ERYTHROCYTE METALLOTHIONEIN RESPONSE TO DIETARY ZINC IN HUMANS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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by

Elizabeth A. Thomas
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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<td>AAS</td>
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<td>deciliter</td>
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<td>deoxyribonucleic acid</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>RBP</td>
<td>retinol binding protein</td>
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<td>Recommended Dietary Allowance</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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ERYTHROCYTE METALLOTHIONEIN RESPONSE TO DIETARY ZINC IN HUMANS

By

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August 1993

Chairperson: Dr. Lynn B. Bailey
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The response of erythrocyte metallothionein to dietary zinc in human subjects was evaluated in a controlled metabolic protocol including standard indices of zinc status. Fifteen male subjects, age 25 ± 3.6 y, participated in a 90-d, four-phase study consisting of acclimation (7 d; 15 mg Zn/d), treatment (6 wk; either 3.2, 7.2 or 15.2 mg Zn/d), depletion (12 d; 0.55 mg Zn/d) and supplementation (30 d; self-selected diet plus 50 mg Zn/d) phases.

During the treatment phase erythrocyte metallothionein decreased in the group fed 3.2 mg Zn/d only. Erythrocyte metallothionein decreased during the depletion phase, to below our normal value, in all groups and increased overall during the supplementation phase. The percent decrease in erythrocyte metallothionein observed during the depletion phase was significantly greater in the 15.2 mg Zn/d versus
the 3.2 mg Zn/d treatment group. These data suggest that erythrocyte metallothionein can be used as a measure of status in severe zinc depletion and that the change in erythrocyte metallothionein concentration over time can be used to differentiate between low and adequate levels of zinc intake.

Plasma zinc concentration decreased only in the group fed 3.2 mg Zn/d during the treatment phase. In all groups, erythrocyte zinc decreased during the depletion phase and increased during the supplementation phase. Urinary zinc excretion decreased only in the 3.2 mg Zn/d group during the treatment phase. During the depletion phase, urinary zinc excretion decreased in the 7.2 and 15.2 mg Zn/d groups, but not in the 3.2 mg Zn/d group, relative to the end of the treatment phase. Activities of plasma ACE and serum alkaline phosphatase, and serum concentrations of albumin and RBP were unchanged in all groups throughout the study.

As a component of this study, the effect of zinc depletion of folate and copper status were assessed. Folate and copper intake remained constant throughout all phases of the study. Serum folate increased and erythrocyte folate tended to decrease during the depletion phase only in the group that previously consumed 3.2 mg Zn/d for six weeks. Copper status, as assessed by erythrocyte SOD activity, was not affected by changes in dietary zinc intake. Folate status may fluctuate in response to severe zinc restriction.
CHAPTER 1
INTRODUCTION

Although zinc has long been recognized as an essential nutrient for humans (Prasad et al. 1963), the amount of dietary zinc required to maintain normal zinc status has not been determined. As a component of every living cell in the human body, zinc has a multitude of diverse functions. Zinc is critical for the proper functioning and structural integrity of numerous enzymes in the body (Hambidge 1986). Zinc plays important roles in protein and nucleic acid metabolism, lipid and carbohydrate metabolism and support of the immune system (Hambidge et al. 1986). Some of the biological actions of zinc include effects on growth, reproduction, appetite, taste acuity and night vision.

Evaluation of zinc nutriture has been difficult due to the lack of a marker for zinc status that is both sensitive and specific to changes in dietary zinc intake. The difficulties associated with assessing zinc status have been reviewed (Golden 1989). Plasma and serum zinc are the measurements most frequently used to assess zinc status and in some instances seem to reflect zinc nutriture. However, circulating zinc does not necessarily reflect total body
zinc status (Baer & King 1984; Solomons 1979). Other indices that have been proposed as measures of zinc status include the concentration of zinc in tissues such as hair, skin, erythrocytes and leukocytes, and in fluids such as semen, sweat, saliva and urine. Several biochemical tests that measure the activity of zinc enzymes, and examination of physiological functions such as taste acuity, dark adaptation and immune response, have also been proposed as indicators of zinc status. Despite the vast number of measurements proposed to evaluate zinc nutriture, none has proven to be a sensitive and specific indicator of zinc status. The need for a new and sensitive method of assessing zinc status is well recognized.

A potential new method for assessing zinc status is the measurement of erythrocyte metallothionein concentration. Metallothionein is a low molecular weight (6000-7000 Da) protein that selectively binds heavy metal ions. The biological functions of metallothionein are not clearly resolved but may include important roles in protection against and detoxification of heavy metals and, in the case of the nutrients zinc and copper, regulation of their metabolism and perhaps function (Dunn et al. 1987). The tissue concentration of metallothionein has been shown to be dependent on dietary zinc intake (Blalock et al. 1988). Dietary zinc intake regulates metallothionein mRNA
levels (Cousins & Lee-Ambrose 1992), presumably through interaction of zinc with a trans-acting nuclear factor (Thiele 1992). In a recent study, it was demonstrated that erythrocyte metallothionein was responsive to acute dietary zinc deficiency and supplementation in humans (Grider et al. 1990). These results suggested that erythrocyte metallothionein may be a reliable measure for assessing zinc nutriture.

Severe zinc deficiency is characterized by retarded growth, depressed immune function, anorexia, dermatitis, altered reproductive performance, skeletal abnormalities, diarrhea and alopecia (Hambidge et al. 1986). In contrast to these manifestations of severe zinc deficiency, the effects of moderate zinc deficiency are not as obvious. It has been postulated that marginal zinc status is responsible for disturbances of taste and smell acuity, delayed wound healing and declining immune function (Greger 1989). Marginal states of zinc nutrition have been reported to exist in specific populations of the United States (U.S.). In a survey of apparently healthy children, Hambidge et al. (1972) observed low hair zinc levels, suboptimal growth, poor appetite and impaired taste acuity in children. All symptoms improved following zinc supplementation. In humans with low plasma zinc levels, accelerated rates of wound healing have been observed as a result of increased zinc
intake, suggesting that the zinc requirement of these subjects was not fully met by their diets (Sandstead 1985). The long-term effects of impaired zinc status are not known.

Since a reliable measure of zinc is not available, assessment of zinc status has not been included in the large national nutrition surveys (National Center for Health Statistics 1988; U.S. Department of Health and Human Services and U.S. Department of Agriculture 1986). Therefore, information about the zinc status of the U.S. population remains speculative.

Data concerning the human zinc requirement was recently reviewed by the Food and Nutrition Board, National Academy of Sciences-National Research Council in the tenth edition of the Recommended Dietary Allowances (RDAs) (National Research Council 1989). It was concluded that the lack of sensitive indicators of zinc status results in an uncertain estimation of zinc requirement and dietary allowances. Several investigators have reported populations consuming dietary zinc levels below the RDA (Greger 1989; Moser-Veillon 1990; Pennington et al. 1984; Swanson et al. 1988). However, due to the controversy involved in setting the RDAs for zinc, this information must be interpreted with caution. Before a true requirement for zinc can be established, it is necessary to determine a method to assess zinc status that is sensitive and specific, as well as responsive to moderate
changes in dietary zinc intake.

An additional complicating factor in determining zinc requirement is the adaptive responses to change in dietary zinc that occur in humans. Both the efficiency of absorption of dietary zinc and the excretion of endogenous and exogenous zinc are responsive to changes in zinc intake (Baer & King 1984; Jackson et al. 1984; Wada et al. 1985; Ziegler et al. 1989). When assessing a possible parameter of zinc status, it cannot be assumed that the response to a short-term change in zinc intake would be reflective of a chronic zinc intake at that level. Milne et al. (1983) demonstrated that a constant zinc intake must be fed for five to six weeks to overcome the effects of these adaptive responses.

The goal of this study was to determine whether a new measure of zinc status may be more sensitive than previously used indices to changes in dietary zinc intake. The primary objective was to evaluate the response of erythrocyte metallothionein, as compared to standard indices of zinc status, to levels of dietary zinc commonly consumed. The study was designed to provide the subjects adequate time to adjust to effects of homeostatic adaptation to changes in zinc intake. In addition, the study permitted the evaluation of the effect of previous dietary zinc intake on
the response to acute zinc depletion and supplementation. The effects of zinc intake on folate and copper status were also investigated.
CHAPTER 2
REVIEW OF THE LITERATURE

Zinc

Body Pools and Homeostasis

The adult human body contains approximately 1.5-2.5 grams of zinc. Compared with other trace elements, zinc is second only to iron in terms of its amount in the human body (Smith 1988). Zinc is present in virtually all organs, tissues, fluids and secretions of the body (Jackson 1989). In general, the concentration of zinc in tissues is 10-100 ug/g wet weight and is remarkably consistent within specific tissues. Relatively large amounts of zinc are deposited in bone and muscle, but these sources are not in rapid equilibrium with the rest of the organism (Cousins & Hempe 1990).

Over 95% of total body zinc is found bound to metalloenzymes within cells or cell membranes. In contrast, relatively little zinc is found in extracellular fluids (Jackson 1989). In the blood, zinc primarily (75-88%) exists in red blood cells, predominantly in the form of the enzyme carbonic anhydrase, followed by the plasma (12-22%) with the remainder of zinc in leukocytes and platelets.
Plasma zinc is distributed in three pools: 18% tightly bound to alpha-2-macroglobulin and 80% less tightly associated with albumin. The remaining 2% is bound to other proteins such as transferrin and ceruloplasmin and to amino acids such as histidine and cysteine (Hambidge et al. 1986).

The body pool of readily available zinc appears to be small and to have a rapid turnover rate, as shown by the prompt appearance of deficiency signs in laboratory animals (Golden 1989). Compared with the effects of most nutritional deficiencies, relatively few days are required for morphological evidence of the existence of, or recovery from, a zinc deficient state (Diamond et al. 1971). This strongly suggests the absence of zinc stores that can be mobilized when zinc-deficient diets are fed. Most tissues, including skeletal muscle, have no measurable decline in zinc concentration, even in severe zinc deficiency (Jackson 1989).

Despite the lack of large, readily mobilizable stores of zinc, the body has developed an efficient homeostatic mechanism to control body zinc levels (Golden 1989; King & Turnlund 1989). Two mechanisms for control of zinc homeostasis were first suggested in 1962 by Cotzias et al. They suggested that zinc homeostasis is regulated by higher absorption at low zinc intakes and increased excretion in
response to high zinc intakes. Adaptive responses in both the efficiency of absorption of dietary zinc (Jackson et al. 1984) and the rate of excretion of endogenous and exogenous zinc (Baer & King 1984; Wada et al. 1985) have been demonstrated. The relatively rare incidence of overt zinc deficiency or excess in man, in spite of wide variations in dietary intakes of zinc, is evidence of the body’s homeostatic regulation of zinc (Jackson 1989).

Biochemical Functions

Clinical manifestations of dietary zinc deficiency are ultimately the result of altered zinc metabolism and/or biochemical functions. As a component of every living cell in the body, zinc has a multitude of diverse functions. This trace element is best known for its involvement with enzymes. Zinc is critical for the proper functioning and structural integrity of numerous enzymes, encompassing each enzyme class. Zinc-dependent enzymes play important roles in the metabolism of proteins and nucleic acids, lipids and carbohydrates. The first zinc metalloenzyme to be identified was carbonic anhydrase in 1940. Today, about 60 zinc-dependent enzymes have been identified in humans (Hambidge et al. 1986).

A role for zinc in cell division was first hypothesized by Fujii (1954) who showed, by histological methods, that
zinc was present in the nucleus of animal cells. The observations by several investigators that zinc deficiency resulted in significant growth retardation and developmental abnormalities led to the hypothesis that the primary locus of zinc deficiency was at the level of deoxyribonucleic acid (DNA) synthesis (Clegg et al. 1989). Numerous studies were designed to quantify DNA and/or to measure the incorporation of radioactive thymidine into DNA under conditions of zinc deficiency (Dreosti et al. 1972; Eckhert & Hurley 1977; Prasad & Oberleas 1973). These studies consistently concluded that both total DNA synthesis and thymidine incorporation rates were significantly reduced as a consequence of zinc deficiency (Clegg et al. 1989). Subsequently, Duncan and Hurley (1978) showed that zinc deficiency in rats resulted in reduced activities of DNA polymerase and thymidine kinase. Despite the abundance of evidence of zinc's role in DNA synthesis, the precise function of zinc in cell division has not been defined.

Although Vallee showed that zinc is a normal constituent of ribonucleic acid (RNA) in 1959, Scrutton et al. (1971) were the first to discuss the presence and possible role of zinc in RNA polymerase. Subsequently, Terhune and Sandstead (1972) showed that RNA polymerase activity was significantly lower than normal in liver from the postnatally zinc-deficient rat. Reduced levels of RNA
in zinc-deficient rat testes have been related to an increased activity of ribonuclease (Macapinlac et al. 1968). Taken together, these reports suggest zinc involvement in both synthesis and degradation of RNA.

While the total amount of protein in zinc-deficient animals is lower than normal because of decreased growth, it has not been clearly demonstrated that zinc deficiency has a direct effect on protein synthesis at the level of translation (Clegg et al. 1989). A number of investigators have reported that neither the rate of incorporation of various radioactive amino acids into protein nor the concentration of protein were decreased by a zinc-deficient state (Burke et al. 1981; O’Neal et al. 1970; Southon et al. 1985). Other investigators found that the rate of amino acid incorporation (Hsu et al. 1969) or protein synthesis (Giugliano & Millward 1987) in zinc deficiency varied depending on the tissue studied. These investigators suggest a possible role for hormones in regulating protein synthetic and degradation rates in zinc-deficient tissues.

The interaction of zinc with nuclear proteins that bind to promoter sequences of specific genes, and thus regulate transcription, suggests another broad spectrum of biological effects for zinc (Cousins & Hempe 1990). Chesters (1978) has proposed "that the critical process for growth requires not, as has been supposed, a zinc metalloenzyme but
participation of freely exchangeable zinc in a process required for alteration in the expression of the genetic information stored in the cell's chromatin". Zinc dependency in the control of gene transcription may also be explained through zinc fingers of transcriptional factors (Klug & Rhodes 1987). Zinc fingers are repeated cysteine- and histidine-containing domains of DNA-binding proteins that bind zinc in a tetrahedral configuration, providing a structural role required for binding to DNA (Cousins & Hempe 1990). Zinc fingers have been identified in nuclear transcription factors from many mammalian cells (Chavrier et al. 1988). The effect of dietary zinc on zinc fingers is unclear.

Zinc is thought to have a role in stabilization of biomembranes. Chvapil (1973) found that treating a suspension of erythrocytes in vitro with a zinc-chelating agent resulted in an increased susceptibility of the erythrocyte to hemolysis when incubated with detergent. When zinc was added back to the medium, the fragility of the erythrocyte membrane appeared normal. Based on these observations Chvapil proposed that membrane-bound zinc alters the fluidity and stabilization of membranes. O’Dell et al. (1987) demonstrated that zinc deficiency increased the sensitivity of rat erythrocytes to osmotic shock. The underlying mechanism for the role of zinc in membrane
function has not been elucidated. However, many of the manifestations of zinc deficiency could be related to membrane dysfunction (Cousins & Hempe 1990).

Studies have shown that certain tissues undergo lipid peroxidation during zinc deficiency (Burke & Fenton 1985; Sullivan et al. 1980). Zinc was shown to suppress free radicals in isolated cells after initiation of lipid peroxidation (Coppen et al. 1988). Zinc thiolate clusters in metallothionein are particularly efficient at scavenging hydroxyl free radicals (Thornalley & Vasak 1985). Metallothionein induction by zinc has been correlated with free-radical suppression (Thomas et al. 1986). Hydroxyl radicals are reported to be scavenged by metallothionein in vitro (Thornalley & Vasak 1985). Free radical-mediated lipid peroxidation of erythrocyte membranes was strongly inhibited by zinc (Girotti et al. 1986) and Zn-metallothionein (Thomas et al. 1986). Transfer of metal ions from metallothionein to the erythrocyte membranes during oxidation may play a major antioxidant role. These findings are of interest because many pathological conditions associated with zinc deficiency may be related to oxidative damage caused by free radicals (Cousins & Hempe 1990).

Clinical zinc deficiency in humans causes many immune system dysfunctions. In humans, zinc is essential for the
blast transformation of both T and B lymphocytes, for cell mediated immunity, including delayed dermal hypersensitivity responses to a large number of test antigens, for antibody-mediated responses to both T-cell dependent and T-cell independent antigens, for the development of normal distribution of lymphocyte subsets including natural killer cells, for some aspects of humoral immunity, and for proper function of phagocytic cells (Gershwin et al. 1985; Meydani 1990; Tapazoglou et al. 1985). Immunological dysfunctions associated with zinc deficiency are greater than those seen with deficiencies of any other micronutrient (Chandra 1991).

**Dietary Intake and Sources**

Two important factors that determine zinc nutriture are the amount of zinc provided by the food supply and the zinc content of the foods individuals eat. Generally, it is estimated that the U.S. food supply provides an average of 12.3 mg zinc per day per person (Moser-Veillon 1990). Pennington et al. (1984), in a survey of U.S. foods, found 13.2 mg zinc in a 2,850 kcal diet. Infant and toddler diets containing 880 and 1,300 kcal contained 5.5 and 8.5 mg zinc, respectively. Elderly people have generally been found to consume 7-10 mg zinc daily (Greger 1989).
Foods vary widely in zinc content. Major dietary sources of zinc include animal products such as shellfish (especially oysters), meats, liver, poultry, eggs and dairy products (Smith 1988). The zinc content of meat varies according to the type of animal and specific tissue. In general, the amount of zinc is higher in dark meat than in white meat (Sandstrom 1989). Cereals and vegetables not only contain lower amounts of zinc than animal products, but the zinc is generally less available for absorption. Considerable variation in the zinc content of the same type of food can occur because of differences in zinc content of the soil (Hambidge et al. 1986).

Absorption and Bioavailability

In general, about 20% of zinc consumed from a usual mixed diet is absorbed. Zinc is absorbed primarily in the mammalian small intestine, although the relative contribution of the individual segments toward overall zinc absorption is not clear (Lonnerdal 1989). There is general consensus that absorption of zinc involves two kinetic processes, a carrier mediated component, which is saturable at higher luminal zinc concentrations, and a nonsaturable diffusion component (Cousins & Hempe 1990). Several zinc-binding ligands, such as fatty acids, prostaglandins, picolinic acid and citric acid, have been suggested to have
roles in the zinc absorption process (Cousins 1985). Although zinc-binding ligands may affect the bioavailability of zinc, it is unlikely that the presence of an intraluminal ligand is required for zinc to be absorbed (Cousins & Hempe 1990).

The bioavailability of zinc in different foods varies widely. Various dietary factors have been thought to affect zinc availability. Phytate and oxylates are substances that can form insoluble complexes with zinc, thus inhibiting its absorption (Sandstrom & Lonnerdal, 1989). Phytate (myoinositol hexaphosphate) is commonly found in whole grains (rye, barley, oatmeal, wheat) and soy products (Hambidge et al. 1986). Animal studies have indicated that a phytate:zinc molar ratio exceeding 12:1-15:1 results in decreased bioavailability of dietary zinc as reflected by a decrease in growth rate and tissue concentration of zinc (Morris & Ellis 1980). High concentrations of phytate may have great practical importance worldwide, but probably not in the U.S., where the phytate content of the average diet is not high enough to impair the utilization of zinc (Erdman et al. 1987). The effect of fiber on zinc absorption is controversial. Differences may be due to the type of fiber as well as interaction with other dietary components. Many foods that contain fiber also contain phytate. High levels of dietary phytate (Reinhold et al. 1973) and fiber (Ismail-
Beigi et al. 1977) have been indicted as major factors in zinc deficiency in adolescents in Iran.

Calcium has been shown to accentuate the effect of phytate in decreasing the bioavailability of zinc in rats (Erdman et al. 1987). Studies in humans have shown that dietary calcium alone had no effect on zinc absorption and retention from a mixed meal (Wood & Zheng 1990).

The simultaneous ingestion of equal amounts of ferrous iron and zinc sulfates depressed zinc absorption in humans, but no such effect occurred with heme iron or when a food source of zinc was used (Solomons & Jacob 1981). Thus, it is unlikely that iron-zinc interaction has a major influence on zinc requirements under most dietary conditions (Solomons & Cousins 1984).

Similar to iron, a competitive interaction has been observed between copper and zinc. Studies in rats using isolated, vascularly perfused rat intestines, fed dietary concentrations of 5, 30 and 180 mg/kg zinc and 1, 6 and 36 mg/kg copper, have, however, not shown mutually interactive effects on their absorption (Oestreicher & Cousins 1985). In human subjects, Valberg et al. (1984) found no effect of 5 mg copper on the absorption of 0.5 mg zinc in water. Human diets generally contain significantly more zinc than copper. Therefore, copper is not likely to affect zinc absorption in humans (Sandstrom & Lonnerdal 1989). There is
conflicting evidence that other nutrients, such as phosphorus (Heth et al. 1966; Spenser et al. 1984), tin (Valberg et al. 1984; Solomons et al. 1983) and folic acid (Milne et al. 1984; Ghishan et al. 1986), impair zinc absorption.

Requirements and Allowances

Because of the lack of sensitive indicators of zinc status, the estimation of a zinc requirement and the setting of recommended allowances are beset with uncertainties. The current Recommended Dietary Allowances for zinc are shown in Table 2-1 (National Research Council 1989). The dietary zinc requirement is defined as the minimum zinc intake that will support optimal growth and metabolism (Smith et al. 1983).

Human zinc requirements have been assessed from balance studies, from measurements of tissue and endogenous losses and from the functional response to a marginal zinc intake (King 1986). Of these methods, the balance study has been used most frequently to study zinc requirement. Balance studies assume that the requirement is equal to the lowest intake that replaces all sources of loss and that zinc balance will be negative if intake falls below that level. The results of balance studies show that both positive and negative zinc balance were detected when zinc intake ranged
<table>
<thead>
<tr>
<th>Category</th>
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<th>Zinc (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant</td>
<td>0.0 - 0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.5 - 1.0</td>
<td>5</td>
</tr>
<tr>
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<td>1 - 3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>4 - 6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>7 - 10</td>
<td>10</td>
</tr>
<tr>
<td>Males</td>
<td>11 - 14</td>
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<tr>
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<td>1st 6 months</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>2nd 6 months</td>
<td>16</td>
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</tbody>
</table>

from 1.5-19 mg/day (Colin et al. 1983; Johnson et al. 1982; Johnson et al. 1993; Milne et al. 1983; Patterson et al. 1984; Robinson et al. 1973; Spenser et al. 1979; Spenser et al. 1983; Spenser et al. 1984). No single level of zinc intake clearly separated negative from positive zinc balances. The variation in the length of the balance studies, which ranged from six to more than 100 days, may have contributed to the lack of a consistent relationship between zinc intake and zinc balance. Since humans adapt to changes in zinc intake by increasing or decreasing zinc absorption and/or excretion (King & Turnlund 1989), short-term balance periods may not allow enough time for complete adaptation to the zinc intake. Milne et al. (1983) fed a group of healthy men 3.2 mg Zn/d for 18 weeks. In that study, the men achieved zinc balance for the first five or six weeks, but then balance became negative and remained negative for the last 12 weeks. Those results indicate that it is necessary to study a dietary zinc level for at least six weeks in order to assess its adequacy for balance.

When the factorial method is used to determine zinc requirements, the requirement is defined as the lowest intake that replaces the endogenous loss. This method requires two steps: quantitation of endogenous zinc loss and measurement of fractional zinc absorption from the usual diet. The dietary requirement is computed by dividing the
endogenous zinc loss by the fractional zinc absorption from the usual diet (King 1986). Data collected during the measurement of endogenous zinc loss in men and women suggested that tissue zinc status influences endogenous zinc excretion and, therefore, the dietary zinc needs (Baer & King 1984; Hess et al. 1977). Individuals in good zinc status may require higher amounts of zinc in their diets than individuals in poor status. Determination of the functional response to marginal zinc intake is difficult because of the lack of a specific, sensitive indicator of zinc status.

Assessment of Zinc Status

An index of zinc nutriture with adequate sensitivity and specificity has yet to be identified (Golden 1989; Hambidge 1989; King & Turnlund 1989). In the search for a reliable measure of zinc status, different body tissues, cells and functional tests have been examined. Studies designed to assess zinc status in response to various conditions/treatments often use more than one of these methods as indices of zinc nutriture. The limitations of these methods and the diversity of tests employed from study to study make it difficult to compare research findings.
Plasma and Serum Zinc

Plasma and serum zinc are the parameters used most frequently to assess zinc status and, in some instances, appear to reflect zinc nutriture (Prasad et al. 1978; Ruz et al. 1991). However, circulating zinc does not necessarily reflect total body zinc status (Baer & King 1984; Solomons 1979). For example, low concentrations of plasma zinc have been reported in patients with normal intracellular levels of zinc (Speich et al. 1987), while other researchers have noted marked intracellular zinc deficiencies without corresponding changes in plasma zinc (Strobel et al. 1978). Ladefoged and Hagen (1988) found that the concentration of erythrocyte and muscle zinc did not differ between patients with subnormal, normal or increased plasma zinc concentrations. Jones et al. (1981) also failed to demonstrate any correlation between plasma zinc content and muscle zinc concentrations.

In a study designed to evaluate zinc utilization in young men fed adequate and low zinc intakes, Wada et al. (1985) found that serum zinc levels did not fall when dietary zinc was decreased to 5.5 mg/d. Similarly, serum zinc levels did not decline during an 18 week study of subjects consuming a low zinc diet (3.4 mg/d), even though zinc balance became negative after the first six weeks of
the study (Milne et al. 1983). Acute versus chronic zinc depletion may affect the circulating zinc response (Ladefoged & Hagen 1988), as does protein status (Solomons 1979). Additionally, plasma zinc is subject to acute and chronic changes related to hormonal control (Solomons et al. 1978), disease (Cousins 1989), diurnal variation (Gordon et al. 1982) and short term fasting (Henry & Wilmes 1975). The changes in circulating zinc in these conditions are due to metabolic disturbances within the free pool and not to changes in zinc status. Therefore plasma and serum zinc concentrations are not very specific measures of zinc deficiency.

Erythrocyte Zinc

Erythrocyte zinc concentrations decreased significantly when volunteers consumed 0.6-1.0 mg Zn/d for 78 days (Bueck et al. 1973). Prasad et al. (1978) also noted significant decreases in erythrocyte zinc in three of four subjects after consuming 2.7 or 3.5 mg Zn/d for two months, although these changes were slow to appear. Conversely, Baer and King (1984), in a zinc depletion study (0.28 mg Zn/d) lasting four to nine weeks, and Rabbani et al. (1987), in a zinc stabilization/depletion/repletion study lasting 56 weeks, failed to show a change in mean erythrocyte zinc concentrations. Ladefoged and Hagen (1988) evaluated the
correlation between concentrations of zinc in erythrocytes and muscle tissue and concluded that erythrocyte and muscle tissues are poor indicators of total body zinc stores. Grider et al. (1990) found erythrocyte zinc unresponsive to a zinc deficient diet (0.7 mg Zn/d) fed to human subjects for eight days.

Leukocyte Zinc

Leukocytes have a high zinc content; and, compared to erythrocytes, they are more metabolically active and have a shorter life span. As a result, it was hypothesized that acute zinc status might be reflected more promptly in these cells (Whitehouse et al. 1982). One of the first studies to use leukocyte zinc to assess zinc status was conducted by Kumer and Rao (1974). These researchers compared the zinc status of normal and diabetic subjects; and, although they found no difference in erythrocyte zinc between these groups, leukocyte zinc in the diabetic subjects was half that of the normal subjects. Jones et al. (1981) noted a significant correlation between the leukocyte and muscle zinc concentration of normal subjects and patients with generalized diseases. In response to experimentally induced zinc depletion and repletion, Prasad et al. (1978) noted a decrease and an increase, respectively, in the leukocyte zinc concentration of their subjects. Data from animal
studies (i.e., rats and pigs) suggest that leukocytes are not sensitive to changes in zinc status (Crofton et al. 1983; Milne et al. 1985). Leukocyte zinc is not a generally accepted indicator of zinc status.

**Urinary Zinc**

Zinc deficient dwarfs in the Middle East were reported by Prasad et al. (1963) to have low urinary zinc levels. Human studies of experimental zinc depletion demonstrated that long-term moderate (4 mg Zn/d for 6 wks; Ruz et al. 1991) or severe (0.28 mg Zn/d for 4-9 wks; Baer & King 1984) zinc restriction resulted in decreased urinary zinc excretion. Baer and King (1984) found that urinary zinc responded more rapidly to changes in zinc intake than plasma zinc and suggested that the former might be useful for evaluating zinc nutriture. Neither of these zinc restriction protocols examined the effect of previous diet on the response of urinary zinc to zinc depletion. In a study designed with a less severe zinc restriction (5.5 mg Zn/d for 54 days), Wada et al. (1985) found no significant changes in urinary zinc excretion.

A potential problem with the use of urinary zinc as a measure of zinc status is that hyperzincuria has been reported to exist concomitantly with hypozincemia. This situation has been associated with disease states such as

Hair Zinc

The zinc content of hair has been proposed as a chronic index of zinc status (Klevay 1970). Studies comparing hair zinc concentration to circulating zinc levels or zinc content of tissues/organs have produced varying results. When hair zinc concentration was compared with serum zinc, a significant correlation was found (Klevay 1970). However, a comparison between hair and plasma zinc concentrations by other researchers failed to show the same effect (McBean et al. 1971). During experimental zinc depletion, mean hair zinc concentration was unaffected (Baer & King 1984). The lack of consistent changes in hair zinc concentration with respect to zinc status may be due to a variety of factors. For example, hair zinc concentration can be influenced by environmental contaminants, gender, age of subject, season, hair color, hair treatment, rate of hair growth and rate of zinc delivery to the hair root (Hambidge 1982). Additionally, zinc deficiency may impair hair growth and thus prevent a decline in hair zinc concentration (Solomons
1979). These factors limit the use of hair zinc to assess zinc status.

Biochemical Tests

Functional biochemical tests that measure the activity of certain zinc metalloenzymes or zinc-activated enzymes have been investigated as possible parameters of zinc status. Measurement of serum alkaline phosphatase (EC 3.1.3.1) activity has been used by some researchers as a tool for assessing zinc nutriture (Prasad et al. 1963, Kay et al. 1976). Low serum alkaline phosphatase activity has been reported in human zinc deficiency due to acrodermatitis enteropathica (Prasad et al. 1963) and unsupplemented total parenteral nutrition (Kay et al. 1976). In a study of experimental zinc deficiency and repletion, Prasad et al. (1978) demonstrated that plasma alkaline phosphatase activity slowly decreased during zinc restriction and increased following supplementation. However, Ruz et al. (1991) saw no consistent change in plasma alkaline phosphatase activity during an eight week experimental zinc depletion study. Recently, Ruz et al (1992) reported a significant decrease in alkaline phosphatase activity in erythrocyte membranes during a controlled zinc depletion study involving healthy male subjects.
The activity of angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is stimulated by zinc as well as cobalt (Ryan 1983). White et al. (1984) observed that both plasma zinc and ACE activity were low in rats fed zinc deficient diets, and that adding zinc in vitro to the assay mixture stimulated significantly more ACE activity in zinc-deficient animals than in controls (White et al. 1986). In a study designed to compare the effect of zinc deficiency on ACE activity in the lung and testis of rats, Reeves and O'Dell (1988) found testicular ACE activity significantly lower, but lung ACE activity significantly higher, in the zinc-deficient versus zinc-adequate rats. Dalheim et al. (1989) reported zinc deprivation in rats significantly reduced plasma and testicular ACE activities, but stimulated aortic ACE while lung values remained constant. In a study of moderate zinc deficiency in humans, Ruz et al. (1991) found the activity of plasma ACE unchanged. Likewise, Milne et al. (1987) did not observe any change in plasma ACE activity in postmenopausal women fed a zinc-depleted diet containing 2.6 mg Zn/d for 6 months. No studies have examined the effect of acute, severe zinc depletion on ACE activity in humans.

Since zinc plays an important role in protein and nucleic acid metabolism (Mills 1989) measurement of serum proteins may be a nonspecific indicator of zinc status.
Smith et al. (1974) reported significantly lower plasma and liver retinol binding protein (RBP) levels in zinc-deficient versus zinc-adequate rats. Wada and King (1986) found serum levels of prealbumin, albumin and RBP decreased significantly when a low zinc diet was fed to adult men for 54 days. Since these serum proteins reflect protein status (Ingenbleek et al. 1975), the authors suggested that the low zinc intakes affected protein utilization through an impairment of protein synthesis or an increase in protein catabolism.

Erythrocyte Metallothionein

It is clear that a single reliable method for diagnosing zinc deficiency and assessing zinc reserves has not been identified. Recently metallothionein has been proposed as a possible measure of zinc status. Metallothionein is a low molecular weight (6000-7000 Dalton) cytosolic metalloprotein that was first identified in 1960 (Kagi & Vallee 1960). Metallothionein has been characterized both physicochemically and physiologically. It consists of a 61 amino acid single polypeptide chain with cysteine as the predominant amino acid residue. Other structural features include the absence of disulfide bridges and aromatic amino acids. Most vertebrate tissues contain two major isoforms of metallothionein, designated as
metallothionein-I and metallothionein-II based on their elution position during ion-exchange chromatography. The relative proportions of the different metallothionein isoforms vary depending on species, tissue, physiological state and exposure to metals. Metallothionein-II is usually the predominant form (Dunn et al. 1987). However, analysis of metallothionein isoform mRNA expression showed metallothionein-I is the primary gene expressed in the bone marrow of rats (Huber & Cousins 1993a). Metallothionein selectively binds heavy metals (7-12 metal atoms/molecule of protein), such as the nutritionally essential trace elements zinc and copper and the potentially toxic elements cadmium and mercury (Dunn et al. 1987).

Although metallothionein has been isolated from most tissues, it is particularly abundant in the liver, kidney and intestine. The concentration of metallothionein in tissues is dependent on the zinc status of the animal, with low concentrations in zinc deficient animals and increased quantities following zinc repletion (Cousins 1985). This relationship can be attributed to the fact that metallothionein is an inducible protein that responds to zinc as well as other dietary constituents (Blalock et al. 1988) and hormonal stimuli (Cousins et al. 1986). Metallothionein induction is regulated at the transcriptional level, as demonstrated by the fact that
prior administration of actinomycin D can inhibit its induction (Richards & Cousins 1975). Recently, Cousins and Lee-Ambrose (1992) demonstrated that dietary zinc level regulates metallothionein mRNA levels in rats. Research conducted by Huber and Cousins (1988) suggests that transcriptional control is highly regulated. Metallothionein gene expression and synthesis in rat fetal liver was altered by zinc intake of the dam during gestation, with both marginal and severe zinc deficiency resulting in decreased expression of metallothionein (Huber & Cousins 1988). Presumably, dietary regulation is through interaction with a trans-acting nuclear factor (Thiele 1992).

The physiological significance of metallothionein is not certain, but functions commonly attributed to this protein include homeostasis of copper and zinc metabolism, heavy metal detoxification, free radical scavenging (Dunn et al. 1987), and zinc distribution in rapidly proliferating tissues (Huber & Cousins 1993b).

Sato et al. (1984) studied the effects of stress and of zinc status on plasma metallothionein concentration in rats. These investigators found that reduced plasma levels of both zinc and metallothionein were indicative of zinc deficiency and suggested that determination of plasma metallothionein could be valuable in the diagnosis of compromised zinc
status. To establish whether metallothionein measurements could be of value in the diagnosis of zinc deficiency, Bremner et al. (1987) conducted studies investigating the effects of low dietary zinc intake and of certain types of stress on metallothionein concentrations in the blood and urine of rats. Because copper can induce synthesis of metallothionein in liver and other tissues (Bremner et al. 1978) and selenium can apparently bind to the protein (Paliwal et al. 1982), this study also investigated the effects of copper and selenium deficiency on blood and urinary metallothionein levels. Marginal zinc deficiency rapidly caused a major decrease in metallothionein levels in the blood cells and to a lesser extent in urine (Bremner et al. 1987). The metallothionein in the blood cells was associated mainly with the erythrocytes. No changes in blood or urine metallothionein levels were found in copper- and selenium-deficient rats. Neither cold stress nor restriction of food intake for 24 hours had any significant effect on metallothionein levels in the blood cells or urine. Endotoxin injection increased urinary metallothionein excretion in both zinc-adequate and zinc-deficient rats but did not affect blood cell metallothionein levels in either group of animals (Bremner et al. 1987).

Recently, Grider et al. (1989) developed an enzyme-linked immunosorbent assay (ELISA) for human
metallothionein-1. Grider et al. (1990) evaluated the response of serum and erythrocyte metallothionein to zinc intake in two groups of human subjects. Seven subjects received an egg-white based, semipurified diet deficient in zinc (0.46 mg Zn/d) for eight days. Six other subjects consumed normal diets and received a zinc supplement (50 mg Zn/d) for 63 days. Erythrocyte metallothionein decreased significantly by day eight in the zinc deficient group and increased significantly by day seven in the supplemented group. Based on these preliminary findings, it was concluded that erythrocyte metallothionein levels may be useful, in conjunction with other parameters, to assess zinc nutriture in humans (Grider et al. 1990).

The potential advantages of metallothionein assessment include increased specificity compared to other biochemical techniques used to diagnose zinc deficiency (Sato et al. 1984, Bremner et al. 1987), as well as the ability to distinguish hypozincemia due to zinc deprivation from other causes such as stress, infection and protein deficiency (Cousins 1985, Bremner et al. 1987). To determine the practical implications of measuring erythrocyte metallothionein concentrations to assess zinc nutriture of individuals, it is necessary to study its response to commonly consumed levels of zinc. Specifically needed are studies in which erythrocyte metallothionein is measured in
individuals chronically consuming zinc levels that are thought to result in moderate zinc deficiency.

Selected Zinc-Nutrient Interactions

Zinc-Folate Interaction

Information on a zinc-folate interaction has accumulated rapidly. Research efforts have been directed toward determining the effect of high folate intakes on zinc status; however, the effect of zinc status on folate absorption has also been investigated. Dietary folates mainly consist of reduced forms of pteroylpolyglutamates that are hydrolyzed by intestinal folate conjugase (hydrolase) (EC 3.4.22.12) to folate monoglutamate forms before absorption from the intestine (Tamura 1978). Silink et al. (1975) showed that bovine hepatic folate conjugase is a metalloenzyme containing 4.2-4.5 zinc atoms per molecule. Reports on the effect of zinc deficiency on intestinal mucosal folate hydrolase and the absorption of polyglutamates in rats are conflicting. Canton et al. (1989) reported significantly less polyglutamate absorption in zinc-deficient rats than in either zinc-supplemented pair-fed or ad libitum-fed controls. However, the intestinal mucosal folate hydrolase activity of zinc-deficient rats was not significantly reduced compared with
two control groups in a study conducted by Tamura et al. (1991).

Tamura et al. (1978) studied the effect of severe experimental zinc depletion on the intestinal absorption of mono- and heptaglutamyl folates in six healthy male subjects. These investigators measured the increase in serum folate after an oral dose of mono- and heptaglutamyl folate before and after initiation of a zinc-deficient diet. At the beginning of the study, no differences were detected in the absorption of mono- and heptaglutamate. After zinc depletion, the rise in serum folate after an oral dose of the heptaglutamate was reduced by an average of 53%, while monoglutamate absorption was unchanged. Based on these data, Tamura et al. (1978) suggested that severe zinc deficiency impairs utilization of folate polyglutamate. Chandler et al. (1986) showed that activity of purified human intestinal brush border folate hydrolase was zinc dependent.

Ghishan et al. (1986) reported a mutually inhibitory effect of zinc and folate on intestinal transport in the rat. These studies indicated that zinc and folate formed insoluble complexes at the acidic pH of the stomach. Although these complexes dissolved at the higher pH of the duodenum, the authors concluded that, under normal
physiological conditions, a mutual inhibition between folate and zinc existed at the site of intestinal transport.

Recently Bailey et al. (1993) studied the effects of zinc restricted (3.5 mg Zn/d) and zinc adequate (14.5 mg Zn/d) diets on folate utilization in 12 male subjects. Each subject was supplemented with 800 ug/d folic acid and consumed an identical diet with the exception of zinc content. Subjects were randomly assigned to received either 3.5 or 14.5 mg Zn/d for 25 days. No significant differences in mean serum, erythrocyte or urine folate concentrations were detected between the subjects fed 3.5 or 14.5 mg Zn/d. These data suggested there is no difference in the utilization of supplemental folic acid in subjects receiving zinc adequate or zinc restricted diets.

Zinc-Copper Interaction

The relationship between zinc and copper nutrition has generally been studied in regard to the effect of zinc intake on copper status. Large quantities of ingested zinc can interfere with copper absorption and as copper is necessary for iron metabolism, anemia may result (Patterson et al. 1985). In a balance study of adult males, Sandstead et al. (1982) showed that as the amount of zinc in the diet increased, so did the amount of copper required to maintain balance. These observations were made at levels of zinc
intake near the RDA. A positive association between copper excretion and dietary zinc intake has been observed in young adult males (Festa et al. 1985), adolescent females (Greger et al. 1978) and the elderly (Burke et al. 1981). The amount of zinc fed in all these studies was near the RDA (Fosmire 1990). Fisher et al. (1984) studied the effect of zinc supplements on copper status. They reported a decrease in erythrocyte Cu,Zn-superoxide dismutase (SOD) when healthy males were fed two daily doses of 25 mg zinc for six weeks. Erythrocyte Cu,Zn-SOD is a metalloenzyme that has been shown to be more sensitive to copper deficiency than plasma copper or ceruloplasmin (Fosmire 1990). Similar results were shown by Yadrick et al. (1989) when adult females were fed 50 mg Zn/day for ten weeks. Erythrocyte SOD activity declined to 53% of pretreatment values, although ceruloplasmin concentrations did not differ.

Recently, Ruz et al. (1991) reported a significant reduction in erythrocyte Cu,Zn-SOD activity in men after seven weeks on a zinc-deficient diet. However, this change was attributed to the dietary regimen used to achieve zinc deficiency rather than a direct effect of zinc deficiency. Taylor et al. (1988) studied the effect of dietary zinc or copper deficiency on liver Cu,Zn-SOD in rats. In copper-deficient rats, liver Cu,Zn-SOD activity was significantly
lower than in either pair-fed or ad libitum-fed controls. Zinc deficiency had no effect on Cu,Zn-SOD activity. These findings support the hypothesis that Cu,Zn-SOD activity is directly influenced by the tissue concentration of copper, but not zinc (Taylor 1988). Therefore, the observed decreases in Cu,Zn-SOD in response to increased zinc intake must be an indirect effect, presumably through a decrease in copper availability.

The mechanism by which zinc could adversely affect copper homeostasis is not clear. However, Cousins (1985) provided a hypothesis that would explain some of this interaction. Once within the absorptive cell, copper and zinc interact within the intracellular pools. Synthesis of metallothionein is strongly induced by zinc (Dunn et al. 1987). The binding affinity of metallothionein for copper is much greater than it is for zinc; consequently, copper may be sequestered within the absorptive cells of the intestine and thus unavailable for utilization elsewhere in the body (Cousins 1985). This hypothesis suggests that the greater the intake of zinc and the lower the intake of copper (absolutely or relatively), the greater the potential for copper sequestration, and ultimately, copper deficiency (Fosmire 1990). This relationship is exploited to alleviate excess copper accumulation in patients suffering from Wilson’s Disease; an autosomal-recessive disorder in which
decreased biliary excretion of copper leads to neurological and hepatic problems (Danks 1989). Wilson’s Disease patients given daily zinc supplements exhibited negative copper balance due to reduced copper absorption (Cousins & Hempe 1990).
CHAPTER 3
MATERIALS AND METHODS

Subjects

Fifteen male subjects were selected from approximately 50 candidates. Candidates were recruited through the use of flyers posted at the University of Florida in the J.H. Miller Health Center, the Department of Food Science and Human Nutrition and the Center for Exercise and Human Performance. Following an initial telephone interview, which included a general description of the study and subject responsibilities, the candidate completed an in-depth questionnaire addressing a variety of topics, including demographic information, medical and medication history, activity status and diet history. Criteria for subject selection is outlined in Table 3-1. Blood chemistry profiles were performed for all candidates that met the selection criteria (SmithKline Laboratories, Gainesville, FL). Candidates with one or more blood chemistry measurements outside the normal range for sex and age were excluded.

A description of the 15 subjects selected to participate in the study is shown in Table 3-2.
TABLE 3-1
Criteria for Subject Selection

30-35 years of age
Caucasian
Non-smoker
No history of chronic disease
Appropriate weight for height
No competitive athletes
Non vegetarians
No chronic drug use
Normal plasma zinc concentration
Blood chemistry parameters
within normal range

TABLE 3-2
Description of Subjects

<table>
<thead>
<tr>
<th></th>
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<th>Range:</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
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<tr>
<td>Weight (kg)</td>
<td>75.4 ± 11.2</td>
<td>62.6 - 101.1</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>178 ± 5.8</td>
<td>167 - 185</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>32 ± 6.5</td>
<td>22 - 42</td>
</tr>
</tbody>
</table>

Normal Dietary Intake:

<table>
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<tr>
<th></th>
<th>Mean ± SD:</th>
<th>Range:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcal/d*</td>
<td>2696 ± 1028</td>
<td>1481 - 4230</td>
</tr>
<tr>
<td>Protein (g/d)</td>
<td>110 ± 31</td>
<td>74 - 145</td>
</tr>
<tr>
<td>Fat (% MJ)</td>
<td>33.4 ± 6.6</td>
<td>20 - 43</td>
</tr>
<tr>
<td>Zinc (mg/d)</td>
<td>17.5 ± 7.1</td>
<td>6.8 - 34.4</td>
</tr>
</tbody>
</table>

* 1 Millijoule = 239 kilocalories
Anthropometric measurements monitored throughout the study included body weight and three-site (tricep, chest and subscapula) skinfold thickness measurements (Jackson & Pollock 1985). To minimize error, all measurements were performed by the same person.

The study was approved by the Institutional Review Board of the University of Florida. All subjects signed informed consent forms and a contract addressing specific responsibilities related to participation in the study. Benefits to the subjects included a $1000.00 stipend and all food and beverages for 60 days. Several steps were taken to ensure optimal compliance to the study protocol. Researchers maintained continual personal interaction with the subjects to reinforce the importance of strict adherence to the diet regimen. Subjects completed a daily checklist addressing compliance and problems encountered. An anonymous survey was completed at the end of the protocol to identify any deviations from the protocol.

**Experimental Design**

The 90-day study was divided into four phases as shown in Figure 3-1. The subjects were free-living throughout the study. During the first 60 days of the study (acclimation, treatment and depletion phases), all food and beverages were provided in the Clinical Research Center (CRC) at the
Figure 3-1. Overview of study protocol. Numbers indicate the zinc content of diets fed to experimental groups during each phase of study. ** Indicates 50 mg Zn/d in supplementation phase was in addition to the zinc content of a self-selected diet.
The diagram illustrates the zinc intake (mg/day) from Days 1 to 91, with specific periods labeled as Acclimation, Treatment, Depletion, and Supplementation. The horizontal axis represents the Day of Study, while the vertical axis shows the zinc intake in milligrams per day. During the Acclimation period, zinc intake is shown to be 15 mg/day, followed by a Treatment period at 15.2 mg/day. The Depletion phase shows a zinc intake of 3.2 mg/day, and the Supplementation phase has a value of 50 mg/day.
University of Florida College of Medicine. Breakfast and dinner were served and consumed in the CRC. Lunch and an evening snack were given to the subjects to be eaten on their own. All meals were consumed "metabolic style", which included scraping, rinsing and licking clean all food containers and utensils.

The first phase (seven days) was an acclimation period during which all subjects received a diet that provided the RDA for zinc (15 mg Zn/d). During the treatment phase, the subjects were randomly divided into three groups and received diets providing either 3.2, 7.2 or 15.2 mg Zn/d (by analysis) for 42 days. These levels were chosen to represent a range from low to adequate levels of dietary zinc intake (Moser-Veillon 1990; Pennington et al. 1984). The lowest level of 3.2 mg Zn/d is considered a realistic estimate of the amount of zinc that would be consumed by a strict vegetarian. The level of 15.2 mg Zn/d, or the RDA is an estimate of the zinc intake of individuals that consistently include zinc-rich foods, such as red meat, in their diet.

Following the treatment phase, all subjects received a diet providing 0.55 mg Zn/d for 12 days (depletion phase). At this point, the metabolically controlled portion of the study ended, and a 30 day supplementation phase followed. During the supplementation phase, all subjects took a
supplement providing 50 mg/d elemental zinc (The Solgar Vitamin Co. Inc., Lynbrook, N.Y.), in addition to a self-selected diet.

A major strength of the design of this protocol is that the treatment phase lasted for six weeks which allowed enough time to overcome the homeostatic adaptations that occur in response to changes in dietary zinc intake (Milne et al. 1983). Also, the study was designed such that dietary treatment preceded depletion to determine whether maintaining different levels of zinc for extended periods would alter the response of erythrocyte metallothionein and other indices to zinc depletion and/or supplementation following acute depletion.

**Diets**

Nutrient composition of the menus used in the acclimation and treatment phases is shown in Table 3-3 and Appendix A. Menus used in these phases are shown in Table 3-4 (recipes and procedures are included in Appendix B). These menus included three meals and one snack and consisted primarily of conventional foods. Food was purchased in case lots to ensure uniformity, and MilliQ® water (Continental Water Systems Corp., San Antonio, TX) (doubly deionized, distilled water; no detectable zinc) was used for food
**TABLE 3-3**

Nutrient composition of four-day cycle menu from acclimation and treatment phases of protocol.*

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy (kcal)</strong></td>
<td>3179</td>
<td>3059</td>
<td>2892</td>
<td>2988</td>
</tr>
<tr>
<td><strong>Protein (g)</strong></td>
<td>73.1</td>
<td>71.4</td>
<td>76.2</td>
<td>89.2</td>
</tr>
<tr>
<td><strong>Carbohydrates (g)</strong></td>
<td>452</td>
<td>501</td>
<td>446</td>
<td>456</td>
</tr>
<tr>
<td><strong>Fat (g)</strong></td>
<td>131</td>
<td>89.5</td>
<td>101</td>
<td>89.2</td>
</tr>
<tr>
<td><strong>Zinc (mg)</strong></td>
<td>3.26</td>
<td>3.02</td>
<td>3.56</td>
<td>2.83</td>
</tr>
</tbody>
</table>

* Daily supplement provided 150 ug retinyl acetate, 60 mg ascorbic acid, 1.5 mg thiamin, 1.7 mg riboflavin, 20 mg niacin, 10 ug ergocalciferol, 20 mg dl-a-tocopherol, 2 mg vitamin B₆, 0.4 mg folic acid, 6 ug cyanocobalamin, 10 mg pantothenic acid, 2 mg biotin, 18 mg iron, 50 mg magnesium, 400 mg calcium and 2 mg copper.

** 1 Millijoule = 239 kilocalories

*** By analysis, all other values determined by Food Processor II nutrient and diet analysis system (ESHA Research, Salem, OR).
# TABLE 3-4

Daily menus used for acclimation and treatment phases of protocol.

<table>
<thead>
<tr>
<th>Day 1:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
</tr>
<tr>
<td>Trix®</td>
</tr>
<tr>
<td>Coffee Rich®</td>
</tr>
<tr>
<td>Raisin Bread</td>
</tr>
<tr>
<td>Jelly</td>
</tr>
<tr>
<td>Lemon Shake</td>
</tr>
<tr>
<td><strong>Lunch</strong></td>
</tr>
<tr>
<td>Turkey Sandwich</td>
</tr>
<tr>
<td>Corn Chips</td>
</tr>
<tr>
<td>Vanilla Wafers</td>
</tr>
<tr>
<td>Pineapple</td>
</tr>
<tr>
<td>Grape Juice</td>
</tr>
<tr>
<td><strong>Dinner</strong></td>
</tr>
<tr>
<td>Crispy Chicken</td>
</tr>
<tr>
<td>Mashed Potatoes</td>
</tr>
<tr>
<td>Salad</td>
</tr>
<tr>
<td>French Dressing</td>
</tr>
<tr>
<td>Bread</td>
</tr>
<tr>
<td>Margarine</td>
</tr>
<tr>
<td>Ice Cream</td>
</tr>
<tr>
<td>Fruit Shake</td>
</tr>
<tr>
<td>Orange Juice</td>
</tr>
<tr>
<td>Twinkies®</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
preparation. Food and beverages were weighed before being served, and subjects were required to consume all items provided. Care was taken to avoid trace mineral contamination. This included wearing powder free gloves for all preparation and serving, using only zinc-free containers for preparation and serving, using only MilliQ® water for preparation of foods and beverages, wiping all trays and preparation and storage containers with MilliQ® water prior to use and washing plastic items in radiacwash (Atomic Products Inc., Shirley, NY) solution to remove mineral contamination.

An egg-white based formula (Table 3-5) similar to one used previously (Turnlund et al. 1984) was provided during the acclimation, treatment and depletion phases to supplement energy and protein. During the acclimation and treatment phases, zinc sulfate (J.T. Baker Chemical Co., Phillipsburg, NJ) was added to the formula to provide appropriate levels of zinc (see Figure 3-1). The depletion diet consisted primarily of the egg-white based formula with additional energy provided by pudding and ice cream made from low zinc ingredients. To limit zinc absorption during the depletion phase, 1.4 g/d sodium phytate from corn (SIGMA, St. Louis, MO) was added to the formula; the phytate to zinc molar ratio was approximately 270 (Gordon et al. 1982). MilliQ® water, Diet 7-Up® (Pepsi-Cola Co.) (8ug
### TABLE 3-5
Recipe and nutrient composition of formula used in acclimation, treatment and depletion phases of protocol.*

<table>
<thead>
<tr>
<th>Recipe:</th>
<th>Nutrient Composition:</th>
</tr>
</thead>
<tbody>
<tr>
<td>(360 g = 1 serving)</td>
<td></td>
</tr>
<tr>
<td>75 g Egg Whites</td>
<td>408 kcal</td>
</tr>
<tr>
<td>130 g Coffee Rich®</td>
<td>7.1 g Protein</td>
</tr>
<tr>
<td>110 g MilliQ Water</td>
<td>64.1 g Carbohydrate</td>
</tr>
<tr>
<td>45 g Flavor Powder**</td>
<td>14.9 g Fat</td>
</tr>
<tr>
<td></td>
<td>0.17 mg Zinc</td>
</tr>
</tbody>
</table>

* Determined by Food Processor II® nutrient and diet analysis system (ESHA Research, Salem, OR).

** Koolaid®, Countrytime Lemonade® or Tang®.

*** 1 Millijoule = 239 kilocalories.
Zn/L) and Carefree® (Planters Lifesaver, Co., Winston-Salem, NC) sugarless gum were given ad libitum during all phases. Vitamin (E.R. Squibb & Sons, Inc., Princeton, NJ) and mineral (Mission Pharmacal Co., San Antonio, TX; Schiff Products, Inc., Moonachie, NJ) supplements were provided during all phases to provide recommended levels of all nutrients (National Research Council 1989) with the exception of zinc. Supplemental biotin (Puritan’s Pride, Bohemia, NY) was given daily to counteract the effect of biotin binding by avidin present in egg albumin (Green 1975).

The zinc content of all diets was determined by direct analysis. Composites of treatment phase diets and the depletion diet were collected in trace element-free containers, homogenized, weighed and frozen. Composites were freeze dried and aliquots were subjected to dry ashing (Menden 1977). Zinc was measured by flame atomic absorption spectrophotometry (AAS) (Model 500 AS-50 Autosampler, Perkin-Elmer Co., Norwalk, CT) utilizing atomic absorption zinc standards, covering the range of 0 to 6 ppm and made in a solution consisting of 10% nitric acid, 0.85% hydrochloric acid and 2.5 g of potassium sulfate per liter (Menden 1977). With the exception of zinc, nutrient composition of all diets was calculated using the Food Processor II® Nutrient and Diet Analysis System (ESHA Research, Salem, OR).
Sample Collection and Processing

Blood samples were collected on the first day of each phase and once per week throughout the study. Fasting venous blood samples were drawn in trace element-free tubes (Sarstedt, Newton, NC) containing heparin (Lyphomed, Inc., Rosemont, IL). Hematocrit measurements were done in duplicate by capillary centrifugation (IEC Model MB Centrifuge, International Equipment Co., Needham Heights, MA). For measurement of whole blood folate concentration, 500 ul of whole blood was aliquoted into a tube containing 5 mg ascorbate and 4.5 ml MilliQ® water and frozen at -20°C. Whole blood was centrifuged at 1500 X g for 10 min to separate the plasma. Plasma for measurement of zinc concentration was transferred to microvials and frozen at -20°C. The buffy coat was removed from the erythrocyte pellet and the cells were washed twice with ice-cold 0.15 mol/L NaCl. The washed cells were lysed by addition of ice-cold MilliQ® water (1:1.4). The erythrocyte lysate was aliquoted into three microvials for measurement of erythrocyte zinc, erythrocyte metallothionein and total protein. Erythrocyte lysates were stored at -70°C.

Additional blood was collected, and serum was separated by centrifugation at 1200 X g for 30 minutes. Serum was transferred to microvials for ferritin and folate analysis.
and frozen at -20°C. Twenty-four hour urine samples were collected in acid-washed plastic containers at the end of the acclimation, treatment and depletion phases. Urine collection started after the first void in the morning and included the first void of the following morning. Total volume of urine collections was measured and recorded. Three 10 ml samples of each urine collection were aliquoted and frozen at -20°C.

**Biochemical Analysis**

**Erythrocyte Metallothionein**

Erythrocyte metallothionein concentrations were determined by a human metallothionein ELISA that was developed at the University of Florida (Grider et al. 1989 & 1990). The method is a competitive assay using IgG raised in sheep injected with human liver metallothionein-I. Purified metallothionein-I and sheep anti-human metallothionein-I IgG were provided by Dr. R.J. Cousins’ laboratory (University of Florida). Donkey anti-sheep IgG/alkaline phosphatase conjugate and reagents for buffers were purchased from SIGMA (St. Louis, MO). Phosphate-buffered saline solution (10 mmol/L Na₂HPO₄, 13.5 mmol/L NaCl, 3 mmol/L KH₂PO₄, 0.02% NaN₃; pH 7.2) with mercaptoethanol (4 ul/2 ml) was used for the coating buffer.
As a washing buffer, the phosphate-buffered saline solution also contained Tween 20 (0.5%; v/v). Bovine serum albumin (0.15 mmol/L) was added to the washing buffer to make the diluting buffer. The substrate solution was 4 mol/L p-nitophenyl phosphate in carbonate buffer (pH 9.6). The metallothionein ELISA procedure is illustrated in Figure 3-2. ELISA plates (NUNC, USA Scientific, Ocala, FL) were coated overnight with human metallothionein-I (100 ng/ml), then washed three times. Diluting buffer (300 ul) was added for 30 minutes to block nonspecific binding sites, followed by three washings. Purified human metallothionein-I or unknown sample (50 ul) was added to appropriate wells. Eight serial dilutions of standards and four serial dilutions of samples were made. Sheep anti-human metallothionein-I IgG (1:4000, 50 ul; primary antibody) was then added to all wells. Duplicates of each sample were assayed. Plates were incubated for four hours at room temperature, washed three times and incubated with donkey anti-sheep IgG/alkaline phosphatase conjugate (2 ul/ml buffer, 100 ul per well; secondary antibody) for 30 minutes at room temperature. Plates were washed three times and then incubated with substrate (200 ul/well) at 37°C. After 60 minutes, absorbance at 405 nm was measured by a microplate reader (Molecular Devises Corp., Menlo Park, CA).
Figure 3-2. Illustration of erythrocyte metallothionein assay.
Concentrations of unknown samples were determined by linear regression of the standard curve after logit transformation (Grider et al. 1989). Metallothionein concentration of each sample was calculated as mean ± SEM from eight possible values. Data were excluded if the value did not fall within the range of the standard curve, if the absorbance value did not show a serial dilution effect, or if the metallothionein concentration of an individual well was greater than two standard deviations from the mean for that sample. The average number of values used to determine erythrocyte metallothionein concentration for individual samples was 6.2 ± 1.4. The interassay coefficient of variation of the erythrocyte metallothionein ELISA, determined from repeated measurements of normal erythrocyte lysates, was 22.4%; and the intraassay coefficient of variation was 17.8%.

Previously, erythrocyte metallothionein concentrations were determined in a population of 50 healthy males between 20 and 35 years of age. These values were used to determine our laboratory specific normal value for erythrocyte metallothionein.

Total protein concentrations of erythrocyte lysates were measured with folin phenol reagent (SIGMA, St. Louis, MO) following the Lowry method (Lowry et al. 1951). Erythrocyte metallothionein values were expressed as nmol metallothionein per gram erythrocyte protein. Ferritin
concentration, as a marker of protein synthesis, was measured spectrophotometrically at 450nm using an ELISA method as specified by the manufacturer (Dako Corp., Carinteria, CA).

**Plasma, Erythrocyte and Urinary Zinc**

Plasma and erythrocyte lysate were diluted 5-fold and 10-fold respectively with MilliQ® water, and zinc concentration was determined by flame AAS (Smith et al. 1979). Urinary zinc concentration was determined by flame AAS by direct aspiration. Standards corresponding to apparent zinc concentrations of 500, 1000, 1500 and 2000 µg zinc per liter were prepared with zinc atomic absorption standard solution (1005 ug/ml in 0.27 mol/L HCl; SIGMA, St. Louis, MO) in glycerol/water solutions (5/95; v/w). A standard curve was prepared daily using fresh standards, and zinc concentrations were calculated directly from the curve. Pooled plasma served as a reference to monitor inter-assay reproducability; that is, the pooled plasma was assayed after every 10 samples assayed.

**Additional indicators of zinc status**

Serum alkaline phosphatase activity was measured enzymatically. Alkaline phosphatase hydrolyzes p-nitrophenylphosphate to form p-nitrophenol and phosphate.
The rate at which p-nitrophenol is formed is proportional to alkaline phosphatase activity (Bowers & McComb 1966). Serum albumin concentration was measured coloremetrically. The procedure is based on the dye-binding capabilities of serum albumin. Albumin reacts with bromcresol green at pH 4.2 to form a green color (Doumas et al. 1971). Plasma was sent to Dr. T. Tamura at the University of Alabama at Birmingham for analysis of retinol binding protein concentration (LC Partigen Kit, Behring Diagnostics, Somerville, NJ) and angiotensin I-converting enzyme activity (Rosenthal et al. 1984). In vitro zinc stimulation of ACE was determined by measuring ACE activity with (1.7 mmol/L) and without the addition of zinc to the assay buffer. In vitro stimulation was calculated by dividing ACE activity with zinc by ACE activity without zinc.

Serum and Erythrocyte Folate

Serum folate and whole blood folate were determined by a microbiological assay using Lactobacillus casei (ATCC 7469) (Tamura 1990). Freeze-dried cultures of L. casei were obtained from Difco Laboratories (Detroit, MI) and prepared as described by Tamura (1990). A 96-well microplate assay procedure was used. Samples were diluted with a 0.1 M phosphate buffer (pH 6.3) containing 1 mg/ml ascorbic acid to obtain final concentrations of samples between 0.01-0.2
ng/100 ul. The volume of samples was adjusted to 150 ul using the same buffer. Six serial dilutions of samples and standards were made; then 150 ul of medium (Difco Laboratories, Detroit, MI) containing 10ul/well of a suspension of the assay organism was added to all wells. After inoculation at 37°C for 18 h, the contents of each well were suspended by repeated aspiration and flushing until the bacterial suspension became homogenous. Bacterial growth was measured by reading the turbidity of each well at 600 nm using a microplate reader interfaced with a personal computer. Data were reduced using Microplate Manager data analysis software (Bio-Rad, Atlanta, GA), and concentrations of original samples were calculated. To check inter-assay reproducibility, folate levels of two pooled plasma samples were measured each time the assay was performed. The interassay coefficient of variation for the folate microbiological assays was 12.7%. The following calculation was used to derive red blood cell folate concentrations:

\[
\text{RBC folate} = (\text{whole blood folate}) - [\text{serum folate} \times (1 - \text{hct}/100)] / \text{hct}/100
\]
Superoxide Dismutase

Superoxide Dismutase activity was measured by a kinetic assay monitoring inhibition of autoxidation of pyrogallol (SIGMA, St. Louis, MO) as originally described by Marlund and Marlund (1974) and modified by Prohaska et al. (1983). The rate of autoxidation of pyrogallol is inhibited 99% by SOD. A 96 well microplate was used for this assay which was performed as follows: 50 ul Tris Buffer (50 mM Tris/1 mM DPTA, pH 8.2) was added to all wells. MilliQ® water (50 ul) was added to the first well of the autooxidation columns and 50 ul sample was added to the first well of the sample columns. Four serial dilutions were performed, giving a range of one to eight times the dilution of the samples. Tris buffer (50ul) was then added to each well, followed by 50 ul Pyrogallol (0.2 mM Pyrogallol/1 mM DPTA). The plates were immediately read at 340 nm for three minutes on the microplate reader. Results are expressed as units of SOD per gram of hemoglobin, with one unit defined as that amount of enzyme causing half the maximum autoxidation of pyrogallol. Hemoglobin concentrations were determined colorimetrically following reaction with Drapkin’s reagent (Sigma, St. Louis, MO).
Statistical Analysis

For each parameter measured, paired difference t-tests were performed to assess the significance of observed change in individual treatment groups during each phase of the study using the SAS statistical computer package (SAS Institute, Inc. 1985). Two sample t-tests were used to identify differences between treatment groups at the completion of each phase of the study. A probability value of less than 0.05 was considered to be significant. All values in the text, tables and figures are reported as means ± standard error.
Anthropometric parameters monitored are shown in Table 4-1. No subject had a greater than 5% change in body weight during the treatment phase. Although the mean change was not statistically significant, all subjects lost weight during the depletion phase, with an average loss of $1.77 \pm 0.8$ kg (2.4% of body weight). No changes in body composition, as determined by three-site skinfold measurements, occurred.

**Erythrocyte Metallothionein**

Erythrocyte metallothionein concentrations decreased significantly in the group fed 3.2 mg Zn/d during the treatment phase, but did not change in the groups fed 7.2 or 15.2 mg Zn/d during that phase (Figure 4-1). These findings indicate that a change in erythrocyte metallothionein concentration was a successful method for distinguishing between low versus adequate levels of dietary zinc. At the end of the treatment phase, the mean erythrocyte metallothionein concentration of the group fed 3.2 mg Zn/d was not different from the values of the other treatment
### TABLE 4-1
Body weight and sum of skinfold-thickness measurements taken throughout each phase of the study.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (kg)</th>
<th>Sum of skinfold-thickness (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>77.3 ± 2.8</td>
<td>32.1 ± 2</td>
</tr>
<tr>
<td><strong>Treatment phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>week 1</td>
<td>75.9 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>week 2</td>
<td>75.7 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>week 3</td>
<td>75.3 ± 2.7</td>
<td>32.4 ± 2</td>
</tr>
<tr>
<td>week 4</td>
<td>75.6 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>week 5</td>
<td>75.6 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>week 6</td>
<td>75.1 ± 2.5</td>
<td></td>
</tr>
<tr>
<td><strong>Depletion phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>75.1 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>day 5</td>
<td>74.1 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>day 8</td>
<td>73.9 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>day 12</td>
<td>73.3 ± 2.3</td>
<td>33.4 ± 1.7</td>
</tr>
</tbody>
</table>

* Taken at chest, tricep and subscapular sites.
** Measurement taken on first day of the week.

...
Figure 4-1. Erythrocyte metallothionein concentrations of individual treatment groups. Bars represent values at the completion of each phase of the study. When compared with previous or following study phase, bars with like letters are significantly different (p < 0.05).
did not increase significantly in any one group during the supplementation phase. When all groups were considered, however, there was a significant increase during the supplementation phase (Figure 4-2). At the end of the depletion phase, all treatment groups had erythrocyte metallothionein concentrations significantly lower than our laboratory established normal value. This suggests that a single erythrocyte metallothionein measurement was indicative of zinc depletion. The average decrease in erythrocyte metallothionein \((46 \pm 11\%)\), observed for all subjects during the depletion phase relative to the end of the treatment phase of this study, is comparable to that observed by Grider et al. (1990), who reported a 68\% decrease in erythrocyte metallothionein in human subjects after seven days of consuming a 0.46 mg Zn/d diet. Although the mean erythrocyte metallothionein concentration increased during the supplementation phase of this study, it was not of the magnitude reported by Grider et al. (1990). The difference is probably due to the fact that our subjects were depleted of zinc prior to supplementation.

During the depletion phase the percent decrease in erythrocyte metallothionein in the group fed 3.2 mg Zn/d during the treatment phase \((38 \pm 4\%)\) was significantly less than the decrease in the group fed 15.2 mg Zn/d during the treatment phase \((50 \pm 2\%)\) (Table 4-2). This suggests a
Figure 4-2. Laboratory established normal value for erythrocyte metallothionein and mean erythrocyte metallothionein concentrations for all subjects. Bars represent values at the completion of each phase of the study. Bars with like letters are significantly different (p<0.05).

**TABLE 4-2**

Percent change in erythrocyte metallothionein concentration for individual treatment groups during each phase of the study.

<table>
<thead>
<tr>
<th>Treatment group:</th>
<th>Treatment</th>
<th>Depletion</th>
<th>Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 mg Zn/d</td>
<td>-15.9 ± 5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>-38.8 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+51.4 ± 8</td>
</tr>
<tr>
<td>7.2 mg Zn/d</td>
<td>+11.8 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-48.7 ± 3</td>
<td>+68.6 ± 2</td>
</tr>
<tr>
<td>15.2 mg Zn/d</td>
<td>+23.5 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-49.5 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+75.5 ± 12</td>
</tr>
</tbody>
</table>

*Mean percent changes are compared between treatment groups for each phase of the study. + indicates percent increase and - indicates percent decrease. Within each study phase, values with like letters are significantly different (p < 0.05).

A curious observation was the apparent direct relationship between pre-depletion (treatment phase) zinc intake and the mean erythrocyte metallothionein concentrations following the supplementation phase. Although the erythrocyte metallothionein values were not significantly different between groups, the correlation between mean erythrocyte metallothionein concentration and treatment phase zinc intake was significant (r=0.989, p<0.05). This trend was observed following a 12 day zinc
depletion. It is possible that under certain conditions the response of erythrocyte metallothionein to zinc supplementation would reflect previous zinc intake. This warrants further investigation. The lack of change in the concentrations of serum ferritin (Table 4-3) suggests that the changes observed in erythrocyte metallothionein were specific and not an indirect effect on iron metabolism or general decrease in protein synthesis.

### Table 4-3

Mean serum ferritin concentrations of individual treatment groups at the completion of each phase of the study.

<table>
<thead>
<tr>
<th>Treatment group:</th>
<th>Study Phase</th>
<th>Acclimation</th>
<th>Treatment</th>
<th>Depletion</th>
<th>Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 mg Zn/d</td>
<td>Acclimation</td>
<td>167 ± 52</td>
<td>156 ± 43</td>
<td>162 ± 40</td>
<td>150 ± 50</td>
</tr>
<tr>
<td>7.2 mg Zn/d</td>
<td>Treatment</td>
<td>172 ± 70</td>
<td>172 ± 80</td>
<td>171 ± 70</td>
<td>179 ± 70</td>
</tr>
<tr>
<td>15.2 mg Zn/d</td>
<td>Depletion</td>
<td>170 ± 70</td>
<td>162 ± 86</td>
<td>176 ± 60</td>
<td>163 ± 90</td>
</tr>
<tr>
<td></td>
<td>Supplementation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Normal values = 18 - 300 ug/L (Young 1993). No significant changes were found within treatment groups.

### Plasma Zinc

Plasma zinc decreased significantly during the treatment phase in the group that received 3.2 mg Zn/d but did not change significantly in the groups fed 7.2 or 15.2 mg Zn/d during that phase (Figure 4-3). Although the decrease was not significant (p<0.10), there was a declining
Figure 4-3. Plasma zinc concentrations of individual treatment groups. Bars represent values at the completion of each base of the study. When compared with previous or following study phase, bars with like letters are significantly different (p < 0.05). * Indicates values are different at p < 0.10 significance.
trend in plasma zinc in the group fed 7.2 mg Zn/d during the treatment phase. These changes indicate that plasma zinc was responsive to the dietary zinc intakes provided during the treatment phase. Our results are similar to the recent findings of Ruz et al. (1991), but differ from the results of several previous studies (Jones et al. 1981, Milne et al. 1983, Wada et al. 1985) in that there was no decrease in plasma or serum zinc in response to low dietary zinc intake. No subject had a mean plasma zinc concentration below the reported normal range (12-18 umol/L) (Shapcott 1992) at any time during the study.

Interestingly, plasma zinc concentrations did not decrease during the depletion phase. In fact, there was a slight, nonsignificant increase in the plasma zinc concentrations during the depletion period in the groups that received 3.2 and 7.2 mg Zn/d during the treatment phase. This is in contrast to the study of Gordon et al. (1982) in which plasma zinc decreased significantly by day five in males subjects consuming a diet similar to our depletion diet. However these findings are similar to those of Grider et al. (1990) who did not observe a significant decrease in plasma zinc levels during an eight day zinc depletion study. Our failure to observe a decrease in plasma zinc in response to zinc depletion may be explained by the diurnal variations of plasma zinc. Gordon et al.
reported a large diurnal variation in plasma zinc, peaking after an overnight fast and decreasing soon after the morning meal. Henry and Wilmes (1975) measured plasma zinc concentrations in nine human subjects at 24, 48 and 72 hours during a 72 hour period of starvation. They found significantly higher concentrations of plasma zinc for each time during the starvation period. Postprandial plasma samples were not collected during this study, however, all blood samples collected were fasting. If diurnal variation had an effect on the plasma zinc levels measured during the depletion phase, it would be expected that comparable effects would be exerted on the levels measured at other phases of the study. In addition, the form of diet provided during the depletion phase was different from the treatment phase (i.e. formula versus conventional foods). It is possible that the form of diet could have had an effect on the rate and percent of zinc absorbed which may have had an affect on fasting plasma zinc concentrations.

Erythrocyte Zinc

Erythrocyte zinc decreased significantly in all groups during the depletion phase and increased significantly during the supplementation phase (Figure 4-4). No changes in erythrocyte zinc were seen during the treatment phase. Studies of the response of erythrocyte zinc to zinc
Figure 4-4. Erythrocyte zinc concentrations of individual treatment groups. Bars represent values at the completion of each phase of the study. When compared with previous or following study phase, bars with like letters are significantly different (p < 0.05).
depletion have been conflicting, with documented decreases in some (Bueck et al. 1973, Jones et al. 1981) and no change in others (Baer & King 1984, Rabbani et al. 1987).

A portion of the observed decrease in erythrocyte zinc may be related to a pool of exchangeable erythrocyte zinc. In erythrocytes, zinc is a component of carbonic anhydrases, superoxide dismutase and δ-aminolevulinate dehydratase (EC 4.2.1.24). In addition, zinc is bound to the erythrocyte membrane, hemoglobin, and other proteins, as well as to small molecules. The less stable zinc complexes in the erythrocyte may exchange the metal with that present in the plasma solute fraction, forming a pool of exchangeable erythrocyte zinc (Van Wouwe et al. 1990). Van Wouwe et al. (1990) demonstrated that plasma zinc and the intracellular zinc pool exchange until equilibrium is achieved. This exchange may explain our failure to find a decrease in plasma zinc during the depletion phase of the protocol.

Ohno et al. (1985) estimated the free zinc pool in the erythrocyte to amount to 7-8% of the total erythrocyte zinc. They define free zinc as zinc not bound to carbonic anhydrases or superoxide dismutase. In an in vitro study of erythrocytes from healthy volunteers, Van Wouwe et al. (1990) found the exchangeable zinc in human erythrocytes to amount to only 2-3% of the total erythrocyte zinc. No information is available regarding the effect of zinc
deficiency on the exchangeable zinc pool. It can be assumed that as erythrocyte metallothionein is degraded as a result of zinc deficiency, the zinc that was bound to the metallothionein would enter the free pool of zinc. In our study, erythrocyte metallothionein decreased an average of 0.5 umol/L RBC. Since 1 mol of metallothionein binds 7 mol of zinc, this would add 3.5 umol Zn/L to the zinc pool. The zinc derived from the metallothionein would account for only approximately 10% of the decrease seen in erythrocyte zinc. It is possible that a zinc deficiency may induce degradation of other zinc binding molecules, thus further increasing the pool of free zinc. Additionally, since membrane-bound zinc may alter the fluidity and stabilization of membranes (Chvapil 1973), a zinc deficiency may allow the exchange of some small zinc bound molecules which would further decrease the intracellular zinc concentration.

**Urinary Zinc**

Urinary zinc excretion decreased significantly during the treatment phase only in the group fed 3.2 mg Zn/day (Figure 4-5), indicating that this parameter was successful in distinguishing between groups receiving different levels of dietary zinc within a narrow range. Our finding of decreased urinary zinc excretion in the lowest zinc intake group is similar to the recent report by Ruz et al. (1991).
Figure 4-5. Urinary zinc concentrations of individual treatment groups. Bars represent values at the completion of each phase of the study. When compared with previous or following study phase, bars with like letters are significantly different (p < 0.05).
These investigators reported a significant decrease in urinary zinc excretion in human subjects who received a 0.6 mg Zn/day diet for one week followed by 4 mg Zn/day for six weeks.

The primary homeostatic mechanism for maintaining zinc balance appears to be regulation of absorption. When Wada et al. (1985) decreased the dietary zinc of six healthy men from 16 to 5.5 mg/d, all but one achieved zinc balance within nine days. Balance was accomplished by a 70% reduction in fecal zinc; urinary zinc losses were unchanged. In a separate study (Baer & King 1984), when the dietary zinc of six healthy men was reduced from 16 to 0.3 mg/d, the subjects' adaptation was remarkable even though zinc balance was not achieved. In this study, fecal zinc excretion decreased 95%, but urinary zinc excretion also decreased by 60% (Baer & King 1984). Since we demonstrated a decreased urinary zinc excretion in the treatment group that received 3.2 mg Zn/d, it can be assumed that this level of zinc intake is too low for balance to be maintained only by an increase in zinc absorption. In contrast, a zinc intake of 7.2 mg/d appears to be sufficient to maintain balance via absorptive mechanisms.

During the depletion phase, urinary zinc excretion decreased in the groups fed 7.2 and 15.2 mg Zn/day, but did not decrease in the group fed 3.2 mg Zn/day (Figure 4-5).
Therefore, urinary zinc was not a consistent indicator of zinc intake in the present study. That is, the effect of zinc depletion on urinary zinc excretion was dependent upon pre-depletion diet. It appears that there is a minimal level of zinc that will be excreted in the urine even in severe zinc restriction.

**Zinc Enzymes**

No significant changes were observed in the activity of alkaline phosphatase in any treatment group during any phase of this study (Table 4-4). This finding is consistent with some (Kasarkis & Shuna 1980; Ruz et al. 1991) but not all (Kay et al. 1976; Nanji & Anderson 1983; Prasad et al. 1978; Weisman & Hoyer 1985) previous reports. The differences in reported effects of zinc intake on alkaline phosphatase activity are probable attributable to differences in study design. Of those studies which demonstrated a significant response in alkaline phosphatase activity to zinc intake, none were conducted on healthy subjects. When a mild zinc deficiency, comparable to our 3.2 mg Zn/d treatment group, was induced in 15 healthy young adult males (Ruz et al. 1991), no significant changes in alkaline phosphatase activity were found.
TABLE 4-4
Serum alkaline phosphatase activity of individual treatment groups at the completion of each phase of the study.

<table>
<thead>
<tr>
<th>Study Phase</th>
<th>Treatment group: 3.2 mg Zn/d</th>
<th>7.2 mg Zn/d</th>
<th>15.2 mg Zn/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acclimation</td>
<td>79 ± 5</td>
<td>79 ± 8</td>
<td>78 ± 10</td>
</tr>
<tr>
<td>Treatment</td>
<td>83 ± 3</td>
<td>79 ± 10</td>
<td>82 ± 10</td>
</tr>
<tr>
<td>Depletion</td>
<td>87 ± 4</td>
<td>83 ± 7</td>
<td>84 ± 11</td>
</tr>
<tr>
<td>Supplementation</td>
<td>90 ± 7</td>
<td>85 ± 8</td>
<td>82 ± 9</td>
</tr>
</tbody>
</table>

*Normal value = 20 - 140 U/L. No significant changes were found within treatment groups.

Acute zinc depletion had no effect on plasma angiotensin converting enzyme activity (Table 4-5). In vitro zinc stimulation of plasma ACE activity was not significantly different in any treatment group when measured before and after a 12 day zinc depletion (Table 4-6). Ruz et al. (1991) found no effect of a seven week moderate zinc depletion on basal ACE activity. Taken together, these findings suggest that ACE activity is not an indicator of either moderate or severe zinc restriction. Recently, Johnston et al. (1993) determined zinc levels and ACE activities in plasma of 146 pregnant women. Plasma zinc levels decreased as pregnancy progressed. Although in vitro zinc stimulation of ACE did not change significantly throughout pregnancy, this measurement was significantly correlated with plasma zinc levels. These investigators concluded that in vitro stimulation of plasma ACE activity...
may be used as a functional test to compliment the traditional methods for evaluating zinc status. However, since plasma zinc is not a reliable indicator of zinc status, the fact that a functional measurement correlates with plasma zinc would not necessarily be evidence that the measurement is an indicator of zinc status.

**TABLE 4-5**

Plasma angiotensin converting enzyme activity of individual treatment groups before and after a 12 day severe zinc depletion.

<table>
<thead>
<tr>
<th>Treatment group:</th>
<th>Before Depletion</th>
<th>After Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without Zn: with Zn:</td>
<td>without Zn: with Zn:</td>
</tr>
<tr>
<td>3.2 mg Zn/d</td>
<td>153±13 168±12</td>
<td>118±9 124±10</td>
</tr>
<tr>
<td>7.2 mg Zn/d</td>
<td>126±18 141±20</td>
<td>126±26 134±27</td>
</tr>
<tr>
<td>15.2 mg Zn/d</td>
<td>158±12 166±14</td>
<td>132±9 136±14</td>
</tr>
</tbody>
</table>

No significant changes found within treatment groups.

**TABLE 4-6**

In vitro zinc stimulation of serum angiotensin converting enzyme activity of individual treatment groups before and after a 12 day severe zinc depletion.

<table>
<thead>
<tr>
<th>Treatment group:</th>
<th>Before Depletion</th>
<th>After Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before Depletion</td>
<td>after Depletion</td>
</tr>
<tr>
<td>3.2 mg Zn/d</td>
<td>1.11 ± 0.3</td>
<td>1.05 ± 0.1</td>
</tr>
<tr>
<td>7.2 mg Zn/d</td>
<td>1.12 ± 0.5</td>
<td>1.08 ± 0.1</td>
</tr>
<tr>
<td>15.2 mg Zn/d</td>
<td>1.05 ± 0.4</td>
<td>1.02 ± 0.6</td>
</tr>
</tbody>
</table>

No significant changes found within treatment groups.
Serum Proteins

Serum albumin concentration was not affected by zinc intake (Table 4-7). This finding is in contrast to the report by Wada and King (1986) who found significantly decreased albumin and prealbumin concentrations in six young men after 28 day of consuming a 5.5 mg Zn/d diet. However, the mean serum albumin concentration, following 54 days on the moderate zinc-deficient diet, was not significantly different than the predepletion concentration (Wada & King 1986). No information was given concerning protein intake prior to beginning the study. The reported changes in serum albumin may have been related to changes in protein, rather than zinc intake.

### Table 4-7

Serum albumin concentration of individual treatment groups at the completion of each phase of the study.

<table>
<thead>
<tr>
<th>Study Phase</th>
<th>Treatment group:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acclimation</td>
</tr>
<tr>
<td></td>
<td>(g/L)*</td>
</tr>
<tr>
<td>3.2 mg Zn/d</td>
<td>46 ± 0.6</td>
</tr>
<tr>
<td>7.2 mg Zn/d</td>
<td>46 ± 0.5</td>
</tr>
<tr>
<td>15.2 mg Zn/d</td>
<td>44 ± 0.7</td>
</tr>
</tbody>
</table>

*Normal value = 40 - 60 g/L. No significant changes found within treatment groups.
Acute, severe zinc depletion did not affect serum retinol binding protein concentration (Table 4-8). Wada and King (1986) found significantly decreased serum RBP in humans following 54 days of consuming a moderate zinc-deficient diet. However, this is the first study to examine the effect of a short term, severe zinc restriction on serum RBP. Although it is clearly established that zinc is required for protein synthesis, serum proteins were not sensitive to changes in zinc intake in the present study.

<table>
<thead>
<tr>
<th>Treatment group:</th>
<th>Before Depletion</th>
<th>After Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 mg Zn/d</td>
<td>48.6 ± 7.4</td>
<td>46.2 ± 6.2</td>
</tr>
<tr>
<td>7.2 mg Zn/d</td>
<td>46.4 ± 12.3</td>
<td>46.8 ± 6.0</td>
</tr>
<tr>
<td>15.2 mg Zn/d</td>
<td>42.6 ± 6.4</td>
<td>40.8 ± 6.6</td>
</tr>
</tbody>
</table>

No significant changes found within treatment groups.

Serum and Erythrocyte Folate

Serum folate concentration increased significantly during the depletion phase in the group that previously consumed 3.2 mg Zn/d for six weeks (Figure 4-6). Acute zinc
Figure 4-6. Serum folate concentration of individual treatment groups. Bars represent values before and after a 12 day severe zinc restriction. Bars with like letters are significantly different (p < 0.05).
depletion had no effect on serum folate concentration in either of the groups that consumed higher levels of zinc during the treatment phase. Similarly, acute zinc depletion had an effect on the erythrocyte folate concentration in the 3.2 mg Zn/d treatment group only. Although the change was not significant, erythrocyte folate tended to decrease (P<0.10) during the depletion phase in the group that previously consumed 3.2 mg Zn/d (Figure 4-7). Erythrocyte folate and serum folate concentrations were negatively correlated (r= -0.687, P<0.05) in the 3.2 mg Zn/d treatment group during the depletion phase. Since folate intake remained constant throughout the entire study, the observed changes in folate concentration during the depletion phase can be directly attributed to zinc status.

It appears that a chronic moderate zinc restriction resulted in an increased susceptibility to the effects of severe zinc restriction. It is possible that the long-term moderate zinc restriction followed by acute zinc depletion resulted in changes in the stability of the erythrocyte membrane, and perhaps in vivo hemolysis, resulting in erythrocyte folate being released into the serum. This explanation for the effect of zinc depletion on circulating folate concentrations is different from previously reported zinc-folate interactions, which suggested zinc restriction decreased folate polyglutamate absorption (Tamura et al.)
Figure 4-7. Erythrocyte folate concentrations of individual treatment groups. Bars represent values before and after a 12 severe zinc restriction. Bars with like letters indicates different at p < 0.10 significance.
1978). If the zinc depletion achieved in our 3.2 mg Zn/d treatment group after a severe zinc restriction resulted in reduced erythrocyte stability, similar changes in the erythrocyte and serum concentrations of other molecules would be expected. This is an intriguing concept that warrants further investigation.

Recently, Bailey et al. (1993) found no significant effect of zinc restriction on folic acid utilization. The level of zinc restriction (3.5 mg Zn/d) provided by Bailey et al. is not comparable to the severe zinc restriction provided during the depletion phase of this study, but it is comparable to the level of zinc provided to our lowest treatment group (3.2 mg Zn/d) during our six week treatment phase. Similar to the findings of Bailey et al. (1993), we found no change in serum zinc concentrations in the 3.2 mg Zn/d group during the treatment phase (Table 4-9). We did find increases in serum folate concentration in the 7.2 mg Zn/d group (Table 4-9) and erythrocyte folate concentration in all treatment groups during the treatment phase (Table 4-10). The changes observed during the treatment phase are probably due to an increase in folate intake during the study versus prestudy folate intake. The subjects' average folate intake before beginning the study was 430 ± 308 ug/d. During the study subjects received approximately 750 ug folate per day (350 ug from diet, 400 ug from supplement).
TABLE 4-9
Serum folate concentrations of individual treatment groups at the completion of each phase of the study.

<table>
<thead>
<tr>
<th>Study Phase</th>
<th>Treatment group:</th>
<th>Acclimation (nmol/L)</th>
<th>Treatment (nmol/L)</th>
<th>Depletion (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.2 mg Zn/d</td>
<td>32.0 ± 8.9</td>
<td>23.7 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.4 ± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7.2 mg Zn/d</td>
<td>13.3 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.4 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.1 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>15.2 mg Zn/d</td>
<td>25.8 ± 8.9</td>
<td>28.2 ± 6.2</td>
<td>37.5 ± 4.6</td>
</tr>
</tbody>
</table>

* Normal value > 13.2 nmol/L (Brody 1991)
<sup>a</sup> Within treatment group, indicates values are significantly different (p < 0.05).

TABLE 4-10
Erythrocyte folate concentrations of individual treatment groups at the completion of each phase of the study.

<table>
<thead>
<tr>
<th>Study Phase</th>
<th>Treatment group:</th>
<th>Acclimation (umol/L)</th>
<th>Treatment (umol/L)</th>
<th>Depletion (umol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.2 mg Zn/d</td>
<td>0.91 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.38 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.95 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7.2 mg Zn/d</td>
<td>0.64 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.05 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>15.2 mg Zn/d</td>
<td>0.72 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.15 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.91 ± 0.3</td>
</tr>
</tbody>
</table>

* Normal value > 0.36 umol/L (Brody 1991)
<sup>a</sup> Within treatment group, indicates values are significantly different (p < 0.05).
<sup>b</sup> Within treatment group, indicates values are different at p < 0.10.
Copper status as assessed by erythrocyte superoxide dismutase activity was not affected by changes in dietary zinc intake (Table 4-11). Mean erythrocyte SOD activities were not significantly different between groups following six weeks of consuming 3.2, 7.2 or 15.2 mg Zn/d intake. Likewise, no treatment group had significantly lower erythrocyte SOD activity following a 12 day severe zinc restriction. These findings were not unexpected. Taylor et al. (1988) demonstrated that dietary zinc deficiency had no effect on the Cu,Zn-SOD activity of rats. Our data suggest that zinc deficiency has no effect on erythrocyte SOD activity of humans.

TABLE 4-11  
Erythrocyte superoxide dismutase activity of individual treatment groups before and after a 12 day severe zinc depletion.

<table>
<thead>
<tr>
<th>Treatment group:</th>
<th>Before Depletion</th>
<th>After Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(U/mg hgb)</td>
<td></td>
</tr>
<tr>
<td>3.2 mg Zn/d</td>
<td>142 ± 7.8</td>
<td>138 ± 9.3</td>
</tr>
<tr>
<td>7.2 mg Zn/d</td>
<td>132 ± 7.1</td>
<td>122 ± 13.4</td>
</tr>
<tr>
<td>15.2 mg Zn/d</td>
<td>147 ± 8.7</td>
<td>121 ± 4.5</td>
</tr>
</tbody>
</table>

No significant changes were found within treatment groups.
CHAPTER 5
SUMMARY AND CONCLUSIONS

The need for a sensitive and specific indicator of zinc status is well recognized. Several factors have made the identification of a "gold standard" for assessment of zinc status difficult. These include a strong homeostatic control of absorption and excretion to maintain zinc balance, even at very low zinc intake; and the interaction of zinc with other nutrients.

Because of the multitude of diverse functions of zinc in the human body, it is imperative that a method for accurate zinc assessment be identified. Specifically, an appropriate assessment parameter is required for identification of moderate zinc deficiency, which is believed to be prevalent in many population groups in the U.S. and throughout the world. Additionally, until a reliable indicator of zinc status is identified, the setting of the Recommended Dietary Allowances for zinc will remain beset with uncertainties.

Although we cannot conclude from this research that the illusive sensitive and specific indicator of zinc status has been identified, valuable information concerning a potential
new method for assessing zinc status, erythrocyte metallothionein, as well as several standard indices of zinc status was gained. Unique information resulted from the novel study design used. Specifically, we provided our subjects with the treatment levels of dietary zinc for an adequate period of time to overcome the effects of homeostatic adaptation. Additionally, our subjects consumed a diet severely zinc-deficient following six weeks of consuming a constant zinc intake. This provided the opportunity to examine the effects of previous zinc intake on the response of indicators of zinc status to zinc depletion.

Our data confirmed the responsiveness of erythrocyte metallothionein to dietary zinc intake. When utilizing a laboratory specific normal value, erythrocyte metallothionein was successful in identifying zinc depletion. In addition, when evaluating change over time, erythrocyte metallothionein concentration was successfully used to distinguish between zinc intakes equal to the RDA and a low level comparable to that seen in populations considered to be at risk for moderate zinc deficiency. The effect of zinc depletion on erythrocyte metallothionein concentration appeared to be influenced by prior zinc intake. Likewise, the response of erythrocyte metallothionein to zinc supplementation was correlated with
predepletion zinc intake. These findings provide an impetus for further investigation. Perhaps the effect of a zinc load on stimulation of erythrocyte metallothionein synthesis could be investigated as an indicator of zinc status.

As expected, based on previous reports, both plasma zinc concentration and urinary excretion of zinc decreased in our lowest treatment group following six weeks of a moderately zinc-deficient diet. Our study design allowed us to demonstrate that the response of urinary zinc excretion to zinc depletion was dependent upon previous dietary zinc intake.

Erythrocyte zinc, when expressed as µmol/L erythrocyte lysate, decreased in all treatment groups in response to a severe zinc depletion. However, the change in erythrocyte zinc observed during depletion did not appear to be dependent upon predepletion diet. The decrease in erythrocyte zinc may explain our demonstrated lack of response of plasma zinc to zinc depletion. Zinc deficiency may have led to an increased exchange of erythrocyte zinc to the plasma.

The conflicting results reported in the literature of the measurement of static zinc concentrations are most likely attributable to differing experimental protocols; however it is evident that a definitive indicator of zinc status in humans has not emerged from among these
measurements. King (1990) proposed that assessment of zinc status in humans could be accomplished by combining the results of plasma zinc and erythrocyte metallothionein measurements. Our results from this study suggest that plasma zinc concentrations and erythrocyte metallothionein concentrations both decrease during moderate zinc restriction (3.2 mg Zn/d). These are derived from two different zinc-dependent mechanisms. Plasma zinc concentrations represent the equilibrium established between regulated zinc absorption processes and regulated tissue uptake and efflux (Dunn & Cousins 1989). These can be up-or down-regulated by dietary and hormonal stimuli. Erythrocyte metallothionein concentration is most likely the result of continuous stimulus of zinc in reticulocyte progenitor cells of the bone marrow to up-regulate expression of the metallothionein gene in that organ system (Grider 1990, Huber & Cousins 1993a).

As a ubiquitous component of the human body, zinc is involved in the utilization and/or function of a variety of nutrients. The influence of dietary zinc on the status of these nutrients is of particular interest. Our data suggest that folate status may fluctuate in response to severe zinc restriction depending on previous zinc intake. However, the changes in serum and erythrocyte folate observed in response to zinc deficiency may be due to an alteration in the
erythrocyte membrane rather than a direct effect of zinc deficiency on folate absorption or metabolism. Unlike zinc supplementation, which was previously shown to influence Cu,ZnSOD activity, acute zinc depletion does not appear to affect copper status in free living subjects.
# APPENDIX A

## NUTRIENT COMPOSITION OF DIETS

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Acclimation Menu</th>
<th>Depletion Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcals</td>
<td>3029</td>
<td>2888</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>77.5</td>
<td>68</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>464</td>
<td>510</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>103</td>
<td>64</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin A (RE)</td>
<td>912</td>
<td>0</td>
</tr>
<tr>
<td>Thiamin (mg)</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Riboflavin (mg)</td>
<td>2.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Niacin (mg)</td>
<td>31</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin B₆ (mg)</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Vitamin B₁₂ (ug)</td>
<td>1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Folacin (ug)</td>
<td>354</td>
<td>280</td>
</tr>
<tr>
<td>Pantothenic acid (mg)</td>
<td>2.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>309</td>
<td>166</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>389</td>
<td>176</td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>17.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>194</td>
<td>56</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>909</td>
<td>673</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>2670</td>
<td>1408</td>
</tr>
<tr>
<td>Selenium (ug)</td>
<td>146</td>
<td>107</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>2810</td>
<td>1358</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>3.2</td>
<td>0.55</td>
</tr>
</tbody>
</table>

*Values are the average of the four daily menus served during the acclimation and treatment phases.*
APPENDIX B
PROCEDURES AND RECIPES

I. Items purchased individually packaged:
   Trix® Cereal 21 g
   Corn Flakes 38 g
   Grape Juice 120 g
   Apple Juice 120 g
   Cranberry Juice 120 g
   Orange Juice 120 g
   Margarine 5 g
   Jelly 14 g
   Cream Cheese 21 g
   Apple Sauce 120 g
   Corn Chips 21 g
   Potato Chips 21 g
   Twinkies® 43 g
   French Dressing 24 g

II. Weighed in advance and individually wrapped and frozen:
   Vanilla Wafers 30 g
   Lorna Doones® 30 g
   White Bread 25 g
   Raisin Bread 50 g
   Bagel 70 g
   Turkey (white meat) 40 g
   Chicken (white meat) 60 g
   Fish (Halibut) 130 g
   Blueberries (frozen) 40 g
   Strawberries (frozen) 50 g
   Cool Whip® 10 g
   Angel Food Cake 40 g

III. Prepared in advance, individually wrapped and frozen:

   A. Corn Bread
      Recipe: 280 g Corn Meal Mix
              25 g Vegetable Oil
              50 g Egg Whites
              275 g Coffee Rich®
              20 g Sugar
-Weigh all ingredients into large bowl
-Mix for 2 minutes at high speed
-Weigh 60 g into individual baking cups
-Bake for 24 minutes at 350°F.

B. Cupcakes
Recipe: 520 g White Cake Mix
99 g Egg Whites
73 g Vegetable Oil
300 g MilliQ® Water

-Weigh all ingredients into large bowl
-Mix for 2 minutes at high speed
-Weigh 40 g into individual baking cups
-Bake for 19 minutes at 325°F

C. Ice Cream
Recipe: 420 g Coffee Rich®
100 g Sugar
3 g Salt
16 g Corn Starch
460 g Cool Whip®

-Weigh all ingredients into large bowl
-Blend for 5 minutes at high speed
-Weigh 140 g into individual serving bowls
-Cover tightly and freeze

IV. Prepared Daily:

A. Day 1:
Coffee Rich®
-Weigh 120 g

Turkey Sandwich
-50 g White Bread
-12 g Mayonnaise
-40 g Turkey

Pineapple
-Weigh 120 g
(see procedure)

Crispy Chicken
-60 g Chicken
-5 g Margarine
-5 g Corn Flakes
-Bake for 22 minutes at 350°F
B. Day 2:

**Coffee Rich®**  
- Weigh 120 g

**Banana**  
- Weigh 25 g

**Peanut Butter & Jelly Sandwich**  
- Weigh 120 g  
- 25 g Tomato  
- 24 g French Dressing

**Baked Chicken**  
- Weigh 60 g  
- 5 g Margarine  
- Bake for 22 minutes at 350°F

**Rice**  
- Weigh 120 g

**Green Beans**  
- Weigh 80 g

**Gelatin Dessert**  
- Weigh 100 g

**Vanilla Pudding**  
- Weigh 120 g

C. Day 3:

**Fruit Cocktail**  
- Weigh 120 g

**Turkey Sandwich**  
- Weigh 120 g  
- 50 g White Bread  
- 12 g Mayonnaise  
- 40 g Turkey

**Pear Halves**  
- Weigh 120 g
BBQ Chicken
- 60 g Chicken
- 5 g Margarine
- 15 g BBQ Sauce
- Bake for 22 minutes at 350°F

Mashed Potatoes
- Weigh 140 g
  (See Recipe)

Carrots
- Weigh 80 g
  (See Procedure)

D. Day 4:

Coffee Rich®
- Weigh 120 g

Tuna Salad
- 50 g White Bread
Sandwich
- 12 g Mayonnaise
- 30 g Tuna (drained)

Rice
- 120 g
  (See Recipe)

Butterscotch Pudding
- 120 g
  (See Recipe)

E. Procedures:

1. Canned Fruit:
   Pineapple
   Pear Halves
   Fruit Cocktail

   - Wipe top of can with sanitized cloth
   - Rewipe with cloth sprayed with MilliQ® water
   - Drain fruit in colander for 10 minutes
   - Place 3 Kimwipes® on a tray. Put drained fruit on the tray for 30 minutes.
   - After drained, weigh fruit into serving cups.

2. Canned Vegetables:
   Carrots
   Green Beans

   - Wipe top of can with sanitized cloth
   - Rewipe with cloth sprayed with MilliQ® water
   - Drain vegetables in colander for 20 minutes
-After drained, weigh vegetables into serving bowls
-At serving time, heat at high power in microwave for 2 minutes (three at a time)

3. Lettuce

-Remove core and any objectionable leaves from head of lettuce
-Submerge lettuce into a plastic container filled with MilliQ® water for 10 minutes
-Drain lettuce in colander for 10 minutes
-Weigh lettuce into serving bowls for salad

G. Recipes:

1. Mashed Potatoes

   396 g Potato Buds®
   900 g Hot MilliQ® Water
   900 g Coffee Rich®
   180 g Margarine
   27 g Salt

   -Weigh ingredients individually
   -Combine all ingredients in large plastic bowl
   -Stir until well moistened
   -Cover bowl tightly with plastic wrap and microwave on high power for 5 minutes
   -Weigh 140 g into individual serving bowls
   -At serving time, reheat in microwave for 2 minutes (three at a time)

2. Rice:

   570 g Minute® Rice
   1440 g MilliQ® Water
   30 g Margarine
   8 g Salt

   -Weigh ingredients individually
   -Combine all ingredients into large plastic bowl
   -Cover bowl tightly with plastic wrap and microwave on high power for 12 minutes
   -Mix with fork and weigh 120 g into individual serving bowls
   -At serving time, reheat in microwave for 2 minutes (three at a time).
3. Pudding:  
Vanilla  
Butterscotch

440 g  Instant Pudding Mix  
1880 g  Coffee Rich®

- Weigh ingredients individually  
- Combine ingredients into large plastic bowl and mix on high speed for three minutes  
- Weigh 120 g into individual serving bowls  
- Cover tightly and freeze
REFERENCES


Sandstead, H.H. (1985) Are estimates of trace element requirements meeting the needs of the user? In: Trace Elements in Man and Animals (Mills, C.F., Bremner, I. & Chester, J.L. eds.) pp. 875-878. TEMA5, Commonwealth Agricultural Bureau, Farnham Royal, United Kingdom.


BIOGRAPHICAL SKETCH

Elizabeth Ann Thomas was born in Pittsburgh, PA, on March 25, 1963 and moved to Vero Beach, FL, in 1965 where she graduated from high school in 1981. She is the daughter of Nancy Thomas Moon and Glen Thomas and has three older brothers. She began college at Clemson University in Clemson, SC, and in 1983 transferred to Florida State University in Tallahassee, FL where she received her B.S. in 1985 and her M.S. in 1987 in human nutrition. She was a nutrition trainee at the Pediatric Pulmonary Center in Gainesville, FL, in 1987 and became a registered dietitian in 1988.

Beth was a pediatric dietitian at Phoenix Children’s Hospital in Phoenix, AZ, from 1988-1990. While in Phoenix, she conducted clinical research on cystic fibrosis and bronchopulmonary dysplasia, which she presented at the International Cystic Fibrosis Conference and the World Conference on Lung Health, respectively.

In 1990 Beth began studying under the guidance of Dr. Lynn B. Bailey at the University of Florida. She was awarded an assistantship from the Center for Nutritional Sciences via an award from the Pew National Nutrition Program to pursue her studies. She has presented her
doctoral research at the annual meeting of the American Society for Clinical Nutrition in 1992 and at the Experimental Biology meetings in 1993. After completion of her doctorate Beth plans to pursue an academic career. She has accepted a visiting assistant professor position in the Human Nutrition and Foods Department at Virginia Polytechnic Institute and State University, which she will begin in August, 1993.