The transport of iron and copper across the cell membrane: different mechanisms for different metals?

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Iron and copper are both essential elements and deficiencies in either lead to a wide variety of symptoms. They also appear to be linked to each other biologically. One of the most characteristic hallmarks of Cu deficiency is a hypochromic, microcytic anaemia. The mechanism involved has not been worked out, but it seems that Cu, especially and perhaps exclusively as ceruloplasmin (*EC* 1.16.3.1), is necessary to maintain the flow of Fe through the hepatocyte (for a recent review, see Linder, 1991).

So much work has been done on the steps involved in Fe uptake that the number of examples used to illustrate any point has been restricted. Since the mechanism is essentially the same in all tissues studied thus far, the present discussion will use the placenta as the major example. Information gained from other tissues and cell types will be discussed where appropriate, however. In contrast, since little is known about Cu transport, data from a variety of different cell types will be discussed.

Fe in plasma is almost exclusively carried on transferrin (Tf). Tf is a serum glycoprotein, molecular weight about 80 000. It can carry two Fe atoms per molecule, which it binds in two extremely-high-affinity sites, one in the N-terminal domain and the other in the C-terminal domain (for a recent review, see de Jong *et al.* 1990). Tf is the major source of Fe for most cells, although there is evidence that some cells, especially in human and guinea-pig, can utilize the Fe carried in serum ferritin (Blight & Morgan, 1987; Lamparelli *et al.* 1989).

Most of the plasma Cu is also protein bound. The majority is associated with ceruloplasmin, an α-globulin which has a sky-blue colour (hence the name) (Holmberg & Laurell, 1947, 1948). The Cu is incorporated into the protein in the liver. There are six or seven Cu atoms per molecule (Messerschmidt & Huber, 1990), none of which are readily exchangeable. They are found as three types: I, II and III; respectively, these are the 'blue' Cu (type I), Cu attached to histidyl imidazoles similar to the low-molecular-weight histidine–Cu complexes (type II), and the type III Cu, a pair forming a diamagnetic complex (Calabrese *et al.* 1989). In vitro, only the blue Cu can be exchanged. This requires the presence of an oxidizing agent, such as vitamin C, and Cu with which to exchange. Removal of the other Cu atoms necessitates very harsh treatment (Herve, 1985; Winyard *et al.* 1989). In addition to ceruloplasmin, significant (perhaps as much as 20%), and probably biologically important amounts of Cu are found attached to a specific site on the N-terminal end of albumin (Lau & Sarkar, 1971) and to amino acids, primarily histidine (Harris & Sass-Kortsak, 1967; Neumann & Sass-Kortsak, 1967). A high-molecular-weight plasma protein, transcuprein, has been described by Linder and co-workers (Weiss *et al.* 1985; Wirth *et al.* 1985) which may also be involved in Cu uptake, especially by hepatocytes. Neither Cu nor Fe are present as the free ion.

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PLACENTAL Fe UPTAKE

The transfer of Fe from mother to fetus can be considered as occurring in three stages: uptake across the placental membrane into the placental cell, transfer across the cell and release into the fetal circulation. In the present review, neither the transplacental carriage of Fe, nor the release of the metal to the fetal circulation will be examined, but the discussion will be restricted only to uptake. Similarly, neither Cu efflux nor transcellular Cu transport will be considered, concentrating only on Cu uptake into the cell.

Tf binding to the Tf receptor. The first stage in uptake is the binding of Tf to the Tf receptor on the chorionic microvilli at the maternal surface of the placenta.

The concept of a receptor for Tf was first suggested for reticulocytes by Jandl & Katz (1963). Abundant evidence was provided for the existence of a receptor, but despite vigorous attempts, the receptor eluded isolation (for a review, see Morgan, 1981). Trowbridge & Omary (1981) reported that a monoclonal antibody raised against membranes of a human leukaemic cell line was immunoreactive against a protein they showed to be the transferrin receptor (TfR) and the protein sequence has now been elucidated (McClelland *et al.* 1984). TfR is a glycoprotein with a molecular weight of 190 000, consisting of two identical dimers, linked by disulphide bridges and with the N-terminals inside the cell.

Diferric Tf binds to the receptor with an affinity variously estimated as being between 10^6/M and 10^9/M (Bleil & Bretscher, 1982; Harding *et al.* 1983; Iacopetta & Morgan, 1983; McArdle *et al.* 1984*b*; Page *et al.* 1984; Bierings *et al.* 1988; de Jong *et al.* 1990). No clear explanation has been found for the different estimates, but it may be relevant that 'normal' cells, such as reticulocytes and primary cultures of placenta and liver, have a lower affinity than transformed or permanent cell lines.

Binding is pH dependent, with the diferric Tf having a high affinity at pH 7·4 and a low one at pH 5·5. At lower pH values, the affinities are reversed. The biological relevance of this observation will be discussed later (p. 201). The number of binding sites varies from cell to cell, with dividing cells usually having higher numbers than quiescent cells (Musgrove *et al.* 1984).

Internalization of the Tf–TfR complex. Following binding, the Tf–TfR complex is internalized. The process involves accumulation into coated pits, followed by sealing into a coated vesicle (Harding *et al.* 1983; Hopkins, 1983; Hanover *et al.* 1985). The vesicle, which can contain different receptors and their ligands, then goes through a complex sorting process. The mechanisms involved in the sorting are outwith the scope of the present review but are outlined in de Jong *et al.* (1990).

Internalization of the Tf–TfR complex as a necessary precursor to Fe accumulation was first suggested in 1969 (Morgan & Appleton, 1969) and, after some debate, has now been accepted as the major way in which Tf is processed (Bleil & Bretscher, 1982; Harding *et al.* 1983; Hopkins & Trowbridge, 1983; Iacopetta & Morgan, 1983). Alternative pathways may also operate in the liver, where 'non-specific' Tf and Fe uptake can become quantitatively important (e.g. see Morley & Bezkorovainy, 1985; Trinder *et al.* 1990).

In the placenta, internalization into coated vesicles was indirectly demonstrated by Booth & Wilson (1981), who isolated the vesicles from human placenta and showed that they contained Tf and immunoglobulin G. Further support was provided by Pearse (1982). Direct evidence that placental cells operate the same mechanism as other cell

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types had to wait, however, until placental cells were grown in culture and Tf and Fe uptake measured under these controlled and controllable conditions.

This was first accomplished by our group in 1984 (McArdle *et al.* 1984*a*, 1985) using rat placental cells. The results obtained were, in essence, the same as those described for other cell types. Since then, several groups have refined the culture process, and data has now been presented for cultured human placental cells, both cytotrophoblast and syncytiotrophoblast (Bierings *et al.* 1988; Bierings, 1989; Douglas & King, 1990). In rat placental cells the ratio of receptors on the surface:those inside the cell was lower than in other cells; 10% internalized at any one time as opposed to 50% (Bierings, 1989) or 60% (Douglas & King, 1990) in human trophoblast cells in culture, 70% in human fibroblasts (Wiley & Kaplan, 1984) and up to 80–90% in reticulocytes and erythrocyte precursors (Morgan, 1981).

Fe release from Tf. Following internalization into vesicles, the pH decreases to between 5·5 and 6·4 (van Renswoude *et al.* 1982; Paterson *et al.* 1984). The mechanism involved in the decrease is uncertain, but it is likely that a hydrogen ion pump is activated. The decrease has several effects. First, the receptor undergoes a conformational change resulting in self association, which in turn decreases sensitivity to protease activity (Turkewitz *et al.* 1988). Second, the decrease results in a drop in the affinity of Tf for its Fe, so that, in the presence of a chelator, citrate or EDTA, for example, the Fe can be removed for transfer across the vesicle membrane and intracellular processing. Third, apoTf has a high affinity for the receptor at low pH. Thus, conversion of the Tf to the apoprotein means that it stays bound to the receptor, again increasing protection against proteolysis and allowing recycling to the membrane surface (Dautry-Varsat *et al.* 1983; Rao *et al.* 1983; Paterson *et al.* 1984).

There is some evidence that, in the reticulocyte and placenta at least, removal of the metal is either accompanied by or preceded by, reduction of the ferric ion to ferrous ion. Fe^{2+} chelators such as 2,2′-bipyridine and 1,10 phenanthroline, which block uptake of Fe from Tf, complex the Fe while it is still within the vesicles in reticulocytes (Baker *et al.* 1985) and placental cells in culture (Wong *et al.* 1987).

There is limited evidence that the transfer of Fe across the vesicle membrane is a protein-mediated process, and Morgan (Bowen & Morgan, 1987) has suggested that this protein is defective in the Belgrade rat, which has a genetic deficiency resulting in anaemia.

Exocytosis of the Tf–TfR complex. Following uptake into endosomes, most ligands are degraded. As discussed previously, however, Tf remains attached as the receptor is returned to the membrane surface where the apoTf is released. The TfR is, thus, free to undergo another cycle, and it has been estimated to perform as many as 300 cycles before being broken down (Omary & Trowbridge, 1981).

Cycling continues whether or not the receptor is occupied (Hopkins & Trowbridge, 1983). The rate of cycling of the receptor is different for different cell types and appears to depend on the Fe requirement of the cell. In reticulocytes, cycling is about 3 min, but can be as much as 10 min in other cells (Karin & Mintz, 1981; Hopkins, 1983; Hopkins & Trowbridge, 1983; Iacopetta & Morgan, 1983; McArdle *et al.* 1984*a*).

CELLULAR Cu UPTAKE

Since so little is known about Cu transfer across the placenta, it will not be possible to concentrate on this or any other cell type in particular. This part of the review, therefore,

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will draw on data derived from a variety of cell types. At the moment, there appears to be two major mechanisms operating: one system for hepatocytes and another for non-hepatocyte cells. However, as the following discussion will show, at the molecular level the uptake processes may be very similar.

Cu uptake by hepatocytes. Following transport across the gut, Cu is first associated with albumin. The protein has a Cu-specific binding site located at the N-terminal (Rakhit & Sarkar, 1981). Critical to the specificity of this site is a histidyl residue at the third amino acid position (Lau *et al.* 1974). In those species where the histidine has mutated to tyrosine (dogs and pigs, for example), the specificity of binding has been lost (Appleton & Sarkar, 1971; I. Bremner, personal communication).

Mechanism of uptake. Cu uptake is a carrier-mediated process not dependent on metabolic energy (Weiner & Cousins, 1980, 1983; Darwish *et al.* 1983; McArdle *et al.* 1988). Michaelis constant (K_m) values vary between 4 and 20 μM and the maximum velocity (V_{max}) shows similar variation. Using hepatocytes in suspension culture, Ettinger's group (Darwish *et al.* 1983; Schmitt *et al.* 1983) showed that uptake was probably from the Cu–histidine ($CuHis_2$) fraction of plasma, an observation confirmed by ourselves (McArdle *et al.* 1988). The histidine is not itself taken into the cell and dissociation of the $CuHis_2$ complex takes place at the membrane surface.

The role of albumin in Cu uptake by hepatocytes. Ettinger and co-workers (Schmitt *et al.* 1983; Darwish *et al.* 1984) also showed that the uptake was 'inhibited' by adding albumin. van den Berg & van der Hamer (1984) obtained similar results and further demonstrated that the albumin:Cu ratio was important. When high ratios were present, adding extra albumin made little difference, while at lower ratios the 'inhibition' was more marked. While we agree with the data, however, I believe that the use of the word 'inhibited' is not a good one. The serum albumin concentration is around 600 μM and the maximum exchangeable (i.e. non-ceruloplasmin) Cu concentration is no more than 2 μM. Thus, under physiological conditions, the ratio is such that the maximal 'inhibition' would always be present.

Mixing albumin with Cu at pH 7·4 results in labelling the protein not only on the high-affinity site, but also on other sites on the protein (Breslow, 1964). In order to label the protein exclusively on the specific site, it is necessary either to use high albumin:Cu ratios, to leave the two together for a considerable period of time, or to mix at pH 5·5 and then raise the pH to 7·4. This fact was exploited to study the uptake of Cu by hepatocytes more closely. It was shown that if all the Cu was on the specific site, which would probably not be the case in the experiments mentioned previously, uptake was higher than if it was on lower-affinity sites (McArdle *et al.* 1990). The effect was specific for albumins with the specific binding site, since dog albumin, which has no histidine at position 3, did not show any pH-dependent effect. Further, if histidine was added, causing the formation of the highest-affinity Cu–histidine–albumin (CuHisAlb) complex (Lau & Sarkar, 1971) then uptake was maximal (McArdle *et al.* 1990).

Conclusions. All these findings can be reconciled by the following hypothesis. It is probable that, at the molecular level, all three complexes look similar, with the Cu atom coordinating with the imidazole-N of the histidine residues and the rest of the molecule being relatively unimportant (McArdle *et al.* 1990). The Cu is removed from the complex (see p. 204) and transferred to the cell separately.

Cu uptake by non-hepatocyte cells. Which substrate is recognized by the transporter? When Cu is transported across the gut, it appears first bound to albumin. Most of the Cu

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is taken up by the liver, but as much as 40% can pass the portal system (Danks, 1988). Thus, in systemic blood, Cu exists as ceruloplasmin (synthesized in the liver), from which it cannot readily be removed, and an exchangeable pool, comprising Cu–albumin (CuAlb), Cu–amino acids, primarily Cu–histidine ($CuHis_2$) (Harris & Sass-Kortsak, 1967) and transcuprein (Weiss *et al.* 1985; Wirth *et al.* 1985). Any or all these, as well as metallothionein, which appears in small but significant amounts in plasma (Mehra & Bremner, 1983), could be the substrate for uptake.

Cu uptake from ceruloplasmin. Findings were presented from the 1960s onward (for review, see Owen, 1980) suggesting that ceruloplasmin was a likely substrate for uptake. However, this possibility was complicated by several factors. Ceruloplasmin has several other roles in the body. It is an enzyme (Gutteridge, 1983) and an acute-phase protein (Aldred *et al.* 1987). Serum levels are elevated by steroids and rise during pregnancy (Danks, 1988). Finally, Frieden (1980) showed that ceruloplasmin had a central role in the liver in Fe mobilization. Several workers used these findings as part of the argument against a transporter role for ceruloplasmin: how could such a multi-functional protein also have a role as a transporter?

Nonetheless, many of the findings were compelling. The changing concentrations of ceruloplasmin could well be related to changing demands of the different tissues. Cu, and hence ceruloplasmin, may have a role in the acute-phase response and the increase during pregnancy could be related to the increased Cu requirement of the developing fetus. Experimental evidence for a role for ceruloplasmin was first presented by Owen (1980) and Marceau & Aspin (1972, 1973) who showed that ^{67}Cu was accumulated in different tissues following injection of the labelled protein. Linder & Moor (1977) showed that both Cu from ceruloplasmin and the protein itself were accumulated in the heart and other organs of the rat.

Dameron & Harris (1987*a*,*b*) showed that Cu from ceruloplasmin could be not only taken up by aortic endothelial cells, but that the Cu was incorporated primarily into Cu,Zn superoxide dismutase (*EC* 1.15.1.1). However, they showed that Cu from albumin and amino acids could also be incorporated into the enzyme.

In K562 cells, an erythroleukaemic cell line, there was strong evidence for a role for ceruloplasmin during differentiation. Percival & Harris (1989, 1990) demonstrated that the amount of ceruloplasmin bound to the cells went up markedly when the cells were induced to differentiate with hemin. The number of sites increased to a maximum at about 72 h and, interestingly, the number of TfR decreased as the ceruloplasmin-binding-site numbers increased (Percival & Harris, 1988). Cu was only accumulated at 37° and not at 4° and was found first in a vesicular fraction separate from the protein moiety of the ceruloplasmin and later associated with superoxide dismutase. There was no evidence that the protein itself was either internalized or taken into the cell at all.

Finally, our group adopted the approach that if other plasma Cu transporters could be excluded, whatever was left had to be the substrate for uptake. Accordingly, we demonstrated that albumin was not involved in Cu uptake in fibroblasts (McArdle *et al.* 1987), a view supported by others (Laurie & Pratt, 1986) and further showed that histidine also acted as an inhibitor, the degree of inhibition being related to the amount of amino acid present. The data obtained could only be explained if it was assumed that free (i.e. ionic) Cu was the substrate for uptake (McArdle *et al.* 1987). This is extremely unlikely in vivo, so that it was concluded that ceruloplasmin was indeed a very plausible carrier of Cu for uptake into cells.

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Cu uptake from the exchangeable pool. At the same time, however, findings were being presented to suggest that low-molecular-weight Cu was also important in transport. Herd *et al.* (1987) had shown that $HisCu_2$ could be taken up by human lymphoblasts. Uptake from low-molecular-weight complexes has been used for many years to identify cells from patients with Menkes' disease, a genetic disorder of Cu metabolism (Danks, 1988; van den Berg *et al.* 1990*a*).

Recently, it has been shown that Cu from $CuHis_2$ can cross the placental barrier, and that the transport shows developmental change (McArdle & Erlich, 1991). This work has been extended and it has been demonstrated that vesicles isolated from human placenta accumulate Cu from $CuHis_2$ (McArdle & van den Berg, 1991).

Barnea *et al.* (1988) had been studying hormone release from median eminence and showed that Cu, presented as $CuHis_2$, could stimulate release. They studied this process carefully and found that there were two $CuHis_2$ transport systems, a high-affinity one which operated at low Cu:His ratios (1:2000) and a low-affinity one which operated at higher ratios (1:20). The effect is specific for L-histidine, suggesting that there may be a biological relevance to the observation. More recently, they have examined the effect of different amino acids on uptake, and have compiled a list with relative affinities. From the data, they have been able to suggest that the Cu recognition site requires at least two N and possibly three N co-ordinating with the metal (Katz & Barnea, 1990).

Release of Cu from its binding complex. The release of Cu from the complex can be accomplished by reducing the cupric ion to cuprous ion. Several pieces of evidence suggest that this occurs. Percival & Harris (1989) have shown that ascorbic acid increases uptake of Cu into K562 cells and that Cu^{+} chelators block the effect. Cu^{2+} chelators, in contrast, have no effect. Similar findings have been obtained in mouse hepatocytes and it has also been shown that vitamin E acts to inhibit the stimulation (H. J. McArdle and S. M. Gross, unpublished results). Further, vitamin C supplementation in rats results in an increased retention of Cu, possibly in the liver (van den Berg *et al.* 1990*b*). A membrane reductase has been described which could fulfil this function (Sun *et al.* 1987).

Identification of a ceruloplasmin–Cu-binding protein. A ceruloplasmin receptor has now been described in a variety of cell types. Stevens *et al.* (1984) showed specific binding in membrane fragments from aortic endothelia and Barnes & Frieden (1984) have shown that they exist in erythrocytes. They isolated a protein of 60 kDa molecular weight which may be a ceruloplasmin receptor. These receptors have been carefully studied by Saenko & Yaropolov (1990) who have shown them to be possibly part of the glycophorin complex of the erythrocyte.

Using membrane fragments from different tissues, and cultured cells, Orena *et al.* (1986) showed that ceruloplasmin bound specifically and that Cu could be accumulated from the protein, but also showed that low-molecular-weight Cu complexes such as Cu–nitrilotriacetate (NTA) could interfere with uptake. We have made similar observations in human placental vesicles (McArdle & van den Berg, 1991).

These observations can be put together to build a model for Cu uptake in non-hepatocyte cells which is essentially the same as for hepatocytes.

A SINGLE HYPOTHESIS FOR Cu UPTAKE IN MAMMALIAN CELLS

This hypothesis is based on the fact that the molecular appearance of most Cu complexes are similar. In most cases, the Cu coordinates with N which can be derived from

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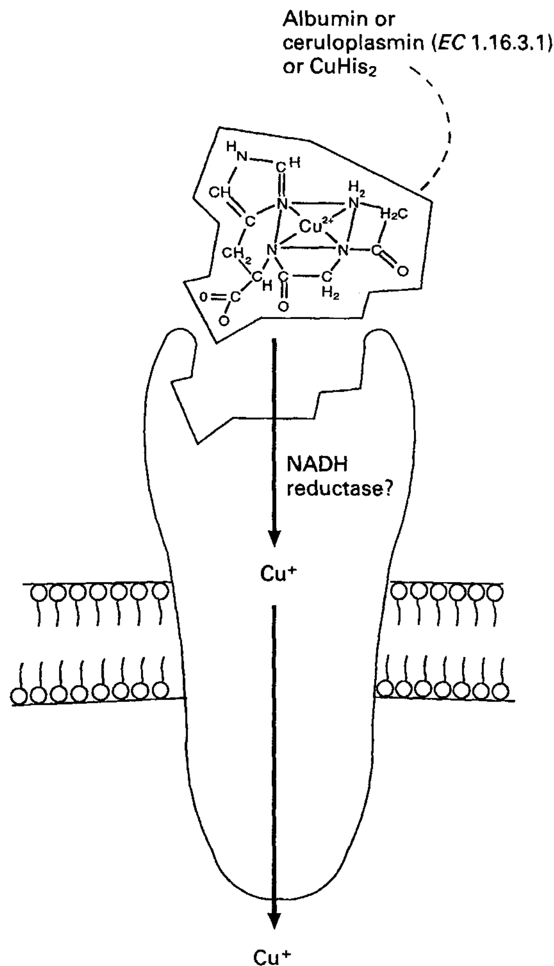

Fig. 1. A model for copper uptake by mammalian cell. The binding site on the carrier recognizes a Cu–imidazole-nitrogen complex; the remainder of the binding moiety being irrelevant. Following binding, the cupric ion is reduced, possibly by a membrane-bound NADH reductase, dissociates from its nitrogenous complex and is transferred across the cell membrane. This model should not be taken to infer that the NADH reductase necessarily is the carrier itself. It should also be noted that other reducing agents, vitamin C, for example, may also be able to act in a similar manner. $CuHis_2$, Cu–histidine complex.

histidine-imidazoles, α-amino groups of amino acids, or N of peptide bonds. If the recognition epitope is small, just the Cu-binding complex, then the membrane carrier would be able to accumulate and utilize Cu from a variety of substrates: $CuHis_2$, CuAlb, CuHisAlb or ceruloplasmin. Following binding, the Cu^{2+} is reduced to Cu^{+}, possibly by a reductase or perhaps by vitamin C, and then carried across the membrane into the cell (Fig. 1).

Clearly, this hypothesis is speculative, but it does have considerable heuristic value and fits the findings collected thus far, as well as explaining both hepatocyte and

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non-hepatocyte Cu uptake in terms of a single mechanism, the final substrate of which varies according to the relative concentration of the substrates rather than any genetic differences.

CONCLUSIONS

Clearly, there are many differences in the mechanisms involved in the transfer of Fe and Cu across the cell membrane. However, there are also important similarities. Both metals are carried on chelating molecules which are important in the recognition system. Both are taken up by carrier systems, although the types of system may be different. It is likely that both are reduced during the uptake process and it is almost certain that, if we apply the lessons learnt in Fe transport, we will rapidly understand more about the processes involved in the uptake of Cu.

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