Biol Metals (1990) 3:141–145

BIOLOGY OF METALS

Copper transfer through the intestinal wall

Serosal release of metallothionein

Klaus Felix, Wolfgang Nagel, Hans-Jürgen Hartmann, and Ulrich Weser

Anorganische Biochemie, Physiologisch-chemisches Institut der Universität Tübingen, Hoppe-Seyler-Strasse 4, W-7400 Tübingen, Federal Republic of Germany

Summary. The elucidation of the molecular side of copper transport in biological systems is a promising task. In this context the transfer of ingested copper into the portal blood plasma was examined. Intralumenal addition of 200 μM copper caused the release of Cu-thionein into the venous effluent. This Cu-thionein became detectable after prior perfusion of the porcine small bowel using a modified isotonic phosphate-buffered saline (P_i/NaCl) medium. The protein was characterized by gel chromatography, luminescence, electronic absorption and immunological identification. ELISA and immunoblotting employing a murine monoclonal antibody to rat liver metallothionein-I proved to be most convenient. Using buffer-loaded sacs of porcine jejunum into which Cu^{2+}, Zn^{2+} and Cd^{2+} were added, the release of metallothionein into the serosal fluid was successfully seen by ELISA. The observed excretion of metallothionein into the portal compartment may be a genuine metal transport system for many biochemically active metals.

Key words: Copper transfer – Metallothionein – Mammalian intestine – ELISA – Immunoblotting

Introduction

Little is known on the molecular mechanism of copper transport across the intestinal wall and the release of this biologically important transition metal into the blood. There are several reports on the fate of orally ingested copper (Evans 1973; Ettinger 1984; Cousins 1985; Bremner 1987). It has been shown that the absorption of copper, and particularly its transfer from the intestinal mucosa into the blood, is a regulated process which is affected by the physiological state (Cohen et al. 1979). Orally administered ^{64}Cu was predominantly bound to the albumin of blood plasma (Bush et al. 1955; Crane and Hunt 1983). Approximately 1% of plasma copper was found to bind to amino acids, especially histidine, mixed amino acid complexes [e.g. His(CuII)Thr] and to form ternary complexes [(CuII)His-albumin] (Sarkar and Kruck 1966; Neumann and Sass-Kortsak 1967). A growth-modulating plasma tripeptide Gly-His-Lys was found to be a promising copper-binding compound (Pickart et al. 1980). However, this tripeptide is a poor vehicle for transporation of Cu(I). The thiolate sulphur, known to be richly abundant in metallothioneins (MTs), is a far better ligand to sequester copper in this oxidation state.

The important role of MT in intestinal metal absorption is not surprising. This protein was identified in chicken and rat duodenum and was found to bind Cd, Zn and Cu in the intestinal mucosa (Starcher 1969; Cousins 1985; Elmes et al. 1987). Orally administered ^{64}Cu is also incorporated into rat or bovine duodenal MT (Evans et al. 1970). Furthermore, dietary Zn-deficiency is associated with enhanced absorption and binding of both zinc and copper to mucosal MT (Smith and Cousins 1980).

Apart from its participation in absorption processes, MT is proposed to be actively involved in the mucosal-to-serosal flux of copper and zinc. It is considered to be a much better candidate for functioning as a safe transport system as it coordinates cuprous copper in the form of stable Cu(I)-thiolate clusters. Thus, the many undesired reactions of unspecifically bound copper are minimized (Felix et al. 1989). Observations on the release of intact Cu(I)-thionein from leucocytes and yeast cells (Hartmann et al. 1989; Felix et al. 1989) encouraged the search for similar transport processes in intestinal cells. Ligated, perfused segments from porcine small intestines which were intralumenally exposed to copper salts were used to investigate a possible serosal release of Cu-thionein into the portal fluid. Chemical, physicochemical and immunological methods including ELISA and immunoblot seemed appropriate to identify and characterize the excreted protein in the portal compartment.

Offprint requests to: U. Weser

Materials and methods

Small bowel perfusion. Three porcine small intestine (jejunum) segments, of 75 cm length each, were resected with their mesenteric blood supply. The mesenteric arteries were cannulated and the small intestines flushed with phosphate-buffered saline (P_i/NaCl) containing heparin (2000 U/l) at 4° C until the venous effluent remained clear. After intralumenal washing with P_i/NaCl, the ligated segments were charged with 500 ml P_i/NaCl containing 5.5 mM glucose, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 200 µM $CuCl_2$. The small intestine segments were kept in a P_i/NaCl bath and perfused at 20° C with O_2-saturated P_i/NaCl at a flow rate of 0.6 ml/min. Venous effluent was collected for 5 h at 20° C, lyophilized and separated on both Sephadex G-75 and G-50 equilibrated with 20 mM Tris/HCl pH 7.4 in the presence of 0.1% (by vol.) 2-mercaptoethanol.

Immunology. Murine monoclonal antibodies to monomeric rat liver MT-I were prepared in the authors' laboratory (Nagel et al. 1989). The monoclonal antibody II-10a was employed in the ELISA. By way of contrast, this antibody reacted exclusively with low-M_r MT in the immunoblot. Thus, for immunostaining the monoclonal antibody, I-8h was used as this antibody proved to react with both high-M_r and low-M_r MT species.

ELISA. This was carried out according to Engvall (1980). The microtiter plates were coated with 200 µl sample solution at pH 9.6 for 4 h at 37° C.To all samples a constant concentration of purified monoclonal antibody (2.6 µg/well) was added. Unspecific binding sites were blocked with milk powder (5% mass/vol.) in P_i/NaCl. Alkaline-phosphatase-conjugated goat anti-mouse IgG (Sigma, Munich) with a final dilution of 1:2000 was used as second antibody. The enzymic activity was measured on a Biotek EL 309 spectrophotometer at 405 nm.

Electrophoresis and immunoblotting. Samples were separated on two parallel running PAGE (15% mass/vol. acrylamide) according to Laemmli (1970). One gel was stained with Coomassie blue, the other was equilibrated in cathode solution (40 mM 6-amino-*n*-hexanoic acid, 20% aqueous methanol) prior to the protein transfer to nictrocellulose (Schleicher & Schüll 0.1 µm). An LKB Multiphor II Nova Blot apparatus was run for 2.5 h at 1.2 mA/cm^2 in the discontinuous Tris buffer system. The blotting efficiency was examined by staining the nitrocellulose with Ponceau S after the protein transfer. The nitrocellulose was blocked with P_i/NaCl containing 5% (mass/vol.) milk powder for 1 h at 4° C under constant agitation. The following procedures were carried out as described by Blake et al. (1984) with modifications. Incubation of the monoclonal antibody was performed in P_i/NaCl, containing 0.02% (mass/vol.) bovine serum albumin and 0.005% (by vol.) Tween 20 for 4 h at 22° C. The second antibody was the same as described above in the ELISA. A dilution of 1:3000 was appropriate. The nitrocellulose was incubated with the reaction solution, consisting of 5 mg nitroblue tetrazolium, 2.5 mg 5-bromo-4-chloro-3-indoxyl phosphate *p*-toluidine in 100 ml buffer containing 50 mM $NaHCO_3$, 4 mM $MgCl_2$, pH 9.6.

Analytical. Copper, zinc and cadmium were quantified by flameless atomic absorption spectrometry on a Perkin-Elmer Zeeman 3030 spectrometer. Protein was analysed according to the procedure of Bradford (1976). Electronic absorption was measured on a Beckman DU-40 spectrophotometer.

Results and discussion

Intralumenal exposure of both porcine and bovine small intestine (jejunum) segments to 100–200 µM Cu^{2+} in P_i/NaCl exerted an orange-red luminescence of the mucosal layer within an hour. This luminescence was conveniently generated by light in the 300-nm region at 20° C (Beltramini and Lerch 1981). Moreover, time-dependent increase of the same fluorescence emission was observed in the lumenal fluid. This phenomenom can best be explained by the formation of Cu-thionein in the intact mucosal cells and its excretion into the intestinal lumen. Similar observations have been made in the case of leucocytes and yeast after Cu-incubation (Hartmann et al. 1989; Felix et al. 1989). The luminescent compound in the cell medium was characterized as Cu-thionein.

It is well established that MT is involved in the homeostatic control of Cu and Zn absorption (Cousins 1985). High intestinal MT levels of Zn-loaded animals have been suggested to be part of a mechanism for secretion of zinc into the lumen (Smith and Cousins 1980). However, convincing data on the secretion of MT into the serosal fluid are missing.

Perfusion experiments on segments of intestinal copper-exposed porcine small bowel were used to elucidate whether or not intact Cu-thionein is released into the portal compartment. A 160-ml portion of the perfusate was collected for the first 5 h. Approximately 90% of the total perfusion volume was recovered as serosal fluid. An almost identical Cu concentration of 1 µM ± 5% was noticed in all fractions. Upon chromatography of the lyophilized samples on Sephadex G-75, three Cu-binding fractions including the exclusion volume, the 8–12-kDa region and the 1-kDa region were separated. The 8–12-kDa eluate was attributed to MT and was rechromatographed on Sephadex G-50 where it migrated as one single Cu-containing band. The amino acid analyses and the electronic absorption spectrum in the ultraviolet region of this fraction were similar to those of earlier Cu-thionein preparations (Hartmann and Weser 1977; Krauter et al. 1989). After Sephadex G-50 gel filtration, the yield of MT which was secreted from a 75-cm bowel segment during a 5-h perfusion was calculated to be 0.5–1 mg. It is common knowledge that, in the case of the extensive preparation processes, considerable loss of metals is experienced (Hartmann and Weser 1977). Thus it was not surprising to detect only some 3%–5% of Cu and Zn normally present in the intact monomeric MT. Furthermore, it is concluded that this low metal/protein stoichiometry is due to oxidation of the Cu,Zn-thiolate clusters and subsequent loss of the metals during the perfusion and purification processes. Thus, portions of the copper appearing in the approximately 1-kDa peak eluted from the Sephadex G-75 column may have originally been bound to MT. This assumption is supported by the observed characteristic orange-red fluorescence emission which increased considerably when Cu(I) was added again to the chromatographed protein.

Decisive evidence of the protein side of the serosal-released Cu-thionein was expected from the immunological identification. Fortunately monoclonal antibody II-10a to rat liver MT-I crossreacted with intestinal MT. ELISA of the Sephadex G-75 eluate as the coating antigen revealed two immunopositive fractions in both the exclusion volume of the column and in the 8–12

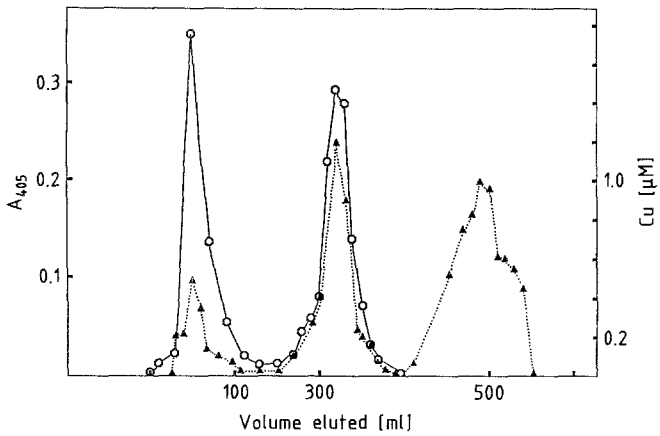

Fig. 1. Immunoreaction and Cu content of chromatographed serosal fluid. ELISA for MT (○) and copper concentration (▲) of the Sephadex G-75 eluate from the serosal fluid of a perfused intestinal segment. A 75-cm porcine intestinal sac, which was previously loaded intralumenally with 200 µM Cu, was perfused for 5 h at 20° C; 160 ml excreted serosal fluid was lyophilized. The sample was chromatographed on Sephadex G-75 (2.5 × 90 cm) at 4° C. The column was operated with 1 mM Tris/HCl pH 7.4 in the presence of 0.1% 2-mercaptoethanol. Immunoreactivity of MT was measured using ELISA as described in Materials and methods. Data are expressed as mean values from four independent measurements. The standard deviation was less than 8%

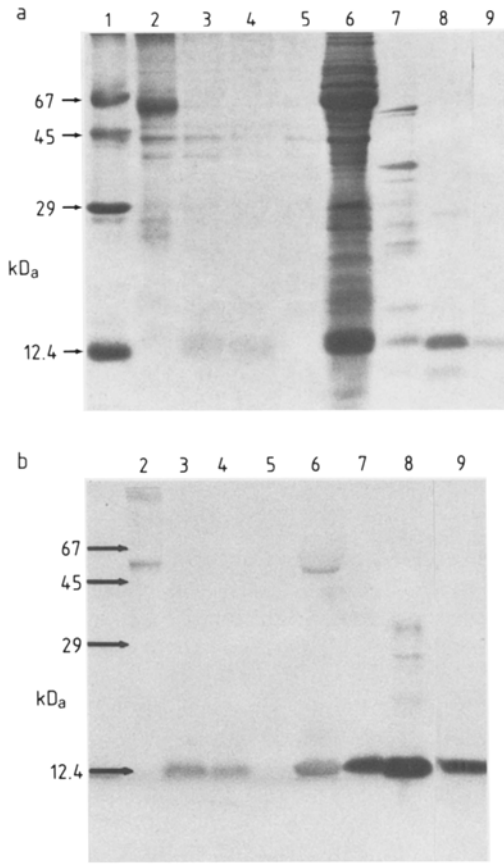

Fig. 2. SDS/PAGE (**a**) and immunoblot (**b**) of serosal protein fractions. Serosal fluid was obtained as in the legend to Fig. 1. SDS/PAGE (**a**) was performed according to Laemmli (1970) using a separation gel of 15% (mass/vol.) acrylamide. Lanes from right to left: (1) molecular mass standards: bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa); (2–5) Sephadex G-75 chromatography, fractions at different volumes eluted: (2) 160 ml (60–70 kDa), (3) 285 ml (18 kDa), (4) 315 ml (11 kDa), (5) 350 ml (7 kDa), (6) crude 5-h perfusare (lyophilized), (7) supernatant of pig liver homogenate, (8) isolated pig liver MT, (9) rat liver MT-I. For the immunoblot (**b**), the same protein banding was transferred from the second SDS/PAGE run in parallel onto nitrocellulose

kDa region (Fig. 1). The high M_r immunoreactive band indicates the formation of polymeric MT species in the serosal fluid during the course of the perfusion process. This polymerization of Cu-thionein did not lead to the complete loss of copper. Minor portions were still bound, possibly in an unspecific biuret manner.

Additional proof for the existence of metallothionein was obtained when immunoblotting was carried out. Prior to blotting, the samples were separated on SDS/PAGE. Samples of hepatic porcine MT from the supernatant of liver homogenate, isolated pig liver MT and rat liver MT-I were used as references. A clear immunoreactivity in the 12-kDa region was noticed with the samples obtained directly after the time-dependent fractionation of the Cu-thionein-containing serosal fluid and after chromatography on Sephadex G-75 (Fig. 2). In addition, bands corresponding to MT species of higher molecular mass were also recognized on the immunoblot.

Emphasis should be placed on the phenomenom that the ellipsoid-shaped 6-kDa MT always migrates like a 10–12-kDa globular protein during gel filtration and SDS/PAGE.

In order to support the results of a mucosal to serosal flux of thionein-bound copper, the intestinal transfer of Zn and Cd was additionally examined. Recently, the intestinal transfer of Cd in everted sacs from small intestine was reported (Ohta et al. 1989). However, no data of an interaction of the metal with MT was given. A similar model system was used in the present investigation on a possible Cu-, Zn- and Cd-MT excretion. Jejunum sacs of 30 cm length, previously charged with 200 ml P_i/NaCl and in the presence of 200 µM $CuCl_2$, $ZnCl_2$ and $CdCl_2$, respectively, were kept for 5 h in 200 ml 2.5 mM potassium phosphate pH 7.4 at 20° C. Specimens containing no additional metal served as the control. Prior to ligating both ends of the inestinal bowel, segments were turned inside to prevent the release of MT and/or mucosal cells into the serosal compartment. To maintain tissue vitality, no oxidation protection reagent was added to the serosal fluid. According to histological analysis no tissue damage was noticed. Moreover no significant change of lactate dehydrogenase release into serosal fluid was noticed during the experiment. The serosal concentration of this enzyme can be used as an indicator for intestinal tissue damage (Toledo-Pereyra et al. 1974). The concentrations of the transferred metals after 5 h ranged over 2–9 µM, whereas a similar protein concentration of approximately 1.75 mg/ml was measured throughout (Table 1).

An identical aliquot of either freeze-dried sample was chromatographed using FPLC on Superose 12 and

Table 1. Metal and protein concentrations in the serosal compartment of differently intralumenally metal-treated intestinal sacs after 5-h incubation at 20° C

Intestinal metal content (200 μM each)	Metal concentration [μM]			Protein concentration [mg × ml^{-1}]
	Cu	Zn	Cd	
Control	0.34	3.51	—	1.55
Cu	2.80	3.50	-	1.90
Zn	0.30	9.18	—	1.75
Cd	0.24	3.90	6.50	1.60

The volume was 200 ml

the fractions were immunologically analysed for MT by ELISA. A clear immunoreaction was noticed when the Cu-, Zn- and Cd-pretreated intestinal segments were used (Fig. 3). A similar elution pattern to that observed during Sephadex G-75 gel filtration was seen. There was one exception in that an additional high-M_r fraction was present after FPLC. This was attributed to the extended separation range of Superose 12. In addition to the 8–12-kDa region, the occurrence of high-M_r proteins can be explained by the well known inter- and intra-molecular polymerization of MT.

Unlike the fairly stable Cu- and Cd-thioneins, low-M_r MT species are absent in the case of Zn-thionein. Nevertheless, it is obvious that, similar to the Sephadex G-75 chromatographed perfusate, the copper and cadmium concentrations parallelled the immunopositive fractions of the Superose 12 effluent. Essentially the same immunoreactivity pattern was observed in the control experiment. However, the intensities of the ELISA values were substantially lower.

General conclusion

In the present study our interest was aimed at copper transport. Earlier work favoured the concept that albumin and/or amino acids are the ligands capable of transporting copper in the portal circulation after being transferred from the intestinal lumen and through absorptive cells. For example, intralumenal addition of histidine caused substantial uptake and serosal release of copper and reconfirmed the earlier studies mentioned above. It should be emphasized that in the histidine-treated specimen no detectable rise of MT release was noticed. Unlike the involvement of glutathione in the synthesis of cytosolic Cu-thionein (Freedman et al. 1989), no improved Cu-thionein concentration in the serosal fluid was observed. This result was not surprising as glutathione is barely present extracellulary.

The present results unequivocally demonstrate that indeed part of the ingested copper enters portal circulation in the form of Cu-thionein. MT has been detected by radioimmunoassay in peripheral blood plasma from untreated rats in low concentrations (1–8 ng/ml), dependent on factors such as age and gender (Bremner et al. 1987). Increased secretion of MT-I into blood may also occur after imposition of certain types of stress, metal status and dietary supply of several metals (Sato et al. 1984). It is emphasized that plasma MT levels respond to a wide range of physiological stimuli (Bremner et al. 1987). The liver is suggested to be the main source of the peripheral plasma MT as there is

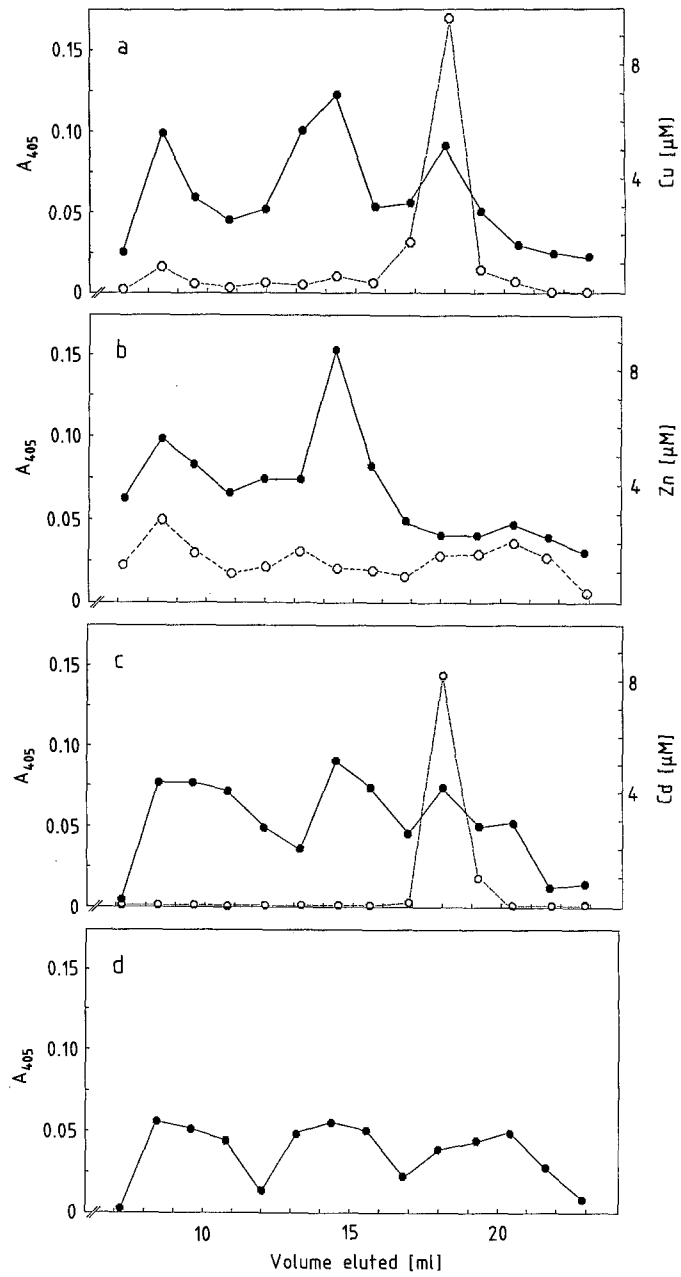

Fig. 3 a–d. Immunological assay for MT in the serosal compartment by ELISA. Into the lumen of ligated porcine 30-cm intestinal segments, 200 μM Cu^{2+}, Zn^{2+} and Cd^{2+} in P_i/NaCl were each added. They were kept for 5 h in 2.5 mM potassium phosphate pH 7.4 at 20° C. In the control experiments no metals were added. Identical aliquots of the lyophilized samples were chromatographed by FPLC on Superose 12 equilibrated with 50 mM Tris pH 7.4. Addition of (**a**) Cu, (**b**) Zn, (**c**) Cd, (**d**) no metal; immunoreactivity (●), metal concentration (○). All ELISA measurements were carried out in quadruplicate. The standard deviation was better than 8%

often a close linear relationship between hepatic MT and blood MT concentrations (Bremner et al. 1987). No function of this protein in blood plasma is known so far. However, the occurrence of Cu-MT in portal plasma after dietary copper uptake can be considered a genuine extracellular transport system for the biologically prominent transition metals copper and zinc. At the same time, less beneficial metals including Cd or Hg are expected to follow the same route.

Acknowledgement. This study was supported by the *Deutsche Forschungsgemeinschaft* We 401/21.

References

Beltramini M, Lerch K (1981) Luminescence properties of neurospora copper metallothionein. FEBS Lett 127:201–203

Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC (1984) A rapid, sensitive method for detection of alkaline-phosphatase-conjugated anti-antibody on Western blots. Anal Biochem 136:175–179

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bremner I (1987) Nutritional and physiological significance of metallothionein. In: Kägi JHR, Kojima Y (eds) Metallothionein II. Birkhäuser, Basel, pp 81–107

Bremner I, Mehra RK, Sato M (1987) Metallothionein in blood, bile and urine. In: Kägi JHR, Kojima Y (eds) Metallothionein II. Birkhäuser, Basel, pp 507–517

Bush JA, Mahoney JP, Markowitz H, Gubler CJ, Cartwright GE, Wintrobe MM (1955) Studies on copper metabolism. XVI. Radioactive copper studies in normal subjects and in patients with heptolenticular degeneration. J Clin Invest 36:1766–1778

Cousins RJ (1985) Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol Rev 65:238–309

Cohen DI, Illowsky B, Linder MC (1979) Altered copper absorption in tumor-bearing and estrogen-treated rats. Am J Physiol 236 (Endocrinol Metab Gastrointest Physiol 5):E309–315

Crane IJ, Hunt DM (1983) A study of intestinal copper-binding proteins in mottled mice. Chem Biol Interact 45:113–124

Elmes ME, Clarkson JP, Jasani B (1987) Histological demonstration of immunoreactive metallothionein in rat and human tissues. In: Kägi JHR, Kojima Y (eds) Metallothionein II. Birkhäuser Verlag, Basel, pp 533–537

Engvall E (1980) Enzyme immunoassay ELISA and EMIT. Methods Enzymol 70:419–439

Ettinger MJ (1984) Copper metabolism and diseases of copper metabolism. In: Lontie R (ed) Copper proteins and copper enzymes. CRC Press, Boca Raton FL, pp 175–229

Evans GW (1973) Copper homeostasis in mammalian system. Physiol Rev 53:535–570

Evans GW, Majors PF, Cronatzer WE (1970) Mechanism for cadmium and zinc antagonism of copper metabolism. Biochem Biophys Res Commun 40:1142–1148

Felix K, Hartmann H-J, Weser U (1989) Cu(I)-thionein release from copper-loaded yeast cells. Biol Metals 2:50–54

Freedman JH, Ciriolo MR, Peisach J (1989) The role of glutathione in copper metabolism and toxicity. J Biol Chem 264:5598–5605

Hartmann H-J, Weser U (1977) Copper thionein from fetal bovine liver. Biochim Biophys Acta 491:211–222

Hartmann H-J, Schechinger T, Weser U (1989) Copper-thionein in leucocytes. Biol Metals 2:40–44

Krauter B, Nagel W, Hartmann H-J, Weser U (1989) Copper-thionein in melanoma. Biochim Biophys Acta 1013:212–217

Laemmli K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–865

Nagel W, Hartmann H-J, Weser U (1989) Monoclonal antibodies to monomeric metallothionein (abstr.). Int Chem Congr Pacific Basin Soc, Part I, No 0497

Neumann PZ, Sass-Kortsak A (1967) The state of copper in human serum: evidence for an amino acid bound fraction. J Clin Invest 46:646–65

Ohta H, De Angelis MV, Cherian MG (1989) Uptake of cadmium and metallothionein by rat everted intestinal sacs. Toxicol Appl Pharmacol 101:62–69

Pickart L, Freedman JH, Loker WJ, Peisach J, Perkins CM, Stenkamp RE, Weinstein B (1980) Growth-modulating plasma tripeptide may function by facilitating copper uptake into cells. Nature 288:715–717

Sarkar B, Kruck TPA (1966) Copper-amino-acid complexes in human serum. In: Peisach J, Aisen P, Blumberg WE (eds) The biochemistry of copper. Academic Press, New York, pp 183–196

Sato M, Mehra RK, Bremner I (1984) Measurement of plasma metallothionein-I in the assessment of the zinc status of zinc-deficient and stressed rats. J Nutr 114:1683–1689

Smith KT, Cousins RJ (1980) Quantitative aspects of zinc absorption by isolated, vasculary perfused rat intestine. J Nutr 110:316–323

Starcher BC (1969) Studies on the mechanism of copper absorption in the chick. J Nutr 97:321–326

Toledo-Pereyra LH, Simmons RL, Najarian JS (1974) Two-to-three day intestinal preservation utilising hypothermic pulsatile perfusion. Ann Surg 179:454–459