MINIREVIEW

Copper Transport: An Overview (43171B)

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Copper is an essential element required by all living systems, more specifically, for some 30 enzymes that use copper as a co-factor. Classified as a "trace metal," copper's beneficial impact on cells occurs in the micromolar range. Copper is also one of the more toxic elements. This places a special burden on systems that regularly transport copper between organs. Such systems must handle very low levels of copper, yet operate with high specificity and complete safety. Over the years, our understanding of the properties of copper transport systems has increased dramatically. From these studies has come the realization that normal transport, storage, and metabolism of copper are inextricably linked to systems that safeguard the cell against copper toxicity. Understandably, there is much that is unique about element 29 as it interphases with the sensitive organic elements of life. This review attempts to relate some of the more recent information on extracellular, membrane, and intracellular phases of the transport mechanism.

Early History

Transferrin and ceruloplasmin, two metal-binding globulins in plasma, were isolated and named almost simultaneously (1, 2). Transferrin is one of a family of iron-binding proteins and keystone to plasma iron transport. Ceruloplasmin (the prefix "cerulo" denotes its heavenly blue color) is the major copper-binding protein in plasma. Its discovery led investigators immediately to speculate on parallel mechanisms for transporting iron and copper to cells. Ceruloplasmin is also an enzyme, having well-documented ferroxidase, amine oxidase, and superoxide dismutase activities (3). In the same era, albumin was shown to have a unique copper-binding site, giving rise to the speculation that serum albumin may also participate in copper transport (4, 5). Neumann and Sass-Kortsak (6) in Toronto called attention to what they called the third plasma copper fraction, that bound to amino acids. Amino acids competed favorably with albumin for plasma copper. On the basis of this and other evidence (7), the proposal was made that a close functional cooperativity exists between albumin and amino acids which gave rise to a "labile" and, therefore "mobile" form of copper. This small copper fraction was considered the form taken up by cells. A strength of the proposal was the demonstration that albumin yielded copper to histidine when the two reacted *in vitro* (8). This made an exchange mechanism feasible, but by no means certain. Ceruloplasmin was ruled out because it could not exchange copper ions with amino acids. In that same decade, however, Owen (9) had shown that numerous rat organs are recipients of copper from ceruloplasmin (9). This finding was later supported by Marceau and Aspin (10) who show that copper from ceruloplasmin bound to cytocuprein (superoxide dismutase) and cytochrome oxidase, thus giving physiologic significance to the ceruloplasmin-mediated transfer. By 1970, albumin and ceruloplasmin had been identified and efforts began in earnest to learn how each functioned in copper transport. More recent work has shown that cells exchange copper atoms with both proteins. The field is split with regard to which is major. Criticisms for an albumin-based (11) and ceruloplasmin-based delivery system (12) have been published. There is also concern that not all plasma copper transport agents have been identified.

An Overview of Copper Metabolism and Transport Agents

Studies with radioactive copper have supported a mechanism whereby absorbed copper ions pass from albumin to ceruloplasmin before being released by the liver (9, 13, 14). Thus, unlike iron, which has but one protein, transferrin, copper employs at least two, acting asynchronously, to move the metal from the intestine to body cells. Other discrepancies between copper and iron systems are shown in Table I. Adult humans normally require about 2 mg of copper/day to sustain

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Table I. Major Proteins in Iron and Copper Transport and Storage

Metal	Transport	Storage
Iron	Transferrin	Ferritin
Copper	Ceruloplasmin	Metallothionein
	Albumin	

a body load of 80–100 mg. Highest organ concentrations are in liver, kidney, heart, brain, and pancreas. Liver, the major homeostatic organ for copper, is also a major storage site for copper. In liver, copper levels remain relatively constant. Biliary excretion and release of ceruloplasmin are the major homeostatic mechanisms for controlling copper levels in liver. Less than 60 μg are excreted daily in urine. ^{64}Cu administered orally or intravenously is transported to liver as a simple complex with albumin. This may be a ternary complex of albumin, copper, and amino acids (15). Albumin-bound copper is highest in the portal circulation immediately after ingesting a copper load. Albumin-bound copper has a half-life in serum of less than 10 min (10, 16). Albumin's main function, therefore, seems to be to transport copper from the gut to the liver. Its role in extrahepatic transport, however, cannot be excluded. Meeting this function, albumin has at least one high specific binding site for copper on the N terminus of the protein (5). This site has been postulated to exchange copper ions with tissues via an intermediary complex with histidine (8).

Copper is released from the liver as a component of ceruloplasmin, and, in time, taken up by extrahepatic organs (9). Concluding that ceruloplasmin is a transport agent for copper justifies its presence in plasma at 30 mg/dl and its high copper-binding capacity (6–7 copper atoms/molecule). Ceruloplasmin also accounts for between 70 and 90% of the approximately 1 μg/ml copper in plasma. Are the copper atoms in ceruloplasmin destined for cellular uptake or are they to allow ceruloplasmin to function as an oxidase enzyme? As an oxidase, ceruloplasmin catalyzes a four electron transfer from its substrate to molecular oxygen. Oxidative functions require a full complement of copper. Transport functions require ceruloplasmin to be dismantled and its copper redistributed to cellular enzymes. The proposed functions are mutually exclusive. Faced with the dilemma, some investigators willingly concede that ceruloplasmin has no singular function, but instead serves the needs of the organism in many ways, copper transport just one of them.

The recent discovery of a 270-kDa copper-containing protein has raised speculation that there may be other serum factors that transport copper to cells (17). The protein, called "transcuprein," has been shown to compete with albumin for copper binding. Transcuprein is also believed to function in portal circulation as a donor of copper to albumin. The existence of transcuprein or proteins with a similar description in portal circulation has been challenged (18).

Membrane Transporters for Copper

Kinetics and Properties. Kinetic studies have supported the existence of membrane transporters that bind and convey copper ions into the cytosol. These transporters appear to be fixed proteins with a special property for binding copper ions reversibly. They may also form the channels through which the copper ions pass. To date, membrane transporters for copper have been analyzed *in situ* but have yet to be characterized as purified entities. Primary hepatocytes isolated fresh from collagenase perfusates of rat liver have been used extensively to study the properties of membrane transport systems. Their ability to absorb copper ions rapidly and show saturation with increasing copper in the medium has made it possible to use kinetic analysis to obtain binding and rate constants as well as assess factors that inhibit or facilitate copper uptake. Copper is introduced into the medium as a free ion and that which is retained by the cells after washing is considered absorbed.

Table II summarizes a number of studies where values for K_m and V_{max} of free copper transport have been determined. The K_m values are uniformly in the low micromolar range whereas values for V_{max} show wide discrepancies. This may reflect variations in incubation conditions and medium compositions. For example, a V_{max} of around 3000 pmol of copper/min/

Table II. Characteristics of Free Copper Transport Systems

	K_m (μM)	V_{max} (pmol/min/mg)	Reference
Cells			
Hepatocytes (mouse)[a]	4.7	—	(22)
Hepatocytes (mouse)[a]	13–18	210–230	(88)
Hepatocytes (mouse)[b]	17–19	270–290	(88)
Hepatocytes (rat)	11	3000	(19)
Hepatocytes (rat)[c]	—	140	(20)
HepG2	3.3	7.1	(21)
Lymphoblasts (human)[a]	6.5	11.9	(23)
Lymphoblasts (human)[d]	4.8	18.2	(23)
Tissues			
Duodenum (mouse)	4.3	—	(32)
Duodenum (rat)	21.0	47.5[e]	(36)
Hypothalamus (rat)	6.0[f]	23	(27)
	40.0[g]	425	(27)

[a] Normal.
[b] Expressing the Brindled phenotype.
[c] Incubated in the presence of 0.2% albumin.
[d] Expressing the Menkes' phenotype.
[e] Per cm of duodenal tissue.
[f] Determined at low histidine to copper ratios.
[g] Determined at high histidine to copper ratios.

mg cell protein is seen when rat hepatocytes are incubated with $^{64}CuCl_2$ in albumin-free medium (19). When albumin is added, the rate drops to one-twentieth the value (20). The type of cell used is also a factor. Apparent binding constants for HepG2 cells show three times greater affinity for copper (K_m = 3 μM), yet transport the metal maximally at only 7.1 pmol/min/mg protein, literally a fraction of the rate of hepatocytes (21). Otherwise, HepG2 cells show saturable and temperature-dependent uptake, the same characteristics as hepatocytes. Zinc and other divalent cations impede copper transport into hepatocytes (19, 22). In terms of effectiveness, $Cd^{2+} > Mn^{2+} > Mo^{2+} > Zn^{2+}$, suggesting the putative membrane carrier could be more generic than specific for copper. Neither zinc nor cadmium ions, however, interfer with copper transport in HepG2 cells (21). Whereas rat hepatocytes rely almost entirely on a carrier, lymphoblasts give evidence for both diffusion and carrier-mediated mechanisms to absorb copper (23). Likewise, mouse hepatocytes show a rapid exponential phase followed by a slower linear phase when these cells absorb copper ions from an albumin-free medium (22). As a consequence, copper uptake by some cells may have the characteristic of a biphasic mechanism. Careful kinetic analysis has shown that the initial uptake may be carrier mediated followed by a slower linear rate attributable to diffusion (23).

A Role for Histidine. Whether membrane carriers recognize free ionic copper or an amino acid-copper complex is still unclear. Histidine facilitates copper uptake by some, but not all cells, suggesting that this amino acid may form part of the complex recognized by the carrier. Histidine, however, is neither transported with copper, nor does copper have any affect on the rate of histidine uptake (24). The two are treated separately once transport commences. In mouse hepatocytes, saturation of the carrier with copper occurs only when histidine is added to the medium (25). In sharp contrast to these observations is the report that HepG2 cells and mouse fibroblasts are refractory to histidine's presence (21, 25). Copper absorption into cells of the intestinal mucosal is also obvious to histidine. Histidine appears to have no facilitating or inhibiting role in copper efflux (26). Thus, the role of histidine in copper transport is likely to be cell specific and, at present, lacks mechanistic insights.

Brain thalamic explants transport copper at a rate that critically depends on complexation with amino acids, histidine the most effective (27). Here, the histidine to copper ratio determines net copper accumulation by the explants. Two transport systems, low affinity-high capacity, and high affinity-low capacity, have been so characterized through histidine. Tests of effectiveness of specific analogues show that L-His > D-His > Me-3-*N*-His by both carrier systems. Histidine at low concentrations is more effective that 14 other amino acids; at high, the differences are less distinguishable (28). Whereas histidine facilitates, pencillamine, a potent and medicinally important chelator of Cu^{2+}, neither aids nor impedes copper uptake by mouse hepatocytes. On the other hand, two compounds (Sar and Diamsar) which encapsulate copper ions in an organic cage strongly block its accumulation by cells (29).

Albumin Regulates Free Copper Transport. As shown in Table II, the rate of ionic copper uptake by hepatocytes, all conditions being uniform, is slower when small amounts of albumin (0.2% w/v) are in the medium. Albumin at a ratio of 3:1 or less renders kinetic analysis ineffectual and is avoided for this reason (24). Practically all cell types absorb copper ions more slowly when albumin is present. Thus, albumin may govern the rate at which cells absorb copper ions brought to the membrane as amino acid complexes. Too fast an entry could be as harmful as no entry at all. An alternative is that cells have receptors that recognize albumin-copper complexes. Kinetic arguments based on copper to albumin ratios have been used to support this idea (30). Kinetic studies have also shown a reduced amount of free copper when albumin is in solution (24). Histidine reverses the inhibition while other amino acids are less effective (24). Although the absorption velocity is retarded, the quantity of copper retained by cells is greater when albumin is in the medium.

Albumin-bound copper, however, has some disturbing properties. An albumin-copper complex has been shown to exert immunosuppressive action on lymphocytes in the G1 and S phase of the cell cycle (31). Metal-free albumin or free copper alone have no effect. Whether this is significant physiologically has not been determined.

Luminal Transport of Copper. A specific carrier for copper ions has been postulated for intestinal epithelial cells. Postulating a carrier helps explain why only a fraction (25–60%) of the copper in a food source is absorbed into bodily fluids. Present understanding is that absorption depends on many factors including, but not limited to, competing metal ions and complexing agents that may be present. Copper absorption is faster from the duodenum than more distal sections of the intestine. Whether duodenum has superior numbers of copper carriers is not known. Evidence for carriers is also supported by kinetic studies. Saturable mechanisms appear to operate in mouse duodenum (32). Moreover, amino acids strongly influence copper uptake by mucosal cells (15). On the other hand, ascorbic acid (vitamin C) is one of the strongest deterrents to copper uptake from the intestines. The vitamin strongly antagonizes short-term absorption (33). When given in a postabsorption period, however, ascorbic acid strongly enhances tissue copper utilization as measured by the activation of copper-dependent enzymes (34).

High dietary iron exacerbates the impairment brought on by the vitamin alone, suggesting that ascorbate blocks copper by enhancing iron uptake (35). Wapnir and Stiel (36) have drawn attention to a possible dependency on sodium ions and the proper pH for optimal copper transport across the brush border membrane.

It is well established that a mutual antagonism between copper and zinc ions lowers the rate of either ion across an intestinal barrier (37). Zinc, however, is a more effective antagonizer of copper than copper of zinc. This makes it unlikely that antagonism reflects competition for a common entry portal. Rather, the antagonism is more likely to be expressed internally. The binding of copper to metallothionein renders copper ions less able to transfer across the serosal membrane (38, 39). Zinc elevates intestinal metallothionein concentration, effectively providing the "trap" that captures copper ions in transit (37). Copper-zinc antagonism, extended to humans, is one reason patients who are given high zinc supplements to correct a suspected zinc deficiency, sometimes develop a mild copper deficiency (40). Therapeutically, the mutual antagonism between copper and zinc has proven to be a most effective strategy for treating patients suffering Wilson's disease. Such patients regularly absorb high amounts of copper. High zinc effectively blunts the copper intake of Wilson's disease patients (41).

Critique of Free Copper Transport. Because free copper ions have the capacity to disrupt membrane systems (42, 43) and become foci for peroxidation reactions affecting phospholipids of the bilayer (44), transmembrane systems must never "see" the copper ions they transport. This implies that copper must be in a sequestered form as it passes within or among sensitive membrane lipids. It is by nature a most delicate maneuver. We presently lack a clear picture of how the sequestering of metal ions is accomplished, especially at the level of micromolar or nanomolar quantities that exist in cells. We also know very little mechanistically as to how trace amounts of copper find their way into enzymes. With regard to membrane transport, the data, still inchoate, suggest a first-order reaction stimulated in some cells by amino acids (45) but requiring no concomitant expenditure of cellular energy. Copper ions accumulate passively against a concentration gradient. The driving force for the thermodynamically uphill movement of copper ions has not been identified. One must also consider that a rapid uptake as would be manifested by movement of large amounts of copper, although favorable for thermodynamic considerations, could spell disaster for cell functions. Copper is highly toxic and excessive transport will disrupt sensitive membrane and cytosolic redox systems. A large influx will also trigger metallothionein synthesis (see below). One is compelled to consider uptake a slow, assiduous process, responsive to low copper. Moreover, cells such as hepatocytes, which are in direct line with copper from the gut, would be expected to experience the highest localized concentration of copper. Their mechanism for coping with copper would be expected to differ from nonhepatic cells. Hepatocytes must solve the problem of copper ions dissociating from albumin. Nonhepatic cells see a different environment of copper and may deal with ceruloplasmin for their copper. Thus, mechanism operating in fibroblasts, for example, would be expected to be different from those in hepatocytes, and recent evidence has tended to support these notions (25).

Copper Transport from Ceruloplasmin

Ceruloplasmin's role in copper transport is well established. Experiments have shown that ^{67}Cu from ^{67}Cu-labeled ceruloplasmin is transferred to cells and into intracellular copper enzymes (46–48). Levels of lysyl oxidase in chick aorta, responding to copper depletion and refeeding, correlate with the plasma concentrations of ceruloplasmin (49). A significant correlation has been found in leukocyte cytochrome *c* oxidase levels and plasma ceruloplasmin in humans manifesting the symptoms of Wilson's disease and heterozygous carriers (50). Moreover, holo-ceruloplasmin activates cytochrome *c* oxidase activity in tissues of rats suffering from dietary copper deficiency (51), showing a direct metabolic connection between ceruloplasmin and copper enzymes. Ceruloplasmin is a preferred source of copper for tumor cells and donates copper ions to numerous organs and tissues (52). With six to seven copper atoms massed within its structure, ceruloplasmin is a most effect vehicle for delivering clusters of copper atoms to a variety of cells and tissues.

Liver Copper Transport and Ceruloplasmin. Drawing parallels to iron transport, one may consider that ceruloplasmin uses an endocytotic mechanism similar to transferrin to deposit copper atoms within cells. Indeed, ceruloplasmin labeled with colloidal gold and infused into the portal vein of rats will be absorbed by cells of the sinusoidal endothelium (53) and hepatocytes (54). The mechanism is not a direct one, however. The native ceruloplasmin molecule absorbed by the endothelial cells is first desialated and released from the endothelial membranes (54). Only then can the desialated ceruloplasmin be absorbed by hepatocytes, apparently by membrane receptors that recognize the exposed galactosyl recognition site. The fate of the bound copper atoms has not been determined. How (or if) absorption of ceruloplasmin by liver cells is for transport of copper has not been made clear. It could be a means for removing spent or partially degraded ceruloplasmin molecules from circulation. Nonetheless, the liver endothelium seems unique in having the ability to endocytose native ceruloplasmin molecules.

As will become clear, nonhepatic tissue employs a different mechanism for obtaining copper from ceruloplasmin.

Ceruloplasmin Receptors. A specific interaction between ceruloplasmin and the cell membrane is a requirement for ceruloplasmin-mediated uptake of copper. This has led to a search for membrane receptors for ceruloplasmin. Stevens *et al.* (55) were the first to characterize ceruloplasmin-binding sites in membranes. These workers used plasma membranes from chick heart and aorta. The partially purified membranes readily bound ^{125}I-labeled ceruloplasmin both specifically (high affinity) and nonspecifically. The latter was about 60% of the total bound. Binding of ^{125}I-labeled ceruloplasmin reached equilibrium in 12 hr at 4°C, showed saturation with picomole amounts of ceruloplasmin, and, at equilibrium, was displaced by nonlabeled, homologous (chick) ceruloplasmin. High affinity sites for ceruloplasmin have since been reported in a number of tissues and cells including erythrocytes (56, 57), liver endothelium (58), lymphocytes, monocytes, and granulocytes (59), Chinese hamster ovary cells (60), and membranes of rat organs (60). As shown in Table III, binding constants measured by Scatchard analysis (K_d) generally are in the nanomolar range, literally three orders of magnitude more sensitive than free copper (Table II). Thus, arguments favor ceruloplasmin as a superior transport-delivery agent because of its much greater sensitivity. Receptor numbers tend to vary with cell type, white blood cell membranes apparently showing the richest concentration of cells examined thus far. Native ceruloplasmin fails to show specific binding to hepatocytes and noninduced K562 erythroleukemic cells. In all instances where specific binding is observed, the binding is reversed (or blocked) by excess unlabeled ceruloplasmin. Boiling or digesting chick aorta and heart membranes with trypsin abolishes specific binding activity (55). As to specificity, competition assays have shown that orosomucoid, fetuin, their asialo derivatives and transferrin (55), superoxide dismutase, β-globin (56), hexokinase, and hemoglobin (60) fail to compete with ceruloplasmin from high affinity sites. Excess of copper as a complex with nitrilotriacetate (NTA), however, will displace ceruloplasmin; Zn-NTA has no effect (60). In general, binding reaches saturation when less than 1 pmol/ml ceruloplasmin is in the medium (55, 56). At this very low level, copper as ionic copper, shows no specific binding to cell membranes (61).

Properties of Ceruloplasmin Receptors. Thus far little is known about the physical properties of ceruloplasmin receptors. Barnes and Frieden (56) used affinity chromatography combined with detergent extraction to obtain a 60-kDa protein from erythrocyte membranes. The protein migrated as a single band on gel electrophoresis. The glycoprotein character of the receptor has since been determined. Based on sialic acid content and response to specific proteases, ceruloplasmin receptors have been considered to be part of the glycophorin components of erythrocyte membranes (57). This conclusion is based on observing that the dimeric (PAS1) and monomeric fragment (PAS2) fragment from glycophorin appear to have ceruloplasmin-binding functions. Moreover, the molecular mass of the PAS1 (58

Table III. Ceruloplasmin Receptors in Tissues and Cells

	K_d (n*M*)	Receptor Concentration (pmol/mg protein)	B_{max} (fmol/10^6 cells)	Reference
Tissue				
Chick aorta	66	7.3		(55)
Chick heart	33	0.7		(55)
Rat heart	100			(60)
Rat brain	100			(60)
Rat liver[a]				(60)
Cells				
Human RBC	5	144	0.24–0.48	(56)
Rabbit RBC	4	294	0.49	(56)
Granulocytes	48	280,000	460	(59)
Monocytes	16	100,000	160	(59)
Lymphocytes	21	40,000	60	(59)
Endothelial	100	210,000	347	(58)
Hepatocytes[b]				(58)
CHO[a]				(60)
K562[b]				(62)
K562-induced[c]	0.2	2,050		(62)

[a] Detected, but quantitative information not reliable.
[b] No specific binding detectable.
[c] Variable with exposure time to hemin.

kDa) suggests identity with the 60-kDa protein described by Barnes and Frieden (56). There is evidence that the sialic acid component in the receptor protein may be an important determinant of the recognition site. The biantennary structure of ceruloplasmin's oligosaccharide units also appears to play a recognition role. Monomeric fucose of *N*-acetylglucosamine disaccharides prevent ceruloplasmin binding to erythrocyte membrane ghosts. The binding may also require calcium ions. Ceruloplasmin treated with ascorbate loses its ability to bind specifically to the membrane of K562 cells (62). A possible role for copper atoms in the binding of the native molecule is suspected.

It should be noted that ceruloplasmin receptors may serve needs other than copper transport. Frieden and Barnes (56), for example, noted that in erythrocytes from several species, those that bound more ceruloplasmin tended to have greater protection against metal-ion-induced lysis of erythrocytes. Implied in these observations is that the binding of ceruloplasmin and its antioxidant activity may play a role in prevention of membrane lipid peroxidation.

Mechanism of Copper Transport-Delivery from Ceruloplasmin. Cells that bind ceruloplasmin are in a position to engage in the transmembrane delivery of copper to the cell. There are several ways of accomplishing the delivery, one is by endocytosis of the holoceruloplasmin molecule. The idea that nonhepatic cells take up bound ceruloplasmin via coated pits and vesicles has not received experimental support, however. At least three different cell types that bind ceruloplasmin fail to incorporate the protein moiety into the cell. The first evidence that copper transport did not involve ceruloplasmin penetration came from Linder's laboratory (60). Cu-NTA was shown to interfer with the uptake of copper from ceruloplasmin into Chinese hamster ovary cells prompting the argument that copper dissociates from the protein before uptake. A similar conclusion was reached for erythrocytes (57). Stronger evidence for a dissociation has come from K562 cells, a human erythroleukemic cell line. These cells bind ^{67}Cu-ceruloplasmin at 4°C. The reaction occurs at pmol levels of copper and ceruloplasmin and is not affected by albumin (3%) in the medium. At 37°C, the ^{67}Cu is no longer removable by a mild acid washing, suggesting internalization of the ^{67}Cu-labeled ceruloplasmin may have occurred. ^{67}Cu entering the cell is localized in a cell fraction that has the buoyant properties (Percoll gradient) of an endosome. Repeating the experiment with ^{125}I-labeled ceruloplasmin, however, fails to localize the ^{125}I in the buoyant fraction. In fact, cells in general fail to incorporate any of the ^{125}I label. The data imply that the copper has dissociated from the protein prior to uptake. Ceruloplasmin-mediated transport is totally inhibited by 1.0 m*M* bathocuproine disulfonate, a chelator of Cu(I). On the other hand, EDTA, a chelator of Cu(II), has no effect (48). Iproniazide, which blocks ceruloplasmin's oxidase activity, also inhibits the uptake of ^{67}Cu from ceruloplasmin. In contrast, L-ascorbate and D-isoascorbate strongly stimulate the uptake of copper from ceruloplasmin (63). The data clearly imply that copper atoms in ceruloplasmin are reduced before they are liberated, i.e., Cu(I) is the form of copper taken up by the cells. In effect, a reduction of Cu(II) to Cu(I) had been predicted by Frieden (3) to be the essential event leading to a fascile discharge of copper atoms from ceruloplasmin. Inhibition by iproniazide further suggests that oxidase activity of the ceruloplasmin is used to draw electrons into the protein and initiate the chemically reduced state of copper. Studies with double-labeled (^{67}Cu, ^{125}I) ceruloplasmin have confirmed that copper and not the ^{125}I-labeled protein appear inside the cell (61). Thus, the function of ceruloplasmin in the transport mechanism ends at the cell surface. The pathways of copper and ceruloplasmin protein diverge at this site.

Sulfhydryl Groups Seem Critical to Membrane Copper Transport. Figure 1 shows that the uptake of ^{67}Cu from ceruloplasmin is inhibited by iodoacetamide and *N*-ethylmaleimide, two reagents that bind sulfhydryl groups. Moreover, treating cells alone with *N*-ethylmaleimide followed by dialysis to remove excess reagent impairs their ability to transport ^{67}Cu from ceruloplasmin. The data imply that a sulfhydryl factor(s), possibly in the membrane, is critical to the transport. The sulfhydryl factor may aid the binding of ceruloplasmin to the cell surface or facilitate the subsequent discharge of copper from the protein. Interestingly, ionic copper uptake by hepatocytes and lymphocytes is also inhibited by sulfhydryl-blocking agents (20, 22, 64). This suggests an overlap in some stages of the ceruloplasmin-mediated and ionic copper transport mechanisms. The inhibitory effects of Cu-NTA complex cited above (60) may have worked through the

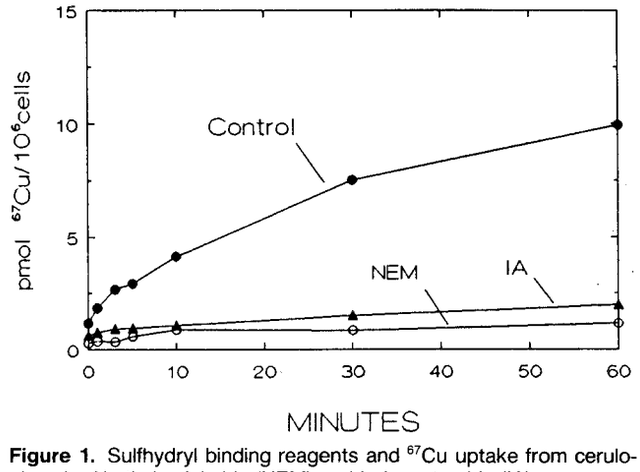

Figure 1. Sulfhydryl binding reagents and ^{67}Cu uptake from ceruloplasmin. *N*-ethylmaleimide (NEM) and iodoacetamide (IA) were present at 1.0 m*M*.

critical membrane sulfhydryls, since Cu-NTA effectively oxidizes sulfhydryl groups.

Ascorbate as a Factor in Copper Transport. Ascorbic acid interferes with the intestinal absorption of copper (see above). Ascorbate added to diets low in copper tends to hasten the onset and severity of copper deficiency symptoms (33). Using activation of lysyl oxidase, a copper-dependent enzyme, as criterion for copper transport, DiSilvestro and Harris (34) have shown that ascorbate given with copper or 75 min before impairs the activation of lysyl oxidase. When ascorbate is given 75 min after copper, the enzyme activation is much greater. It was proposed at the time that ascorbate had a postabsorption role in copper transport. The site of ascorbate action was not identified. Ascorbate stimulation *in vivo* correlates with a 3-fold increase in plasma ceruloplasmin concentration. In the *in vitro* system for studying copper transfer from ceruloplasmin, 100–200 μM ascorbate stimulates copper absorption by cells 2- to 10-fold (63). Thus, ascorbate interaction with copper may be directed at cellular absorption of copper from ceruloplasmin. Is the vitamin a factor in the transport of copper and will a deficiency in ascorbate impair copper utilization? These questions open inquiries on a possible connection between deficiencies of the vitamin (which lead to scurvy) and deficiencies of copper. As shown in Table IV, there is reason to suspect an overlap in the pattern of symptoms expressed for the two seemingly unrelated nutrition deficiencies.

Figure 2 summarizes events at the membrane surface when ceruloplasmin discharges its bound copper atoms into cells. The scheme is hypothetical but based on a composite of data. Ascorbate is shown as a factor in the release of copper. The role of the sulfhydryl component, although not defined, may be to assist physically the transfer of copper ions through the membrane or maintain the reduced copper state. Released copper in the cuprous form [Cu(I)] enters a cellular compartment that has buoyant properties similar to an endosome. The protein moiety of ceruloplasmin stays outside the cell.

Intracellular Copper Transport

Less is known about intracellular transport of copper than any other phase of its metabolism. Intracellular transport and prevention of copper toxicity are pathways interwoven into all cells. So overlapping are they that one cannot be studied without the other. Workers continue to seek out steps where pathways for copper destined to bind enzymes diverge from pathways leading to metal-binding and storage proteins.

Intracellular Compartments for Copper. Copper ions in bound form are distributed among organelles and cytosolic components. Evidence that intracellular copper may be localized in discrete, interacting, and noninteracting cellular components has come from several sources. Copper destined for biliary excretion (the major excretory route for ingested copper) is separated from stored copper and copper awaiting incorporation into ceruloplasmin (35). Copper in the cytosol is further resolvable into a number of discrete proteins including superoxide dismutase and metallothionein (47). Once absorbed, some of the copper is subject to rapid efflux from cells (26). The efflux rate seems to vary with the length of time copper is internalized, suggesting subsequent metabolic steps lock copper within (26). Metallothionein, a cysteine-rich, metal-binding protein, is the major sequestering protein for copper and as such is a major biochemical factor in preventing a toxic copper build-up.

A Central Role for Metallothionein and Glutathione. The binding of copper to metallothionein has been studied extensively, and is perhaps the best understood intracellular metabolic process for copper. One of the less understood functions of metallothionein is the demonstration that copper (and zinc) bound to metallothionein can be transferred to apoenzymes *in vitro* (66–70). Whether this pathway represents a significant way of placing copper into the structure of enzymes *in*

Table IV. Symptoms of Copper Deficiency and Scurvy

Scurvy	Copper deficiency
Weakness, lassitude	Weakness, lassitude
Vague aches	Vague pain in joints
Impaired growth	Impaired growth
Hyperkeratotic skin	
Osteoporosis	Osteoporosis
Petechial hemorrhaging	Petechial hemorraging
	Arterial aneurysms
Poor wound healing	Poor wound healing
Fever, susceptibility to infection	Central nervous system degeneration
	Neutropenia
Hemacytic, hypochromic anemia	Hemacytic, hypochromic anemia
	Achromatism
Coiled hair	Twisted "kinky" hair

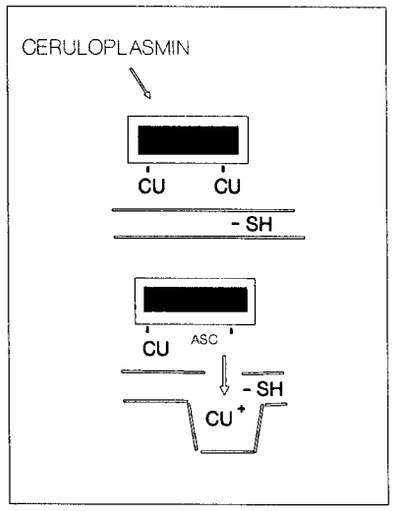

Figure 2. Copper uptake from ceruloplasmin. Schema shows that uptake occurs after ceruloplasmin has bound to the membrane. A sulfhydryl group (−SH) in the cell membrane may assist in the release and movement of copper through the membrane. The role of ascorbate (ASC) is not defined, but may facilitate the breaking of bonds holding copper to ceruloplasmin. Copper is pictured entering the cell sequestered in a vesicle and in the cuprous [Cu(I)] form.

vivo has not been determined. In the initial stages, the pathways for storage and functional redistribution of copper seem to converge. Recently, Freedman and Peisach (71) have shown that glutathione, a cysteine-containing tripeptide, may also function in intracellular copper transport. These workers used a copper-resistant hepatoma cell line to show that copper binds to glutathione before engaging metallothionein or cellular enzymes (71). Cells depleted of glutathione by treatment with L-buthionine sulfoximine fail to bind copper to metallothionein (72). We have also determined that K562 cells treated with L-buthionine sulfoximine, while retaining full viability, are nonetheless impaired in their ability to incorporate copper into copper-zinc-superoxide dismutase (Harris and White, unpublished data). The copper donor function of glutathione remains to be firmly established.

Copper destined for enzyme synthesis may transverse a pathway separate from copper binding to metallothionein. The possibility has been difficult to determine experimentally because low levels of copper will cause the induction of metallothionein synthesis. Because ceruloplasmin can deliver pmol amounts of copper without stimulating metallothionein synthesis, it has been possible to study intracellular movement of copper when metallothionein levels are negligible. K562 cells show a direct incorporation of copper from ceruloplasmin into copper-zinc superoxide dismutase. That reaction is severely deterred by pretreating the cells with 25 μM zinc ions, which cause a strong elevation in intracellular metallothionein levels (Harris and Redell, unpublished data). The data suggest that in K562 cells, the binding of metallothionein is a pathway away from copper incorporation into copper-zinc-superoxide dismutase, i.e., a possible divergence in competing pathways for copper in these cells.

Copper Transport and Genetic Regulation. One of the more exciting new areas of intracellular copper transport has focused on copper as a regulator of metal-binding protein biosynthesis. A transport system for copper that regulates metallothionein gene expression has been identified in yeast cells (*Sacchromyces cerevesiae*). The specific metallothionein locus, designed CUP1, encodes metallothionein, a 6570 molecular weight metal-binding protein (73). Strains deleted of *CUP1* gene, while viable, are hypertensive to low levels of copper in the growth medium (74). The CUP1 promoter does not respond directly to copper. Rather, this is a property of an upstream-activating sequence (UAS) present as a tandem sequence designated UAS_p and UAS_d located between −105 to −180 bp from the transcription start site (75).

In responding to heavy metal ions, copper in particular, UAS_p and UAS_d bind a 225-residue protein designated the ACE1 protein. High resolution DNase I footprinting shows that the ACE1 protein binds to two upstream sites located at −121 to −144 and −230 to −290, respectively (76). A consensus sequence in the CUP1 UAS is 5′-TCTTTTTGCT-3′ (75). The ACE1 protein has an N-terminal domain rich in basic residues and a C-terminal domain characterized by an unusually high number of cysteine residues, eight of which are in the Cys-X-Cys or Cys-X-Y-Cys sequence reminiscent of metallothionein. Binding of ACE1 protein to CUP1 UAS critically depends on copper which favorably alters the conformation of the protein upon binding. EDTA cannot remove copper from ACE1 protein, but potassium cyanide and dithiothreitol, both movalent chelators, are effective (77). Binding of ACE1 protein is enhanced in those strains of yeast with multiple copies of the ACE1 locus and is not seen in cells with a nonfunctional ACE1 locus (76). Binding of the ACE1 protein is also enhanced if copper is added to the medium.

At least two other proteins have been isolated from nuclear extracts and shown by gel retardation analysis to bind specifically to the upstream-activating sequence in two regions −137 to −159 and −168 to −180 bp, respectively (78). Unlike ACE1, these proteins do not respond to copper in the medium.

Menkes' Disease

A rare X-linked disorder causes cells to accumulate massive amounts of copper selectively, disrupting copper homeostasis as a result. Fibroblasts and lymphoblasts from patients suffering from Menkes' disease (79, 80) or mice afflicted with the mottled mutation (81) express the defect. With as little as 0.01 μg/ml copper

ions in the medium, cells accumulate copper against a concentration gradient whose inward movement and retention may be a function of metal-binding proteins within (64, 82). The mutant cells are very intolerant to moderate levels of copper in the medium (83, 84). The excessive amount of copper within the cell is bound to metallothionein (64). At first, it was postulated that afflicted cells synthesize a structurally modified form of metallothionein which either binds greater amounts of copper or turns over copper more slowly. That theory was discounted with the discovery that metallothionein in humans and mice was synthesized on autosomal chromosomes (85, 86). A second consideration was that regulation of the metallothionein expression may be hypertensive to copper. Supporting this notion was the observation that external copper must be at least 200 μM in order to induce metallothionein in normal cells but only 50 μM for Menkes' cells (82). A close examination, however, revealed that the intracellular copper concentration needed to trigger metallothionein synthesis is the same in normal and Menkes' cells (87). This means that enhanced binding to metallothionein is secondary to the build up of an accessible pool of copper in the cytosol. Menkes' and normal lymphoblasts show about the same binding affinity and transport rate for copper ions (23), suggesting membrane components involved in recognition and uptake are not affected. Hepatocytes from brindled mice also show nearly identical kinetic parameters as normal hepatocytes (88). Thus, by these criteria, the site of the lesion must lie beyond membrane transport. A recent study has found that normal fibroblasts with elevated metallothionein (induced with copper, zinc, or dexamethasone) do not accumulate excessive amounts of copper and show normal efflux rates (89). Understandably, attention has now turned away from metallothionein, away from membrane transport, and been directed to intracellular pathways as the site of the defect. The rationale is that an interruption in the flow of copper ions to enzymes will feedback and induce metallothionein synthesis thus shifting copper flow to metallothionein.

Conclusions and New Areas for Research

Copper transport continues to show unexpected and, in some cases, surprising departures from traditional mechanisms. Although both iron and copper bind to plasma proteins, the recent data suggest that each works through separate and distinct mechanisms for gaining access to cells. Ceruloplasmin and albumin as postulated transport carriers have withstood the test of time. One must concede, however, that there may be other serum proteins that perform a copper transport function. One thought not to be forgotten is that in postulating specific transport proteins for copper, one is showing an "animal bias." Plants and some microorganisms also require copper, but there have yet to be found specific proteins that perform copper transport functions in these organisms. There is an immediate and pressing need to clarify the nature of the component(s) that conveys copper ions through the cell membrane. We stand to learn how copper is shielded from doing harm to membrane components and perhaps apply this information to other aspects of copper metabolism. The multifaceted nature of a copper transport mechanism seems to find unity at the membrane surface. Ceruloplasmin, working through specific receptors and membrane dissociating agents is able to convey copper atoms to cells. Similarly, albumin, utilizing complexes with amino acids, conveys copper ions to the membrane transports. The transport, in turn, may show different recognition functions depending on cell type. There is always the concern that copper may "borrow" an existing transport system for its own transport purposes. Diseases like Menkes' syndrome rule against this possibility and tend to fortify the belief that copper transport is unique and of its own.

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