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REVIEW ARTICLE

The role of transferrin in the mechanism of cellular iron uptake

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INTRODUCTION

The role of transferrin and its cell surface receptor in cellular iron metabolism has received considerable interest during the last decade. Hence, over this period several reviews covering various aspects of the topic have been published.

In this review we attempt to summarize the new knowledge and controversies encountered during the last 4–5 years. The focus is on iron metabolism by the hepatocyte. We also view the concept of transferrin–cell interactions from angles which previously may not have been given particular attention, and we include some aspects of iron metabolism not normally covered by reviews of this type. Central parts of what may be regarded as well-established knowledge either have been entirely omitted, or are only briefly described or mentioned to the extent considered relevant to the particular topic discussed. Important aspects of iron metabolism such as iron adsorption, haemochromatosis, ferritin iron and iron in infection and neoplasia are not covered in the present paper. Instead, the reader is referred to one or more of the recently published reviews [1–5] and references therein.

TRANSFERRIN AND THE TRANSFERRIN RECEPTOR

The structure, properties and functions of transferrin and the transferrin receptor have been reviewed in several recent papers [6–14]. For an extensive treatment the interested reader is referred to these publications.

Table 1 lists some characteristics of human serum transferrin.

Table 1. Characteristics of transferrin

Data are compiled from [9,10] and references therein.

Property	Value
No. of amino acid residues	679
Molecular weight	79570
Carbohydrate content	6%
Shape	Prolate ellipsoid
Dimensions	11.0 nm × 5.5 nm [a]
Max. no. of HCO_3^- bound	2
$\log K_a(HCO_3^-)$	2.7, 1.8
Iron binding ligands	Tyr (2), His (1), Asp (1)
Max. no of Fe bound	2
$\log K_a(Fe^{3+})$, pH 7.4	20.2 [b]
$\log K_a(Fe^{2+})$, pH 7.0	3.2
$\log K_a(Fe^{3+})$, pH 5.5	12.6 [c]
$E_0(Fe^{III}\text{–}Tf/Fe^{II}\text{–}Tf)$, pH 7.0–7.4	−310 mV [d] −400 mV [e] −520 mV [f]

[a] Diferric transferrin
[b] C-terminal.
[c] Calculated.
[d] Linear free energy relation.
[e] Chemical.
[f] Electrochemical.

The amino acid residues involved in the specific binding of iron by transferrin appear to have been unequivocally identified by X-ray crystallography. At both the *N*- and *C*-terminal region of transferrin, iron is directly co-ordinated to two tyrosines, one histidine and one aspartic acid, and indirectly co-ordinated to an arginine via the (bi)carbonate anion. The last co-ordination position of iron is occupied by a water molecule or a hydroxyl ion. Identical results have been obtained with rabbit serum transferrin and human lactoferrin [15,16]. Recent Mössbauer studies have also confirmed the similarity between the two iron-binding sites in human transferrin [17].

For the first time a direct electrochemical determination of the reduction potential of transferrin-bound iron has been reported, yielding a value near −520 mV [18]. This is in contrast to results obtained previously by indirect methods or by chemical reduction which gave results in the range −280 mV to −400 mV [19,20]. The latter value, however, when recalculated taking into account the binding of ferrous iron by transferrin, yields a value near −500 mV [9].

Table 2 lists some properties of the transferrin receptor. The receptor consists of two identical subunits, organized into three domains; the *N*-terminal cytoplasmic tail of 62 amino acid residues, the transmembrane segment of 26 amino acid residues and the large extracellular *C*-terminal region making up the rest of the polypeptide. The transmembrane segment contains three

Table 2. Characteristics of the transferrin receptor

Data are compiled from [7–14,21] and references therein.

Property	Value
No. of subunits	2
Molecular mass/subunit	90000
No. of amino acids/subunit	760
No. of amino acids in cytoplasmic tail	62
transmembrane segment	26
N-Terminal	Cytoplasm
No. of carbohydrate chains/subunit	3
Location	Extracellular
No. of fatty acid chains/subunit	1
Location	Cytoplasm
No. of subunit-linking S–S bridges	2
Location	Membrane
Principal phosphorylation site	Ser-24
No. of transferrin bound/subunit	1
K_a (diferric transferrin), pH 7.4	$(0.34\text{–}1.6) \times 10^7$ M^{-1} [a] 1.1×10^8 M^{-1} [b] 1.4×10^8 M^{-1} [c]
K_a (diferric transferrin), pH 5.5	7.7×10^7 M^{-1} [c]
K_a (monoferric transferrin), pH 7.4	2.6×10^7 M^{-1} [b]
K_a (apotransferrin), pH 7.4	4.6×10^6 M^{-1} [b]
K_a (apotransferrin), pH 5.5	7.7×10^7 M^{-1} [c]

[a] Rat hepatocytes.
[b] Rabbit reticulocytes.
[c] Human HepG2.

Abbreviations used: RME, receptor-mediated endocytosis; FITC, fluorescein isothiocyanate; PCT, porphyria cutanea tarda.

cysteine residues involved in the binding of a fatty acid chain and the formation of two subunit-linking disulphide bridges. The cytoplasmic tail contains four potential phosphorylation sites, but only one (Ser-24) appears to be a target for protein kinase C-mediated phosphorylation.

Major recent contributions to our understanding of the transferrin receptor have been made through studies at the level of molecular biology of the receptor. The mRNA for the receptor has been shown to encode a peptide of 760 amino acid residues. Expression of both transferrin receptor and ferritin appears to be regulated by iron-responsive elements located to the 3'- and 5'-untranslated regions of the respective mRNAs [13]. Transferrin receptor expression is apparently regulated differently during changes in iron status compared with proliferative changes of the cell [14].

CELLULAR UPTAKE OF IRON FROM TRANSFERRIN

Receptor-mediated endocytosis

The general mechanism by which cells acquire iron from transferrin was apparently solved when the concept of receptor-mediated endocytosis (RME) was worked out. This model has been thoroughly discussed in several recent reviews [9,21–23]. Its main features are as follows.

Cellular uptake of iron from transferrin is initiated by the binding of transferrin to the transferrin receptor at the cell surface. Via coated pits and coated vesicles the transferrin–transferrin receptor complex becomes trapped within endocytic vesicles termed endosomes. Through the action of a proton-pumping ATPase of the endosomal membrane, the vesicle's lumen is rapidly acidified (pH 5–5.5). The low pH facilitates iron mobilization from transferrin and the iron is transported across the endosomal membrane into the cytosol. At the pH of the endosomal lumen the apotransferrin formed binds tightly to the transferrin receptor. Through unknown processes the apotransferrin–transferrin receptor complex is sorted into exocytic vesicles, hence escaping lysosomal degradation. The exocytic vesicle fuses with the plasma membrane and the apotransferrin–transferrin receptor complex is exposed to the extracellular pH. At this pH the apotransferrin has a very low affinity for the transferrin receptor. As a result apotransferrin dissociates from the receptor leaving it ready for another cycle of transferrin binding and endo-/exo-cytosis.

Although the RME model gives a good overview of the main features of cellular uptake of iron from transferrin it fails to explain certain experimental observations. For instance, at the pH of the endosome complete release of iron from transferrin *in vitro* may take hours [24]. Yet, in the endosome in which transferrin resides only a few minutes, the release of iron is highly efficient. Thus, there must be more to the iron release process than a mere pH lowering. A recent study on reticulocytes has suggested the presence of an Fe(III)-binder in the membrane of endocytic vesicles [25]. The association constant (K_a) for the binding of ferric iron to the membrane protein was reported to be 3.63×10^9 M^{-1} at pH 5. The calculated K_a for the binding of ferric iron by transferrin at this pH is 4×10^{10} M^{-1}. Thus, the presence of the ferric iron binder alone may not be sufficient to explain the rapid release of iron from transferrin by the cells. However, the association constant for the ferric iron binder was measured with iron citrate as iron donor. It would appear unlikely that this is the form of iron presented to the binder *in vivo*. Another problem with the RME model is to explain why iron uptake from transferrin continues to increase when the extracellular concentration of transferrin is far in excess of the concentration needed to saturate all the transferrin receptors.

Finally, a most crucial question left unanswered by the RME model (or any other model for that matter!) is how iron is transported across the biological membranes into the cytosol. In addition, it must be made clear that the RME model, derived from work mainly on immature erythroid cells and established cell lines in culture, apparently does not apply to all cell types. In particular, the hepatocyte appears to be an exception from the RME model. Consequently, from experiments on isolated hepatocytes evidence of mechanisms in addition to RME has accumulated.

Reductive iron release

It is well accepted that iron is most efficiently loaded into ferritin, the main iron acceptor in the hepatocyte, when presented to the protein in ferrous form. However, following entry into the protein core ferrous iron is oxidized to the ferric state, and release of iron from ferritin is best achieved under reducing conditions [26]. Furthermore, biosynthesis of haem requires ferrous iron for insertion into the porphyrin moiety [27]. Thus, intracellular iron apparently undergoes frequent redox cycles during transit between cellular and molecular compartments.

The concept of a mechanism of iron uptake from transferrin by the hepatocyte involving reduction of iron has evolved through accumulation of results that apparently do not fit into the RME model. Some of these observations, together with a critical treatment of the evidence in favour of RME, was reviewed by Morley & Bezkorovainy in 1985 [22]. Since then, new information has been added and the model has been further developed. On the other hand, some problems relating to the basis of this model have also been pointed out (see below).

It must be held in mind that to date most of the work on reductive iron uptake has been performed on isolated hepatocytes in suspension. These cells lack the polarity present in the cells *in situ* in the liver.

A striking feature of the uptake of iron from transferrin by isolated hepatocytes is the pronounced increase in iron uptake observed when the concentration of oxygen in the incubation medium is lowered [28,29]. These observations were originally made with rat hepatocytes in suspension using human transferrin as the iron source but the results have since been reproduced with rat transferrin and rat hepatocytes in culture (K. Thorstensen, unpublished work). The initial interpretation was that low oxygen concentration facilitated reduction of iron by preventing autoxidation and rebinding of iron to transferrin [28]. Later (see below), the oxygen effect has been supplemented with additional data in favour of a reductive release mechanism. It must be mentioned, however, that the possibility exists that the method of oxygen removal (i.e. substitution of the air above the cell suspension with nitrogen gas in a medium without any added bicarbonate) may have disturbed the binding of bicarbonate to transferrin and hence affected the binding of iron to transferrin. On the other hand, the concentration of bicarbonate, as calculated from the pCO_2 of the cell suspensions, was in the range 1–4 mM (K. Thorstensen, unpublished work).

Hypoxia has also been reported to increase iron absorption in mice and rats both *in vivo* and *in vitro*, and increased hepatic uptake of iron has also been reported in young rats subject to hypoxic conditions [30–32].

A number of reports concluding that cellular uptake of iron from transferrin involves the reduction of iron have relied on the ability of strong Fe(II) chelators to inhibit cellular iron uptake from transferrin or to pick up iron released from transferrin in the presence of cell membranes and NAD(P)H [22,28,29,33–37]. This approach has been used with reticulocytes as well as hepatocytes, and both hydrophobic and hydrophilic chelators have been employed. Generally, hydrophobic but not hydrophilic Fe(II) chelators inhibited reticulocyte iron uptake from trans-

ferrin whereas both types of chelators were effective inhibitors of hepatocyte iron uptake. From this type of experiment it was concluded that iron is reduced during cellular uptake from transferrin, and that in the hepatocyte the site of iron reduction is the plasma membrane [29,36]. However, there is a serious experimental problem associated with such studies. By introducing a strong Fe(II) chelator into a system containing diferric transferrin and a reductant (e.g. the cell) the chelator functions as a strong thermodynamic driving force which shifts the equilibrium greatly in favour of formation of the Fe(II)–chelator complex, even when the reductant in itself is unable to reduce transferrin-bound iron [38]. Thus, no inferences to whether transferrin-iron is reduced by the cell can be made from such experiments. At best, due to the fact that only hydrophobic chelators are effective inhibitors of reticulocyte iron uptake, the chelator experiments [36] indicate that the reticulocyte and the hepatocyte release iron from transferrin at different locations.

Although the argument for a reductive mechanism of cellular iron uptake relies heavily on the effect of Fe(II) chelators, there are additional lines of evidence in favour of the redox mechanism. One such line of evidence is related to the description and characterization of a plasma membrane redox system apparently ubiquitously distributed throughout the animal and plant kingdom (for review see [39]). The system, termed ferricyanide reductase or NADH:ferricyanide oxidoreductase, has been shown to be able to reduce extracellular electron acceptors by furnishing reducing equivalents from cytosolic NADH to the cell surface. Associated with this redox system is a proton-pumping activity directing an efflux of protons from the cell. The proton efflux seems to occur via the plasma membrane Na^+/H^+ antiport [40,41]. The oxidoreductase readily reduces extracellular ferricyanide and apparently also transferrin-bound iron [37]. This latter finding is hampered by the use of bathophenanthroline disulphonate to assay iron reduction, and a later study was unable to reproduce the finding [38]. However, under anaerobic conditions rat liver plasma membranes were apparently able to release iron from transferrin in the absence of bathophenanthroline disulphonate but in the presence of NADH [37]. The rate of NADH oxidation by the membranes was also increased upon addition of transferrin to the system [37], and this finding has been independently confirmed [38].

In hepatocytes, inhibitors of the NADH:ferricyanide oxidoreductase also inhibit iron uptake from transferrin [29]. Furthermore, a low oxygen concentration, which stimulates hepatocyte iron uptake, increases the cellular NADH concentration and the activity of the redox system [29,42]. Lastly, ferricyanide inhibits uptake of iron from transferrin by hepatocytes in suspension [29] as well as in culture (K. Thorstensen, unpublished work), whereas transferrin inhibits the reduction of ferricyanide [29]. Thus, a pronounced correlation exists between the activity of the redox system and the rate of iron uptake from transferrin by the hepatocyte.

A serious obstacle to the model of reductive release of iron from transferrin is the fact that at neutral pH the reduction potential for transferrin iron is much more negative than that of NADH. For that reason, the model has incorporated a hypothesis of destabilization of the iron–transferrin bond following the binding of transferrin to the receptor. It should be clear that to date no direct evidence for such destabilization and its assumed effect on the iron reduction has been reported. However, it may be calculated that by lowering the pH from 7.4 the overall equilibrium constant for the dissociation and subsequent reduction of transferrin iron by NADH changes in favour of reduction. The magnitude of the change will, of course, depend on the pH fall and the presence of any iron-binding compounds.

Another question left largely unanswered is how iron is transported across cellular membranes, i.e. what is the iron carrier and in which valency state is iron during transport? Peters *et al.* [43] have presented results suggestive of free fatty acids playing a role in membrane iron transport in isolated brush border vesicle membranes, and Glaus & Schneider [44] have proposed mixed-ligand copper(II) complexes as models of membrane iron binders. As to the valency state of iron, Peters *et al.* [43] found that the fatty acids bound Fe(II). We have favoured the ferrous state because iron uptake correlates with the hepatocyte membrane's redox activity, and also because iron uptake is inhibited by divalent cations of ionic radii similar to that of ferrous iron [29,36]. However, others favour the Fe(III) state [22,25,35] and the data accumulated so far do not allow any firm conclusions to be drawn.

Another interesting problem relates to the effect of calcium. Calcium is virtually obligatory to the uptake of iron from transferrin (T. Nilsen, unpublished work) (see also [29]), as well as from non-transferrin iron complexes [45]. In the hepatocyte the effect of calcium cannot be ascribed to effects on transferrin binding, plasma membrane redox-activity, proton pumping or endocytosis (T. Nilsen & K. Thorstensen, unpublished work). This opens an interesting possibility so far not explored—the effect of calcium on membrane fluidity [46] and the significance of membrane fluidity to transmembrane iron transport. In fact, preliminary experiments do suggest that calcium enhances iron uptake by modulating membrane fluidity [I. Romslo & T. Nilsen, unpublished work].

The redox model as presented in Fig. 1 starts, as does the RME model, with the binding of transferrin to the cell surface receptor. From this step the two models diverge; in the redox model the concerted action of protons and reducing equivalents furnished by the redox system in close proximity to the transferrin receptor evoke the destabilization of the transferrin–iron bond and the reduction of iron. The ferrous iron is bound by a membrane binder/carrier specific for Fe^{2+}. Iron is then translocated across the membrane to the cytosolic side where it is picked up by cytosolic iron acceptors.

In view of the redox model as independent of RME but dependent on transferrin binding by the transferrin receptor, some aspects of hepatocyte iron uptake unaccounted for by the RME model may be explained. For example, weak bases or ionophores which disrupt the low pH of the endosomal compartment and inhibit the uptake of iron from transferrin by most cell types have no or very little effect on hepatocyte iron uptake [22,29]. The effect depends on the concentration of transferrin and also on any additional effects of the weak base. For instance, methylamine raises the pH of endocytotic compartments containing FITC–transferrin but has no effect on hepatocyte iron uptake from transferrin [29] unless the concentration of transferrin is low [47,48]. By comparison, the weak base chloroquine which, in addition to its pH-raising effect, also inhibits the hepatocyte plasma membrane NADH:ferricyanide oxidoreductase, reduces hepatocyte iron uptake from transferrin by 45–50% regardless of the transferrin concentration [47]. Such results may be interpreted to mean that in the hepatocyte the pH-raising effect works only on a minor part of iron uptake via classical RME whereas inhibition of the redox system affects the main pathway of iron uptake.

Transferrin internalized by RME needs a few minutes to traverse a complete cycle of endo-/exo-cytosis and during this time no uptake of iron in excess of transferrin should occur. Yet, observations made in this laboratory have shown that already during the first minute of incubation of isolated hepatocytes with transferrin at 37 °C the iron/transferrin ratio is significantly increased [47]. Also, following only 60 s of incubation at 37 °C more iron than transferrin is unavailable to Pronase and some

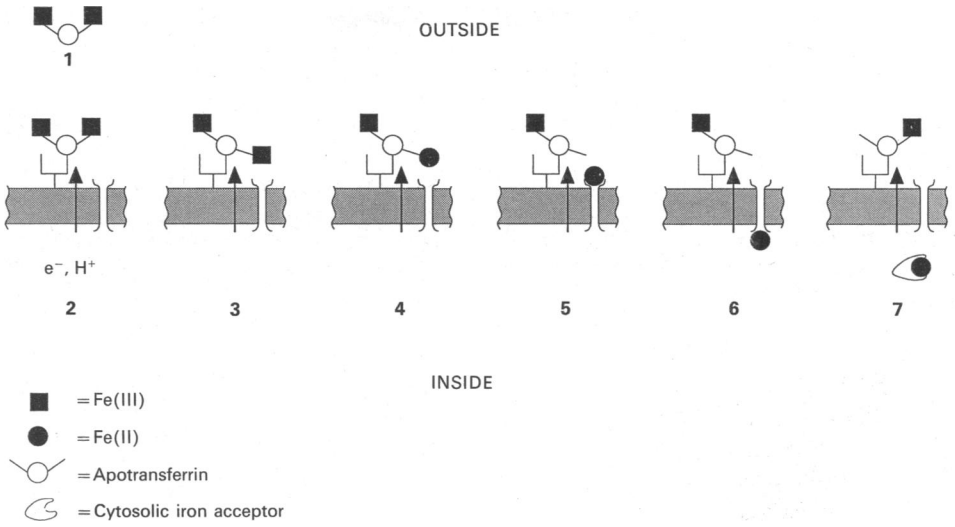

Fig. 1. The redox model

The redox model starts, as does the RME model, with the binding of transferrin to the cell surface transferrin receptor (**1**). From this step the two models diverge; in the redox model, before the transferrin–transferrin receptor complex becomes trapped within endocytic vesicles, the concerted action of protons and reducing equivalents (**2**) furnished by the NADH:ferricyanide oxidoreductase in close proximity to the transferrin receptor evokes the destabilization of the transferrin–iron bond (**3**) and the reduction if iron (**4**). The ferrous iron is bound by a membrane binder/carrier specific for Fe^{2+} (**5**). Iron is then translocated across the membrane to the cytosolic side (**6**) where it is picked up by cytosolic iron acceptors (**7**).

30–40% of cell-associated iron is found in cytosol eluting as ferritin upon h.p.l.c. gel filtration [49]. Such observations are not readily explained by the RME model but may be consistent with the redox model.

Other models

In addition to the models of hepatocellular uptake of iron from transferrin described above, another type of model implies that transferrin and/or the transferrin receptor is of minor importance to the hepatocyte iron uptake.

The number of transferrin receptors on the hepatocyte plasma membrane is relatively few [7], and at 37 °C cellular uptake of iron continues to increase when the extracellular concentration of transferrin is increased far in excess of the concentration needed to saturate all transferrin receptors [34,48,50,51]. This has led some investigators to conclude that adsorptive or fluid-phase endocytosis is the main mechanism of hepatocyte uptake of iron from transferrin [48,50,51]. Such mechanisms would involve a receptor-independent release of iron from transferrin. Since hepatocyte iron uptake from transferrin is insensitive to agents which raise pH [22,29], release of iron at acidic pH is probably not the mechanism. The model also implies that the sorting of transferrin to avoid lysosomal degradation of the protein is independent of the binding of transferrin to the receptor. This may be accomplished by extensive recycling from prelysosomal compartments, as recently demonstrated to occur in rabbit hepatocytes [52]. Alternatively, pinocytic vesicles containing transferrin may fuse with endocytic vesicles containing unoccupied transferrin receptors (see [53] and references therein). If iron and transferrin are indeed separated in fluid-phase endocytosis compartments, then the components needed to transport iron may not be found exclusively in the endosomal membrane or the plasma membrane. The process of iron transport across the membrane would be independent of the transferrin receptor. In line with this is the observation that the hepatocyte is able to accumulate iron not bound to proteins [42,45], and hence by a mechanism independent of endocytosis. The efficiency of this type of iron uptake is often much greater than iron uptake from transferrin. The ability to utilize iron from simple iron salts has also been described for other cell types, e.g. reticulocytes [54–56], and L1210 cells [57].

In a series of reports Tavassoli and co-workers have described experiments aimed at elucidating the interplay between the hepatocyte and the endothelial cell of the liver in the uptake of iron from transferrin [58–62]. Through these studies a model was suggested in which modification of transferrin by the endothelial cell is a prerequisite for hepatocyte uptake of iron from transferrin. In this model circulating diferric transferrin binds to transferrin receptors on the endothelial cell, whose cell surface transferrin receptor number is in the order of 2×10^6 per cell [59] (or even as high as 14×10^6 per cell according to their estimates [58]). Transferrin is then internalized by RME but iron is not separated from transferrin. Instead, transferrin is desialylated and diferric asialotransferrin is released from the endothelial cell into the space of Dissé. The asialotransferrin is then bound by the hepatocyte asialoglycoprotein receptor and taken into the cell by RME. Iron is released from asialotransferrin, which in turn is partly degraded and partly resialylated and released to the extracellular medium. The model thus renders the direct contribution of circulating transferrin to the hepatocyte iron uptake very much a minor one.

It should be mentioned that the estimates of endothelial cell surface transferrin receptor made by Tavassoli and co-workers are very much in discrepancy with other studies reporting 5.5×10^3 receptors per cell [63]. Furthermore, 5 min after the injection of ^{125}I-labelled rat diferric transferrin into rats, 55–67% of liver ^{125}I is found in hepatocytes, and preinjection of unlabelled transferrin 2 min ahead of the labelled transferrin reduces the above figure by 50% [64]. Finally, a rabbit anti-(rat transferrin receptor) antibody has been shown to decrease the binding of transferrin to cultured rat hepatocytes and to reduce the uptake of iron from transferrin [65]. This demonstrates that at least part of the hepatocyte uptake of iron from transferrin occurs via specific interaction with transferrin receptors.

Aisen and co-workers have presented results which indicate that transferrin and the transferrin receptor are of minor importance to the hepatocyte iron uptake [66–69]. They have described a process which relies on the co-operation of Kupffer

cells and hepatocytes to supply the latter cell with iron via ferritin. In their model, Kupffer cells digest effete erythrocytes. The iron in haemoglobin is released from the Kupffer cell in the form of ferritin and partly in a form which can be picked up by apotransferrin. The ferritin binds to hepatocyte ferritin receptors and is subsequently delivered to lysosomes via RME. Here, the ferritin protein shell is degraded and iron is released to cytosolic ferritin and mithochondria. The rate of ferritin endocytosis appears to be significantly lower than the endocytosis of transferrin but the number of iron atoms delivered to the cell by each ferritin is so large that the resultant iron delivery by ferritin exceeds that by transferrin by an order of magnitude.

RELEASE OF IRON FROM HEPATOCYTES

The liver is the major iron-storage organ and functions as a depot from which iron, stored in ferritin, may be withdrawn in times of increased demands for iron by the erythron. As a consequence, the hepatocyte must have mechanisms for the release of iron from intracellular ferritin to circulating transferrin. The number of studies covering this important aspect of hepatocellular iron metabolism is, however, remarkably small. Moreover, existing data are often conflicting.

The release of iron from the liver is normally a slow process. A recent study on rats injected with ^{59}Fe-labelled ferritin shows a release of iron from the liver of approx. 6% per day [70]. In various systems of hepatocytes in culture or suspension the basic release rate varies between 2.5 and 10% per h. However, by addition of chelators to the extracellular medium the rate of iron release may be increased, although usually only modestly. The effects of various additions or manipulations on the incubation conditions are summarized in Table 3. The basic release rate varies considerably depending on experimental factors (e.g. incubation medium, iron loading time and source of iron). Only high concentrations of citrate or hypoxia induced by N_2 gas produce iron release outside the range of basic release rates.

A finding which appears to be consistent throughout is that the relative amount of iron released from the hepatocyte increases when the preloading time decreases. This suggests that the iron most readily mobilized is in a state of transit between intracellular compartments, and not sequestered in ferritin. Apotransferrin, the ultimate physiological iron acceptor, appears to function as a passive extracellular iron acceptor without influencing the intracellular steps of iron mobilization.

The mechanism by which hepatocyte iron release is regulated remains completely unknown. The finding that low oxygen concentration also increases iron release from hepatocytes, together with the fact that iron is most readily mobilized from ferritin under reducing conditions [26], may be indicative of a reduction process. Speculations in this direction have been presented in discussions on iron release from BeWo choriocarcinoma cells [77]. Another interesting finding of possible physiological relevance is that serum from anaemic rats apparently contains factors that increase iron mobilization from normal rat hepatocytes [76]. The lack of effect of inhibitors of endo-/exo-cytosis is a strong argument against the involvement of the endocytic system in the iron release process [73].

Thus, the bottom line is that our knowledge regarding the mechanism of hepatocyte iron release is scarce.

Table 3. Release of iron from hepatocytes

Data are compiled from [70–76] and references therein.

Additions	Concentration	Release rate (%/h)
None	–	2.5–10
Apotransferrin	0.1–3.0 mg/ml	0–7
ATP	2 mM	0
Pyrophosphate	2 mM	2
Citrate	2.2–25 mM	2.2–45
Desferrioxamine	0.05–1 mM	1.1–10
Nitrilotriacetate	1 mM	1.2
Hypoxia (N_2)	–	30
Hypoxia (CO_2)	–	2
Hyperoxia (O_2)	–	7
Serum	50% (v/v)	0.9–2.5
Anaemic serum	50% (v/v)	6.5

TRANSFERRIN AND THE REGULATION OF CELL GROWTH

Transferrin has long been recognized as an essential factor for cell growth, and a number of studies have shown that the number of transferrin receptors on the cell surface is closely regulated by the cell's proliferation state as well as its iron status (see for example [12,14,78,79]). Antibodies directed against the transferrin receptor inhibit cell growth provided that the antibody interferes with the binding of transferrin to the receptor or the endocytotic cycle of the receptor [80].

Changes in iron status appear to provoke changes in transferrin receptor synthesis whereas, at least in the regenerating liver, changes in proliferation status result in redistribution of the transferrin receptor from intracellular compartments to the cell surface. [81]. Thus, it is widely accepted that cell growth is regulated via the transferrin receptor by transferrin's ability to bind to the receptor and deliver iron to the cell by means of RME.

An alternative, more controversial, hypothesis exists to explain transferrin's role in growth regulation: modulation of the cell's ability to transfer reducing equivalents from cytosol to extracellular electron acceptors regulates cell growth. Transferrin iron would be (one of) the physiological electron acceptor(s). Hence, the cell, by regulating its number of cell surface receptors for transferrin, regulates not only its ability to sequester iron from transferrin but also its potential ability to donate electrons to transferrin iron. What would be the foundation of such a hypothesis? In 1983 Ellem & Kay [82] demonstrated that ferricyanide could sustain the growth of human melanoma cells under serum-free conditions. This effect was apparently due to the ability of ferricyanide to function as a sink for electrons furnished through the plasma membrane NADH:ferricyanide oxidoreductase. The growth-promoting effect of ferricyanide (as well as other ferric chelates) could not be ascribed to their iron content, since non-iron extracellular oxidants were later shown to have similar effects provided their reduction potential was more positive than −125 mV [83].

Based on these findings it has been demonstrated that all the characteristic intracellular signals and responses associated with cell growth — i.e. alkalinization of the cytosol through proton extrusion via the Na^+/H^+ antiport [84], increase in cytosolic free calcium concentration [84], changes in intracellular NAD^+/NADH ratio [85] and activation of 'immediate early genes' (e.g. c-*myc* and c-*fos*) [86] — may be triggered by stimulation of the NADH:ferricyanide oxidoreductase [40,41,87–90].

Furthermore, growth inhibitors such as adriamycin, bleomycin and retinoic acid are also inhibitors of the redox system [91–93], and cell growth promoters also stimulate the NADH:ferricyanide oxidoreductase [94,95]. Finally, transferrin stimulates the redox system and induces proton efflux via the Na^+/H^+ antiport [40,41,96], whereas some antibodies against the transferrin receptor inhibit the redox system [97]. Thus, variations in the cell's

ability to donate electrons to extracellular electron acceptors may play a vital role in regulating cell growth. *In vivo* the physiological electron acceptor is transferrin. Transferrin's ability to accept electrons from the cell would in turn be dictated by the number of transferrin receptors.

It must be noted that Ellem *et al.* [98] recently proposed an alternative explanation for the growth-promoting effect of at least some of the extracellular electron acceptors, i.e. oxidation of H_2O_2 commonly found in synthetic cell culture media, particularly following light exposure.

TRANSFERRIN AND ALCOHOLIC LIVER DISEASE

Ethanol affects iron homeostasis in a number of ways, the most conspicuous clinical manifestations being fatty liver, liver cirrhosis and hepatic siderosis [99–101]. The mechanisms behind liver iron accumulation associated with excess ethanol intake are obscure. This arises largely from the fact that the mechanisms may change during the progress of the condition from normality to manifest and life-threatening liver failure [99–101]. Also, one has to cope with the problem of extrapolating from findings in experimental animals to the situation *in situ* in man.

Human transferrin contains two glycans at positions 413 and 611 [102,103]. The terminal part of the glycans may be branched, giving rise to bi-, tri- or tetra-antennary structures which are normally terminated by a sialic acid residue. However, when one or more of the sialic acid residues are missing transferrin shows micro-heterogeneity, and following isoelectric focusing up to nine different electrophoretic fractions may be observed [103]. The main transferrin component in normal human serum contains 4 mol of sialic acid per mol of transferrin (tetrasialotransferrin). Transferrins with different sialic acid contents are much less abundant [104]. In serum from alcoholics, however, abnormal microheterogeneity of transferrin is observed [105–107]. The most striking feature is a marked increase in disialo-transferrin and to a lesser extent monosialo- and asialotransferrin. Interestingly, changes in the carbohydrate moiety of plasma proteins other than transferrin have been reported in alcoholics [108].

Regoeczi *et al.* [109] have shown that compared with fully sialylated transferrin both iron and the protein are more readily accumulated when normal rat hepatocytes are incubated with diferric asialo-transferrin. In a recent paper Irie & Tavassoli [110] have shown that transferrin is desialylated during transport of transferrin through the endothelial cells of the liver. This desialylation affects almost exclusively the tri-antennary transferrin component, leaving the bi- and tetra-antennary transferrin unaffected. The authors forward the interesting idea that alcohol abuse not only changes the degree of sialylation but possibly the glycan structure of transferrin as well, hence increasing the relative amounts of tri-antennary transferrin. The excess tri-antennary transferrin would then readily be desialylated during passage through the endothelial cell giving rise to transferrin species which are effectively cleared by the hepatocyte asialoglycoprotein receptor. However interesting, the model is still a hypothetical one. In fact, to explain liver iron excess during alcoholism only through additional uptake of asialotransferrin is hardly tenable for several reasons. Firstly, a number of alcoholics do not have excess liver iron [99,111]. Secondly, Petrén & Vesterberg [112] found that in patients known to abuse alcohol, and who had a decreased total serum transferrin level and increased disialo-transferrin level, total transferrin increased but the disialo-transferrin level did not drop significantly during 10 days of abstinence. The authors concluded that excess ethanol consumption increased transferrin synthesis *de novo* and deranged the clearing of desialylated transferrin from the circulation by the hepatocyte. This conclusion is in line with previous studies which showed increased transferrin synthesis as well as transferrin turnover in patients with alcoholic fatty liver [101], and the loss of hepatocyte cell surface receptors for asialoglycoproteins in ethanol-fed rats [113].

Finally, liver iron varies with the pathological changes induced by alcohol consumption [100] and the uptake of iron from asialotransferrin is, at least *in vitro*, too fast to explain the rather modest increase in liver iron stores in alcoholism. Thus, if the content of sialic acid in transferrin is a pathogenetic factor to liver iron storage in alcoholism, additional mechanisms must also be looked for.

An interesting idea is that the ethanol-exposed hepatocyte itself is responsible for the abnormal accumulation of iron in the alcoholic liver. Nunes and co-workers [114] studied the effect of ethanol on iron uptake by isolated rat hepatocytes. In their experiments ethanol reduced iron uptake, and at the same time produced a significant decrease of the pH of the incubation medium. In a subsequent paper these findings were examined in more detail [115]. A most unexpected finding was that iron uptake from transferrin decreased with decreasing pH. This finding is at variance with the well-known behaviour of transferrin-bound iron at low pH, i.e. dissociation of iron from transferrin at low pH. In a more recent study, Stenberg & Romslo [116] could not confirm the findings of Nunes and co-workers [114,115]; in fact hepatocyte uptake of iron from transferrin was increased in the presence of ethanol and iron uptake was inversely correlated to pH. Ethanol had no effect on transferrin binding by the cells and the effect of ethanol was inhibited by the alcohol dehydrogenase inhibitor 4-methylpyrazole. The reasons for the discrepancy between these two studies are unclear but may relate to differences in experimental conditions. It should also be mentioned that the data of Nunes *et al.* [115] indicate that in their system fluid-phase endocytosis, as assessed by measuring accumulation of inulin, was approx. 10-fold higher than values reported by other investigators [34,48,117]. Furthermore, in the study of Nunes *et al.* [115] the time course of iron uptake from transferrin was non-linear and the average rate of iron uptake could be calculated to approximately 50 pmol of Fe/h per 10^6 cells. This corresponds to approximately 490000 Fe atoms/min per cell. By comparison, the rate of iron uptake by the reticulocyte is approximately 100000 Fe atoms/min per cell. In none of these studies, however, are data regarding the sialic acid content of the transferrin disclosed.

Sherlock and co-workers [118] have speculated that ethanol could somehow interfere with the efflux of iron from hepatocytes. As far as we are aware, experiments to explore this possibility in more detail have not yet been reported.

Other possible mechanisms for excess liver iron during alcohol abuse are increased iron intake and absorption, deranged intermediary metabolism and hepatocyte damage [99–101, 118–120]. As yet, definite proofs for their significance are lacking. As to the mechanism by which iron accumulation may interfere with hepatocyte function, most studies focus on the avidity with which iron may accelerate the formation of toxic oxygen species [121]. Iron and ethanol have additive effects on lipid peroxidation [121–123]. A possible sequence of reactions is the following: intake of ethanol produces NADH via alcohol dehydrogenase, this in turn mobilizes iron from ferritin, and non-haem iron thus mobilized catalyses the production of toxic oxygen species, of which $OH^{\cdot}$ is the most potent.

IRON AND PORPHYRIA CUTANEA TARDA

The most consistent abnormality of iron metabolism observed

in patients with porphyria cutanea tarda (PCT) is the presence of hepatocellular and Kupffer cell siderosis which is found in more than 80% of individuals with significant uroporphyrinuria [124]. As yet, however, the relationship between excess liver iron and the clinical manifestations of PCT remains controversial. We do not know whether liver iron accumulation is caused by the primary defect or results from secondary changes [124]. Neither do we know if excess iron is obligatory to the clinical manifestations of PCT [125]. What we do know, however, is that liver iron depletion through phlebotomy improves the condition of patients with PCT [125,126].

Much effort has been devoted to explore the relationship between haemochromatosis and PCT but as yet no definite answer can be given [127]. In general, patients with PCT (sporadic or familiar) do have significantly less liver iron than patients with haemochromatosis [128]. According to Lundvall *et al.* [129], in patients with manifest PCT, liver iron averaged 2–3 times that of healthy controls, whereas, in individuals with latent PCT, liver iron was no different from that of controls.

To explain increased liver iron one has to consider increased intake, increased absorption, deranged internal iron distribution or decreased iron losses. Increased iron intake as an aggravating factor to the manifestations of PCT has been amply documented in the Bantus [130], but these findings do not explain the preponderance of excess liver iron in PCT in other areas. Turnbull *et al.* [131] and Reizenstein *et al.* [132] reported increased iron absorption in overt PCT and this was found also in the presence of excess liver iron. As to the relationship between decreased uroporphyrinogen decarboxylase activity and excess liver iron, most studies suggest an inverse relationship between enzyme activity and liver iron [133–135] although Blekkenhorst *et al.* [136] claimed that ferrous iron enhances uroporphyrinogen decarboxylase activity. According to Elder & Sheppard [137], in PCT there is loss of enzyme activity but no loss of enzyme protein.

Evidence has been presented that in PCT there is a tendency of uncoupling of oxidative phosphorylation, and this is more pronounced if iron excess coexists. The effect is explained through lipid peroxidation with the generation of (presently) unknown metabolites [138]. In a recent paper Jacobs *et al.* [139] have shown that uroporphyrinogen oxidation by hepatic microsomes was increased by the addition of iron, and Elder *et al.* [128] have forwarded a model in which iron operates in concert with a specific cytochrome *P*-450 isoenzyme that generates reactive oxygen species and produces an inhibitor of uroporphyrinogen decarboxylase.

So far, however, the role of iron, be it a primary or a secondary factor to the manifestation of PCT, remains to be established. Also, it remains to be established if transferrins with different glycan chains exist or if PCT gives rise to excess non-transferrin-bound iron.

FUTURE RESEARCH

Under this heading we try to summarize and localize those parts of the process of cellular iron uptake and metabolism which still remain largely unknown or controversial and hence need future research effort. Such areas we designate as 'black boxes' and they are presented in Fig. 2.

Strikingly, most of the 'black boxes' relate to the dynamics of intracellular iron transport. These boxes represent the question of whether the interaction of transferrin with the transferrin receptor provokes destabilization of the transferrin-iron bond (box **1**), how iron is transported across cellular membranes (be it the plasma membrane, the endosomal membrane or the mitochondrial inner membrane) and how this transport is regulated

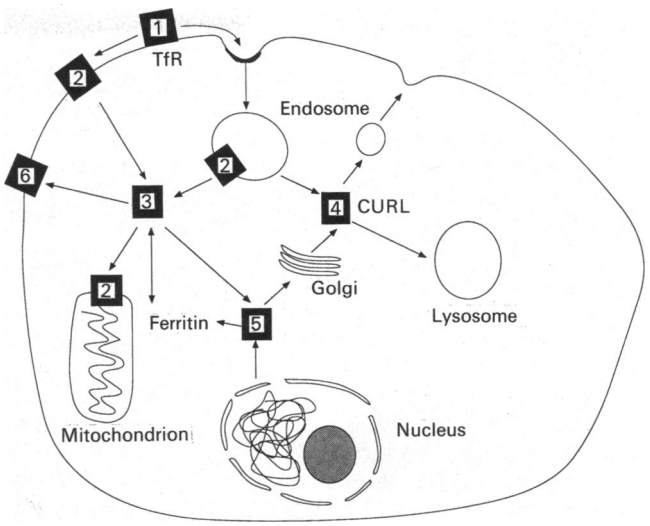

Fig. 2. 'Black boxes'

This Figure localizes some problem areas related to the process of cellular uptake of iron from transferrin. The 'black boxes' signify steps in the process which are mostly unresolved and hence need future research effort to be clarified. **1.** Is binding of transferrin to the transferrin receptor (TfR) an obligatory step, and does the binding provoke destabilization of the transferrin–iron bond? **2.** How and in which form (ferric or ferrous) is iron transported across cellular membranes? **3.** What is (are) the cytosolic iron acceptor(s)? **4.** How is apotransferrin sorted for exocytosis and not degradation? (CURL is defined in the text.) **5.** How and in what form does intracellular iron exert its regulatory function on both ferritin and transferrin receptor synthesis via the iron responsive elements? **6.** What is the mechanism of hepatocyte iron release?

by such variables as oxidation state, membrane potential, intra- and extra-cellular ions, membrane components and transmembrane ion fluxes (boxes marked **2** and **6**), what is (are) the intracellular iron acceptor(s) (box **3**) and how is the interplay between the iron acceptor(s) and ferritin and the iron-responsive elements involved in the regulation of both ferritin and transferrin receptor synthesis (box **5**). On the transferrin side of the Figure the most prominent question appears to be how apotransferrin is sorted in the compartment of uncoupling and recycling of ligand (CURL) to escape routing to the lysosomes for degradation and instead is recycled to the cell surface (box **4**).

Thus, at present, in spite of our increasing knowledge of what is going on in cellular iron metabolism, we are still left with more questions than answers.

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