomonas toxin, and viruses such as influenza (Stahl and Schwartz, 1986). The receptors for these molecules are found in clathrin coated pits, clathrin being a protein that supports the receptors in the cell membrane. It appears that a number of ligands enter the cell via the same coated pit (Carpentier et al, 1982).

Once the ligand has bound to its receptor, the clathrin coated pit invaginates, loses the clathrin and forms a prelysosomal endosome, known as a receptorsome. The receptorsome is then transported by saltatory motion within the cell cytoplasm along microtubule and microfilament tracts (May and Cuatrecasas, 1985). The process appears to be energy dependent and is also sensitive to changes in temperature. Almost no endocytosis occurs at 4°C, with endocytosis increasing as the temperature is raised. Endocytosis is inhibited at temperatures above 40°C (Morgan, 1981). The mechanism responsible for this may be the denaturation of membrane proteins involved in endocytosis. That

transferrin endocytosis is blocked by a variety of inhibitors of cellular metabolism such as dinitrophenol and rotenone is evidence that endocytosis is linked to a supply of metabolic energy (Morgan, 1981).

Factors inducing internalization of the transferrin receptor, other than transferrin, include monoclonal antibodies to the transferrin receptor (McArdle and Morgan, 1984) and the phorbal esters. These esters, which are structurally unrelated to transferrin, induce internalization of the transferrin receptor, even in the absence of transferrin (May et al, 1984; 1985). These esters are thought to act by stimulating the production of calcium dependent protein kinase C. Protein kinase in turn appears to phosphorylate the transferrin receptor.

May and coworkers (1985) showed that cytoskeleton inhibitors could inhibit transferrin receptor internalization induced by phorbol esters without inhibiting transferrin receptor phosphorylation. This suggests that phosphorylation of the transferrin receptor is not the only factor involved in stimulating receptor endocytosis. Endocytosis can also be blocked by microtubular and microfilament inhibitors such as the vinca alkaloids and colchicine (Hemmaplardh et al, 1974). The foregoing discussion indicates that transferrin receptor internalization involves complex interactions between cell membranes, clathrin coated pits, the cytoskeleton, microtubules and microfilaments and the enzymes regulating phosphorylation of these structures.

1.3.1.4. Iron release from transferrin

After endocytosis of the diferric transferrin receptor complex, the interior of the endocytic vesicle becomes acidified. This occurs by an active protonation process which is energy dependent (Wileman et al, 1985). The acidified vesicle is known as the compartment of uncoupling of receptor and ligand (CURL) (Goldstein et al, 1979). Iron dissociates from transferrin in the acidified vesicle, leaving the iron-free apotransferrin in the vesicle. The efficient translocation of iron through the membrane barrier between the site of its release from transferrin in the acidified vesicle and the site of its utilization, is by a recently described high affinity carrier mediated iron transport system present in the membrane of the vesicle (Egyed, 1988).

Iron delivery to cells can be inhibited by interfering with the acidification of the endocytic vesicle. Weak bases such as chloroquine, ammonium chloride and methylamine diffuse across membranes into cells in their uncharged, lipid soluble forms. They can accumulate in the endocytic vesicle where they become protonated. This in turn can buffer the endocytic vesicle against acidification (Wileman et al, 1985).

Iron delivery to cells can also be disturbed by inhibitors of energy metabolism. The mechanism of this also appears to be interference with endosomal acidification (Kailis and Morgan, 1974). An example of such a substance is the metabolic inhibitor 2,4 dinitro-phenol.

1.3.1.5. Transferrin release from the cell

The endocytosis of most receptor-ligand complexes results in their intracellular catabolism within secondary lysosomes which are formed as a result of the fusion of the endocytic vesicle with intracellular lysosomes. An example is that of epidermal growth factor whose receptor undergoes digestion in the endocytic vesicle (Wileman et al, 1985). This is not the case for transferrin and its receptor which are recycled. The variable pH dependent affinities of diferric- and apotransferrin for the transferrin receptor is fundamental to transferrin recycling through the cell.

As mentioned, acidification of the endocytic vesicle results in dissociation of iron from transferrin, leaving the iron free apotransferrin in the vesicle. Apotransferrin has a low affinity for the transferrin receptor at physiological pH. However, in the acidic environment of the endocytic vesicle, apotransferrin has very high affinity for the receptor. This has been shown in hepatoma cells (Dautry-Varsat et al, 1983), in reticulocytes (Morgan, 1983b) and in erythroleukaemia cells (Klausner et al, 1983). After release of the iron from the endocytic vesicle, the vesicle returns to the cell surface where it fuses with the cell membrane. In the process, the contents of the vesicle are exposed to physiological pH. At this pH, the apotransferrin receptor dissociates from the receptor and is available for further iron transport.

1.3.2. Regulation of iron uptake by erythropoietic cells

The rate of iron uptake by immature erythroid cells is affected by both extracellular and intracellular factors.

The major extracellular determinant of iron uptake is the supply of transferrin-bound iron. The rate of iron uptake by reticulocytes in vitro increases as the concentration of transferrin-iron is raised, until saturation of the iron uptake process occurs. At iron concentrations below the saturating level, the supply of iron to cells will be submaximal and could be the rate limiting factor for haemoglobin synthesis (Morgan, 1981).

Cellular factors affecting the rate of iron uptake are the degree of maturation of cells and the regulatory effects of intracellular free haem. More mature cells show slower rates of uptake of iron. This is probably related to the number of transferrin receptors on the cell. With maturation of the erythroid precursors, there is a decrease in the number of transferrin receptors and thus a decrease in the capacity for iron uptake by the cell (van Bockxmeer and Morgan, 1979).

The concentration of free haem in reticulocytes may act as a regulator of iron uptake. The addition of haem to reticulocytes stimulates globin synthesis (London et al, 1964) but inhibits haem synthesis and iron uptake by the reticulocyte (Ponka and Neuwirt, 1969; 1971). Inhibition of haem synthesis with isonicotinic acid hydrazide results

in increased iron uptake by cells, while inhibition of globin synthesis reduces iron uptake by cells, probably as a result of the accumulation of free haem in the cells (Ponka and Neuwirt, 1969; 1971). Haem does not appear to inhibit transferrin uptake by the cell, but probably acts by blocking iron dissociation from transferrin (Ponka et al, 1974).

1.3.3. Erythrocyte ageing

Normal human erythrocytes survive in the circulation for about 120 days. Senescent and damaged erythrocytes are selectively removed from the circulation by the reticuloendothelial system, in particular by macrophages in the spleen and liver (Bennett and Kay, 1981). This selectivity implies that the macrophage can recognize age or damage-dependent changes on the surface of the red cell. While considerable data exist describing the mode of destruction of abnormal red cells, relatively little is known about red cell ageing and the mode of destruction of senescent red cells. The major obstacle to solving this problem has been the lack of suitable methods for separating young cells from old cells (Bunn, 1972). In spite of this a variety of changes have been described in the red cell as it ages. Their significance, if any, in the final destruction of the cell is unknown.

The uniformity of the lifespan of the erythrocyte suggests that its destruction is the result of age dependent factors. The essential steps of erythrocyte senescence occur in the cytosol (Bocci, 1981). In the anucleated erythrocyte, there is a progressive decline of

metabolism, as evidenced by a fall of ATP concentration (Bunn, 1972). ATP depletion is followed by a progressive increase of intracellular calcium and it has been suggested that this increase in calcium may be the crucial event triggering an irreversible change in the cytoskeletal system of the erythrocyte and a profound redistribution of integral membrane proteins (Sheetz and Singer, 1977; Shotton et al, 1978). The changes of the erythrocyte membrane occurring as a result of the cytosolic events mentioned may be detected by macrophages and may account for the removal of senescent red cells from the circulation.

The formation of fragments or vesicles as the erythrocyte ages also leads to important changes of the erythrocyte membrane which may be responsible for removal of senescent red cells from the circulation. Weed and Reed (1966) defined fragmentation as "loss from the cell of a piece of membrane which may or may not contain haemoglobin". The loss of portions of the red cell membrane during ageing is associated with an alteration of the lipid-protein ratio of the erythrocyte membrane and accounts for changes in the activities of membrane associated enzymes found in the erythrocyte (Kadlubowski and Agutter, 1977). Also this membrane loss may account for decreased membrane fluidity and deformability (Shiga et al, 1979). Since the viability of the red cell in the circulation is dependent on its ability to withstand changes in its shape (Weed, 1970), the decreased membrane deformability consequent upon loss of portions of the erythrocyte membrane as it ages may be an explanation for red cell destruction with ageing. In addition, it appears that older red cells are smaller than younger ones and they

have an increased density (Ganzoni et al, 1971). This may be another reason why older red cells are more rigid and less able to withstand changes in their shape.

It has been reported that older red cells contain less sialic acid than do young red cells (Aminoff et al, 1980; Bocci, 1981). Red cells depleted of sialic acid enzymatically have been found to have a shortened survival (Durocher et al, 1975). It was thus suggested that the decrease in sialic acid content of older red cells may be a factor responsible for erythrocyte destruction. However this has been a controversial issue with divergent results being obtained in experiments involving artificial desialyation of red cells. The reason for this may be that senescence of red cells induces changes more complex than those obtained by artificial desialyation of red cells and therefore the physiological meaning of those results is doubtful (Bocci, 1981). The facts of the matter are that during ageing loss of carbohydrate with portions of the red cell membrane are occurring. This may result in a decreased sialic acid content of older erythrocytes, but the surface-charge density and hence the electrophoretic mobility of the erythrocyte remains unchanged throughout the whole lifespan of the erythrocyte (Luner et al, 1977; Seaman et al, 1977).

Though the loss of sialic acid residues from the membranes of mature red cells may not alter the surface charge density of the cell, the cleavage of these sialic acid residues may mediate the removal of

senescent red cells from the circulation by another mechanism. It has been suggested that younger red cells express terminal N-acetylneuraminic acid residues on the glycoproteins of their surface membranes, whereas older red cells appear to lack such sialic acid residues on their glycoproteins (Alderman et al, 1981). The loss of the terminal sialyl residues may reveal Ig-binding sites on these desialyated glycoproteins. Antibodies, for example IgG, are directed against these binding sites (Kay, 1975). The antibody-coated senescent red cells are then opsonized and subjected to phagocytic removal by reticuloendothelial macrophages. It may be that the antibodies accumulate on the red cell surface as the cell ages and that upon reaching a critical concentration, the red cell may be sufficiently coated with antibodies to be recognised by the macrophage and be phagocytosed (Kay, 1975). In addition macrophages are well documented as exhibiting an asialoglycoprotein receptor on their surfaces (Hamilton et al, 1984).

Advanced glycosylation endproducts (AGE) on erythrocyte cell surfaces have recently been shown to promote the clearance of aged erythrocytes (Vlassara et al, 1987). The advanced glycosylation endproducts, formed as a result of the nonenzymatic reaction of glucose with amino groups of proteins (including haemoglobin), accumulate in extracellular and membrane proteins as a function of time and glucose concentration (Brownlee et al, 1984). A recently identified membrane-associated macrophage receptor that recognizes proteins modified by this process of long term nonenzymatic AGE formation has been implicated in the

preferential removal of senescent macromolecules (Vlassara et al, 1984; 1985) and may also mediate the endocytosis of erythrocytes with advanced glycosylation endproducts formed on their surface (Vlassara et al, 1987).

The loss of membrane lipid during the lifespan of mature red cells has also received attention as a possible cause for the destruction of senescent red cells. Reticulocytes are rich in membrane lipids and this excess lipid, present both in intracellular organelles and plasma membranes is lost during reticulocyte maturation (Shattil and Cooper, 1972).

Recently a new technique has been developed in an attempt to identify the basis for selective elimination of senescent red blood cells by macrophages. This technique involves the use of IC-21 cells, an established line of Simian virus 40-transformed mouse peritoneal macrophages (Walker et al, 1984). These macrophages bind and ingest populations of homologous red cells that are aged in vivo by serial hypertransfusion. It is hoped that by altering the aged red cells in various ways, the IC-21 macrophage line, used with the in vivo aged erythrocytes, will afford a model system for identifying more precisely the mechanisms of macrophage mediated phagocytosis of senescent red cells (Walker et al, 1984).

1.4 THE RETICULOENDOTHELIAL SYSTEM (RES)

1.4.1. Introduction

The RES is made up of the scattered and histologically heterogeneous cells in the body which have in common the property of phagocytosis. There are three main components of the RES. They are the reticulum cells which produce reticulin fibres, the phagocytic endothelial cells lining the blood capillaries of the liver, splenic sinusoids, lymph nodes, bone marrow, adrenal and pituitary glands and the macrophages of tissues, also known as histiocytes, and of blood, known as monocytes. After leaving the bone marrow, mononuclear phagocytes travel through the blood as monocytes before reaching those target tissues where they constitute the resident macrophage population. Resident tissue macrophages or histiocytes exist in protean forms, including the hepatic kupffer cell, alveolar macrophage of the lung, giant cell of granulomas, dermal Langerhans cell, microglial cell of granulomas, peritoneal and pleural macrophage and probably the osteoclast (Abramson et al, 1977; Cline et al, 1978). Although macrophages, endothelial cells and fibroblasts are anatomically associated in the RES, these cell types are not developmentally related. Macrophages are derived from marrow haemopoietic progenitors, whereas endothelial cells and fibroblasts are somatic cells derived from the entoderm and mesenchyme respectively.

The RES plays an important role in the internal iron economy of the body. This function is effected by the uptake and catabolism of effete and senescent red cells, the removal of iron from the haemoglobin and the return of this iron to the plasma where it is bound by transferrin and transported to the bone marrow (Lynch et al, 1974). In the bone marrow, the iron is utilised in haemoglobin synthesis. In addition to being the main source of iron entering the plasma, the RES is also concerned with the storage of iron which is not immediately required for the synthesis of metabolically active compounds. Under normal circumstances, the reticuloendothelial cells of the spleen, liver and bone marrow are those most concerned with iron metabolism.

The following section will deal with 3 aspects of reticuloendothelial-iron interactions, namely iron entry into reticuloendothelial cells, haemoglobin degradation and iron metabolism in reticuloendothelial cells.

1.4.2. Iron entry into reticuloendothelial (RE) cells

The RES derives most of its iron directly from catabolised haemoglobin. Two-thirds of this iron comes from red blood cells sequestered by the RES at the end of their lifespan. The remainder is derived from haemoglobin released as a result of cellular wastage during erythropoiesis and from non-haem iron that has been removed from viable erythrocytes (Cook et al, 1970).

Extracellular haemoglobin present in plasma does not reach the RES. It is largely bound to haptoglobin and haemopexin and is removed from the circulation by hepatocytes (see section 1.5.3.1.) or by the kidney (Noyes et al, 1960). Small quantities of ferritin within erythrocytes transferred directly to RE cells also provide source of iron for the RES (Deiss and Cartwright, 1970).

Another source of iron entering the RE cell is transferrin iron. Transferrin receptors have been found on human macrophages and it has been shown that transferrin iron is taken up by these cells.

1.4.2.1. Phagocytosis of aged, abnormal or damaged erythrocytes

Senescent, damaged and abnormal red blood cells are selectively removed from the circulation and catabolised by cells of the RES, in particular by macrophages in the spleen, liver and to a lesser extent by cells in the bone marrow (Bennett and Kay, 1981). The most commonly used method of the study of the sites of phagocytosis of red cells involves the infusion of damaged red cells, usually labelled with radioiron. This is followed either by surface counting or by direct counting of extripated organs to identify sites of concentration of the isotope (Deiss, 1983). Several modes of cell damage have been used, including ageing, heat damaging and chemical and immune injury to the cell. As discussed previously, the actual alterations in senescent red cells that determine the end of their lifespan have not yet been fully elucidated (section 1.3.3.). While the phagocytic function of

mononuclear phagocytes is generally acknowledged, the mechanisms resposible for the ingestion of aged erythrocytes remain a matter of debate.

Most of the phagocytosis of senescent red cells occurs in the sinusoids of the liver and spleen which are large capacitance systems. Thus the slow passage of blood through these sinusoids, combined with the presence of large numbers of phagocytes with extended dendritic processes provides favourable conditions for macrophages to recognise and phagocytose cells (Kay, 1975).

The contact between the erythrocyte and the macrophage induces the subsequent events. A small area on the surface of the erythrocyte initially attaches to the macrophage. This is followed by sphering of the red cell and its envelopment by a hyaloplasmic veil from the phagocyte (Lynch et al, 1974). After the disintegration of the erythrocyte, the haemoglobin is initially located mainly in endocytic vacuoles known as phagosomes. These then merge with primary lysosomes which supply lytic enzymes capable of degrading haemoglobin (Lynch et al, 1974).

1.4.2.2. Transferrin receptor activity on macrophages

Initially it was believed that iron entered the RES exclusively by the phagocytosis of red cells and their waste products. However, it became clear that RE cells could also take up transferrin-bound iron in vitro.

Macsween and coworkers (1969) were the first to demonstrate that pulmonary and peritoneal macrophages and Kupffer cells assimilated transferrin-bound iron from a prepared medium. These findings are in keeping with subsequent observations of the progressive uptake of transferrin by cultured macrophages (Summers and Jacobs, 1976; Wyllie, 1977; Sizemore and Bassett, 1984; Baynes et al, 1987c).

Macrophages appear to be heterogeneous in their expression of transferrin receptors. Human monocytes and resident and activated macrophages have absent or diminished transferrin receptor expression. Transferrin receptor expression appears to be enhanced on responsive macrophages and cultured macrophages (Andreesen et al 1983; Parmley et al, 1983; Hamilton et al, 1984). The resident tissue macrophage represents a quiescent cell in that it does not respond to lymphokine or endotoxin by becoming cytolytic . A responsive macrophage is one that becomes cytolytic when exposed to endotoxin after prior lymphokine stimulation. An activated macrophage is fully cytolytic.

Until recently iron uptake by cultured macrophages has been assumed to be by receptor-mediated endocytosis. There was however little direct evidence for this. A recent study by Baynes and colleagues (1987c) confirmed the existence of differic transferrin receptors on the surface of cultured human blood monocytes and demonstrated the receptor mediated uptake of transferrin iron by cultured macrophages. This uptake was shown to be by an endocytic pathway very similar to that noted in erythroid precursors.

The reason for the existence of such a pathway in RE cells is unclear, since these cells acquire large amounts of iron from the haemoglobin of effete and senescent cells. It may be that the interaction between transferrin iron and macrophages plays a role in regulating the differentiation and proliferation of resident macrophages (Andreesen et al, 1983; Galbraith et al, 1980; May et al, 1985).

1.4.3. Haemoglobin degradation

Following phagocytosis and red cell lysis, haemoglobin (Fe^{2+}) is converted into methaemoglobin (Fe^{3+}). After globin is split from methaemoglobin it is hydrolysed to its constituent amino acids, which mix with the general amino acid pool (Ehrenreich and Cohn, 1968). Iron is released from the haem (Fe^{3+}) by the action of microsomal haem oxygenase (Tenhunen, 1972). This enzyme is demonstratable in peritoneal macrophages, blood monocytes, Kupffer cells, macrophages of the bone marrow and lung, in brain glial cells, as well as in hepatocytes and renal tubular epithelial cells. Activity of haem oxygenase is highest in the spleen, followed by the bone marrow and then the liver (Tenhunen et al, 1970). Activity in all these cells may be induced by exposing them to haem compounds.

Microsomal haem oxygenase utilises molecular oxygen and NADPH in its degradation of haem. The NADPH is generated through an NADPH dependent cytochrome c reductase (Elder, 1980). Haem oxygenase disrupts the alpha carbon bridge of the tetrapyrrolic haem molecule. For every

molecule of haem thus degraded one molecule of biliverdin and one molecule of carbon monoxide result. The biliverdin is promptly converted to bilirubin by a second enzyme, biliverdin reductase, which is present in the soluble fraction of the same cells in which biliverdin is formed from haem (Tenhunen, 1972).

1.4.4. Iron metabolism in the RE cell

Once iron is freed from haemoglobin, it may either return to the plasma or become incorporated in iron stores. The proportion following each route is influenced by various factors. The incorporation of iron into iron stores is covered in section 1.6. and the factors influencing iron release from cells is discussed in section 1.7.

Initial studies showed that after the release of iron from haem, part of the red cell iron processed by the RE cell is rapidly returned to the plasma, and another portion exchanges with RE stores and is slowly reutilised (Noyes et al, 1960; Lipschitz et al, 1971c). This gave rise to the concept of the early release pool, also known as the "pre-release" pool or the labile iron pool, and a slow storage pool. The kinetics of these pools were further characterized by Fillet and coworkers (1974), who showed that in dogs there was an initial processing period within the RE cell of radioiron derived from labelled, heat damaged red cells. After this the radioiron was either rapidly returned to the circulation ($t^1/_2$ = 34 minutes) or was transferred to a slowly exchanging pool of storage iron within the RE

cell ($t^1/_2$ release to plasma of 7 days). These pathways were of equal magnitude in the normal dog.

While the presence of an intermediate "labile iron pool" had been postulated, it is only recently that this pool has been better characterized. This labile iron pool or low molecular weight iron pool as it is now known is dealt with in section 1.8.

The passage of iron through the RE cell can be summarized diagramatically (figure 1; May and Williams, 1980). The input flux refers to iron entering the cell as a result of catabolism of haemoglobin from effete or senescent red cells. This iron enters the rapidly exchangeable iron pool which is made up of low molecular weight and labile protein- or polypeptide-bound iron. Most of the "chelatable iron" in cell cytoplasm is derived from this rapidly exchangeable iron pool. The slowly exchangeable iron pool represents ferritin. Iron may also be taken up by the cell from transferrin, or may be donated to transferrin.

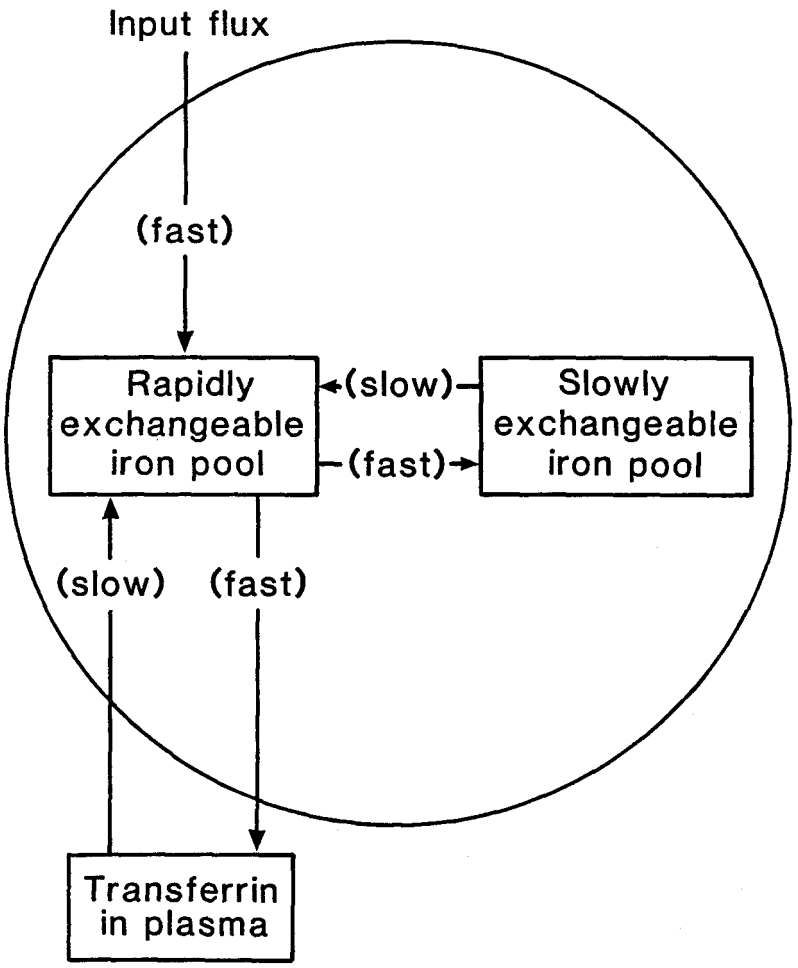

Figure 1. Schematic diagram of the passage of iron through the RE cell.

1.5 HEPATOCYTE IRON UPTAKE

1.5.1. Introduction

Virtually all aspects of iron metabolism are represented in the liver. The liver is the principal organ of iron storage in ferritin (Kondo et al, 1988). It contains two major cell types, namely reticuloendothelial cells, also known as Kupffer cells, and parenchymal cells or hepatocytes. The Kupffer cell, like other cells of the reticuloendothelial system, is capable of processing and releasing iron acquired by phagocytosis of red blood cells, and is a major site for restoring iron from senescent erythrocytes to the circulation (Kondo et al, 1988).

The hepatic parenchymal cell is second only to the erythroid bone marrow in its capacity for iron exchange with plasma transferrin (Morgan and Baker, 1986). Hepatocytes take up transferrin iron, haem-haemopexin iron, haptoglobin-haemoglobin iron and ferritin iron (Hershko et al, 1972). Another transferrin iron uptake pathway has been described which involves the interaction of desialated transferrin with non-specific asialoprotein receptors on hepatocyte surfaces (Young et al, 1983; Dekker et al, 1985; Tavassoli et al, 1986; Rudolph and Regoeczi, 1988). Recently attention has focused on hepatic clearance of the small pool of low molecular weight iron complexes (Wright et al, 1988).

The following section will deal with the various routes utilized by hepatocytes in their acquisition of iron.

1.5.2. Uptake of transferrin-bound and asialotransferrin-bound iron

Plasma transferrin is thought to be the most significant source of iron for the hepatocyte, with some 10-20% of plasma iron turnover in rats being directed at the hepatocyte (Page et al, 1984). A number of mechanisms are responsible for hepatocyte iron uptake from transferrin. These include a combination of receptor mediated endocytosis, which is most effective at lower transferrin concentrations, and non-saturable transferrin binding which occurs mainly at higher concentrations of transferrin (Page et al, 1984; Trinder et al, 1986). Since transferrin iron uptake by hepatocytes is inhibited by the energy metabolism blocking agent cyanide (Thorstensen and Romslo, 1986), a large part of this uptake must involve energy dependent processes.

Recent work has indicated that when compared to erythroid precursors hepatocytes have much higher concentrations of surface membrane ferro-reductase activity. This observation may suggest that in the case of hepatocytes, after diferric transferrin has bound to the surface receptor, the iron may be liberated at the cell surface and reduced to the ferrous state whereafter transmembrane transport of this iron can occur (Thorstensen, 1988). After transferrin-iron is taken up by normal cultured hepatocytes, approximately two-thirds of the endocytosed iron accumulates in ferritin (Trinder et al, 1986).

The mechanism whereby asialotransferrin delivers its iron to hepatocytes is complex and incompletely understood. There is disagreement as to whether the asialotransferrin is taken into the hepatocyte via the conventional diferric transferrin receptor or whether it enters the hepatocyte via an asialoprotein receptor (Young et al, 1983; Dekker et al, 1985; Rudolph and Regoeczi, 1988). The hepatocyte appears to have an asialoprotein receptor known as the asialoglycoprotein-binding lectin which seems to be concerned with the removal of desialated proteins from the circulation (Ashwell and Harford, 1982). Tavassoli and coworkers (1986) showed that hepatic endothelium possesses the ability to transport endocytosed transferrin from the vascular lumen to the hepatocyte side of the endothelium. In the process the transferrin was desialated and was subsequently taken up by hepatic asialoprotein receptors. Young and coworkers (1983) suggested that asialotransferrin can be endocytosed by suspended hepatocytes via either the transferrin receptor or the asialoprotein receptor. They concluded that the net uptake of asialotransferrin was the sum of both pathways. Another group of investigators (Dekker et al, 1985) proposed that iron uptake from rat asialotransferrin occurs via transferrin receptors, and that when taken up by the asialoprotein receptor, the transferrin is recycled without unloading its iron. More recently it has been proposed that the asialoprotein receptor facilitates capture of the asialotransferrin by the same binding sites that are normally available for transferrin rather than that it functions as an alternate pathway (Rudolph and Regoeczi, 1988).

1.5.3. Uptake of non-transferrin-bound iron

Under normal circumstances, non-transferrin-bound iron represents only a minor source of hepatocyte iron when compared with transferrin, but the quantitative significance of this form of iron increases in various settings.

1.5.3.1. Haptoglobin and haemopexin

Haemoglobin introduced into the plasma following intravascular haemolysis binds to haptoglobin, or to haemopexin if the haptoglobin binding capacity of the plasma is exceeded as occurs when intravascular haemolysis increases greatly in haemolytic diseases and those disorders associated with high levels of ineffective erythropoiesis (Morgan and Baker, 1986). Both haptoglobin-haemoglobin and haem-haemopexin complexes are taken up by hepatocytes (Hershko et al, 1972). This uptake process appears to be by receptor mediated endocytosis, with the process showing several similarities to that of transferrin-iron uptake (Smith and Morgan, 1981). Within the hepatocyte, these complexes are catabolized and iron is released from the porphyrin ring of haem by haem oxygenase. The iron then enters an intracellular pool common to that derived from transferrin and ferritin (Morgan and Baker, 1986). Haem bound to albumin as methaemalbumin is also taken up by hepatocytes but without binding of the albumin to the cells. It is likely that the uptake mechanism involves transfer of the haem to haemopexin, which is

then taken up by the hepatocyte in the usual fashion (Morgan and Baker, 1986).

1.5.3.2. Serum ferritin

As is discussed in section 1.6.3. small quantities of ferritin are present in plasma under normal conditions, and higher amounts are present in plasma when iron stores are increased. It has been shown that ferritin introduced into the plasma is rapidly cleared from the circulation by hepatocytes. This is a receptor-mediated process (Mack et al, 1983; Morgan and Baker, 1986) and probably involves endocytosis followed by fusion of the endocytic vesicles with lysosomes and degradation of the protein (Unger and Hershko, 1984). Plasma ferritin with a high iron content, as occurs under conditions of tissue necrosis, has to be considered as a potential source of iron uptake by the liver (Bacon and Tavill, 1984). Also, in pathological conditions in which iron stores are elevated, plasma ferritin probably represents an important source of hepatocyte iron and contributes to parenchymal iron overload (Morgan and Baker, 1986).

Recent experiments suggest that in addition to its role in intracellular iron storage, ferritin may also serve as an intrahepatic carrier of iron between Kupffer cells and hepatocytes (Kondo et al, 1988; Sibille et al, 1988). The avidity with which hepatocytes take up ferritin released from Kupffer cells may explain the susceptibility of the liver to iron overload (Sibille et al, 1988).

In contrast to the conservation of transferrin during its interaction with hepatocytes, the protein shell of the ferritin molecule is rapidly degraded into trichloracetic acid-soluble fragments (Sibille et al, 1988).

1.5.3.3. Low molecular weight iron complexes

Although most of the iron in plasma is bound to transferrin, there is increasing evidence that low molecular weight iron complexes present in plasma are an important source of iron for the rat liver (Brissot et al, 1985; Wright et al, 1986). The plasma concentration of this form of iron, which is thought to consist of a mixture of iron complexes with ascorbate, carbonate, certain amino acids and to a lesser extent plasma proteins such as albumin, is extremely small in normal individuals. However much higher concentrations have been reported in iron overload when the specific iron binding sites of transferrin are saturated and iron added to the plasma cannot bind to transferrin (see section 1.2.3.3.).

Hepatic clearance of low molecular weight plasma iron complexes appears to be an extremely efficient process, with 58-75% removed in a single pass through the liver (Brissot et al, 1985). Autoradiography has confirmed that most iron removed in this process is cleared by parenchymal cells (Brissot et al, 1985).

Unlike uptake of transferrin, hepatic uptake of these low molecular weight iron complexes is not reduced by iron loading (Wright et al, 1986). In addition, this transport mechanism appears to be carrier-mediated as evidenced by saturation, competitive inhibition by other transition metal ions and temperature dependence. Iron uptake by this mechanism is also strongly calcium dependent and is relatively insensitive to inhibitors of cellular energy metabolism (Wright et al, 1986).

The very high efficiency of hepatic uptake of this form of iron far exceeds the rate of transport of physiologic ions in serum (Wright et al, 1988). The basis for this high efficiency is largely unknown. Recent data has confirmed that hepatic uptake of these low molecular weight iron complexes is mediated by a membrane carrier in the rat liver and strongly suggests that uptake occurs by an electrogenic transport mechanism that is driven by the potential difference across the liver cell membrane (Wright et al, 1988). Hepatic accumulation of other transition metal ions such as copper, zinc, and manganese may be driven by similar forces since there is evidence that these ions share a common carrier with iron (Brissot et al, 1985; Wright et al, 1986).

1.6 STORAGE IRON

1.6.1. Introduction

Iron that is not immediately needed to mediate one of its many biological functions in cells and that is present in excess quantities in the cell is diverted into iron stores. This surplus iron is stored in one of two related forms, namely a soluble mobile fraction known as ferritin, or as insoluble aggregates known as haemosiderin (Worwood, 1982). The role of ferritin in different cell types includes both specialized functions and intracellular housekeeping functions. Examples of specialized functions are the recycling of iron in macrophages and short and long term iron storage as in red cells of embryos or hepatocytes of adults. Intracellular housekeeping functions include providing a reserve of iron for cytochromes, nitrogenase, ribonucleotide reductase, and for detoxification of excess iron that may enter the cell (Theil, 1987). Recently it has been suggested that ferritin may serve as an intrahepatic carrier of iron between Kupffer cells and hepatocytes (Kondo et al, 1988: Sibille et al, 1988). Although the iron in haemosiderin is not as immediately accessible to the cell as is the iron from ferritin, it is readily mobilisable in the face of increased body needs (Bothwell et al, 1979; Halliday and Powell, 1984). Iron stored as ferritin in the parenchymal cells of the liver accounts for approximately a third of the total storage iron (Powell and Halliday, 1982). The remainder of the body's reserve iron is stored as haemosiderin within the cells of the reticuloendothelial

system in the liver, spleen, bone marrow and skeletal muscle (Bothwell et al, 1979; Powell and Halliday, 1982).

1.6.2. Ferritin

1.6.2.1. Structure

The ferritin molecule comprises a globular protein shell called apoferritin, with a central cavity within which iron accumulates (Harrison,1980). Apoferritin is a colourless molecule, whereas ferritin has a deep red-brown colour when it carries a full compliment of iron (Bothwell et al, 1979; Worwood, 1980). Apoferritin has a molecular weight of between 450-480000 dalton, is water soluble and relatively heat stable (Harrison et al, 1980). It is composed of 24 polypeptides or subunits arranged in a symmetrical fashion to form the hollow, almost spherical shell (Harrison, 1980). Hydrophobic and hydrophilic channels exist between the subunits. Small molecules, including iron and sucrose enter into the central cavity of the molecule through these channels (Crichton and Charloteaux-Wauters, 1987). Up to 4500 iron atoms can be included in the core along with variable amounts of phosphate (Mann et al, 1986).

Ferritin is not a homogeneous protein, but consists of up to 20 isoferritins (Bomford and Munro, 1980). The evidence for this has been the finding that ferritin from different organs isofocuses over a pH range of 4.8 to 5.8 and gives several bands of isoferritins (Drysdale

et al, 1977; Arosio et al, 1978). On sodium sulphate or polyacrylamide gel electrophoresis, two major subunit bands are evident. It is variations in the proportions of these two subunits, which differ in size, amino acid composition, surface charge and immunoreactivity that give rise to the heterogeneity of ferritin (Drysdale, 1977). The two subunits are designated H and L respectively and have molecular weights of 21000 and 19000 (Arosio et al, 1978). The range of isoferritins found varies from tissue to tissue (Worwood, 1980). Isoferritins from the heart, lymphocytes and red blood cells consist mainly of H subunits and are relatively acidic in nature, while isoferritins from the liver, spleen and placenta consist mainly of L subunits and are more basic (Worwood, 1986). The acidic H subunits appear to contain a higher proportion of iron rich molecules (Treffrey et al, 1984). The H and L subunits are synthesized as independent polypeptide chains (Watanabe and Drysdale, 1981). While it has been discovered that the H and L subunits are coded for by genes on chromosomes 11 and 19 respectively (Worwood et al, 1985), H and L sequences have also been found on other chromosomes (Cragg et al, 1985; Mcgill et al, 1987).

1.6.2.2. Synthesis

The stimuli for ferritin synthesis are decreasing levels of ferritin in the cell and excess iron that enters the cell (Finch et al, 1986). For ferritin to fulfil its role of storing excess iron, there must be a mechanism whereby the excess iron can be rapidly incorporated into

ferritin so as to protect the cell from toxic levels of iron. While some of the iron is incorporated into pre-existing apoferritin, the high levels of intracellular iron induce ferritin synthesis in a unique fashion (Bomford and Munro, 1980).

There exists a cytoplasmic store of ferritin L and H subunit mRNA. In response to increased intracellular levels of chelatable iron, there is movement of this ferritin mRNA from ribonucleoproteins to polyribosomes, with resulting increased ferritin mRNA translation (Aziz and Munro, 1986; Hentze et al, 1987; Rogers and Munro, 1987; Casey et al, 1988; Dickey et al, 1988; Leibold and Munro, 1988). Recent studies have shown that the translational regulation of ferritin synthesis in storage cells is achieved by the interaction of iron with a soluble protein mediator that is attached to the ferritin mRNA (Leibold and Munro, 1988). The area to which the protein mediator is attached is called the iron responsive element. It is a highly conserved stem loop structure on the 5'-untranslated region of the ferritin mRNA and as a result of the attachment of iron to the protein mediator, the mediator is removed from the iron responsive element (Hentze et al, 1987; Aziz and Munro, 1987). The unmasked region that results then interacts with a high affinity site on the polyribosome and translation of the mRNA occurs. Recent evidence would seem to indicate that enhanced transcription may also play a minor role in the induction of ferritin subunit synthesis by iron (White and Munro, 1988).

However, because ferritin functions both as a housekeeping protein (iron stored for intracellular use) and a specialized-cell protein (iron stored for use by other cells), mechanisms of regulation of ferritin synthesis may vary depending on the cell type (Theil, 1987). In contrast to storage cells which contain abundant ferritin mRNA that iron recruits for translation, in non-specialized cells which only require ferritin for metabolic needs, the concentration of ferritin mRNA is low and increased intracellular iron induces a change in the mRNA concentration by enhancing transcription or by altering mRNA stability (Cairo et al, 1985; Dickey et al, 1987).

1.6.2.3. Iron incorporation and release

The exact mechanism whereby iron is incorporated into and released from apoferritin in vivo has not been elucidated (Halliday and Powell, 1984). Iron is taken up into the apoferritin shell through its structural channels (Harrison et al, 1980). In vitro studies have shown that this iron is in the ferrous form and that it is then oxidized to ferric iron (Bothwell et al, 1979). The apoferritin shell catalyses this reaction in which molecular oxygen functions as the electron acceptor (Bomford and Munro, 1980). Following hydrolysis the iron is deposited in the interior of the apoferritin shell in the form of inorganic hydrated ferric oxide phosphate micelles (Harrison et al, 1980). These micelles form a nucleation centre upon which further micellar growth takes place, the amount of iron taken up by ferritin being determined by the available surface area of the iron core

(Harrison et al, 1980). This has been confirmed by kinetic studies which have shown that iron uptake by apoferritin increases progressively until a 25% saturation level is approached after which time iron uptake decreases (Harrison et al, 1980).

Iron contained in ferritin is readily available for use in functional iron compounds when required (Bothwell et al, 1979). Iron release from the iron core occurs such that the iron atoms are stripped off the iron core layer by layer, with the iron atoms on the outermost layer being the most available for release (Harrison et al, 1980). Studies where radioiron incorporated onto ferritin last is the first to be released have confirmed this "last-in-first-out" concept of iron mobilization from ferritin (Hoy et al, 1974).

Iron release from ferritin may occur by reduction of ferric iron to ferrous iron in the presence of the appropriate chelator, or it may occur by direct chelation of ferric iron (Crichton and Charloteaux-Wauters, 1987). In vitro studies have shown that ferritin iron release by reduction of ferric iron to the ferrous forms occurs more rapidly than does the direct chelation of ferric iron (Crichton and Charloteaux-Wauters, 1987). Reduced flavin nucleotides have been implicated as the chelators which may mediate the ferric-ferrous reduction of iron (Harrison et al, 1980). It is thought that the reduced flavins, with their small diameters of 1.3 nm, are capable of traversing the channels of the apoferritin shell and reacting with the iron contained within the crystalline lattice (Hoy et al, 1974).

However doubt has been cast on the role played by reduced flavins in iron release from ferritin by the finding that in vivo the intracellular concentration of free flavins is very low (Crichton and Charloteaux-Wauters, 1987).

1.6.2.4. Ferritin catabolism; haemosiderin

Apoferritin has a finite lifespan and must constantly be renewed (Bothwell et al, 1979). When laden with iron, the protein has a half life of only a few days. Studies utilizing rat liver apoferritin have shown a half life of 50-75 hours for this ferritin (Drysdale and Munro, 1966).

Tissue ferritin appears to be taken up and degraded by the liver (Siimes and Dallman, 1974; Unger and Hershko, 1974), probably by a process of receptor mediated endocytosis (Mack et al, 1983; Morgan and Baker, 1986). The ferritin is degraded by lysosomes, either to its constitutive amino acids or to form haemosiderin (Bothwell et al, 1979). Thus haemosiderin appears to be the degradation product of ferritin. It is formed within secondary lysosomes by the protease induced decomposition of the ferritin molecule (Richter, 1984). Within the lysosomes, the solubilizing protein coat of ferritin is degraded, resulting in the water insoluble aggregates of hydrated iron oxide and organic constituents that make up haemosiderin (Bothwell et al, 1979; Harrison et al, 1980). Recently it has been shown that ascorbate can

delay the transition of ferritin into haemosiderin (Bridges and Hoffman, 1986).

The ratio of ferritin to haemosiderin in storage organs varies according to the amount of iron present. At lower concentrations, ferritin predominates but at higher concentrations most iron is found as haemosiderin (Shoden et al, 1958). Haemosiderin deposits are used as a histologic means of assessing the size of the body iron stores.

1.6.3. Serum ferritin

It was not surprising to find ferritin present in the circulation in situations of damage to organs containing large amounts of ferritin, for example hepatic necrosis (Bothwell et al, 1979; Worwood, 1986). The development of sensitive immunological techniques to measure ferritin brought with them the finding of ferritin in the circulation of normal healthy individuals (Addison et al, 1972; Worwood, 1986). This serum ferritin has been shown to be different from tissue ferritin in a number of ways.

Serum ferritin has a very low iron content. Immunologically it resembles liver or spleen ferritin and is rich in L subunits (Worwood, 1986). It has been found that a high proportion of ferritin in normal serum binds to concanavalin A, a lectin which forms complexes with polysaccharides and glycoproteins (Worwood et al, 1979). This finding suggests that the range of isoferritins of serum ferritin is the result

of glycosylation of ferritin and isnot due to variable proportions of H and L subunits, as is the case for tissue ferritin (Worwood, 1986). Another point of difference between serum ferritin and tissue ferritin is their rate of clearance from the circulation, with serum ferritin being cleared from the circulation at a slower rate than tissue ferritin (Worwood, 1982). This difference too may result from the fact that serum ferritin is glycosylated.

The origin of serum ferritin is not clear. While all body cells probably secrete ferritin, the liver and RES appear to be the main source of serum ferritin production (Mack et al, 1981). The origin of serum ferritin and the isoferritins present in serum also differ in different states of health. In normal individuals much of the serum ferritin is glycosylated and this suggests secretion of ferritin is possibly from phagocytic cells degrading haemoglobin (Worwood, 1986). A reticuloendothelial cell origin is supported by experiments performed in rats in which the entry of ferritin into the plasma was followed with radioiron (Siimes and Dallman, 1974). However, in conditions of tissue damage another mechanism becomes important, that of the direct release of cellular ferritin through damaged cell membranes (Prieto et al, 1975). Supporting this is the finding in patients with ferritinaemia due to liver necrosis, of very little of the plasma ferritin binding to concanavalin A (Worwood et al, 1979).

Serum ferritin appears to be cleared exclusively by hepatic parenchymal cells. This occurs by a specific receptor mediated process (Mack et al, 1983; Morgan and Baker, 1986).

Under normal circumstances the amount of ferritin secreted into the circulation is thought to be representative of the amount of ferritin synthesized in the major iron storage sites of the body, namely the RES and the liver (Finch et al, 1986). This close relationship between serum ferritin levels and the amount of storage iron in the body is used clinically in that, taken in conjunction with other parameters, measurement of serum ferritin allows for discrimination between simple iron depletion, iron deficient erythropoiesis and iron deficiency anaemia (Bothwell et al, 1979). Also, it provides a non-invasive and moderately accurate way of assessing the total amount of iron stored in the body (Worwood, 1980).

The chief restriction to the clinical use of the plasma ferritin concentration for assessing iron nutrition is the fact that it is influenced by other factors. Concentrations higher than justified by the amount of stored iron are found in acute and chronic infections (Baynes et al, 1986a; 1986b), inflammation (Baynes et al, 1986a; Baynes et al, 1987a), neoplastic conditions (Bezwoda et al, 1985; Worwood, 1986), heavy alcohol ingestion (Meyer et al, 1984), liver disease (Worwood, 1986) and after unusually heavy exercise (Taylor et al, 1987). A raised serum ferritin concentration is thus a relatively non-specific finding in clinical situations. However, a low plasma

ferritin concentration of less than 12 ug/l can only be due to iron deficiency.

1.6.4. The ferritin receptor

The ferritin receptor has been described in guinea pig reticulocytes (Pollack and Campana, 1981; Blight and Morgan, 1983), rat hepatocytes (Mack et al, 1983) and human placental cells (Takami et al, 1986). However its precise physiological role remains unclear. It has been suggested that the ferritin receptor may transfer iron from ferritin to haem in the mitochondria (Ulvik, 1982).

Most recently it has been suggested that the ferritin receptor on hepatocytes may serve to take up ferritin released from Kupffer cells (Kondo et al, 1988; Sibille et al, 1988). In this setting ferritin may function as an intrahepatic carrier of iron between Kupffer cells and hepatocytes. It can also be seen that the ferritin receptor on the hepatocyte, by binding ferritin released by the Kupffer cell, may account for the resistance of the liver to iron deficiency, as well as the susceptibility of the liver to iron overload (Sibille et al, 1988).

1.6.5. Red cell ferritin

While erythrocytes contain both H and L isoferritins, the ferritin within erythrocytes has been found to be approximately ten times more reactive with antibody to heart ferritin than to spleen ferritin

(Peters et al, 1983). The two isoferritins appear to have different metabolic functions within erythroid cells. The acidic H isoferritin appears to be an intermediate in the transfer of iron from the plasma membrane to the mitochondria for incorporation into haem (Cazzola et al, 1983). The basic L isoferritin within the erythrocyte acts as an intracellular depot of iron which is in excess of that required for haem synthesis. It also reflects changes occurring in tissues in iron deficiency and iron overload (Cazzola et al, 1983; Peters et al, 1983).

1.7 IRON RELEASE FROM RETICULOENDOTHELIAL (RE) CELLS AND HEPATOCYTES

1.7.1. Introduction

Under normal circumstances, the amount of iron transferred from plasma to cells each day is balanced by an equal amount returning in the opposite direction (Morgan, 1981). Compared to what is known about iron uptake by cells, relatively little is understood about the events and mechanisms of iron release from cells, and about the factors influencing this iron release.

The major source of iron returning to the plasma is the reticuloendothelial system (Morgan, 1981). Thus much of the research into iron release from cells has been performed using the RE cell. The

liver and gastrointestinal mucosal cells make a smaller but significant contribution to plasma iron (Morgan, 1981). This discussion will concentrate on iron release from RE cells and hepatocytes to transferrin.

1.7.2. Iron release to apotransferrin

After donation of its iron to cells by a process of receptor mediated endocytosis, transferrin is released as apotransferrin from the cells to which it has donated its iron (Morgan, 1981; Huebers and Finch, 1987). Apotransferrin is then available to bind iron and be involved in many more plasma to cell cycles, as is indicated by its long plasma half life of 7-10 days (Katz, 1961). Transferrin iron is principally directed to red cell precursors in the bone marrow. Iron released from RE cells in vivo is bound randomly by any available vacant transferrin iron-binding site, both on monoferric transferrin and apotransferrin (Groen et al, 1982). Iron released from isolated rat hepatocytes also binds randomly to any empty iron-binding sites on transferrin (Young, 1982). Since diferric transferrin is a better iron donor in vitro than monoferric transferrin (Huebers et al, 1983a), it is likely that in vivo, transferrin must acquire 2 atoms of iron before it becomes an effective iron donor (Young and Bomford, 1984).

Much debate exists as to whether apotransferrin binds iron at the cell membrane or extracellularly at a site distal to the cell membrane. Clinical observations are in favour of an active mechanism regulating

iron release from cells, since iron is released from RE cells and cells of the gastrointestinal tract in proportion to body needs. However iron is retained in RE cells and gut mucosal cells in the anaemia of chronic disorders despite a low plasma iron and transferrin saturation (Roeser, 1980; Lee, 1983). In contrast, proportionately small amounts of iron are held up in the RE cells and gut mucosal cells relative to the striking overload of parenchymal cells and plasma transferrin in idiopathic haemochromatosis (Brink et al, 1976). These two disturbances of cellular iron handling appear to suggest that iron release or retention is an active process since in both of them iron is retained or released from cells despite significant concentration gradients.

The mechanism of iron release from the liver was investigated using the isolated perfused rat liver and rat hepatocyte suspensions (Baker et al, 1977). It was found that the addition of apotransferrin to a transferrin-free medium produced an increase in the rate of iron release from both types of preparations. However, other iron chelators such as citrate and desferrioxamine were also shown to be effective as mobilizers of cellular iron. This suggested that transferrin may be functioning in a non-specific way by providing an iron-binding component in the extracellular fluid which can bind iron after its release from the cell. Alternatively, the citrate and desferrioxamine may aid iron release by a different mechanism than that of transferrin (see section 1.9.).

In favour of apotransferrin acting as a passive acceptor of iron, was the finding of no specific receptors for apotransferrin on hepatocytes (Young and Aisen, 1981), a conclusion also reached by others using the intact perfused rat liver (Baker et al, 1980). However, specific apotransferrin receptors have been identified on cultured rat peritoneal macrophages (Nishisato and Aisen, 1982; Saito et al, 1986). Iron binding to these receptors was not influenced by diferric transferrin but was displaced by cold apotransferrin (Nishisato and Aisen, 1982). In addition it has been reported that the incubation of iron loaded macrophages in media containing apotransferrin resulted in the progressive saturation of the apotransferrin with iron (Saito et al, 1986). The implication of these studies was that apotransferrin receptors existed to which apotransferrin bound in its acquisition of iron from cells. In these studies, macrophages were found to release considerable amounts of iron as ferritin, raising the question as to whether the results obtained were consequent upon leakage occurring from the cells as a result of their decreased viability. Indeed a recent study failed to identify the presence of apotransferrin receptors at physiological pH of 7.4 on cultured human blood monocytes (Baynes et al, 1987b). In this study it appeared that the small amount of apotransferrin binding that did occur resulted from iron contamination. It is possible that the discrepancy between these findings and the previous findings of apotransferrin receptors on rat peritoneal macrophages may be due in part to minor degrees of iron contamination in the latter studies.

On current evidence it seems unlikely that apotransferrin receptors are required in the normal transport of iron from RE cells to plasma. Were apotransferrin receptors to be of physiological significance, it might be anticipated that they would be present on RE cells in large numbers, since RE cells deliver as much iron daily into the plasma as is removed by erythroid precursors (Baynes et al, 1987b).

1.7.3. The effect of a saturated transferrin

Since transferrin is the major iron binding protein in the plasma, studies have been performed whereby the saturation of transferrin has been altered in an attempt to further elucidate factors affecting iron release from cells. These studies have also attempted to determine the role played by transferrin in iron release from cells. Such studies in the past have utilized the parenteral infusion of elemental iron to saturate transferrin and thus block transferrin binding sites and iron release. The results of these studies have not been in agreement with one another. In one study, iron as iron-nitrilotriacetic acid was administered to rats in amounts which exceeded the available binding sites of transferrin (Lipschitz et al, 1971c). This resulted in marked hyperferraemia and diminished release by RE cells of iron from subsequently infused damaged red cells. Studies in dogs found that there was little difference in the pattern of iron release from RE cells after heat damaged erythrocytes were injected into control dogs and dogs in which the plasma transferrin was saturated by injecting ferric ammonium citrate (Fillet et al, 1974). In a third study,

ferrous ammonium sulphate was infused into rats to saturate plasma transferrin and in that study the inability of RE cells and hepatocytes to release iron in the transferrin-saturated animal was again demonstrated (Bergamaschi et al, 1986).

In addition to discordant results being obtained, another problem of these studies has been their use of infused elemental iron to saturate transferrin. This method of saturating transferrin is unphysiological and may result in supersaturation of transferrin with consequent circulating free iron present in the plasma. Thus the results of these studies must be viewed with suspicion.

One aspect of the experimental work of this thesis is aimed at saturating transferrin in a more physiological fashion and evaluating the regulation of RE and hepatic parenchymal iron release in the presence of a physiologically saturated transferrin.

1.7.4. The role of caeruloplasmin and ascorbic acid

Release of iron from cells may also be dependent upon the availability of caeruloplasmin. When the livers from copper-deficient baby pigs or adult dogs were perfused with serum free solutions containing human apotransferrin, little or no iron was released to the protein. However incorporation of caeruloplasmin into the perfusing medium resulted in a rapid efflux of iron from the livers, the iron appearing bound to transferrin (Osaki et al, 1971). Although the deleterious effects of

copper deficiency on iron metabolism and the capacity of caeruloplasmin to correct these effects have long been appreciated, the mechanism by which caeruloplasmin acts remains controversial (Williams et al, 1974). It may be that iron release from cells depends on its oxidation from the ferrous to the ferric form, the oxidation state bound by transferrin, and that this change is dependent upon the ferroxidase activity of caeruloplasmin (Frieden and Osaki, 1974).

Ascorbic acid also appears necessary for the release of iron from cells, possibly by maintaining iron in its appropriate oxidation state to be bound by transferrin. Ascorbate deficiency exerts a marked effect on the metabolism and storage of iron, as evidenced by clinical studies in scorbutic Black patients (Bothwell et al, 1964), as well as in guinea pigs (Banerjee and Chakrabarty, 1965; Lipschitz et al, 1971a). These studies showed that scurvy resulted in a block in RE iron release associated with a shift of iron stores from ferritin to haemosiderin and a reduction in serum iron levels. Ascorbate treatment in scorbutic patients resulted in a dramatic increase in serum iron concentration (Bothwell et al, 1964) followed by an increase in serum ferritin levels (Cohen et al, 1981; Chapman et al, 1982). Other studies illustrating the role that ascorbic acid plays in iron metabolism showed that while the plasma iron concentration is elevated in Black South Africans with dietary siderosis, such patients that had coexistent ascorbic acid deficiency were found to have normal or even low plasma iron concentrations. Hyperferraemia occurred promptly following administration of ascorbic acid to these patients (Wapnick et

al, 1970). The ascorbate deficiency in iron overload results from its accelerated oxidative catabolism (Lynch et al, 1967).

Recent in vitro studies in K562 cells have shown that ascorbate delays the transition of ferritin iron into the insoluble haemosiderin compartment, and provided evidence that this delay is associated with expansion of the chelatable low molecular weight intracellular iron pool (Bridges and Hoffman, 1986).

1.8 THE LOW MOLECULAR WEIGHT (LMW) INTRACELLULAR IRON POOL

1.8.1. Introduction

There is a considerable amount of information available on the cellular acquisition of iron, the release of iron from its transport protein transferrin, and its incorporation into its final biochemical form. However the pathway that iron actually follows after its release from transferrin and prior to its reaching its target in the cell is not well understood. It appears that iron passes through a labile, intracellular iron pool at least partially comprised of low molecular weight complexes, and that this pool can be tapped by extracellular complexing agents. The concept of an intracellular pool of LMW iron ligands is appealing as it serves to rationalize intracellular iron exchange (Jacobs, 1977). However direct evidence for this pool has until recently been largely speculative.

1.8.2. Characterization

Indirect evidence for a highly chelatable form of iron which is a normal metabolic intermediate in reticuloendothelial cells was provided by a number of studies using the chelator desferrioxamine (DFO) (Cumming et al, 1967; Karabus and Fielding, 1967; Lipschitz et al, 1971b). A reduction in iron flow through the reticuloendothelial cell produced by suppression of erythropoiesis reduced the amount that could be chelated (Lipschitz et al, 1971b) and an increase in iron flow induced by haemolysis increased chelation (Cumming et al, 1967). Rat experiments confirmed that ferritin and haemosiderin were not important direct donors of iron to DFO and that compounds in the pathway between storage compounds and transferrin are the probable source of the iron chelated by DFO (Lipschitz et al, 1971b).

Within the erythrocyte, evidence was found for the existence of short-lived nonhaem intermediates. Zail and coworkers (1964) presented serum-bound ^{59}Fe to cultures of human marrow cells and found a nonferritin non-haemoglobin precursor from which iron was rapidly removed for haem synthesis. In marrow cultures from iron-deficient patients there was a transient labelling of the intermediate fraction before complete incorporation into haemoglobin took place. The presence of this soluble intracellular iron compound in erythroid cells that served as a source of iron for haemoglobin and ferritin was confirmed by other workers (Primosigh and Thomas, 1968).

More recent data regarding the intracellular labile iron pool in non-erythroid cells was obtained from experiments using cultured Chang cells as a model system. About 30% of the iron taken up by these cells from transferrin is membrane bound and the remainder is found in the cytosol. Chang cells synthesize ferritin actively in response to an iron load and most of the cytosol iron is present in this form (White et al, 1976a). However about 35% of this iron is in a non-haem, nonferritin dialyzable form (White et al, 1976a). Gel filtration of the membrane-free supernatant after centrifugation of the sonicated Chang cells showed that the LMW fraction could be readily chelated by DFO, EDTA or transferrin. When Chang cells were labelled with ^{59}Fe transferrin so that the radioiron was normally distributed through the cell fractions, subsequent transfer of the culture to a medium containing DFO resulted in rapid iron depletion of the cell (White et al, 1976b). The ^{59}Fe ferritin content fell more rapidly than did the LMW ^{59}Fe, but as DFO has been shown not to chelate ferritin iron directly, mobilization presumably occurred via the LMW pool. Similar mobilization of intracellular iron was observed when the cells were incubated with transferrin, the amount removed being inversely related to the transferrin saturation (White et al, 1976b).

The incubation of pure cultures of human polymorphonuclear leukocytes, lymphocytes and monocytes under the same conditions as those used in Chang cell experiments showed that despite wide differences between the different cell types and variations in disease states, "cytosol" iron was present in both ferritin and non-ferritin forms (Summers and

Jacobs, 1976). Thus, while a variety of cells appeared to contain this LMW iron pool which was easily available for uptake by ferritin, haem, transferrin and chelators, the evidence for its existence was indirect and its chemical nature was poorly characterized until recently.

1.8.3. Chemical nature of the LMW pool

For a long while the chemical nature of the intermediate or labile iron pool was unknown. It appeared to be a low molecular weight complex of iron or a mixture of such complexes (Jacobs, 1977). The ligands in this LMW pool could have included almost any of the LMW species present in the cytosol (Pollack et al, 1985). A reducing agent has been found to be necessary in the transfer of iron from transferrin to ferritin (Miller and Perkins, 1969) and it was postulated that this agent was to be found in the LMW pool. The reducing agent functions to form an intermediate ferrous chelate with iron once iron has been freed from transferrin. It ensures that the iron is available in the ferrous state for uptake by ferritin (Miller and Perkins, 1969). Ascorbic acid appears to fulfil this role effectively and citrate, sugars, amino acids and nucleotides may all function as intermediate ligands in iron transfer. Identification of these intermediate iron compounds not only depends on their chemical characterization but also depends on their ability to display the appropriate functions. While ferric citrate can be utilized directly by the reticulocyte and the same is true for a number of LMW chelates such as nitriloacetate (Morgan, 1971), these chelates are however nonphysiologic.

The first direct demonstration of a LMW iron pool in the cell was by Pollack and Campana (1980). This was achieved using a buffer with minimal affinity for metal and without adding chelate. This LMW iron in the reticulocyte has recently been characterized (Pollack et al, 1985). On thin layer chromatography this LMW iron is distinguishable from the iron complexes of a variety of nucleotides, sugars and amino acids. On paper chromatography it comigrates with a 250nm absorbing, orcinol-positive material. The eluted count peak contains phosphorous. Continuously gassing the buffer used in these experiments with nitrogen improved recovery of the LMW iron, suggesting that the iron is in the ferrous oxidation state (Pollack et al, 1985). One of the aims of the present investigation is to further characterize the nature of the iron in this labile intermediate pool.

1.8.4. Clinical significance

The intracellular LMW iron pool occupies a focal position in intracellular iron metabolism. It provides iron for mitochondria which are the site of haem synthesis either for mitochondrial cytochromes, for extramitochondrial cytochromes such as cytochrome P450 and for specialised proteins such as haemoglobin or myoglobin (Jacobs, 1977; Bomford et al, 1986). A wide variety of processes, such as DNA synthesis (Hoffbrand et al, 1976) are inhibited by DFO and may be assumed to depend on the availability of iron in the LMW iron pool. In addition all cells contain non-haem enzymes which appear to depend on an adequate tissue iron status for their function (Finch et al, 1976),

and presumably such enzymes must depend on the metabolically functional intracellular labile iron pool.

In addition ferritin synthesis is induced by enlargement of this iron pool (Lynch et al, 1974; Young et al, 1985; Bomford et al, 1986), a finding that is addressed by the present investigation. The cellular basis for the regulation of ferritin synthesis has recently been delineated (see section 1.6.2.2. previously). This may provide an explanation for the finding that enlargement of the LMW pool appears to be a transient phenomenon, as evidenced by the fact that with the passage of time, less iron is present in this form and more is present in the form of functional and storage compounds (Bridges and Cudkowicz, 1984; Young et al, 1985; Bomford et al, 1986).

Finally, the interaction of this small molecular weight pool with extracellular transferrin and thus similar pools in other tissues provides a mechanism whereby an equilibrium can be established not only between the various metabolic processes in the cell but also between different organs in the body (Jacobs, 1977).

1.9 CHELATABLE IRON AND CHELATING AGENTS

1.9.1. Introduction

Evolution has provided efficient mechanisms for the acquisition, transfer and storage of iron, but has not provided mechanisms for promoting the excretion of excess iron. The clinical consequence of continued iron accumulation is the abnormal function of a number of organs, the most important of which are the heart, liver and pancreas. While the manifestations of chronic iron overload are potentially curable by the effective mobilization of storage iron by phlebotomy (Easley et al, 1972; Short et al, 1981), the effective administration of iron chelators such as desferrioxamine can prevent and even reverse the clinical manifestations of iron overload (Hershko and Weatherall, 1988).

In normal subjects, most of the body iron is unavailable for chelation. Iron in haemoglobin, representing over two thirds of all iron, is unavailable for chelation and transferrin-bound-iron is a poor source of iron for chelation. The most likely sources of chelatable iron include that stored in tissues in the form of ferritin or haemosiderin and the labile iron compartment constituting the LMW iron pool that is in equilibrium with ferritin (Hershko and Weatherall, 1988). The increased availability of iron for chelation found in conditions of increased catabolism of ferritin and haemoglobin-bound iron (Cumming et al, 1967; Karabus and Fielding, 1967; Lipschitz et al, 1971b) lent

support to the existence of a LMW chelatable iron pool. That this pool rather than ferritin or haemosiderin is the most likely source of iron bound by chelators has been confirmed by rat experiments (Lipschitz et al, 1971b). A consequence of the accessibility of the LMW pool to chelators is that knowledge of the mechanism of action of these chelators can be utilized in an attempt to characterize the nature of the iron in this LMW pool. It is against this background that various chelators have been employed in the investigations performed in this thesis. What follows is a short overview of the chelators utilized in this study.

1.9.2. Desferrioxamine (DFO)

DFO is a colourless crystalline substance produced by Streptomyces Pilosus. It consists of a chain of three hydroxamic acids terminating in a free amino acid group, which enables it to form salts with organic and inorganic acids (Keberle, 1964). It is capable of combining with ferric iron at a 1:1 molar ratio and with a stability constant of 10^{31}. The affinity of DFO for ferrous iron and other metal ions such as zinc, copper, magnesium and calcium is much lower.

There are fundamental differences in the behaviour of DFO as compared to ferrioxamine, the DFO-iron complex. As a result of the change in configuration following interaction with iron, ferrioxamine becomes an extremely stable compound resistant to enzymatic degradation (Meyer-Brunot, 1967). In contrast to DFO which is capable of penetrating

various tissues, ferrioxamine is distributed in the extracellular space and is unable to penetrate cells.

DFO is able to interact in vitro with iron located in cultured hepatocytes (White et al, 1978; Octave et al, 1983; O'Connell et al, 1985), cardiac cells (Sciortino et al, 1980; Link et al, 1985) and monocytes (Esparza and Brock, 1981). Direct evidence for the cellular uptake of DFO is available only in hepatocytes (O'Connell et al, 1985) where it interacts in situ with hepatocellular iron which is then excreted via the biliary tract. The contribution of RE cells to DFO-induced iron excretion is limited (Hershko et al, 1973; Pippard et al, 1982; Kim et al, 1985), and it is not entirely clear as to whether RE-derived iron is chelated by DFO within RE cells or following its release into the plasma (Pippard et al, 1982). The iron derived from the RES by DFO is subsequently excreted via the urine. Urinary DFO is excreted by both glomerular filtration and tubular secretion, whereas ferrioxamine is partly reabsorbed following glomerular filtration (Peters et al, 1966).

The gastrointestinal absorption of both DFO and ferrioxamine is poor (Keberle, 1964). Oral administration of DFO is effective in blocking the intestinal absorption of inorganic iron but has only a small effect on urinary excretion. The intestinal absorption of orally administered iron is inhibited by the parenteral administration of DFO.

The rapid clearance of DFO from plasma, its effective catabolism and its active tubular secretion severely limit the effectiveness of single bolus injections given at long intervals. However the fact that it is distributed in over 60% of the total body volume indicates that it has access to intracellular compartments in diseases where interaction with chelatable intracellular iron is beneficial (Hershko and Weatherall, 1988).

1.9.3. Diethylenetriamine penta-acetic acid (DTPA)

DTPA is a synthetic compound belonging to the group of polyanionic amines. It was first used for the treatment of iron overload in humans by Fahey and coworkers (1961). It was subsequently shown to be as effective as DFO in promoting urinary iron excretion (Bannerman, 1962). It is highly soluble in water but is unable to penetrate cells. Iron chelated by DTPA is derived from RE cells and is excreted solely in the urine (Hershko et al, 1978a). It is ineffective by oral administration. Its highest affinity is for ferric iron (Bridges and Cudkowicz, 1984). Calcium-DTPA is well tolerated when administered by slow subcutaneous or intravenous infusion. Zinc depletion after prolonged DTPA treatment is a significant problem, but it can be prevented by oral zinc supplementation.

1.9.4. 2,2'bipyridine

This is a hydrophobic iron chelator which partitions into cell membranes and binds ferrous iron as it passes through this lipid environment (Nunez et al, 1983; Morgan, 1983a). The bipyridine-iron complex is however hydrophilic and unable to cross cell membranes (Nunez et al, 1983; Morgan, 1983a; Bridges and Cudkowicz, 1984). 2,2'bipyridine is also able to limit erythroid iron uptake by blocking transmembranous ferrous iron transport from the acidified endocytic vesicle into the cell cytosol and returning the bipyridine-ferrous iron hydrophilic complex back to the extracellular environment (Morgan, 1983a; Baynes et al, 1988a). The iron-chelator is released from the cell by a process which is very similar to that of transferrin release from cells with respect to kinetics and sensitivity to incubation temperature and the effects of metabolic inhibitors and other chemical reagents (Morgan, 1983a).

There is some disagreement as to whether 2,2'bipyridine acts within the cytosol in its chelation of ferrous iron. Morgan (1983a) hypothesized that 2,2'bipyridine blocked cell iron uptake by gaining entrance to the cytosol, chelating ferrous iron and then diffusing out of the cell. The results of investigations by Nunez and coworkers (1983) were incompatible with the hypothesis of 2,2'bipyridine chelating iron from the cytosol compartment. They showed that the chelator partitions into the membrane compartment of reticulocytes, chelates membrane-associated iron and then exits into the extracellular milieu without entering the

cell cytosol in the process. The change in solubility when the chelator binds iron affords an explanation for the fast onset of the inhibition of iron uptake (Nunez et al, 1983).

CHAPTER TWO

METHODS AND MATERIALS

2.1. Introduction

Much is known of the nature of transferrin iron delivery to erythroid and other cells (Morgan, 1981; Huebers and Finch, 1987). In contrast there is a paucity of knowledge of the factors regulating RE iron metabolism and release. The rate of return of red cell derived iron, liberated from catabolized haemoglobin in the RE cell, to the circulation is governed by partitioning between an early release pool and a slow storage pool (Noyes et al, 1960). The factors regulating this partitioning are incompletely understood. Previous work involving the parenteral infusion of elemental iron to block transferrin binding sites for iron suggested that cellular iron release was inhibited in the presence of a highly saturated transferrin (Lipschitz et al, 1971c; Bergamaschi et al, 1986). A criticism of this work is that the use of a parenteral infusion of iron to saturate transferrin is unphysiological in that it may have resulted in supersaturation of transferrin with consequent circulating free iron being present.

In the current investigation the enteral administration of carbonyl iron (Huebers et al, 1986) was employed in an attempt to saturate transferrin in a more physiological fashion. In addition, the effects on serum iron of single and repeated transfusions of heat damaged red cells, and of haemoglobin were investigated.

Radioiron-tagged heat damaged red cells were employed for the study of RE behaviour in the setting of altered transferrin saturations,

enhanced erythropoiesis, prior injection of non-tagged haemoglobin and infusion of homologous tissue ferritin. The nature of the radioiron derived from the heat damaged red cells and held up in the RES as a consequence of a highly saturated transferrin was characterized by chromatographic analyses of splenic and hepatic extracts. Chelators with various modes of action were included in splenic and hepatic preparations to further characterize the intracellular chelatable pools of radioiron. Finally, the findings of the in vitro inclusion of chelators in splenic and hepatic preparations were confirmed by a series of in vivo chelator studies.

All investigations included in the study have been approved by the Animal Ethics Committee of the University of the Witwatersrand.

2.2. Sprague-Dawley rats

The study utilized normal male Sprague-Dawley rats, weighing 250-300g each. All rats were maintained on a standard commercial cube diet (Epol, Johannesburg, South Africa).

2.3. Carbonyl iron

2.3.1. Background

Carbonyl iron is a small particle preparation of highly purified metallic iron. It is inert and incapable of reacting with strong

chelators of iron such as transferrin and desferrioxamine (Huebers et al, 1986). The manufacturing process involves the heating of gaseous iron pentacarbonyl ($Fe(CO)_5$), which results in the deposition of metallic iron as submicroscopic crystals that form microscopic spheres. The term "carbonyl" describes the abovementioned manufacturing process and not the composition of the iron particles. When administered orally, carbonyl iron is much less toxic than ionized forms of iron such as ferrous sulphate (Crosby, 1978). The reason for this and the manner in which the metallic particles of carbonyl iron are made available for absorption and utilization was not fully understood. A study recently undertaken to determine the mechanism of carbonyl iron absorption and the reason for its lower toxicity (Huebers et al, 1986) revealed that solubilization of carbonyl iron by gastric acid is a prerequisite for its subsequent absorption and that the slow rate of solubilization of carbonyl iron resulted in its more prolonged absorption and its low toxicity. Once it has been solubilized , the subsequent pathway of absorption of carbonyl iron by the intestinal mucosa and the amount absorbed is similar to that of ferrous ammonium sulphate (Huebers et al, 1986). Little or no carbonyl iron is absorbed in vitro and in vivo unless it is exposed to a pH of less than 2 (Huebers et al, 1986).

2.3.2. Administration

The carbonyl iron used was 3 to 4 μm in particle size, of SF grade and was obtained from GAF Corporation, New York. Each dose of carbonyl

iron was administered in 1 ml acidified saline pH 2.0. It was introduced into the stomachs of fasted rats with an olive tipped canula. On day 1, after an overnight fast during which time they had free access to water, the animals were fed 400mg of carbonyl iron, followed by 200mg carbonyl iron 24 hours later. The animals were fasted during this 24-hour period, but had free access to water. Two animals were exsanguinated by cardiac puncture under anaesthesia at various time intervals after the first and second doses of carbonyl iron. The blood was separated by centrifugation and serum iron, unsaturated iron-binding capacity, total iron binding capacity and percentage saturation of transferrin were measured (International Committee for Standardization in Haematology (Iron Panel), 1978 a; b). The time period when near complete steady state transferrin saturation occurred was determined.

2.4 Heat Damaged Red Cells (HDRC)

Similar time dependent studies examining the effects of HDRC on serum iron concentration and transferrin saturation were performed.

2.4.1. Preparation

HDRC were prepared after the method of Lipschitz and coworkers (1971b). Blood removed from a donor rat was centrifuged at 2000 rpm for 20 minutes, with the removal of plasma. The red cells were washed twice in normal saline and were then suspended in four times their volume of

a 1:1 mixture of isotonic saline and ACD (5g citric acid, 13.8g sodium citrate and 13g dextrose per litre of solution). The red cells in the resulting solution, with a haematocrit of 20%, were denatured by heating for 20 minutes in a waterbath at 40°C.

2.4.2. Transfusion

The HDRC were transfused via the tail vein within one hour of preparation. The effect of a single transfusion of a 3 ml/kg 20% haematocrit solution of heat damaged homologous erythrocytes and of 3 repeated transfusions of 6 ml/kg of a similar solution on serum iron, unsaturated iron-binding capacity, total iron-binding capacity and percentage saturation of transferrin was determined (International Committee for Standardization in Haematology (Iron Panel), 1978a; b). Animals were again exsanguinated by cardiac puncture under anaesthesia.

2.5. Radioiron tagging of erythrocytes

The haemoglobin of rat red cells was uniformly labelled by repeated injections of radioiron (^{59}Fe). These radioiron-tagged erythrocytes were then heat damaged as described and were employed for the study of RE and hepatic iron handling in various experimental settings.

Radioiron tagging of red cells in vivo was accomplished as follows. Rats whose erythropoiesis was stimulated by removing 4 ml of blood by cardiac puncture on the sixth and fifth days prior to the first

injection of radioiron, were given an initial intravenous injection of 200 µCi ^{59}Fe as ferric chloride. 50 µCi of ^{59}Fe as ferric chloride was administered every week thereafter. After sufficient time had elapsed to permit uniform tagging, these rats were bled under anaesthesia to obtain radiolabelled erythrocytes for the preparation of HDRC. Each animal was not venesected more than once a month.

2.6. Serum iron (SI) concentration

The SI concentration was determined utilizing the method recommended by the International Committee for Standardization in Haematology (ICSH, 1978a). Initially the plasma was acidified with a mixture of 1 molar HCl and 10% trichloracetic acid in order to dissociate the iron-transferrin complex and to precipitate proteins. The simultaneous addition of a 3% thioglycollic acid solution ensured complete reduction of the dissociated transferrin iron to the ferrous form. Deproteinization is an important step in measuring serum iron as it removes substances such as haemoglobin, bilirubin and plasma lipids which would otherwise increase the optical density of the plasma (Bothwell et al, 1979). The optically clear supernatant was then treated with buffered bathophenanthroline sulfonate and the absorption of the ferrous complex was measured at 535 nm. Two ml of a standard iron solution containing 40 µmol/l iron in 5 mmol/l HCl, and two ml of distilled water for a blank were treated in the same way as the serum samples. The serum iron concentration was then calculated by

multiplying by 40 the ratio of the differences of the absorption of the test sample and the blank to the standard sample and the blank.

2.7. Unsaturated iron-binding capacity (UIBC)

This was measured utilizing the method recommended by the International Committee for Standardization in Haematology (ICSH, 1978b), and involved an adsorbent method in which iron in excess of the binding capacity of transferrin is added. The compound used to saturate transferrin was radio-labelled ferric chloride in a dilute HCl solution. Magnesium carbonate which strongly adsorbs the unbound ionic iron was added, and the samples were centrifuged to remove the iron. The unsaturated iron-binding capacity was measured directly by counting the radioactivity present in the supernatant. The ratio of the radioactive counts in the test sample to those of two ml of the saturating iron solution yielded the UIBC value, after background activity was subtracted.

2.8. Total iron-binding capacity (TIBC)

The TIBC was calculated by summing the values obtained for serum iron concentration and UIBC. The TIBC reflects the level of transferrin and gives the total number of iron binding sites on the transport protein, while the serum iron concentration reflects the number of iron atoms actually bound (Kimber et al, 1983).

2.9. Percentage saturation of transferrin

Percentage saturation was calculated by multiplying the ratio of serum iron to total iron binding capacity (TIBC) by 100.

2.10. Analytical ^{59}Fe organ distribution

Radioiron tagged HDRC were injected into both control rats and into experimental rats via the tail vein within one hour of preparation. The volume of tagged HDRC used was 3 ml/kg of a 20% haematocrit solution. This corresponded to a dose of iron of approximately 0.8 mg/kg. At various time intervals after the injection of the tagged HDRC, rats were anaesthetized, exsanguinated by cardiac puncture and washed out with normal saline. The percentage distributions of ^{59}Fe in the liver, spleen, blood, kidneys, and marrow was determined. The ^{59}Fe activity of one ml of blood, both kidneys, one femur, and the spleen and liver were counted in a Packard Autogamma scintillation spectrometer model 5650 (Packard Autogamma Co., Downers Grove, Illinois). Total marrow activity was estimated by multiplying the counts of one femur by 13 (Hershko et al, 1972). The activity of the blood was calculated from the one ml of blood counted, assuming that the total blood volume of a rat is 65 ml/kg.

The organ distribution of ^{59}Fe was assessed in experiments conducted both shortly after the initial dose of carbonyl iron and at a time when steady state increased transferrin saturation had been achieved. Organ

distribution of radioiron was also studied in animals that had enhanced erythropoiesis prior to tagged-HDRC injection. The effect of prior injection, one hour before tagged HDRC infusion, of an equivalent amount of non-tagged free haemoglobin on organ distribution of ^{59}Fe was also assessed. Studies were also conducted after concomitant infusion of homologous tissue ferritin together with the tagged HDRC.

2.11. Enhanced erythropoiesis

Erythropoiesis was stimulated by removing 4 ml of blood via cardiac puncture under anaesthesia on the sixth and fifth days prior to commencement of the study. Enhanced erythropoiesis was confirmed by reticulocyte counting.

2.12. Free haemoglobin production

Free haemoglobin was produced by the method of Pippard and coworkers (1982). Blood removed from a donor rat was centrifuged at 2000 rpm for 20 minutes, with the removal of plasma. To the resulting red cells was added 4 times the volume of sterile water. The resulting solution was centrifuged at 1000 rpm for 10 minutes to remove cell debris, and the resulting solution containing free haemoglobin was utilized.

2.13. Homologous tissue ferritin

Homologous tissue ferritin was produced by the method of Huebers and coworkers (1976). Donor rats were anaesthetized, exsanguinated by cardiac puncture and washed out using normal saline perfusion. The livers of these rats were removed and subjected to coarse homogenization in 4 times their volume of cold distilled water. The homogenate was heated to 75-80°C in a waterbath with constant stirring. After heating for 10 minutes, the homogenate was cooled on ice and was centrifuged at 2000 rpm for 20 minutes. The resulting supernatant, with pH corrected to 4.9 by the addition of 25% acetic acid, was stored at 4°C for 12 hours, after which time it was again centrifuged at 2000 rpm for 20 minutes. To the resulting supernatant was added an equal volume of saturated ammonium sulphate. After standing for a further 12 hours at 4°C, the solution was centrifuged at 2000 rpm for 20 minutes. The resulting pellet was dissolved in the smallest volume of normal saline and was dialysed against 2 litres of normal saline for 36 hours. The normal saline was changed every 12 hours during the 36 hour period. After this time the dialysate was centrifuged for 10 minutes at 1000 rpm and the resulting supernatant was utilized as the homologous tissue ferritin.

A limulus lysate assay for endotoxin was performed by Sabax Ltd, Johannesburg on this tissue ferritin preparation.

2.14. Sizing column chromatography

Hepatic and splenic tissue was prepared for sizing column chromatography in an attempt to characterize the nature of ^{59}Fe, derived from prior infusion of tagged HDRC, which was present in the RE cells of the spleen and the RE and parenchymal cells of the liver in the presence and absence of a highly saturated transferrin and after prior transfusion with non-tagged HDRC. In an attempt to define the nature of iron released from RE cells, whole sera obtained after tagged HDRC infusion into animals with normal and saturated transferrins were also fractionated on AcA 44 column chromatography.

The buffer and column procedure were those of Pollack and coworkers (1985), namely an AcA 44 ultrogel column (LKB, Bromma, Sweden) and a 0.05 M saline, 0.02 M hepes buffer, pH 7.0. The precautionary procedure of bubbling nitrogen through the buffer to prevent iron-oxygen complexes from forming was employed as described (Pollack et al, 1985). The hydrophobic ferrous chelator 2,2'bipyridine and the hydrophilic ferric chelator desferrioxamine were included in hepatic and splenic preparations to characterize the intracellular chelatable pools of ^{59}Fe. At each time interval studied, a sample of liver and a sample of spleen was subjected to coarse homogenization in running buffer (0.05 M NaCl; 0.02 M Hepes; pH 7) containing soybean trypsin inhibitor (100 μg/ml) and either desferrioxamine (5 mg/ml) or 2,2'bipyridine (1.39 mg/ml). The homogenized sample was centrifuged at 1000 rpm for 10 minutes, after which time the resulting supernatant,

which contained near single cell suspensions, was sonicated with 3 bursts of ultrasound of 30 seconds each at medium intensity from an M.S.E. ultrasonicator (Crawley, Sussex, U.K.). Post sonication the sample was subjected to ultracentrifugation at 25000 rpm for 40 minutes at 4°C. The resulting supernatant, a membrane and cytosol enriched sample, was fractionated on AcA 44 ultrogel chromatography. The fractions collected were counted for radioactivity in a Packard Autogamma scintillation spectrometer model 5650. Prior to its use, the sizing column was calibrated with radiolabelled ferritin and transferrin. Between each sample application the column was washed with 12 ml 1% triton X-100 and 12 ml 0.1 M EDTA. At least 3 volumes of buffer were then run through the column prior to the next sample application.

Detergent solubilized preparations of hepatic and splenic tissue were also subjected to sizing chromatography on an AcA 44 ultrogel column. Coarse homogenization of splenic and hepatic tissue in running buffer containing soybean trypsin inhibitor but no chelators was followed by centrifugation of the homogenized samples at 1000 rpm for 10 minutes. Running buffer containing 2% triton X-100 was added to the resulting pellet. After agitation at 4°C for 2 hours, the sample, representing a membrane enriched fraction, was subjected to ultracentrifugation at 20000 rpm for 1 hour at 4°C. The supernatant was fractionated on an AcA 44 ultrogel chromatography column, and the fractions collected were counted for radioactivity as before.

The colours of the various fractions were also of use in confirming the nature of the peaks. The ferritin peak (confirmed by quantitative immune precipitation) was brown in colour and appeared at the void volume. This was followed by the haemoglobin peak which was red in colour. The small molecular weight desferrioxamine (brown) or 2,2' bipyridine (pink) peaks were the last ^{59}Fe containing fractions to elute from the column. Immune precipitation was by the methods of Morrow and co-workers (1986) and Enler and co-workers (1985) (see section 2.16. below).

2.15. In vivo chelator studies

These studies were conducted to confirm the fractionation findings of the in vivo inclusion of chelators in hepatic and splenic preparations. 2,2'bipyridine and desferrioxamine were administered in doses of 50 ml/kg intramuscularly one hour, 3 hours, 5 hours, and 7 hours after the intravenous injection of tagged HDRC to normal rats and rats whose transferrin had been saturated with carbonyl iron. The hydrophilic extracellular ferric chelator diethylenetriamine penta-acetic acid (DTPA) was also used in the in vivo studies. It was administered by the intravenous route. In between injections the rats were kept in metabolic cages. At the end of 8 hours, the rats were exsanguinated by cardiac puncture and were perfused with normal saline. The percentage distribution of ^{59}Fe in the liver, spleen, blood, kidneys, marrow, and urine and faeces collected over the period since chelator administration began was determined as was done previously.

2.16. Immune precipitation

Immune precipitation was by the methods of Morrow and co-workers (1986) and Enler and co-workers (1985).

A solution containing 0.5 ml of sample in running buffer (0.02 M Hepes; 0.05 M NaCl; pH 7), 0.5 ml 0.2 M EDTA, 0.2 ml rat ferritin (2 mg/ml) and 0.5 ml Ig G solution was incubated for one hour at 37°C. PEG solution (12% PEG 6000 in 0.2 M EDTA; pH 8.5) was added to a final concentration of 3%. The resulting solution was left to stand overnight at 4°C. The following day it was centrifuged for 20 minutes at 2000 rpm. The pellet was washed with 3% PEG solution and was counted in a Packard Autogamma scintillation spectrometer model 5650.

2.17. Statistical analyses

These were by means of Student t tests. Bonferroni corrections were applied when more than two groups were compared. When analysis variance was applied, an F value was generated. The level of statistical significance was taken to be 0.05.

CHAPTER THREE

RESULTS

3.1. Serum iron and unsaturated iron binding capacity following:

3.1.1. Carbonyl iron administration

The time dependent effect of the oral administration of carbonyl iron to rats on SI and TIBC is shown in figure 2. It can be seen that a single dose of 400 mg of carbonyl iron resulted in a transient increase in the percentage saturation of transferrin. A second dose of 200 mg of carbonyl iron 24 hours later resulted in a more protracted elevation in the percentage saturation of transferrin. The fact that the percentage saturation of transferrin never exceeded 100% when estimated either purely colorimetrically or by a combination of SI colorimetrically and unsaturated iron binding capacity by radioisotopic assay indicated that the carbonyl iron was not causing supersaturation of transferrin with consequent circulating free iron.

3.1.2. HDRC infusions

The time dependent effects of a single 3 ml/kg infusion of HDRC and 3 repeated 6 ml/kg infusions of HDRC on the same iron related parameters are also indicated on figure 2. The single 3 ml/kg HDRC infusion was followed by a transient increase in transferrin saturation maximal at 3 hours post infusion. The first of the repeated 6 ml/kg infusions was followed by a similar increase in the transferrin saturation. The subsequent two infusions had no effect on transferrin saturation.

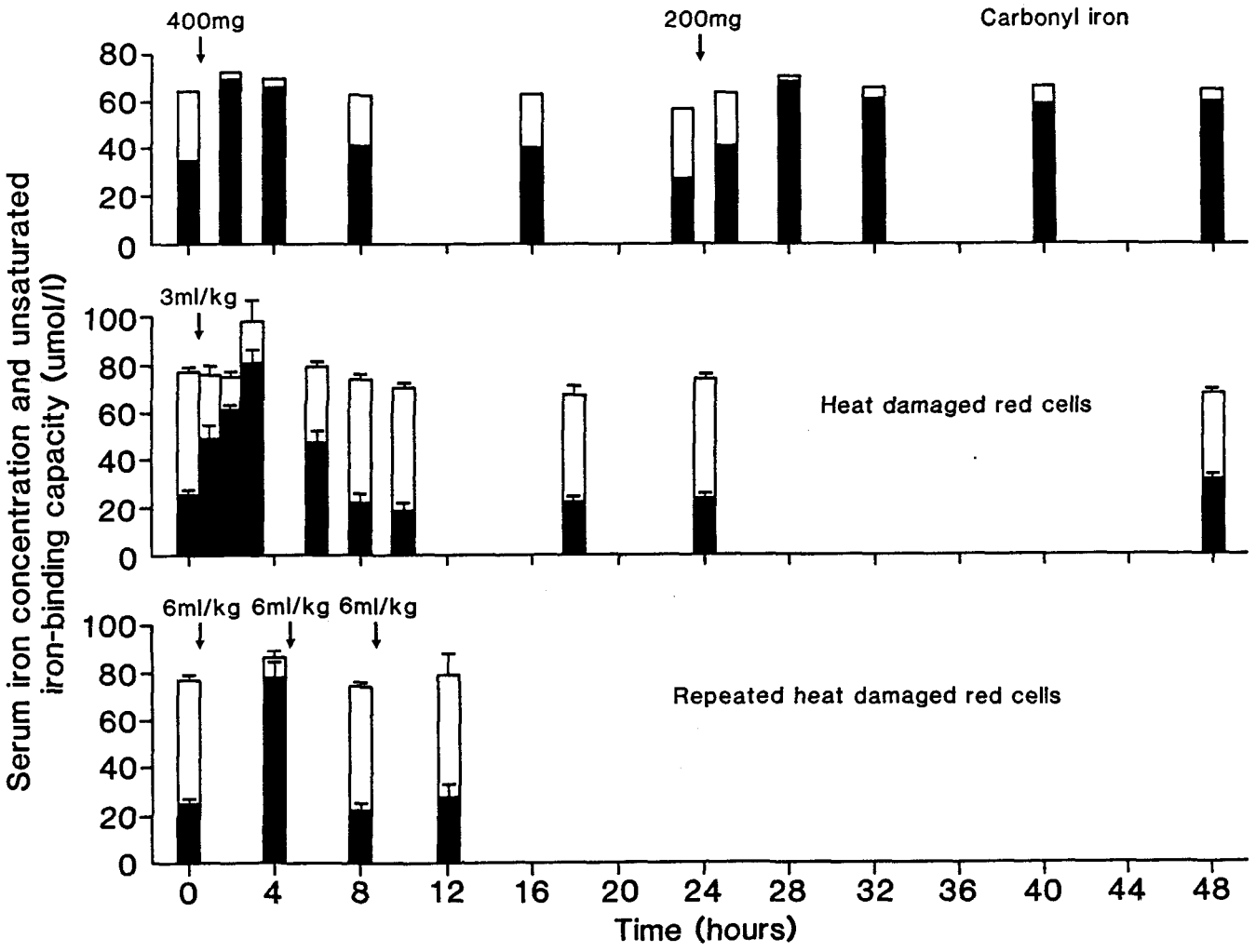

Figure 2. The time dependent effect on SI (shaded bar) and UIBC (open bar) of 2 doses of carbonyl iron (top frame), a single infusion of 3 ml/kg HDRC (middle frame) and 3 repeated infusions of 6 ml/kg HDRC (bottom frame).

3.2. Analytical organ distribution of ^{59}Fe HDRC in various experimental settings

3.2.1. Two doses of carbonyl iron

The effect of two doses of carbonyl iron (24 hours apart) on the fate of tagged HDRC (3 ml/kg of a 20% haematocrit solution) administered four hours after the second dose of carbonyl iron is shown as a function of time post infusion of the cells in Table I. In the control group of animals, the rate of clearance of HDRC was rapid, with greater than 60% of HDRC cleared by 30 minutes. The organ specific ^{59}Fe activity was greatest in the spleen early on. With the passage of time there was a progressive loss of radioiron from the spleen with a concomitant rise of ^{59}Fe activity in the blood and marrow. Whether this was due to direct transfer of iron from RE cells to marrow erythroid precursors could not be answered directly from the control data.

In animals with saturated transferrins, the splenic pattern of radioiron uptake and release was almost identical to the control group of animals. By contrast though, the radioiron present in the blood and marrow was significantly reduced when compared to control animals, and there was a significantly increased amount of ^{59}Fe present in the liver. This suggests that there is a significant pathway of iron transport from spleen to liver, particularly in the presence of a saturated transferrin.

TABLE I COMPARISON OF THE FATE OF ^{59}FE HDRC IN 3 EXPERIMENTAL SETTINGS

	Time post infusion (hours)	Marrow	Liver	Spleen	Blood
	0.5	3.2 (0.1)	16.6 (0.9)	45.1 (9.7)	35.2 (10.5)
	1.5	6.3 (0.7)	26.0 (1.8)	47.8 (3.5)	19.9 (3.0)
Control	4.0	16.4 (1.2)	22.9 (2.1)	37.8 (1.7)	22.9 (1.7)[a]
	8.0	26.7 (1.2)	29.0 (3.2)	29.3 (2.7)	13.4 (1.4)
	24.0	26.9 (1.8)	14.6 (3.3)	33.9 (1.5)	24.7 (0.1)
	0.5	3.8 (2.2)	19.7 (4.2)	30.1 (2.7)	46.5 (0.7)
Carbonyl	1.5	6.7 (0.8)	39.0 (2.2)[a]	40.9 (3.3)	11.1 (2.9)
iron	4.0	10.4 (1.3)[c]	44.0 (3.9)[c]	36.3 (2.5)	9.3 (2.4)
	8.0	15.7 (1.2)[c]	48.1 (1.8)[a]	28.6 (1.9)	7.6 (0.7)[c]
	24.0	14.7 (1.0)[d]	43.7 (4.3)[d]	33.2 (5.2)	8.6 (0.1)[d]
	0.5	5.8 (1.0)	26.6 (6.8)	56.7 (2.8)	11.1 (4.9)
Heat damaged	1.5	7.0 (0.4)	25.6 (0.4)	63.6 (0.6)[b]	3.8 (0.2)
red cells	4.0	11.3 (3.0)	29.5 (2.2)	53.5 (5.4)[a]	11.3 (0.3)
	8.0	18.0 (0.8)	21.6 (3.9)	52.2 (2.6)[a]	8.3 (0.6)

Note. ^{59}Fe HDRC (3 ml/kg) were infused into control rats, into animals in which the transferrin saturation had been maximally raised by 2 prior oral administrations of carbonyl iron and into rats which had received a pretransfusion of heat damaged red cells (6 ml/kg). Mean (± SEM) organ counts of ^{59}Fe at various times are expressed as percentages of recovered counts.

a Statistically different ($p < 0.05$) from the other two groups which are statistically similar
b Statistically different from group given carbonyl iron ($p < 0.05$)
c Statistically different from control group ($p < 0.05$)
d First 2 groups different at 24 hours ($p < 0.05$)

3.2.2. Pretransfusion of HDRC

The effects of an infusion of unlabelled HDRC (6ml/kg) 8 hours prior to the infusion of the regular dose of ^{59}Fe HDRC on the organ distribution of ^{59}Fe are also shown in Table I.

In contrast to the results obtained following the administration of carbonyl iron where there was hold up of radioiron in the liver, pretransfusion of HDRC was associated with a hold up of HDRC derived ^{59}Fe in the spleen and a consequent reduction of blood and marrow activity. The fact that less than 1.5% of recovered counts were present in the kidney indicated that significant intravascular haemolysis of the HDRC had not occurred. This finding of splenic retention of iron is in keeping with the observation that following HDRC infusions, only the first infusion of HDRC was associated with a rise in transferrin saturation. The lack of rise in transferrin saturation following subsequent infusions of HDRC is due to the fact that the RES is conditioned by the first load of HDRC to store iron rather than release it into the circulation.

3.2.3. Single dose of carbonyl iron

That the altered organ distribution was not a function of a protracted (>24 hours) increased transferrin saturation was demonstrated when tagged HDRC were infused into rats 1 hour after the first feeding of oral carbonyl iron and the animals were sacrificed four hours later.

The organ distribution was similar to that noted when a high percentage saturation was maintained for greater than 24 hours with two doses of carbonyl iron, namely reduced marrow and blood ^{59}Fe with a striking increase in hepatic ^{59}Fe activity (Table II).

3.2.4. Enhanced erythropoiesis

In the next series of experiments the effect of enhanced erythropoiesis, induced by prior venesection, on the ^{59}Fe organ distribution 4 hours after the infusion of tagged HDRC was assessed. The enhanced erythropoiesis was confirmed by circulating reticulocyte counts in the region of 20% compared to controls of less than 2%. The effect of the increased erythropoiesis was to markedly increase ^{59}Fe counts in the blood and marrow. This was at the expense of the spleen predominantly, the ^{59}Fe content of which was strikingly reduced. The ^{59}Fe content of the liver was little changed (Table III). The effect of this enhanced erythropoietic rate was nullified by the oral administration of the 2 doses of carbonyl iron. In the latter experiments, although the reticulocyte count was again increased to the level of approximately 20%, transferrin saturation was increased to the region of 90-95% by the 2 doses of carbonyl iron. This resulted in the organ distribution of ^{59}Fe not being significantly different from animals with normal erythropoiesis and enhanced transferrin saturations. These experiments were terminated at 4 hours post infusion of tagged HDRC.

TABLE II THE FATE OF ^{59}FE HDRC 4 HOURS AFTER THEIR INFUSION INTO CONTROL ANIMALS AND ANIMALS WHOSE TRANSFERRIN SATURATION WAS TRANSIENTLY ELEVATED BY THE ORAL ADMINISTRATION OF CARBONYL IRON ONE HOUR PRIOR TO HDRC INFUSION.

	Marrow	Spleen	Liver	Blood
Control rats	16.4 (1.2)	37.8 (1.7)	22.9 (2.1)	22.9 (1.7)
Rats with saturated transferrin	5.9 (0.7)	28.1 (4.6)	59.8 (3.8)	6.2 (0.1)
t value	5.2555	2.5469	9.0742	9.6970
p value	<0.0005	<0.0314	<0.0001	<0.0001

Note: Results are shown as means (SEM) of percentage recovered counts by organs

TABLE III COMPARISON OF THE FATE OF ^{59}FE HDRC INFUSED INTO CONTROL RATS AND ANIMALS WITH ENHANCED ERYTHROPOIESIS

	Marrow	Liver	Spleen	Blood
Control	16.4 (1.2)	22.9 (2.1)	37.8 (1.7)	22.9 (1.7)
Control plus carbonyl iron	10.4 (1.3)	44.0 (3.9)	36.3 (2.5)	9.3 (2.4)
Venesection	27.3 (1.1)[a]	18.6 (6.8)[c]	23.8 (3.0)[a]	30.4 (2.7)[b]
Venesection plus carbonyl iron	11.4 (1.8)[c]	44.6 (1.9)[c]	34.2 (3.6)[c]	10.0 (0.2)[c]

Note. ^{59}Fe labelled heat damaged red cells were infused into control rats and into animals which had been venesected of 4 ml blood 6 and 5 days prior to the study. A further control and venesected group were fed two prior doses of oral carbonyl iron to increase the percentage saturation of transferrin. Mean (+ SEM) ^{59}Fe organ counts are expressed as percentages of recovered counts at 4 hours after the infusion.

a Significantly different from the control group ($p < 0.01$)
b Not significantly different from the control group ($p < 0.08$)
c Not significantly different from the appropriate control group.

3.2.5. Prior carbonyl iron, pretransfusion with untagged HDRC, a combination of both, and prior carbonyl iron plus free haemoglobin infusion 8 hours after ^{59}Fe HDRC injection

In an attempt to further define the pathways in internal iron exchange, the organ distribution of ^{59}Fe HDRC at 8 hour was tested in several groups of 3 animals each. The groups included a control group, one which had received prior carbonyl iron, one which had received prior unlabelled HDRC, one which had received both and one which had received prior carbonyl iron and unlabelled free hemoglobin (Table IV). The prior administration of both carbonyl iron and unlabelled HDRC was associated with splenic retention, which is the pattern obtained in a previous experiment when HDRC were given alone (Table I). In contrast, when prior carbonyl iron and free haemoglobin were administered together, the pattern of increased hepatic counts was similar to that obtained with carbonyl iron alone. Marrow and blood activity were reduced by all the manoeuvres and the administration of free haemoglobin was associated with an increase in kidney counts.

3.3. Sizing column chromatography

3.3.1. Introduction

In an attempt to understand the effects of a saturated transferrin and prior transfusion on reticuloendothelial and parenchymal cell iron processing from HDRC, splenic and hepatic cytosolic extracts were

TABLE IV THE EFFECTS OF PRIOR CARBONYL IRON, PRETRANSFUSION WITH UNLABELLED HDRC, A COMBINATION OF BOTH, AND PRIOR ORAL CARBONYL IRON PLUS AN INFUSION OF FREE HAEMOGLOBIN ON THE ORGAN DISTRIBUTION OF ^{59}FE 8 HOURS AFTER THE INJECTION OF ^{59}FE LABELLED HEAT DAMAGED RED CELLS.

	Marrow	Spleen	Liver	Blood	Kidney
Control	35.4 (3.9)[d]	20.6 (2.3)	35.0 (5.0)[b]	9.0 (0.2)[a]	2.2 (0.7)
Carbonyl iron	18.0 (0.6)	23.5 (1.0)	52.1 (0.7)	6.4 (0.7)	2.9 (1.4)
HDRC	15.7 (0.7)	39.1 (1.1)[c]	40.9 (2.4)[b]	4.3 (1.0)	1.2 (0.9)
Carbonyl iron plus HDRC	15.9 (1.6)	34.2 (7.0)[c]	45.2 (6.3)	4.7 (0.3)	3.3 (1.4)
Carbonyl iron plus free haemoglobin	20.7 (1.4)	20.6 (1.0)	54.4 (2.1)	4.3 (0.5)	5.5 (0.2)

Note. Mean (+ SEM) organ counts of ^{59}Fe 8 hours after the injection are expressed as percentages of recovered counts

a Statistically different from the other groups which are statistically equivalent ($F = 34.9$; $p < 0.0001$)
b Statistically similar but different from the other groups ($F = 12.7$; $p < 0.006$)
c Statistically similar but different from the other groups which were similar ($F = 24.0$; $p < 0.0006$)
d Statistically different from the other groups which were statistically equivalent ($F = 61.7$; $p < 0.0001$)

submitted to sizing chromatographic analysis on AcA 44 ultrogel chromatography. The nature of the ^{59}Fe peaks obtained off the column was identified by comparison to calibration with known iron transport and storage proteins, by immune precipitation and by utilization of the characteristic colours of ferritin, haemoglobin, ferrioxamine and the 2,2' bipyridine-iron complex. The relative sizes of these peaks were calculated as a percentage of the counts recovered from the column. The 2,2' bipyridine-iron peak was of slightly lower molecular size (shifted to the right of) than the ferrioxamine peak. Representative chromatograms for splenic and hepatic cytosolic preparations and for hepatic detergent extracted membrane enriched fractions compared to calibration of the column, are shown in figure 3.

3.3.2. Time dependent fractionation of splenic extracts

The results of the time dependent analysis of splenic cytosolic preparations in terms of ^{59}Fe from HDRC as a function of a normal transferrin, a saturated transferrin or prior transfusion are shown in Table V. In all groups there was a progressive shift of ^{59}Fe from the haemoglobin pool into the ferritin pool with a relatively consistent small molecular weight component. Overall there were no major differences between groups in the percentage distribution of the iron peaks. The claim that the control group was one with a normal transferrin saturation is somewhat of an oversimplification since infusion of the HDRC per se did produce an increase of transferrin saturation as the iron (roughly 0.8 mg/kg) from these red cells was

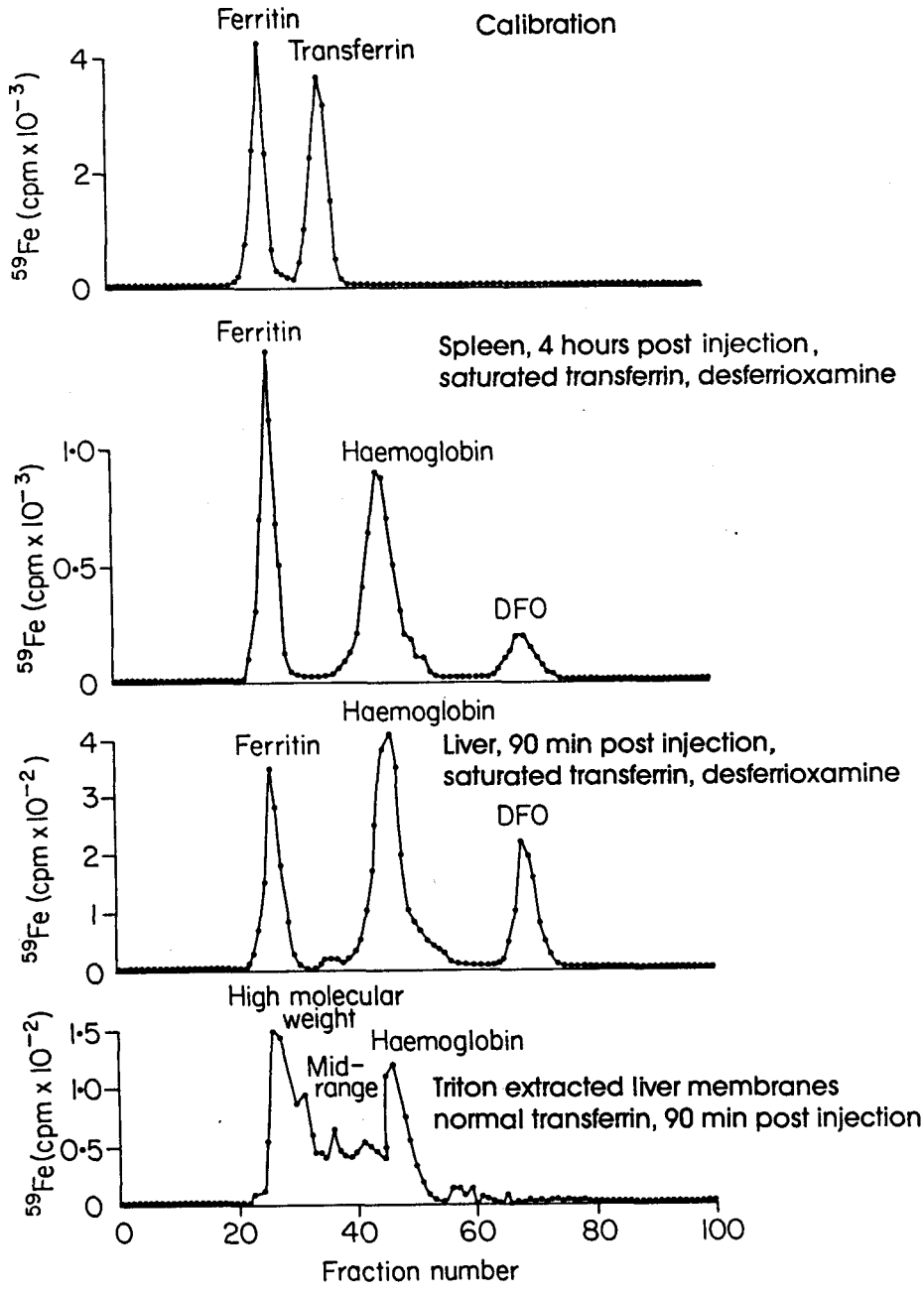

Figure 3. Representative chromatograms for splenic (second frame) and hepatic (third frame) cytosolic preparations and for hepatic detergent extracted membrane enriched fraction (bottom frame), compared to calibration of the column with ferritin and transferrin (top frame).

TABLE V TIME DEPENDENT FRACTIONATION OF SPLENIC EXTRACTS

	Time post infusion (min)	Ferritin	Haemoglobin	Small molecular weight	
				Ferric	Ferrous
Control		1.6 (0.5)	88.1 (0.5)	6.0 (0.2)	-
Carbonyl iron	30	1.9 (0.2)	86.9 (0.9)	4.0 (0.4)	-
Heat damaged red cells		4.0 (1.3)	87.9 (2.3)	6.1 -	2.4 (0.1)
Control		9.9 (1.1)	80.7 (1.3)	5.7 (1.5)	2.7 (0.2)
Carbonyl iron	90	11.9 (0.4)	75.0 (1.4)[a]	8.5 (0.7)	7.6 (2.1)
Heat damaged red cells		12.5 (1.3)	79.7 (1.2)	3.7 (0.1)	2.3 (0.2)
Control		19.2 (2.8)[b]	62.7 (3.6)	4.8 (0.3)	5.2 (0.2)
Carbonyl iron	240	37.2 (3.3)	51.4 (3.1)	5.6 (0.8)	3.6 (0.4)
Heat damaged red cells		42.0 (2.4)	51.4 (3.0)	3.4 (0.3)	2.1 (0.2)
Control		73.8 (3.7)	18.5 (3.5)	3.6 (0.3	-
Carbonyl iron	480	86.0 (1.9)	9.5 (1.5)	2.5 (0.1)	-
Heat damaged red cells		72.7 (0.9)	22.7 (1.2)	2.4 (1.5)	2.2 (0.9)

Note. Splenic tissue was fractionated into ferritin, haemoglobin and small molecular weight peaks after the giving of ^{59}Fe labelled HDRC. The groups include control rats, animals which had received prior oral carbonyl iron and those which had received prior unlabelled HDRC. Mean (+ SEM) organ counts of ^{59}Fe at various times are expressed as percentages of recovered counts. Statistical comparisons refer only to ferritin and hemoglobin as the small molecular weight characterisations were performed on only small numbers of samples.

a Statistically different from control group ($p < 0.05$)
b Statistically different from carbonyl iron and heat damaged red cell groups ($p < 0.05$)

processed (figure 2). Clearly this may have led to a somewhat exaggerated ferritin peak on cytosolic ^{59}Fe analysis of splenic material in the allegedly normal saturation animals.

3.3.3. Time dependent fractionation of hepatic extracts

Time dependent hepatic cytosolic ^{59}Fe analysis post HDRC infusion in animals of normal transferrin saturation, increased transferrin saturation and prior infusion of HDRC are shown in Table VI. There were striking differences between splenic fractionation (Table V) and hepatic fractionation. At the times studied the liver contained strikingly less haemoglobin ^{59}Fe but more ferritin and small molecular weight ^{59}Fe than did the spleen. The carbonyl iron group had significantly more hepatic small molecular weight iron, (both in percentage and absolute terms) than the normal or pretransfusion groups while ^{59}Fe ferritin was increased in both the carbonyl iron and prior transfused groups.

3.3.4. Fractionation of solubilized cell and membrane pellets

In an attempt to investigate the cellular level at which the saturated transferrin was exerting its effect, detergent solubilized extracts of hepatic and splenic cell and membrane pellets obtained 90 minutes after HDRC infusions into control animals and animal with saturated transferrins were fractionated for ^{59}Fe activity on AcA 44 ultrogel sizing column chromatography (Table VII). These detergent extracts

TABLE VI TIME DEPENDENT FRACTIONATION OF HEPATIC EXTRACTS

	Time post infusion (min)	Ferritin	Haemoglobin	Small molecular weight Ferric	Small molecular weight Ferrous
Control		5.0 (0.6)[a]	61.9 (3.5)[b]	17.3 (2.4)	17.9 (0.9)
Carbonyl iron	90	20.0 (1.9)[a]	42.2 (3.3)	28.3 (4.8)	24.9 (0.9)
Heat damaged red cells		36.3 (1.8)[a]	38.8 (2.0)	15.9 (0.3)	16.0
Control		47.4 (2.7)	17.8 (3.6)	18.5 (5.6)	20.8 (3.7)
Carbonyl iron	240	51.4 (7.1)	10.8 (1.5)	22.0 (9.6)	28.4 (11.9)
Heat damaged red cells		81.2 (8.2)[c]	7.1 (3.0)	6.9	4.3

Note. Hepatic tissue was fractionated into ferritin, haemoglobin and small molecular weight peaks after the giving of ^{59}Fe labelled HDRC. The groups include control rats, animals which had received prior oral carbonyl iron and those which had received prior unlabelled heat damaged red cells. Mean (± SEM) organ counts of ^{59}Fe at various times are expressed as percentages of recovered counts. Statistical comparisons refer only to ferritin and hemoglobin peaks as the small molecular weight comparisons were performed on small numbers of samples.

a Each statistically different from the other 2 groups ($p < 0.05$)

b Statistically different from the other 2 groups ($p < 0.05$)

c Statistically different from control group ($p < 0.05$)

TABLE VII FRACTIONATION OF SOLUBILIZED CELL AND MEMBRANE PELLETS

		High molecular weight peak	Mid-range ^{59}Fe activity	Haemoglobin peak
	Control	32.4 (3.6)	29.6 (2.7)	27.4 (4.6)
Liver				
	Carbonyl iron	45.9 (1.5)	19.0 (4.9)	26.5 (2.7)
	Control	13.6 (1.5)	10.8 (3.6)	69.0 (7.2)
Spleen				
	Carbonyl iron	19.1 (2.0)	13.8 (3.1)	61.9 (2.9)

Note. The mean (+ SEM) percentage distribution of ^{59}Fe hepatic and splenic activity in solubilized cell and membrane pellets 90 minutes after the administration of ^{59}Fe labelled HDRC in controls and in animals which had received prior oral carbonyl iron. The pellets were solubilized with triton and fractionated on an AcA ultrogel sizing column.

revealed 3 regions of ^{59}Fe activity, namely a high molecular weight (void) fraction, a haemoglobin fraction and a midrange fraction between these two peaks. No statistically significant differences were noted between the total ^{59}Fe activity in the pellets (not shown in table) or in the distribution between the radioiron peaks, in controls as compared with animals in which the transferrin saturation had been raised by prior administration of carbonyl iron.

3.4. Parenteral administration of chelators

In an attempt to further elucidate the spleen to liver cycle of radioiron, the effect of various chelators on the organ distribution of ^{59}Fe 8 hours after the infusion of labelled HDRC into control animals and animals with increased transferrin saturations was studied. The chelators employed were desferrioxamine, an intracellular hydrophilic ferric chelator, DTPA, an extracellular hydrophilic ferric chelator and 2,2' bipyridine, a membrane hydrophobic ferrous chelator.

The effects of the parenterally administered chelators on the distribution of ^{59}Fe HDRC are shown in Table VIII. In the control animals all 3 chelators caused a modest but statistically insignificant reduction in the ^{59}Fe hepatic pool. In control animals given carbonyl iron the size of the hepatic pool was increased and the chelators caused a significant reduction in its size. Marrow uptake was significantly decreased by 2,2' bipyridine in the control group and there was a concomitant increase in blood activity. A similar, though

TABLE VIII THE EFFECT OF VARIOUS CHELATORS ON THE DISTRIBUTION OF ^{59}FE IN ORGANS

	Chelator	Marrow	Liver	Spleen	Blood	Non-recovered
Control	None	20.0 (2.3)	20.8 (3.6)	24.3 (3.4)	9.7 (1.7)	25.3 (2.7)
	2,2' bipyridine	5.7 (0.4)[b]	15.1 (1.7)	15.6 (0.3)	22.3 (3.4)[b]	41.4 (3.5)
	Desferrioxamine	20.8 (2.2)	14.6 (1.7)	22.0 (0.5)	10.7 (1.1)	31.6 (4.1)
	DTPA	14.0 (3.2)	17.4 (2.0)	14.5 (2.0)[b]	9.8 (1.3)	43.3 (8.3)
Prior carbonyl iron	None	13.3 (3.4)	34.0 (3.5)	18.2 (1.1)	6.4 (0.2)	28.2 (3.9)
	2,2' bipyridine	6.4 (2.0)	16.9 (0.3)[a]	18.2 (0.9)	19.0 (1.1)[b]	39.6 (0.4)
	Desferrioxamine	9.8 (0.2)	17.4 (0.2)[a]	20.2 (0.5)	5.8 (1.2)	46.9 (1.7)[b]
	DTPA	7.5 (0.5)	21.0 (3.8)[a]	14.8 (3.3)	7.3 (1.0)	49.5 (0.0)[b]

Note. The effect of various chelators on the 8 hour organ distribution of ^{59}Fe labelled HDRC in control rats and in animals which had received prior oral carbonyl iron. The chelators were administered parenterally starting 1 hour after the infusion of the red cells. Mean (± SEM) organ counts are expressed as percentages of recovered counts.

a All three statistically equivalent but together significantly different from liver counts in the carbonyl group which did not receive any chelators ($f = 23.5$; $p < 0.005$)

b Significantly different from the appropriate group which did not receive any chelator ($p < 0.05$)

less marked trend was noted in the carbonyl group. DTPA significantly reduced the splenic pool in the control group and a similar but not significant reduction was noted in the animals given prior carbonyl iron.

3.5. Fractionation of serum

Serum obtained 30 minutes after the administration of the ^{59}Fe HDRC was fractionated on AcA 44 ultrogel chromatography. Organ distribution in the control and carbonyl iron groups was similar. The transferrin saturations were 51.0 (SEM 1.5)% and 91.3 (SEM 5.5)% respectively. In the serum of the control animals there was an equal distribution of radioiron between ferritin and transferrin, while in the animals which had received carbonyl iron the transferrin peak was very small (Figure 4).

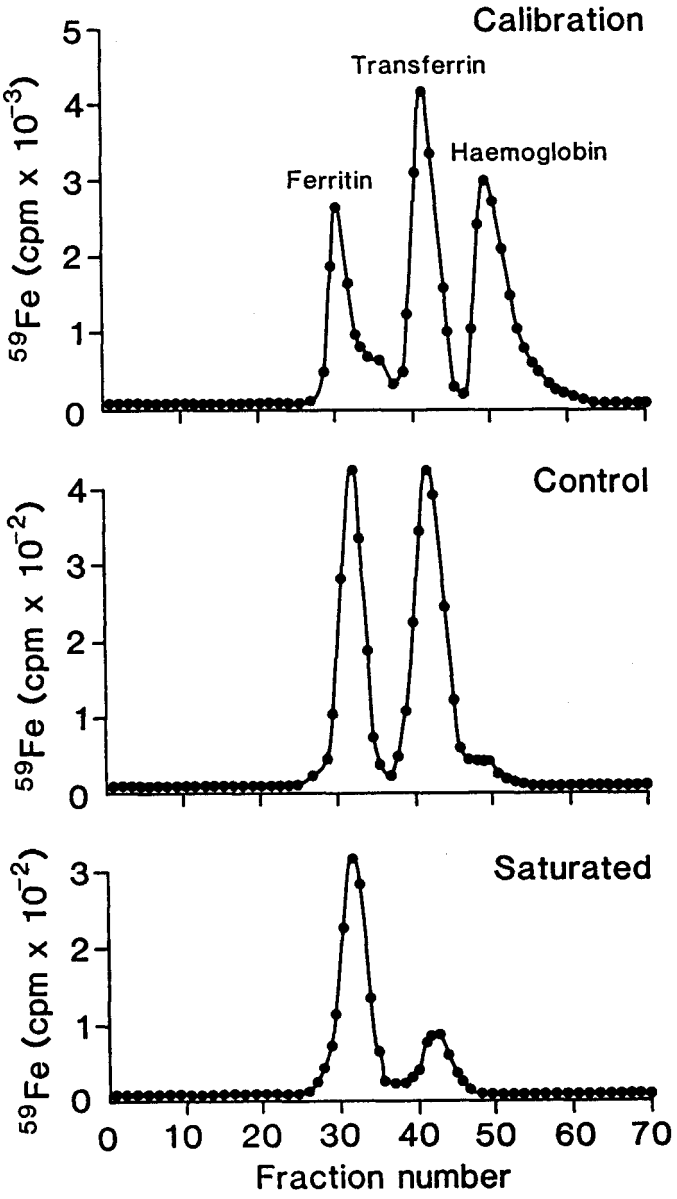

Figure 4. Fractionation on an AcA 44 ultogel column of equal volumes of whole serum 30 min post ^{59}Fe HDRC infusion in normal rats (middle frame) and in rats given prior oral carbonyl iron (bottom frame). Calibration of the column for ferritin, transferrin and haemoglobin is shown in the top frame.

CHAPTER FOUR

DISCUSSION

4.1. The effect of a saturated transferrin

In the current investigation the enteral administration of carbonyl iron proved to be an effective way of producing an increased transferrin saturation without the risk of supersaturation. This manoeuvre was used as the method for evaluating the effect of transferrin saturation on the regulation of the internal exchange of erythrocyte derived iron.

The results of previous work have shown that saturation of transferrin with iron causes a marked reduction in iron release from the reticuloendothelial system (Lipschitz et al, 1971c; Bergamaschi et al, 1986). In the present study results were somewhat different, possibly due to the fact that transferrin saturation was produced in a more physiological way. Whereas both acute and more sustained increases in transferrin saturation had no effect on the splenic retention of iron derived from HDRC, an increase in hepatic retention of iron was noted.

The heat damaged red cell infusion did per se result in a transient increase in transferrin saturation which may to a degree have modified the pattern of internal iron exchange in animals with the so called control transferrin saturation. Splenic retention of radioiron was however enhanced by the prior infusion of unlabelled HDRC. This latter observation was supported by another experiment in which the percentage saturation of transferrin was measured after repeated injections of

HDRC. Only the first infusion was associated with a rise in the transferrin saturation. The lack of any subsequent rises was presumably due to a conditioning of the reticuloendothelial cells by the first load of HDRC to store the released iron rather that to deliver it back to the plasma.

Stimulation of erythropoiesis by venesection in animals that had not received carbonyl iron caused a mobilization of iron from both liver and spleen and its transfer to red cell precursors. Saturating transferrin by the prior administration of carbonyl iron prevented this enhanced cellular iron release despite the increased erythropoietic activity.

4.2. The nature of iron transport from the spleen to the liver

An attempt was made to explain the nature of the iron transport from spleen to liver, particularly in the presence of a saturated transferrin. Prior infusion of an equivalent amount of haemoglobin as free haemoglobin did not alter the redistribution of ^{59}Fe from spleen to liver in carbonyl treated rats. This argues strongly against this circuit being a haptoglobin-haemoglobin one consequent upon intravascular haemolysis in the spleen (Bergamaschi et al, 1986). If the haptoglobin-haemoglobin pathway was the explanation it still would not explain why intravascular haemolysis should be increased in the setting of the increased transferrin saturation and why the major hold-

up should still not be in the spleen if haemolytic rates were equivalent.

The splenic and hepatic fractionation studies gave a further clue as to the nature of the splenic-hepatic circuit. The fractionation studies showed that while in both organs there was a progressive shift of ^{59}Fe from haemoglobin into ferritin, possibly through a small molecular weight chelatable pool, at any given time the haemoglobin fraction in the liver was significantly smaller than that in the spleen. By contrast the small molecular weight and ferritin peaks were much larger at any given time in the liver. The question then is whether the nature of the ^{59}Fe transport was as ferritin or some small molecular weight non-transferrin bound form of iron.

In this regard recent work has shown that isolated macrophages release iron in the form of ferritin (Sibille et al, 1988) and that this ferritin is rapidly taken up by isolated hepatocytes (Sibille et al, 1988) presumably by a receptor mediated pathway (Mack et al, 1983). Non-transferrin iron has also been shown to be very effectively taken up by hepatocytes (Wright et al, 1988). The fractionation studies indicate, in absolute terms, that in the presence of a saturated transferrin, at 90 minutes, 8% of recovered counts were present in ferritin and roughly 10% in the small molecular weight pool in the liver. This compares to roughly 1% and 4.5% in the control animals. By 240 minutes these values were 22.5% and roughly 11% in the saturated

transferrin group while the control group had values of 11% and roughly 4.5%.

To further evaluate whether the ^{59}Fe transport from spleen to liver was as ferritin or as some small molecular weight non-transferrin bound form of iron, injections of homologous rat tissue ferritin were administered an hour prior to and 2 hours post HDRC administration. Organ distribution of ^{59}Fe was assessed at 8 hours post HDRC infusion. The results showed that both control and saturated transferrin groups of animals exhibited marked splenic ^{59}Fe retention with little ^{59}Fe being released into the blood and transported to the liver. The explanation for this related to consistently high titres of endotoxin being detected on limulus lysate assay of the homologous rat tissue ferritin preparation. Endotoxin itself causes major perturbations of internal iron transport (Torrance et al, 1978).

Whole serum fractionation studies at 30 minutes post HDRC infusion showed that there was no increase in the circulating ferritin ^{59}Fe peak in the animals with saturated transferrin. Since this was evaluated in systemic blood rather than portal blood the possibility still exists that enhanced hepatic ferritin clearance might have limited a rise in the circulating ferritin peak. The existence of such a circulating ^{59}Fe ferritin peak after HDRC infusion has previously been well documented (Siimes and Dallman, 1974). In the animals with a saturated transferrin whole serum fractionation indicated an absence of the transferrin ^{59}Fe peak.

The study involving various classes of chelators shed some further light on possible iron release mechanisms and the nature of the spleen to liver iron pathway. In animals with normal transferrin saturations, the extracellular hydrophilic chelator DTPA markedly enhanced splenic iron release. This, together with the studies on animals with enhanced erythropoiesis, suggests that reticuloendothelial iron release can be enhanced by increased unsaturated iron binding capacity. The finding that in control animals, the membranous ferrous chelator 2,2' bipyridine was associated with an increased amount of ^{59}Fe in the blood and reduced ^{59}Fe in the marrow is compatible with the observation that 2,2' bipyridine decreases iron uptake from diferric transferrin by a number of cell types, including erythroid precursors (Morgan, 1983; Nunez et al, 1983; Baynes et al, 1988a; 1988b). The increased hepatic retention of iron derived from ^{59}Fe labelled HDRC noted in animals with saturated transferrins was reduced by all three chelators. Each, however, must have obtained its iron from a different pool. 2,2' bipyridine presumably removed ferrous iron from splenic or hepatic cellular membranes or both, DTPA obtained ferric iron released by the spleen, while desferrioxamine procured its iron from a ferric pool predominantly in hepatocytes (Pippard et al, 1982).

There was one further point of interest. Fractionation of solubilized membrane enriched fractions from spleens and livers after the infusion of ^{59}Fe HDRC did not show any accumulation of radioiron activity in the hepatic membranes of animals with a saturated transferrin. This

finding suggests that membrane transport is not a rate limiting step in iron release from the liver.

4.3. The small molecular weight labile iron pool

The findings of this study have contributed to the knowledge of the nature of the small molecular weight labile iron pool. The cytosolic fractionation studies confirmed that the iron in this pool appears to be of a low molecular weight and illustrated that the iron in this pool is made up of both ferrous and ferric components. Enlargement of this pool following the infusion of HDRC into animals with a saturated transferrin appears to be a transient phenomenon, as evidenced by the finding that with the passage of time, less iron was present in this form and more was present in the form of ferritin. The transient nature of this pool has been documented by others (Young et al, 1985; Bomford et al, 1986).

There are in vitro and in vivo data to suggest that enlargement of the intracellular small molecular weight iron pool is a transient phenomenon because this iron induces a redistribution of cytosolic ferritin mRNA from being free within the cytosol to being attached to polyribosomes (Aziz and Munro, 1986; Rogers and Munro, 1987). This results in enhanced ferritin mRNA translation and in increased ferritin synthesis, which in turn would tend to reduce free low molecular weight iron by incorporating this iron into the newly synthesized ferritin.

The finding in this investigation of an increase in intracellular ferritin ^{59}Fe content soon after the infusion of HDRC into animals with saturated transferrins is in keeping with the observation that new ferritin production is mediated at a translational level in iron storage cells (Aziz and Munro, 1986). The redistribution of ferritin mRNA has recently been demonstrated to be mediated by an iron responsive element on the ferritin mRNA which effects the shift of the cytoplasmic ferritin mRNA to the polyribosomes (Aziz and Munro, 1986; Rogers and Munro, 1987; Hentze et al, 1987; Leibold and Munro, 1988; Casey et al, 1988; Dickey et al, 1988).

4.4. Implications of this investigation

The findings of this investigation can be seen as further evidence of the important role that non-transferrin iron has to play in internal iron exchange, especially in conditions of a saturated transferrin.

The documentation by others (Hershko et al, 1978; Anuwatanakulchai et al, 1984; Gutteridge et al,1985; Wang et al, 1986) of the presence of much higher concentrations of total iron circulating in the form of low molecular weight complexes not bound to transferrin in the plasma of patients with severe iron overload, together with the documentation of the highly efficient hepatic clearance of this form of iron (Brissot et al, 1985) have highlighted the importance of this form of iron in the hepatic iron loading and toxicity that characterizes many of the iron loading states. In the current investigation, the results of the

fractionation studies of hepatic extracts from control animals and those with saturated transferrins suggest that differences in the amount of iron present in the form of low molecular weight complexes are contributing to the altered organ distribution of iron derived from HDRC. These results also serve to confirm and emphasize the central role that the low molecular weight complexes are playing in the aetiology of hepatic iron loading, especially in conditions of a saturated transferrin.

While it is known that ferritin is rapidly cleared from the circulation by hepatocytes, probably by a receptor mediated process (Mack et al,1983; Morgan and Baker, 1986), what proportion of non-transferrin iron ferritin constitutes and the role it may play in hepatic iron loading is still not clear. Recently it has been suggested that ferritin may serve as an intrahepatic carrier of iron between Kupffer cells and hepatocytes (Kondo et al,1988; Sibille et al, 1988) and that this may explain the susceptibility of the liver to iron overload.

While serum fractionation studies performed in this investigation were unable to show an increase in circulating ferritin in animals with saturated transferrins, this was evaluated in systemic blood rather than portal blood. The possibility exists that ferritin may be playing an important part in iron transport from the spleen to the liver in conditions of a saturated transferrin and may be partly responsible for hepatic iron loading. Enhanced hepatic ferritin clearance may limit a rise in circulating ferritin levels, but fractionation of serum

obtained from the portal vein by its cannulation may shed more information on the role that ferritin has to play in iron transport from the spleen to the liver.

4.5. A model of internal iron exchange

Based on the results of the investigations carried out in this study, a model to explain the regulation of the internal exchange of HDRC derived iron was arrived at.

The reticuloendothelial cell typified by the splenocyte catabolizes haemoglobin at a relatively constant rate. It also releases its iron at a constant rate. This is in agreement with previous data of Noyes and coworkers (1960). The iron is transported across the cell membrane as ferrous iron. Extracellularly it undergoes redox state change to ferric iron.

This ferric iron is taken up by either transferrin or a non-transferrin iron binding mechanism. This non-specificity of binding is in keeping with previous work from this laboratory showing the absence of a specific apotransferrin-macrophage interaction (Baynes et al, 1987b). A ferric chelator is also able to gain access to the released iron.

The rate of release of iron from the splenocyte is not modified by a saturated transferrin. The reason for this may relate to the well documented relative lack of transferrin receptors on tissue macrophages

(Hamilton et al, 1984). The rate of release can be increased by increasing extracellular unsaturated iron binding capacity. Prior transfusion, by moving more iron through the reticuloendothelial cell is able to enhance splenocyte iron storage capacity.

Diferric transferrin resulting from splenocyte iron release then undergoes tissue specific distribution in direct relation to transferrin receptor density on tissues, with the major recipient tissue being the erythroid marrow. Non-transferrin iron (released largely in the setting of saturated transferrin) is rapidly cleared by the hepatocytes. Ferritin release from splenocytes and uptake by hepatocytes may contribute to the spleen to liver circuit in animals with a saturated transferrin. Ferritin has been shown to contribute to the circuit between the spleen and the liver in circumstances associated with red cell breakdown, but its quantitative significance is not clear (Siimes and Dallman, 1974). Once the iron reaches the liver it is retained there in the setting of a saturated transferrin. This difference between the hepatocyte and reticuloendothelial cell in the handling of iron in the setting of a saturated transferrin may relate to the well developed transferrin receptor system on the hepatocyte (Page et al, 1984; Trinder et al, 1986). The mechanism whereby transfusion increases reticuloendothelial iron storage capacity and whereby a saturated transferrin increases hepatocyte iron storage capacity more than likely involves increased intracellular iron effecting a shift of cytoplasmic ferritin mRNA to the polyribosomes by the iron responsive element (Aziz and Munro, 1986; Rogers and Munro,

1987; Hentze et al, 1987; Leibold and Munro, 1988; Casey et al, 1988; Dickey et al, 1988).

The apparent discrepancy between the current data and those of Bergamaschi and co-workers (1986) in relation to increased transferrin saturation increasing splenocyte iron retention may relate to these worker's use of infused iron which supersaturated the animals sera and may have resulted in depletion of the non-transferrin iron binding capacity.

REFERENCES.

Abramson S, Miller RG, Phillips RA. The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems.
J Exp Med 145: 1567-1579, 1977.

Addison GM, Beamish MR, Hales CN, Hodgkins M, Jacobs A, Llewellin P. An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. J Clin Pathol 25: 326-329, 1972.

Aisen P, Leibman A. Citrate-mediated exchange of Fe^{3+} among transferrin molecules. Biochem Biophys Res Commun 32: 220-226, 1968.

Aisen P, Leibman A. Lactoferrin and transferrin: A comparative study. Biochim Biophys Acta 257: 314-323, 1972.

Alderman EM, Fudenberg HH, Lovins RE. Isolation and characterization of an age-related antigen present in senescent human red blood cells. Blood 58: 341-349, 1981.

Aminoff D, Anderson J, Dabich L, Gathmann WD. Sialic acid content of erythrocytes in normal individuals and patients with certain hematologic disorders. Am J Hematol 9: 381-389, 1980.

Andreesen R, Bodemann H, Bross KJ, Costabel U, Osterholz J, Lohr GW. Human monocyte-macrophage differentiation: intracellular ferritin and

membrane receptors for transferrin at late states of maturation. Blood (supplement) 62: 148a, 1983.

Anuwatanakulchai M, Pootrakul P, Thuvasethakul P, Wasi P. Non-transferrin plasma iron in beta thalassaemia /Hb E and haemoglobin H diseases. Scand J Haematol 32: 153-158, 1984.

Arosio P, Adelman TG, Drysdale JW. On ferritin heterogeneity. Further evidence for heteropolymers. J Biol Chem 253: 4451-4458, 1978.

Ashwell G, Harford J. Carbohydrate-specific receptors of the liver. Annu Rev Biochem 51: 531-554, 1982.

Aziz N, Munro HN. Both subunits of rat liver ferritin are regulated at a translational level by iron induction. Nucleic Acids Res 14: 915-927, 1986.

Aziz N, Munro HN. Iron regulates ferritin mRNA translation through a segment of its 5'-untranslated region. Proc Natl Acad Sci USA 84: 8478-8482, 1987.

Bacon BR, Tavill AS. Role of the liver in normal iron metabolism. Semin Liver Dis 4: 181-191, 1984.

Baker E, Vicray FE, Huehns ER. Iron mobilisation from isolated rat hepatocytes. In: Proteins of Iron Metabolism. Brown EB, Aisen P,

Fielding J, Crichton RR (eds). Grune and Stratton, New York. 1977, pp327-334.

Baker E, Morton AG, Tavill AS. The regulation of iron release from the perfused rat liver. Br J Haematol 52: 631-640, 1982.

Banerjee D, Flanagan PR, Cluett J, Valberg LS. Transferrin receptors in the human gastrointestinal tract. Relationship to body iron stores. Gastroenterology 91: 861-869, 1986.

Banerjee S, Chakrabarty AS. Utilization of iron by scorbutic guinea pigs. Blood 25: 839-844, 1965.

Bannerman RM, Callender ST, Williams DL. Effect of desferrioxamine and DTPA in iron overload. Br Med J 2: 1573-1577, 1962.

Barnes D, Sato G. Serum-free cell culture: A unifying approach. Cell 22: 649-655, 1980.

Baynes R, Bezwoda W, Bothwell T, Khan Q, Mansoor N. The non-immune inflammatory response: serial changes in plasma iron, iron binding capacity, lactoferrin, ferritin and c-reactive protein. Scand J Clin Lab Invest 46: 695-704, 1986a.

Baynes RD, Flax H, Bothwell TH, Bezwoda WR, MacPhail AP, Atkinson P,

Lewis D. Haematological and iron related measurements in active pulmonary tuberculosis. Scand J Haematol 36: 280-287, 1986b.

Baynes RD, Bothwell TH, Bezwoda WR, Gear AJ, Atkinson P. Hematologic and iron related measurements in rheumatoid arthritis. Am J Clin Pathol 87: 196-200, 1987a.

Baynes RD, Bukofzer G, Bothwell TH, Bezwoda WR. Apotransferrin receptors and the delivery of iron from cultured human blood monocytes. Am J Hematol 25: 417-425, 1987b.

Baynes R, Bukofzer G, Bothwell T, Bezwoda W, Macfarlane B. Transferrin receptors and transferrin iron uptake by cultured human blood monocytes. Eur J Cell Biol 43: 372-376, 1987c.

Baynes RD, Friedman BM, Bukofzer GT, Bothwell TH, Macfarlane BJ, Lamparelli RD. The effect of ferrous and ferric chelators on transferrin iron-macrophage interactions. Am J Hematol 29: 27-32, 1988a.

Baynes R, Friedman B, McNamara L, Bothwell T, Bukofzer G, Macfarlane B. Transferrin iron interactions with cultured hepatocellular carcinoma cells. Eur J Cell Biol 46: 282-288, 1988b.

Bennett GD, Kay MMB. Homeostatic removal of senescent murine erythrocytes by splenic macrophages. Exp Hematol 9: 297-307, 1981.

Bergamaschi G, Eng MJ, Huebers HA, Finch CA. The effect of transferrin saturation on internal iron exchange. Proc Soc Exp Biol Med 183: 66-73, 1986.

Bessis M, Lessin L, Beutler E. Morphology of the erythron. In: Hematology. Williams WJ, Beutler E, Erslev AJ, Lichtman MA (eds). Mcgraw-Hill, New York. 1983. pp257-279.

Bezwoda WR, Derman DP, Bothwell TH, Baynes R, Hesdorffer C, MacPhail AP. Serum ferritin in Hodgkins disease. Scand J Haematol 25: 505-510, 1985.

Bezwoda WR, MacPhail AP, Bothwell TH, Baynes RD, Derman DP, Torrance JD. Failure of transferrin to enhance iron absorption in achlorhydric human subjects. Br J Haematol 63: 749-752, 1986.

Blight GD, Morgan EH. Ferritin and iron uptake by reticulocytes. Br J Haematol 55: 59-71, 1983.

Bocci V. Determinant of erythrocyte aging: a reappraisal. Br J Haematol 48: 515-522, 1981.

Bomford AB, Munro HN. Biosynthesis of ferritin and isoferritins. In: Iron in Biochemistry and Medicine II. Jacobs A, Worwood M eds. Academic Press, London and New York. 1980. pp173-202.

Bomford AB, Munro HN. Transferrin and its receptor: Their roles in cell function. Hepatology 5: 870-875, 1985.

Bomford A, Young S, Williams R. Intracellular forms of iron during transferrin iron uptake by mitogen-stimulated human lymphocytes. Br J Haematol 62: 487-494, 1986.

Bothwell TH, Bradlow BA, Jacobs P, Keeley K, Kramer S, Seftel H, Zail S. Iron metabolism in scurvy with special reference to erythropoiesis. Br J Haematol 10: 50-58, 1964.

Bothwell TH, Charlton RW, Cook JD, Finch CA. Iron metabolism in man. Oxford: Blackwell Scientific Publications, 1979.

Bottomley SS, Wolfe LC, Bridges KR. Iron metabolism in K562 erythroleukemic cells. J Biol Chem 260: 6811-6815, 1985.

Bridges KR, Cudkowicz A. Effect of iron chelators on the transferrin receptor in K562 cells. J Biol Chem 259: 12970-12977, 1984.

Bridges KR, Hoffman KE. The effects of ascorbic acid on the intracellular metabolism of iron and ferritin. J Biol Chem 261: 14273-14277, 1986.

Brink B, Disler P, Lynch S, Jacobs P, Charlton R, Bothwell T. Patterns of iron storage in dietary iron overload and idiopathic hemochromatosis. J Lab Clin Med 88: 725-731, 1976.

Brissot P, Wright TL, Ma W-L, Weisiger RA. Efficient clearance of non-transferrin-bound iron by rat liver. Implications for hepatic iron loading in iron overload states. J Clin Invest 76: 1463-1470, 1985.

Brock JH, Esparza I. Failure of reticulocytes to take up iron from lactoferrin saturated by various methods. Br J Haematol 42: 481-483, 1979.

Brownlee M, Vlassara H, Cerami A. Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann Intern Med 101: 527-537, 1984

Bunn HF. Erythrocyte destruction and hemoglobin catabolism. Semin Hematol 9: 3-17, 1972.

Cairo G, Bardella L, Schiaffonati L, Arosio P, Levi S, Bernalli-Zazzera A. Multiple mechanisms of iron-induced synthesis in Hela cells. Biochem Biophys Res Commun 133: 314-321, 1985.

Carpentier JL, Gorden P, Anderson RGW, Goldstein JL, Brown MS, Cohen S, Orci L. Co-localization of ^{125}I experimental growth factor and ferritin low-density lipoprotein in coated pits: A quantitative electron

microscopic study in normal and mutant human fibroblasts, J Cell Biol 95: 73-77, 1982.

Casey TL, Hentze MW, Koeller DM, Caughman SW, Rouault TA, Klausner RD, Harford JB. Iron responsive elements: regulatory RNA sequences that control mRNA levels and translation. Science 240: 924-928, 1988.

Cazzola M, Dezza L, Bergamaschi G, Barosi G, Bellotti V, Caldera D, Ciriello MM, Quglini S, Arosio P, Ascari E. Biological and clinical significance of red cell ferritin. Blood 62: 1078-1087, 1983.

Chapman RW, Hussein MAM, Gorman A, Laulicht M, Politis D, Flynn DM, Sherlock S, Hoffbrand AV. Effect of ascorbic acid deficiency on serum concentrations in patients with beta-thalassaemia major and iron overload. J Clin Pathol 35: 487-491, 1982.

Cline MJ, Lehrer RI, Territo MC, Golde DW. Monocytes and macrophages: Functions and diseases. Ann Intern Med 88: 78-88, 1978.

Cohen A, Cohen IJ, Schwartz E. Scurvy and altered iron stores in thalassemia major. N Eng J Med 304: 158-160, 1981.

Cook JD, Marsaglia G, Eschbach JW, Funk DD, Finch CA. Ferrokinetics: a biological model for plasma iron exchange in man. J Clin Invest 49: 197-205, 1970.

Cragg SJ, Drysdale J, Worwood M. Genes for the "H" subunit of human ferritin are present on a number of human chromosomes. Hum Genet 71: 108-112, 1985.

Crichton RR, Charloteaux-Wauters M. Iron transport and storage. Eur J Biochem 164: 485-506, 1987.

Crosby WH. Prescribing iron? Think safety. Arch Intern Med 138: 766-767, 1978.

Cumming RLC, Goldberg A, Morrow J, Smith JA. Effect of phenylhydrazine-induced haemolysis on the urinary excretion of iron after desferrioxamine. Lancet 1: 71-74, 1967.

Dautry-Varsat A, Ciechanover A, Lodish HF. pH and the recycling of transferrin during receptor mediated endocytosis. Proc Natl Acad Sci USA 80: 2258-2262, 1983.

Deiss A, Cartwright CB, Ferritin metabolism in reticulated-siderocytes. J Clin Invest 49: 517-523, 1970.

Deiss A. Iron metabolism in reticuloendothelial cells. Semin Hematol 20: 81-90, 1983.

Dekker CJ, Kroos MJ, van der Heul C, van Eijk HG. Uptake of asialotransferrins by isolated rat hepatocytes. Comparison of a heterologous and a homologous system. Int J Biochem 17: 701-706, 1985.

Delaney TA, Morgan EH. Chemical but not functional differences between the iron-binding sites of rabbit transferrin. Biochim Biophys Acta 701: 295-304, 1982.

Dickey LF, Sreedharan S, Theil EC, Didsbury TR, Wong YH, Kaufman RE. Differences in the regulation of messenger RNA for housekeeping and specialized cell ferritin. J Biol Chem 262: 7901-7907, 1987.

Dickey LF, Wang YH, Shull GE, Wartman IA, Theil EC. The importance of the 3' untranslated region in the translational control of ferritin mRNA. J Biol Chem 263: 3071-3074, 1988.

Drysdale JW, Munro HN. Regulation of synthesis and turnover of ferritin in rat liver. J Biol Chem 241: 3630-3637, 1966.

Drysdale JW, Adelman TG, Arosio P, Casareale D, Fitzpatrick P, Hazard JT, Yokota M. Human isoferritins in normal and disease states. Semin Hematol 14: 71-88, 1977.

Drysdale JW. Ferritin phenotypes: structure and metabolism. In: Iron Metabolism. Ciba Foundation Symposium 51 (New Series) Amsterdam: Elsevier/ Excerpta Medica/ North Holland. 1977. pp41-57.

Durocher JR, Payne RC, Conrad ME. Role of sialic acid in erythrocyte survival. Blood 45: 11-20, 1975.

Easley RM, Schreiner BF, Yu PN. Reversible cardiomyopathy associated with hemochromatosis. N Eng J Med 287: 866-867, 1972.

Ehrenreich BA, Cohn ZA. Fate of hemoglobin pinocytosed by macrophages in vitro. J Cell Biol 38: 244-248, 1968.

Egyed A. The significance of transferrin-bound bicarbonate in the uptake of iron by reticulocytes. Biochim Biophys Acta 304: 805-813, 1973.

Egyed A. Carrier mediated iron transport through erythroid cell membrane. Br J Haematol 68: 483-486, 1988.

Elder GH. Haem synthesis and breakdown. In: Iron in Biochemistry and Medicine II. Jacobs A, Worwood M (eds). Academic Press, London. 1980. pp563-587.

Enler HH, Kern P, Loffler H, Dietrich M. Precipitable immune complexes in healthy homosexual men, acquired immune deficiency syndrome and the related lymphadenopathy syndrome. Clin Exp Immunol 59: 267-275, 1985.

Enns CA, Sussman HH. Physical characteristics of the transferrin receptor in human placenta. J Biol Chem 256: 9820-9823, 1981.

Esparza I, Brock JH. Release of iron by resident macrophages following ingestion and degradation of transferrin-antitransferrin immune complexes. Br J Haematol 49: 603-610, 1987.

Fahey JL, Roth CE, Princiotto JV, Brick IB, Rubin M. Evaluation of trisodium calcium diethylenetriamine pentaacetate in iron storage disease. J Lab Clin Med 57: 436-449, 1961.

Fairbanks VF, Beutler E. Iron metabolism. In: Hematology. Williams WJ, Beutler E, Erslev AJ, Lichtman MA (eds). Mcgraw-Hill, New York. 1983. pp300-310.

Fillet G, Cook JD, Finch CA. Storage iron kinetics VII. A biological model for reticuloendothelial iron transport. J Clin Invest 53: 1527-1533, 1974.

Finch CA, Miller LR, Inandar Ar, Person R, Seiler K, Mackler B. Iron deficiency in the rat; Physiological and biochemical studies of muscle dysfunction. J Clin Invest 58: 447-453, 1976.

Finch CA, Huebers H. Perspectives in iron metabolism. N Eng J Med 306: 1520-1528, 1982.

Finch CA, Bellotti V, Stray S, Lipschitz DA, Cook JD, Pippard MJ, Huebers HA. Plasma ferritin determination as a diagnostic tool. West J Med 145: 657-663, 1986.

Frieden E, Osaki S. Ferroxidases and ferrireductases: Their role in iron metabolism. Adv Exp Med Biol 48: 235-265, 1974.

Galbraith RM, Werner P, Arnaud P, Galbraith GMP. Transferrin binding to peripheral blood lymphocytes activated by phytohemagglutinin involves a specific receptor. J Clin Invest 66: 1135-1143, 1980.

Ganzoni AM, Oakes R, Hekkman RS. Red cell aging in vivo. J Clin Invest 50: 1373-1378, 1971.

Goldstein JL, Anderson RGW, Brown MS. Coated pits, coated vesicles and receptor mediated endocytosis. Nature 279: 679-685, 1979.

Groen R, Hendricksen P, Young SP, Leibman A, Aisen P. Molecular ferrokinetics in the rabbit. Br J Haematol 50: 43-53, 1982.

Gutteridge JMC, Rowley DA, Griffiths E, Halliwell B. Low-molecular-weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis. Clin Sci 68: 463-467, 1985.

Halliday JW, Powell LW, Mack U. Iron absorption in the rat: the search for possible intestinal mucosal carriers. Br J Haematol 34: 237-250, 1976.

Halliday JW, Powell LW. Ferritin metabolism and the liver. Semin Liver Dis 4: 207-216, 1984.

Hamilton TA, Weiel JE, Adams DO. Expression of the transferrin receptor is modulated in macrophages in different stages of functional activation. J Immunol 132: 2285-2290, 1984.

Harrison PM, Clegg GA, May K. Ferritin structure and function. In: Iron in Biochemistry and Medicine II. Jacobs A, Worwood M (eds). Academic Press, London. 1980. pp131-171.

Hemmaplardh D, Kailis SG, Morgan EH. The effects of inhibitors of microtubule and microfilament function on transferrin and iron uptake by rabbit reticulocytes and bone marrow. Br J Haematol 28: 53-65, 1974.

Hemmaplardh D, Morgan EH. Transferrin uptake and release by reticulocytes treated with proteolytic enzymes and neuraminidase. Biochim Biophys Acta 426: 385-398, 1976.

Hemmaplardh D, Morgan EH. The role of calcium in transferrin and iron uptake by reticulocytes. Biochim Biophys Acta 468: 423-436, 1977.

Hentze MW, Caughman SW, Rouault TA, Barriocanal JG, Dancis A, Harford JB, Klausner RD. Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. Science 238: 1570-1573, 1987.

Hershko C, Cook JD, Finch CA. Storage iron kinetics. II. The uptake of hemoglobin iron by hepatic parenchymal cells. J Lab Clin Med 80: 624-634, 1972.

Hershko C, Cook JD, Finch CA. Storage iron kinetics III. Study of desferrioxamine action by selective radioiron labels of RE and parenchymal cells. J Lab Clin Med 81: 876-886, 1973.

Hershko C, Grady RW, Cerami A. Mechanism of iron chelation in the hypertransfused rat: definition of two alternative pathways of iron mobilization. J Lab Clin Med 92: 144-151, 1978a.

Hershko C, Graham G, Bates GW, Rachmilewitz EA. Nonspecific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. Br J Haematol 40: 255-263, 1978b.

Hershko C, Weatherall DJ. Iron chelating therapy. CRC Crit Rev Clin Lab Sci 26: 303-345, 1988.

Hoffbrand AV, Ganeshaguru K, Tattersall MHN, Tripp E. The effect of iron deficiency on DNA synthesis. Br J Haematol 33:517-526, 1976.

Hoy TG, Harrison PM, Shabbir M. Uptake and release of ferritin iron: surface effects and exchange within the crystalline core. Biochem J 139: 603-607, 1974.

Huebers H, Huebers E, Rummel W, Chrichton RR. Isolation and characterization of iron-binding proteins from rat intestinal mucosa. Eur J Biochem 66: 447-455, 1976.

Huebers H, Bauer W, Huebers E, Csiba E, Finch C. The behavior of transferrin iron in the rat. Blood 57: 218-228, 1981.

Huebers HA, Finch CA. Introduction: clinical aspects of iron deficiency. Semin Hematol 19: 3-5, 1982.

Huebers HA, Csiba E, Huebers E, Finch CA. Competitive advantage of diferric transferrin in delivering iron to reticulocytes. Proc Natl Acad Sci USA 80: 300-304, 1983a.

Huebers HA, Huebers E, Csiba E, Rummel W, Finch CA. The significance of transferrin for intestinal iron absorption. Blood 61: 283-290, 1983b.

Huebers HA, Huebers E, Csiba E, Finch CA. Heterogeneity of the plasma iron pool: explanation of the Fletcher-Huehns phenomenon. Am J Physiol 247: 280-283, 1984.

Huebers KA, Brittenham GM, Csiba E, Finch CA. Absorption of carbonyl iron. J Lab Clin Med 108: 473-478, 1986.

Huebers HA, Finch CA. The physiology of transferrin and transferrin receptors. Physiol Rev 67: 520-582, 1987.

Idzerda RL, Huebers H, Finch CA, McKnight GS. Rat transferrin gene expression: Tissue specific regulation by iron deficiency. Proc Natl Acad Sci USA 83: 3723-3727, 1986.

International Committee for Standardization in Haematology (Iron Panel). Recommendations for the measurement of serum iron in human blood. Br J Haematol 38: 281-290, 1978a.

International Committee for Standardization in Haematology (Iron Panel). The measurement of total and unsaturated iron-binding capacity in serum. Br J Haematol 38: 291-294, 1978b.

Jacobs A. Low molecular weight intracellular iron transport compounds. Blood 50: 433-439, 1977.

Jandl JH, Inman JK, Simmons RL, Allen DW. Transfer of iron from serum iron-binding protein to human reticulocytes. J Clin Invest 38: 161-185, 1959.

Jandl JH, Katz JH. The plasma-to-cell cycle of transferrin. J Clin Invest 42: 314-326, 1963.

Jordan SM, Morgan EH. Plasma protein synthesis by tissue slices from pregnant and lactating rats. Biochim Biophys Acta 174: 373-379, 1969.

Kadlubowski M, Agutter PS. Changes in the activities of some membrane-associated enzymes during the in vivo ageing of the normal human erythrocyte. Br J Haematol 37: 111-125, 1977.

Kailis SG, Morgan EH. Transferrin and iron uptake by rabbit bone marrow cells in vitro. Br J Haematol 28: 37-52, 1974.

Karabus CD, Fielding J. Desferrioxamine chelatable iron in haemolytic, megaloblastic and sideroblastic anaemias. Br J Haematol 13: 924-933, 1967.

Katz JH. Iron and protein kinetics studied by means of doubly labelled human crystalline transferrin. J Clin Invest 40: 2143-2151, 1961.

Kay MMB. Mechanism of removal of senescent cells by human macrophages in situ. Proc Natl Acad Sci USA 72: 3521-3525, 1975.

Keberle H. The biochemistry of desferrioxamine and its relation to iron metabolism. Ann N.Y. Acad Sci 119: 758-768, 1964.

Kim BK, Huebers H, Pippard MJ, Finch CA. Storage iron exchange in the rat as affected by desferoxamine. J Lab Clin Med 105: 440-448, 1985.

Kimber RJ, Rudzki Z, Blundeu RW. Clinching the diagnosis 1. Iron deficiency and iron overload. Serum ferritin and serum iron in clinical medicine. Pathology 15: 497-503, 1983.

Klausner RD, Ashwell G, Van Renswoude J, Harford JB, Bridges KR. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. Proc Natl Acad Sci USA 80: 2263-2266, 1983.

Kondo H, Saito K, Grasso JP, Aisen P. Iron metabolism in the erythrophagocytosing Kupffer cell. Hepatology 8: 32-38, 1988.

Lee GR. The anemia of chronic disease. Semin Hematol 20: 61-80, 1983.

Leibold EA, Munro HM. Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light- subunit mRNAs. Proc Natl Acad Sci USA 85: 2171-2175, 1988.

Link G, Pinson A, Hershko C. Heart cells in culture: a model of myocardial iron overload and chelation. J Lab Clin Med 106: 147-153, 1985.

Lipschitz DA, Bothwell TH, Seftel HC. The role of ascorbic acid in the metabolism of storage iron. Br J Haematol 20: 155-163, 1971a.

Lipschitz DA, Dugard J, Simon MO, Bothwell TH, Charlton RW. The site of action of desferrioxamine. Br J Haematol 20: 395-404, 1971b.

Lipschitz DA, Simon MO, Lynch SR, Dugard J, Bothwell TH, Charlton RW. Some factors affecting the release of iron from reticuloendothelial cells. Br J Haematol 21: 289-303, 1971c.

Lipschitz DA, Cook JD, Finch CA. An evaluation of serum ferritin as an index of iron stores. N Eng J Med 290: 1213-1216, 1974.

London IM, Bruns GP, Karabian D. The regulation of hemoglobin synthesis and the pathogenesis of dome hypochromic anemias. Medicine (Balt) 43: 789-802, 1964.

Luner SJ, Szklarek D, Knox RJ, Seaman GVF, Josefowicz JY, Ware BR. Red cell charge is not a function of cell age. Nature 269: 719-721, 1977.

Lynch SR, Seftel HC, Torrance JD, Charlton RW, Bothwell TH. Accelerated oxidative catabolism of ascorbic acid in siderotic Bantu. Am J Clin Nutr 20: 641-647, 1967.

Lynch SR, Lipschitz DA, Bothwell TH, Charlton RW. Iron and the reticuloendothelial system. In: Iron in Biochemistry and Medicine. Jacobs A, Worwood M (eds). Academic Press, London. 1974. pp563-587.

MacGillivray RTA, Mendez E, Shewale JG, Sinha S, Lineback-Zins J, Brew K. The primary structure of human serum transferrin. J Biol Chem 258: 3543-3553, 1983.

Mack U, Cooksley WGC, Ferris RA, Powell LW, Halliday JW. Regulation of plasma ferritin by the isolated perfused rat liver. Br J Haematol 47: 403-412, 1981.

Mack U, Powell LW, Halliday JW. Detection and isolation of a hepatic membrane receptor for ferritin. J Biol Chem 258: 4672-4675, 1983.

MacSween RNM, MacDonald RA. Iron metabolism by RE cells. In vitro uptake of transferrin-iron by rat and rabbit cells. Lab Invest 21: 230-235, 1969.

Mann S, Bannister JV, Williams RJP. Structure and composition of ferritin cores isolated from human spleen, limpet (Patella vulgata) hemolymph and bacterial (Pseudomonas aeruginosa) cells. J Mol Biol 188: 225-232, 1986.

May PM, Williams DR. THe inorganic chemistry of iron metabolism. In: Iron in Biochemistry and Medicine. Jacobs A, Worwood M (eds). Academic Press, London, 1980, pp1-28.

May WS, Jacobs S, Cuatrecasas P. Association of phorbol ester induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL60 cells. Proc Natl Acad Sci USA 81: 2016-2020, 1984.

May WS, Cuatrecasas P. Transferrin receptor: Its biological significance. J Membr Biol 88: 205-215, 1985.

May WS, Sahyoun N, Jacobs S, Wolf M, Cuatrecasas P. Mechanism of phorbol diester-induced regulation of surface transferrin receptor involves the action of activated protein kinase c and an intact cytoskeleton. J Biol Chem 260: 9419-9426, 1985.

McArdle HJ, Morgan EH. The effect of monoclonal antibodies to the human transferrin receptor on transferrin and iron uptake by rat and rabbit reticulocytes. J Biol Chem 259: 1398-1400, 1984.

McGill JR, Naylor SI, Sakagushi QY, Moore CM, Boyd D, Barret KJ, Shows TB, Drysdale JW. Human ferritin H and L sequences lie on 10 different chromosomes. Hum Genet 76: 66-72, 1987.

McKnight GS, Lee DS, Hemmaplardh D, Finch CA, Palmitter RD. Transferrin gene expression: effects of nutritional iron deficiency. J Biol Chem 255: 144-147, 1980a.

McKnight GS, Lee DC, Palmitter RD. Transferrin gene expression. Regulation of mRNA transcription in chick liver by steroid hormones and iron deficiency. J Biol Chem 255: 148-153, 1980b.

Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J, Jolles P. Human lactoferrin: amino acid sequence and structural comparisons with other transferrins. Eur J Biochem 145: 659-676, 1984.

Meyer TE, Kassianides C, Bothwell TH, Green A. Effect of heavy alcohol consumption on serum ferritin concentrations. S Afr Med J 66: 573-575, 1984.

Meyer-Brunot HG, Keberle H. The metabolism of desferrioxamine B. Biochem Pharm 16: 527-535, 1967.

Miller JPG, Perkins DJ. Model experiments for the study of iron transfer from transferrin to ferritin. Eur J Biochem 10: 146-151, 1969.

Montreuil J, Tonnelat J, Mullet S. Preparation and properties of lactosiderophilin (lactotransferrin) of human milk. Biochim Biophys Acta 45: 413-421, 1960.

Morgan EH. A study of iron transfer from rabbit transferrin to reticulocytes using synthetic chelating agents. Biochim Biophys Acta 244: 103-116, 1971.

Morgan EH. Transferrin, biochemistry, physiology and clinical significance. Mol Aspects Med 4: 1-123, 1981.

Morgan EH. Chelator mediated iron efflux from reticulocytes. Biochim Biophys Acta 733: 39-50, 1983a.

Morgan EH. Effects of pH and iron content of transferrin on its binding to reticulocyte receptors. Biochim Biophys Acta 762: 498-502, 1983b.

Morgan EH, Baker E. Iron uptake and metabolism by hepatocytes. Fed Proc 45: 2810-2816, 1986.

Morrow WJW, Wharton M, Stricker RB, Levy JA. Circulating immune complexes in patients with acquired immune deficiency syndrome contain the AIDS-associated retrovirus. Clin Immunol Immunopathol 40:515-524, 1986.

Muller-Eberhard U, Javid J, Liem HH, Hanstein A, Hanna M. Plasma concentrations of hemopexin, haptoglobin and heme in patients with various hemolytic diseases. Blood 32: 811-815, 1968.

Myhre E. Iron uptake by human erythroid cells in vitro. Scand J Clin Lab Invest 16: 201-211, 1964.

Nagel RL, Gibson QH. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. J Biol Chem 246: 69-73, 1971.

Nishisato T, Aisen P. Uptake of transferrin by rat peritoneal macrophages . Br J Haematol 52: 631-640, 1982.

Noyes WD, Bothwell TH, Finch CA. The role of the reticuloendothelial cell in iron metabolism. Br J Haematol 6: 43-55, 1960.

Nunez M-T, Cole ES, Glass J. The reticulocyte plasma membrane pathway of iron uptake as determined by the mechanism of α-α'dipyridyl inhibition. J Biol Chem 258: 1146-1151, 1983.

O'Connell MJ, Ward RJ, Baum H, Peters TJ. The role of iron in ferritin- and haemosiderin-mediated lipid peroxidation in liposomes. Biochem J 229: 135-139, 1985.

Octave JN, Schneider YJ, Crichton RR, Trouet A. Iron mobilization from cultured hepatocytes: effect of desferrioxamine B. Biochem Pharm 32: 3413-3418, 1983.

Osaki S, Johnson DA, Frieden E. The mobilisation of iron from the perfused mammalian liver by a serum copper enzyme, ferrioxidase I. J Biol Chem 246: 3018-3023, 1971.

Page MA, Baker E, Morgan EH. Transferrin and iron uptake by rat hepatocytes in culture. Am J Physiol 246: G26-G33, 1984.

Pan BT, Johnstone R. Selective externalization of the transferrin receptor by sheep reticulocytes in vitro. J Biol Chem 259: 9776-9782, 1984.

Parmley RT, Hajdu I, Denys FR. Ultrastructural localization of the transferrin receptor and transferrin on marrow cell surfaces. Br J Haematol 54: 633-641, 1983.

Parmley RT, Barton JC, Conrad ME. Ultrastructural localization of transferrin receptor and iron binding sites on human placental and duodenal microvilli. Br J Haematol 60: 81-89, 1985.

Peters G, Keberle H, Schmid K, Brunner H. Distribution and renal excretion of desferrioxamine and ferrioxamine in the dog and in the rat. Biochem Pharm 15: 93-109, 1966.

Peters SW, Jacobs A, Fitzsimons E. Erythrocyte ferritin in normal subjects and patients with abnormal iron metabolism. Br J Haematol 53: 211-216, 1983.

Pippard MJ, Johnson DK, Finch CA. Hepatocyte iron kinetics in the rat explored with an iron chelator. Br J Haematol 52: 211-224, 1982.

Pollack S, Lasky FD. A new iron-binding protein isolated from intestinal mucosa. J Lab Clin Med 87: 670-679, 1976.

Pollack S, Campana T. Low molecular weight nonheme iron and a highly labeled heme pool in the reticulocyte. Blood 56: 564-566, 1980.

Pollack S, Campana T. Immature red cells have ferritin receptors. Biochem Biophys Res Commun 100: 1667-1672, 1981.

Pollack S, Campana T, Weaver J. Low molecular weight iron in guinea pig reticulocytes. Am J Hematol 19: 75-84, 1985.

Ponka P, Neuwirt J. Regulation of iron entry into reticulocytes. I. Feedback inhibitory effect of heme on iron entry into reticulocytes and on heme synthesis. Blood 33: 690-707, 1969.

Ponka P, Neuwirt J. Regulation of iron entry into reticulocytes. II. Relationships between hemoglobin synthesis and entry of iron into reticulocytes. Biochim Biophys Acta 230: 381-392, 1971.

Ponka P, Neuwirt J, Borova J. The role of heme in the release of iron from transferrin in reticulocytes. Enzyme 17: 91-99, 1974.

Powell LW, Halliday JW. Iron, ferritin and the liver. Prog Liver Dis 7: 599-614, 1982.

Prieto J, Barry M, Sherlock S. Serum ferritin in patients with iron overload and acute and chronic liver diseases. Gastroenterology 68: 525-533, 1975.

Primosigh JV, Thomas ED. Studies on the partition of iron in bone marrow cells. J Clin Invest 47: 1473-1482, 1968.

Rabin M, McClelland A, Kuhn L, Ruddle FH. Regional localization of the human transferrin receptor to 3q 26.2-q ter. Am J Hum Genet 37: 1112-1116, 1985.

Richter GW. Studies of iron overload. Rat liver siderosome ferritin. Lab Invest 50: 26-35, 1984.

Roeser HP. Iron metabolism in inflammation and malignant disease. In: Iron in Biochemistry and Medicine II. Jacobs A, Worwood M (eds). London, Academic Press. 1980. pp605-640

Rogers J, Munro H. Translation of ferritin light and heavy subunit mRNAs is regulated by intracellular chelateable iron levels in rat hepatoma cells. Proc Natl Acad Sci USA 84: 2277-2281, 1987.

Rudolph JR, Regoeczi E. Interaction of rat asialotransferrin with adult rat hepatocytes: Its relevance for iron uptake and protein degradation. J Cell Physiol 135: 539-544, 1988.

Saito K, Nishisato T, Grasso JA, Aisen P. Interaction of transferrin with iron-loaded rat peritoneal macrophages. Br J Haematol 62: 275-286, 1986.

Schneider C, Sutherland R, Newman R, Greaves M. Structural features of the cell surface receptor for transferrin that is recognised by the monoclonal antibody OKT 9. J Biol Chem 257: 8516-8522, 1982.

Schneider C, Owen MJ, Bonville D, Williams JG. Primary structure of human transferrin receptor deduced from the mRNA sequence. Nature 311: 675-678, 1984.

Sciortino CV, Byers BR, Cox P. Evaluation of iron-chelating agents in cultured heart muscle cells. Identification of a potential drug for chelation therapy. J Lab Clin Med 96: 1081-1085, 1980.

Sciot R, Paterson AC, Van den Oord JJ, Desmet VJ. Lack of hepatic transferrin receptor expression in haemochromatosis. Hepatology 7: 831-837, 1987.

Seaman GVF, Knox RJ, Nordt FJ, Regan DH. Red cell aging. I. Surface charge density and sialic acid content of density-fractionated human erythrocytes. Blood 50: 1001-1011, 1977.

Seligman PA, Schleicher RB, Allen RH. Isolation and characterization of the transferrin receptor from human placenta. J Biol Chem 254: 9943-9946, 1979.

Shattil SJ, Cooper RA. Maturation of macroreticulocyte membranes in vivo. J Lab Clin Med 79: 215-227, 1972.

Sheetz MP, Singer SJ. On the mechanism of ATP-induced shape changes in human erythrocyte membranes. I. The role of the spectrin complex. J Cell Biol 73: 638-646, 1977.

Shiga T, Maeda N, Suda T, Kon K, Sekiya M. The decreased membrane fluidity of in vivo aged, human erythrocytes. A spin label study. Biochim Biophys Acta 553: 84-95, 1979.

Shoden A, Gabrio BW, Finch CA. The relationship between ferritin and haemosiderin in rabbits and man. J Biol Chem 204: 823-830, 1958.

Short EM, Winkle RA, Billingham ME. Myocardial involvement in idiopathic hemochromatosis. Morphological and clinical improvement following venesection. Am J Med 70: 1275-1279, 1981.

Shotton D, Thompson K, Wofsy L, Branton D. Appearance and distribution of surface proteins of the human erythrocyte membrane. An electron microscope and immunochemical labeling study. J Cell Biol 76: 512-531, 1978.

Sibille JC, Kondo H, Aisen P. Interactions between isolated hepatocytes and Kupffer cells in iron metabolism: A possible role for ferritin as an iron carrier protein. Hepatology 8: 296-301, 1988.

Siimes MA, Dallman PR. New kinetic role for serum ferritin in iron metabolism. Br J Haematol 28: 7-18, 1974.

Simon M, MacPhail P, Bothwell T, Lyons G, Baynes R, Torrance J. The fate of intravenously administered hepatic ferritin in normal, phenylhydrazine-treated and scorbutic guinea-pigs. Br J Haematol 65: 239-243, 1987.

Sizemore DJ, Bassett ML. Monocyte transferrin-iron uptake in hereditary hemochromatosis. Am J Hematol 16: 347-354, 1984.

Smith A, Morgan WT. Hemopexin-mediated transport of heme into isolated rat hepatocytes. J Biol Chem 256:10902-10909, 1981.

Stahl P, Schwartz Al. Receptor mediated endocytosis. J Clin Invest 77: 657-662, 1986.

Summers MR, Jacobs A. Iron uptake and ferritin synthesis by peripheral blood leucocytes from normal subjects and patients with iron deficiency and anaemia of chronic disease. Br J Haematol 34: 221-229, 1976.

Takami M, Mizumoto K, Kasuya I, Kohsuke K, Sussman HH, Tsunoo H. Human placental ferritin receptor. Biochim Biophys Acta 884: 31-38, 1986.

Tavassoli M, Kishimoto T, Soda R, Kataoka M, Harjes K. Liver endothelium mediates the uptake of iron-transferrin complex by hepatocytes. Exp Cell Res 165: 369-379, 1986.

Taylor C, Rogers G, Goodman C, Baynes RD, Bothwell TH, Bezwoda WR, Kramer F, Hattingh J. Hematologic,iron-related and acute phase protein responses to sustained strenuous exercise. J Appl Physiol 62: 464-469, 1987.

Tenhunen R, Marver HS, Schmid R. The enzymatic catabolism of hemoglobin: Stimulation of microsomal heme oxygenase by hemin. J Lab Clin Med 75: 410-421, 1970.

Tenhunen R. The enzymatic degradation of heme. Semin Hematol 9: 19-29, 1972.

Theil EC. Ferritin: Structure, gene regulation, and cellullar function in animals, plants and microorganisms. Annu Rev Biochem 56: 289-315, 1987.

Thorstensen K, Romslo I. Uptake of iron from transferrin by isolated hepatocytes. Relationship to cellular energy metabolism. Scand J Clin Lab Invest 46: 107-113, 1986.

Thorstensen K. Hepatocytes and reticulocytes have different mechanisms for the uptake of iron from transferrin. J Biol Chem 263: 16837-16841, 1988.

Torrance JD, Charlton RW, Simon MO, Lynch SR, Bothwell TH. The mechanism of endotoxin-induced hypoferraemia. Scand J Haematol 21: 403-410, 1978.

Treffrey A, Lee PJ, Harrison PM. Functional studies on rat liver isoferritins. Biochim Biophys Acta 785: 22-29, 1984.

Trinder D, Morgan E, Baker E. The mechanisms of iron uptake by fetal rat hepatocytes in culture. Hepatology 6: 852-858, 1986.

Trowbridge IS, Omary MB. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Proc Natl Acad Sci USA 78: 3093-3043, 1981.

Trowbridge IS, Newman RA, Domingo DL, Sauvage C. Transferrin receptors structure and function. Biochem Pharmacol 33: 925-932, 1984.

Ulvik RJ. Relevance of ferritin-binding sites on isolated mitochondria to the mobilization of iron from ferritin. Biochim Biophys Acta 715: 42-51, 1982.

Unger A, Hershko C. Hepatocellular uptake of ferritin in the rat. Br J Haematol 28: 169-179, 1984.

van Bockxmeer FM, Morgan EH. Identification of transferrin receptors in reticulocytes. Biochim Biophys Acta 468: 437-450, 1977.

van Bockxmeer FM, Yates GK, Morgan EH. Interaction of transferrin with solubilized receptors from reticulocytes. Eur J Biochem 92: 147-154, 1978.

van Bockxmeer FM, Morgan EH. Transferrin receptors during rabbit reticulocyte maturation. Biochim Biophys Acta 584: 76-83, 1979.

Van der Heul C, Kroos MJ, Ban Noort WL, Van Eijk HG. No functional differences of the two iron-binding sites of human transferrin in vitro. Clin Sci 60: 185-190, 1981.

Vlassara H, Brownlee M, Cerami A. Accumulation of diabetic rat peripheral nerve myelin by macrophages increases with the presence of advanced glycosylation endproducts. J Exp Med 160: 197-207, 1984.

Vlassara H, Brownlee M, Cerami A. High-affinity receptor-mediated uptake and degradation of glucose-modified proteins: A potential mechanism for the removal of senescent macromolecules. Proc Natl Acad Sci USA 82: 5588-5592, 1985.

Vlassara H, Valinsky J, Brownlee M, Cerami C, Nishimoto S, Cerami A. Advanced glycosylation endproducts on erythrocyte cell surface induce receptor-mediated phagocytosis by macrophages. J Exp Med 166: 539-549, 1987.

Walker WS, Singer JA, Morrison M, Jackson CW. Preferential phagocytosis of in vivo aged murine red blood cells by a macrophage-like cell line. Br J Haematol 58: 259-266, 1984.

Wang WC, Ahmed N, Hanna M. Non-transferrin-bound iron in long-term transfusion in children with congenital anemias. J Pediatr 108: 552-557, 1986.

Wapnick AA, Bothwell TH, Seftel H. The relationship between serum iron levels and ascorbic acid stores in siderotic Bantu. Br J Haematol 19: 271-276, 1970.

Ward HP, Tauxe WN Kiely JM. ^{59}Fe-autoradiography of bone marrow culture from normal subjects and patients with uraemia and pernicious anaemia. Br J Haematol 12: 99-104, 1966.

Watanabe N, Drysdale J. Evidence for distinct mRNAs for ferritin subunits. Biochem Biophys Res Commun 98: 507-514, 1981.

Weed RI, Reed CF. Membrane alterations leading to red cell destruction. Am J Med 41: 681-698, 1966.

Weed RI. THe importance of erythrocyte deformability. Am J Med 49: 147-150, 1970.

Weinberg ED. Iron withholding: A defense against infection and neoplasia. Physiol Rev 64: 65-102, 1984.

White GP, Bailey-Wood R, Jacobs A. The effect of chelating agents on cellular iron metabolism. Clin Sci Mol Med 50: 145-152, 1976a.

White GP, Jacobs A, Grady RW, Cerami A. The effect of chelating agents on iron mobilisation in Chang cell cultures. Blood 48: 923-929, 1976b.

White GP, Jacobs A. Iron uptake by Chang cells from transferrin, nitriloacetate and citrate complexes: the effects of iron-loading and chelation with desferrioxamine. Biochim Biophys Acta 543: 217-225, 1978.

White K, Munro HN. Induction of ferritin subunit synthesis by iron is regulated at both the transcriptional and translational levels. J Biol Chem 263: 8938-8942, 1988.

Wileman T, Harding C, Stahl P. Receptor mediated endocytosis. Biochem J 232: 1-14, 1985.

Williams DM, Lee GR, Cartwright GE. Ferroxidase activity of rat ceruloplasmin. Am J Physiol 227: 1094-1097, 1974.

Worwood M, Cragg SJ, Wagstaff M, Jacobs A. Binding of human serum ferritin to concanavalin A. Cli Sci 56: 83-87, 1979.

Worwood M. Serum ferritin. In: Iron in Biochemistry and Medicine. II. Jacobs A, Worwood M (eds). Academic Press, London. 1980. pp203-244.

Worwood M. Ferritin in human tissues and serum. Clin Haematol 11: 275-307, 1982.

Worwood M, Brook JD, Cragg SJ, Hellkuhl B, Jones BM, Perera P, Roberts SH, Shaw DJ. Assignment of human ferritin genes to chromosomes 11 and 19q 13.3-19qter. Hum Genet 69: 371-374, 1985.

Worwood M. Serum ferritin. Cli Sci 70: 215-220, 1986.

Wright TL, Bissot P, Ma W-L, Weisiger RA. Characterization of non-transferrin-bound iron clearance by rat liver. J Biol Chem 261: 10909-10914, 1986.

Wright TL, Fitz JG, Weisiger RA. Non-transferrin-bound iron uptake by rat liver. Role of membrane potential difference. J Biol Chem 263: 1842-1847, 1988.

Wyllie JC. Transferrin uptake by rabbit alveolar macrophages. Br J Haematol 37: 17-24, 1977.

Yang F. Lum JB, McGill JR, Moore Cm, Naylor SL, Ban Bragt PH, Baldwin WD, Bowman BH. Human transferrin: cDNA characterization and chromosomal localization. Proc Natl Acad Sci USA 81: 2752-2756, 1984.

Young SP, Aisen P. Transferrin receptors and the uptake and release of iron by isolated hepatocytes. Hepatology 1: 114-119, 1981.

Young SP. Evidence for the functional equivalence of the iron-binding sites of rat transferrin. Biochim Biophys Acta 718: 35-41, 1982.

Young SP, Bomford A, Williams R. Dual pathways for the uptake of rat asialotransferrin by rat hepatocytes. J Biol Chem 258: 4972-4976, 1983.

Young S, Bomford A. Transferrin and cellular iron exchange. Cli Sci 67: 273-278, 1984.

Young SP, Roberts S, Bomford A. Intracellular processing of transferrin and iron by isolated rat hepatocytes. Biochem J 232: 819-823, 1985.

Zail SS, Charlton RW, Torrance JD, Bothwell TH. Studies of the formation of ferritin in red cell precursors. J Clin Invest 43: 670-680, 1964.