CHARACTERISTICS OF INTRACELLULAR IRON TRANSPORT IN ERYTHROID CELLS

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LIST OF PUBLICATIONS

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Articles

Analysis of iron-binding components in the low molecular weight fraction of rat reticulocyte cytosol.

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Release of iron from endosomes is an early step in the transferrin cycle.

D.L. Bakkeren, C.H.M. De Jeu-Jaspars, M.J. Kroos and H.G. Van Eijk. Int. J. Biochem. **19** *No. 2, pp 179-186 (1987).*

Characteristics of iron release from isolated heavy and light endosomes.

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Abstracts

Isolation of endosomes from human erythroleukemia cells (K 562) and some characteristics of iron mobilization from these endosomes.

D.L. Bakkeren, C.H.M. De Jeu-Jaspars and H.G. Van Eijk.
Voordracht voor de Europeans Iron Club Meeting, 18-20 september 1985, Amersfoort.

Release of iron from endosomes is an early step in the transferrin cycle.

D.L. Bakkeren, C.H.M. De Jeu-Jaspars, M.J. Kroos and H.G. Van Eijk.
Poster voor de Europeans Iron Club Meeting, 22-25 september 1986, Pavia, Itali.

Iron release from endosomes to mitochondria in vitro.

D.L. Bakkeren, C.H.M. De Jeu-Jaspars and H.G. Van Eijk.
Voordracht voor de Federatieve Vergadering, 23 april 1987, Nijmegen.

Study on the characteristics of iron release from isolated heavy and light endosomes.

D.L. Bakkeren, C.H.M. De Jeu-Jaspars, M.J. Kroos and H.G. Van Eijk.
Poster voor de Europeans Iron Club Meeting, 19-22 september 1987, Glasgow, Schotland.

ABBREVIATIONS

α_2-M	α_2-macroglobulin
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
BPS	bathophenantroline disulphonate
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
DNP	2,4-dinitrophenol
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
GTP	guanosine-5'-triphosphate
Hepes	N-2-hydroxy-ethylpiperazine-N'-2 ethane sulfonic acid
IgG	immunoglobulin G
INH	isonicotinic acid hydrazide
K 562	human erythroleukemic cell line
L	light mitochondrial, lysosome rich (fraction)
LDL	low density lipoprotein
LMWF	low molecular weight fraction
LP	combined light mitochondrial/particulate (fraction)
M	molar (mol/litre); mitochondrial (fraction)
Mw	molecular weight
N	nuclear (fraction)
n	number of observations
NAD(H)	(reduced) nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
P	particulate (fraction)
PBS	phosphate buffered saline
PIH	pyridoxal isonicotinoyl hydrazone
RPMI	Roswell Park Memorial Institute (tissue culture medium)
P(C)	reversed phase (chromatography)
S	(final) supernatant (fraction)
S.D.	standard deviation
SE(C)	size exclusion (chromatography)
Tf	transferrin
Tris	tris(hydroxymethyl)-aminomethane
w/v	weight/volume

Contents

CHAPTER 1

GENERAL INTRODUCTION

1. Iron in the human body

Iron is one of the most abundant elements in the earth's crust and important to almost every form of life. The only exception probably are the lactic acid bacteria. There are two possible states of oxidation of iron: the (Fe(II)) state and the (Fe(III)) state . At neutral pH and under aerobic conditions Fe(II) compounds are easily oxidised to Fe(III) compounds. The solubility of Fe(III) compounds is very low (solubility product of $Fe(OH)_3 = 4 \times 10^{-38}$ mol/l), leaving a free Fe(III) concentration of 10^{-17} mol/l. This property, therefore, makes the acquisition and handling of iron a difficult prospect for cells and whole organisms. The human body contains about 4 grams of iron, the distribution of which is shown in table 1.1. In the body iron is important because of its role in the transport of oxygen (hemoglobin and myoglobin), electrons (cytochromes and ferredoxins) and its functions in enzymes such as dehydrogenases, peroxidases and enzymes committed to DNA replication. Iron exchange between different iron pools in the body totals up to 30 mg (5×10^{-4} mol) per day.

Table 1.1 **Distribution of iron in the human body**

70 %	in hemoglobin
20 %	as stored in ferritin in liver, spleen and bone marrow
4 %	in myoglobin
0.3 %	in enzymes (cytochromes, ferredoxins, peroxidases)
0.1 %	as transport iron bound to transferrine
0.1 %	in hemosiderine in the liver

The human body does not possess an active excretion mechanism for iron and therefore the balance between uptake and loss is entirely regulated by modulation of the iron uptake by the mucosal cells of the duodenum and proximal jejunum, though the exact mechanism is still poorly understood. Approximately 1 - 2 mg iron is lost daily from the body through the kidneys, gut, sweat, blood loss, hair, nails and skin scales. Two forms of iron are present in food: heme iron, especially present in meat (hemoglobin and myoglobin) and non-heme iron. Generally a greater percentage of heme iron from animal origin is absorbed than of non-heme iron of either animal or plant food. Other constituents of food may positively

(ascorbic acid, low molecular weight oranids) or negatively (tea, coffee, wheat bran, egg) influence the uptake of dietary iron (Morris, 1983; Morck *et al.*, 1983).

2. Proteins of iron transport and storage

Three major problems are associated with the availability of iron in mammalian tissues: the maintenance of adequate stores of iron in a non-toxic form (as iron can be extremely toxic by stimulating lipid peroxidation); the direction of iron to the sites of synthesis of iron-proteins such as hemoglobin, myoglobin and enzymes at concentration much higher than 10^{-17} mol/l; and the diversion of iron from sites were iron catalyzed reactions may result in damage. The two major proteins that help overcoming these problems are transferrin and ferritin.

3. Transferrin

Transferrin is the principle iron transport protein in the plasma, where it is found at concentrations of between 2.4 and 7.2 g/l. This 79,570 Dalton glycoprotein consists of one bisymmetrically folded polypeptide chain, giving rise to the two so-called N-terminal and C-terminal domains (MacGillevray *et al.*, 1984; Uzan *et al.*, 1984). Two carbohydrate chains, comprising 6 % of the molecular mass, are bound to the C-terminal domain and have two, three or, exceptionally, four antennas each. Each domain contains one binding site for a Fe(III) atom, giving rise to the so-called N-terminal and C-terminal binding sites. The complexation of iron within a binding site involves two histidine and two tyrosine residues, with an arginine residue being involved in the binding of the anion. For each iron atom bound three protons are released. Simultaneously a (bi)carbonate anion (synergistic anion) associates with the protein to form a part of the ligand binding site (Schlabach and Bates, 1975). The synergistic anion is thought to aid the positioning of the iron atom within the binding cavity of the molecule. Under normal physiological conditions transferrin in the plasma is only 30 % saturated, resulting in a mixed population with different iron loadings: transferrin without iron (apotransferrin), monoferric transferrin, with iron on either of the two binding sites (Fe_NTf) and ($TfFe_C$) and diferric transferrin. The two binding sites differ in their affinity for Fe(III) depending on the pH and ionic composition of the medium. The binding sites appear to be independent and may have slightly different affinities for iron (affinity constant $1\text{-}6 \times 10^{22}$ M^{-1}) (Aisen and Leibman, 1978). The N-terminal site binds iron at pH 7-8, while above pH 8 progressively more C-terminal sites are occupied. The C-terminal site is therefore called the acid-labile site, the N-terminal site the acid-stable site. Analysis of transferrin from the plasma of normal people has revealed that iron is not randomly distributed between the

specific binding sites of transferrin. There is a considerable range of variation in the ratio of monoferric Fe_NTf and $TfFe_C$ though predominantly the N-terminal sites are occupied (Williams and Moreton, 1980; Huebers *et al.*, 1984; van Eijk and van Noort, 1986). It seems paradoxic that the acid-labile binding sites of transferrin are preferentially occupied under physiological conditions. An explanation for this phenomenon has not yet been found, but binding to this sight might be influenced by a low molecular weight allosteric modulator. It is also possible that the affinity of the two iron bindingsites is altered at the site of iron uptake and the N-sites are preferentially labelled. *In vitro* there is no exchange of iron between the two binding sites. The mechanism of iron removal from transferrin is still a matter of debate. There are several possibilities: the affinity of transferrin for iron may be reduced by the addition of protons to the amino acid residues involved in iron binding. *In vitro* all iron is released from transferrin, below ±pH 5, provided there are acceptor molecules present. Alternatively, ferric iron might be reduced to ferrous iron for which transferrin has only low affinity. The third possibility does not exclude the first but may be complementary. It suggests the extraction of the synergistic bound anion (carbonate or bicarbonate) from the complex (Patch and Carrano, 1982), allowing the formation of an intermediate mixed-ligand complex of ferric transferrin with for example pyrophosphate (Cowart *et al.*, 1986). The carbohydrate chains of transferrin normally have terminal sialic acid residues. The sialic acid residues are removed one after another on aging of the molecule. Therefore a number of isotransferrins are found in serum and other biological fluids differing in their carbohydrate chains (two two-antennary chains or one two-antennary chain and one three- or even four antennary chain) each with a different number of terminal sialic acid residues (van Eijk *et al.*, 1982, 1983). The pattern of isotransferrins in isoelectric focussing and crossed immunoelectrophoresis appears greatly changed in certain physiological condition such as pregnancy and in certain pathological condions such as cancer and rheumatoid arthritis (van Eijk *et al.*, 1987). So far neither the physiological origin or consequences of this phenomenon are understood.

The major transferrin synthesising cell is the hepatocyte (Aisen, 1984), although other sites of transferrin synthesis are: the lactating mammary glands (Hochwald *et al.*, 1964; Jordan and Morgan, 1969) and cells beyond blood-tissue barriers such as oligodendrocytes in the central nervous system (Bloch *et al.*, 1985; Dickson *et al.* 1985) and Sertoli cells in the testes (Skinner and Griswold, 1983). Iron can be absorbed by the body in the absence of transferrin, as is the case in the genetic disorder, atransferrinemia. The virtual absence of transferrin results in an extremely low plasma iron, severe iron-deficiency anemia and yet abundant storage iron in the liver. For efficient distribution of iron through the body tissues, transferrin is required.

4. Ferritin

The storage protein ferritin is present throughout the phylogeny, while transferrin is restricted to chordates. Ferritin is a complex molecule consisting of 24 globular protein subunits forming the empty near-spherical shell (apoferritin), giving a molecular weight of 450,000 D. Each ferritin molecule is able to deposit up to 4,000 iron (Fe(II)) atoms in the central cavity as small crystalline particles, which are essentially inorganic ferric oxyhydroxide polymers with some phosphate (Ford *et al.*, 1984). Although present in all cells as well as in the plasma, most ferritin is found in the parenchymal cells of the liver and the reticulo-endothelial cells of the spleen. There are two types of ferritin subunits: the H- or heart type and the L- or liver type subunit. Ferritin molecules are made up of a composition of both subunit types. It is not clear yet what determines the molecular composition and if there is a physiological reason for the difference between the H-subunit rich isoferritin and L-subunit rich type, but it has been established that molecules with a higher portion of H subunits have a lower isoelectric point and take up iron most rapidly (Wagstaff *et al.*, 1978). Ferritin in the plasma may be derived from damaged cells or be excreted by cells as part of a mechanism to control an iron surplus within cells. Under conditions of normal cell turnover serum ferritin is directly correlated to the body's iron stores. A high serum ferritin concentration implies with pathologic iron deposition (hemochromatosis), whereas a low concentration indicates iron deficiency anemia. It remains unclear whether iron stored in ferritin can be utilized directly for heme synthesis. Iron incorporation by ferritin may be a one-way process to prevent potentially toxic levels of high intracellular iron, while ferritin degradation in lysosomes or exocytosis of ferritin might then complete the cycle (Roberts *et al.*, 1987; Mostert *et al.*, 1987).

5. Iron metabolism

Fletcher and Huehns (1967, 1968) proposed that the difference between the N-terminal and C-terminal iron binding site of transferrin inferred a different physiologic behaviour. The C-terminal site might be the iron donor for the erythropoietic system, while the N-terminal site may donate its iron to the iron storage cells. Van der Heul and others, however, have since proved that there is no difference between the C-terminal and N-terminal sites in donating iron to the erythropoietic system or storage cells (van der Heul *et al.*, 1981; Huebers *et al.*, 1978; Morgan *et al.*, 1978). By binding to transferrin, iron can be transported through the plasma at a concentration of 10^{-30} μmol, a 10^{11} times greater concentration than would be feasible according to the solubility product of $Fe(OH)_3$.

The major portion of body iron is found in the iron protoporphyrin complex of hemoglobin. This means that the anabolic erythron and the catabolic reticulo-endothelial system are the most active ferrokinetic compartments. In the bone marrow transferrin delivers iron to erythroid precursor cells where it is incorporated into protoporphyrin molecules resulting in heme formation. Old and senescent erythrocytes are sequestered by macrophages of the reticulo-endothelial system of spleen and liver. After breakdown of hemoglobin the iron is conserved by being rebound to transferrin in the circulation where it is put to immediate use or taken away for storage.

Conflicting views exist about the entry of iron into cells. Does transferrin release iron at the extracellular side of the plasma membrane or is internalization of ferric-transferrin a prerequisite sitesitein the normal metabolism of iron? The fact is that the processes concerned with iron uptake and intracellular release are very rapid, which makes it difficult to study sequential phases of the pathway. In erythroid cells iron is detected in micropinocytotic vesicles within 30 seconds of incubation and in hemoglobin after 3 minutes (Primosigh and Thomas, 1968; Sullivan *et al.*, 1976). In 1963 Jandl and Katz proposed that plasma-transferrin does not enter the cell, but that iron is removed from transferrin by a plasma membrane receptor (Jandl and Katz, 1963) This hypothesis has been supported by a number of other investigators (Egyed, 1977; Loh *et al.*, 1977; Speyer and Fielding, 1977; Glass *et al.*, 1980; Woodworth *et al.*, 1982; Morley *et al.*, 1983). In an autoradiographic study Morgan and Appleton (1969) showed the internalization of transferrin in reticulocytes. This study led to the concept of receptor-mediated endocytosis as the physiological process by which iron enters the cell. The pathway of receptor-mediated endocytosis is discussed in greater detail in chapter 6. Morley and Bezkorovainy described a hypothesis in which internalization does not occur but ferric-transferrin releases its iron after combination with a cell surface transferrin receptor. As a result of the transferrin-receptor interaction conformational changes are thought to bury the complex within the plasma membrane. Because transferrin is hidden within the membrane it is not, then, accessible to extracellular proteolytic enzymes (sensitivity of transferrin for extracellular proteolytic enzymes is often used as evidence for the internalization of transferrin). It is proposed that then iron is removed from the transferrin to an unknown membrane iron binding molecule involving a reduction step, although iron is bound to this iron binding molecule in the ferric state. The iron binder then releases iron to the cytosol or to intracellular transferrin or ferritin (Morley *et al.*, 1985; Morley and Bezkorovainy, 1985). The authors do not deny the existence of receptor-mediated endocytosis as a way to introduce iron into the cell, but advocate their hypothesis as an alternative pathway for hepatocytes (Morley and Bezkorovainy, 1985). It must be stressed that many points in this controversial theory are still unproven.

Both theories acknowledge the existence of the same specific transferrin receptor molecules at the plasma membrane. The transferrin receptor has been characterized as a transmembrane glycoprotein consisting of two identical 90,000 Dalton subunits linked by disulphide bonds (reviewed by Newman, 1982). The N-linked glycan chains are added in the rough endoplasmic reticulum while further processing and addition of peripheral monosaccharides to complex oligosaccharides takes place in the Golgi. A late event in the biosynthesis is the addition of palmitine residues to the cytoplasmic domain of the protein. This domain contains several serine residues acting as phosphorylation sites. The function of phosphorylation does not seem to be in receptor internalization since deletion mutants, lacking this part of the cytoplasmic domain, are internalized normally. Phosphorylation may act as a signal for marking aging molecules (Hunt et al, 1984; Rothenberger *et al.*, 1987). The biosynthetic rate of turnover of transferrin receptor molecules has been calculated from pulse chase experiments to be approximately 60 hours (Omary and Trowbridge, 1981). This may, however, vary greatly among different cell types since K 562 cells have a transferrin receptor turnover of 7 hours (Mattia *et al.*, 1984; Forsbeck and Nilsson, 1985). As the transferrin cycle has a $t_{1/2}$ of 5 - 10 minutes (Ciechanover, 1983), this suggests availability for many cycles of transferrin uptake, though this has not yet been shown directly. The precise route followed by transferrin is not clear and this in part is the subject of this thesis. Transferrin receptors are found on all cell types with exception of mature erythrocytes. The number of cell surface transferrin receptors varies greatly among different cell types depending on their function. Large number of cell surface transferrin receptors are found on: erythroid precursor cells because of extensive heme synthesis, ranging from 500,000 on polychromatic normoblasts to about 100,000 on young reticulocytes in rat fetal liver (Iacopetta *et al.*, 1983); and all rapid proliferating cell types such as activated macrophages and tumor cells. The number of cell surface receptors is dependent on the availability of iron to the cells. In K 562 cells the usual distribution of transferrin receptor between the cell surface and intracellular locations is 30 % on the membrane and 70 % within the cell. Immediately after addition of diferric transferrin to K 562 cells the number of surface receptors decreases as shown by decreased sensitivity to pronase digestion, acid wash or enzyme mediated receptor iodination. It seems this is not due to receptor downregulation, but a consequence of greater receptor internalization (Hunt *et al.*, 1984; Watts, 1985). Long term regulation of the number of transferrin receptors is still a matter of dispute. It may be regulated by either the intracellular heme concentration (Ward *et al.*, 1984; Ponka and Schulman, 1985) or by the intracellular or extracellular iron concentration (Cudkowicz *et al.*, 1984; Rao *et al.*, 1985; Rudolph *et al.*, 1985). The expression of transferrin receptors is also modulated by the cell cycle. Use of intracellular iron chelators such as desferrioxamine promotes increased expression of surface transferrin receptors even when cell proliferation is impaired. An increa-

sed supply of any form of iron to cells leads to receptor-down regulation. High surface receptor densities were found on cells in S-phase and low densities on cells in G_1-phase. Severe limitation of iron arrests proliferating cells in S-phase when receptors are maximally expressed (Bomford *et al.*, 1986). However, it is clear that regulation is mediated on the level of transcription (Rao *et al.*, 1986).

In addition to iron uptake from transferrin, cells can, with more or less effort, take up iron from a number of other compounds such as: low molecular weight iron chelators, the natural chelators: fructose, citrate and ascorbic acid or the artificial chelators such as pyridoxal isonicotinoyl hydrazone and derivatives; heme derivatives or ferritin. As shown by Titeux *et al.* (1984) cultured K 562 cells can proliferate for several generations in artificial serum-free, transferrin-free medium supplemented with ferric ammonium sulphate or ferric ammonium citrate. Little is known about the routes of entry these compounds. It may involve: receptor-mediated endocytosis (hemopexine mediated uptake of hemin; Taketani *et al.*, 1986), simple diffusion or fluid-phase endocytosis. An important difference between the bioavailability of iron through transferrin or iron chelators may be the accessability of oxygen to iron in some of the latter compounds, which may cause lipid peroxidation with all its deleterious effects on biomembranes and enzymes (Mostert *et al.*, 1986).

6. Intracellular iron metabolism

As mentioned above, the uptake of iron by cells is dependant upon the cell type, phase of the cell cycle and a variety external conditions. The selectivity in iron uptake from transferrin by the erythron is illustrated by the fact that in erythroid cells the amount of iron is normally ten to hundred times greater than in other tissues (Morgan, 1974). The iron sequestered by these developing red cells is used into heme synthesis in the mitochondria. The metabolic pathway(s) of iron after its penetration into the cell is (are) unknown. Some possible routes taken by iron are depicted schematically in fig. 1.1. After uptake iron is incorporated rapidly in heme or ferritin depending on the cell type studied. Developing erythroid cells incorporate iron almost exclusively into hemoglobin, whereas cells not committed to extensive heme synthesis incorporate the majority of the iron into ferritin. The latter is the case in the erythroleukemic cell line K 562.

The substance(s) involved in the delivery of iron at the sites of utilization or storage are unknown. There are several reasons to suggest that iron in the cell is temporarily associated with a protecting ligand. One reason is that it is difficult for charged molecules to cross apolar membranes. Indeed to reach the mitochondria, iron molecules must pass either the en-

dosomal membrane (or the plasma membrane) and the mitochondrial membranes. It is probable that such a ligand neutralizes the iron atom's charge. If so, it will be an anionic substance. A second reason to suppose a protecting ligand is the high intracellular oxygen concentration, especially in erythroid cells. The ligand must protect iron from reacting with oxygen, which would result in the formation of insoluble ferric hydroxide polymers. A third reason is that the amount of iron taken up by cells is much greater than the calculated free concentration of 10^{-17} M. A forth reason is that free iron atoms would react with all kinds of other molecules while traversing the cytosol, before reaching their specific targets. It is clear that iron in the cytosol can not exist in a free soluble form. Initially it was suggested that transferrin delivered iron to the mitochondria. This idea was derived from in vitro incubation experiments with transferrin and isolated mitochondria (Neuwirt *et al.*, 1975; Ponka *et al.*, 1977). This hypothesis implies that there is either an association of plasma membrane bound transferrin with mitochondria at the inner membrane site (in case the concept of endocytosis is rejected); or of transferrin containing endosomes with mitochondria; or of free cytosolic transferrin and mitochondria. There is, however, neither biochemical nor electron microscopic evidence, for the association of any transferrin containing compartment with mitochondria *in vivo*. Other iron transporting compounds suggested over the years are: ferritin and many low molecular weight molecules such as: citrate (Morley and Bezkorovainy, 1983), nucleotides (ATP, GTP, ADP etc.), or amino acids (glycine, cysteine). Mazur and Carleton supported by other investigators suggested that ferritin was the intracellular transferrin iron acceptor and the subsequent iron donor to mitochondria (Mazur and Carleton, 1963; Fielding and Speyer, 1977; Speyer and Fielding, 1979). However, Grasso and coworkers (1984) demonstrated convincingly that ferritin is neither an obligatory intermediate in heme synthesis nor a cytosolic transport molecule involved in the mobilization of iron from the transferrin-receptor complex.

7. Tools in this study: reticulocytes and erythroleukemia cells

Though much research in iron metabolism has been done using rat or rabbit reticulocytes, working with tissue cultures of neoplastic cells has a number of important advantages over the use of animal cells. First of all from the ethical point of view, it should be every investigator's aim to use as few animals as possible with minimal discomfort to the animals concerned. Raising reticulocytes through injection with phenylhydrazin is unpleasant for the animals. Rats have to be exsanguinated to obtain enough reticulocytes, and the result is a mixture of reticulocytes of different state of maturation in every animal. When working with cell cultures many differences are eliminated. Under strictly controlled conditions of logarithmic growth there is little variation between individual cells. Besides, working with human

neoplastic cells has the advantage that much is known about the structure and chemistry of human transferrin. Another benefit of the use of cultured erythroleukemic cells is that they take up large amounts of iron and produces considerably less hemoglobin (which is often considered a contaminating protein during purification procedures) than reticulocytes. Obviously there are differences in iron metabolism, as in erythroleukemic cells iron uptake is high and hemoglobin synthesis is low, resulting in the majority of the iron being sequeste-

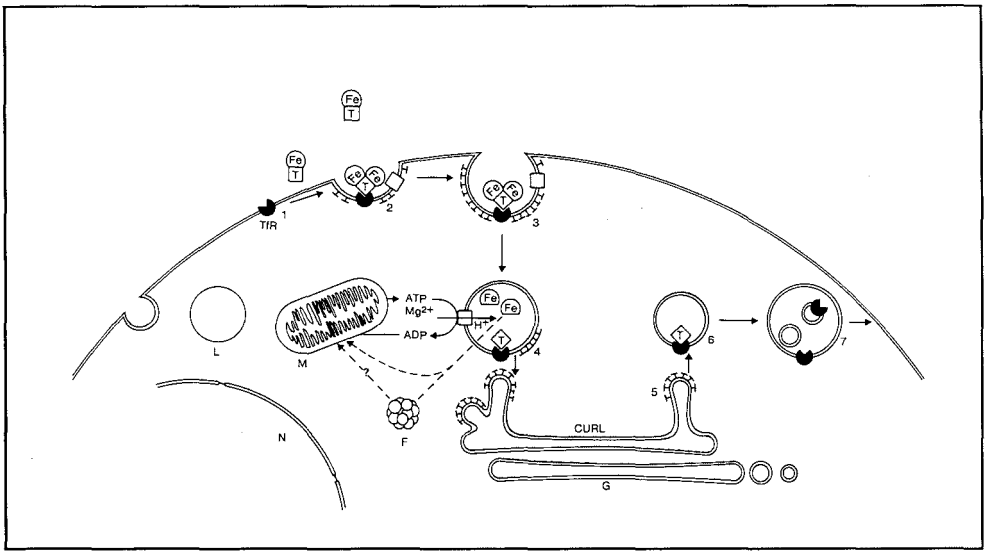

Fig 1.1 *The drawing above is a schematic representation of the processes involved in the intracellular transport of iron. Iron in the medium or plasma is bound to transferrin. Diferric and monoferric transferrins bind with high affinity to transferrin receptor molecules that are present in specialised areas of the cell surface called 'coated pits'. The coated pits are pulled into the cytosol by the clathrin coat proteins. The coated vesicles lose most of their coat proteins and are then called primairy endosomes. Iron release from these vesicle starts as soon as the vesicles have left the cell surface. Iron release from transferrin, like release of other ligands from their receptors, involves ATP dependent acidification of the internal milieu of endosomes. Since coated pits do not discriminate between the cell surface receptors, an intracellular sorting mechanism must provide for proper directioning of the ligands to their different destinations. Receptor sorting is thought to occur in the secondary endosome (also called 'CURL' or transGolgi). After release from endosomes, iron is transported through the cytosol to ferritin molecules where it is stored or to mitochondria where is combined with protoporphyrin IX to give heme. The transferrin-transferrin receptor complex is externalised undegraded, whereas other receptor complexes are degraded in lysosomes. T = transferrin; Fe = iron; TfR = transferrin receptor; H^+ = proton; F = ferritin; L = lysosome; N = nucleus; G = Golgi; CURL = Compartment for Uncoupling Receptor and Ligand, equal to the secondary endosome compartment.*

red in ferritin contrary to normal erythroid precursor cells. Nevertheless, for many studies, especially those looking at qualitative aspects of intracellular iron metabolism, it is still the most elegant solution. Some details of the K 562 cell line are described below.

8. Details on the K 562 cell line

K 562 cells were isolated from the pleural effusion of a patient with chronic myeloid leukemia (CML) in terminal blast crisis (Lozzio and Lozzio, 1975). There has been a lot of discussion about the commitment of the K 562 cell line. The human erythroleukemia cell line K 562 synthesizes embryonic and fetal hemoglobin chains and thus iron is incorporated into heme. Glycophorin, the major sialoglycoprotein of the erythroid cell surface was detected in large amounts (Gahmberg *et al.*, 1979; Jokinen *et al.*, 1979). A granulocytic nature was considered on the basis of the presence of granulocytic antigens, although other cytological and cytochemical characteristics of granulocytic differentiation were absent. The K 562 cell line arises from the proliferation of bipotent stem cells and therefore posses variable capacities of differentiation towards erythroid and megakaryocytic cell lineages depending on the inducers of differentiation (Vainchenker *et al.*, 1981). Normally the commitment to heme synthesis is not very high. About 70 - 80 % of iron internalized by these cells is immediately incorporated into ferritin. K 562 cells can be directed to increase hemoglobin synthesis by addition of differentiating agents to the culture medium. Among these agents are: hemin; protoporphyrin IX; 5-azacytidine; butyric acid; DMSO and phorbol esters (Cioe *et al.*, 1981; Hunt *et al.*, 1984; Gambari *et al.*, 1984). Differentiation of erythroid cells increases the amount of iron incorporated into heme and decreases the amount incorporated into ferritin. At the same time proliferation slows down and the cells become red. Removal of the differentiating agent reverses these changes.

9. SUMMARY AND SCOPE OF THIS THESIS

Transferrin mediated uptake of iron is the physiological and most efficient way for introduction of iron into vertebrate cells. Though not accepted by all investigators, receptor-mediated endocytosis of ferric-transferrin is thought to be the normal mechanism of iron uptake. However, the concept of this pathway does not answer many elementary questions about the internalization of iron, its release from transferrin, its transfer to the cytosol, and the possible existence of a transport intermediate. To contribute to the knowledge of intracellular iron metabolism we aimed to obtain answers to the following questions: What is the nature of the low molecular weight iron binding fraction found in reticulocytes? At what point in the transferrin cell cycle does iron leave the endosome; and what are the conditions neces-

sary for endosomal iron release? In chapter 3 the composition of the low molecular weight iron binding fraction is analyzed to determine the existence of physiologically important low molecular weight iron transporting species in the cytosol. Chapter 4 discusses in the phase of the transferrin cycle at which iron is removed from endosomes. In chapter 5 the experimental work is described which explores the presence of endogenous acceptor molecules for iron released from isolated endosomes.

REFERENCES

Aisen P. (1984) Transferrin metabolism and the liver. Seminar Liver Dis. **4**:192-206.

Aisen P. and A. Leibman (1978) Thermodynamic and accessibility factors in the specific binding of iron to human transferrin. In: Transport by proteins (G. Blauer and H. Sund eds.) Walter de Gruyter, Berlin.: 277-294.

Bloch, B., T. Popovici, M.J. Levin, D. Tuil and A. Kahn. (1985) Transferrin gene expression visualized in oligodendrocytes of the rat brain by using in situ hybridization immunohistochemistry. Proc. Natl. Acad. Sci. USA **82**: 6706-6710.

Bomford A., J. Isaac, S. Roberts, A. Edwards, S. Young and R. Williams. (1986) The effect of desferrioxamine on transferrin receptors, the cell cycle and growth of human leukemic cells. Biochem. J. **236**: 243-249.

Cioe L., A. McNab, H.R. Hubbell, P. Meo, P.Curtis and G. Rovera. (1981) Differential expression of the globin genes in human leukemia K562(S) cells induced by hemin or butyric acid. Cancer Res. **41**: 237-243.

Cowart R.E., S. Swope, T.T. Loh, N.D. Chasteen and G.W. Bates. (1986) The exchange of Fe^{3+} between pyrophosphate and transferrin. J. Biol. Chem. **261**: 4607-4614.

Cudkowicz A., R.D. Klausner and K. Bridges. (1984) Regulation of the transferrin receptor in K562 erythroleukemia cells. The Red Cell: Sixth Ann Arbor Conference. Alan R. Liss, Inc., New York N.Y. USA: 509-519.

Dickson P.W., A.R. Aldred, P.D. Marley, T. Guo-Fen, G.T. Howlett and G. Schreiber. (1985) High prealbumin and transferrin mRNA levels in the choroid plexus of rat brain. Biochem. Biophys. Res. Commun. **127**: 890-895.

Eijk H.G. van, W.L. van Noort and C. van der Heul. (1982) Microheterogeneity of human serum transferrins: a consequence for immunochemical determinations? Clin. Chim. Acta **126**: 193-195.

Eijk H.G. van, W.L. van Noort, M.L. Dubelaar. C. van der Heul. (1983) The microheterogeneity of human transferrins in biological fluids. Clin. Chim. Acta **132**: 167-171 .

Eijk H.G. van, and W.L. van Noort. (1986) A non-random distribution of transferrin iron in fresh human sera. Clin. Chim. Acta **30**: 299-303. .

Eijk H.G. van, W.L. van Noort, G. de Jong and J.F. Koster. (1987) Human serum sialo transferrins in diseases. Clin. Chim. Acta **165**: 141-145.

Fielding J., and B.E. Speyer. (1977) Ferritin as a cytosol transport protein: does transferrin enter reticulocytes? In: Proteins of iron metabolism (Eds.: E.B. Brown, P. Aisen, J. Fielding and R.R. Chrichton) Grune and Stratton Publ.: 311-318.

Fletcher J. and E.R. Huehns. (1968) Function of transferrin. Nature **218**: 1211-1214.

Fletcher J. and E.R. Huehns. (1967) The significance of the binding of iron by transferrin. Nature **215**: 584586.

Ford, G.C., P.M. Harrison, D.W. Rice, J.M.A. Smith, A. Treffrey, J.L. White and J. Yariv. (1984) Phil. Trans. R. Soc. Lond. **B304**: 551-556.

Forsbeck K., and K. Nilsson. (1985) The dynamic morphology of the transferrin-transferrin receptor system in human leukemia/lymphoma cell lines and its relation to iron metabolism and cell proliferation. Scan. J. Haematol. **35**: 145-154.

Gahmberg C.G., M. Jokinen and L.C. Andersson. (1979) Expression of the major red cell sialoglyco-

protein, glycophorin A, in the continuous cell line K 562. J. Biol. Chem. **254**: 7442-7448.

Gambari R., L. del Senno, R. Barbieri, L. Viola, M. Tripodi, G. Rashella and A. Fantoni. (1984) Human leukemia K-562 cells: induction of erythroid differentiation by 5-azacytidine. Cell Diff. **14**: 87-97.

Heul C. van der, M.J. Kroos, W.L. Van Noort and H.G. van Eijk. (1981) No functional difference of the two iron-binding sites of human transferrin in vitro. Clinical Science **60**: 185-190.

Hochwald, G.M., E.B. Jacobson and G.J. Thorbecke. (1964) C^{14}-amino acid incorporation into transferrin and $_{2A}$-globin by ectodermal glands in vitro (abstr.) Federation Proc. **23**: 557..

Huebers H., E. Huebers E., E. Csiba and C.A. Finch. (1978) Preferential utilization in vitro of iron bound to diferric transferrin by rabbit reticulocytes. Biochem. J. **166**:175-179.

Huebers H.A., B. Josephson, E. Huebers, E. Csiba and C.A. Finch. (1984) Occupancy of the iron binding sites of human transferrin. Proc. Natl. Acad. Sci. USA **81**: 4326-4330.

Hunt R.C., R. Ruffin and Yih-Sheng Yang. (1981) Alterations in the transferrin receptor of human erythroleukemic cells after induction of hemoglobin synthesis. J. Biol. Chem. **259**: 9944-9952.

Iacopetta B.J., E.H. Morgan and G.C.T. Yeoh. (1983) Receptor mediated endocytosis of transferrin by developing erythroid cells from fetal rat liver. J. Histochem. Cytochem. **31**: 336-344.

Jandl J.H., and J.H. Katz. (1963) The plasma-to-cell cycle of transferrin. J. Clin. Invest. **42**: 314-326.

Jokinen M., C.G. Gahmberg, L.C. Andersson. (1979) Biosynthesis of the major red cell sialoglycoprotein, glycophorin A, in a continuous cell line. Nature **279**: 604-607.

Jordan, S.M. and E.H. Morgan. (1969) Plasma protein synthesis by tissue slices from pregnant and lactating rats. Biochim. Biophys. Acta **174**: 373-379.

Lozzio, C.B. and B.B. Lozzio. (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood **45**: 321-334.

MacGillevray, R.T.A., E. Mendez, J.G. Sinha, M.R. Sutton, J. Lineback-Zins and K. Brew. (1982) The complete amino acid sequence of human serum transferrin. Proc. Natl. Acad. Sci. USA **79**: 2504-2508.

Mattia E., K. Rao, H.H. Sussman, R.D. Klausner. (1984) Biosynthetic regulation of the human transferrin receptor by desferrioxamine in K 562 cells. J. Biol. Chem. **259**: 2689-2692.

Mazur A., and A. Carleton. (1963) Relation of ferritin iron to heme synthesis: in marrow and reticulocytes. J. Biol. Chem. **238**: 1817-1824.

Morck, T.A., S.R. Lynch and J.D. Cook. (1983) Inhibition of food iron absorption by coffee. Am. J. Clin. Nutr. **37**:416-420.

Morgan E.H., and T.C. Appleton. (1969) Autoradiographic localization of ^{125}I-labelled transferrin in rabbit reticulocytes. Nature **223**: 1371-1372.

Morgan E.H. (1974) Transferrin and transferrin iron. In "Iron in biochemistry and medicine". A. Jacobs and M. Worwood eds. Academic press London and New York: 29-71.

Morgan E.H., H. Huebers and C.A. Finch. (1978) Differences between the binding sites for iron-binding and release in human and rat transferrin. Blood **52**: 1219-1228. .

Morley C.G.D., K.A. Rewers and A. Bezkorovainy. (1983) Iron metabolism pathways in the rat hepatocyte. Clin. Physiol. Biochem. **1**: 3-11.

Morley C.G.D., and A. Bezkorovainy. (1983) Identification of the iron chelate in hepatocyte cytosol. ICRS Med. Sci. **11**: 1106-1107.

Morley C.G.D., L. Solberg and A. Bezkorovainy. (1985) The removal of iron from transferrin by isolated plasma membranes of rat hepatocytes. Proteins of iron storage and transport. Proceedings of the seventh international conference on proteins of iron metabolism, Villeneuve d'Ascq. Spik G. et al., ed., Elsevier Science Publ. B.V., Amsterdam: 175-178.

Morley C.G.D., and A. Bezkorovainy. (1985) Cellular iron uptake from transferrin: is endocytosis the only mechanism? Int. J. Biochem. **17**: 553-564. .

Morris E. (1983) An overview of current information on bioavailability of dietary iron to humans. Symposium: Bioavailability of essential and toxic trace elements. Federation Proceedings **42**: 1716-1719.

Mostert L.J., G. de Jong, J.F. Koster and H.G. van Eijk. (1986) Iron mobilization from isolated hepatocytes. Int. J. Biochem. **18**: 1061-1064.

Mostert L.J., M.I. Cleton, J.F. Koster and H.G. van Eijk. (1987) Iron mobilization after repeated phlebotomy. Submitted for publication.

Neuwirt J., J. Borova and P. Ponka. (1975) Intracellular iron kinetics in erythroid cells. In: Proteins of iron storage and transport in biochemistry and medicine. Ed. R.R. Chrichton. Elsevier North-Holland, Amsterdam: 161-166.

Omary M.B. and I.S. Trowbridge. (1981) Biosynthesis of the human transferrin receptor in cultured cells.J. Biol. Chem. **256**: 12888-12892.

Patch M.G. and C.J. Carrano. (1982) An affinity label for the anion binding site in ovotransferrin implications for iron release to reticulocytes. Biochim. Biophys. Acta **700**: 217-220.

Ponka P., J. Borova, J. Neuwirt and O. Fuchs. (1977) Studies on the mechanism of iron supply in the regulation of hepatic transferrin sysnthesis. In: Iron metabolism. Ciba Foundation Symposium, Elsevier Amsterdam **51**: 167-168.

Ponka P., and H.M. Schulman (1985) Regulation of heme synthesis in erythroid cells: Hemin inhibits transferrin iron utilization but not protoporphyrin synthesis. Blood **65**: 850-857.

Primosigh J.V. and E.D. Thomas. (1968) Studies on the partition of iron in bone marrow cells. J. Clin. Invest. **47**: 1473-1483.

Rao K., J.B. Harford, T. Raoult, A. McClelland, F.H. Ruddle and R.D. Klausner. (1986) Transcriptional regulation by iron of the gene for the transferrin receptor. Molec. Cell. Biol. **6**: 236-240.

Rao K.K., D. Shapiro, E. Mattia, K. Bridges and R.D. Klausner. (1985) Effects of alterations in cellular iron on biosynthesis of the transferrin receptor in K 562 cells. Molec. Cell. Biol **5**: 595-600.

Roberts S., and A. Bomford. (1987) Dynamics of intracellular iron in human leukemic cells. European Iron Club Meeting 1987, Glasgow.

Rothenberger S., B.J. Iacopetta and L.C. Kühn. (1987) Endocytosis of the transferrin receptor requires the cytoplasmic domain but not its phosphorylation site. Cell **49**: 423-431.

Rudolph N.S., B.M. Ohlson-Wilhelm, J.F. Leary and P.T. Rowley. (1985) Regulation of K 562 cell transferrin receptors by exogenous iron. J. Cell. Physiol. **122**: 441-450.

Schlabach M.R. and G.W. Bates. (1975) The synergistic binding of anions and Fe^{3+} by transferrin. J. Biol. Chem. **250**: 2182-2188.

Skinner, M.K. and M.D. Griswold. (1983) Sertoli cells synthesize and secrete transferrin-like protein. J. Biol. Chem. **255**: 9523-9525.

Speyer B., and J. Fielding. (1979) Ferritin as a cytosol iron transport intermediate in human reticulocytes. Brit. J. Haematol. **42**: 255-267.

Sullivan A.L., J.A. Grasso and L.R. Weintraub. (1976) Micropinocytosis of transferrin by developing red cells. An electron microscopic study utilizing ferritin conjugated transferrin and ferritin conjugated antibody to transferrin. Blood **47**: 133-143.

Taketani S., H. Kohno and R. Tokunaga (1986) Receptor-mediated heme uptake from hemopexin by human erythroleukemia k 562 cells. Biochem. Int. **13**: 307-312.

Titeux M., U. Testa, F. Louache, P. Thomopoulos, H. Rochant and J. Breton-Gorius. (1984) The role of iron in the growth of human leukemic cell lines. J. Cell. Physiol. **121**: 251-256.

Uzan, G., M. Frain, I. Park, C. Besmond, G. Maessen, J.S. Trepat, M.M. Zakin and A. Kahn. (1984) Molecular cloning and sequence analysis of cDNA for human transferrin. Biochem. Biophys. Res. Commun. **119**: 273-281.

Vainchenker W., U Testa, J. Guichard, M. Titeux and J. Breton-Gorius. (1981) Hetrogeneity in cellular commitment of a human leukemic cell line: K 562. Blood Cells **7**: 357-375.

Wagstaff M., M. Worwood and A. Jacobs. (1978) Properties of human tissue isoferritins. Biochem. J. **173**: 969-977.

Ward J.H., I. Jordan, J.P. Kushner and J. Kaplan. (1984) Heme regulation of HeLa transferrin receptor number. J. Biol. Chem. **259**: 13235-13240.

Watts C. (1985) Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. J. Cell Biol. **100**: 633-637.

Williams J. and K. Moreton. (1980) The distribution of iron between the metal-binding sites of transferrin in human serum. Biochem. J. **185**: 483-488.

Woodworth R.C., A. Brown-Mason, T.G. Christiansen, D.P. Witt and R.D. Comeau. (1982) An alternative model for the binding and release of diferric transferrin by reticulocytes. Biochem. **21**: 4220-4225.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

1. MATERIALS

1.1. Chemicals

All chemicals used were of analytical grade or of the highest purity available. Bovine serum albumin (BSA), aprotinin, pronase B, dithiothreitol and acridine orange were obtained from Sigma, St. Louis MO, USA. AcA 34 gel was from LKB, Bromma, Sweden. Methanol (gold-label), was from Janssen Pharmaceuticals, Beerse, Belgium. Sephadex G-100, G-50 medium, Sephacryl S-1000, Percoll and Density Marker Beads were obtained from Pharmacia, Uppsala, Sweden. Triton X-100 and 2,4-Dinitrophenol (DNP) were from BDH, Poole United Kingdom. The citric acid test-kit containing the enzyme citric acid lyase and adenosine triphosphate tri-sodium salt (ATP) were from Boehringer Mannheim, Germany. Lithium lactate was from K & K Laboratories Inc., Plainview, N.Y. USA. Ethylene diamine tetraacetic acid (EDTA), bathophenanthroline disulphonate di-sodium salt (BPS), N-ethylmaleimide (NEM), isonicotinic acid hydrazide (INH), potassium cyanide, phenylhydrazin, N-2-hydroxy-ethylpiperazine-N'-2 ethane sulfonic acid (Hepes) and all other chemicals were from Merck, Darmstad, Germany. Pyridoxal isonicotinoyl hydrazone (PIH) was synthesized as described by Ponka *et al.* (1984).

1.2. Media

RPMI 1640 medium (without Hepes) and Dulbecco's minimal essential medium (DMEM) were obtained from Gibco, Breda, Nederland. Phosphate buffered saline pH 7.45 (Oxoid, Basingstoke, UK) was used to wash reticulocytes and K 562 cells during labelling procedures.

1.3. Isotopes

$Na^{125}I$ and ^{59}Fe-citrate were from Radiochemical Centre Amersham, United Kingdom. In this thesis the term ^{59}Fe is often used to indicate iron atoms in general, not to indicate a specific effect of the ^{59}Fe isotope.

1.4. Transferrins

Rat transferrin was isolated as described by Van Noort *et al.* (1977). Rat transferrin was made iron free by twice dialyzing against 50 mM acetic acid, 20 mM EDTA pH 5.0. EDTA was removed from transferrin through dialyses against 20 mM phosphate buffer pH 7.4. Human transferrin was obtained as apotransferrin from Behring AG, Marburg, Germany.

1.5. Cells

1.5.1. Rat reticulocytes

Male Wistar rats were once injected subcutaneously with 4.5 mg phenylhydrazin (Merck, Darmstadt, Germany) in 1 ml PBS. On day 2 and 5 the rats were bled by orbita punction to remove the bulk of phenylhydrazin damaged erythroid precursor cells. On day 6 the rats were sacrificed and the blood was sampled. The cells were washed 4 times in ice-cold saline containing 1 mM Tris, pH 7.45. Reticulocyte counts were between 85 and 95 %.

1.5.2. K 562 cell culture

The erythroleukemia cell line K 562 (Lozzio and Lozzio, 1975) was a gift from Dr. A. Hagemeijer-Hausman (Dept. of Genetics, Erasmus University Rotterdam). Cell cultures were maintained in exponential growth between 1 x 10^5 and 1 x 10^6 cells/ml by dilution into fresh RPMI 1640 medium, 10 % newborn calf serum plus antibiotics, three times a week. Cell counts were determined with a Sysmex CC-130 cell counter as white blood cells. Viability was assessed by Trypan Blue exclusion and was always greater than 95 %.

2. METHODS

2.1. Radiolabelling of transferrin

Transferrin was labelled with $^{59}FeCl_3$ to give diferric transferrin using a 100 fold excess of nitrilotriacetate relative to ^{59}Fe and a 50 fold excess of $NaHCO_3$ at pH 8.4 for 30 min at 37°C or overnight at 4°C (van der Heul *et al.*, 1978). Excess ^{59}Fe was removed by dialysis against 0.025 M Hepes pH 7.45 for 24 hours with one change of buffer. Full saturation with iron was indicated by an E_{470}/E_{280} ratio of 0.045 (Klausner *et al.*, 1983). Diferric transferrin was either labelled with Bolton and Hunter reagent (N-succinimidyl 3-(4 hydroxy 5(^{125}I)iodophenyl propionate) (Bolton and Hunter, 1962) (chapter 3) or iodinated using carrier-free $Na^{125}I$ and chloramine-T according to Hunter and Greenwood (1962) (chapter 4 and 5). Using the chloramine-T method, excess $^{125}I^-$ was removed by ion exchange chromatography over IRA 400 Cl at pH 7.45. Radioactivity was measured with a Packard Autogamma 500-C. ^{125}I counts were corrected afterwards for ^{59}Fe spill-over (13 % of ^{59}Fe counts).

2.2. Reticulocytes: procedures

The procedures described below were used in the experiments described in chapter 3.

2.2.1. Reticulocyte labelling

After the last wash the reticulocytes were pelleted and the packed cells were diluted 1:1 in Egyed's incubation medium (Egyed, 1982) containing 120 mM NaCl, 20 mM Hepes, 10 mM inosine and 10 mM phosphate at pH 7.45. To stop heme synthesis 40 μmol isonicotinic acid hydrazide (INH) was added to 4 ml of 1:1 diluted cells and the cells were incubated for 15 min at 37°C under 5 % CO_2. Subsequently ^{125}I-Tf^{59}Fe was added to a final concentration of 10 μM and incubated in presence of INH for 30 min at 37°C under 5 % CO_2. In most experiments, however, INH was replaced by 1 mM potassium cyanide, and 0.25 mM 2,4-dinitrophenol (DNP), as described by Egyed (1982).

2.2.2. Reticulocytes: Lysis and cytosol preparation

Incubation was stopped by washing the cells with ice-cold saline containing 1 % BSA followed by three washes with ice-cold saline. Cells were lysed by adding 3 volumes of 1mM Hepes, pH 7.40 to one volume of packed cells. After 10 min at room temperature KCl was added until osmolarity was restored, thus preventing rat hemoglobin from precipitating (Condò, 1981). The lysate was centrifuged at 12,000 g for 5 min in a Beckman J-21 centrifuge with a JA-21 rotor. The resulting supernatant was saved and called cytosol. Radioactivity was measured with a three channel Packard autogamma 500-C ^{125}I counts were corrected afterwards for ^{59}Fe spill-over (13 % of ^{59}Fe counts).

2.3. K 562 cells: procedures

2.3.1. K 562 cells: Labelling of cell surface transferrin receptors

K 562 cells were harvested from a log phase culture and washed 2 times with phosphate buffered saline (PBS). In the experiments described in chapter 4, the cells were pre-incubated for 30 min in 50 ml DMEM/0.1 % BSA (hereafter called DMEM/BSA) at 37°C under 5 % CO2 to remove intracellular transferrin from the cells. The cells were counted and the viability, which was determined by Trypan Blue exclusion, was always greater than 95 %. Cell surface transferrin receptors were saturated with transferrin by incubation with double labelled transferrin (0.1 mg/10^9 cells) for 15 - 60 min at 4°C in 10 - 20 ml DMEM/BSA.

Excess transferrin was removed by washing the cells 4 times with 50 ml ice-cold PBS.

2.3.2. K 562 cells: Endocytosis of transferrin saturated cell surface transferrin receptors

The cells were resuspended in 10 ml ice cold DMEM/BSA and transferred to a glass vessel with a relatively large bottom area which allowed rapid warming up. At t=0 10 ml DMEM/BSA of 37°C was added and the vessel was placed in a 37°C water bath with mild agitation for the time indicated in each experiment. Endocytosis was stopped by adding 10 ml ice cold DMEM/BSA and swirling the vessel in melting ice until the temperature had dropped to 6°C.

2.3.3. Pronase digestion of non-internalized ^{125}I-Tf^{59}Fe

^{125}I-Tf^{59}Fe still bound to the extracellular side of the plasma membrane, was removed by pronase digestion. Cells were resuspended in ice-cold DMEM containing 1 mg/ml pronase B for 30 min at 4°C, followed by three washes with ice cold DMEM/BSA. The final pellet was resuspended in 10 ml homogenization buffer containing: 10 mM Hepes pH 7.2, 0.25 M sucrose and 1 % aprotinin. The homogenization buffer used in experiments described in chapter 4 additionally contained 0.2 mM EDTA.

2.3.4. Homogenization

The cell suspension was transferred to a pre-cooled 40 ml cavitation bomb (Parr, type 4639, Molina USA). In experiments in which remaining cell surface transferrin receptors were removed by pronase digestion, 5 μl octane-2-ol was added to the thready cell suspension to secure a more homogeneous suspension after cavitation. The bomb was pressurized with nitrogen to 200 psi. After 30 min at 4°C the suspension was slowly released from the bottom of the bomb into a cooled glass tube and vortexed thoroughly to release the contents of the broken cells. Cell disruption was checked under a microscope and found to be greater than 98 %. Although virtually all cells were broken by cavitation the suspension was homogenized with 10 strokes of a glass Dounce homogenizer with tight fitting pestle, to tear apart the plasma membranes of the cells and to release the cellular contents more completely. The volume of the homogenate was determined. A 0.5 ml fraction was saved for γ-counting then frozen at -20°C for subsequent determination of the protein content and enzyme composition

2.3.5. Cell fractionation

2.3.5.1. Procedure I

This procedure is a modification of the fractionation procedure for KB cells described by Dickson *et al.* (1983). Briefly: all steps were carried out at 0-4°C. The homogenate was centrifuged for 10 min at 500 g to remove whole cells, nuclei and cell debris. The supernatant was saved while the pellet was resuspended in 2 ml homogenization buffer and pottered with 5 strokes at 200 rpm to a homogeneous suspension using a Potter/Elvehjem homogenizer with a tight teflon pestle. The volume was made up to 10 ml and centrifuged as before. The supernatants were pooled and combined with iso-osmotic Percoll-sucrose to make a solution with starting density of 1.055 g/ml. An identical counter-tube contained Density Marker Beads. The gradient was formed by 1 hour centrifugation at 17,400 rpm in a Beckman 60 Ti rotor. The gradient was fractionated by inserting a glass capillary from the top to the bottom of the tube, collecting 1 ml fractions. Percoll, Golgi material and soluble proteins were removed from the pooled ^{125}I-Tf and ^{59}Fe containing fractions by gelfiltration.

2.3.5.2. Procedure II

This procedure is a slight modification of the fractionation procedure described by De Duve *et al.* (1955). Briefly: all manipulations were performed at 4°C. The homogenate was transferred to a cooled centrifuge tube and centrifuged for 5 min in a Beckman JA-20 rotor and a Beckman J-21 centrifuge at 4,000 rpm. The supernatant was saved and the pellet was washed twice in 10 ml homogenization buffer and centrifuged for 5 min at 3,200 rpm in a Beckman JA-20 rotor. The supernatants were pooled and the pellet was called the **N** (nuclear) fraction. The pooled supernatants were centrifuged for 5 min at 11,000 rpm in the JA-20 rotor. The resultant supernatant was saved and the pellet was twice resuspended in 5 ml of buffer and centrifuged in the JA-20 rotor at 11,000 rpm. The pellet was saved and called **M** (mitochondrial) fraction. The supernatants were pooled and centrifuged for 15 min in the JA-20 rotor at 17,400 rpm. The pellet was twice in resuspended in 5 ml of buffer and centrifuged for 15 min in the JA-20 rotor at 17,400 rpm. The pellet at this stage was saved and called **L** (light mitochondrial) fraction. The pooled supernatants were centrifuged for 110 min at 25,000 rpm in a Beckman 35 rotor in a Beckman L8-70 centrifuge. The supernatant and pellet were separated. The pellet was called **P** (particulate) fraction and the supernatant was called final **S**(supernatant) fraction. After mea-

suring the volumes of each fraction, the radioactivity in each fraction was determined using a Packard autogamma 500 C using appropriate spill-over correction of ^{59}Fe in the ^{125}I channel. All fractions were analyzed for total protein and their content of lysosomal and Golgi enzymes.

2.3.5.3. Procedure III

In experiments described in chapter 5, procedure II was modified following the separation of the mitochondrial fraction. Instead, the post-mitochondrial supernatant was precipitated by 47 min centrifugation at 40,000 rpm (6.6×10^{10} rad^2/s) in a Beckman 60 Ti rotor. The final supernatant is the same as the De Duve S fraction. The pellet (LP fraction) contained lysosomes, endosomes, ER vesicles and Golgi cisternae. Endosomes were further purified by rapid discontinuous sucrose gradient centrifugation.

2.4. Enzymes

Enzyme activities were determined in all fractions derived during cell fractionation. γ-glutamyl transferase was determined as described by Persijn *et al.* (1976) to identify plasma membrane at 25°C with a kit from Boehringer cat. no. 543080, Boehringer Mannheim, Germany (Persijn and van der Slik, 1976; Szasz and Persijn, 1974). As Golgi marker: galactosyl-transferase as described by Hudgin and Ashwell and Beaufay *et al.* using ^{14}C-UDP-galactose at a specific activity of 0.4 mCi/mmol and a 60 min incubation time (Beaufay *et al.*, 1974; Hudgin and Ashwell, 1974). The mitochondrial enzyme -GPDH (glycerol-3-phosphate dehydrogenase) was determined as described by Meyer (Meyer *et al.*, 1977). Glucose-6-phosphatase as endoplasmic reticulum marker was determined as described by Schrijver *et al.* (1975). Two lysosomal enzymes were determined: acid phosphatase according to Walter and Schütt and b-glucosidase as described by Galjaard using 4-methylumbelliferine-glucopyranoside as substrate (Galjaard, 1980). Protein was determined according to a modified Lowry as described by Markwell (Markwell, 1978). In some experiments protein was determined according to Bradford (1976). In either case bovine serum albumin was used as the standard.

2.5. Sucrose gradient centrifugation

2.5.1. Discontinuous sucrose gradients

Samples from the L and P fraction were layered on the surface of discontinuous sucrose gradients consisting of 41 %, 26.5 %, 20 %, 15 % and 10.5 % (w/w) sucrose in 10 mM Hepes pH 7.2, 1mM dithiothreitol as described by Flatmark *et al.* (1985). The gradients were centrifuged for 3 hours at 27,000 rpm in a Beckman SW 27 rotor or alternatively for 70 min at 40,000 rpm in a Beckman SW 40 rotor. The gradients were fractionated from the bottom of the gradient. Fractions of 2 ml and 1 ml respectively were collected from the bottom.

2.5.2. Isopycnic sucrose gradient centrifugation

Isopycnic sucrose gradient centrifugation was performed using a 14 - 41 % sucrose gradient in 10 mM Hepes pH 7.2. Samples were layered on the gradients and centrifuged for 18 hours at 37,000 rpm in a Beckman Ti 70 rotor. 1 ml fractions were collected from the bottom.

2.5.3. Rapid discontinuous sucrose gradient centrifugation

Endosomes were further purified by rapid discontinuous sucrose gradient centrifugation. The pellet was carefully resuspended in a minimum volume (about 2 ml). The suspension was layered over two discontinuous sucrose gradients made up of the following sucrose/buffer solutions: 1 ml 66 %, 2.5 ml 45 %, 2.5 ml 33 %, 2.5 ml 27 % and 2.5 ml 11 % sucrose in 1 mM Hepes pH 7.4. Low buffer strength was used throughout the homogenization procedure to avoid a high buffer capacity inside endosomes, thereby preventing proper internal acidification. The gradients were centrifuged in a Beckman SW 40 rotor for 1 hour at 40,000 rpm and 4°C. The gradients were unloaded from the top by a glass capillary connected to a peristaltic pump. 0.5 ml fractions were collected and counted (see fig. 4.1). The heavy endosome fractions were found in the density range of 1.140 -1.125 g/ml (average 1.131 g/ml) and the light endosome fraction in the density range of 1.115 - 1.095 g/ml (average 1.105 g/ml). In the subsequent procedure care was taken to gradually lower the osmolarity of the heavy and light endosome fraction. The yield of purified endosomes was 4.2 $\pm$ 0.1 mg protein/10^9 cells for light endosomes and 1.9 $\pm$ 0.1 mg protein/10^9 cells for heavy endosomes. The approximate ratio of 2:1 for light : heavy endosomes was consistent in all experiments.

2.6. Density measurements of endosome fractions

All density measurements of fractions of the sucrose gradients were made with a Zeiss, Abbe type, refractometer at room temperature.

2.7. Measurement of endosome acidification by acridine orange fluorescence quenching

Endosomes were isolated according to cell fractionation procedure I from ^{125}I-Tf^{59}Fe loaded cells after 5 min incubation at 37°C followed by pronase digestion to remove ^{125}I-Tf^{59}Fe still bound to the plasma membrane. Heavy endosomes (density 1.051 ± 0.002, n = 4 on the Percoll gradient) were gelfiltrated over Sephacryl S-1000. The void volume fractions contained the endosome peak. Fluorescence quenching was measured in 1.5 ml of buffer containing: 1 mM Hepes pH 7.2, 3 mM $MgCl_2$, 200 mM sucrose, 45 mM NaCl, and 3 μM acridine orange. To this buffer 50 μl of concentrated endosome suspension was added, containing 4 mg protein/ml. Measurements were performed at room temperature in a Perkin-Elmer MPF 44 A spectrofluorometer with excitation wavelength set at 490 nm and emission wavelength set at 540 nm (Lee and Forte, 1978). After recording the baseline level, quenching measurements were started by the addition of 10 μl 1.5 M ATP carefully adjusted to pH 7.2. In order to reverse the proton gradient 5 μl of 1.0 M ammonium chloride was added (Galloway *et al.*, 1983; Schneider *et al.*, 1981; Stone *et al.*, 1983; Gluck *et al.*, 1982).

2.8. Incubation of endosomes

2.8.1. Incubation of endosomes with ATP and/or PIH

Preliminary experiments concerning the influence of ATP and the iron chelator PIH on iron release from endosomes are described in chapter 4. Each (duplicate) tube contained 1 ml of heavy endosome suspension (isolated as described for the fluorescence measurements) containing at least 1500 cpm ^{59}Fe and 8,000-25,000 cpm ^{125}I-Tf in a buffer consisting of 10 mM triethanolamine (or 10 mM Hepes) pH 6.8, 250 mM sucrose, 1 mM $MgCl_2$ pH 6.8. A 30 min incubation was started with the addition of 2 ml buffer containing ATP and/or PIH. Incubation was stopped by cooling the suspension to 4°C and endosomes and supernatant were separated at 4°C by 1 hour centrifugation at 100,000 g in a Beckman Ti 70.1 rotor.

2.8.2. Incubation of purified endosomes with iron chelators

Pooled sucrose gradient fractions containing either the heavy or light endosomes were gradually diluted with the incubation solution consisting of 130 mM KCl, 20 mM NaCl and 6mM $MgCl_2$ in aqua dest. to a final volume of 10 ml. Endosomes were incubated with either: BPS, a hydrophylic chelator of iron(II) ions; PIH, a hydrophylic chelator of iron(III)-ions or lactate, a hydroxyacid, and possible physiologic iron(III) chelator. BPS and lactate form chelates with iron in a 1:3 ratio (Fe:chelator), while PIH and iron form complexes in a 1:2 ratio (Fe:chelator). The concentrations of the chelator were high with respect to the iron concentration in each incubation. The concentration of PIH was calculated to be at least 3,000 times higher than the iron concentration as calculated from the ^{59}Fe activity (1500 times higher bearing in mind the 1:2 ratio of chelation). The same applies for the other chelators used. Incubations were carried out in 50 ml glass erlenmeyers in a water bath at 30°C and shaking at 60 cycles/min. Incubations were composed of the following stock solutions: heavy or light endosome suspension (5,000 µl, heavy endosome suspension average 0.48 mg/-ml protein, light endosome suspension average 1.05 mg/ml protein); 500 mM ATP in 5.0 mM Hepes, carefully adjusted to pH 7.2, final concentration 4 mM; 1.0 mM PIH, final concentration 10 µM; 3.3 mM BPS, final concentration 10 mM, 650 mM lactate in 2 mM Hepes pH 7.2, final concentration 6.5 mM; 0.1 M NEM, final concentration 5 mM; 1.0 mM NADH and 0.1 mM NAD^+ in a 1:1 ratio, final concentrations 1.0 µM and 0.1 µM respectively, as indicated in the individual experiments; the pH of the incubation mixtures was 7.0. The total volume of each incubation was 12.0 ml. Those incubations containing NEM (the inhibitor of the H^+/ATPase of endosomes) were preincubated with NEM at 30°C for 5 min before receiving PIH and ATP. The first samples (t=0) were taken after a mixing period of 15 seconds after addition of the last constituent. Duplicate samples of 1.0 ml were taken at 0, 10, 20, 40 and 80 min. Iron release was stopped through instant dilution of each sample in 9.0 ml ice-cold incubation solutions containing 100 µl 1.0 M NH_4Cl to dissipate any proton gradient (Galloway *et al.*, 1983). Endosomes and supernatants were separated by ultracentrifugation for 20 min at 40,000 rpm (1.72×10^{10} rad^2/s) in two SW 40 rotors in a Beckman L5-50 and a L8-65 ultracentrifuge. Finally the supernatants were decanted, whereas the stable pellets remained at the bottom of the polyallomer centrifuge tubes. Pellets and supernatants were counted in a Packard Autogamma 500-C. Care was taken to adjust the tube position to the centre of the

3" crystal for full (supernatant) and empty (pellet) tubes. ^{125}I counts were corrected afterwards for ^{59}Fe spill-over (13 % of ^{59}Fe counts in the ^{125}I channel).

2.8.2.1. Calculations

For each incubation containing an iron chelator and ATP, a blank incubation was carried out lacking ATP. Data from the blank incubations were subtracted from the incubation containing the chelator/ATP. Some unavoidable loss of endosome integrity occurs, particularly in the step were the LP pellet is homogenized after centrifugation, therefore, all data were normalized for iron release at t=0 to be 0. In vivo it has been shown that as iron is released from endosomes, transferrin remains stably bound to its receptor and is recycled to the plasma membrane (Van Bockxmeer and Morgan, 1979; Karin and Mintz, 1981; Octave *et al.*, 1981; van Renswoude *et al.*, 1982). However, during prolonged in vitro incubation some transferrin is lost from disrupted endosomes. Therefore the proportional iron release to the supernatant was corrected by subtracting the proportional release of ^{125}I-transferrin to the supernatant. This was then termed: the (proportional) ATP specific endosomal iron release.

2.9. Chromatography

2.9.1. Soft-gel chromatography

2.9.1.1. Gelfiltration of reticulocyte cytosol

Gelfiltration of rat reticulocyte cytosol was performed on either of two parallel Sephadex G-100 (Pharmacia, Uppsala, Sweden) columns (100 x 3 cm) using 0.1 M Tris, 0.5 M NaCl, pH 8.10 as eluent. Flow rates were 3.5 $ml/cm^2.hr^{-1}$ and 5.1 $ml/cm^2.hr^{-1}$, respectively. The effluents were collected in fractions of 7 ml.

2.9.1.2. Gelfiltration over Sephacryl S-1000 of a crude endosome suspension

A crude endosome suspension containing Percoll, Golgi vesicles and soluble endosomes was purified by gelfiltration over a 50 ml (1 x 30 cm) Sephacryl S-1000 column as described by Dickson *et al.* (1983 b) using a buffer of 10 mM triethanolamine pH 6.8 (or 10 mM Hepes pH 7.2) and 0.25 M sucrose. Non-specific binding to the column was minimised by pre-running the column with a plasma membrane fraction of K 562 cells. Endosomes eluted in the columns void volume, whereas all other components eluted later as determined by gamma-counting and enzyme determinations (results not shown).

2.9.1.3. Gelfiltration of solubilized endosomes containing ^{125}I-Tf and ^{59}Fe

Endosomes were isolated from ^{125}I-Tf^{59}Fe loaded cells after 7 min incubation at 37°C according to procedure I. A sample from the heavy endosome suspension was mixed with Triton X-100 to a final concentration of 0.5 % and gelfiltrated on an AcA 34 column (2.5 x 40 cm) in a buffer containing 10 mM Hepes, 0.25 M sucrose pH 7.2 and 0.5 % Triton X-100 at a flow of 11 ml/hr 3 ml fractions were collected and counted. Recovery of ^{125}I-Tf was greater than 98 % and of ^{59}Fe greater than 92 %. Molecular weight calibrations were performed using horse spleen ferritin, human IgG, ^{125}I-Tf^{59}Fe and ovalbumin.

2.9.2. High Performance Liquid Chromatography (HPLC)

2.9.2.1. Equipment

Equipment (LKB, Bromma, Sweden) consisted for the following components: a 2152 HPLC controller, two 2150 pumps with a stationary capillary mixer and 2 meters of damping capillary coils, a 2154-100 injector with interchangeable loops from 100 to 500 µl, a 2138 Uvicord S with 8 µl, 0.5 cm cell or a 2151 Variable Wavelength monitor with 10 µl, 1.0 cm cell, a 2210 two channel recorder and a 2211 Superrac fraction collector.

2.9.2.2. Columns

Reversed phase chromatography was performed on a LiChrosorb 250-4 RP-18 column with 10 µm modified irregular silica particles (Merck, Darmstadt, Germany) at a flow rate of 1.0 ml/min. Size-exclusion chromatography was performed on a 2135-330 and a 2135-360 LKB TSK G-3000 SW Ultropac column in series, or on a 2135-260 LKB TSK G-2000 SW Ultropac column, all with a 2135-075 LKB TSK GWSP Ultropac pre-column. Flow rates were between 0.1 and 1.0 ml/min.

2.9.2.3. Elution buffers

Reversed phase chromatography; 0.1 M ammonium acetate/acetic acid, pH 5.0, 50 mM NaCl with an adapted methanol gradient from 0 to 50 %. Size-exclusion chromatography; 0.1 M phosphate buffer, pH 6.8, 50 mM NaCl and 0.005 % NaN_3 (w/v).

2.10. Spectrophotometric analysis

^{59}Fe containing fractions from the HPLC reversed phase separations were analyzed on a model 25 Beckman spectrophotometer with recorder in Ultrosil quartz cuvettes (0.4 ml, 1 cm). Elution buffers were used as blanks.

2.11. Concentration

Pooled fractions from the Sephadex columns were concentrated by Amicon ultrafiltration. Large quantities were concentrated using an Amicon model 402 with an UM05 membrane (cut off 500 Mw) or an YM2 membrane (cut off 1,000 Mw). Small quantities were concentrated using a No. 5106 Amicon model 3 with a YC05 membrane (cut off 500 Mw).

2.12. Protein determination

Protein was determined after Amicon ultrafiltration according to Bradford (Bradford, 1976), modified by Brogdon and Davis (1983), with a bovine serum albumin standard.

2.13. Citric acid determination

Citric acid was determined after Amicon ultrafiltration of the low molecular weight iron-containing fractions from the Sephadex gelfiltration columns with a commercial citric acid testkit using the enzyme citric acid lyase (Boehringer, Mannheim, Germany). Alteration of the NADH concentration was spectrophotometrically detected with a model 25 Beckman spectrophotometer.

2.14. Pyrophosphate determination

Pyrophosphate was determined as described by Heionenen *et al.* (1981).

2.15. Amino acid analysis

Amino acid analysis was carried out on the concentrated low molecular weight fractions from the Sephadex columns, as on the ^{59}Fe-containing fractions from the reversed phase separations of these low molecular weight fractions. From each fraction a quantitative determination was made of free amino acids and of the total amino acid content. For free amino acid analysis protein in each fraction was precipitated with a final concentration of 10 % trichloroacetic acid. The supernatant was recovered after a short centrifugation and excess TCA was eliminated by triple diethylether extraction. The pH was adjusted to 2.2. To measure the total amino acid content each fraction was hydrolyzed for 24 hr at

110°C in 6.0 M HCl in a vacuum glass ampulla. Determinations were performed on a Beckman Multichrom M 4327, as described previously (van Eijk *et al.*, 1976).

REFERENCES

Beaufay H., E. Aman-Costesec, D. Feytmans, W.M. Thinès-Sempoux, H. Robbi and J. Berthet (1974) Analytical study of microsomal and isolated subcellular microsomes from rat liver. J. Cell Biol. **61**: 188-200.

Bolton A.E., and W.M. Hunter (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. **133**: 529-539.

Bradford M. M. (1976) A rapid and sensitive method for quantitation of micrograms of protein utilizing the principle of protein dye binding. Analyt. Biochem. **72**: 248-254.

Bockxmeer F.M. van, and E.H. Morgan (1979) Transferrin receptors during rabbit reticulocyte maturation. Biochim. Biophys. Acta **584**: 76-83.

Brogdon W.G. and C.M. Dickinson (1982) A micro assay system for measuring esterase activity and protein concentration in small samples and in high pressure liquid chroma-tography eluate fractions. Anal. Biochem. **131**: 499-503.

Condò S.G., B. Giardina, D. Barra, S.J. Gilh and M. Brunori (1981) Purification and functional properties of hemoglobin components from the rat (Wistar). Eur. J. Biochem. **116**: 243-247.

Dickson R.B., I. Beguinot, J.A. Hanover, N.D. Richert, M.C. Willingham and I. Pastan (1983) Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. Proc. Natl. Acad. Sci. **80**: 5335-5339.

Duve C. de, B.C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans (1955) Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J., **60**: 604-617.

Egyed A. (1982) Studies on the partition of transferrin-donated iron in rabbit reticulocytes. I. The kinetics of iron distribution between stroma and cytosol. Br. J. Haematol. **52**: 475-483.

Eijk H.G. van, and W.L. van Noort (1976) Isolation of rat transferrin using CNBr-activated Sepharose-4B. J. Clin. Chem. Clin. Biochem. **14**: 475-478.

Galjaard H. (1980) Genetic metabolic diseases. Elsevier, North Holland, 817-825.

Galloway C.J., G.E. Dean, M. Marsh, G. Rudnick and I. Mellman (1983) Acidification of macrophage and fibroblast endocytic ves-icles in vitro Proc. Natl. Acad. Sci. U.S.A. **80**: 3334-3338.

Harmsen E., de Tombe P. Ph. and de Jong J.W. (1982) Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high performance liquid chromatography. J. Chrom **230**: 131-136.

Heionenen J.K., S.H. Honkasalo and E.I. Kukko (1981) A method for the concentration and for the colorimetric determination of nanomoles of inorganic pyrophosphate. Analyt. Biochem. **117**: 293-300.

Heul C. van der, C.M.H. de Jeu-Jaspars, M.J. Kroos and H.G. van Eijk. (1978) Binding sites of iron transferrin on rat reticulocytes: inhibition by specific antibodies. Biochim. Biophys. Acta **511**: 430-441.

Hudgin R.L. and G. Ashwell (1974) Studies in the role of glycosyltransferases in the hepatic binding of asialoglycoproteins. J. Biochem. **249**: 73697372.

Hunter W.M. and F.C. Greenwood (1962) Preparation of Iodine-131 labelled human growth factor of high specific activity. Nature **194**: 495-496.

Karin M. and B. Mintz (1981) Receptor-mediated endocytosis of transferrin in developed totipotent

mouse teratocarcinoma cells. J. Biol. Chem. **257**: 5969-5976.

Klausner R.D., J. van Renswoude, G. Ashwell, C. Kempf, A.N. Schechter, A. Dean and K.R. Bridges (1983) Receptor-mediated endocytosis of transferrin in K 562 cells. J. Biol. Chem. **258**: 4715-4724.

Lozzio C.B. and B.B. Lozzio (1975) Human chronic myelogenous leukemia celline with positive Philadelphia chromosome. Blood **45**: 321-334.

Markwell M.A.K., S.M. Hags, L.L. Bieber and N.E. Tolbert (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Analyt. Biochem. **193**: 265-275.

Noort W.L. van, and H.G. van Eijk (1977) Affiniteitschromatografie met behulp van CNBr-geactiveerde Sepharose. Mogelijkheden en moeilijkheden. Tijdschrift v. Med. Anal. **32**: 184-196.

Octave J.-N., Y.-J. Schneider, R.R. Crighton and A. Trouet. (1981) Transferrin uptake by cultured rat embryo fibroblasts. Eur. J. Biol. **115**: 611-618.

Persijn J. P. and W. van der Slik (1976) A new method for the deterination of gamma-glutamyltransferase in serum. J. Clin. Chem. Clin Biochem. **14**: 421-427.

Ponka P., R.W. Grady, A. Wilczynska and H.M. Schulman (1984) The effect of various chelating agents on the mobilization of iron from reticulocytes in the presence and absence of pyridoxal isonicotinoyl hydrazone. Biochim. Biophys. Acta **802**: 477489.

Renswoude J. van, K.R. Bridges, J.B. Harford and R.D. Klausner (1982) Receptor mediated endocytosis of transferrin and the uptake of Fe in K 562 cells: Identification of a nonlysosomal acidic compartment. Proc. Natl. Acad. Sci. U.S.A. **79**: 6186-6190.

Schrijver J., J.F. Koster and W.F. Hülsmann (1975) Insignificance of gluconeogenesis in human platelets. Eur. J. clin. Invest. **5**: 7-14.

Szasz G. and J.P. Persijn (1974) New substrates for measuring gamma-glutamyltranspeptidase activity. Z. klin. Chem. klin. Biochem. **12**: 228-229.

CHAPTER 3

ANALYSIS OF IRON-BINDING COMPONENTS IN THE LOW MOLECULAR WEIGHT FRACTION OF RAT RETICULOCYTE CYTOSOL

Chapter 3

1. INTRODUCTION

Despite years of research the intracellular iron metabolism of erythroid cells has not been unravelled. Many investigators now believe that iron-transferrin the iron-donor to the erythroid cells, together with the transferrin receptor are taken up by receptor-mediated endocytosis (Van Renswoude *et al.*, 1982; Iacopetta and Morgan, 1983; Klausner *et al.*, 1983; Dickson *et al.*, 1983a; Dickson *et al.*, 1983b). After release of iron from transferrin within the low pH endocytic vesicle, iron atoms traverse the cytosol. In doing so iron atoms have to cross three membrane barriers, viz. the endosome membrane and the outer and inner mitochondrial membrane. Since transport of uncomplexed iron is very unlikely, it is presumed that a carrier is involved. Many cytosolic iron-binding molecules have been described since the early sixties (reviewed by Romslo, 1983). The molecular weights of these iron binding compounds have varied from a few hundred to about half a million Dalton. In 1977 Jacobs suggested the presence of a labile low molecular weight iron pool in the cytosol, maintaining a dynamic equilibrium between iron uptake, iron storage and iron utilization into hemoglobin (Jacobs, 1977). Opinions are divided about the existence of such a low molecular weight iron pool. In our laboratory we isolated an iron-containing low molecular weight fraction (LMWF) from rat reticulocyte cytosol (van der Heul *et al.*, 1982). In this paper we present the results of analysis of the iron containing LMWF by modern high performance liquid chromatography (HPLC), reversed phase chromatography (RPC) and size-exclusion (SEC) chromatography. Furthermore, the iron-containing LMWF was examined for the presence of peptides, amino acids, nucleotides, pyrophosphate and citrate.

2. RESULTS

2.1. Gelfiltration of reticulocyte cytosol

The reticulocyte cytosol showed the following elution profile when separated on the Sephadex G-100 column (Fig. 3.1). The four radioactive peaks represent: (I) ferritin, near the columns void volume; (II) transferrin = ^{125}I peak; (III) hemoglobin; and (IV) the low molecular weight fraction, ^{59}Fe always slightly preceded the protein and ^{125}I peak. Ferritin, transferrin and hemoglobin eluted together near the columns void volume in preparative separation on Sephadex G-50. Recovery of ^{59}Fe activity was usually between 75 and 85 %. It is known that the ^{59}Fe activity containing components in the LMWF have some affinity to Sephadex (Jacobs, 1977) even in the presence of 0.5 M NaCl. This non-specific bound ^{59}Fe could be removed from the column by 0.1 mmol ATP or an acid wash.

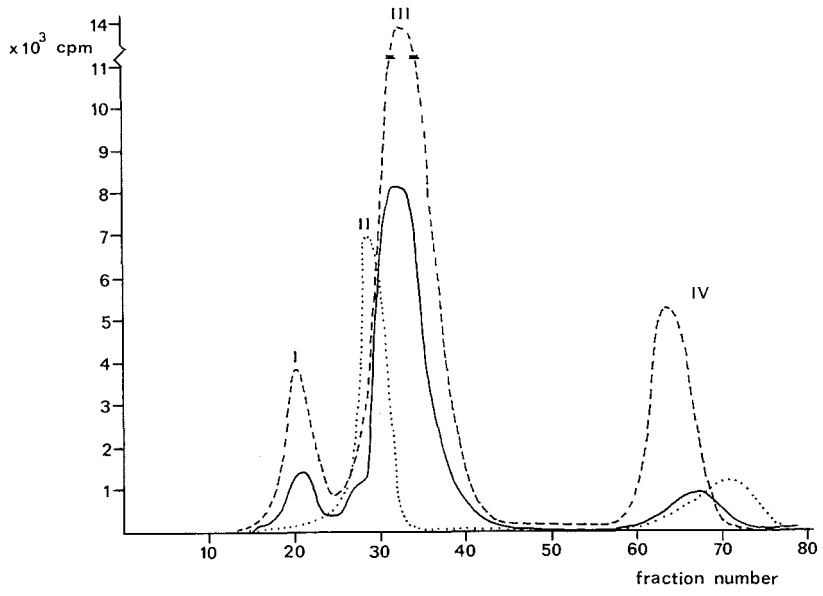

Fig. 3.1 *Rat reticulocyte were preincubated with 1 mM KCN and 0.25 mM DNP for 15 min at 37°C under 5 % CO_2 to stop heme synthesis and to block the oxidative phosphorilation. Subsequently the cells were incubated with ^{125}I-Tf ^{59}Fe at a final concentration of 10 μM for 30 min at 37°C under 5 % CO_2. After hypotonic lysis in 3 vol. 1 mM Hepes, pH 7.4, the cytosol was separated from the stroma by centrifugation. The cytosol was subjected to gelfiltration on two parallel Sephadex columns (100 x 3 cm) using 0.1 M Tris, 0.5 M NaCl, pH 8.1 as eluens at a flow rate of 3.5 ml cm^{-2} .hr^{-1}. Fractions of 7 ml were sampled and the radioactivity was counted in a γ-counter. = —— E_{280} profile; - - - - = ^{59}Fe activity; .... = ^{125}I-TF activity.*

Under our experimental conditions the percentage of ^{59}Fe activity recovered in the LMWF was about 7 % when 10^{-2} M INH was used to inhibit the heme synthesis. Without INH only 2.5 % ^{59}Fe activity could be found in the LMWF (van der Heul *et al.*, 1981). The simultaneous use of 1 mM cyanide to inhibit the heme synthesis and 0.25 mM DNP to uncouple the oxidative phosphorylation resulted in a dramatic rise in the ^{59}Fe activity recovered in the LMWF up to 39 %.

2.2. HPLC reversed phase separation of the ^{59}Fe-LMWF

A 100-fold concentration of the LMWF by Amicon ultrafiltration resulted in 98 % recovery of the ^{59}Fe activity and 2-4 % of the ^{125}I activity in the concentrate when a UM05 membrane was used. ^{125}I activity probably originates from transferrin breakdown products formed

during fractionation and is of no importance in the LMWF. Six ml of packed cells gave 18 ml cytosol after lysis with 18 ml 5 mM Hepes. Preparative gelfiltration of the cytosol gave about 350 ml solution containing the LMWF. The 100-fold concentrated LMWF contained between 121 and 169 μg/ml protein. The LMWF was separated by HPLC reversed phase chromatography (Fig. 3.2) to purify the ^{59}Fe activity from the applied LMWF eluted directly from the column even before the methanol gradient was started and showed one broad peak in which 56 % of the recovered ^{59}Fe activity was found. The rest of the ^{59}Fe activity was found randomly distributed through the chromatogram just above the background level. Typically ^{59}Fe recovery was 70 %. The non-specifically bound iron could not be removed from the column with more apolar organic solutes such as tetrahydrofuran or methanol : chloroform (1:1). However, it could be removed with 0.1 M HCl. The chromatogram showed that the ^{59}Fe-containing fraction was separated from the bulk of non-iron-containing material. However, the fact that the broad ^{59}Fe peak overlapped a number of E 280

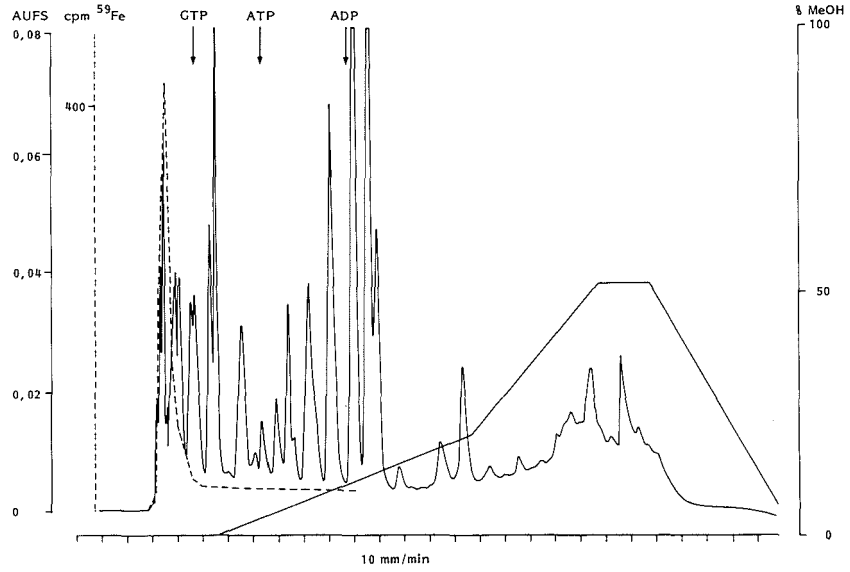

Fig. 3.2. *Reversed phase chromatogram of the ^{59}Fe-containig LMWF derived after gelfiltration chromatography on Sephadex G-100. A 100 μl sample was injectedon a RP-18 column (25 x 0.4 cm) in a starting buffer of 0.1 M ammonium acetate and 50 mM NaCl pH 5.0 at 1.0 ml/min. After 4.9 ml an adapted methanol gradient was started, running from 0 to 50 % (solid line). Detection was performed at 280 nm at a sensitivity of 0.08 AUFS. The dashed line represents the ^{59}Fe activity found after counting 0.25 ml fractions in a γ-counter. On top of the chromatogram it is shown where the nucleotides GTP, ATP and ADP elute when run under identical conditions.*

nm peaks suggests that the ^{59}Fe-containing fraction consists of a group of polar components not showing primary extinction at 280 nm. Although this reversed phase separation was not able to separate the ^{59}Fe-containing components from each other, it was useful to remove the bulk of the more apolar non iron-containing molecules. Anion nor cation exchange HPLC showed proper separation of the iron-containing component(s) in the LMWF (results not shown).

2.3. Molecular weight estimations

Molecular weight estimations of the low molecular weight components were made using Amicon ultrafiltration and HPLC size exclusion chromatography. Ultrafiltration showed that all the ^{59}Fe activity passed through a membrane with a Mw cut off of 10,000 Dalton, but 90 % of the ^{59}Fe activity was recovered in the concentrate when a membrane with a Mw cut off of 1,000 Dalton was used. The size-exclusion column (Fig. 3.3) was calibrated with globular protein covering a range from 160,000 to 3,000 Dalton while tyrosine was used to mark the columns total permeation volume. The apparent molecular weight of the ^{59}Fe containing components of the LMWF was consistently found to be 5,500 Dalton. The same molecular weight was found with PAGE electrophoresis. The apparent Mw of 5,500 Dalton for the iron-containing components was checked further on a Sephadex G-25 medium column that excludes molecules with Mw higher than 5,000 Dalton. It was found that the ^{59}Fe containing peak from the LMWF was fully excluded on this matrix. When a dilute solution of ^{59}Fe-citrate was separated on the HPLC size-exclusion column it eluted in the same volume as the ^{59}Fe-containing fraction of the LMWF.

Table 3.1 **Dominant amino acid species in the low molecular weight fraction.**

	Free amino acids	Total amino acids
Iron containing LMWF	aspartic acid	aspartic acid
	serine	
	glutamic acid	glutamic acid
	glycine	glycine
	$^1/_2$ cystine	$^1/_2$ cystine
Iron peak after HPLC-RPC	aspartic acid	aspartic acid
	glycine	glycine
	$^1/_2$ cystine	$^1/_2$ cystine

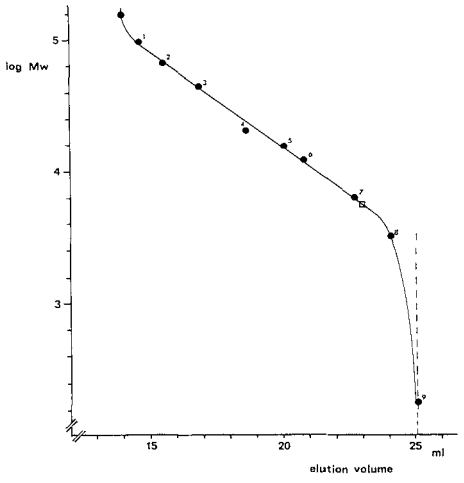

Fig. 3.3. *A lineair relationship exists between the log Mw and the elution vlolume of the proteins in the range of Mw 100,000 - 4,000 Dalton on the TSK G-2000 SW Ultropac column. The HPLC size exclusion column was calibrated with globular proteins to determine the molecular weight of the ^{59}Fe containing LMWF. Calibration was performed with: 0 = human γ-globuline, 160,000 D; 1 = Ovalbumin dimer, 90,000 D; 2 = human serum albumin, 68,000 D; 3 = Ovalbumin, 45,000 D; 4 = Trypsin, 21,500 D; 5 = myoglobin, 16,000 D; 6 = Cytochrome C, 12,500 D; 7 = Aprotinin, 6,500 D; 8 = Insulin-bchain, 3,000 D; 9 = Tyrosine, 180 D; = ^{59}Fe-containing LMWF.*

2.4. Amino acid analysis

The 100-fold concentrated iron-containing LMWF and the iron-containing fraction derived from the LMWF after HPLC-RPC were analyzed for their amino acid content. The presence of free amino acid was determined after TCA precipitation of protein followed by centrifugation and removal of the excess TCA from the supernatant. The total amino acid content was determined after complete hydrolysis of the samples. The wide range in the concentrations of the amino acids found in different LMWF fractions and subsequent iron-containing fractions after HPLC-RPC treatment emphasized once again the heterogeneous and variable nature of the LMWF's. All amino acids were found in hydrolysates of the 100-fold concentrated LMWF's, but the majority were the neutral species. The iron-peak derived from the LMWF after HPLC-RPC contained few free basic amino acids. Therefore only the most dominant amino acids are mentioned in table 3.1. Because of great variability in the LMWF's the dominant amino acids are listed only qualitatively, in order of increasing iso-electric points as they were repeatedly (n = 4) found in the iron-containing LMWF and in the iron peak derived from the LMWF after HPLC-RPC.

2.5. Pyrophosphate determination

The 100-fold concentrated iron-containing LMWF was analyzed for the presence of pyrophosphate. Using the method described in chapter 2, no pyrophosphate could be detected in the concentrated LMWF. The detection limit of this assay is 5 nmol/l (Heionenen *et al.*, 1981).

2.6. Nucleotide determination

The iron-containing fraction, separated from the LMWF's by HPLC reversed phase chromatography, was assayed for the presence of nucleotides with special attention to ATP, ADP and GTP, which are known to chelate Fe(II)(Strickland *et al.*, 1965). No nucleotides at all could be detected in this iron containing fraction. Dilute solutions of ATP, ADP and GTP (0.16 nmol/50 ml) were injected on the HPLC reversed phase column under conditions identical to the separations of the LMWF. ATP eluted at 4 % methanol, ADP at 10 % methanol, while GTP eluted before the methanol gradient was started. The positions are indicated in fig. 3.2.

2.7. Citric acid determination

The 100-fold concentrated LMWF was tested for the presence and concentration of citric acid. In all LMWF citric acid was present and the concentrations found varied between 12.4 and 23.1 nmol/ml. Citric acid is known to be present as dimers (Spiro *et al.*, 1967) and can be concentrated using an Amicon membrane (UN05) or YC05). This was tested with a dilute solution of ^{59}Fe-citrate in which citrate is present in 240-fold molar excess.

3. DISCUSSION

3.1. Molecular weight estimation by gelfiltration

The aim of the study was to learn more about the iron-binding components present in the low molecular weight fraction of the reticulocyte cytosol that has been described by several authors. The cytosol iron content could be enlarged dramatically by the simultaneous use of cyanide and DNP as inhibitors of heme synthesis and uncouplers of oxidative phosphorylation (Borova, 1973; Egyed, 1982, 1983). Up to 39 % of the recovered ^{59}Fe activity was present in the LMWF. Molecular weight determinations by HPLC size-exclusion chromatography gave only approximate molecular weights, as these small iron-binding molecules probably do not have a globular shape. The calibration of the column was made with a set of globular proteins. The apparent molecular weight of the iron-binding fraction of the LMWF was 5,500 Dalton. These iron-binding components were also found to be excluded on a Sephadex G-25 which has an exclusion limit of 5,000 Dalton. It should be noted, however, that a dilute solution of ^{59}Fe-citrate (240-fold molar excess of citrate) appeared to have a similar molecular weight to the iron-binding components of the LMWF when determined by HPLC size-exclusion chromatography. The molecular weight of the iron-binding components of the LMWF was not altered by oxidation or reduction (results not shown) as was found in hepatocytes by Morley and Bezkorovainy (1983).

3.2. Amino acids and nucleotides determination

The concentrated LMWF was analyzed for the presence of known iron chelators such as amino acids, nucleotides, pyrophosphate and citrate. Amino acid analysis showed that the LMWF and HPLC-RP purified ^{59}Fe peak contained significant quantities of iron chelating amino acids. The most dominant iron chelating amino acid found was glycine. Glycine was present mostly as a free amino acid. Apart from glycine all fractions contained the $^{1}/_{2}$cystine amino acid residue that is known to complex readily with iron. In the cell there are many iron-sulphur proteins. The high energy nucleotides, ATP, ADP and GTP, which may also chelate iron, are unlikely to be detected in the LMWF, because of the length of time needed for gelfiltration and concentration and the short turnover time of the nucleotides. Even if they were present ATP and ADP would elute from the HPLC-RP column at respectively, 4 % and 10 % methanol, while GTP would elute before the methanol gradient has started. However, several HPLC-RP separated ^{59}Fe peaks were tested for the presence of these nucleotides, none of these nucleotides was found in any of the tested samples.

3.3. Pyrophosphate determination

The 100-fold concentrated LMWF's were analyzed for the presence of pyrophosphate, since Konopka and Romslo (1980, 1981) claimed that pyrophosphate is the intermediate for the uptake of iron from transferrin and the donor of iron to mitochondria. In our concentrated LMWF's no pyrophosphate could be detected within the range of the assay's detection limit.

3.4. Analyses for the presence of citric acid

Citric acid was determined only in the 100-fold concentrated LMWF, because the detection limit of this determination is 2 nmol/ml. Prior to this determination it was shown with a dilute ^{59}Fe-citrate solution that citrate could be concentrated by 99 % using an Amicon YC05 membrane (cut off 500 Mw). According to Spiro *et al.* (1967) iron is chelated by an excess of citrate in the low molecular weight form $Fe(cit)_5^{2-}$ with a Mw of 500 Dalton. It was shown that the concentrated LMWF did contain citric acid in concentrations varying between 12.4 and 23.1 nmol/ml.

3.5. Conclusions

In this study we used a blockade of the heme synthesis and oxidative phosphorylation hoping this would result in the accumulation of an iron transporting molecule in the cytosol. However, even with the use of the HPLC-SEC and HPLC-RPC no specific low molecular weight iron-binding protein was found in the reticulocyte cytosol. Inste-

ad it was shown that this LMWF contained the non-specific iron-chelating molecules citric acid, glycine and $^{1}/_{2}$cystine. Other non-specific iron-chelating molecules such as pyrophosphate and the nucleotides ATP, ADP and GTP were not found. The results lead to two possible interpretations. The first is that specific proteineous iron-transporting molecules in the cytosol do not exist and that the iron is transported to the mitochondria through a variety of iron chelating amino acids and salts. However, it seems unlikely that after a sequence of highly specific interactions, such as iron-binding to transferrin, transferrin-binding to its receptor, receptor-mediated endocytosis and iron release in an acid endosomal milieu, iron transport would proceed via several aspecific amino acids, salts and nucleotides. The second, most plausible, explanation is that there is a specific iron-binding and transporting factor with a very high turnover, that looses its iron during the isolation and fractionation procedure to the 'next best' iron chelators, namely glycine and citrate. The nucleotides, ATP, ADP and GTP that have also been shown to chelate iron (Strickland *et al.*, 1965) decay during these procedures and are therefore not found in the LMWF. A better possible way of discovering the specific iron-binding and transporting factor might be the isolation of the Tf^{59}Fe carrying endosomes. The interior of the endosome or its membrane may possibly contain the iron-transporting factor. It is also possible that iron remains free in the endosomal acid interior and that the iron-transporting factor resides in the CURL (compartment for uncoupling receptor and ligand). Therefore our further investigations focused on the isolation and characterization of iron and transferrin containing endosomes.

REFERENCES

Borova J., P. Ponka and J. Neuwirt (1973) Study of the intracellular iron distribution in rabbit reticulocytes with normal and inhibited heme synthesis. Biochim. Biophys. Acta **320**: 143-156.

Dickson R.B., J.A. Hanover, M.C. Willingham and I. Pastan (1983a) Prelysosomal divergence of transferrin and epidermal growth factor during receptor-mediated endocytosis. Biochem. **22**: 5667-5674.

Dickson R.B., L. Beguinot, J.A. Hanover, N.D. Richert, M.C. Willingham and I. Pastan (1983b) Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. Proc. Natl. Acad. Sci. USA **80**: 5335-5339 (1983).

Egyed A. (1982) Studies on the partition of transferrin-donated iron in rabbit reticulocytes.I. The kinetics of iron distribution between stroma and cytosol. Br. J. Haematol. **52**: 475-483.

Egyed A. (1983) Studies on the partition of transferrin donated iron in rabbit reticulocytes. II. Distribution and kinetics of non-haem iron in cytosol. Br. J. Haematol. **53**: 217-225.

Heul C. van der, C.M.H. de Jeu-Jaspars, M.J. Kroos and H.G. van Eijk (1982) Intracellular iron transport in rat reticulocytes. In: The Biochemistry and Physiology of Iron (edited by Saltman P. and Hegenauer J.), pp. 189-191. Elsevier, North Holland.

Heul C. van der, M.J. Kroos, C.M.H. de Jeu-Jaspars and H.G. van Eijk (1981) Intracellular iron transport and iron-binding components of reticulocytes. In: Proteins of Iron storage and Transport Ed. P. Saltman.

Iacopetta B. and E.H. Morgan (1983) An electron microscope autoradiographic study of transferrin endocytosis by immature red cells. Eur. J. Cell Biol. **32**: 17-28.

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

Klausner R.D., J. van Renswoude, G. Ashwell, Chr. Kemp, A.N. Schecter, A. Dean and K.R. Bridges (1983) Receptor-mediated endocytosis of transferrin in K562 cells. J. Biol. Chem. **258**: 4715-4724.

Konopka K. and I. Romslo (1980) Uptake of iron from transferrin by isolated rat-liver mitochondria mediated by phosphate compounds. Eur. J. Biochem. **107**: 433-439.

Morley C.G.D. and A. Bezkorovainy (1983) Identification of the iron chelate in hepatocyte cytosol. IRCS Med. Sci. **11**: 1106-1107.

Renswoude J. van, K.R. Bridges, J.B. Harford and R.D. Klausner (1982) Receptor-mediated endocytosis of transferrin and uptake of Fe in K562 cells: Identification of a non-lysosomal acidic compartment. Proc. Natl. Acad. Sci. USA **79**: 6186-6190.

Romslo I. (1983) Intracellular transport of iron. In iron in Biochemistry and Medicine II (Edited by A. Jacobs and M. Worwood) Chap. 9. Academic Press, London..

Spiro T.G., G. Bates and P. Saltman (1967) The hydrolytic polymerization of ferric citrate. II. The influence of excess citrate. J. Am. chem. Soc. **89**: 5559-5562.

Strickland E.H. and B.C. Davis (1965) Fe^{3+} uptake by rat-liver mitochondria. Biochim. Biophys. Acta **104**: 596-599.

CHAPTER 4

RELEASE OF IRON FROM ENDOSOMES IS AN EARLY STEP IN THE TRANSFERRIN CYCLE

Chapter 4

1. INTRODUCTION

In mammalian cells, iron is taken up by the process of receptor-mediated endocytosis of ferric-transferrin. In this universal process of ligand uptake, mono- and diferric transferrin are bound to the transferrin receptors present in the coated pit areas in the plasma membrane. The coated pits then transform to intracellular coated vesicles, which rapidly loose their clathrin coat and become endosomes. This process has been described in detail for some ligands such as LDL, EGF, some viruses, toxins and transferrin (Morgan et al., 1969; Anderson et al., 1977; Dickson et al., 1983 a,b; Dales, 1973; Helenius and Marsh, 1982; Keen et al., 1982; Karin and Mintz, 1981; van Renswoude et al., 1982). Transferrin, however, behaves differently from the other ligands mentioned, because it is not routed to the lysosomal compartment after the ligand sorting, but is recycled undegraded to the medium. Many investigators have contributed to our current knowledge regarding internalization of transferrin (reviewed most recently by Hanover and Dickson, 1985), but little progress has been made concerning the intracellular iron transport. Still unanswered questions are: at which stage of the transferrin cycle is iron released from transferrin and by which mechanism or carrier is iron released from endosomes? (Bakkeren et al., 1984). In this paper we report the in vivo release of iron from endosomes at an early stage of the transferrin cycle, using subcellular fractionation experiments. In an earlier paper we analyzed a low molecular weight iron fraction from reticulocyte cytosol to determine the presence of one clear iron carrying substance. However, several low molecular weight candidates were found, without one seeming very specific. Therefore we turned to the intracellular environment, more specifically the endosomal system, where the intracellular movement of iron becomes obscure. Endosomes were isolated from a human erythroleukemic cell line, because physiologically they resemble reticulocytes. The isolated endosomes were functionally tested for their capability to acidify their lumen. We also tested the hypothesis that acidification alone is sufficient to release iron from isolated endosomes.

2. RESULTS

Cells, preloaded with double labelled transferrin, showed a decreased sensitivity to pronase digestion with respect to transferrin after 3 and 5 min incubation at 37°C. The decrease is greater for ^{59}Fe than for ^{125}I-Tf (see table 4.1). This indicates that ^{125}I-Tf^{59}Fe rapidly disappeared from the plasma membrane to an intracellular location after incubation at 37°C. Cells were homogenized after endocytosis and pronase digestion and a post-nuclear supernatant was separated by 1.055 g/ml Percoll gradients. After 3 and 5 min endocytosis two populations of ^{125}I-Tf and ^{59}Fe vesicles were found. The heavy population was found

at a density of 1.051 ± 0.002 g/ml (n=4), while the light population was found at a density of 1.042 ± 0.002 g/ml (n=4), as determined with Density Marker Beads. As the contribution of plasma membrane vesicles containing ^{125}I-Tf^{59}Fe was minimised by pronase digestion, it is likely that virtually all ^{125}I-Tf and ^{59}Fe was present in pronase resistant endocytic ves-icles. Lysosomes were predominantly found at densities higher than 1.06 g/ml as indicated by the presence of the lysosomal enzymes acid phosphatase and β-D-glucosidase. On Percoll gradients with a starting density of 1.07 g/ml only one ^{125}I-Tf and ^{59}Fe containing fraction was found at about 1.045 g/ml (results not shown). A relatively high background of ^{59}Fe activity was found throughout the Percoll gradient on which the ^{59}Fe activity of the endosome fractions was superimposed. It was therefore not possible to determine accurately the ^{125}I/^{59}Fe ratio of the two endosome fractions. To determine the source of the ^{59}Fe activity and to separate it from the endosome fractions, homogenates were fractionated by differential centrifugation (procedure II). Results from gelfiltration of solubilized endosomes presented in fig. 4.1 show that the majority of ^{125}I-Tf was found at a molecular weight corresponding to the transferrin-transferrin receptor complex (distinct from the ferritin elution position) and still carrying ^{59}Fe. About 30 % of ^{59}Fe present in the endosomes was in a low molecular weight form.

2.1. Subcellular fractionation by differential centrifugation

Subcellular fractionation was performed after 0, 3 and 5 min incubation at 37°C. At t=0 the L and P fraction contained ^{125}I-Tf and ^{59}Fe with the highest relative specific activity. The distribution of ^{125}I-Tf and ^{59}Fe between the L and P fraction, however, was variable depending upon whether the fluffy pellet overlying the compact pellet of the L fraction was left with the L fraction or transferred partially or completely to the P fraction (see table 4.2). Therefore data from the L and P fraction were taken together in all experiments. The S fractions at t=0 showed the highest ^{125}I/^{59}Fe ratio of all fractions, 27.7 ± 1.6 % higher than in the homogenates (the ^{125}I/^{59}Fe ratio of the homogenates was standardised to be 1). At t=3 the percentage of ^{125}I-Tf activity found in the LP and S fraction was not significantly changed. The ^{59}Fe activity on the other hand was decreased in the LP fraction by 10.6 ± 6.4 % and increased in the S fraction by 14.0 ± 3.2 %. These changes were accompanied by a decrease in the ^{59}Fe relative specific activity in the LP fraction and an increase in the S fraction. At t=5 there was a further decrease by 9.0 ± 6.4 % of ^{59}Fe activity in the LP fraction coupled to an increase by 11.0 ± 6.2 % in the S fraction. The distribution of ^{125}I-Tf activity was again virtually unchanged.

We investigated using discontinuous sucrose gradient centrifugation, whether the ^{125}I-Tf and ^{59}Fe found in the L and P fraction was in vesicles of distinct densities. To this end ^{125}I-

Tf and ^{59}Fe loaded cells were incubated for 5 min at 37°C and fractionated according to procedure II. Samples from the L fraction and the P fraction were layered on top of discontinuous sucrose gradients and centrifuged. Differences between the L and P fraction are shown in fig. 4.2. The L fraction showed one large peak of ^{125}I-Tf and ^{59}Fe activity at the 1.180 - 1.110 g/ml interface (peak 1) and a smaller shoulder at the 1.110 -1.080 g/ml interface (peak 2). The P fraction clearly showed two peaks; one at the 1.180 - 1.110 g/ml interface (peak 1) and the second at the 1.110 - 1.080 g/ml interface (peak 2). The buoyant densities of these fractions were determined with isopycnic sucrose gradients. Peak 1 in the L and P fraction had the same buoyant density of 1.133 g/ml (range 1.121 - 1.145 g/ml) and peak 2 also showed the same buoyant density of 1.105 g/ml (range 1.101 - 1.109 g/ml) in both fractions. These data are summarized in table 4.3.

Release of ^{125}I-Tf and ^{59}Fe from K 562 cells by pronase digestion after 0, 3 and 5 minutes of endocytosis

	Time of endocytosis (minutes)		
Label	0	3	5
^{125}I-Tf	88 ± 4	53 ± 5	49 ± 7
^{59}Fe	83 ± 9	50 ± 9	36 ± 6

All values are expressed as percentage relative to the total activity (±SEM, n=4). Recoveries of radioactivity were about 95 %.

Table 4.1 *Cells were incubated for 60 min with double lablled transferrin (0.1 mg/10^6 cells) at 4°C. Unbound transferrin was removed by four washes in ice-cold PBS. Cells were incubated for 3 or 5 minutes at 37°C to allow endocytosis, followed by rapid cooling or were kept at 4°C for 30 minutes using 0.1 % pronase in DMEM without BSA. Digestion was terminated by three washes with ice-cold DMEM/0.1% BSA/1.0% Aprotinin.*

2.2. Are isolated endosomes still intact functional vesicles?

To answer this question two types of experiments were performed. In the first cells were incubated with 0.1 % pronase after endocytosis, to remove non-internalized ^{125}I-Tf^{59}Fe from the plasma membrane. After 5 min of endocytosis 49 % of ^{125}I-Tf and 36 % of ^{59}Fe could be removed from the plasma membrane (see table 4.1). Subsequently, endosomes were isolated according to procedure I. Heavy endosomes (Density: 1.051 0.002 g/ml on the Percoll gradient) were chromatographed over Sephacryl S-1000. Endosomes eluting near the columns void volume were incubated for 30 min at 37°C with 0.1 % pronase to determine whether ^{125}I-Tf and/or ^{59}Fe were sensitive to digestion. The results are presented in table 4.4.

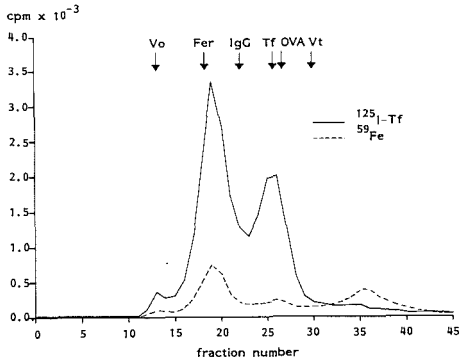

Fig. 4.1. *Endosomes were isolated according to procedure I and suspended in 10 mM Hepes (pH 6.5), 0.25 M sucrose, 0.5 % Triton X-100 final concentration. Gelfiltration was performed on a 2.5 x 40 cm AcA 34 column at a flow rate of 11 ml/hr. Fractions of 3 ml were counted. Displayed are: Fer = ferritin (450,000 D); IgG = (160,000 D); Tf = diferric transferrin (80,000 D); OVA = ovalbumin (45,000 D).*

The functioning of isolated endosomes was tested by measuring the ability of endosomes for ATP/Mg^{2+} dependent acidification of their interior, using the acridine orange fluorescence quenching technique (Stone et al., 1983; Lee and Forte, 1978; Gluck et al. 1982; Galloway et al., 1983) as shown in fig. 3. Quenching reached an equilibrium 10 min after addition of ATP. Addition of extra ATP at equilibrium did not alter the observed quenching, indicating that the steady state was not due to a lack of ATP. The fluorescence reversed imme-

Distribution of ^{125}I-Tf and ^{59}Fe over the LP and S fraction at early stages of endocytosis

Label	fraction	Time of endocytosis (minutes) 0 (n=3)	3 (n=3)	5 (n=4)
^{125}I-Tf	LP	38.7 ± 3.8	42.6 ± 1.0	43.5 ± 9.0
	S	16.6 ± 0.6	13.0 ± 2.9	16.7 ± 3.5
^{59}Fe	LP	42.4 ± 4.9	31.8 ± 4.1	22.8 ± 1.7
	S	13.0 ± 1.5	27.0 ± 2.8	38.0 ± 5.6

All values are expressed as percentage relative to the homogenate (± SEM). Recoveries ranged from 80-95 %.

Table 4.2 *Cells were incubated for 1 hour at 4°C with ^{125}I-Tf ^{59}Fe, followed by four washed with ice-cold PBS to remove unbound ^{125}I-Tf ^{59}Fe. Endocytosis was allowed by quickly warming the cells to 37°C for the indicated times. Endocytosis was stopped by adding 10 ml ice-cold DMEM/0.1 % BSA and swirling the vessels in melting ice. Alternatively cells were processed without incubation at 37°C (0 minutes). Subcellular fractionation was performed according to a modified De Duve procedure. Fractions were couted for ^{125}I-Tf and ^{59}Fe activity with appropriate spill-over corrections for 59Fe in the ^{125}I channel. Data from L and P fractions were taken together (see text).*

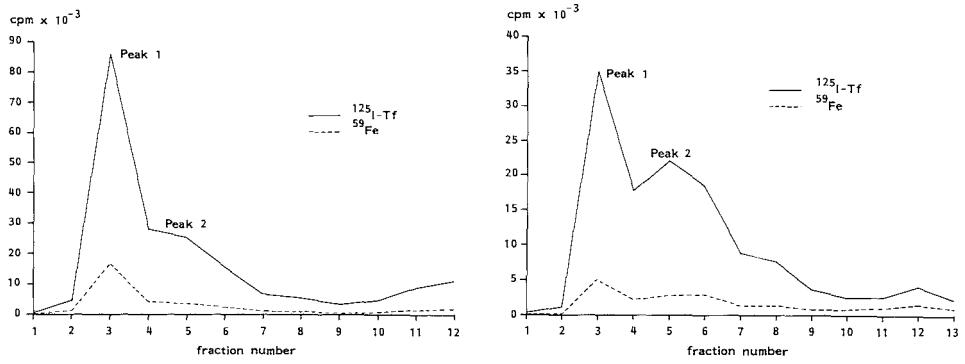

Fig. 4.2 A,B. *Samples from the L and P fraction derived from endosome isolation procedure II were layered on discontinuous gradients of 41, 26.5, 20, 15, and 10.5 % sucrose (w/v) in 10 mM Hepes pH 7.2, 1 mM DTT and centrifuged for 3 hr at 27,000 rpm in a Beckman SW 27 rotor or 70 min at 40,000 in a Beckman SW 40 rotor. The gradients were fractionated from the bottom and 2 ml respectively 1 ml fractions were collected and counted for ^{125}I-TF and ^{59}Fe activity.* **A**: *(left) L fraction;* **B**: *(right) P fraction.*

diately to al level slightly above that of the original after addition of 5 µl 1 mM NH_4Cl. NH_4Cl is known to neutralize the proton gradient.

The finding that acidification of the endosomal interior and the release of 59Fe from the endosomes are very early processes in the endocytic pathway raised the question whether acidification alone would be the mechanism responsible for iron release from endosomes. Endosomes were incubated with two concentrations of ATP/Mg^{2+} or with PIH (table 4.5 A). In the second set of experiments endosomes were incubated with ATP/Mg^{2+} and

Average densities of isolated endosome fractions

	Percoll gradient	isopycnic sucrose gradient
Heavy endosomes	1.051 ± 0.002	1.133 ± 0.012
Light endosomes	1.042 ± 0.002	1.105 ± 0.004

Values are expressed in g/ml (± SEM; n = 4)

Table 4.3 *Average buoyant densities of pooled fractions containing heavy and light endosomes separated by Percoll and isopycnic sucrose gradients. Densities in the Percoll gradient were determined with Density Marker Beads, in the sucrose gradients they were deter-*

PIH (table 4.5 B). Two types of blank experiments were performed; one containing 1 ml endosomes suspension and 2 ml buffer only, the second containing 1 ml endosome suspension and 2 ml pronase solution at a final concentration of 0.1 %. In the first blank experiment 94.5 ± 1.6 % of ^{125}I-Tf and 87.7 ± 0.3 % of ^{59}Fe was found in the pellet. In the pronase digestion experiments 88.4 ± 3.0 % of ^{125}I-Tf and 84.0 ± 1.7 % of ^{59}Fe was found in the pellet. Incubation of endosomes with 0.05 mM or 1.0 mM ATP showed only a slight increase in ^{59}Fe activity in the supernatant, while incubation with the iron chelator PIH led to a large increase in the release of iron from the endosomes. However, in both cases ^{125}I-Tf was found in the endosome pellet. When endosomes were incubated with 1.0 mM PIH and 2.4 mM ATP release of ^{59}Fe was less than when incubated with 1.5 mM PIH alone. After incubation of endosomes with 1.5 mM of both ATP and PIH release of ^{59}Fe seemed increased, but at the same time there was an increase in ^{125}I-Tf activity in the supernatant. When results were corrected for the blank experiments, there was no significant difference between the two incubation experiments.

3. DISCUSSION

Cells take up iron from ferric transferrin at 37°C by receptor mediated endocytosis (Van Renswoude et al. 1982; Klausner et al., 1982; Iacopetta and Morgan, 1983; Dickson et al. 1983 a,b; Rao et al, 1983; van der Heul et al., 1984). It has been shown that transferrin is recycled intact to the extracelular medium after donation of its iron to the cell (Karin and Mintz, 1981). There is evidence that endosomes are equipped with an ATP/Mg^{2+} dependent mechanism to acidify their interior, a condition suitable, if not a prerequisite for the liberation of iron from transferrin (Tycko and Maxfield, 1982; Galloway et al., 1983; Van Renswoude et al., 1983; Ciechanover et al.,1983; Yamashiro et al. 1984). Morphologic and biochemical evidence has been provided for the presence of transferrin in endosomes (receptosomes) of KB cells and reticulocytes within 5 min after warming up to 37°C (Willingham and Pastan, 1982; Dickson et al., 1983 b; Harding et al., 1983). However, it is not known at which stage in the transferrin cycle iron is released from endosomes and by what mechanism or carrier this is accomplished. Our pronase digestion experiments confirm a rapid internalization of transferrin and iron at 37°C. These results with regard to ^{125}I-Tf are comparable to those of Klausner and Harding using acid wash or proteolytic digestion (Klausner et al., 1983; Harding et al., 1984). After 3 min at 37°C already 50 % ^{125}I-Tf and ^{59}Fe was inaccessible for pronase digestion and therefore no longer present at the plasma membrane. The fact that after 5 min of endocytosis more ^{125}I-Tf than ^{59}Fe can be removed, means that already some ^{125}I-Tf has recycled, while ^{59}Fe has accumulated in the cell. We routine-

Percentage of activity found in pellet and supernatant after pronase digestion of endosomes.

Label	fraction	blank	pronase
^{125}I-Tf	Pellet	94.5 ± 1.6	88.4 ± 3.0
	Supernatant	5.5 ± 1.6	84.0 ± 1.7
^{59}Fe	Pellet	87.7 ± 0.3	84.0 ± 1.7
	Supernatant	12.4 ± 0.3	16.0 ± 1.7

Table 4.4 *Endosomes were incubated with or without 0.1 % pronase at 37°C. Incubation was stopped by cooling to 4°C and endosomes and supernatant were separated by centrifugation for 1 hour at 100,000 g and 4°C. Radioactivity was determined in pellet and supernatant.*

ly used pronase digestion after endocytosis to minimalize the contribution of contaminating plasma membrane vesicles containing ^{125}I-Tf^{59}Fe.

Subcellular fractionation by differential centrifugation showed a rapid decrease of ^{59}Fe activity with respect to ^{125}I-Tf from the combined endosome containing fractions (LP) to the supernatant (S) fraction. In incubation experiments performed at 4°C (t=0) ^{59}Fe and ^{125}I-Tf were not separated from each other as indicated by an ^{125}I/^{59}Fe ratio close to or slightly lower than that of the corresponding homogenate. Similar results were obtained by Rao (Rao et al., 1983) using K 562 cells and Triton X-100 solubilized cell extracts. Even though there is some degree of synchronization in the formation of endosomes by the sudden temperature rise, there will be a difference in the ^{125}I/^{59}Fe ratio of the first formed endosomes and endosomes formed just prior to the cooling. The ^{125}I/^{59}Fe ratio measured in the endosome fraction after 3 and 5 min incubation at 37°C will therefore be an average value of all stages. These results suggest that iron release from endosomes is an early event in the transferrin endocytic cycle.

Differential centrifugation and sucrose gradient centrifugation experiments demonstrated that two vesicle populations were present in the L and P fraction (peak 1 and 2). However, these endosome populations were incompletely separated from each other by differential centrifugation. The largest part of peak 1 is found in the L fraction together with a small part of peak 2, while the bulk of peak 2 and part of peak 1 are found in the P fraction. As mentioned in section 3 there was considerable variation in the distribution of the two endosome populations over the L and P fraction.

Comparing the results of the Percoll gradient purification of endosomes after 5 min of endocytosis with the differential centrifugation and sucrose gradient purification of endosomes both, it appeared that in both, the ^{125}I/^{59}Fe ratio of the heavy endosome fraction (1.051 g/ml, Percoll gradient; 1.133 g/ml sucrose gradient) was greater than that of the light endo-

Release of iron from isolated endosomes after incubation with ATP and/or PIH								
(A)	Blank		0.05 mM ATP		1.0 mM ATP		1.5 mM PIH	
	^{125}I-Tf	^{59}Fe	^{125}I-Tf	^{59}Fe	^{125}I-Tf	^{59}Fe	^{125}I-Tf	^{59}Fe
Endosomes	99-95	97-95	97	89	91	86	9	25
Supernatant	1-5	3-5	3	11	9	14	7	74

(B)	Blank		1.0 mM PIH 2.4 mM ATP		1.5 mM PIH 1.5 mM ATP	
	^{125}I-Tf	^{59}Fe	^{125}I-Tf	^{59}Fe	^{125}I-Tf	^{59}Fe
Endosomes	96	88	98	35	86	3
Supernatant	4	12	2	64	14	97

All values are expressed as percentage of the sum of the recovered activity in pellet and supernatant.

Table 4.5 A,B. *Heavy endosomes were isolated according to procedure I and incubated for 30 minutes at 37°C with buffer (blank), ATP or PIH (**A**) or with ATP and PIH (**B**) at the indicated concentration. Incubation was stopped by cooling on ice. Endosomes and supernatant were separated by 1 hour centrifugation at 100,000 g and 4°C. Radioactivity was determined in the pellet and supernatant.*

some fraction. Also in absolute quantities the heavy endosome fraction contained more ^{125}I-Tf than the light endosome fraction.

Fluorescence quenching measurements of the heavy endosome fraction, having low lysosomal enzyme activity, showed these isolated endosomes to be capable of ATP dependent acidification of their interior. The heavy endosome fraction, however, was not devoid of Golgi material as indicated by the presence of galactosyltransferase. After the discovery of the prelysosomal acidic compartment it became clear that an acid interior was not an exclusive lysosomal property, but also present in coated vesicles and endosomes (Schneider et al., 1981; Okhuma et al., 1982). The proton pumping ATPases of these organelles have much in common. They are all N-ethylmaleimide sensitive, but can not be inhibited by inhibitors of the mitochondrial ATPase and Na^+/K^+ ATPase (Galloway et al., 1983). To our knowledge no specific inhibitor has been found that discriminates between the lysosomal and prelysosomal (coated vesicle and endosomal) ATPase.

Endosome fractions with similar density properties were also isolated and characterized from total rat liver by Evans (Evans et al., 1985 a,b). In rat liver endosomes there was a shift of ^{125}I-asialotransferrin between 1-2 min and 10 min after in vivo injection from heavy endosomes (Evans notation: DN-2; 1.12 - 1.13 g/ml in sucrose gradients) to light endosomes (DN-1; 1.07 - 1.12 g/ml) 10 min after injection. Gelfiltration experiments of solubilized endosomes (fig. 4.1) demonstrated that beside transferrin-transferrin receptor complexes they contained free transferrin and low molecular weight iron. The free transferrin found possibly represents apotransferrin that has lost its affinity for the receptor during fractionation and chromatography (pH 6.8), and at the same time regained affinity to bind iron. About 30 % of ^{59}Fe was found at molecular weight smaller than 20,000 D. It has not yet been possible to further characterize this low molecular fraction, due to its limited quantity. It does show, however, that conditions during the short time of endocytosis favoured the dissociation of ^{59}Fe from transferrin. The fact that, evenso, about 30 % of ^{59}Fe was actually present in a low molecular weight form, could mean that the release of iron from the endosome is the rate limiting step in the transport of iron from endosome to cytosol and not the liberation of ^{59}Fe from transferrin. When endosomes were incubated with ATP/Mg^{2+} at 37°C there was no significant release of ^{59}Fe to the supernatant. However, incubation of endosomes with the iron chelator PIH led to a release of ^{59}Fe to the supernatant. Ponka recently showed PIH to chelate both Fe(II) and Fe(III) in an 2:1 ratio (PIH:Fe). The $Fe(PIH)_2$ complexes were able to cross the reticulocyte plasma membrane (Ponka et al., 1984). Incubation of ATP and PIH did not lead to a further increase in the release of iron from endosomes. The conclusion can be that ATP dependent acidification alone is not enough to release iron from isolated endosomes. PIH is able to release ^{59}Fe from endosomes probably by chelating the low molecular weight iron pool inside endosomes.

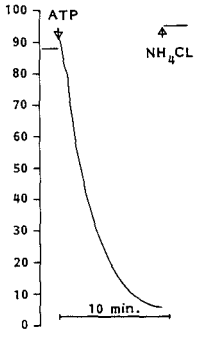

Fig. 4.4 *Heavy endosomes, isolated according to procedure I were tested for their ability to acidify their interior by measuring the quenching of acridine orange fluorescence. After recording the baseline of the incubation buffer (see chapter 2) for 1 minute, measuremens were started by addition of 10 µl of 1.5 M ATP (pH 7.2). Equilibrium was reached within 10 min after addition of ATP. To check whether the measured quenching was indeed caused by a proton gradient over the endosomal membrane, 5 µl of 1.0 M NH_4Cl was added to reverse the proton gradient. The fluorescence intensity is given in arbitrary units.*

REFERENCES

Anderson R.G., M.S. Brown and J.L. Goldstein (1977) Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein. Cell **10**: 2434-2438.

Bakkeren D.L., C.M.H. de Jeu-Jaspars, C. van der Heul, H.G. van Eijk. (1984) Analysis of iron-binding components in the low molecular weight fraction of rat reticulocyte cytosol. Int. J. Biochem. **17**: 925-930.

Ciechanover A., A.L. Schwartz, A. Dautry-Varsat and H.F. Lodish. (1983) Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. J. Biol. Chem. **258**: 9681-9689.

Dales S. (1973) Early events in cell-animal virus interactions.Bacteriol. Rev. **37**: 103-135.

Dickson R.B., J.A. Hanover, M.C. Willingham and I. Pastan (1983a) Prelysosomal divergence of transferrin and epidermal growth factor during receptor mediated endocytosis. Biochem. **22**: 5667-5674.

Dickson R.B., L. Beguinot, J.A. Hanover, N.D. Richert, M.C. Willingham and I. Pastan (1983b) Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. Proc. Natl. Acad. Sci U.S.A. **80**: 5335-5339.

Duve C. de, B.C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans (1955) Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J., **60**: 604-617.

Evans W.H. and N. Flint (1985a) Subfractiona-tion of hepatic endosomes in Nycodenz gradients and by free-flow electrophoresis: Separation of ligand-transporting and receptor-enriched membranes. Biochem. J. **232**: 25-32.

Evans W.H. and W.G.M. Hardison (1985b) Phospholipid, cholesterol, polypeptide and glycoprotein composition of hepatic endosome subfractions. Biochem. J. **232**: 33-36.

Galloway C.J., G.E. Dean, M. Marsh, G. Rudnick and I. Mellman (1983) Acidification of macrophage and fibroblast endocytic vesicles in vitro. Proc. Natl. Acad. Sci. U.S.A. **80**: 3334-3338.

Gluck S., S. Kelly and Q. Al-Awquati (1982) The proton translocating ATPase responsible for urinary acidification. J. Biol. Chem. **257**: 9230-9233.

Hanover J.A. and R.B. Dickson (1985) Transferrin: receptor-mediated endocytosis and iron delivery. In: Endocytosis. I. Pastan and M.C. Willingham editors Plenum Press NY, 341-387.

Harding C., J. Heuser and Ph. Stahl (1983) Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J. Cell Biol. **97**: 329-339.

Harding C., J. Heuser and Ph. Stahl (1984) Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. Eur J. Cell Biol. **35**: 256-263.

Helenius A. and M. Marsh (1982) Endocytosis of enveloped animal viruses. Ciba Found. Symp. **92**: 59-76.

Heul C. van der, A. Veldman, M.J. Kroos, H.G. van Eijk (1984) Two mechanisms are involved in the process of iron-uptake by rat reticulocytes. Int. J. Biochem. **16**: 383-389.

Iacopetta B. and E.H. Morgan (1983) An electron-microscope study of transferrin endocytosis by immature erythoid cells. Eur. J. Cell Biol. **32**: 17-23.

Keen J.H., F.R. Maxfield, M.C. Hardegree and W.H. Habig (1982) Receptor-mediated endocytosis of diphtheria toxin by cells in culture. Proc. Natl. Acad. Sci. U.S.A. **79**: 2912-2916.

Karin M. and B. Mintz (1981) Receptor-mediated endocytosis of transferrin in developed totipotent mouse teratocarcinoma cells. J. Biol. Chem. **256**: 3245-3252.

Klausner R.D., J. van Renswoude, G. Ashwell, C. Kempf, A.N. Schechter, A. Dean and K.R. Bridges (1983) Receptor-mediated endocytosis of transferrin in K 562 cells. J. Biol. Chem. **258**: 4715-4724.

Lee H.C. and J.G. Forte (1978) study of H+ transport in gastric microsomal vesicles using fluorescent probes. Biochim. Biophys. Acta **508**: 339-356.

Morgan E.H. and Appleton T.C. (1969) Autoradiogrphic localization of ^{125}I-labeled transferrin in rabbit reticulocytes. Nature **223**: 1371-1372.

Okhuma S., Y. Moriyami and T. Takano (1982) Identification and characterization of a proton pump on lysosomes by fluorescein isothiocyanate-dextran fluorescence. Proc. Natl. Acad. Sci. USA **79**: 2758-2762.

Ponka P., R.W. Grady, A. Wilczynska and H.M. Schulman (1984) The effect of various che-lating agents on the mobilization of iron from reticulocytes in the presence and absence of pyridoxal isonicotinoyl hydrazone. Biochim. Biophys. Acta **802**: 477-489.

Rao K., J. van Renswoude, C. Kempf, R.D. Klausner (1983) Separation of Fe^{3+} from transferrin in endocytosis. FEBS lett. **160**: 213-216.

Renswoude J. van, K.R. Bridges, J.B. Harford and R.D. Klausner (1982) Receptor mediated endocytosis of transferrin and the uptake of Fe in K 562 cells: Identification of a nonlysosomal acidic compartment. Proc. Natl. Acad. Sci. USA **79**: 6186-6190.

Schneider D. (1981) ATP-dependent acidification of intact and disrupted lysosomes. J. Biol. Chem. **256**: 3858-3864.

Stone D.K., X. Xie, E. Racker (1983) An ATP-driven proton pump in clathrin coated vesicles. J. Biol. Chem. **258**: 4059-4062.

Tycko B. and F.R. Maxfield (1982) Rapid acid-ification of endocytic vesicles containing α_2-macroglobulin. Cell **28**, 643-651.

Willingham M.C. and I. Pastan (1982) The transit of epidermal growth factor through coated pits of the Golgi. J. Cell Biol. **94**: 207-212.

Yamashiro D.J., B. Tycko, S.R. Fuss, F.R. Maxfield (1984) Segragation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. Cell **37**: 789-800.

CHAPTER 5

CHARACTERISTICS OF IRON RELEASE FROM ISOLATED HEAVY AND LIGHT ENDOSOMES

Chapter 5

1. INTRODUCTION

The receptor-mediated uptake of ferric-transferrin is the major pathway by which erythroid cells acquire iron *in vivo* (Hemmaplard and Morgan, 1973, 1977; Karin and Mintz, 1981; van Renswoude et al., 1982). The first step in receptor-mediated uptake is the binding of transferrin to the transferrin receptors present on the plasma membrane. The transferrin receptors are clustered in coated pits, areas of the plasma membrane specialized to internalize extracellular ligands according to the cells needs (Lamb et al., 1983; Iacopetta and Morgan, 1983; Hopkins and Trowbridge, 1983; Hopkins, 1985; Dickson et al, 1983). The coated pits are pulled from the cell surface into the cytoplasm through assembly of the clathrin coat. The resulting coated vesicles rapidly loose their clathrin coat and become primary endosomes (Pearse, 1976; Heuser, 1980). Primary endosomes travel along tracks of microtubuli to the perinuclear area where they fuse with an intracellular tubulo-vesicular system called CURL (compartment for uncoupling of ligand and receptor) or transreticular Golgi in which ligands are uncoupled from there receptors at low pH (Geuze et al., 1983; Willingham et al., 1984). Coated vesicles, endosomes and CURL generate a low intravesicular pH (pH 5.0-5.7) by proton pumping Mg^{2+}-dependent ATPases present in all these membrane vesicles, as well as in lysosomes (Schneider, 1981; Tycko and Maxfield; 1982; Galloway et al, 1983; Stone et al., 1983; Veldman et al., 1986; Lake et al., 1987). In the CURL sorting takes place according to the different intracellular destinations of the ligands, since coated pits do not select ligands (Blight and Morgan, 1983; Courtoy et al., 1985). The determinants that effect the sorting of ligands are not yet known, but they are most probably receptor determinants. Vesicle acidification could be important for iron release from transferrin, as, *in vitro*, iron binding to transferrin is highly pH dependent. At pH 5.0 no iron remains bound to transferrin in the presence of other iron acceptors. Though most ligands dissociate from their receptor at low pH, apotransferrin binds more strongly to its receptor at pH 5.0 (Klausner et al., 1983). Apotransferrin is recycled to the medium (pH 7.4), where it rapidly exchanges with more the avid binding species diferric transferrin. It is obvious that the primary interest of cells focuses on iron, not on transferrin, although transferrin might also play a role as maturation factor (Evans et al., 1986). In a previous study we have shown that iron release from transferrin is an early step in the transferrin cycle that starts as soon as the coated pit has been internalized (Bakkeren et al., 1987). Presumably iron release continues in subsequent organelles along the transferrin endocytic pathway. Eventually, iron released from transferrin has to be transported through the endosomal membrane, through the cytosol, to ferritin for storage or to mitochondria for heme synthesis. Many investigators report that at acidic pH iron is released from endosomes to the cytosol, though both the mechanism of this iron transport and the intracellular iron acceptor are completely unknown.

The aim of the present study is to provide insight in the characteristics of endosomal iron release. We describe the isolation and purification of heavy and light endosomes from K 562 cells. In addition we present the results of *in vitro* incubations of isolated heavy and light endosomes with iron chelators.

2. RESULTS

Cells labelled with ^{125}I-Tf^{59}Fe were allowed 3 minutes of endocytosis at 37°C. Differential centrifugation of the homogenates generated by disruption of these cells demonstrated ^{125}I-Tf and ^{59}Fe to be present predominantly in the LP fraction, though not to the same extent. Clearly some iron has already been separated from transferrin (table 5.1).

The presence of transferrin in two endosome populations isolated by fast discontinuous sucrose gradient centrifugation revealed as early as 3 minutes after the onset of endocytosis. Both endosome fractions had similar characteristics as the fractions isolated by Percoll gradient centrifugation and equilibrium centrifugation using continuous sucrose gradients described in a previous study (Bakkeren et al., 1987). The present procedure is far less time consuming and separation was improved. Heavy endosomes were found at a sucrose density of 1.133 g/ml (range 1.121-1.145 g/ml), light endosomes were found at sucrose density of 1.105 g/ml (range 1.101-1.109 g/ml, see fig 5.1). In table 5.2 the distribution of ^{125}I-Tf and ^{59}Fe in the heavy and light endosome fraction is presented.

Distribution of transferrin and iron over the fractions derived by differential centrifugation after 3 minutes endocytosis.

	^{125}I-Transferrin	^{59}Fe
N fraction	3.2 ±0.8	8.4 ±5.2
M fraction	12.6 ±4.9	17.8 ±3.2
LP fraction	70.3 ±8.0	57.3 ±8.3
S fraction	13.9 ±5.9	16.6 ±9.1

All values are expressed as percentage of the total recovered activity (± S.D.; n=8)

Table 5.1 *Cells were labelled, washed and incubated at 37°C as described in chapter 2. Cells were disrupted by cavitation (30 min at 200 psi and 4°C) followed by 10 strokes in Dounce homogenizer. The N or nuclear fraction was the pellet derived after 5 min centrifugation at 4000 rpm in a Beckman JA-20 rotor with two washes of the pellet. The M (mitochondrial) fraction was derived after 5 min centrifugation at 11,000 rpm with one wash of the pellet. The LP (light mitochondrial/particulate) fraction was the pellet and the S (final supernatant) fraction was the supernatant derived after 47 min centrifugation at 40,000 rpm in Beckman Ti 60 rotor.*

2.1. Change in $^{125}I/^{59}Fe$ ratio of isolated endosomes relative to the transferrin used to label the cells

We determined the change in the $^{125}I/^{59}Fe$ ratio of the heavy and light endosome fraction relative to the $^{125}I/^{59}Fe$ ratio of the transferrin used for labelling the cells, 3 min after the onset of endocytosis of a single cohort of receptor-bound transferrin (table 5.3). The average $^{125}I/^{59}Fe$ ratio of the heavy endosome fractions was 4.4 % ± 2.9 % (n=4) higher than the $^{125}I/^{59}Fe$ ratio of the transferrin used for labelling the cells, meaning some iron has already been released from these endosomes. The average $^{125}I/^{59}Fe$ ratio of the light endosomal fractions, on the contrary, was 25.1% ± 3.9% (n=4) lower than the $^{125}I/^{59}Fe$ ratio of the transferrin used for labelling the cells.

Change in the endosomal $^{125}I/^{59}Fe$ ratio relative to that of the transferrin used for labelling.

Heavy endosomes	+ 4.4 % ± 2.9 %
Light endosomes	− 25.1 % ± 3.9 %

All values are expresssed as the percentage change (± S.D.; n=4).

Table 5.2 *The preparation of the endosomal fractions is described in the legends of table I and II. The average $^{125}I/^{59}Fe$ ratio of the pooled fractions of heavy and light endosomes were compared with the $^{125}I/^{59}Fe$ ratios of the transferrins used for labelling the cells.*

Distribution of transferrin and iron in heavy and light endosomes after 3 minutes endocytosis.

	^{125}I-Tf	^{59}Fe
Heavy endosomes	40.0 ± 9.2	29.5 ± 5.7
Light endosomes	33.2 ± 5.8	34.9 ± 6.4

All values are expressed as percentage of the total recovered activity (± S.D.; n=8)

Table 5.3*The preceding steps are descrbed in the legend of table 5.1. The LP fraction was separated on a discontiuous sucrose gradient consisting of 1 ml 66 %, 2.5 ml 45 %, 2.5 ml 33 %, 2.5 ml 27 % and 2.5 ml 11 % sucrose in 1 ml Hepes pH 7.4. After fractionation of the gradient all fractions were counted and corresponding fractions were pooled giving a heavy and light endosome fraction(fig.5.1). The $^{125}I/^{59}Fe$ ratio of the isolated fractions was compared with the $^{125}I/^{59}Fe$ ratio of the transferrin used for labelling the cells.*

The interpretation of these results could be that transferrin as well as iron may be released from these endosomes (which are thought to be part of the tubulo-vesicular part of the endosomal system (CURL/transGolgi). From the significant change in the $^{125}I/^{59}Fe$ ratio it seems that more (apo)transferrin than iron is released. Transferrin is known to recycle as apotransferrin from the tubulo-vesicular endosomes to the plasma membrane.

2.2. Incubations with BPS

Heavy and light endosomes were incubated with BPS at a final concentration of 10 μM, with and without the redox couple formed by NADH/NAD$^+$ in a 10:1 ratio. This redox couple has a calculated potential of -0.33 V at pH 7.0 and 30°C. Though this potential is low, it is not sufficient to reduce Fe^{3+}-transferrin to Fe^{2+}-transferrin, which requires a potential of -

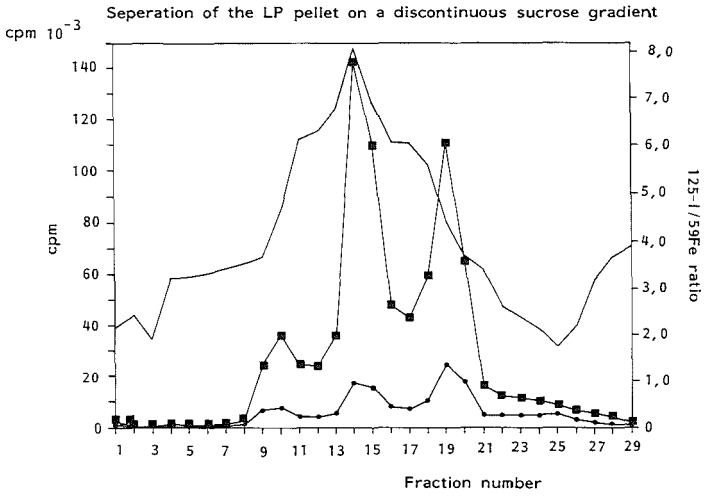

Fig. 5.1 *The LP fraction prepared by differential centrifugation was resuspended in minimal volume of cold incubation solution and counted. It was applied on two identical discontinuous gradients (see table 5.2). The gradients were centrifuged in a Beckman SW 40 rotor for 1 hour at 40.000 rpm (6.6 x 10 ^{10} rad ^{2}/s) and 4°C. The gradients were unloaded from the bottom and 0.5 ml fractions were collected and couted for ^{125}I and ^{59}Fe. ■ = ^{125}I; • = ^{59}Fe; —— = ^{125}I/ ^{59}Fe ratio.*

0.40 V at pH 7.3 and 25°C (equal to -0.418 V at pH 7.0 and 30°C) (Harris et al., 1985). The reason for investigating the influence of a reductive couple was to determine whether there is a period during endosomal iron release, in which an iron intermediate is sensitive to reduction. It could be that, apart from acidification which removes iron from transferrin, reduction plays an additional role in iron release from endosomes.

In the presence of the redox couple NADH/NAD$^+$, the maximum ATP specific iron release from light endosomes to BPS is only 2.5 %, which is reached within 10 min. Maximal ATP

specific iron release from heavy endosomes to BPS is 6 % after 20 min (fig. 5.2A). Incubation of heavy and light endosomes with BPS, but without NADH/NAD^+ lead to ATP specific iron release to BPS of 6.5 % for both heavy and light endosomes after 20 - 30 min respectively (fig. 5.2B). This result indicates that the NADH/NAD^+ redox couple has a negative influence on ATP specific iron release to BPS especially in light endosomes (fig. 5.2C). Heavy endosomes are influenced only slightly by NADH/NAD^+.

2.3. Incubations with PIH

Identical experiments were carried out with PIH instead of BPS. With light endosomes maximal ATP specific iron release to PIH was 4 % in the presence of the redox couple NADH/NAD^+. This was reached within 30 min (fig. 5.3A). Using heavy endosomes the ATP specific iron release to PIH was 7.5 % in 20 min. The same experiments without NADH/NAD^+ showed a maximal ATP specific iron release of 7.5 % from light endosomes after 20 min while iron release from heavy endosomes increased to 11 % after 80 min (fig. 5.3B). The result of these experiments also showed the NADH/NAD^+ redox couple reduced endosomal iron release from light endosomes to half, while from heavy endosomes only two-third of the iron was released (fig. 5.3C).

2.4. Incubations with PIH and NEM

In another set of experiments we used the drug N-ethylmaleimide in combination with PIH. In *in vivo* experiments N-ethylmaleimide has been shown to inhibit the lysosomal and endosomal proton pumping ATPase, thereby reducing the proton gradient between vesicle and cytosol (Galloway et al., 1983). After a 5 min preincubation period of the endosome suspensions with NEM (at 30°C), ATP and PIH were added to the incubation mixtures and the first samples taken. The blank experiments contained only PIH in addition to the endosomes. The experiments containing light endosomes showed maximal ATP specific iron release approximately 4 % after 20 min, while in the experiments containing the heavy endosomes maximal ATP specific iron release was reached (about 8.5 %) up to 80 min (fig. 5.4A). Comparing these data with those obtained with PIH in the absence of NEM (fig. 5.3C) it can be seen that NEM reduces ATP specific iron release to 2 % in heavy endosomes and up to 4 % in light endosomes (fig. 5.4B).

2.5. Incubations with lactate

In similar experiments also employed the potential physiological iron chelator lactate. Incubations were carried out without NADH/NAD^+. However, no significant ATP specific iron release was observed. The result demonstrates that lactate is not able to enhance endosomal iron release by acting as a endogenous iron acceptor (fig. 5.5)

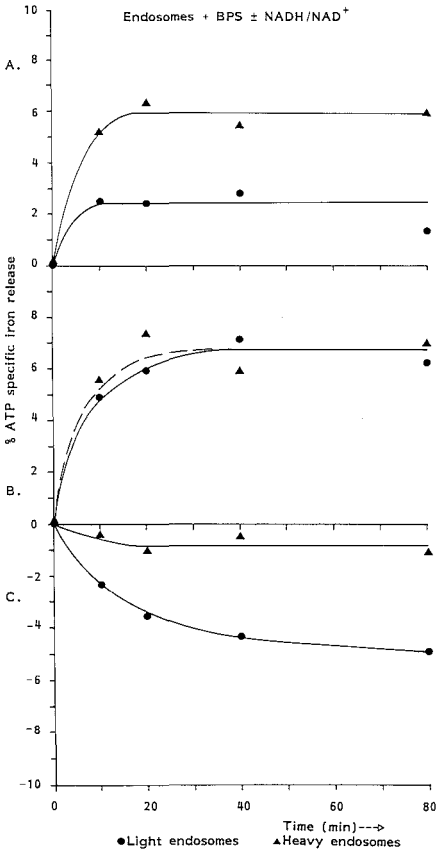

Fig. 5.2 *Endosomes were incubated with 10mM BPS with/ without a redox couple of 1.0/0.1 mM NADH/NAD$^+$ and with/without 4 mM ATP. At the indicated times duplicate samples of 1.0 ml were taken and diluted in 9.0 ml ice-cold incubation solution containing 100 ml 1.0 M NH_4Cl. Endosomes and supernatants were separated by ultracentrifugation as described in chapter 2.* **A***: Heavy and light endosomes with BPS and NADH/NAD$^+$;* **B***: Heavy and light endosomes with BPS , without NADH/NAD$^+$.* **C***: Resultant: influence of NADH/NAD$^+$ redox couple on ATP specific iron release to BPS from heavy and light endosomes.*

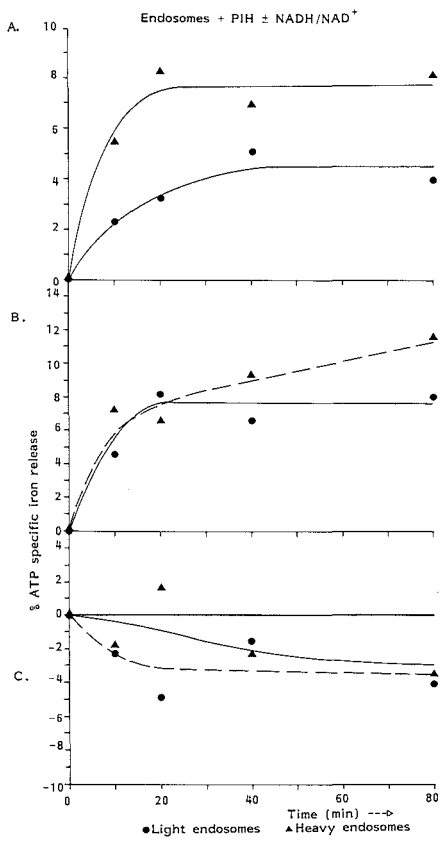

Fig. 5.3 **A***: Heavy and light endosomes with PIH and NADH/NAD$^+$.* **B***: Heavy and light endosomes with PIH, without NADH/NAD$^+$.* **C***: Resultant: influence of NADH/NAD$^+$ redox couple on ATP specific iron release to PIH from heavy and light endosomes. Data from blank experiments (without ATP) were subtracted from ATP containing experiments. Data were normalised for iron release at t = 0 to be 0; proportionaliron release to the supernatant is corrected for the proportional release of transferrin from disrupted endosomes.*
The result s shown are the average of duplicate experiments.

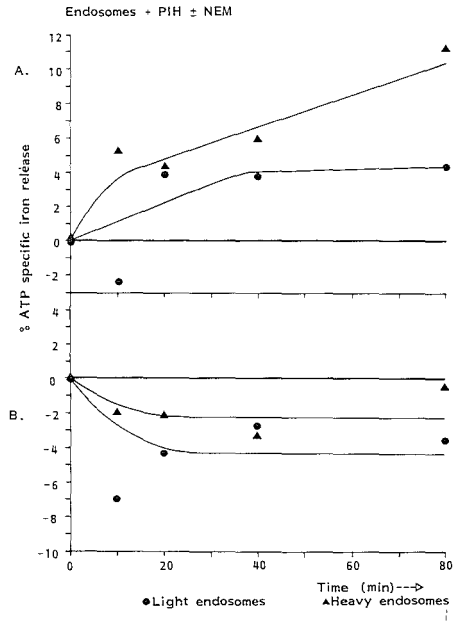

Fig. 5.4 *Endosomes were incubated with PIH with or without NEM.* **A**: *Net ATP specific iron release from heavy and light endosomes incubated with NEM.* **B**: *Influence of NEM; comparison of ATP specific iron release from heavy and light endosomes with PIH and NEM relative to ATP specific iron release with PIH alone.*

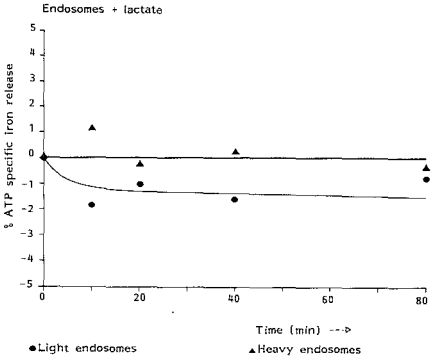

Fig. 5.5 *Lactate was suggested to be a major intermediate in placental iron transfer (Inoue et. al. 1987). Other cell types with high iron metabolism, such as develloping red cells and tumorcells, are known to have raised intracellular lactate concentrations because of their high aerobic glycolysis. Endosomes were incubated with lactate as potential physiological iron acceptor. Incubations were carried out without NADH/NAD$^+$.*

3. DISCUSSION

The results of the determination of the $^{125}I/^{59}Fe$ ratio in heavy and light endosomes sheds light on the dynamics of the endocytic cycle. As the $^{125}I/^{59}Fe$ ratio of the heavy endosome fraction was a little higher than that of the transferrins used for labelling the cells, some iron must have been released from the initial endosome population. The lower $^{125}I/^{59}Fe$ ratio of the light endosome fraction can be explained by a decrease in the amount of ^{125}I-Tf or an increase in the amount of ^{59}Fe. However, data from experiments at longer times during endocytosis suggest a decrease of both ^{125}I-Tf and ^{59}Fe in the light endosome fraction, while the decrease in ^{125}I-Tf activity was larger than that of the ^{59}Fe activity. For the process of

endocytosis this means that acidification and iron release start in the coated vesicle/primary endosome. However, heavy endosomes have only a very short lifetime ($t_{1/2} = \pm 2.5$ min) since they rapidly fuse with the tubulo-vesicular endosomal system (Oka and Weigel, 1983). From the tubulo-vesicular endosomal system vesicles containing apotransferrin coupled to the transferrin receptor pinch off. It appears that transferrin is released faster from the light endosomes than iron. Results from an earlier investigation suggested that iron transport through the endosomal membrane might be a rate limiting factor (Bakkeren et al., 1987).

Preliminary results indicated that incubation of endosomes with ATP alone does not lead to significant endosomal iron release, whereas in incubations with PIH as iron acceptor, iron was released to the supernatant (Bakkeren et al., 1987). We used purified heavy and light endosomes *in vitro* to determine what type of iron acceptors are able to increase endosomal iron release. Artificial chelators were used, because until now no unambiguous intracellular iron chelator has been identified. The chelators used were: bathophenantroline disulphonate; pyridoxal isonicotinoyl hydrazone and lactate.

It was shown, using the Fe(II) chelator BPS with NADH/NAD^+, that heavy endosomes proportionally released over two times more ^{59}Fe than light endosomes. However, omitting NADH/NAD^+ resulted in near equal iron release from heavy and light endosomes. Our conclusion is that the NADH/NAD^+ redox couple does not enhance Fe(II) release from heavy and light endosomes. On the contrary it showed NADH/NAD^+ was shown to reduce iron release especially from light endosomes. When heavy and light endosomes were incubated with PIH and NADH/NAD^+, the proportional ATP specific iron release from heavy endosomes was also about two times higher than the corresponding iron release from light endosomes. Omitting NADH/NAD^+ in these experiments also restored iron release from light endosomes to a level that was about two-third that of heavy endosomes.

With PIH as an iron acceptor there was an increase in iron was released from both heavy and light endosomes, in the presence of absence of NADH/NAD^+. However, as was most noticable with BPS, NADH/NAD^+ exerted a negative effect on iron release that was again more pronounced in the light endosomes than in the heavy endosomes population.

Experiments in which NEM was used showed a decrease in ATP specific iron release, again predominantly in the light endosomes. Contrary to the results found *in vivo* NEM does not produce total inhibition of endosomal iron release. There may be a number of reasons to explain this behaviour. Firstly, *in vivo*, heavy endosomes have only a short lifetime before they fuse with the tubulo-vesicular endosomal system. This implies that the amount of iron

that can be released from the heavy endosome compartment is limited. For unknown reasons the light endosome compartment may be more sensitive to perturbing drugs. The fact that a high NEM concentration does not completely inhibit iron release, could indicate that release of iron already removed from transferrin inside the endosome, continues even when the pH rises to the cytosolic pH. More investigations are necessary to answer the question.

In a recent article Inoue and co-workers (1987) suggested the iron-lactate chelate to be the major transit intermediate in placental iron transfer (Inoue et al., 1987). Developing red cells and tumor cells are known to have raised lactate concentrations because of their aerobic glycolysis (Lehninger, 1976). We found lactate not to be a suitable iron acceptor in this *in vitro* system. These experiments confirm our earlier observation that *in vitro* in the absence of a suitable acceptor the net ATP specific iron release is negligible and in the presence of ATP alone there is no net iron release (Bakkeren et al., 1987).

Our conclusions are that endosomal iron release is dependent both on acidification and the presence of a suitable iron acceptor. BPS and PIH are able to act as such iron acceptors, though to different extents. PIH is a better iron acceptor than BPS. The redox couple $NADH/NAD^+$ does not increase iron release from endosomes to the Fe(II) chelator BPS, as might be expected. On the contrary the $NADH/NAD^+$ redox couple reduces iron release to PIH and BPS. *in vitro* most iron was released from heavy endosomes to both BPS and PIH, though *in vivo* this might not be the case, due to the limited lifetime of heavy endosomes before fusing with the tubulo-vesicular light endosomes. Despite extensive investigations over the last twenty years, no consistent cytosolic iron transfer factor or acceptor has been found. The factors found were either not physiologically acceptable, or if they were, follow up reports on the isolates were lacking (Romslo, 1981). It is clear that more research has to be done to elucidate the mechanism of endosomal iron release and subsequent cytosol iron transport.

REFERENCES

Bakkeren D.L., C.M.H. de Jeu-Jaspars, M.J. Kroos and H.G. van Eijk (1987) Release of iron from endosomes is an early step in the transferrin cycle. Int. J. Biochem. **19**: 179-186.

Blight C.D. and E.H. Morgan (1983) Receptor-mediated uptake of ferritin by reticulocytes. Brit. J. Haematol. **55**: 59-71.

Bockxmeer F.M. van, and E.H. Morgan (1979) Transferrin receptors during rabbit reticulocyte maturation. Biochim. Biophys. Acta **584**: 76-83.

Courtoy P.J., J. Quintart, N. Limet, C. de Roe and P. Baudhuin (1985) Polymeric IgA and galactose-specific pathways in rat hepatocytes. In: Endocytosis, I. Pastan and M.C. Willingham eds. Plenum Press, New York. Chapt. 6, 163-188.

Dickson R.B., I. Beguinot, J.A. Hanover, N.D. Richert, M.C. Willingham and I. Pastan (1983) Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. Proc. Natl. Acad. Sci. **80**: 5335-5339

Evans W.H., S.M. Wilson and M.G. Mage (1986) Transferrin induces maturation of neutrophil granulocyte precursors in vitro. Leuk-emia Res. **10**: 429-436.

Galloway C.J., G.E. Dean, M. Marsh, G. Rudnick and I. Mellman (1983) Acidification of macrophage and fibroblast endocytic vesicles *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **80**: 3334-3338.

Geuze H.J., J.W. Slot, G.J. Lodish and A.L. Schwartz (1983) Intracellular site of asialoglycoprotein-ligand uncoupling: Double-label immunoelectron microscopy during receptor-mediated endocytosis. Cell **32**: 277-287.

Harris D.C., A.L. Rhinehart, D. Hereld, R.W. Schwartz, F. Burke and A.P. Salvador (1985) Reduction potential of iron in transferrin. Biochim. Biophys. Acta **838**: 295-301.

Hemmaplardh D. and E.H. Morgan (1977) The role of endocytosis in transferrin uptake by reticulocytes and bone marrow cells. Brit. J. Haematol. **36**: 85-96.

Hemmaplardh D. and E.H. Morgan (1974) Transferrin and iron uptake by human cell in culture. Exp. Cell Res. **87**: 207-212.

Heul C. van der, C.M.H. de Jeu-Jaspars, M.J. Kroos and H.G van Eijk (1978) Binding sites of iron transferrin on rat reticulocytes: inhibition by specific antibodies. Biochim. Biophys. Acta **511**: 430-441.

Heuser J.E. (1980) Three-dimensional visualization of coated vesicle formation in fibro-blasts. J. Cell Biol. **84**: 560-583.

Hopkins C.R. (1985) The appearance and internalization of transferrin receptors at the margins of spreading tumor cells. Cell **40**: 199-208.

Hopkins C.R., and I.S. Trowbridge (1983) Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. J. Cell Biol. **97**: 508-521.

Iacopetta B., and E.H. Morgan (1983) An electron microscope autoradiographic study of transferrin endocytosis by immature red cells. Eur. J. Cell Biol. **32**: 17-28.

Inoue T., N. Toh and E. Kimoto (1987) Studies on the major intermediate transit iron complex in the human placenta. Inorg. Chim. Acta **135**: 23-26.

Karin M. and B. Mintz (1981) Receptor-mediated endocytosis of transferrin in developed totipotent mouse teratocarcinoma cells. J. Biol. Chem. **257**: 5969-5976.

Klausner R.D., J. van Renswoude, G. Ashwell, C. Kempf, A.N. Schechter, A. Dean and K.R. Bridges (1983) Receptor-mediated endocytosis of transferrin in K 562 cells. J. Biol. Chem. **258**: 4715-4724.

Lake J.R., R.W. van Dyke and B.F. Scharschmidt (1987) Acidic vesicles in cultured rat hepatocytes. Gastroenterology **92**: 1251-1261.

Lamb J.E., F. Ray, J.H. Kushner and J. Kaplan (1983) Internalization and subcellular local-ization of transferrin and transferrin receptors in HeLa cells. J. Biol. Chem. **258**: 8751-8758.

Lehninger A.L. (1976) Biochemistry, Worth Publishers N.Y. 2th edition: 849.

Octave J.-N., Y.-J. Schneider, R.R. Crighton and A. Trouet (1981) Transferrin uptake by cultured rat embryo fibroblasts. Eur. J. Biol. **115**: 611-618.

Oka J.A. and P.H. Weigel (1983) Recycling of the asialoglycoprotein receptor in isolated rat hepatocytes. J. Biol. Chem. **258**: 1025-1030.

Pearse B.M.F. (1976) Clathrin: A unique protein associated with intracellular transfer of membrane by coated vesicles. Proc. Natl. Acad. Sci. USA **73**: 1255-1259.

Renswoude J. van, K.R. Bridges, J.B. Harford and R.D. Klausner (1982) Receptor mediated endocytosis of transferrin and the uptake of Fe in K 562 cells: Identification of a nonlysosomal acidic compartment. Proc. Natl. Acad. Sci. U.S.A. **79**: 6186-6190.

Romslo I. (1981) Intracellular transport of iron. In: Iron in biochemistry and medicine II. A. Jacobs and M. Worwood eds. Chapt. 9, 325-362.

Schneider D. (1981) ATP-dependent acidification of intact and disrupted lysosomes. J. Biol. Chem. **256**: 3858-3864.

Stone D.K., X. Xie and E. Racker (1983) An ATP-driven proton pump in clathrin-coated vesicles. J. Biol. Chem. **258**: 4059-4062.

Tycko B. and F.R. Maxfield (1982) Rapid acidification of endocytic vesicles containing α_2-macroglobulin. Cell **28**: 643-651.

Veldman A., C. van der Heul, M.J. Kroos and H.G. van Eijk (1986) Fluorescence probe measurement of the transferrin microenvironment during iron uptake by rat bone marrow erythroid cells. Brit. J. Haematol. **62**: 155-162.

Willingham M.C., J.A. Hanover, R.B. Dickson and I. Pastan (1984) Morphologic character-ization of the pathway of transferrin endocytosis and recycling in human KB cells. Proc. Natl. Acad. Sci. USA **81**: 175-179.

CHAPTER 6

GENERAL DISCUSSION

Chapter 6

1. GENERAL DISCUSSION

Cells take up molecules from the extracellular medium for a number of reasons, such as: nutrition, defence or regulatory functions. Iron is one of the metals essential to virtually all forms of life. Its sequestration is a problem for animal and plants, due to the chemistry of iron in our atmosphere. As discussed in the previous chapters there is no agreement about the precise mechanism of transferrin-mediated iron uptake in mammalian cells. Either ferric-transferrin donates its iron to the cell without internalization or the protein is internalized, iron is removed from transferrin within the cell and the protein is externalized. The results of described in this thesis support the concept of endocytosis as the primary mechanism of iron uptake and some characteristics of the subsequent intracellular pathway are discussed. In this chapter a compilation of the current knowledge concerning endocytosis is given, especially that dealing with the endocytosis of ferric-transferrin.

2. Outline of receptor-mediated endocytosis

Endocytosis is the process by which cells take up macromolecules into membrane bound vesicles, and encompasses three types of processes: 1) in phagocytosis, typically seen in macrophages which cells internalize large particles such as bacteria or damaged cells by engulfing them. The resultant vesicle is called phagosome. 2) pinocytosis; the uptake of extracellular fluid into small vesicles called pinosomes. 3) receptor-mediated endocytosis. In the latter type the cell 'selects' from the environment those macromolecules that fit highly specialized receptors on the cell surface. This is seen for example with low density lipoproteins (LDL), α_2-macroglobulin (α_2-M), epidermal growth factor (EGF) and transferrin (Tf). The receptors for most ligands are randomly distributed over the cell surface when unoccupied, however, those of LDL and Tf tend to cluster in specialized areas of the plasma membrane at the shallow depressions formed are termed coated pits (Goldstein *et al.*, 1979; Willingham and Pastan, 1984). The term 'coated' refers to its characteristic appearance under the electron microscope. A coated pit shows as a regular bristlelike layer of protein at the cytoplasmic side of the pit. They are found in all eukariotic cells, of invertebrates and vertebrates, protozoa and yeast, including platelets, but not in mature erythrocytes. In cultured cells there are 500-1,000 coated pits per cell, covering about 1 % of the cell surface. Kinetic data indicate that each coated pit gives rise to an endosome every 20 seconds (Pastan and Willingham, 1981). Soon after receptor clustering the coated pits sink into the intracellular space and eventually pinch off the plasma membrane forming an isolated intracellular vesicle, which contains the membrane anchored ligand-bearing receptors. The formation of an endosome (or receptosome) is brought about by the coat protein clathrin. The signal for the coat assembly is not yet known but may either be the result of ligands cross-

linking two or more cell surface receptors or may be a continuous process that does not need a signal to start. Many different ligand-occupied receptors eventually gather in the same coated pit, making it difficult to study whether cross-linking of one specific receptor influences the formation to a coated vesicle. This consideration is sometimes overlooked in publications suggesting that there is continuous internalization of unoccupied transferrin receptor molecules (Watts, 1985). Clathrin, the protein present on the intracellular side of the coated pit, consists of a 180,000 Dalton heavy chain and two light chains, 36,000 Dalton and 33,000 Dalton respectively, forming a triskelion structure. The clathrin monomer triskelions can rapidly associate to form a cage of pentagonal and hexagonal arrangement, pulling the base of the coated pit inward from the cell surface. Finally, when the barrel-shaped structure is completed the coated vesicle is pinched off from the plasma membrane. After the formation of the isolated coated vesicle the clathrin polymer dissociates and the monomers cycle back to the plasma membrane to be reutilized. The uncoated vesicle is called endosome. Endosomes can be considered to be outside-in vesicles. The primary endosomes have a diameter of approximately 200 nm. Primary endosomes formed at the plasma membrane move centripetally, first by saltatory motion, and later with continuous unidirectional motion to the perinuclear area (Herman and Albertini, 1984). It is thought that endosomes move through the gel-like cytoplasm along tracks of microtubules. Some investigators found that drugs that disruptmicrotubule formationinhibits endosome cycling, while others found little or no effect in reticulocytes when using the same drug. As a consequence of a physical and temporal separation primary endosomes can only fuse with other primary endosomes formed at the same time. The resultant is a secondary endosome with a diameter of up to 600 nm (Ajioka and Kaplan, 1986).

Coated pits do not discriminate between different receptors, thus all internalized ligands are found in the same endosome. There must, therefore, be a compartment where ligands are sorted according to their final destinations e.g. lysosomes, cytosol, nucleus, mitochondria or vesicles involved in exocytosis. It is important to bear in mind that the process of receptor-mediated endocytosis is not specially designed to suit iron uptake, but that is forms a universal route for the selective uptake of macromolecules to which iron metabolism has adapted itself during the evolution.

Essential to iron uptake is the existence of the specialised transferrin receptor. The molecule is a dimeric transmembrane glycoprotein with identical subunits of 90,000 Dalton connected by a single disulphide bond, close to or in the membrane. About 6% of its mass consists of carbohydrates containing the same sugars as transferrin: mannose, N-acetyl glucosamine, galactose and sialic acid. The N-linked glycan chains are added in the rough

endoplasmic reticulum while further processing and addition of peripheral monosaccharides to complex oligosaccharides takes place in the Golgi. The hydrophobic domain of each subunit is linked to a palmitine fatty acid chain which may play a role in anchoring the receptor in the membrane. Each subunit is able to bind one transferrin molecule with a dissociation constant of 2-8 x 10^{-9} M (Klausner et al, 1983b). Diferric transferrin has been shown to have a 10 - 100 times higher affinity for the transferrin receptor than apotransferrin. The cytoplasmic domain contains several serine residues acting as phosphorylation sites. The function of phosphorylation does not seem to be involved in receptor internalization, as deletion mutants lacking this part of the cytoplasmic domain are internalized normally. Phosphorylation may form a signal marking aging molecules (Hunt et al, 1984; Rothenberger *et al.*, 1987). Several studies indicate that cross-linking of the transferrin receptors in the coated pits, either by transferrin or anti-transferrin IgG antibodies, enhances or triggers transferrin receptor internalization (reviewed by Newman, 1982; Hopkins and Trowbridge, 1983; Enns *et al.*, 1983). At 37°C the half-life of internalization varies between 2 - 5 minutes depending on the cell type (reviewed by Testa, 1985). The intracellular routes taken by transferrin and iron are not yet clear. There is evidence that transferrin may follow more than one route: a short route, identifiable by pulse chase labeling and a longer route that becomes labelled upon longer incubation with the tracer (Hopkins & Trowbridge, 1983). This longer route may be associated with resialization of transferrin in Golgi. New formed endosomes move by saltatory motion, but in a second phase they stop saltating and translocate centripetally, with constant velocity, to the Golgi at the centre of the cell. The intracellular translocation of endosomes seems to follow microtubule tracks, during both endocytosis and exocytosis, since microtubule depolymerizing agents immediately halt their motion (Huang and Ponka P., 1983). These antimitotics block the route to ligand sorting sites and, therefore, their subsequent degradation in lysosomes or recycling to the plasma membrane. However, internalization of coated pits is not prevented (Oka and Weigel, 1983; Herman and Albertini, 1984. Contrary to the findings of Oka and Weigel, Morgan and Iacopetta (1987) found only vinblastine, but not other microtubule inhibitors blocked transferrin endocytosis and thus iron uptake by reticulocytes. Therefore no definitive conclusion can be drawn about the intracellular movement of endosomes.

Generally the cell is very economic with its receptors and they are not degraded during endocytosis. Endosomes probably do not contain proteolytic enzymes, though recently there have been some reports describing certain proteolytic enzymes in macrophage endosomes (Diment and Stahl, 1985). Most internalized ligands are sent to the lysosomes to be degraded. Soon after internalization the endosome interior becomes acidic (pH 5.0 - 6.5) (Tycko and Maxfield, 1982; Dickson *et al.*, 1983; Yamashiro *et al.*, 1984). With the excep-

tion of transferrin the low pH causes the ligands to dissociate from their receptors (chapter 5 of this thesis). Endocytosed diferric transferrin releases its iron in the endosomal lumen at low pH, but at the same time the association of apotransferrin to its receptor at pH 4.8 is 2 times stronger than at pH 7.2. (Ecarot-Charrier *et al.*, 1977; Klausner *et al.*, 1983). When the intravesicular pH is neutralized by amines such as monensin or methylamine, segregation of iron and transferrin is blocked, but endocytosis proceeds (Egyed *et al.*, 1986 Harford *et al.*, 1983 van der Heul *et al.*, 1984; Veldman *et al.*, 1986). This supports the hypothesis that an acidic compartment is required for the separation of iron and transferrin. Since apotransferrin binds relatively strongly to its receptor at pH 5.5 it remains bound to the receptor as it recycles to the plasma membrane. The exocytic vesicles that leave the site of ligand separation (CURL or trans-Golgi) may be 'single vesicular endosomes' (SVE) or 'multi vesicular endosomes' (MVE) (also called 'multi vesicular bodies' (MVB)), as they appear und er the electron microscope as large vesicles, filled with many very small vesicles (Harding *et al.*, 1985). It is not yet understood how MVE are formed and from which compartment they originate. The transferrin receptor can be shown to be present on the surface of the inner vesicles and on the intravesicular side of the MVE membrane (Harding *et al.*, 1983). It may be that some of the apparent vesicles seen in MVE are in fact cytoplasmic protrusions into the complex organelle judged to the equal staining of the cytoplasm and the lumen of some of these apparent vesicles. MVE are not seen in all cell types; they are mostly described in reticulocytes. During the differentiation of erythroid precursor cells the number of cell surface transferrin receptors is reduced from 500,000/cell for polychromatic normoblast to zero in erythrocytes. Therefore reticulocytes, the final stage in the differentiation process before the erythrocyte, are in the process of reducing their numbers of transferrin receptors. There are two possible ways of achieving such a reduction. One way is the physiological degradation of the aging receptor, while the synthesis of new receptor molecules is inhibited at the transcription level. Transferrin receptors have a half-life of several hours; aging (possibly indicated by phosphorylation of the receptor) may lead to degradation in lysosomes. The second way is that of receptor shedding. In this recently described process the inclusion vesicles of MVE are released at the cell surface at a rate that accounts for the required receptor reduction and at the same time reduces excess membrane (Pan and Johnstone, 1983). This may occur only in reticulocytes as they are in the process of eliminating organelles and longer possess clearly identifiable lysosomes (Veldman *et al.*, 1984). The complete transferrin cycle takes 3-7 min (Iacopetta and Morgan,1983). However, the route through the MVE takes longer - about 32 minutes. In the normal transferrin cycle apotransferrin is lost from the cell when the exocytotic vesicle associates with the plasma membrane and the transferrin-receptor complex is exposed to an environment of about pH 7.4. Comparison of the dissociation constants of diferric- and apotransferrin has that di-

ferric transferrin binds 25 times more stronger to the transferrin receptor in K 562 cells (pH 7.2) (Klausner *et al.*, 1983) and 14 times more stronger to rabbit reticulocytes (pH 7.4) (Morgan, 1983) than the apo-protein does. Consequently through competitive interaction of diferric- or monoferric transferrin, apotransferrin dissociates from the receptor ($t^1/_2$ = 17 s) (Ciechanover et al., 1983).

2.1. Conclusions

In this thesis two classes of endosomes were isolated, both containing transferrin and iron. These endosomal compartments differed from each other in their buoyant density and $^{125}I/^{59}Fe$ ratio. The heavy compartment consists of the initial compartment entered, as was shown by its $^{125}I/^{59}Fe$ ratio, which closely paralleled that of the transferrin used for labelling. The light compartment probably comprises the sorting part of the endosomal system. This is the part in which transferrin is separated from ligands destined for degradation in lysosomes. It was shown in vitro that isolated primary (or heavy) endosomes were capable of internal acidification. Therefore it is thought that iron release from endosomes starts early in the endocytic cycle. However, as there still is a considerable amount of iron in the secondary endosome and since iron uptake into erythroid cells is nearly 100 % efficient, this implies that the rest of the iron is released in the secondary or sorting endosomes. Studies on the mechanism of sorting are still young and no clear theory has yet emerged. Sorting may occur at several locations. First, acidification releases acid-dissociable ligands from their receptor, leaving an acidic fluid phase and a solid membrane-associated phase containing the receptors (Palade, 1982). Further sorting may relate to the lipid composition of domains in the sorting compartment. Depending on the cytoplasmic domain of each receptor, sorting could take place to the different protrusions of the tubular compartment and thus mediate sorting into different 'leaving' vesicles and/or addressing of the latter to the appropriate destination. However, this is still hypothetical.

The results in chapter 4 suggest that in solubilized heavy endosomes about 30 % of the iron was present in a apparently free low molecular weight form. It is presumed, though, that iron is associated with some acceptor in the endosome. Considering the small internal volume of the endosome, dissociation of iron atoms from transferrin increases the free iron concentration enormously. One free iron atom in an endosomal volume of 10^{-17} litre yields a concentration of 6.0 μM. Usually there are more iron atoms per endosome, though they may not be all released at the same time. The association constant of transferrin for iron dramatically decreases upon lowering of the pH (the exact affinity constant of iron-transferrin at pH 5.0 is not known) though even at pH 5.0 association is probably favoured considering the endosomal iron concentration, unless iron can be bound by a stronger acceptor

at this pH. These considerations prompt the idea that free iron probably does not exist in endosomes even at low pH.

Isolated K 562 endosomes were found to be functional with respect to internal acidification, and many investigators have reported that the internal endosomal milieu is acidic (Klausner *et al.*, 1983; Harding *et al.*, 1985; Paterson *et al.*, 1984; Loh *et al.*, 1985; Dautry-Varsat *et al.*, 1983) What exactly is an 'acid pH'? The classic definition of pH is the negative logarithm of the H^+ concentration (-log $[H^+]$), the number of moles of H^+/litre. This is a statical concept useful in chemistry and many fields of biochemistry and medicine. However, when studying subcellular organelles as small as endosomes this can not be used. Since the internal volume of an endosome can be as small as 10^{-17} litre, pH 5.0 would indicate 60 free protons per endosome. However, pH 6.8 would mean 1 proton per endosome and a physiological pH of 7.4 would signify 0.24 protons per endosome! This is not workable concept. Therefore it seems more suitable to employ a dynamic model of proton activity, namely the velocity of generation of protons per second per volume. Protons pumped into endosomes by the membrane associated ATPases often do not remain free, but associate with many of the proteins present inside endosomes. This for instance is the case with transferrin, which takes up protons and thereby lowers its affinity for iron.

Little work has been done on iron release from endosomes, though this of vital importance for understanding cellular iron uptake. Before the elucidation of receptor-mediated endocytosis investigators were perplexed as to how iron could cross the apolar plasma membrane. Now there is a better understanding as to how iron (and transferrin) enter the cell, however, little is being done to study how iron leaves the endosome. Data presented in chapter 4 indicate that acidification alone is not sufficient to release iron from endosomes. These experiments were therefore extended with isolated and purified heavy and light endosomes. Chapter 5 describs how heavy and light endosomes are incubated with different iron chelators, since it is not clear whether an iron acceptor at the cytoplasmic side of the endosome is a prerequisite for iron release. Analysis of the low molecular weight iron containing fraction of reticulocyte cytosol (described in chapter 3), however, did not indicate the presence of a particular iron binding molecular species. It was noted that such intermediates might have a high turnover rate, thereby losing iron to the "next best" iron chelators. Because until now no unambiguous intracellular iron chelator has been identified, artificial chelators were used to study endosomal iron release. BPS and PIH were both suitable as iron acceptors though to different extends. Proportional ATP specific iron release from heavy and light endosomes to BPS was equal though maximalrelease was 6.5 % which is lower than iron release to PIH. Proportional ATP specific iron release to PIH showed a greater re-

lease from heavy endosomes than from light endosomes. In all experiments with BPS and PIH the presence of a NADH/NAD^+ redox couple reduced iron release from light endosomes approximately to 50 % (from 6.5 % to 2.5 % with BPS and from 7,5 % to 4 % with PIH). If reducing conditions are essential iron release to BPS would be expected to increase rather than to decrease. These results prompted the conclusion that reducing conditions are not beneficial to iron release from endosomes. The release from heavy endosomes was influenced to a much lesser degree by the redox couple. Iron release was always higher from heavy endosomes than from light endosomes, but this might not, however, be the case in vivo since the lifetime of a heavy endosome is limited by its fusion to form a light endosome.

The thiol reagent N-ethylmaleimide completely inhibits acidification of endosomes and lysosomes by the Mg^{2+}-dependent ATPases at a concentration of 1 mM (Galloway et al. 1983; Stone et al., 1983). The effect of NEM on iron release from isolated endosomes was limited. Iron was still released but at a reduced rate. This may be explained by the fact that about 30 % of the iron found in endosomes was not associated with transferrin, but in a low molecular weight form. It is conceivable that this pool can still release iron after acidification has ceased.

Based on chemical and NMR analysis it has been suggested that lactate is a major intermediate in human placental iron transfer. The placenta has a very high glucose metabolism and lactate is transferred to the fetus as a source of energy. Other cells types associated with high iron metabolism are e.g. erythroid precursor cells, tumor cells and liver parenchymal cells. The first two groups have higher intracellular lactate levels due to high aerobic glycolysis. Parenchymal cells are active in conversion of lactate to glucose. Therefore lactate has been studied as a possible physiological iron chelator in vitro. Although lactate is a much weaker chelator than PIH or BPS, the concentration used was considered high enough to be effective in chelation. However, no release of iron was seen with lactate as the iron acceptor. This also confirms that, without a suitable iron chelator, no iron is released from endosomes.

The conclusions are that iron release from endosomes depends on the presence of an iron acceptor. The iron acceptor should preferentially be an Fe(III) acceptor. Heavy endosomes release more iron than light endosomes, though this may be a consequence of the arbitrary conditions used in this study. The presence of the redox couple NADH/NAD^+ reduced iron release predominantly from light endosomes. The reasons for this reduction of iron release are still unknown.

3. Can receptor-mediated endocytosis explain total iron uptake in erythroid cells?

To answer this question one has to make several assumptions based on literature about cell types other than that studied. Rabbit reticulocytes for instance contain 100,000 - 150,000 receptors, which is similar to the number reported for mouse and rat. Transferrin internalization in the rabbit saturates at about 2.5 μM, while the plasma transferrin concentration is about 25 μM (in humans 25-55 μM) (Iacopetta and Morgan, 1983). This indicates that the transferrin cycle always proceeds at maximal rate. There are about 4-8 transferrin receptor molecules in a coated pit (the volume of a primary endosome is about 1×10^{-17} litre, the volume of the receptor-transferrin complex in solution is about 1.4×10^{-19} litre, so if there were be only transferrin receptor complexes in the endosome their would be space for about 70 complexes. (Hu and Aisen, 1978)). Therefore each endosome may contain 8-16 transferrin molecules with 16-32 iron atoms, since diferric transferrin molecules are preferentially bound. If reticulocytes have an average of 750 coated pits/cell giving rise to an endosome every 20 seconds (see chapter 1) then about 72,000 iron atoms per minute are internalized (or 1,200 atoms/s). This closely corresponds to the 1,100 atoms/s found by Iacopetta and Morgan (1983). These figures must be even higher in less mature erythroid cells because of their greater number of receptors. This calculation can be expanded to human erythrocytes to calculate if endocytosis of ferric-transferrin can account for the total iron uptake. At an average erythrocyte MCH of 1,900 amol Fe^{2+}, erythrocytes contain 1×10^{9} iron atoms. When the rate of 1,200 atoms/s is considered as an average the total uptake would take 265 hours = about 11 days. Average maturation of erythroid cells takes 118-255 hours (Wintrobe *et al.*, 1974). Bearing in mind the assumptions made, especially concenrning the number of cell surface transferrin receptors on reticulocytes (polychromatic normoblasts bear upto 500,000 recptors/cell), the above suggests that receptor-mediated endocytosis is the major mechanism of iron internalization and can provide the iron necessary for developing erythroid cells.

REFERENCES.

Ajioka R.S., and J. Kaplan (1986) Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors. Proc. Natl. Acad. Sci. USA 83: 6445-6449.

Ciechanover A., A.L. Schwartz, A. Dautry-Varsat and H.F. Lodish. (1983) Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. J. Biol. Chem. **258**: 9681-9689.

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

Dekker C.J., M.J. Kroos, C. van der Heul and H.G. van Eijk (1985) Uptake of sialo and asialo transferrins by isolated rat hepatocytes: Comparison of a heterologous and a homologous system. Int. J. Biochem. **17**: 701-706.

Dickson R.B., L. Bequinot, J. Hanover, M.C. Willingham and I. Pastan (1983) Isolation and characterization of a highly enriched preparation of receptosomes (endosomes) from a human cell line. Proc. Natl. Acad. Sci. USA **80**: 5335-5339.

Diment S., and P. Stahl (1985) Macrophage endosomes contain proteases which degrade endocytosed protein ligands. J. Biol. Chem. **260**: 15311-15317.

Ecarot-Carrier B., W. Grey, A. Wilczynska and H.M. Schulman (1977) In: Proteins of iron metabolism. Eds. E.B. Brown, P. Aisen, J. Fielding and R.R. Crighton. Grune and Stratton, New York and London.

Egyed A., I. Fodor and G. Lelkes (1986) Coated pit formation and membrane function involved in the regulation of cellular iron uptake. Br. J. Haematol. **64**: 263 269. .

Eijk H.G. van, and W.L. van Noort (1986) A non-random distribution of transferrin iron in fresh human sera. Clin. Chim. Acta **157**: 299-304.

Enns C.A., H. Suomalinen, J. Schroder and H.H. Sussman (1983) Co-migration and internalization of transferrin and its receptor on K562 cells. J. Cell Biol. **97**: 597-585.

Galloway C.J., G.E. Dean, M. Marsh, G. Rudnick and I. Mellman (1983) Acidification of macrophage and fibroblast endocytic vesicles in vitro. Proc. Natl. Acad. Sci. U.S.A. **80**: 3334 3338.

Goldstein J.L., R.G. Anderson and M.S. Brown (1979) Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature **279**: 679-685.

Harding C., J. Heuser and P. Stahl (1983) Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J. Cell Biol. Vol **97**: 329-339.

Harding C., M. Aach and P. Stahl. (1985) Morphological analysis of ligand uptake and processing: the role of multivesicular endosomes and CURL in receptor-ligand processing. Eur. J. Cell Biol. **36**: 230-238.

Harford J., A.W. Wolkoff, G. Ashwell and R.D. Klausner. (1983) Monensin inhibits intracellular dissociation of asialoglycoproteins from their receptor. J. Cell. Biol. **96**: 1824-1828.

Herman B. and D.F Albertini (1984) A time-lapse video image intensification analysis of cytoplasmic organelle movements during endosome translocation. J. Cell Biol. Vol **98**: 565-576. .

Heul C. van der, A. Veldman, M.J. Kroos and H.G. van Eijk. (1984) Two mechanisms are involved in the process of iron uptake by rat reticulocytes. Int. J. Biochem. **16**: 383-389.

Hopkins C. and I.S. Trowbridge (1983) Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. J. Cell Biol. **97**: 508-521.

Hu H.Y., and P. Aisen (1978) Molecular characteristics of the transferrin-receptor complex of rabbit reticulocytes. J. Supramol. Struct. **8**: 349-360.

Huang A.R., and P. Ponka (1983) Study of the mechanism of action of pyridoxal isonicotinoyl hydrazone at the cellular level using reticulocytes loaded with non heme 59Fe. Biochim. Biophys. Acta **757**: 306 315.

Iacopetta B.J., and E.H. Morgan (1983) The kinetics of transferrin endocytosis and iron uptake from transferrin in rabbit reticulocytes. J. Biol. Chem. **258**: 9108-9115.

Klausner R.D., G. Ashwell, J. van Renswoude, J.B. Harford and K.R. Bridges (1983a) Binding of apotransferrin to K562 cells: Explanation of the transferrin cycle. Proc. Natl. Acad. Sci. USA Vol **80**: 2263-2266.

Klausner R.D., J. van Renswoude, G. Ashwell, C. Kempf, A.N. Schechter, A. Dean and K.R. Bridges. (1983b) Receptor mediated endocytosis of transferrin in K562 cells. J. Biol. Chem. **258**: 4715-4724.

Loh T.T., R.Y. Chan and W.F. Fong. (1985) Effects of spermine on transferrin and iron uptake by reticulocytes: II. Changes in intravesicular pH. Cell. Biol. Int. Rep. **9**: 495-500.

Morgan E.H. (1983) Effect of pH and iron content of transferrin on its binding to reticulocyte receptors. Biochim. Biophys. Acta **766**: 498-502.

Morgan E.H., and B.J. Iacopetta (1987) Vinblastine but not other microtubule inhibitors to block transferrin endocytosis and iron uptake by reticulocytes. Clin. Exp. Pharm. Physiol. **14**: 119-126.

Newman R., C. Schneider, R. Sutherland, L. Vodinelich and M. Graves (1982) The transferrin receptor. Trends Biochem Sci. **7**: 397-400.

Oka J.A. and P.H. Weigel (1983) Microtubule-depolymerizing agents inhibit asialo-orosomucoid delivery to lysosomes but not its endocytosis or degradation in isolated rat hepatocytes. Biochim. Biophys. Acta **763**: 368-376.

Palade G.E. (1982) Chairman's closing remarks in: Membrane recycling, CIBA Foundation Symposium Nr 92 (D. Evered ed.), Pitman Press London: 293-297.

Pan B.T., and R.M. Johnstone (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell **33**: 967 978.

Pastan I.H., and M.C. Willingham (1981) Journey to the centre of the cell: Role of the receptosomes. Science **214**: 504-509.

Paterson S., N.J. Armstrong, B.J. Iacopetta, H.J. McArdle and E.H. Morgan. (1984) Intravesicular pH and iron uptake by immature erythroid cells. J. Cell. Physiol. **120**: 225-232.

Stone D.K., X. Xie and E. Racker (1983) An ATP driven proton pump in clathrin coated vesicles. J. Biol. Chem. **258**: 4059-4062.

Testa U. (1985) Transferrin receptors: Structure and function. Current topics in Hematology **5**: 127-161.

Trinder D., E.H. Morgan and E. Baker (1986) The mechanism of iron uptake by fetal rat hepatocytes in culture. Hepatology **6**: 852-858.

Tycko B., and F. Maxfield (1982) Rapid acidification of endocytic vesicles containing α2-macroglobulin. Cell **28**: 643-651.

Veldman A., M.J. Kroos, C. van der Heul and H.G. van Eijk (1984) Are lysosomes directly involved in the iron uptake by reticulocytes Int. J. Biochem. **16**: 39-45.

Veldman A., C. van der Heul, M.J. Kroos and H.G. van Eijk. (1986) Fluorescence probe measurement of the pH of the transferrin microenvironment during iron uptake by rat bone marrow erythroid cells. Br. J. Haematol. **62**: 155-162.

Willingham M.C., J.A. Hanover, R.B. Dickson and I. Pastan (1984) Morphologic character-ization of the pathway of transferrin endocytosis and recycling in human KB cells. Proc. Natl. Acad. Sci. USA **81**: 163-175.

Wintrobe M.M., G.R. Lee, D.R. Boggs, T.C. Bithel, J.W. Athens and J. Foerster (1974) The erythrocyte. In: Clinical hematology, M.M. Wintrobe ed. Lea and Febiger, Philadelphia USA.: 80-135.

SUMMARY

Iron is an essential element for all cells. It is involved in oxygen transport as a constituent of hemoglobin and myoglobin, in electron transport within mitochondria and as a cofactor in many enzymes. Under oxygen rich conditions, such as those in the developing erythroid cell, elemental iron would prove unutilizable. At high concentrations of oxygen and neutral pH Fe(II) - the less oxydised form of the metal - readily oxidises to Fe(III) which is highly insoluable. This would result in concentrations of free Fe(III) that are too low for the required metabolic functions. In the cause of evolution specialized molecules have developed enabling transport and storage of iron at much higher concentrations, without harmful effects. The last decades much research has been done on iron metabolism in man, both on the organism as a whole and at cellular level. Although many questions still lay unanswered, particularly regarding cellular iron metabolism. This thesis presents a study on the characteristics of iron transport within developing erythroid cells.

Chapter 1 introduces the reader to a number of characteristics and facts about iron metabolism, and the structure and function of the most important proteins involved in transport and storage of iron. Subsequently chapter 1 describes the current hypotheses concerning the uptake of transferrin-bound iron into the cell and the scope of this thesis is indicated. Namely: what mechanism releases iron from transferrin after uptake by endocytosis? What are the conditions for translocation of iron from the endosome to the cytosol and what is the nature of the transport factor carrying iron through the cytosol to its targets? Chapter 2 describes the materials and methods used in this study. Chapter 3 deals with the study about the iron transport factor(s) in the cytosol. When double labelled rat transferrin is presented to rat reticulocytes with inhibited heme synthesis, accumulation of ^{59}Fe is found in a fraction of low molecular weight (LMWF). Chapter 3 describes the characteristics and composition of the low molecular weight fraction. Following the conclusions of chapter 3, chapter 4 describes the isolation of endosomes from a human erythroleukemia cell-line (K 562). The results show there were at least two types of endosomes. The heavy and light endosomes had different buoyant densities and there were temporal differences of formation and in the amount and ratio of iron and transferrin in each. Studing their characteristics it was demonstrated that, immediately after internalization of double labelled transferrin, ^{59}Fe is released from the initial endosomal compartment (heavy endosomes). Due to the limited lifetime of heavy endosomes iron release continued after the fusion of heavy endosomes and the formation of the secondary, light, endosomal structure. Previous studies suggest that this is the organelle responsible for the recycling apotransferrin to the plasma membrane. Indeed the observed reduction in the $^{125}I/^{59}Fe$ ratio suggests that ^{125}I-transferrin re-

lease from light endosomes exceeds iron release. In order to determine the nature of possible acceptor molecules involved in endosomal iron release vitro experiments with isolated endosomes and iron chelators described in chapter 5 say something about the nature of acceptor molecules involved in the release of iron from endosomes to the cytosol. In chapter 6 the results described in this thesis are discussed in the light of the current knowledge and theories about uptake and release of molecules by cells through receptor-mediated endocytosis.

SAMENVATTING

IJzer is een belangrijk metaal voor de meeste cellen. Het is betrokken bij de zuurstof voorziening in de vorm van hemoglobine en myoglobine, bij elektronentransport in mitochondriën en als co-factor in enzymen. IJzer is echter een moeilijk te handhaven element voor organismen door de bijzondere eigenschappen van ijzer in zuurstofrijk milieu, met name in zich ontwikkelende erythrohrode cellen. IJzer komt voor in twee oxidatie toestanden: als Fe(II) en Fe(III). Echter onder zuurstofrijke condities en neutrale pH oxideert Fe(II) snel tot Fe(III). Fe(III) verbindingen zijn zo slecht oplosbaar, dat de vrije Fe(III) concentratie in het lichaam veel te laag zou zijn om alle processen naar behoren te laten verlopen. In de loop van de evolutie zijn gespecialiseerde moleculen ontstaan die ijzer transport en ijzer opslag mogelijk maken bij veel hogere concentraties en zonder schadelijke gevolgen. In de afgelopen decennia is er veel onderzoek gedaan aan het ijzermetabolisme bij de mens, zowel wat betreft het metabolisme in het gehele individu als het cellulaire metabolisme. Met name op het gebied van het cellulaire ijzermetabolisme zijn nog steeds een groot aantal vragen onbeantwoord. In dit proefschrift is een onderzoek beschreven over karakteristieken van het ijzertransport binnen jonge rode bloedcellen.

In hoofdstuk 1 wordt de lezer geïntroduceerd in een aantal eigenschappen van en feiten omtrent het ijzermetabolisme en de bouw en functie van de belangrijkste eiwitten betrokken bij transport en opslag van ijzer. Onder meer zijn de hypotheses beschreven die geformuleerd zijn met betrekking tot de opname van transferrinegebonden ijzer in de cel. Tevens wordt in dit hoofdstuk de probleemstelling aangegeven. Deze is als volgt in deelvragen te omschrijven: hoe wordt transferrinegebonden ijzer, nadat het in de cel opgenomen is, van transferrine ontkoppeld; wat zijn de voorwaarden voor translocatie van ijzer vanuit het endosoom naar het cytosol; en als laatste; wat is de aard van de transport factor die ijzer door het cytosol transporteert. In hoofdstuk 2 zijn alle materialen en methoden weergegeven die bij dit onderzoek gebruikt zijn. Hoofdstuk 3 behandelt het onderzoek naar de transport factor(en) in het cytosol. Wanneer dubbelgelabeld rattetransferrine aangeboden wordt aan rattereticulocyten met geïnhibeerde heemsynthese, dan vindt stapeling plaats van ^{59}Fe in een fractie met laag moleculaire gewicht (LMWF). Eigenschappen en samenstelling van deze fractie zijn in dit hoofdstuk beschreven. Voortbouwend op de conclusies van hoofdstuk 3 is in hoofdstuk 4 beschreven hoe endosomen zijn geïsoleerd uit een humane erythroleukemische cel-lijn (K 562). Hierbij werd gevonden dat er sprake is van tenminste twee soorten endosomen. Deze zware en lichte endosomen verschillen o.m.

in dichtheid en in de hoeveelheid en verhouding van ijzer en transferrine in de tijd. Hun karakter werd onderzocht en er werd gevonden dat, onmiddelijk na de internalizatie van dubbelgelabeld transferrine, ^{59}Fe werd vrijgemaakt uit het initiële endosomale compartiment, dat gevormd wordt door de zware endosomen. Door de korte levensduur van de zware endosomen gaat de afgifte van ijzer door na de fusie van de zware endosomen en de vorming van het secundaire, 'lichte', endosomale structuur. Echter, apotransferrine keert van dit organel terug naar de plasmamembraan. De $^{125}I/^{59}Fe$ ratio suggereert dat de afgifte van ^{125}I-transferrine uit de lichte endosomen de ijzer afgifte overtreft, hetgeen leidt tot een lage $^{125}I/^{59}Fe$ ratio. Teneinde uitspraken te kunnen doen over de aard van de acceptor-moleculen betrokken bij de translocatie van ijzer uit endosomen naar het cytosol, zijn de in hoofdstuk 5 beschreven in vitro experimenten uitgevoerd met geïsoleerde zware en lichte endosomen met verschillende artificiële ijzerchelatoren en een mogelijk fysiologische ijzerchelator. In hoofdstuk 6 worden de resultaten beschreven in dit proefschrift bediscussieerd in het licht van de huidige kennis en opvattingen omtrent de opname en afgifte door de cel van molekulen middels receptor gemedieerde endocytose.

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CURRICULUM VITAE

Dirk Bakkeren werd geboren op 16 januari 1957 te s'-Gravenhage. Na het behalen van zijn atheneum B diploma aan het St. Maartens College te Voorburg begon hij in hetzelfde jaar de studie biologie aan de Rijksuniversiteit te Utrecht.

Op 10 maart 1980 behaalde hij het kandidaatsexamen biologie met tweede hoofdvak scheikunde (B4). De studie werd vervolgd met als hoofdvak Experimentele Immunologie (Prof. Dr. J. Willers), de bijvakken Microbiologie (Prof. Dr. W. Hoekstra) en Virologie (Prof. Dr. J. Smets en Dr. R. Michalides, Antoni van Leeuwenhoekhuis Amsterdam) en de nevenrichting Elektronenmicroscopische Structuuranalyse (Prof. Dr. Elbers). Het doctoraalexamen werd behaald op 24 januari 1984.

Het in dit proefschrift beschreven werk werd verricht aan het Instituut Chemische Pathologie (vakgroep Biochemie) Erasmus Universiteit Rotterdam, waar hij was aangesteld als wetenschappelijk assistent in tijdelijke dienst van 1 mei 1983 tot 1 juli 1987.

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Dirk Bakkeren was born on January 16th 1957 in The Hague, Netherlands. Having obtained his diploma athenaeum B at St. Maartens College in Voorburg he began studying biology at the State university of Utrecht the same year.

On March 10th 1980 he obtained his batchelor's degree in chemical biology. He continued this study with major specialization in Experimental Immunology (Prof. Dr. J. Willers), co-specializations in Microbiology (Prof. Dr. W. Hoekstra) and Virology (Prof. Dr. J. Smets en Dr. R. Michalides, Antoni van Leeuwenhoekhuis, Amsterdam) and Electron Microscopic Structure Analysis (Prof. Dr. Elbers). On january 24th 1984 he graduated.

The work described in this thesis was performed at the Institute of Chemical Pathology (Dept. of Biochemistry) of the Erasmus University Rotterdam, where he was employed as a scientific assistant with a temporary assignment, between May 1st 1983 and July 1st 1987.

Colofon

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