Considerations for determining 'optimal nutrition' for copper, zinc, manganese and molybdenum

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Defining optimal dietary intakes of Cu and Zn throughout the life cycle continues to present a considerable challenge for nutrition scientists. Although the daily intake of these micronutrients is below that currently recommended for many groups, traditional biochemical indicators of nutritional status for these trace metals largely remain within the normal range. Thus, it is unclear whether the recommended daily intakes may be unnecessarily high, or if the commonly-used markers simply lack the necessary sensitivity and specificity that are required for accurately assessing Cu and Zn status. The increasing number of reports that daily supplements with these trace metals enhance the activities of selective metalloenzymes and specific cellular and organ processes further points out the need to differentiate between meeting the requirement and providing optimal nutriture. Results from recent studies suggesting that alternative molecular and functional markers possess sufficient sensitivity to better assess Cu and Zn status are discussed. Likewise, recent studies evaluating the impact of very low and excessive levels of dietary Mn and Mo on selective biochemical and metabolic indicators are reviewed.

Copper: Zinc: Manganese: Molybdenum: Nutritional assessment

Discussion of approaches to define optimal nutrition for Cu, Zn, Mn and Mo presents an interesting challenge when it is considered that the Food and Nutrition Board of the (US) National Research Council (1989) concluded that there was sufficient information to establish a recommended dietary allowance for Zn, but not Cu, Mn or Mo. Instead, Cu, Mn and Mo, along with Cr, fluoride, biotin and pantothenic acid, were assigned to the category that provides a range of daily intakes that are estimated to be safe and adequate for age and sex, i.e. the estimated safe and adequate daily dietary intake (ESADDI). Before discussing recent discoveries about the individual trace metals, it seems instructive to consider several similarities of these nutrients. All four serve as catalytic and/or structural cofactors for specific proteins. Their metabolism is regulated to ensure the delivery of adequate, but not excessive, quantities of the metal to the appropriate cellular compartments for incorporation into the apo-metalloproteins over a range of dietary intakes. The consequences of severe dietary deficiencies and frank toxicity for each element were described initially with experimental animals. The outcomes are quite similar for human subjects who inadvertently experience severe dietary restriction or excessive exposure to these metals, as well as

for individuals with inherited disorders that impair transport or metabolism of a specific trace metal. Insights related to the regulation of metabolism have been gained by the use of radioactive isotopes and the recent development and application of stable-isotope methodology. Likewise, the application of the tools of molecular biology has provided new information about the metabolism and functions of these inorganic nutrients. The development of imaging techniques allows the non-invasive investigation of the responses of tissues and organs to various levels of these trace metals. Thus, the combined information from past, ongoing and future studies will facilitate the development of strategies required to define optimal nutrition of specific populations and, perhaps, even individuals throughout their life cycle. The following discussion focuses on approaches for defining optimal Cu nutriture and then considers Zn, Mn and Mo in less detail.

Copper

The level of Cu intake that has been recommended as safe and adequate (ESADDI) is 1·5–3 mg/d for adults (National Research Council, 1989). Dietary analyses have consistently

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shown that typical Western diets fail to provide the lower limit of the recommended range for adults, as well as children and infants (Pennington & Young, 1991; Van Dokkum, 1995). Estimates for the actual Cu content of daily meals are 1·0–1·2 mg for adults. Assuming that the recommended range is appropriate, Cu deficiency would be expected to be relatively common. However, overt symptoms of Cu deficiency are quite rare. This situation suggests that the lower limit of the recommended range for Cu intake is simply too high, that marginal Cu deficiency may be common but difficult to diagnose, that water provides additional Cu to meet the dietary requirement, or some combination of such factors. Numerous investigators have addressed these possibilities during the past decade without clear resolution of the problem. Regardless of the eventual outcome, it is evident that there is some threshold at which the supply of dietary Cu is inadequate to sufficiently maintain optimal activities of Cu-dependent processes, and that such a response is likely to be influenced by a variety of factors in addition to the level of the metal in the diet. Thus, the need to identify sensitive and specific markers of Cu status will remain a challenging area for inquiry. Before reviewing some specific observations related to the Cu 'requirement', the general biochemical functions and metabolism of the metal will be briefly considered. A series of reports presented at the 1996 International Conference on Genetic and Environmental Determinants of Copper Metabolism provide excellent in-depth reviews of the nutrition, metabolism, functions and clinical aspects of Cu (Lonnerdal & Uauy, 1998) and complement several recent comprehensive reviews on Cu (Linder, 1996; Harris, 1997).

The typical human adult body contains approximately 100 mg Cu. The majority of the Cu that has been characterized is tightly bound to less than two dozen identified proteins. Cu serves as a catalytic cofactor in some of these metalloproteins. These known cuproenzymes participate in diverse cellular and extracellular activities, including ATP production (cytochrome c oxidase; *EC* 1.9.3.1; CCO), O_2 metabolism (superoxide dismutase; *EC* 1.15.1.1; SOD), Fe transport (caeruloplasmin; Cp; enzyme *EC* 1.16.3.1), the maturation of extracellular matrix (lysyl oxidase; *EC* 1.4.3.13) and neuroendocrine peptides (peptidylglycine α-amidating monooxygenase; *EC* 1.14.17.3; PAM), the synthesis of noradrenaline (dopamine-β-monooxygenase; *EC* 1.14.17.1) and melanin (tyrosinase; *EC* 4.1.99.2), and the catabolism of histamine and polyamines (diamine oxidase; *EC* 1.4.3.6; DAO). Obviously, maintenance of these activities depends on the ability of the organism to provide adequate quantities of Cu for incorporation into the apo-cuproproteins.

Foods are believed to represent the major source of Cu for higher animals and human subjects, although water (Olivares & Uauy, 1998) and nutritional supplements (Johnson *et al.* 1998) can contribute considerable quantities of the metal. Generally, 30–60 % of ingested Cu is absorbed across the intestinal mucosa and rapidly transferred to the liver and kidney. Within the liver much of the recently-acquired Cu is incorporated into Cp and returned to the circulation for distribution to other tissues. The recent discovery that tissue levels of Cu are normal in acaerulo-plasminaemic patients (Harris *et al.* 1998) indicates that

there are alternative pathways for cellular acquisition of this metal. Within the cell Cu distribution among organelles and exportation appears to be mediated by P-type ATPases. Defects in these pumps are the basis for Wilson and Menkes diseases. Cu leaving peripheral tissues can re-enter the circulation and be taken up by liver for re-utilization or excretion. Excess hepatic Cu is exported into bile for elimination from the body and can be stored temporarily as metallothionein (Dameron & Harrison, 1998). Urinary losses of Cu are typically quite low.

In a sentinel human study Turnland *et al.* (1989) examined Cu absorption and retention by young healthy men fed on meals that provided low, adequate or high daily intakes of Cu (0·8, 1·7, and 7·5 mg Cu/d respectively). The results demonstrated that the efficiency of Cu absorption varied inversely with dietary Cu content, and most subjects achieved balance within 1 week of the transition to a different level of dietary Cu. Thus, absorption and biliary excretion represent processes subject to homeostatic control over a broad range of Cu intake. This discovery provided initial support for the possibility that the lower limit of the ESADDI was set too high. However, it was not clear if homeostatic adaptation might falter when the nutritional stress was longer in duration, or if the activities of specific cuproenzymes and processes dependent on such activities were altered at the extremes of intake.

Intensive efforts by Milne (1998) and Turnland (1998) and their associates during the past decade have addressed these issues in a series of well-controlled human trials that have investigated the responses of different populations of healthy adults. Numerous indicators known to change in severe Cu deficiency in animals and human subjects have been monitored, and include (a) plasma levels of Cu and Cp protein and enzyme activity, as well as the activities of Cu,Zn-SOD and CCO in plasma and/or blood cells, (b) haematological profile (erythrocytes, neutrophils and platelets), and (c) non-invasive assessments of cardiac activity, blood pressure and immunocompetence. In general, these variables were not altered, changed minimally, or responded inconsistently when daily dietary intake of Cu exceeded 0·75 mg. In contrast, changes in some indicators were noted when daily Cu intake was 0·4–0·7 mg.

Milne & Nielsen (1996) observed significant reductions in erythrocyte SOD and platelet Cu and CCO activity in post-menopausal women fed on a diet containing 0·57 mg Cu for 105 d. The greatest change was a 50 % reduction in the activity of platelet CCO activity between weeks 10 and 13 of the Cu-depletion phase. Plasma and urinary Cu, Cp enzyme activity and protein, and haematological variables were resistant to low Cu intake. Platelet CCO activity remained significantly depressed after subjects were repleted with 2 mg Cu daily for 5 weeks, whereas platelet Cu was restored to control levels during this period. Turnland *et al.* (1997) reported significant declines in levels of Cu content of plasma, polymorphonuclear cells and urine, Cp protein and enzyme activity, and erythrocyte and extracellular SOD activities after feeding healthy men on a diet with only 0·38 mg Cu/d for 6 weeks. The majority of these variables responded to Cu supplementation. There were no significant disorders in heart rate during the depletion and repletion phases of the study. Lysyl oxidase in skin also

declined in response to very low Cu intake and rebounded after supplementation (Werman *et al.* 1997). The results prompted Turnland *et al.* (1997) to conclude that the minimum daily requirement for Cu is between 0·4 and 0·8 mg, a level markedly below current recommendations.

The previously mentioned studies have provided new insights regarding the lower limit of the Cu requirement for healthy adults in controlled settings. However, the task of defining optimal Cu intake for diverse groups of free-living individuals represents a different problem. This is particularly so for individuals with clinical conditions that predispose to Cu deficiency (e.g. prematurity and prolonged total parenteral nutrition without Cu) and the chronic use of Zn supplements (Beshgetoor & Hambidge, 1998). Selection of appropriate biochemical and functional indicators of Cu status needs to be considered carefully before initiating screening. Milne (1994) has critically reviewed the sensitivity and specificity of the traditional indicators of Cu in blood. Low levels of plasma Cu and Cp are good markers of clinical Cu deficiency. Moreover, samples require minimal processing and are stable for extended periods, and the assays are simple. However, they alone cannot be used as indicators of Cu status, since plasma levels are elevated by oestrogen, pregnancy, infection and inflammation. Moreover, the relative insensitivity of these markers to even very low Cu intake in the human trials suggests that the threshold for change is below the level of Cu provided by most diets. Erythrocyte SOD activity seems to respond to rigorous restriction of dietary Cu. However, the degree of change appears to be limited by the slow rate of erythrocyte turnover. Intense physical exercise also may elevate the amount of SOD protein in erythrocytes (Lukaski *et al.* 1990). Milne (1994) favours the use of CCO activity in platelets and leucocytes as a marker, since these cells have short lifetimes and may better reflect the metabolically-active pool of endogenous Cu. However, the isolation of specific cell types is labour intensive, the enzyme is labile, and the assay of its activity is subject to considerable variation. The need to monitor at least several of the previously mentioned biochemical markers to assess Cu status is evident. Identification of additional reliable markers would be beneficial, and several possible candidates are discussed later.

An alternative approach to defining optimal Cu nutriture is to evaluate whether supplementation with the metal enhances Cu-dependent processes. The evident concern with this approach is the potential toxicity of excess Cu. The pathological consequences of Cu overload in animals and individuals with impaired ability to excrete Cu via bile (e.g. Wilson's disease) are instructive for establishing upper limits for Cu intake (Bremner, 1998). There are several reports of particular interest. Adults receiving daily supplements of 10 mg Cu as the gluconate chelate for 12 weeks did not exhibit any signs of toxicity (Pratt *et al.* 1985). Similarly, supplementation of breast-fed and formula-fed infants with Cu via drinking water from 3–12 months of age did not adversely affect growth, liver function or indicators of Cu status (Olivares *et al.* 1998). Ingestion of the high-Cu water by formula-fed infants doubled daily Cu intake (2·5 *v.* 1·2 mg). It has been shown also that short-term supplementation (4 weeks) of hypercholesterolaemic men with 2 mg Cu daily did not increase the oxidation of lipoproteins

in vivo (Jones *et al.* 1997). These results support the likelihood that reasonable doses of supplemental Cu do not exceed the homeostatic capacity to maintain a safe body burden of the metal. Obviously, individuals with impaired biliary activity (e.g. premature and newborn term infants, and patients with cholestatic liver conditions) are at risk for Cu toxicity if supplemented.

Additional potential biomarkers for assessing optimal copper nutriture

Cuproenzymes. Several recent reports suggest that the activity of the cuproenzyme PAM in plasma merits special consideration as a potentially-useful indicator of Cu status. PAM is required for the post-translational modification of numerous neuroendocrine peptides to their bioactive forms which possess an amidated carboxyl terminus (Eipper *et al.* 1992). Representative α-amidated peptides include calcitonin, cholecystokinin, gastrin, neuropeptide Y, oxytocin and vasopressin. Two distinct catalytic activities are required for the covalent modification. The COOH-terminal glycyl residue of the peptide substrate is converted to peptidyl-α-hydroxyglycine by the Cu, ascorbate and molecular oxygen-dependent peptidylglycine α-hydroxylating monooxygenase. The peptidyl-α-hydroxyglycine-α-amidating lyase cleaves the intermediate to produce the α-amidated peptide and glyoxylate. Inhibition of PAM activity increases the concentration of glycine-extended peptides and decreases that of the α-amidated peptides in tissues.

The possibility that Cu availability affects PAM activity was suggested by several early studies. Treatment of murine pituitary cell cultures and rats with disulfiram, the disulfide dimer of the high-affinity Cu chelator diethyldithiocarbamate, inhibited PAM activity and increased the quantities of several glycine-extended peptides (Mains *et al.* 1986). Also, serum activity of PAM was reduced in adult male rats after feeding a Cu-deficient diet for 9 weeks (Main *et al.* 1985). Prohaska (1997) examined the effects of marginal and moderate Cu deficiencies in young rats on the activities of several cuproenzymes in the heart and plasma. The results clearly showed that serum PAM activity, like that of Cp enzyme, was correlated with Cu intake. The activities of PAM, CCO and Cu,Zn-SOD in the heart also were modulated by Cu intake. The observation that *in vitro* addition of Cu^{2+} restored PAM activities in samples from Cu-restricted rats to those of the controls is particularly interesting. In contrast, exogenous Cu failed to increase the activities of Cp enzyme, CCO and Cu,Zn-SOD. Prohaska (1997) proposed that comparison of serum PAM activity in the presence and absence of Cu salt may be useful for assessing Cu status. Additional support for this possibility is available. First, several subjects with a mild variant of Menkes disease were found to have significantly lower levels of plasma Cu and Cp than control subjects (Prohaska *et al.* 1997). Although plasma PAM activity was similar for the two groups, *in vitro* addition of Cu stimulated enzyme activity 3·0- and 1·6-fold in samples from subjects with Menkes disease and control subjects respectively ($P < 0·001$). This finding suggests that the amount of plasma PAM protein increases in response to Cu deficiency.

Second, the stimulatory influence of exogenous Cu on PAM activity in plasma from an adult female with acquired Cu deficiency (Smith *et al*. 1994) decreased in response to Cu supplementation *in vivo* (JR Prohaska, personal communication). Third, it is noteworthy that plasma PAM activity in adult male rats was not altered in response to a variety of changes in endocrine status and was similar in male and female animals (Mains *et al*. 1985). Finally, quantification of PAM activity requires a small volume of plasma that can be obtained from a microhaematocrit tube, thereby eliminating the need for venipuncture (Prohaska, 1997). Further examination of the effects of nutritional, physiological and pathological stresses on plasma PAM activity is warranted in light of the very interesting observations concerning this cuproenzyme.

A second cuproenzyme that has received recent attention as a possible indicator of Cu status is DAO. DAO oxidatively deaminates polyamines such as cadaverine and histamine to aldehydes and ammonium ion with the subsequent production of H_2O_2. It has been proposed that DAO activity provides a barrier that prevents potentially-damaging polyamines from entering the circulation (Wolvekamp & deBruin, 1994). High activities are present in the intestine and other tissues containing rapidly-replicating cells. Low levels of DAO activity of unknown origin are normally present in the plasma of animals and human subjects. Regarding the response of DAO to altered Cu status, DiSilvestro *et al*. (1997) found low activity of plasma DAO in an adult female with diagnosed Cu deficiency, and this activity was elevated to normal by parenteral Cu administration. Similarly, plasma DAO activity was about 6-fold lower in rats fed on a moderately-low-Cu diet for 6 months. The impact of Cu supplementation on plasma DAO activity has been examined in two double-blind placebo-controlled crossover studies. Jones *et al*. (1997) supplemented twenty hypercholesterolaemic adult males with 2 mg glycine-chelated Cu daily for 4 weeks. Cu supplementation significantly increased plasma DAO and erythrocyte SOD activities in those subjects whose plasma Cu levels were below the group median at the start of the trial. The responsiveness of plasma DAO activity to supplementation with a higher level of Cu has been examined in a trial in Northern Ireland (Kehoe *et al*. 1999). Healthy adult men (*n* 12) and women (*n* 12) received supplements of 3 and 6 mg Cu daily for 6-week periods that were interspersed with periods of equal length when placebo was administered. Serum DAO activity was elevated 2- to 5-fold ($P < 0.01$) in subjects at the end of each period of Cu supplementation. In contrast, there were either no responses or limited changes in the activities of cellular SOD and serum Cp to Cu supplementation. Subjects did not experience clinical symptoms of acute Cu toxicity such as nausea or gastrointestinal distress during the supplementation periods. These findings encourage further inquiry concerning the use of plasma DAO as a biomarker of Cu status. However, its utility may be limited, since activity is higher in females than males (JJ Strain, personal communication), elevated in response to pregnancy and some types of cancer, cystic fibrosis, uraemia, intestinal ischaemia and renal dialysis (Wolvekamp & deBruin, 1994; DiSilvestro *et al*. 1997), and lower in subjects with Crohn's and coeliac diseases (Wolvekamp & deBruin, 1994), and severe trauma (Joung *et al*. 1998).

Functional indicators of optimal copper status. Recent studies suggest that selective activities of the skeleton and immune system may serve as helpful markers for defining optimal Cu nutriture. Osteoporotic lesions and increased incidence of infections have been reported for Cu-deficient animals and human subjects (Harris, 1997). Ten years ago, Strain (1988) suggested that mild Cu deficiency had the potential to contribute to the onset and progression of osteoporosis during the ageing process. Studies by Strause *et al*. (1994) demonstrated that chronic daily supplementation with 1000 mg Ca (as citrate malate complex) plus the gluconate salts of Cu (2·5 mg), Mn (5 mg) and Zn (15 mg) significantly arrested spinal bone loss in post-menopausal women; supplementation with only Ca or the trace metal mix were not as effective. The actual contribution of Cu to the maintenance of bone mineral density was unknown. Eaton-Evans *et al*. (1996) addressed this question by conducting a random double-blind study in which women aged 45–56 years received either a placebo or 3 mg glycine-chelated Cu daily for 2 years. Initial and final bone mineral density of the lumbar vertebrae were not different in the Cu-supplemented group, whereas bone mineral density in the group receiving placebo declined significantly ($P < 0.01$). This apparently beneficial effect of the Cu supplement was not associated with a change in the activities of erythrocyte SOD or plasma alkaline phosphatase (*EC* 3.1.3.1). The outcome supports the need for further examination of functional responses to Cu supplementation as a tool for defining optimal Cu intake for specific populations.

The essentiality of Cu for the maturation and signal-mediated activation of immune cells is well recognized (Percival, 1995; Failla & Hopkins, 1998). The observation that *in vitro* DNA synthesis and interleukin 2 (IL-2) production by T-cells and respiratory-burst activity of neutrophils from adult rats chronically fed on a diet marginally low in Cu were impaired is particularly interesting (Hopkins & Failla, 1995). Since traditional indicators of Cu status such as tissue activities of Cu,Zn-SOD and plasma Cp were not altered by the chronic dietary treatment, immunocompetence appears to be very sensitive to marginal Cu deficiency in the rat. Our efforts have focused on defining the role of Cu in modulating the synthesis of the cytokine IL-2 for the following reasons: (a) IL-2 plays a central role in coordinating cell-mediated immunity; (b) the regulation of IL-2 gene expression is well characterized; (c) *in vitro* production of IL-2 is attenuated by decreased cellular Cu; (d) IL-2 production can be restored to normal by supplementing Cu-deficient cells with low levels of Cu, but not other metals (Bala & Failla, 1992; Hopkins & Failla, 1997; Failla & Hopkins, 1998). We have found that reduced Cu content attenuates transcriptional activity, but not IL-2 mRNA stability, using Jurkat cells stably transfected with a luciferase reporter gene driven by the full-length IL-2 promoter–enhancer region (Hopkins & Failla, 1999). Elucidation of the mechanism by which cellular Cu status modulates IL-2 expression may identify novel markers in leucocytes that respond to Cu status in a specific and sensitive manner. It should be noted that the mitogenic reactivity of T-cells isolated from human subjects fed on a

very-low-Cu diet (Kelley *et al.* 1995) and from a woman with clinical Cu deficiency (Smith *et al.* 1994) was impaired. The potential ability of physiological levels of Cu to stimulate *in vitro* mitogenic responsiveness and IL-2 production by human T-cells, as it does for rat cells (Bala & Failla, 1992), also merits consideration as a sensitive biomarker for detecting marginal Cu deficiency.

Zinc

Zn is essential for the activities of an impressive array of enzymes and regulatory proteins. In addition to its role as a catalytic cofactor for all six classes of enzymes, Zn provides structural integrity for the activities of nuclear transcription factors, including the steroid receptor superfamily. The metal also is required by protein kinases that participate in signal transduction processes and for the activation of *trans*-acting proteins that regulate gene expression. With essential roles in such fundamental cellular processes, it is not surprising that the whole-body content of Zn is tightly controlled. During periods of low Zn intake, absorption is enhanced and secretion of endogenous Zn into the gastrointestinal lumen is suppressed. In contrast, high Zn intake is associated with decreased absorption and enhanced secretion of endogenous Zn. Within cells, fluctuations in Zn content are modulated by changes in the amount of the metal associated with the storage protein metallothionein. Urinary losses usually are minimal. Up-to-date details about the biochemistry and metabolism of Zn are provided in several excellent reviews (Cousins, 1996; Chesters, 1997).

Results from the Total Diet Study (Pennington & Young, 1991) indicate that the amounts of Zn provided by typical diets are below the recommended daily allowance for children, adolescent females and women during their reproductive years, and elderly men and women. Results from surveys in a number of European countries are similar (Van Dokkum, 1995). The discrepancy between actual and recommended intakes is the basis for concern that many individuals may lack sufficient cellular pools during times of increased utilization, e.g. growth, reproduction and tissue repair (Aggett & Comerford, 1995). Traditional indicators of Zn, such as levels in plasma and the activities of Zn metalloenzymes in blood, are relatively resistant to changes in dietary Zn, but often altered by other physiological and pathological conditions. Nevertheless, the list of beneficial outcomes of administering Zn supplements to groups believed to be at risk of deficiency continues to increase (for example, see Prasad *et al.* 1993; Goldenberg *et al.* 1995; Sazawal *et al.* 1997). Together, these findings indicate the need for the identification of more sensitive and specific indicators of Zn status. Recent studies support the consideration of several additional factors as potential markers for defining better the optimal Zn status.

It is generally assumed that Zn is non-toxic because of the strong homeostatic regulation of processes controlling the absorption and endogenous secretion of the metal. Very high doses can cause gastrointestinal distress and vomiting. Levels that are present in many Zn nutritional supplements have been found to decrease the activity of SOD in erythrocytes. It is not clear if this alteration is sufficient to increase susceptibility to free-radical-induced damage or may reflect a general decrease in Cu status (Fosmire, 1990).

New potential biomarkers for assessing optimal zinc nutriture

Daily supplementation of post-menopausal women with 30 mg glycine-chelated Zn for 3 weeks increased the activity of the Zn-dependent enzyme 5-nucleotidase by 60 % (Blostein-Fujii *et al.* 1997). In contrast, the activity of this enzyme in plasma significantly decreased when elderly healthy women were fed on a low-Zn (4 mg) diet for 15 d; enzyme activity responded robustly when the subjects ingested 28 mg Zn for 6 d (Bales *et al.* 1994). Thymulin is a Zn-dependent immunomodulatory peptide secreted by thymic epithelial cells. Serum thymulin activity is decreased in mild Zn deficiency associated with either dietary Zn restriction or sickle-cell anaemia (Prasad *et al.* 1988). Thymulin activity was increased following Zn supplementation of the subjects and by *in vitro* addition of Zn to samples from mildly-Zn-deficient subjects. The ability of Zn to enhance *in vitro* activity when serum thymulin is not saturated with Zn provides a potentially-useful tool for examining optimal Zn status. While there are concerns about the labour-intensive nature of the standard bioassay (Chesters, 1997), its use in more laboratories and/or the development of alternative assays to monitor thymulin activity warrants consideration.

There has been considerable interest in the use of plasma or blood cell metallothionein as an indicator of Zn status, just as serum ferritin has been used as a marker of cellular Fe stores. Early studies by Bremner and associates (Sato *et al.* 1984; Bremner *et al.* 1987) showed that metallothionein could be detected in plasma and erythrocytes of rats, that the levels responded as predicted to changes in Zn status, and that endotoxin treatment increased plasma but not erythrocyte metallothionein content. Cousins and associates (Grider *et al.* 1990; Thomas *et al.* 1992) subsequently reported that erythrocyte levels of metallothionein were increased and decreased in adult men in response to Zn supplementation and deprivation respectively. More recently, this group has developed a sensitive method using the competitive reverse transcriptase–polymerase chain reaction to quantify the level of metallothionein mRNA in human monocytes. Monocytic metallothionein mRNA rapidly increased to levels about 4-fold above control levels when subjects received a 50 mg Zn supplement; levels returned to those in control samples within 4 d after discontinuation of the supplement (Sullivan & Cousins, 1997; Sullivan *et al.* 1998). Responses of metallothionein mRNA in monocytes to nutrient manipulations other than Zn and various stresses are needed to ascertain the specificity of the marker. Nevertheless, the possibility that the new method will be a useful tool for optimizing Zn nutriture is exciting.

Finally, a family of Zn transporters that mediate cellular efflux and, possibly, intracellular compartmentalization of the metal may provide additional markers related to Zn status (McMahon & Cousins, 1998*a*). Initial studies have revealed that the level of Zn transporter-1 mRNA in murine intestine and liver increases in response to Zn loading (Davis *et al.* 1998), and Zn transporter-1 mRNA and protein

levels also increase modestly when rats are fed on a high-Zn diet (McMahon & Cousins, 1998b).

Manganese

Mn is required as a catalytic cofactor for mitochondrial SOD, arginase (*EC* 3.5.3.1) and pyruvate carboxylase (*EC* 6.4.1.1). It also is an activator of glycosyltransferases, phospho*enol*pyruvate carboxylase (*EC* 4.1.1.31) and glutamine synthetase (*EC* 6.3.1.2). Foods, and particularly plant foods, represent the principal source of Mn for the activities of these proteins. Small amounts of ingested Mn are absorbed and initially transferred to the liver for utilization, for export back to the plasma for distribution to other tissues (probably via the transferrin endocytic pathway), or secretion into bile for elimination from the body. The metal is not stored in tissues, since biliary elimination of excess Mn is quite efficient. Studies with animals have shown that an insufficient dietary supply causes skeletal abnormalities that are probably due to defective proteoglycan synthesis secondary to the reduced activity of the glycosyl-transferases. Mn deficiency also impairs reproduction, exocrine and endocrine pancreatic activities, and carbohydrate and lipid metabolism. Peroxidative damage, especially in mitochondria, has been demonstrated in Mn-deficient tissues, in conjunction with decreased SOD activity in the organelle. Details concerning the metabolism and biochemistry of Mn are presented in recent reviews (Keen & Zidenberg-Cherr, 1996; Leach & Harris, 1997).

The results from the Total Dietary Study for 1982–9 indicate that the estimated intake for 6–11-month-old infants was 10 % above the upper limit of the ESADDI, while the intake of 14–16-year-old girls was approximately 10 % below the lower limit of the recommended range (Pennington & Young, 1991). Estimated intakes of Mn for young children (2 years), adolescent boys, and young and older adult men and women were all within the ESADDI range. Where monitored, Mn intakes in western European countries are also within the recommended range (Van Dokkum, 1995). Many multivitamin and mineral supplements for adults provide 2·5–5 mg Mn, and liquid nutritional supplements and ready-to-feed infant formulas contain bioavailable $MnSO_4$ (Johnson *et al*. 1998). These findings suggest that Mn intake throughout the life cycle meets the requirement. In support of this conclusion, there is only one report of apparent human Mn deficiency. This condition resulted from unintentional deletion of the metal from a purified vitamin K-deficient diet (Doisy, 1974). Friedman *et al*. (1987) observed that some (five of seven) male subjects developed a mild dermatitis after ingesting a diet with a very low Mn content (0·1 mg/d) for 39 d. However, whole blood and serum Mn did not change significantly in response to severe dietary deprivation of the metal.

The impact of Mn supplementation on possible indicators of Mn status has been carefully investigated in young women (Davis & Greger, 1992). Subjects received either placebo or 15 mg amino acid-chelated Mn daily for 124 d. Serum Mn increased linearly throughout the supplementation period, whereas lymphocyte Mn-SOD activity was slightly elevated only during the fourth month of supplementation. Urinary Mn was not altered by supplementation.

The increases in serum Mn and lymphocyte Mn-SOD activity were not affected by the use of oral contraceptives or stage of the menstrual cycle. However, the responses to Mn supplementation were greater in women consuming diets with more than 31 % of their energy from fat than from those consuming less than 30 % of their energy from fat. It was concluded that the change in cellular Mn-SOD activity reflected exposure to Mn rather than improved Mn status.

The demonstration of Strause *et al*. (1994) that supplementation of post-menopausal women with Ca plus a trace metal mix containing 5 mg Mn arrested the decline in spinal bone mineral density merits consideration. The contribution of Mn, as opposed to Cu and Zn, to the beneficial effect is unknown, and should be investigated.

Excess Mn in the brain adversely affects motor and cognitive function; continued accumulation can lead to severe psychiatric disorders and permanent neurological dysfunction (Keen & Zidenberg-Cherr, 1996; Leach & Harris, 1997). Workers in Mn mines are at high risk. Individuals with cholestatic liver disease are susceptible to Mn overload since they lack the means to excrete the excess metal. It has been demonstrated also that individuals receiving Mn-supplemented total parenteral nutrition are also at risk of Mn toxicity if liver and biliary function are compromised. Fell *et al*. (1996) recently reported a high incidence of hypermanganesaemia in children receiving the standard dose of Mn in parenteral nutrition formulas. This condition was associated with impaired liver function. Mn deposition in the brain was detected by magnetic resonance imaging scans, and the two patients with the highest blood Mn content exhibited movement disorders. Withdrawal of the supplemental Mn from the formula lowered blood Mn and improved liver function in a subgroup with cholestatic liver disease. The investigators recommended a reduction in the Mn level in parenteral formulas, suggesting that Mn toxicity itself can be a factor causing cholestatic liver disease, especially in young children.

Recommended markers for defining optimal manganese nutriture

Greger (1998) recently suggested a battery of potential biomarkers for assessing Mn exposure. Serum Mn, lymphocyte Mn-SOD activity and possibly blood arginase activity are recommended for detecting low Mn intake. Serum Mn, magnetic resonance imaging scans of the brain and a panel of neurofunctional tests are proposed for detecting excess exposure. Potential confounding factors that require attention include the elevation of Mn-SOD activity in response to oxidative stress and pro-inflammatory cytokines, and increased tissue arginase activity in response to high protein intake, diabetes and catabolic states with muscle wasting.

Molybdenum

Mo is required for the activities of aldehyde oxidase (*EC* 1.2.3.1), sulfite oxidase (*EC* 1.8.3.1), xanthine oxidase (*EC* 1.1.3.22) and xanthine dehydrogenase (*EC* 1.1.1.204). Within cells a portion of the metal is converted into a molybdopterin cofactor that binds to the

apomolybdoproteins. Metal that is not utilized in this manner is excreted. The properties of these enzymes and the synthesis of the cofactor are the subject of a recent excellent review (Johnson, 1997). Turnland *et al.* (1995*a*,b) have used stable isotopes to investigate Mo metabolism in healthy men. Mo absorption was efficient (about 90 %) when subjects ingested diets containing five levels of the metal (ranging from 22 to 1490 µg/d) for 24 d each. Excess Mo was rapidly excreted in urine, although whole-body retention was increased when the dietary level of the metal was low.

Results from the Total Diet Study indicate that the daily level of Mo intake by all age-groups and sex groups meets or exceeds the lower limit of the ESADDI (Pennington & Jones, 1987; National Research Council, 1989). Adult men fed on a diet with only 22 µg Mo (one-third of the lower limit of ESADDI) for 102 d did not develop any symptoms of Mo deficiency, leading Turnland *et al.* (1995*b*) to suggest that the minimum daily requirement for this trace element is about 25 µg. In light of the typical intakes, Mo deficiency would be expected to be rare. Indeed, there is only one recorded case of apparent Mo deficiency that occurred in a subject receiving total parenteral nutrition for 18 months due to Crohn's disease (Abumrad *et al.* 1981). The levels of S metabolites and uric acid in urine were restored in response to treatment with 300 µg ammonium molybdate daily (160 µg Mo). A cursory survey of labels on adult multivitamin and mineral formulations revealed that many include 160 µg Mo per tablet!

Insights about markers for defining optimal Mo nutriture are provided by studies with animals and human subjects with inherited disorders in the synthesis of the Mo cofactor (Johnson, 1997). Decreased urinary levels of sulfate and uric acid in conjunction with elevated levels of sulfite, hypoxanthine, xanthine and other S metabolites are indicative of impaired activities of the molybdoenzymes. The absence of toxicity symptoms in men fed on diets with 1490 µg Mo/d for 24 d provides a working upper boundary for further studies.

Conclusion

Widely-used biochemical and functional indicators of essential trace metal status generally lack both the sensitivity and the specificity that are required to define optimal intake at various stages of the life cycle. Recent efforts have provided a number of potential 'sensors' of cellular Cu, Zn and Mn status that merit further evaluation. The judicious application of methods in molecular biology and non-invasive imaging techniques is likely to provide new breakthroughs and rapid advances for the nutrition and biology of the trace metals.

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References

Abumrad NM, Schneider AJ, Steele D & Rogers LS (1981) Amino acid intolerance during prolonged total parenteral nutrition reversed by molybdate therapy. *American Journal of Clinical Nutrition* **34**, 2551–2559.

Aggett PJ & Comerford JG (1995) Zinc and human health. *Nutrition Reviews* **53**, S16–S22.

Bala S & Failla ML (1992) Copper deficiency reversibly impairs DNA synthesis in activated T lymphocytes by limiting interleukin-2 activity. *Proceedings of the National Academy of Sciences USA* **89**, 6794–6797.

Bales CW, DiSilvestro RA, Currie KL, Plaisted CS, Joung H, Galanos AN & Lin PH (1994) Marginal zinc deficiency in older adults: responsiveness of zinc status indicators. *Journal of the American College of Nutrition* **13**, 455–462.

Beshgetoor D & Hambidge M (1998) Clinical conditions altering copper metabolism in humans. *American Journal of Clinical Nutrition* **67**, 1017S–1021S.

Blostein-Fujii A, DiSilvestro RA, Frid D, Katz C & Malarkey W (1997) Short-term zinc supplementation in women with non-insulin-dependent diabetes mellitus: effects on plasma 5′-nucleotidase activities, insulin-like growth factor I concentrations, and lipoprotein oxidation rates in vitro. *American Journal of Clinical Nutrition* **66**, 639–642.

Bremner I (1998) Manifestations of copper excess. *American Journal of Clinical Nutrition* **67**, 1069S–1073S.

Bremner I, Morrison JN, Wood AM & Arthur JR (1987) Effects of changes in dietary zinc, copper and selenium supply and of endotoxin administration on metallothionein I concentrations in blood cells and urine in the rat. *Journal of Nutrition* **117**, 1595–1602.

Chesters JK (1997) Zinc. In *Handbook of Nutritionally Essential Mineral Elements*, pp. 185–230 [BL O'Dell and RA Sunde, editors]. New York: Marcel Dekker Inc.

Cousins RJ (1996) Zinc. In *Present Knowledge in Nutrition*, 7th ed., pp. 293–306 [EE Ziegler and LJ Filer Jr, editors]. Washington, DC: ILSI Press.

Dameron CT & Harrison MD (1998) Mechanisms for protection against copper toxicity. *American Journal of Clinical Nutrition* **67**, 1091S–1097S.

Davis CD & Greger JL (1992) Longitudinal changes of manganese-dependent superoxide dismutase and other indexes of manganese and iron status in women. *American Journal of Clinical Nutrition* **55**, 747–752.

Davis SR, McMahon RJ & Cousins RJ (1998) Metallothionein knockout and transgenic mice exhibit altered intestinal processing of zinc with uniform zinc-dependent zinc transporter-1 expression. *Journal of Nutrition* **128**, 825–831.

DiSilvestro RA, Jones AA, Smith D & Wildman R (1997) Plasma diamine oxidase activities in renal dialysis patients, a human with spontaneous copper deficiency and marginally copper deficient rats. *Clinical Biochemistry* **30**, 559–563.

Doisy EA Jr (1974) Effects of deficiency in manganese upon plasma levels of clotting proteins and cholesterol in man. In *Trace Element Metabolism in Animals-2*, pp. 668–670 [WG Hoekstra, JW Suttie, HE Ganther and W Mertz, editors]. Baltimore, MD: University Park Press.

Eaton-Evans J, McIlrath EM, Jackson WE, McCartney H & Strain JJ (1996) Copper supplementation and the maintenance of bone mineral density in middle-aged women. *Journal of Trace Elements in Experimental Medicine* **9**, 87–94.

Eipper BA, Stoffers DA & Mains RE (1992) The biosynthesis of neuropeptides: peptide amidation. *Annual Review of Neuroscience* **15**, 57–85.

Failla ML & Hopkins RG (1998) Is low copper status immunosuppressive? *Nutrition Reviews* **56**, S59–S64.

Fell JME, Reynolds AP, Meadows N, Khan K, Long SG, Quaghebeur G, Taylor WJ & Milla PJ (1996) Manganese toxicity in children receiving long-term parenteral nutrition. *Lancet* **347**, 1218–1221.

Fosmire G (1990) Zinc toxicity. *American Journal of Clinical Nutrition* **51**, 225–227.

Friedman BJ, Freeland-Graves JH, Bales CW, Behmardi F, Shorey-Kutschke RL, Willis RA, Crosby JB, Trickett PC & Houston SD (1987) Manganese balance and clinical observations in young men fed a manganese-deficient diet. *Journal of Nutrition* **117**, 133–143.

Goldenberg RL, Tamura T, Neggers Y, Copper RL, Johnston KE, DuBard MB & Hauth JC (1995) The effect of zinc supplementation on pregnancy outcome. *Journal of the American Medical Association* **274**, 463–468.

Greger JL (1998) Dietary standards for manganese: Overlap between nutritional and toxicological studies. *Journal of Nutrition* **128**, 368S–371S.

Grider A, Bailey LB & Cousins RJ (1990) Erythrocyte metallothionein as an index of zinc status in humans. *Proceedings of the National Academy of Sciences USA* **87**, 1259–1262.

Harris ED (1997) Copper. In *Handbook of Nutritionally Essential Mineral Elements*, pp. 231–274 [BL O'Dell and RA Sunde, editors]. New York: Marcel Dekker Inc.

Harris ZL, Klomp LWJ & Gitlin JD (1998) Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. *American Journal of Clinical Nutrition* **67**, 972S–977S.

Hopkins RG & Failla ML (1995) Chronic intake of a marginally low copper diet impairs in vitro activities of lymphocytes and neutrophils from male rats despite minimal impact on conventional indicators of copper status. *Journal of Nutrition* **125**, 2658–2668.

Hopkins RG & Failla ML (1997) Copper deficiency reduces interleukin-2 (IL-2) production and IL-2 mRNA in human T-lymphocytes. *Journal of Nutrition* **127**, 257–262.

Hopkins RG & Failla ML (1999) Transcriptional regulation of interleukin-2 gene expression is impaired by copper deficiency in Jurkat human T lymphocytes. *Journal of Nutrition* **129**, 595–601.

Johnson JL (1997) Molybdenum. In *Handbook of Nutritionally Essential Mineral Elements*, pp. 413–438 [BL O'Dell and RA Sunde, editors]. New York: Marcel Dekker Inc.

Johnson MA, Smith MM & Edmonds JT (1998) Copper, iron, zinc, and manganese in dietary supplements, infant formulas, and ready-to-eat breakfast cereals. *American Journal of Clinical Nutrition* **67**, 1035S–1040S.

Jones AA, DiSilvestro RA, Coleman M & Wagner TL (1997) Copper supplementation of adult men: Effects on blood copper enzyme activities and indicators of cardiovascular disease risk. *Metabolism* **46**, 1380–1383.

Joung H, DiSilvestro RA, Burge JC, Choban PS & Flancbaum L (1998) Zinc and copper-related blood parameters in male trauma patients. *Nutrition Research* **18**, 693–701.

Keen CL & Zidenberg-Cherr S (1996) Manganese. In *Present Knowledge in Nutrition*, 7th ed., pp. 334–343 [EE Ziegler and LJ Filer Jr, editors]. Washington, DC: ILSI Press.

Kehoe CA, Turley E, Bonham MP, O'Connor JM, McKeown A, Faughnan MS, Coulter JA, Gilmore WS, Howard AN & Strain JJ (1999) Responses of putative indices of copper status to copper supplementation in human volunteers: The FOODCUE project. *British Journal of Nutrition* (In the Press).

Kelley DS, Daudu PA, Taylor PC, Mackey BE & Turnland JR (1995) Effects of low copper diet on human immune responses. *American Journal of Clinical Nutrition* **62**, 412–416.

Leach RM Jr & Harris ED (1997) Manganese. In *Handbook of Nutritionally Essential Mineral Elements*, pp. 335–356 [BL O'Dell and RA Sunde, editors]. New York: Marcel Dekker Inc.

Linder MC (1996) Copper. In *Present Knowledge in Nutrition*, 7th ed., pp. 307–319 [EE Ziegler and LJ Filer Jr, editors]. Washington, DC: ILSI Press.

Lonnerdal B & Uauy R (1998) Genetic and environmental determinants of copper metabolism. *American Journal of Clinical Nutrition* **67**, 960S–964S.

Lukaski HC, Hoverson BS, Gallagher S & Bolonchuk WW (1990) Physical training and copper, iron, and zinc status of swimmers. *American Journal of Clinical Nutrition* **51**, 1093–1099.

McMahon RJ & Cousins RJ (1998*a*) Mammalian zinc transporters. *Journal of Nutrition* **128**, 667–670.

McMahon RJ & Cousins RJ (1998*b*) Regulation of the zinc transporter ZnT-1 by dietary zinc. *Proceedings of the National Academy of Sciences USA* **95**, 4841–4846.

Mains RE, Myers AC & Eipper BA (1985) Hormonal, drug, and dietary factors affecting peptidylglycine α-amidating mono-oxygenase activity in various tissues of the adult male rat. *Endocrinology* **116**, 2505–2515.

Mains RE, Park LP & Eipper BA (1986) Inhibition of peptide amidation by disulfiram and diethyldithiocarbonate. *Journal of Biological Chemistry* **261**, 11938–11941.

Milne DB (1994) Assessment of copper nutritional status. *Clinical Chemistry* **40**, 1479–1484.

Milne DB (1998) Copper intake and assessment of copper status. *American Journal of Clinical Nutrition* **67**, 1041S–1045S.

Milne DB & Nielsen FH (1996) Effects of a diet low in copper on copper status indicators in postmenopausal women. *American Journal of Clinical Nutrition* **63**, 358–364.

National Research Council (1989) *Recommended Dietary Allowances*, 10th ed. Washington, DC: National Academy Press.

Olivares M, Pizarro F, Speisky H, Lonnerdal B & Uauy R (1998) Copper in infant nutrition: Safety of World Health Organization provisional guideline value for copper content of drinking water. *Journal of Pediatric Gastroenterology and Nutrition* **26**, 251–257.

Olivares M & Uauy R (1998) Limits of metabolic tolerance of copper and biological basis for present recommendations and regulations. *American Journal of Clinical Nutrition* **63**, 846S–852S.

Pennington JAT & Jones JW (1987) Molybdenum, nickel, cobalt, vanadium, and strontium in total diets. *Journal of the American Dietetic Association* **87**, 1644–1650.

Pennington JAT & Young BE (1991) Total diet study nutritional elements. *Journal of the American Dietetic Association* **91**, 179–183.

Pratt WB, Omdahl JL & Sorenson JRJ (1985) Lack of effects of copper gluconate supplementation. *American Journal of Clinical Nutrition* **42**, 681–682.

Percival SS (1995) Neutropenia caused by copper deficiency: possible mechanisms of action. *Nutrition Reviews* **53**, 59–66.

Prasad AS, Fitzgerald JT, Hess JW, Kaplan J, Pelen F & Dardenne M (1993) Zinc deficiency in elderly patients. *Nutrition* **9**, 218–224.

Prasad AS, Meftah S, Abdallah J, Kaplan J, Brewer GJ, Bach JF & Dardenne M (1988) Serum thymulin in human zinc deficiency. *Journal of Clinical Investigation* **82**, 1202–1210.

Prohaska JR (1997) Responses of rat cuproenzymes to variable dietary copper. *Journal of Nutritional Biochemistry* **8**, 316–321.

Prohaska JR, Tamura T, Percy AK & Turnland JR (1997) In vitro copper stimulation of plasma peptidylglycine α-amidating monooxygenase in Menkes disease variant with occipital horns. *Pediatric Research* **42**, 862–865.

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. *Journal of Nutrition* **114**, 1683–1689.

Sazawal S, Black RE, Bhan MK, Sinha A & Bhandari N (1997) Efficacy of zinc supplementation in reducing the incidence and

prevalence of acute diarrhea–a community-based double-blind controlled study. *American Journal of Clinical Nutrition* **66**, 413–418.

Smith D, Hopkins RG, Kutlar A & Failla ML (1994) Diagnosis and treatment of copper deficiency in adult humans. *FASEB Journal* **8**, A4754.

Strain JJ (1988) A reassessment of diet and osteoporosis-Possible role for copper. *Medical Hypotheses* **27**, 333–338.

Strause L, Saltman P, Smith KT, Bracker M & Andon MB (1994) Spinal bone loss in postmenopausal women supplemented with calcium and trace metals. *Journal of Nutrition* **124**, 1060–1064.

Sullivan VK, Burnett FR & Cousins RJ (1998) Metallothionein expression is increased in monocytes and erythrocytes of young men during zinc supplementation. *Journal of Nutrition* **128**, 707–713.

Sullivan VK & Cousins RJ (1997) Competitive reverse transcriptase-polymerase chain reaction shows that dietary zinc supplementation in humans increases monocyte metallothionein mRNA levels. *Journal of Nutrition* **127**, 694–698.

Thomas EA, Bailey LB, Kauwell GA, Lee DY & Cousins RA (1992) Erthrocyte metallothionein response to dietary zinc in humans. *Journal of Nutrition* **122**, 2408–2414.

Turnland JR (1998) Human whole-body copper metabolism. *American Journal of Clinical Nutrition* **67**, 960S–964S.

Turnland JR, Keyes WR, Anderson HL & Acord LL (1989) Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope ^{65}Cu. *American Journal of Clinical Nutrition* **49**, 870–878.

Turnland JR, Keyes WR & Peiffer GL (1995a) Molybdenum absorption, excretion and retention studied with stable isotopes in young men at five intakes of dietary molybdenum. *American Journal of Clinical Nutrition* **62**, 790–796.

Turnland JR, Keyes WR, Peiffer GL & Chiang G (1995b) Molybdenum absorption, excretion, and retention studied with stable isotopes in young men during depletion and repletion. *American Journal of Clinical Nutrition* **61**, 1102–1109.

Turnland JR, Scott KC, Peiffer GL, Jang AM, Keyes AM, Keen CL & Sakanaski TM (1997) Copper status of young men consuming a low-copper diet. *American Journal of Clinical Nutrition* **65**, 72–78.

Van Dokkum W (1995) The intake of selected minerals and trace elements in European countries. *Nutrition Research Reviews* **8**, 271–302.

Werman MJ, Bhathena SJ & Turnland JR (1997) Dietary copper intake influences skin lysyl oxidase in young men. *Journal of Nutritional Biochemistry* **8**, 201–204.

Wolvekamp MC & deBruin RW (1994) Diamine oxidase: an overview of historical, biochemical and functional aspects. *Digestive Diseases* **12**, 2–14.