

**EFFECTS OF DIETARY ZINC DEFICIENCY AND REPLETION  
ON METALLOTHIONEIN AND Ki-67  
IN RAT SMALL INTESTINE, LIVER AND KIDNEY**

By

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A thesis submitted to the Department of Foods and Nutrition  
In partial fulfillment of the requirements for the degree of  
Master of Science

Department of Foods and Nutrition  
University of Manitoba  
Winnipeg, Manitoba, Canada

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**Effects of Dietary Zinc Deficiency and Repletion on  
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**Elżbieta I. Szczurek**

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University  
of Manitoba in partial fulfillment of the requirements of the degree  
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Master of Science**

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## **ABSTRACT**

### **EFFECTS OF DIETARY ZINC DEFICIENCY AND REPLETION ON METALLOTHIONEIN AND Ki-67 IN RAT SMALL INTESTINE, LIVER AND KIDNEY**

**E. I. Szczurek, MSc. Thesis, Department of Foods and Nutrition**

Zinc is a nutrient essential for normal growth and development. Numerous cellular proteins involved in proliferative processes require zinc for their function. Metallothionein (MT), a metal binding protein, may regulate the interactions of zinc with cellular proteins, and thus, it may modulate zinc dependent processes in cell proliferation. Dietary zinc can affect MT tissue concentration, however, the effect of zinc status on MT tissue distribution is unknown. Also, the interactions between dietary zinc and MT in relation to cell cycle activity have not been investigated.

The objective of this study was to examine the effects of zinc deficiency and repletion on MT concentration, and MT and Ki-67 immunohistochemical localization in small intestine, liver and kidney. Weanling Sprague Dawley rats were assigned to zinc deficient (ZD), pair-fed (PF) and control (C) groups for 3 weeks. Half of ZD and PF rats were repleted with the control diet (ZR and PFR groups) for an additional 24 hours. Zinc status, tissue zinc and MT concentration, and immunohistochemical localization of MT and Ki-67, a cell cycle associated marker, were determined.

The ZD group had significantly lower serum, femur and renal zinc concentrations and unchanged intestinal and hepatic zinc concentrations compared to the C group. The ZD group also had significantly reduced intestinal, hepatic and renal MT concentrations compared with the C group. Zinc repletion for 24 hours increased but did not restore

femur and renal zinc concentrations, restored serum zinc concentration, and reduced hepatic zinc concentration. Intestinal, hepatic and renal MT concentrations were restored following 24 hour zinc repletion. The PF group had higher MT concentrations in kidney, and higher MT and zinc concentrations in liver than the C group, and these elevations were lowered by 24 hour calorie repletion. There was a significant positive correlation between tissue zinc and MT concentrations in liver and kidney ( $r = 0.70$ ,  $p = 0.0001$ , and  $r = 0.57$ ,  $p = 0.0001$ , respectively), but not in small intestine. Further, zinc deficiency resulted in the absence of MT staining in small intestine, and the absence of the staining or weak staining in liver and kidney. After zinc repletion, strong MT staining was observed in Paneth cells and surface epithelial cells of small intestine, and the intensity of staining increased in the kidney. Calorie restriction resulted in strong MT staining in liver that diminished after calorie repletion. Zinc deficiency and calorie restriction resulted in less Ki-67 staining in liver and kidney as compared with the C group, and the staining was not affected by zinc and calorie repletion.

The results of this study indicate that changes in zinc status affect intestinal, hepatic and renal MT concentration and distribution, and suggest that MT status is associated with tissue zinc concentration in kidney and liver, but not in small intestine. These findings support the role of MT in zinc metabolism, and the function of zinc and MT in gut immunity and intestinal mucosal turnover. They also imply that cell cycle activity in liver and kidney may be influenced by zinc status.

## ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to my advisor, Dr. Carla Taylor, for her patient guidance and support throughout the project as well as in the editing of this thesis.

I would also like to extend an acknowledgement to Dr. Ranjana Bird and Dr. Ronald Marquardt for serving on the committee.

Many thanks are also given to Marilyn Latta for her technical assistance, and Dr. Erwin Huebner and Chris Bjornsson for their expertise in histology and image analysis. My grateful recognition to Dr. William Halliday, Leo Laveuqe, Mark Patsetka and Virginie Rimbart, who provided invaluable contributions at various stages of the research.

To all fellow graduate students, your encouragement and help are greatly appreciated. A special "thank you" to Diana, Kathy, Jeri-Ann, and Melanie whose warm friendship and sense of humor enlightened the darkest of times.

Much appreciation is also given to my friends and family for helping me maintain the faith that this project could become reality. Special thanks to my mom, dad, sister and Mark. I will always be grateful for your much needed support, love and understanding throughout my university education.

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## **I. Literature Review**

### **Introduction**

The role of zinc in growth and development is well established (Vallee and Falchuk, 1993). Zinc is required for normal growth, development and differentiation of all living organisms. Cell growth and proliferation depend on the availability of zinc in a system. Metallothionein (MT), zinc-binding protein, plays an important role in homeostatic regulation of zinc metabolism. therefore, it may potentially modulate zinc dependent processes in cell proliferation (Vallee and Falchuk, 1993). MT is present in cytoplasm and nuclei of rapidly proliferating cells, and its synthesis is developmentally regulated in human and animals. Further, MT synthesis can be induced by zinc and zinc status can affect the concentration and expression of MT in various tissues (Sato et al, 1984; Blalock et al, 1988; Bremner, 1991; Cousins and Lee-Ambrose, 1992). This chapter will explore the interrelationships between zinc, MT and cell proliferation, and will review current knowledge about the interactions between dietary zinc and MT.

### **Zinc in Growth and Development**

Zinc has been recognized for a long time as a nutrient essential for proper growth and development of all living organisms (Wu and Wu, 1987; Vallee and Falchuk, 1993). In developing humans and experimental animals, inadequate zinc nutrition causes growth retardation, multiple medical complications and teratological abnormalities (Bettger and O'Dell and Revees, 1989; Vallee and Falchuk, 1993).

Zinc deficiency in humans was first described in young Egyptian and Iranian males who showed dwarfism, sexual immaturity, dermatosis, depressed immunocompetence and anemia (Prasad, 1984). While signs of anemia were reversed by iron supplements, other symptoms were corrected only by zinc treatment (Prasad, 1984). Growth retardation, dermatitis, alopecia and susceptibility to infections are also observed in children with *Acrodermatitis Enteropathica*, genetic disorder resulting in zinc malabsorption (Moynahan, 1974; Kelly et al, 1976; Vallee and Falchuk, 1993). Administration of zinc supplements improves growth in these children and reverses other pathologies associated with the disease (Moynahan, 1974; Kelly et al, 1976; Vallee and Falchuk, 1993). Oral zinc supplementation has been also shown to stimulate growth in growth-retarded children during recovery from malnutrition (Ninh et al, 1996).

Similarly, growth inhibition is a major manifestation of zinc deficiency in weanling rodents (O'Dell and Revees, 1989; Dorub et al, 1991; Prescod, 1998). Suppressed growth in zinc deficient rats and mice is evidenced by lower body weights and weight gains (O'Dell and Revees, 1989; Dorub et al, 1991; Prescod, 1998), and decreased linear growth (Prescod, 1998) as compared with controls. Immediately after zinc repletion of zinc depleted animals compensatory growth occurs (Prescod, 1998). Like in humans, zinc supplementation promotes growth and lean body mass gain in undernourished mice recovering from malnutrition (Morgan, 1988). Because of decreased feed intake observed in animals shortly after initiation of a zinc deficient diet (O'Dell and Revees, 1989; Prescod, 1998), it is difficult to separate the effects of zinc deficiency on growth and development from those of calorie depletion. However, studies

using relative pair-feeding as opposed to absolute pair-feeding, show that zinc deficiency per se has restrictive effect on growth independently of anorexia (Dorub et al, 1991).

Teratogenicity of zinc deficiency in animals is well documented. In rats and mice, maternal zinc deficiency results in low rate of survival, altered embryonic and fetal development and growth retardation (Keen et al, 1990; Vallee and Falchuk, 1993; Rogers et al, 1995; Dalton et al, 1996). Only 30% - 40% of embryos survive and become implanted (Vallee and Falchuk, 1993), and the majority of those that survive are malformed and underweight (Keen et al, 1990; Vallee and Falchuk, 1993; Rogers et al, 1995; Dalton et al, 1996). Embryonic and fetal development are affected not only by zinc deprivation throughout pregnancy but even by short-term zinc deficiency. It has been reported that as few as 4 days of zinc deficiency can produce excess embryonic cell death and morphological abnormalities (Rogers et al, 1995). Most severe effects of zinc deficiency appear to occur during the period of organ formation and rapid growth indicating a critical role of zinc in differentiation and organogenesis (Dalton, 1996; Gallant and Cherian, 1987). In human, teratogenic effects of zinc deficiency are difficult to study, and thus, less well documented. However, epidemiological and intervention data reviewed by Keen et al (1990) indicate that marginal to severe maternal zinc deficiency may be a complication of some pregnancies.

## **Zinc and Cell Proliferation**

Zinc is known to be indispensable for normal cell growth and development. Impaired growth in zinc deficient humans and animals occurs as a result of failure of cells to divide and differentiate (Wu and Wu, 1987; Coleman, 1992). Although zinc-

dependent cellular mechanisms responsible for cell division are not fully understood, numerous studies suggest that the metal may be critical for cell signaling, transcription, translation and DNA replication. Many of the proteins involved in these cellular processes have been found or are believed to be zinc metalloproteins/metalloenzymes (Wu and Wu, 1987; Vallee and Auld, 1990; Vallee et al, 1991; Coleman, 1992; Bettger and O'Dell, 1993; Vallee and Falchuk, 1993). A few studies also imply the role of zinc in cell cycle machinery (Chesters et al, 1989; Chesters, 1990; Chesters, 1993). This section will discuss the role of zinc in zinc proteins, and the importance of zinc in the regulation of cell cycle activity.

### ***Zinc Proteins***

Zinc is a catalytic and structural component of numerous metalloenzymes and metalloproteins. More than 300 enzymes are known today to contain zinc (Vallee and Auld, 1990; Vallee and Falchuk, 1993). Zinc-metalloenzymes can be found in all known classes of enzymes including oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (Vallee and Auld, 1990; Coleman, 1992; Vallee and Falchuk, 1993). As a functionally and/or structurally essential component of these enzymes, zinc can affect all aspects of cellular metabolism including synthesis and breakdown of proteins, lipids, carbohydrates and nucleic acids. Approximately 30 – 40% of cellular zinc is localized in the nucleus (Vallee and Falchuk, 1993). As a part of histone proteins, zinc stabilizes the native structures of DNA and RNA (Wu and Wu, 1987). DNA and RNA polymerases are zinc-metalloenzymes, and it is well documented that zinc motifs are essential constituents of many transcription factors (TFs) (Wu and Wu, 1987; Vallee

et al. 1991; Vallee and Falchuk, 1993). As a structural component of TFs, zinc can play an important role in regulation of gene expression. RNA polymerases can transcribe different classes of cellular genes but they have no specificity for gene recognition. However, TFs are able to direct RNA polymerases to specific genes (Wo and Wo, 1987). By binding to the promoter region of a target gene and interacting with RNA polymerase, they can initiate its transcription and ultimately control its synthesis.

Three different classes of DNA-binding zinc-proteins are presently known. These are zinc-fingers, zinc-twists and zinc-clusters (Vallee et al. 1991; Coleman, 1992; Vallee and Falchuk, 1993). In zinc-fingers, zinc is coordinated to two cysteine and two histidine residues. In zinc-twists and zinc-clusters, all four metal ligands are cysteines. The classical example of a zinc-finger protein is fungal TFIIIA, the first zinc-finger protein identified and characterized. Other TFs with zinc-finger motifs are members of Sp1 gene family. Fungal TF GAL4 is a representative of zinc-cluster proteins, and zinc-twists are found in numerous hormone receptors such as glucocorticoid or estrogen receptors. TFIIIA, GAL4 and the glucocorticoid receptor are among the very few DNA-binding zinc-proteins for which native structure has been determined by X-ray crystallographic and NMR analysis (Vallee et al, 1991; Coleman, 1992; Vallee and Falchuk, 1993). They serve as reference standards for identification and characterization of other putative zinc-proteins. The number of TFs with putative zinc-binding sites approximates 500 and is still increasing (Vallee and Falchuk, 1993). Since zinc stabilizes the protein structure of TFs, and zinc-coordination generates a DNA recognition site, the metal may play important role in the regulation of gene expression. In fact, the essentiality of zinc for TF-DNA interactions has been demonstrated for TFIIIA, GAL4 and SP1. Removal of

zinc from the proteins abolishes their binding to DNA and the binding can be restored by the addition of zinc (Coleman, 1992; Vallee and Falchuk, 1993; Shi and Berg, 1996).

Zinc may also be important for TF-protein interactions. Zinc-containing TFs, as opposed to their zinc-free forms, are able to cause conformational changes in DNA upon binding as has been demonstrated recently for TF Sp1 (Shi and Berg, 1996). It has been suggested that these changes in DNA conformation may be important for the interaction of TFs with other proteins. These types of studies contribute to the evidence for the molecular role of zinc in the cell cycle.

### ***Zinc and Cell Cycle***

Before addressing the importance of zinc in the cell cycle, an overview of the cell cycle will be presented followed by a discussion of the approaches used for the assessment of cell proliferation with the emphasis on Ki-67 as a cell cycle-related marker.

#### ***i. Cell Cycle Regulation of Cell Proliferation***

In order for a cell to divide into two daughter cells, it has to pass through four precisely controlled stages of the cell cycle: mitosis (M phase), the first gap of cell cycle (G1 phase), DNA synthesis (S phase) and the second gap of cell cycle (G2 phase) (Murray and Hunt, 1993; Reddy, 1994). Completion of specific metabolic events has to take place in each phase for a cell to progress to the next phase (Reddy, 1994). Multiple checkpoints coordinate different cell cycle events in time and space to ensure their right order (Reddy, 1994; Nigg, 1997).

$G_1$  phase which follows mitosis is the longest phase in cell cycle and a time of rapid cell growth. It is a preparatory stage in which proteins and enzymes required for DNA replication are synthesized (Murray and Hunt, 1993; Reddy, 1994). Completion of  $G_1$  events, and thus, progression to S phase depends on the availability of extracellular factors such as growth hormones and nutrients (Reddy, 1994). Late in  $G_1$ , growth factor-induced signals are converged and the decision to enter S phase is made (Murray and Hunt, 1993). Beyond this point cells are resistant to external stimuli, including nutrient deprivation, and become dependent only on intrinsic mechanisms (Reddy, 1994). In S phase, cell cycle regulatory proteins ensure complete duplication of DNA and they act through  $G_2$  and M phases to ensure successful cell division (Nigg, 1997).

After mitosis, cells can either enter another cell cycle, or they can exit the cell cycle and either undergo differentiation or arrest in  $G_0$  phase (quiescence) (Murray and Hunt, 1993). In most tissues, cells exist in  $G_0$ . They remain viable and metabolically active performing basic maintenance functions (Murray and Hunt, 1993; Reddy, 1994). Unlike terminally differentiated cells,  $G_0$  cells are capable of re-entering the cell cycle and proliferating under certain extracellular conditions (Murray and Hunt, 1993; Alison, 1995). Examples of such conditionally renewing cells are glandular epithelial cells including hepatocytes and renal tubular cells (Alison, 1995; Morrison et al. 1997). Within the body, only a small number of cells have infinite ability for self-renewal to maintain their number. For example, epithelia of gastrointestinal tract consist of constantly renewing populations (Alison, 1995).

## ***ii. Assessment of Cell Proliferation: Ki-67 as a Cell Cycle Associated Marker***

Complete analysis of cell proliferation involves the assessment of the rate and the state of cell proliferation (Alison, 1995). Several methods are now available to estimate proliferative indices, however, none of them by itself can provide information about both the rate and the state of cellular proliferation. In addition, they all have some technical and/or practical disadvantages and suffer from pitfalls that may impact on the accuracy of results. Methods used to assess the proliferative state of cells include thymidine labeling, bromodeoxyuridine labeling and flow cytometry as well as a combination of these techniques (Alison, 1995; Hall and Levison, 1990). They offer accurate assessment of the S-phase fraction and they are objective. However, they are either costly, time consuming and technically complex or require in vivo administration or in vitro incubation of radioactive material or toxic substrates. In addition, they only provide information about the S-phase fraction and do not give assessment of total cycling cells. Furthermore, flow cytometry requires tissue disruption, and thus, does not give information about topographical distribution of proliferating cells. Continuous or pulse labeling and stathmokinetic techniques can be used to measure proliferative rates but like the other methods described here they are lengthy, costly and involve complex techniques. Due to their numerous limitations, the methods discussed above are being increasingly replaced by cheaper, easier and less time consuming immunohistochemical techniques that use antibodies to cell cycle-related antigens. The number and proportion of cycling cells (growth fraction) can be determined in tissue sections by manual counting of immunoreactive cells or by image analysis techniques (Brown and Gatter, 1990. Alison, 1995). Immunohistochemistry is a static assessment of the population of

cycling cells and does not provide information about the actual state or rate of cell proliferation (Alison, 1995). However, besides being cheaper, fast and easy, immunohistochemistry has the important advantage of preserving spatial orientation of cycling cells in histological sections (Brown and Gatter, 1990; Yu et al, 1992; Alison, 1995).

Antibodies to Ki-67 nuclear antigen and proliferating cell nuclear antigen (PCNA) are widely used antibodies for the assessment of cell cycle activity in histological material. Ki-67 is a nuclear antigen associated with cell cycle that can be identified by monoclonal mouse antibody. It is conserved in neoplastic and normal tissues from many species including human, lamb, calf, dog, rabbit and rat. The nature of the antigen is poorly identified and its exact function is not known. Research indicates that it may be a component of the nuclear matrix and some authors suggest that it acts as a timer molecule (Brown and Gatter, 1990). Ki-67 is expressed in all phases of cell cycle except G<sub>0</sub> and early G<sub>1</sub>. Its antigenic expression changes with the progression of cell cycle, rising during S phase and reaching maximum during G<sub>2</sub> and M phases (Brown and Gatter, 1990; Hall and Levison, 1990; Yu et al, 1992; Nagel and Vallee, 1995). Ki-67 provides a more accurate estimation of the growth fraction than PCNA. The major disadvantage of PCNA is that it is detected not only in cycling cells but also in cells that recently left the cell cycle. By including G<sub>1</sub> phase of the cell cycle, Ki-67 staining allows more complete analysis of cell cycle activity than flow cytometry, [<sup>3</sup>H] thymidine labeling or bromodeoxyuridine labeling.

Immunoexpression of Ki-67 has been shown to correlate well with other indices of cell proliferation such as flow cytometry, [<sup>3</sup>H] thymidine incorporation and

bromodeoxyuridine incorporation (Brown and Gatter, 1990, Nagel and Vallee, 1995). Antibodies against Ki-67 have wide application in histopathology and research, and are used in the assessment of the growth fraction of normal, reactive and neoplastic tissues (Brown and Gatter, 1990; Hall and Levison, 1990; Yu et al, 1992). Until recently only antibodies to human Ki-67 were available, and their use was limited to frozen sections. Presently, antibodies cross-reacting with rodent Ki-67 are available such as MMI and MIB-5, and antigen retrieval techniques make their use possible on formalin-fixed, paraffin-embedded tissue sections.

### *iii. Zinc, DNA Synthesis and the Cell Cycle*

The effect of zinc deprivation on DNA synthesis has been demonstrated in vivo and in vitro. A 50% reduction in DNA synthesis, as assessed by [<sup>3</sup>H] thymidine incorporation, was observed in liver, kidney and spleen from weanling rats fed zinc deficient diet (< 0.9 ppm zinc). Because the inhibition was observed within the first 5 days of dietary treatment before food consumption and growth were affected, the study demonstrated an independent effect of zinc on DNA synthesis (Williams and Chesters, 1970). In synchronized cultures of 3T3 cells, chelation of zinc inhibited thymidine incorporation into DNA by up to 90%. Re-addition of Zn<sup>2+</sup>, but not other divalent cations, reversed this effect, and zinc supplementation of the medium prevented chelator-induced inhibition of thymidine incorporation (Chesters et al. 1989).

The nature and timing of zinc requirements for DNA synthesis were further examined in series of experiments conducted by Chesters and his colleagues. Chesters et al (1989) found that in synchronized cultures of 3T3 cells, the major requirement for zinc

was within the period from 8 hours after stimulation of quiescent cells until 3 hours before the start of S phase. This period corresponds to the mid- and late G<sub>1</sub> phase of the 3T3 cell cycle. There was also a minor requirement for zinc during G<sub>1</sub>/S transition. This conclusion was based on the observation that zinc addition to the medium at 8 hr for a short period of time resulted in less chelator-treated cells entering S phase as compared to the cells in which zinc was present from 8 hr onward.

In another study, Chesters et al (1990) demonstrated that the reduction in thymidine incorporation in zinc-deficient 3T3 cells stimulated from quiescence by serum was accompanied by decrease in the activity of thymidine kinase, the enzyme necessary for DNA synthesis. It was further shown that lower activity of the enzyme was associated with a corresponding decrease in its mRNA concentration. The decrease in enzyme activity and its transcription was prevented by the addition of zinc. On the other hand, zinc deprivation had no effect on the activity or expression of ornithine decarboxylase, another enzyme activity that increases in 3T3 cells after serum stimulation. However, the activity of this enzyme reaches maximum between 4 - 6 hours following stimulation as opposed to thymidine kinase which has peak activity between 8 - 12 hours. The researchers concluded that zinc is necessary for the induction of thymidine kinase rather than activation of the enzyme. The authors also proposed that thymidine kinase may be only one specific example of a more general effect of zinc on the induction of the group of enzymes and proteins required for DNA synthesis. Finally, insensitivity of 3T3 cells to lack of zinc during the initial period after serum stimulation supported the earlier demonstrations of zinc-dependent steps during G<sub>1</sub> to S phase transition.

The potential role of zinc in regulating transcription of thymidine kinase was further explored by Chesters et al (1993). Using transfected Syrian hamster fibroblasts, the authors examined whether thymidine kinase expression in response to zinc is dependent on sequences within promoter region on the protein. They found that zinc deprivation inhibited [<sup>3</sup>H] thymidine incorporation and reduced thymidine kinase mRNA concentration in the cells in which the thymidine kinase gene was activated by its own promoter, and inhibition was reversed by zinc supplementation. On the other hand, zinc deprivation had marginal effect on the reduction in thymidine incorporation and thymidine kinase mRNA concentration in the cells in which the thymidine kinase gene was constructed with the SV40 promoter. These findings supported the earlier speculations about a zinc requirement for thymidine kinase induction.

The relationship between zinc requirements, mRNA and protein synthesis, and the cell cycle progression into S phase was investigated by Chesters and Boyne (1991). The authors found that restriction of zinc, inhibition of mRNA synthesis by 5,6-dichlororibofuranosylbenzimidazole, and inhibition of protein synthesis by cycloheximide all delayed the entry of synchronized 3T3 cells into S phase as determined by [<sup>3</sup>H] thymidine pulse labeling techniques. Further, the combination of zinc depletion with inhibition of mRNA synthesis or protein synthesis resulted in the reversion of cells to quiescence. The authors concluded that zinc-dependent processes in the cell cycle require concurrent protein and mRNA synthesis, and that zinc is needed for protein accumulation occurring prior to S phase.

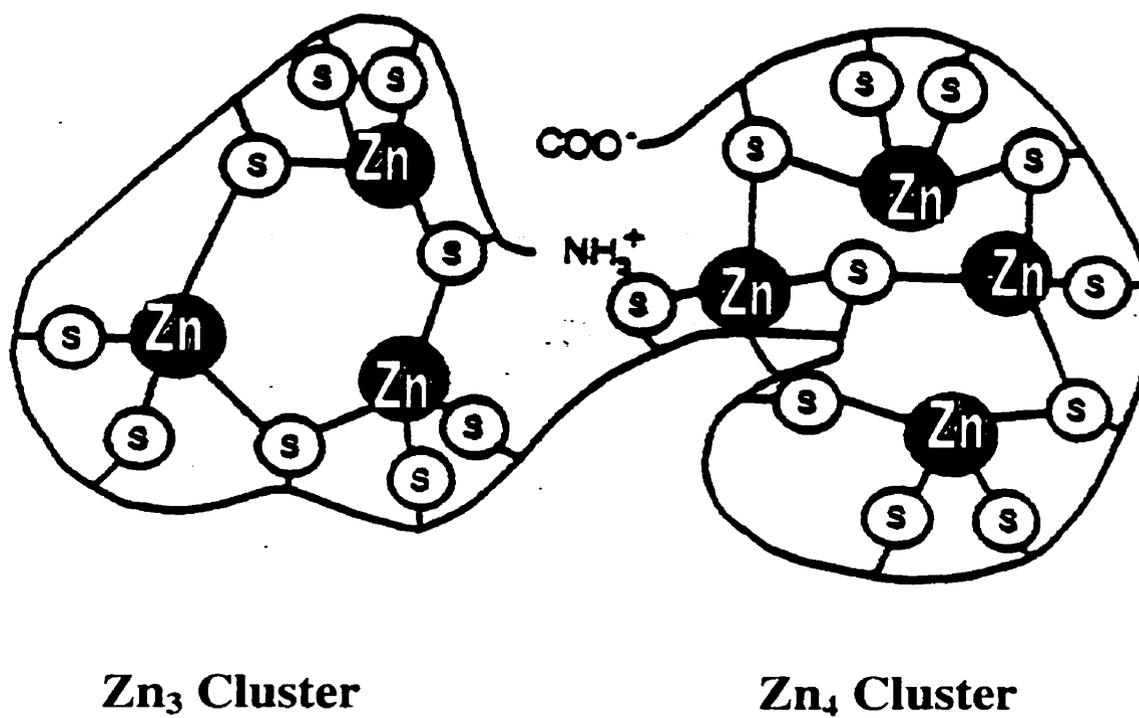
In summary, these studies indicate that zinc is a permissive factor for the cell cycle progression from G<sub>1</sub> to S phase, and that the timing of zinc requirements

corresponds to a period of rapid mRNA and protein synthesis, and precedes or parallels the induction of enzymes required for DNA synthesis.

## **Metallothionein**

MT is a small protein with molecular weight of 6000 - 7000 daltons, composed of single polypeptide chain of 60 - 61 amino acids (Kägi and Schäffer; 1988, Richards, 1989; Kägi, 1991). It can be found in almost all species of animal kingdom and is present in varying amounts in nearly all vertebrate tissues. The unusual amino acid composition and extraordinary metal content make MT unique among all other naturally occurring metalloproteins. One third of MT residues are cysteines, and the protein lacks aromatic and heterocyclic amino acids, histidine residues and disulfide bonds. MT can bind nutritionally essential elements such as zinc and copper, as well as potentially toxic metals including Cd, Pb and Hg. There are 7-g atoms per mole of protein molecule, and all metal ions are coordinated to cysteine residues forming a binuclear metal cluster. The structure of Zn<sub>7</sub>-MT is shown in Figure 1.

It has been 40 years since MT was discovered but its biological function still remains the subject of debate. Establishing a specific biological role for MT is complicated by its extensive polymorphism, the presence of multiple metaloforms of the protein, and the existence of numerous MT inducers (Dunn et al, 1987; Kägi and Schäffer, 1988; Vallee, 1991). Synthesis of the protein can be induced in vitro and in vivo by such factors as heavy metals, hormones, cytokines, cytotoxic agents, and physical and chemical stress including starvation, infection, inflammation, X- and UV-radiation (Danielson et al, 1982; Heilmaier and Summers, 1985; Waalkes and Klaassen, 1985;



**Figure 1.** Zinc thiolate cluster of metallothionein (Kägi and Kojima, 1987).

Cousins et al, 1986; Sato et al, 1984; Templeton and Cherian, 1991; Kägi, 1991).

Therefore, MT has been implicated to play a role in such diverse processes as detoxification of heavy metals, protection against radiation and free radical damage, cellular defense, and regulation of zinc and copper metabolism. The properties of MT and its potential functions have been reviewed in detail by many authors (Dunn et al, 1987; Kägi and Schäffer, 1988; Richards, 1989; Vallee and Auld, 1990; Bremner, 1991; Kägi, 1991; Vallee, 1991). This thesis will focus on the role of MT in cell proliferation.

### ***Role of Metallothionein in Cell Proliferation***

Evidence for MT involvement in cell proliferation comes from three different areas of research: developmental studies, cancer research and cell cycle studies.

#### ***i. Developmental Studies***

The developmental differences in tissue MT synthesis and its cellular localization have been observed in both human and rodents. MT levels are high during fetal and neonatal periods, and MT is present in both cytoplasm and nuclei of cells from fetal and neonatal tissues (Narthey et al, 1987; Nishimura et al, 1989; Cherian, 1994). A dramatic decrease in MT concentration occurs with age, and MT becomes mainly cytoplasmic in adult tissues (Narthey et al, 1987; Nishimura et al, 1989; Cherian, 1994).

Nishimura et al (1989) examined the role of MT in developmental processes using an immunofluorescent technique that employed antibody against MT-1. The researchers studied MT localization in liver, kidney and small intestine from rat fetuses and neonates age 4 - 27 days. They observed pronounced changes in MT staining with

development in all organs examined. In liver, the most intense immunofluorescence was present in both cytoplasm and nuclei of fetal hepatocytes and those of 4 day old rats. By Day 18, the intensity of MT staining in cytoplasm decreased and nuclear staining disappeared. Almost no MT staining was present by Day 27. MT was detectable again in the cytoplasm of many hepatocytes and the nucleus of some hepatocytes of 2 month old rats but the staining was weak. Some MT staining was noted in bile canaliculi. Similar age-related changes in intensity of MT staining and its intracellular redistribution occurred in kidney and small intestine. In all organs, MT staining diminished by Day 18 when the growth pattern in rat begins to change from an increase in cell number to an increase in cell size. The changes in intensity of staining were paralleled by translocation of MT from nuclei (Day 4) to cytoplasm (Day 18). In kidney, staining was observed predominantly in epithelial cells of renal proximal convoluted tubules. The pattern and timing of MT distribution in kidney reflected the events occurring during rat kidney nephrogenesis indicating the involvement of MT in cellular differentiation. For example, strong staining was noted in the developing straight portion of renal tubules while well differentiated proximal convoluted tubules showed little immunoreactivity. In addition, MT staining disappeared on Day 27 when nephrogenesis of rat kidney is completed. In small intestine, MT was localized in surface epithelial cells of villi, goblet cells and Paneth cells. It was concluded that the observed changes in MT during development support its role in cell proliferation. The authors also suggested that the presence of MT in Paneth and goblet cells as well as in bile canaliculi indicates the role of MT in zinc storage, transport and secretion.

Similarly, Narthey et al (1987) found high concentrations of MT in liver and kidney from human fetuses and neonates as measured by the Cd-hemoglobin assay. The levels of MT in fetal liver were 3 times higher than the levels observed in adult liver. Further, using a MT antibody and peroxidase–antiperoxidase technique the researchers demonstrated nuclear and cytoplasmic MT staining in fetal and neonatal hepatocytes but mainly cytoplasmic staining in adult hepatocytes. Similarly, MT in fetal and neonatal kidney was localized in nuclei and cytoplasm of renal proximal tubular cells but predominantly in the cytoplasm of adult kidney. In addition, MT was detected in the lumen of renal proximal tubules.

Developmental changes in hepatic MT concentration and immunolocalization similar to the changes seen in rat and human were also observed in mice (Lau, 1998).

Consistent with the role of MT in cell proliferation, increased MT expression has been also demonstrated in cells during tissue regeneration. Nishimura (1989) found strong cytoplasmic and nuclear MT staining in rat liver after partial hepatectomy. The author noted that MT appeared in nuclei of hepatocytes as early as 12 hours after hepatectomy, and suggested that MT might play an important role in early stages of cell proliferation. Similarly, Tsujikawa et al (1991) reported intense MT staining in nuclei of primary cultured rat hepatocytes after 70% hepatectomy.

## *ii. Cancer Studies*

MT overexpression has been observed in many types of human tumors. Immunochemical studies, as reviewed by Cherian (1994), demonstrated nuclear and cytoplasmic MT localization in certain types of both benign and malignant tumors

including thyroid tumors, testicular germ cell carcinomas, bladder transitional cell carcinomas and salivary gland tumors. The studies indicate that MT is mainly expressed in epithelial cells of tumors, and especially on their proliferating edge. The most intense MT staining is found on the proliferating edge of malignant tumors. In addition, non-differentiated tumors show intense MT staining as compared with little or no MT staining in well differentiated tumors. In their review on the significance of MT overexpression in human tumors, Jasani and Schmid (1997) suggested a relationship between MT expression and invasiveness of certain tumors. They also proposed that MT may play a role in metastatic transformations.

### *iii. Cell Cycle Studies*

Cell cycle studies indicate that changes in MT expression and MT intracellular localization may be cell cycle-specific. Using immunocytochemical techniques, Nagel and Vallee (1995) demonstrated oscillations in MT-1 immunoreexpression during the cell cycle of human colonic cancer cells (HT-29). In  $G_1$ -synchronized continually dividing HT-29 cells, cytoplasmic MT reached a maximum in successive late  $G_1$  phases and at  $G_1/S$  transitions. No nuclear MT staining was observed. On the other hand, Tsujikawa et al (1991) observed cell-cycle specific nuclear translocation of MT in growing primary cultured adult rat hepatocytes stimulated from quiescence ( $G_0$ ) by growth factors. They found cytoplasmic MT staining during  $G_0$  and  $G_1$  phases and nuclear staining in early S phase. The different findings of the two studies may be due to cell type specific MT expression or the time lag in cells entering the cell cycle from  $G_0$  as opposed to exponentially growing  $G_1$  cells.

In summary, the enhanced synthesis of MT and its nuclear translocation in rapidly dividing normal, regenerating and cancer cells implies a role of MT in cell proliferation. Cell-cycle-specific MT expression and intracellular redistribution indicate that MT may be important during early stages of the cell cycle.

## **Metallothionein, Zinc and Cell Proliferation**

### ***Metallothionein and Zinc Metabolism***

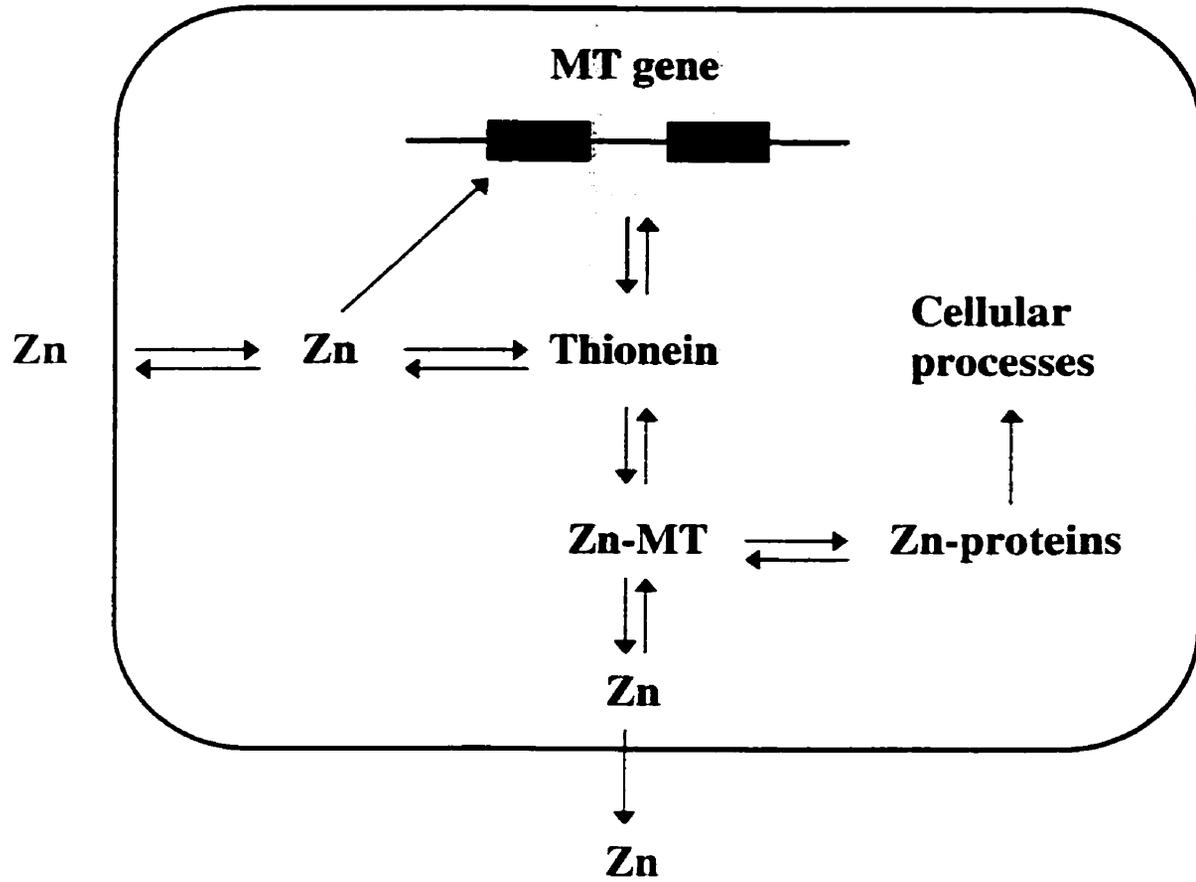
Several observations lead to the idea of a MT role in the regulation of zinc metabolism. MT is present at basal levels in all major mammalian organs, and its synthesis can be induced by heavy metals in the majority of rat tissues (Danielson, 1982; Heilmair and Summer, 1985; Waalkes and Klassen, 1985). However, zinc has been shown to be the most effective inducer of MT among other metals tested, especially in pancreas, liver, intestine and kidney indicating specific roles of MT in zinc absorption, storage and excretion. Presence of MT in body fluids such as plasma and pancreatic juices may suggest a role for MT in metal transport between tissues. While the induction of MT by other heavy metals was observed after IP or IV injections of high nonphysiological metal doses, zinc can increase MT synthesis when administered in physiological doses via the dietary route. Increased concentrations of tissue MT in response to zinc are accompanied by concurrent increases in tissue zinc concentrations (Waalkes and Klaassen, 1985). Further, the native protein is usually isolated as Zn<sub>7</sub>-MT or less frequently as Zn,Cu-MT.

### ***Regulation of Zinc Homeostasis***

The labile pool of zinc in a cell is very small accounting for only about 1 - 2% of total cellular zinc implicating the existence of zinc-storage protein (Vallee and Falchuk, 1993). High metal content, thermodynamic stability and kinetic lability of MT make it a good candidate to serve this function (Kägi and Schäffer, 1988; Kägi, 1991).

A hypothetical model of MT as a regulator of cellular zinc metabolism is presented in Figure 2. Zinc entering a cell from plasma is immediately bound by thionein, the apo-form of the protein, and is either distributed to zinc-metalloproteins and metalloenzymes, or transported for excretion from a cell (Dunn et al. 1987; Richards, 1989; Kägi, 1991). Thus, MT controls cellular zinc uptake, distribution and excretion, and acts as a short-term storage for the metal. The regulation of zinc homeostasis is an autoregulated process since synthesis of thionein is itself induced by zinc (as discussed later).

It can be speculated that by controlling zinc supply to cells and the metal intracellular concentration, MT can regulate the interaction of zinc with cellular proteins. and as such it can potentially modulate any of the zinc-dependent processes in cell proliferation. In fact, several studies have shown that MT may act both as a cellular zinc acceptor and donor. Zaia et al (1998) studied the transfer of zinc from Zn<sub>7</sub>-MT to apo-carbonic anhydrase. The ability of MT to abstract zinc from native zinc-containing forms of TFs was demonstrated for fungal TFIII (Zeng et al, 1991b), Sp1 (Zeng et al, 1991a, Knoepfel et al. 1994) and bacteriophage T4 gene 32 protein (Zaia et al, 1998). Sequestration of zinc from these proteins resulted in loss of their function, and thus, prevented their binding to DNA and transcriptional activation. These effects were



**Figure 2.** Homeostatic regulation of zinc metabolism by metallothionein (adapted from Richards, 1989).

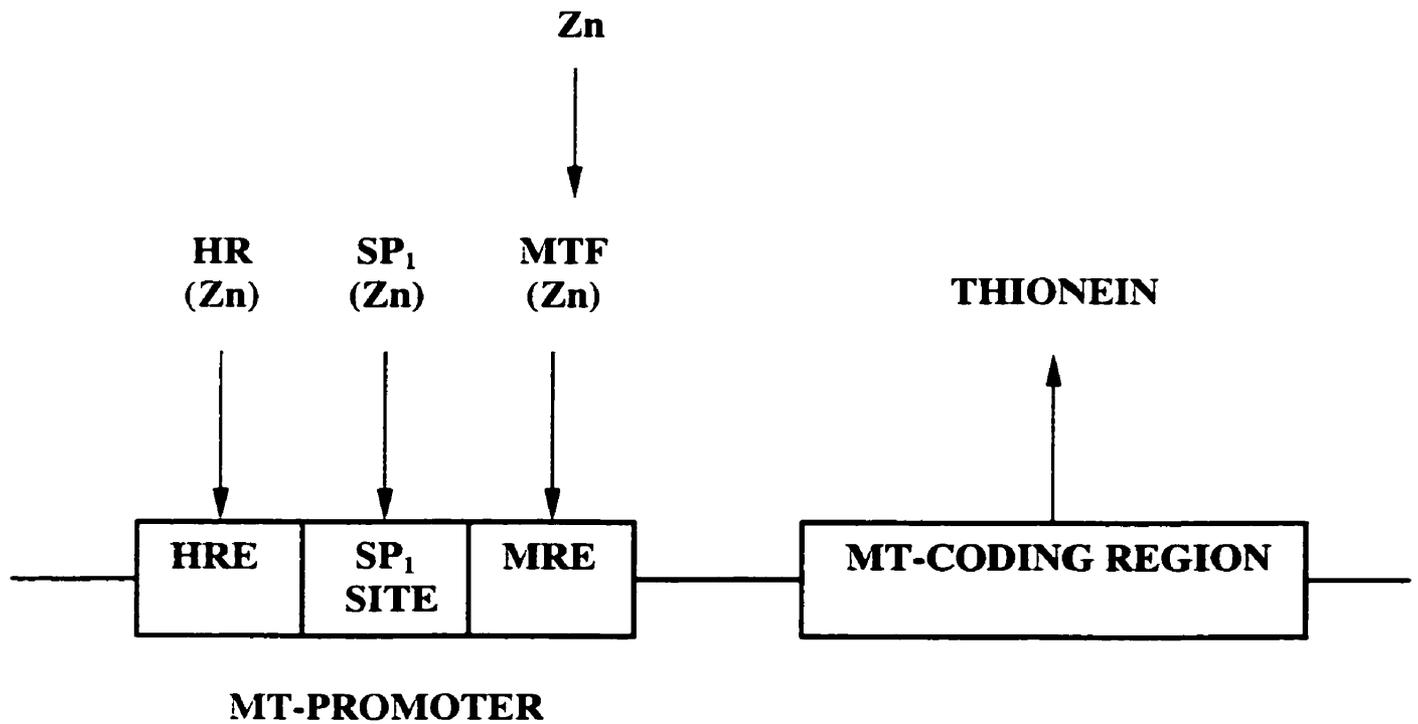
reversed by zinc addition. Several investigators found that MT can be an acceptor or a donor of zinc ions depending on intracellular conditions such as cellular redox and energy state and the presence of other metals (Maret, 1995; Jacob et al, 1998; Jiang et al, 1998).

### ***Regulation of Metallothionein Gene Expression***

The regulation of MT gene expression occurs mainly at the level of transcription initiation (Coleman, 1992; Maret, 1995). Inducers interact with the promoter region of MT gene and activate its transcription. The promoter region of the mammalian MT gene contains several regulatory DNA sequences: steroid response element that binds nuclear hormone receptors, Sp1 binding site that binds TFs of Sp1 'housekeeping' family, and metal response element (MRE) which is regulated by metal-sensitive TFs (MTFs) (Figure 3).

Zinc regulation of MT gene expression involves binding of zinc to MTF which in turn enables MTF to interact with MRE and activate MT transcription (Varshney et al. 1986; Kägi and Schäffer, 1988; Coleman, 1992). Several zinc-proteins that bind to MRE and interact with MT promoter have been recently identified. It has been proposed that by the same mode of action zinc may regulate expression of other genes (Maret, 1995).

Regulation of MT by non-metal inducers involves zinc-dependent TFs as well. This links MT and zinc-dependent processes to different cellular pathways (Dunn and Cousins, 1987; Maret, 1995).



**Figure 3.** Regulation of metallothionein gene expression by zinc and zinc-dependent transcription factors. HRE: hormone response element, HR: hormone receptor, MRE: metal response element, MTF: metal sensitive transcription factor (adapted from Maret, 1995).

In summary, the interactions of MT with zinc-proteins as well as the role of zinc in the regulation of MT gene expression provide a link between MT and zinc-dependent processes in cell proliferation. However, they are supported mostly by in vitro observations and are still highly speculative. Therefore, their relationship to nutrition has yet to be delineated.

### ***Dietary Zinc and Metallothionein***

Dietary zinc has been shown to affect MT concentration in plasma and tissues of growing and adult rats (Bremner, 1991). Zinc status influences MT concentration and its mRNA synthesis in almost all major organs. The protein is particularly sensitive to dietary zinc in organs of absorption, secretion and storage such as liver, kidney and small intestine. Responses of MT to changes in dietary zinc supply are very rapid occurring within the first few days of a dietary treatment. Tissue zinc concentration appears to be a determinant of MT levels in some but not all organs studied (Bremner, 1991). There is an interaction between dietary zinc, MT and zinc-sensitive TFs (Cousins et al, 1994). This interaction can potentially affect zinc-dependent processes in cell proliferation.

Richards and Cousins (1976) investigated the effect of fluctuations in dietary zinc supply on MT synthesis and degradation in adult rat liver and intestine by measuring accumulation and loss of MT-bound zinc. Cytosolic MT was isolated by gel filtration chromatography and the zinc content of MT fraction was determined by zinc absorption spectrophotometry. Rats were fed zinc-deficient (0.8 ppm) or zinc-supplemented diet (150 ppm) on alternating days. The zinc-deficient diet resulted in a significant decrease in hepatic and intestinal zinc concentrations within 24 hours. This was accompanied by a

reduction in MT-bound zinc in these tissues to trace amounts. The repletion with the 150 ppm diet for the next 24 hours significantly increased hepatic and intestinal zinc and MT-bound zinc. Refeeding with the zinc deficient diet significantly decreased both parameters again. In another experiment employing the same dietary treatments and experimental protocol as described above, intestinal and hepatic zinc uptake and MT-bound zinc were determined using  $^{65}\text{Zn}$  tracer. Intestinal MT was inversely related to the  $^{65}\text{Zn}$  absorption rate indicating that intestinal MT is synthesized in response to increases in zinc status. Hepatic  $^{65}\text{Zn}$  uptake was directly correlated with liver MT, and hepatic MT increased in proportion to serum  $^{65}\text{Zn}$  suggesting the uptake and storage function of hepatic MT.

Using radioimmunoassay, Sato et al (1984) found that the plasma and hepatic MT concentration declined to nondetectable levels in weanling rats fed zinc deficient diet (< 1 ppm) for 14 days. The decrease in MT concentration was observed by Day 4 of feeding zinc-deficient diet. By Day 14 of the experiment, plasma zinc levels decreased by 50% in zinc-deficient rats as compared with *ad libitum* and pair-fed controls, but liver zinc concentration was not significantly lower. In pair-fed rats, there was slight increase in plasma MT and significant increase in hepatic MT as compared with controls. In addition, the researchers found that starvation for 24 - 48 hours increased MT in liver by 2 - 3 fold and liver zinc by 20%, and had no effect on plasma zinc and MT.

Other experiments showed that dietary zinc affects not only tissue MT concentration but also MT gene expression. Blalock et al (1988) investigated regulation of MT gene expression by dietary zinc and copper in adult rat liver, kidney, small intestine and brain using 3 x 3 factorial design. Rats were fed diets with 5, 30 and 180

ppm zinc, and 1, 6 and 36 ppm copper for 14 days. MT mRNA was measured by dot blot hybridization. The researchers found that kidney and to a lesser extent intestine were principal organs in which MT mRNA was sensitive to changes in dietary zinc. In kidney, MT mRNA expressed as MT mRNA molecules/cell increased 4- to 5-fold with increasing levels of zinc ( $p < 0.005$ ). Similarly, kidney MT concentration was proportional to dietary zinc levels and it was 50% less in zinc deficient rats than in control and zinc supplemented rats. Copper had a significant effect on kidney MT mRNA but it was less pronounced. In intestine, an increase in MT mRNA occurred only at the highest zinc levels and lowest copper levels ( $p < 0.005$ ) indicating that zinc is a more potent stimulator of MT transcription in intestine at lower copper levels. The increase in intestinal MT mRNA was 7-fold greater than in the group with high supplemental zinc and low dietary copper. Liver MT mRNA increased slightly only when adequate and supplemental levels of zinc were fed (one fold increase,  $p < 0.05$ ) but liver MT concentration was not affected by dietary zinc. Zinc and copper concentration in plasma changed in accordance with changes in levels of the metals in diet with lowest levels of plasma zinc and copper seen in 5 ppm and 1 ppm diet groups, respectively. Brain MT was not affected by the dietary treatments.

Using Northern blot analysis, Cousins et al (1994) found a dose-dependent relationship between dietary zinc and MT mRNA in kidney, liver, intestine, spleen and heart from rats fed diets containing 5, 50 and 180 ppm zinc. The most dramatic increase was observed in kidney, liver and intestine. MT mRNA in thymus and lung did not respond to changes in dietary zinc levels. The researchers further examined the potential mechanisms for dietary zinc control of MT expression. The effect of varying levels of

dietary zinc on MT mRNA, tissue and nuclear zinc uptake, and nuclear distribution of zinc was investigated. The same treatment diets were fed for a 2-hour period except that diets were labeled with  $^{65}\text{Zn}$  tracer. The researchers found a dose-dependent relationship between dietary zinc intake and MT mRNA, and  $^{65}\text{Zn}$  acquired by tissues and nuclear zinc uptake. The greatest effect in MT mRNA was again seen in kidney, liver and small intestine. Tissue uptake of zinc increased in all tissues tested and there was significant direct correlation between dietary zinc level and  $^{65}\text{Zn}$  recovered in tissue ( $p < 0.05$ ). Intestine and liver took up the greatest portion of zinc. There was dose-dependent nuclear  $^{65}\text{Zn}$  accumulation in liver, kidney and spleen ( $p < 0.05$ ). Liver nuclei accumulated the greatest amount of  $^{65}\text{Zn}$ . To examine dietary zinc interactions with intranuclear factors that may be responsible for regulation of MT expression, the authors first isolated MT by chromatographic techniques. Next, they used Western and Southwestern blotting methods to analyze MT fractions for their ability to bind  $^{109}\text{Cd}$ ,  $^{65}\text{Zn}$ , and  $^{32}\text{P}$ -labeled probe for the MRE-coding sequence of MT gene. They found that the binding of  $^{109}\text{Cd}$ ,  $^{65}\text{Zn}$  and  $^{32}\text{P}$ -labeled MRE probe coincided with 2 discrete fractions from nuclear extracts. They concluded that dietary zinc could interact with MRE-binding TF and regulate MT gene expression, and proposed that in the same way dietary zinc might regulate transcription of other genes.

### ***Dietary Zinc, Metallothionein and Development***

The effect of dietary zinc on tissue MT is also observed in the maternal-fetal complex. When maternal rats are given low zinc diets during pregnancy or lactation,

liver MT and mRNA in pups decrease in line with decreased tissue zinc content (Gallant and Cherian, 1987).

In the cross-fostering experiment by Gallant and Cherian (1987), zinc deficient pups were suckled from zinc sufficient dams and vice versa. Both gestational and postpartum zinc deficiency caused significant decreases in hepatic MT and zinc concentrations ( $p < 0.05$ ) postpartum as determined by the  $^{109}\text{Cd}$ -binding MT assay and atomic absorption spectrophotometry. At birth, zinc and MT concentrations in the livers of pups from zinc-deficient dams were 50% lower than those seen in livers of control pups. Hepatic MT and zinc increased with zinc repletion until day 10 postpartum but did not reach the levels observed in control rats. At Day 22 postpartum, liver zinc and MT concentrations of zinc deficient and zinc repleted rats were significantly lower from than controls but were not different from each other, indicating that postpartum zinc repletion may not compensate for gestational zinc deficiency. Consistent with this finding, both gestational and postnatal zinc deficiency resulted in impaired growth. There was increased retention of  $^{65}\text{Zn}$  in all organs of zinc deficient rats and accelerated degradation of hepatic MT. The authors suggested that degradation of liver MT in zinc deficient pups maintains zinc levels in other tissues. This study supports the role of MT in hepatic zinc storage during development, and indicates that during zinc deficiency, liver MT is degraded faster to supply zinc that is distributed by liver to other tissues to maintain normal physiological processes and growth.

A close relationship between tissue zinc and MT during development was also demonstrated in transgenic mice that overexpress MT-1. These mice have 56 copies of the MT-1 transgene, and consequently, accumulate 20 times more MT in pancreas, 16

times more in liver, and 5 times more in intestine and kidney as compared with normal controls. Accordingly, they have pancreatic and liver zinc concentrations increased by 300% and 50%, respectively, and slightly increased zinc in kidney and intestine. In contrast, the copper concentration in liver and pancreas of transgenic mice is similar to normal controls (Iszard et al, 1995). Dalton et al (1996) examined the effect of zinc deficiency on pregnancy outcome in transgenic and control mice that were fed zinc deficient (0.5-1.5 ppm zinc) or zinc adequate (50 ppm zinc) diets for 2 weeks starting at Day 1 of gestation. The authors found that teratogenic effects of zinc deficiency were markedly reduced in fetuses of transgenic mice as compared with normal controls. The fetuses from zinc deficient transgenic mice had significantly lower resorption rates ( $p < 0.001$ ) than those from zinc deficient controls (4% vs. 60%, respectively). In addition, none of the fetuses from transgenic females were morphologically abnormal while 55% of the surviving fetuses from control females were malformed. Neither transgenic nor control mice were able to complete pregnancy. The pancreatic MT concentration of both transgenic and control zinc deficient mice decreased by 99.8% and 98%, respectively. A similar decrease was observed in pancreatic MT mRNA. The study demonstrates that MT overexpression protects against teratogenic effects of zinc deficiency, and the authors suggested that a larger maternal pool of zinc in the transgenic mice allows fetal development to progress normally for longer periods of time. Further, the study shows that pancreatic MT is very sensitive to dietary zinc during pregnancy.

## Summary

The review of current literature indicates that there is a relationship between zinc, MT and cell proliferation. The role of zinc in growth and development is well established (Vallee and Falchuk, 1993). Growth retardation is the main manifestation of zinc deficiency in developing mammals. In cell cultures, cell growth and proliferation depend on the availability of zinc in a culture medium. Biochemical and molecular mechanisms underlying the involvement of zinc in cell growth and proliferation are not fully understood. However, it is well documented that numerous cellular proteins involved in proliferative processes require zinc for proper functioning (Vallee and Auld, 1990; Coleman, 1992). Studies show that MT may regulate the interaction of zinc with these proteins (Zeng et al. 1991a; Zeng et al, 1991b; Knoepfel et al. 1994; Maret, 1995; Jakob et al, 1998; Jiang et al 1998; Zaia, 1998), and as such, it may potentially modulate zinc dependent processes in cell proliferation. Increased concentrations of MT in tissues at times of active growth and development, and its presence in cytoplasm and nuclei of rapidly proliferating cells supports the hypothesis for MT involvement in cell proliferation. Further, MT gene expression is regulated by zinc and zinc-proteins, and studies indicate that there is an interaction between dietary zinc and a zinc-sensitive TF that binds to MRE on the MT gene promoter (Coleman, 1992, Cousins and Lee-Ambrose, 1992). Consistent with these findings, zinc status has been shown to affect MT concentration and its mRNA synthesis in various animal tissues (Sato et al, 1984; Blalock et al, 1988; Bremner, 1991; Cousins and Lee-Ambrose, 1992). Finally, in cell cultures, the timing of zinc requirements and increased MT synthesis appear to be cell cycle

specific and to coincide with each other (Chesters et al, 1989; Tsujikawa, 1991; Nagel and Vallee, 1995).

Although the current literature provides some evidence for the relationship between zinc, MT and cell proliferation, there are no studies available that relate zinc nutrition and MT status to cell cycle activity. Further, the available literature lacks information about the effects of dietary zinc on MT tissue distribution. Previous immunohistochemical studies investigated tissue MT localization in relation to development. Finally, the knowledge about the effects of short-term zinc repletion on MT status, MT tissue distribution, and cell cycle activity is limited. Thus, this thesis further explores the interactions between dietary zinc and MT by examining the effects of dietary zinc on tissue and intracellular MT distribution, and investigates whether these interactions are directly reflected in cell cycle alterations.

## II. Study Rationale

Zinc deficiency results in growth retardation in human and experimental animals, and a lack of zinc in culture systems prevents cell replication. By controlling zinc homeostasis, MT may regulate the interactions of zinc with zinc-proteins, and thus, it may modulate zinc-dependent processes in cell proliferation. In experimental animals, changes in dietary zinc intake influence tissue MT concentration and its mRNA synthesis, and there is an interaction between dietary zinc, MT and zinc-sensitive TFs.

The hypothesis of this study is that MT tissue concentration and distribution change in response to dietary zinc intake, and that these changes are reflected in cell cycle activity. The objective of this study was to investigate the effects of dietary zinc deficiency and 24 hour zinc repletion on MT concentration, and MT and Ki-67 immunohistochemical localization in rat liver, kidney and small intestine. To meet our objective, zinc deficiency was produced in 3-week old rats by feeding them zinc deficient diet for 16 days. Weanling rats were selected for this experiment as they undergo rapid growth and development, and thus, potential cell cycle alterations are expected to be more pronounced in their tissues as opposed to tissues of adult rats. In a rat, the growth pattern begins to change from an increase in cell number to an increase in cell growth at approximately 3 weeks of age, and the development of kidney and small intestine is not complete until 4 weeks of age. Therefore, the initiation of dietary treatment at 3 weeks of age may interfere with the organ development and the achievement of maximal cell number. The rats were subjected to zinc deficient diet containing 1 ppm zinc for 16 days as this level of zinc fed for the duration of approximately 2 weeks has been shown

previously to induce severe zinc deficiency in rats. After the zinc deficiency phase, the rats were repleted with zinc-adequate diet for 24 hours. We felt that the 24 hour period was an appropriate time to account for digestion and zinc absorption, and to allow for MT synthesis to occur in response to increased zinc supply. Similarly, we felt this time to be efficient to reverse the potential cell cycle alterations due to zinc deficiency. The pair-fed groups were included in the study to control for calorie depletion that occurs in zinc deficient animals as a result of anorexia. The effects of dietary zinc on MT were studied in small intestine, liver and kidney. MT in these tissues has been shown to be sensitive to zinc status, and the choice of the organs of absorption, storage and excretion allows for the investigation of the role of MT in zinc metabolism. In addition, since intestinal mucosa is comprised of constantly proliferating cells, the intestine is an ideal tissue for studying the effects of zinc on cell cycle activity.

Previous studies that examined the relationship between zinc status and MT assessed MT concentration and its mRNA expression. In addition to quantitative measurements of MT, this study investigated MT tissue and intracellular distribution in response to dietary zinc manipulations using immunohistochemical techniques. Immunohistochemistry allows for visualization of MT distribution in tissues, and may provide important information about cell type-specific responses of MT to dietary zinc.

Other studies investigating the relationship between dietary zinc and MT focused on MT regulation of zinc metabolism. Despite substantial evidence about the role of zinc and MT in cell proliferation, no attempt has been made to explore the interactions between dietary zinc and MT in relation to cell cycle activity. In this study, the immunolocalization of Ki-67 nuclear antigen was assessed as an indicator of cell cycle

activity. Ki-67 was selected over PCNA, another cell cycle associated marker, since the latter has been shown to be expressed in quiescent cells that have recently left the cell cycle. Ki-67 is expressed exclusively by cycling cells, and thus, provides a more accurate estimation of populations of cycling cells.

### III. Materials and Methods

#### Materials

Citric acid, CdCl<sub>2</sub>, Tris-HCl, hematoxylin, bovine hemoglobin and bovine serum albumin were purchased from Sigma (St.Louis, MO). NaCl, Na<sub>2</sub>HPO<sub>4</sub> • 7H<sub>2</sub>O, NaH<sub>2</sub>PO<sub>4</sub> • H<sub>2</sub>O, reagent alcohol, hydrogen peroxide, formalin, xylene, eosin and Permunt were purchased from Fisher (Fair Lawn, NJ). <sup>109</sup>Cd was purchased from New England Nuclear Life Science Products Inc. (Boston, MA). Sources for all other reagents and materials are specified in the text.

#### Animals and Diets

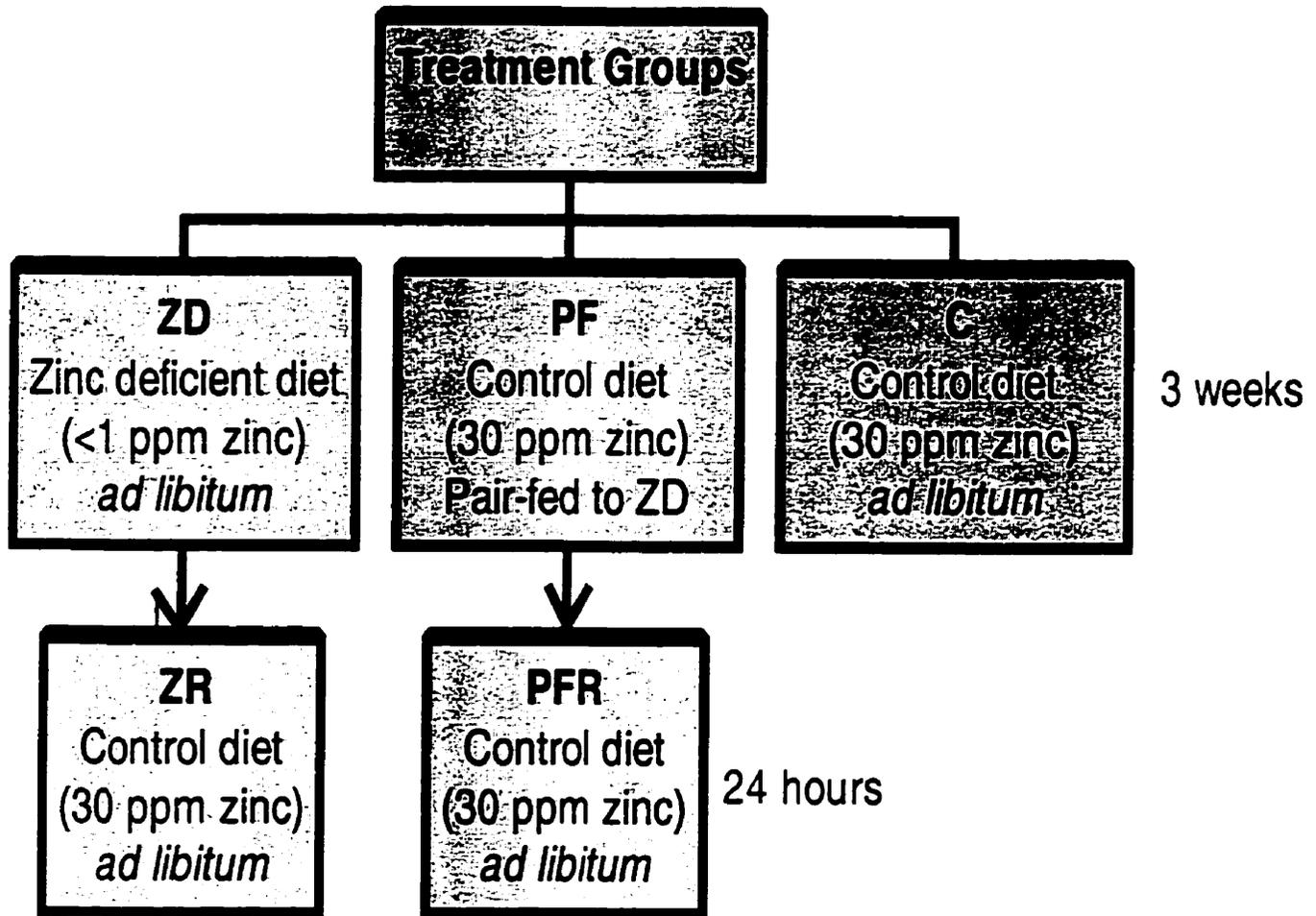
Forty weanling male Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were randomized into treatment groups and maintained in a temperature (21-23<sup>0</sup>C) and humidity (55%) controlled room with lights on from 0700 to 1900 hours. After a three-day acclimatization period to nutritionally complete control diet, rats were fed zinc deficient (ZD group; <1 ppm zinc) and control (30 ppm zinc) diet, either pair-fed to the zinc deficient group (PF group) or fed *ad libitum* (C group). The PF rats were given the amount of feed that was consumed by their ZD counterparts on the previous day. The PF group served as a control for potentially confounding effects of voluntary feed withdrawal and calorie depletion secondary to zinc deficiency. The experimental diets were based on the AIN-93G formulation (Reeves, 1993) and the formulation of the diets is provided in Table 1. Rats received their respective diets for a 16-day period. After 16 days, the C group and half of the rats from ZD and PF groups were terminated.

The remaining ZD and PF rats were repleted with the control diet *ad libitum* (ZR and PFR groups, respectively) for an additional 24 hours (see Figure 4). After the repletion trial, the ZR and PFR rats were terminated. Body weights were obtained at the start, every four days, and at the conclusion of the study as a part of growth assessment. The animal care protocol for this study was approved by the University of Manitoba Protocol and Management Committee.

During the course of experiment, precautions were taken to avoid zinc recycling and contamination. Animals were housed individually in stainless steel hanging cages with wire bottoms. They were allowed free access to double deionized water provided in plastic bottles with stainless steel sipper tubes. The ZD group was located on the top row of the cage rack. In addition, care was taken to prevent cross-contamination of the diets during diet preparation and during feeding times. Zinc content of the diets was verified as described under the method for zinc analysis.

### **Tissue Collection and Storage**

At the end of the 16-day experiment and after the 24-hour repletion trial, rats were terminated by CO<sub>2</sub> asphyxiation in accordance with the Canadian Council on Animal Care guidelines (1993). Body weights, body lengths (from nose to anus) and tail lengths (from anus to tip of tail) were recorded, and trunk blood was collected after decapitation. Blood samples were kept on ice until centrifuged (Beckman TJ-6R centrifuge) at 1290 x g for 15 minutes to obtain serum. The femurs, liver, kidneys and small intestine were dissected. The organs were weighed and the length of intestine was measured from pylorus to rectum. Samples of liver, kidney and small intestine (mid- section) were then



**Figure 4. Experimental Design.** Forty rats were randomly assigned to three treatment groups, and were fed zinc deficient (ZD group) or control diet, either pair-fed to ZD group (PF group) or fed *ad libitum* (C group) for 16 days. Initially, there were 16 rats in both ZD and PF groups and 8 rats in C group. After 16 days, the C group and half of the rats from ZD and PF groups were terminated, while the remaining ZD and PF rats were repleted with control diet *ad libitum* (ZR and PFR, respectively) for an additional 24 hours. After the repletion phase, ZR and PFR rats were terminated.

**Table 1. Diet Formulation (g/kg)**

<b>Ingredients <sup>1</sup></b>	<b>Zinc deficient diet (&lt;1 ppm zinc)</b>	<b>Control diet (30 ppm zinc)</b>
Dextrose	613.6	603.6
Egg White	212.5	212.5
Soybean Oil	70	70
Cellulose	50	50
Mineral Mix (AIN-93M: zinc-free)	35	35
Vitamin Mix (AIN-93)	10	10
Potassium Phosphate	5.4	5.4
Choline Bitartrate	2.5	2.5
Biotin Mix <sup>2</sup>	1	1
Zinc Premix <sup>3</sup>	---	10

<sup>1</sup> Diet ingredients were purchased from Harlan Teklad (Madison, WI) with the exception of dextrose (Moonshiners, Winnipeg, MB), soybean oil (Vita Health, Winnipeg, MB) and potassium phosphate (Fisher Scientific, Fair Lawn, NJ)

<sup>2</sup> Biotin Mix (200 mg biotin/1000 g dextrose)

<sup>3</sup> Zinc Premix (5.775 g zinc carbonate/1000 g dextrose)

excised. After brief rinsing with phosphate buffered saline (PBS, 0.15 M NaCl, 10 mM phosphate; pH 7.4) to remove surface blood and intestinal contents, the tissue samples were fixed in 10% buffered formalin for 24 to 48 hours in preparation for immunohistochemical studies. The remaining tissues were frozen in liquid nitrogen. All tissue samples and serum were stored at  $-80^{\circ}\text{C}$ , with the exception of femurs that were stored at  $-20^{\circ}\text{C}$ .

### **Zinc Analysis**

Femur, liver, kidney, small intestine, serum and experimental diets were assessed for zinc using atomic absorption spectrophotometry. Serum samples of 200–400  $\mu\text{l}$  were analyzed directly after 3 to 5-fold dilution in double deionized water. Two blanks containing deionized water were processed with the serum samples. The femurs, organs and diets underwent acid digestion based on the method of Clegg et al (1981).

Preceding digestion, frozen femurs were defrosted and scraped of all adjacent tissue with a scalpel blade. Cleaned bones and the samples of organs were weighed and placed in a drying oven for 72 hours at  $85^{\circ}\text{C}$ . Dried tissues were weighed immediately after removal from the oven, and were put into zinc-free Pyrex test tubes prewashed with 30% nitric acid. One milliliter of concentrated nitric acid (70%, trace element grade; VWR CanLab, Mississauga, ON) was added to each tube and the tissues were allowed to digest at room temperature for 24 hours followed by heating at  $85^{\circ}\text{C}$  for 8 hours in a dry bath heater. Diet samples of 0.2 g were combined with 1 ml of concentrated nitric acid and digested in the same manner. Depending on the dietary treatment group and the dry weights of the tissue samples, digests were diluted with double deionized water as

follows: femur to 25 or 100 ml, liver to 25 ml, kidney to 10 or 25 ml, and small intestine to 10 or 20 ml. Digested diets were diluted 10-fold (zinc deficient diet) or 20-fold (control diet). A bovine liver reference (0.1 g/10 ml; no. 1577B, U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) was processed as a quality control. Two blanks containing nitric acid were also processed with the samples of each tissue and with the diet samples. Zinc concentration of diluted samples was measured using a Varian Spectra AA-30 Spectrophotometer (Georgetown, ON). Zinc standards (0.1-1 ppm) were prepared from zinc atomic absorption standard (1000 ppm. #H595-01 Mallinckrodt, Paris, Kentucky).

### **Zinc Concentration Calculations:**

#### Tissues and diets:

$$\text{Zinc } (\mu\text{g/g dry weight}) = \frac{\text{Sample zinc concentration} \times \text{dilution factor}}{\text{Sample dry weight}}$$

#### Serum:

$$\text{Zinc } (\mu\text{g/ml}) = \text{Sample zinc concentration} \times \text{dilution factor}$$

### **Metallothionein Assay**

MT concentrations in liver, kidney and small intestine were determined by a modified cadmium saturation assay based on the method of Eaton and Toal (1982). In preparation for the assay, tissue samples were weighed and homogenized in 4 volumes of ice-cold 10 mM Tris-HCl buffer (pH 7.4) at 50 rpm for approximately 30 seconds using a Polytron homogenizer (Kinematica GMBH, Luzern, Switzerland). Homogenates (1.5 ml) were then placed in 1.5 ml microfuge tubes and centrifuged (Micromax Centrifuge, International Equipment Company, Needham Heights, MA) at 10,000 g for

20 minutes at 4<sup>0</sup>C. After transferring to fresh tubes, supernatant fractions were heated for 2 minutes in a boiling water bath, followed by cooling on ice for 10 minutes. The cooled samples were centrifuged for 5 minutes at 4<sup>0</sup>C to separate precipitated proteins. The heat-denatured supernate was removed and aliquots of 250  $\mu$ l were placed in fresh tubes. Two hundred fifty microliters of <sup>109</sup>Cd solution (2  $\mu$ g/ml, 1  $\mu$ Ci/ml; carrier free <sup>109</sup>Cd combined with CdCl<sub>2</sub> in Tris-HCl buffer) was added to each tube, and after careful vortexing, the samples were incubated for 10 minutes at room temperature. To remove unbound cadmium, 125  $\mu$ l of 2% bovine hemoglobin solution was added to the tubes, mixed by vortexing and heated in a boiling water bath for 2 minutes; this was followed by cooling on ice for 10 minutes and centrifuging for 5 minutes. The supernatant fraction was again transferred to clean tubes, and another 125  $\mu$ l aliquot of 2% hemoglobin was added. The heating, cooling and centrifugation of the samples were repeated. Five hundred microliters of the final supernate was removed, placed in a clean polypropylene tube and counted in a gamma counter (Canberra-Packard Canada Ltd., Mississauga, ON). Tubes for assay blanks (buffer in place of a tissue sample) and total counts (buffer in place of a tissue sample; no hemoglobin treatment) were included in each assay. All samples were processed in triplicate.

### **Calculations:**

The concentration of MT (cadmium-binding potential) in a sample was calculated accordingly:

$$\text{nmol Cd bound/ml} = (\text{Cts}_S - \text{Cts}_B) \times (17.8/\text{Cts}_T) \times \text{dilution factor}$$

where Cts<sub>S</sub> stands for the counts per minute in a sample; Cts<sub>B</sub>, counts per minute in an assay blank; and Cts<sub>T</sub>, counts per minute in a total count sample. The factor of 17.8 was

obtained by dividing the grams of cadmium in a sample (0.5  $\mu\text{g}$ ) by the atomic weight of cadmium (0.1124  $\mu\text{g}/\text{nmol}$ ) and the MT sample size (250  $\mu\text{l}$ ) to give units of nanomoles of cadmium per 1 ml of sample. The value calculated from the above equation corresponds to micrograms of MT assuming that 7-g atoms of cadmium bind to a mole of MT, and that the molecular weight of Cd-MT is 7000 (Eaton and Toal, 1982).

MT concentration was expressed per gram tissue wet weight as well as per gram tissue protein. Protein tissue concentration was determined as described below under protein assay.

### **Calculations:**

$$\text{MT } (\mu\text{g}/\text{g wet weight}) = \frac{\text{Sample MT concentration} \times \text{dilution factor (4)}}{\text{Sample wet weight}}$$

$$\text{MT } (\mu\text{g}/\text{mg protein}) = \frac{\text{Sample MT concentration}}{\text{Sample protein concentration}}$$

### **Protein Assay**

Aliquots of the liver, kidney and small intestine homogenates used in the MT assay were analyzed for protein concentration using bicinchoninic acid (BCA) protein assay method (Pierce, Rockford, IL). The method employs principles of the biuret reaction (Lowry, 1951) with the addition of BCA to enhance reaction product.

Prior to the assay procedure, the tissues were homogenized in the manner described earlier under MT assay, and homogenates were diluted with 10 mM TRIS buffer (pH 7.4). Fifty-fold diluted homogenates of kidney and small intestine, and 100-fold diluted homogenates of liver were used. To establish a standard curve, a set of protein standards (0.0625 mg/ml – 1.0 mg/ml) was prepared from bovine serum albumin

(1.0 mg/ml) in TRIS buffer as a diluent. Ten microliters of samples, standards and buffer blanks were pipeted in triplicate into 96-well polystyrene microtiter plate wells (# 25880-96, Corning Glass Works, Corning, NY). Two hundred microliters of the BCA working reagent was then added to the wells using a multi-channel pipet. The samples were shaken for 5 seconds by the SpectraMax microtiter plate reader (Molecular Devices Corporation, Sunnyvale, CA), and incubated for 30 minutes at 37<sup>0</sup>C. Absorbances were read at 562 nm on a SpectraMax 340 spectrophotometer. The Softmax Pro Microplate Analysis Software was used to calculate concentrations of the unknowns.

**Protein Concentration Calculation:**

$$\text{Protein (mg /ml)} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of standard} - \text{Absorbance of blank}} \times \text{Conc. of standard}$$

**Immunohistochemical Localization of MT**

MT localization in liver, kidney and small intestine was studied using the indirect immunoperoxidase technique. The formalin-fixed tissue samples were embedded in paraffin, cut into 5 μm sections and mounted on fixative-coated slides by the Pathology Lab, Health Sciences Center (Winnipeg, MB). For each rat, serial sections were obtained for the comparison of MT staining and Ki-67 staining (described under the Immunohistochemical Localization of Ki-67 section). After deparaffinization in xylene and rehydration in a graded series of ethanol to water, the endogenous peroxidase activity in the tissues was inhibited by immersing slides in 3% hydrogen peroxide for 10 minutes. The reaction was stopped by placing the slides in PBS (0.15 M NaCl, 10 mM phosphate; pH 7.2) bath for 5 minutes. The sections were then treated with 20% normal goat serum (DAKO Corporation, Carpinteria, CA) for 1 hour at room temperature to block

nonspecific binding sites. After blocking, monoclonal mouse anti-MT antibody (clone E9; DAKO) was diluted in PBS (1:50 for liver and small intestine, and 1:100 for kidney), applied to the sections (250  $\mu$ l diluted antibody per slide) and incubated for 1 hour at room temperature (small intestine) or overnight at 4<sup>0</sup>C (liver and kidney) in a humid tray chamber. The sections were then layered with prediluted DAKO Envision System goat anti-mouse/anti-rabbit peroxidase labeled polymer (250  $\mu$ l solution per slide) and allowed to incubate for 15 (kidney), 30 (small intestine) or 45 (liver) minutes at room temperature in a humid tray chamber. The sections were washed with fresh PBS, followed by a 5-minute PBS bath after the incubations with the primary and secondary antibodies. The reaction product was visualized by treating the sections with 5 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB•4HCl; Polysciences, Inc., Warrington, PA) in 10 ml PBS containing 30  $\mu$ l of 3% hydrogen peroxide for 5 minutes at room temperature. The unreacted DAB was briefly washed off with distilled water and the tissues were counterstained with hematoxylin for 15 seconds. The tissues were then rinsed with tap water, dehydrated in graded concentrations of ethanol, cleared in xylene, and mounted with cover slips by using Permount. The small intestine was additionally counterstained with eosin for easier identification of Paneth cells. The specificity of the reaction was confirmed under three control conditions: (1) substitution of anti-MT antibody with mouse IgG1, kappa (clone DAK-GO1; DAKO); (2) omission of anti-MT antibody from the procedure; (3) incubation of the respective tissues from a MT null mouse with anti-MT antibody. The reaction was specific for MT since the negative control procedures gave no staining. The tissue sections from a zinc supplemented (300 ppm zinc diet) rat served as positive controls. Computer images of immunostained sections were obtained

using Northern Eclipse software (Empix Imaging Inc., Toronto, ON). The intensity of MT staining was estimated subjectively at 4 levels: nil, defined as the absence of staining; weak, defined as the staining just visible above the background at lower magnification (10x objective); moderate, defined as the staining easily visible at lower magnification; and strong, defined as the staining easily visible at lowest magnification (2.5x objective). To confirm the identity of Paneth cells, small intestine sections were examined using oil immersion objective.

### **Immunohistochemical Localization of Ki67**

Ki-67 nuclear antigen localization was determined by the same immunohistochemical technique as described previously under immunohistochemical localization of MT. Formalin-fixed, paraffin-embedded sections of liver, kidney and small intestine were processed and stained in a similar manner as the sections stained for MT, with the addition of a heat treatment step to unmask Ki-67 antigen. Briefly, following deparaffinization and rehydration, tissue sections were placed in 10 mM citrate buffer (pH 6.0) and heated in a pressure cooker (DAKO) in a microwave oven (500 watts) on high power for 30 minutes. After a 15 minute cool down at room temperature, the sections were rinsed with PBS and sequentially treated with 20% normal goat serum for 1 hour, monoclonal mouse antibody against Ki-67 nuclear antigen (clone MM1; Novocastra Laboratories) for 1 hour, and DAKO Envision System goat antimouse/antirabbit peroxidase labeled polymer for 10 minutes. Sections of liver and small intestine were incubated with 1:200 dilutions of anti-Ki-67 antibody, while kidney sections were incubated with 1:100 dilutions of the antibody. All incubations were

performed at room temperature. The sections were then treated with DAB•4HCl solution, counterstained with hematoxylin, dehydrated and mounted with Permount. The specificity of reaction was verified by substituting the anti-Ki67 antibody with mouse IgG1, kappa, and by omitting the anti-Ki67 antibody from the procedure. Neonate rat tissue sections were used as positive controls. Computer images were obtained using Northern Eclipse software. The intensity of staining was assessed in the same manner as for MT staining. For liver, Ki-67 positive cells (total number of Ki-67 stained cells) were enumerated in 3 randomly selected areas (approximately 1000 cells) of each tissue section and expressed as a percentage of the total number of nucleated cells. In addition to the total number of Ki-67 stained cells, the number of cells that showed strong Ki-67 staining was determined. The counting was computer-aided using the Northern Eclipse software. The thresholds for total stained cell and strongly stained cell counts and total cell counts were set up subjectively. During counting, hepatocytes were discriminated from other cells based on shape and size. The percentage of a total number of Ki-67 stained cells and the percentage of cells with strong Ki-67 staining were calculated.

**Calculations:**

$$\% \text{ of Ki-67 stained cells} = \frac{\text{Number of stained cells/area}}{\text{Total cell number/area}} \times 100$$

Due to poor morphology, it was not possible to obtain cell counts for small intestine sections, and time constraints did not allow for performing cell counts on kidney sections.

## **Statistical Analysis**

Differences between dietary treatment groups were analyzed by ANOVA using SAS software version 6.04 (SAS institute, Cary, NC). Duncan's multiple range test was used to determine significant differences between treatment group means. Correlation analysis (zinc status versus MT concentration in liver, kidney and small intestine) was conducted using Pearson's correlation coefficient. Repeated measures analysis was performed on body weights. For all analyses, differences were considered significant at  $p < 0.05$ .

## IV. Results

The Results section reports the results for growth and zinc assessments followed by the results for intestine, liver and kidney in separate subsections. Data (mean  $\pm$  SEM) presented in the Figures are also reported in tabular form in Appendix A. The means of ZD, PF and C groups are results obtained at Day 16 of the study, and those of ZR and PFR groups were obtained at Day 17 of the study, unless otherwise specified.

### Growth Assessment

To assess growth of the rats during the zinc deficiency phase of the study, body weights at days 0, 4, 8, 12 and 16 of dietary treatment, and body length, tail length, femur weight and epididymal fat pad weight at termination were determined. To examine the effects of zinc repletion on growth, the body weight, body length, tail length, femur weight and epididymal fat pad weight of ZR and PFR groups were determined on Day 17 of the study. The final body weights of the ZR and PFR groups on Day 17 of the study (after zinc repletion) were compared with their body weights on Day 16 of the study (before zinc repletion), and with the final body weights of ZD, PF and C groups on Day 16 of the study. In addition, the feed intake of ZR and PFR groups on Day 16 of the study (before zinc repletion) was compared with their feed intake on Day 17 of the study (after zinc repletion).

### ***Body Weights During Zinc Deficiency***

Figure 5 depicts body weights of rats at days 0, 4, 8, 12 and 16 of the study, before ZR and PFR groups were repleted with zinc and calorie adequate diets. There were no significant differences in body weight between treatment groups at the start of the experiment. On Day 4, the only significant difference found was between the weights of C and ZR groups ( $87.1 \pm 2.7$  g vs.  $78.1 \pm 1.44$  g, respectively). By Day 8, the C group had significantly higher body weight than the other groups and continued to weigh significantly more until termination. On Day 16 of the study (termination day for ZD, PF and C groups), the mean body weight of C group was almost twice of the weight of ZD and PF groups which were not significantly different from each other ( $183.3 \pm 4.5$  g vs.  $99.3 \pm 7.5$  g and  $104.7 \pm 6.9$  g, respectively). PF and PFR groups weighed significantly more than ZD and ZR groups on Day 8 of the study ( $94.1 \pm 3.1$  g and  $93.9 \pm 2.1$  g vs.  $82.7 \pm 4.6$  g and  $81.6 \pm 1.5$  g, respectively) but this difference disappeared on Day 12. On Day 16, only the PF group weighed significantly more than the ZR group ( $104.7 \pm 6.9$  g vs.  $88.5 \pm 1.5$  g). There were no significant differences in body weight between ZD and ZR groups, and PF and PFR groups at days 4, 8, 12 and 16 of the study.

### ***Body Weight and Feed Intake Before and After Zinc Repletion***

Figure 6 shows the final body weights of the rats after zinc repletion of ZR group and calorie repletion of PFR group (Day 16 for ZD, PF and C groups, and Day 17 for ZR and PFR groups). On Day 17 of the study, after zinc repletion of ZR and PFR groups, the body weight of PFR group was greater but not significantly different from that of PF group on Day 16 ( $119.1 \pm 3.2$  g vs.  $104.7 \pm 6.9$  g), and the body weight of ZR group was

similar to and not significantly different from that of ZD group on Day 16 ( $97.5 \pm 1.5$  g vs.  $99.3 \pm 7.5$  g). While on Day 16 of the study PFR and ZR groups were not significantly different from each other ( $98.5 \pm 2.7$  g vs.  $88.5 \pm 1.5$  g), on Day 17, the PFR group weighed significantly more than ZR group ( $119.1 \pm 3.2$  g vs.  $97.5 \pm 1.5$  g).

Figure 7 depicts body weights (A) and feed intakes (B) of ZR and PFR groups on Day 16 (before zinc repletion) and Day 17 (after zinc repletion) of the study. The body weight and feed intake of ZR group on Day 17 was significantly greater than its body weight and feed intake on Day 16 ( $97.5 \pm 1.47$  g vs.  $88.5 \pm 1.5$  g, and  $14.0 \pm 1.2$  g vs.  $5.9 \pm 1.1$  g, respectively). Similarly, the PFR group had significantly greater body weight and feed intake on Day 17 as compared with Day 16 ( $119.1 \pm 3.2$  g vs.  $98.5 \pm 2.7$  g, and  $20.9 \pm 0.6$  g vs.  $5.9 \pm 1.1$  g, respectively).

### ***Body Length and Tail Length***

Body length and tail length of the rats followed a similar trend to that seen for body weight (Figure 8A and B). The C group had significantly greater body length and tail length than other treatment groups at  $18.7 \pm 0.2$  cm and  $12.8 \pm 0.2$  cm, respectively. There was no significant difference in body length between ZD and PF groups ( $15.7 \pm 0.4$  cm vs.  $15.9 \pm 0.3$  cm) but the body length of ZR group was significantly less than the PFR group ( $15.4 \pm 0.1$  cm vs.  $16.3 \pm 0.2$  cm). The tail length of ZD group was significantly less than the PF group ( $10.8 \pm 0.4$  cm vs.  $11.6 \pm 0.3$  cm) but tail lengths of ZR and PFR groups were not significantly different from each other. There were no significant differences in both parameters between ZD and ZR groups, and PF and PFR groups.

### ***Femur Weight and Femur Weight to Body Weight Ratio***

Femur wet weights and femur wet weight to body weight ratios of the rats are presented in Figure 9A and B. Figure 10A and B depicts femur dry weights and femur dry weight to body weight ratios. Similar to the parameters described above, the femur dry weight of C group was significantly greater than the femur dry weights of all other groups at  $0.24 \pm 0.01$  g (Figure 10A). The femur dry weight of ZD group was not significantly different from the PF group ( $0.19 \pm 0.01$  g vs.  $0.20 \pm 0.01$  g), but the femur dry weight of ZR group was significantly less than the PF and PFR groups ( $0.17 \pm 0.00$  g vs.  $0.20 \pm 0.01$  g and  $0.20 \pm 0.01$  g, respectively). There were no significant differences in femur dry weights between ZD and ZR groups, and PF and PFR groups. In contrast to femur dry weight, the femur dry weight to body weight ratio of C group was significantly less than the femur dry weight to body weight ratios of all other groups at 0.13% (Figure 10B). The PF group had a femur dry weight to body weight ratio that was significantly greater than the ZR and PFR groups (0.20% vs. 0.18% and 0.17%, respectively) but it was not significantly different from the ZD group (0.20% vs. 0.19%). There was no significant difference in femur dry weight to body weight ratio between ZD and ZR. The same significant differences between treatment groups as those observed for the femur dry weight were found for the femur wet weight (Figure 9A). Similar to femur dry weight to body weight ratio, the femur wet weight to body weight ratio of the C group was significantly less than all other groups at 0.30% (Figure 9B). In contrast to femur dry weight to body weight ratio, the femur wet weight to body weight ratios of ZD and PF groups were significantly greater than the ZR and PFR groups (0.41% and 0.42% vs.

0.37% and 0.35%, respectively). The femur wet weight to body weight ratios of ZD and PF groups, and ZR and PFR groups were not significantly different from each other.

#### ***Epididymal Fat Pad Weight and Epididymal Fat Pad Weight to Body Weight Ratio***

The C group had a 4-5.5-fold greater epididymal fat pad weight than the other treatment groups (Figure 11A). The epididymal fat pad weight of C group was  $1.32 \pm 0.07$  g as compared with  $0.29 \pm 0.05$  g for ZD group. ZD, ZR, PF and PFR groups were not significantly different from each other. Similarly, epididymal fat pad weight to body weight ratio of the C group was significantly greater than all other groups and the other groups were not significantly different from each other (Figure 11B).

#### **Zinc Status Assessment**

Serum zinc and femur zinc concentrations were determined in this study as indicators of short term and long term zinc status.

Analysis of the experimental diets showed that they contained the concentrations of zinc in close agreement with those indicated in the Diet Formulation (Table 1). Zinc content of the control diet was 28.82 ppm and of the zinc deficient diet was 1.62 ppm.

#### ***Serum Zinc Concentration***

The ZD group had significantly a lower serum zinc concentration than all other groups (Figure 12). The serum zinc concentration of the ZD rats was more than 4 times lower than the C and PF rats which were not significantly different from each other ( $0.37 \pm 0.11$   $\mu\text{g/ml}$  vs.  $1.62 \pm 0.06$   $\mu\text{g/ml}$  and  $1.60 \pm 0.07$   $\mu\text{g/ml}$ , respectively). Zinc repletion

restored serum zinc concentrations to the value of C group ( $1.53 \pm 0.10 \mu\text{g/ml}$  vs.  $1.62 \pm 0.06 \mu\text{g/ml}$ ). After calorie repletion, the serum zinc concentration of the PFR group was significantly less than the PF group ( $1.30 \pm 0.05 \mu\text{g/ml}$  vs.  $1.60 \pm 0.07 \mu\text{g/ml}$ ).

### ***Femur Zinc Concentration***

Similar to serum zinc concentration, the femur zinc concentration of ZD group was significantly lower than other groups at  $80.1 \pm 5.2 \mu\text{g/g}$ , and it was only 29% and 31% of the C and PF groups, respectively (Figure 13). Zinc repletion for 24 hours did not restore femur zinc concentrations to the value of C group. After 24 hour zinc repletion, the femur zinc concentration of the ZR group was 23% higher than the ZD group. Although the femur zinc concentration of ZR group was significantly lower than the C, PF and PFR groups ( $103.8 \pm 3.8 \mu\text{g/g}$  vs.  $275.1 \pm 5.3 \mu\text{g/g}$ ,  $260.5 \pm 5.3 \mu\text{g/g}$  and  $263.2 \pm 6.3 \mu\text{g/g}$ , respectively), it was significantly higher than the ZD group ( $103.8 \pm 3.8 \mu\text{g/g}$  vs.  $80.1 \pm 5.2 \mu\text{g/g}$ ). C, PF and PFR groups were not significantly different from each other.

## **Intestine**

### ***Weight and Weight to Body Weight Ratio***

Intestine weights and intestine weight to body weight ratios are presented in Figure 14A and B. The C group had an intestine weight significantly higher than all other groups (Figure 14A). The intestine weight of PFR group was significantly lower than the C group ( $10.92 \pm 0.47 \text{ g}$  vs.  $13.30 \pm 0.56 \text{ g}$ , respectively) but it was significantly

higher than the PF, ZR and ZD groups ( $10.92 \pm 0.47$  g vs.  $6.96 \pm 0.62$  g,  $7.69 \pm 0.19$  g and  $7.26 \pm 0.75$  g, respectively) which were not significantly different from each other. In contrast to intestine weights, the ratios of intestine weight to body weight of ZD, ZR and PF groups were not significantly different from the C group (7.2%, 7.9% and 6.6% vs. 7.2%, respectively) (Figure 14B). In addition, the intestine weight to body weight ratio of PF group was significantly less than the ZR group (6.6% as compared with 7.9%). The PFR group had the highest intestine weight to body weight ratio (9.1%) that was significantly different from all other groups.

### ***Length***

Similar to body weight, the C group had the highest intestine length of  $122.6 \pm 3.0$  cm which was significantly different from all other groups (Figure 15). PF and PFR groups had intermediate intestine lengths which were significantly longer than the ZD and ZR groups ( $105.0 \pm 0.9$  cm and  $103.5 \pm 0.6$  cm vs.  $96.9 \pm 2.7$  cm and  $94.2 \pm 1.5$  cm, respectively). There were no significant differences between PF and PFR groups, and ZD and ZR groups.

### ***Zinc Concentration***

Figure 16A depicts the concentrations of zinc in rat small intestine. Interestingly, intestine zinc concentrations of ZD, PF and C groups were not significantly different from each other. The ZR group had a higher intestine zinc concentration than the ZD group, and the PFR group had a higher intestine zinc concentration than the PF group but the differences were not significant ( $134.3 \pm 5.7$   $\mu\text{g/g}$  vs.  $110.9 \pm 7.2$   $\mu\text{g/g}$ , and  $148.1 \pm$

9.7  $\mu\text{g/g}$  vs.  $125.9 \pm 4.5 \mu\text{g/g}$ , respectively). The intestine zinc concentration of the PFR group was significantly higher than the ZD and C groups ( $148.1 \pm 9.7 \mu\text{g/g}$  vs.  $110.9 \pm 7.1 \mu\text{g/g}$  and  $115.7 \pm 13.5 \mu\text{g/g}$ , respectively).

### ***Metallothionein Concentration***

In contrast to zinc concentration, MT concentration in small intestine, measured as  $\mu\text{g}$  per g tissue, was significantly lower in the ZD group ( $4.70 \pm 0.36 \mu\text{g/g}$ ) compared to all other treatment groups (Figure 16B). The ZR group had MT concentration similar to the C and PFR groups ( $12.57 \pm 2.24 \mu\text{g/g}$  vs.  $12.75 \pm 1.64 \mu\text{g/g}$  and  $12.82 \pm 1.61 \mu\text{g/g}$ , respectively). The PF group had the highest MT concentration ( $16.23 \pm 1.33 \mu\text{g/g}$ ) but it was not significantly different from the MT concentrations of PFR, C and ZR groups. The same significant differences were observed when MT concentration was expressed as  $\mu\text{g}$  per mg protein (Figure 16C).

To further examine the relationship between zinc status and MT concentration in small intestine, correlation analysis was performed. No significant correlation was found between zinc concentration and MT concentration measured both as  $\mu\text{g/g}$  and  $\mu\text{g/mg}$  protein ( $r = 0.02$ ,  $p = 0.916$ , and  $r = 0.09$ ,  $p = 0.614$ , respectively). Figure 17 shows a scatter plot of zinc concentration vs. MT concentration measured as  $\mu\text{g}$  per g tissue.

### ***Metallothionein Immunostaining***

Results of the immunoperoxidase staining using mouse monoclonal anti-MT as the primary antibody on rat small intestine are depicted in Figures 18 and 19. The staining was specific for MT since the negative control procedures gave no staining

(Figure 18). Strong nuclear and cytoplasmic MT staining of Paneth cells was observed for all treatment groups with the exception of ZD group (Figure 18). In the ZD group, the staining was absent or weak and limited to very few cells. Similarly, cytoplasm and nuclei of surface epithelial cells, with the exception of goblet cells, stained with varying intensity in all groups but ZD group. The strongest and most consistent staining of epithelial cells was present in PF and C rats. In ZR and PFR rats, there was some individual variability with staining ranging from very weak to strong. Staining of epithelial cells was predominantly localized in proliferative region of villi. No MT staining was detected in lamina propria, submucosa, muscularis, and vasculature of small intestine in any of the treatment groups. Interestingly, in all groups, moderate to strong MT staining was observed in M-cells of lymphoid nodules. Figure 19 shows MT staining in the lymphoid nodules of ZD, PF and C groups.

Morphologically, ZD, ZR, PF and PFR groups had flattened and fewer villi, and thinner submucosa, muscularis and serosa than the C group. The sections of small intestine presented in Figure 18 do not depict true histological differences between the treatment groups as most of them are not complete longitudinal sections. These sections were selected only for the purpose of demonstrating the differences in MT staining.

### ***Ki-67 Immunostaining***

Results of the immunoperoxidase staining for Ki-67 nuclear antigen are presented in Figures 20 and 21. The staining was specific for Ki-67 since negative procedures gave no staining (Figure 20). Strong nuclear staining of surface epithelial cells in the proliferative region of villi was observed in all treatment groups (Figure 20). The

staining intensity diminished toward the tip of villi where no or very light staining was present. Only a few Paneth cells stained for Ki-67. In all groups, strong Ki-67 staining was also demonstrated in lymphoid cells in the germinal center of lymphoid nodules, and in some cells of lamina propria. Figure 21 shows Ki-67 staining in the lymphoid nodules of ZD, PFR and C groups. To confirm the specificity of Ki-67 staining, one intestine section from the ZD group and one from the AL group were treated with PCNA. The pattern of PCNA staining was similar to that observed for Ki-67 staining. There were no differences in the intensity or pattern of PCNA staining between ZD and C groups. The Ki-67 staining and the PCNA staining in the intestine of the C group are compared in Figure 22.

## **Liver**

### ***Weight and Weight to Body Weight Ratio***

Liver weights and liver weight to body weight ratios of the rats are presented in Figure 23A and B. The C group had a significantly higher liver weight than all other groups at  $9.16 \pm 0.52$  g (Figure 23A). ZR and PFR groups had liver weights significantly higher than liver weights of ZD and PF groups ( $5.31 \pm 0.27$  g and  $6.38 \pm 0.74$  g as compared with  $4.13 \pm 0.39$  g and  $3.95 \pm 0.47$  g, respectively). There were no significant differences in liver weight between ZD and PF groups, and ZR and PFR groups. Similar to liver weights, the liver weight to body weight ratios of ZD and PF groups (4.1% and 3.7%) were significantly less than all other groups and they were not different from each other (Figure 23B). In contrast to liver weights, the liver weight to body weight ratios of

ZR and PFR groups were not significantly different from the C group (5.4% and 5.3%, respectively, as compared with 5.0%).

### ***Zinc Concentration***

Figure 24A depicts liver zinc concentrations of the rats. The PF group had the highest liver zinc concentration and it was significantly different from all other groups at  $119.2 \pm 5.5 \mu\text{g/g}$ . ZD and C groups had intermediate liver zinc concentrations that were significantly higher than liver zinc concentrations of ZR and PFR groups ( $89.7 \pm 4.6 \mu\text{g/g}$  and  $91.5 \pm 4.0 \mu\text{g/g}$  vs.  $76.5 \pm 2.6 \mu\text{g/g}$  and  $73.7 \pm 1.9 \mu\text{g/g}$ , respectively). Liver zinc concentrations of ZD and C groups, and ZR and PFR groups were not significantly different from each other.

### ***Metallothionein Concentration***

Similar to liver zinc concentration, liver MT concentration measured both as  $\mu\text{g/g}$  liver and  $\mu\text{g/mg}$  protein was significantly higher in the PF group than in all other groups at  $40.62 \pm 4.66 \mu\text{g/g}$  and  $0.271 \pm 0.035 \mu\text{g/mg}$  protein, respectively (Figure 24B and C). In contrast to liver zinc concentration, liver MT concentrations of ZD, ZR, PFR and C groups measured as  $\mu\text{g/g}$  liver were not significantly different from each other (Figure 24B). When measured as  $\mu\text{g/mg}$  protein, liver MT concentrations of ZR and PFR groups were not significantly different from the C group ( $0.056 \pm 0.010 \mu\text{g/mg}$  protein and  $0.140 \pm 0.016 \mu\text{g/g}$  protein vs.  $0.107 \pm 0.015 \mu\text{g/mg}$  protein) but liver MT concentration of ZR group was significantly lower than the PFR group ( $0.056 \pm 0.010 \mu\text{g/mg}$  protein vs.  $0.140 \pm 0.016 \mu\text{g/mg}$  protein) (Figure 24C). The ZD group had the lowest MT concentration at  $0.042 \pm 0.010 \mu\text{g/mg}$  protein which was significantly different from the PF and C groups

but not significantly different from the ZR group. To further investigate the relationship between liver MT concentration and liver zinc status, Pearson's correlation analysis was conducted. There was a significant positive correlation ( $r = 0.70$ ,  $P = 0.0001$ ) between liver MT concentration measured as  $\mu\text{g/g}$  tissue and liver zinc concentration (Figure 25). A similar positive correlation was found between zinc and MT concentrations when MT was measured as  $\mu\text{g/mg}$  protein ( $r = 0.60$ ,  $P = 0.0001$ ).

### ***Metallothionein Immunostaining***

Results of the immunoperoxidase staining using mouse monoclonal anti-MT as primary antibody on rat liver are depicted in Figure 26. The staining was specific for MT since the negative control procedures gave no staining. Strong and moderate MT staining was demonstrated in hepatocytes of PF and PFR rats, respectively. MT staining was weak in C group. In ZD and ZR groups, MT staining was absent, or weak to moderate staining appeared in only a few cells. In all groups, the staining was localized in cytoplasm and nuclei of hepatocytes. In the PF group, and to a lesser extent in the PFR group, MT staining was concentrated predominantly around central veins. In contrast, the MT staining was scattered throughout the liver of C group.

### ***Ki-67 Immunostaining***

Results of the immunoperoxidase staining for Ki-67 nuclear antigen in rat liver are presented in Figure 27. The staining was specific for Ki-67 since the negative control procedures gave no staining. In all treatment groups, light to moderate Ki-67 staining was observed in the nuclei of majority of hepatocytes. A few hepatocytes with darkly stained nuclei were detected in ZD, ZR, PF and PFR groups. In contrast, in the AL

group, nuclei of many hepatocytes showed very strong Ki-67 staining. For comparison, PCNA staining was performed on one liver section from the ZD and AL groups. In both groups, strong PCNA staining was detected in the nuclei of many hepatocytes but the number of PCNA stained cells was less than the number of Ki-67 positive cells. The Ki-67 staining and the PCNA staining in the liver of C group is presented in Figure 28.

In addition to the microscopic assessment of Ki-67 staining, the percentage of Ki-67 stained cells was determined. The number of total Ki-67 immunoreactive cells and the number of cells with strong Ki-67 staining was obtained by computer-aided nuclear counting and expressed as percent of total nucleated cells (Figure 29A and B). In the ZD group, the percentage of cells with strong Ki-67 staining and total Ki-67 staining ( $2.10 \pm 0.80\%$  and  $67.93 \pm 3.82\%$ , respectively) was not significantly different from percentages of all other groups (Figure 29A and B). Although the percentage of cells with strong Ki-67 staining in the ZD group was not significantly different from the C group, it was only 35% of the C group. The PF group had the lowest percentage of cells with strong Ki-67 staining and it was significantly different from the ZR, PFR and C groups ( $0.66\% \pm 0.15\%$  vs.  $5.52 \pm 1.93\%$ ,  $4.47 \pm 1.36\%$  and  $5.31 \pm 0.26\%$ , respectively) but these groups were not significantly different from each other (Figure 29A). The percentage of cells with strong Ki-67 staining in the PF group was only 12% of the C group. The PF group also had the lowest percentage of total Ki-67 positive cells and it was significantly different from the ZR and PFR groups ( $60.6 \pm 2.7\%$  vs.  $72.2 \pm 2.1\%$  and  $69.2 \pm 2.5\%$ , respectively) but it was not significantly different from the C and ZD groups ( $60.6 \pm 2.7\%$  vs.  $67.8 \pm 1.8\%$  and  $67.9 \pm 3.8\%$ , respectively) (Figure 29B). The percentages of

total Ki-67 positive cells were not significantly different from each other in the ZD, ZR, PFR and C groups.

## **Kidney**

### ***Weight and Weight to Body Weight Ratio***

Kidney wet weights and kidney wet weight to body weight ratios of the rats are presented in Figure 30A and B. The C group had the highest wet kidney weight at  $1.97 \pm 0.01$  g and it was significantly different from all other treatment groups (Figure 30A). PF and PFR groups had kidney weights that were significantly greater than the ZR group ( $1.28 \pm 0.06$  g and  $1.29 \pm 0.05$  g vs.  $1.10 \pm 0.02$  g, respectively) but they were not significantly different from the ZD group ( $1.28 \pm 0.06$  g and  $1.29 \pm 0.05$  g vs.  $1.18 \pm 0.07$  g). No significant differences were found between ZD and ZR groups, and PF and PFR groups. In contrast to kidney wet weight, kidney wet weight to body weight ratio of the PF group was the highest and it was significantly different from the ZR, PFR and C groups (1.23% vs. 1.13%, 1.09% and 1.08, respectively) but these groups were not different from each other (Figure 30B). The ZD group had a kidney wet weight to body weight ratio that was significantly greater than the PFR and C groups (1.20% vs. 1.09% and 1.08%, respectively) but it was not significantly different from the ZR and PF groups (1.20 vs. 1.13% and 1.23%, respectively).

### ***Zinc Concentration***

The ZD group had a significantly lower kidney zinc concentration than all other groups at  $73.7 \pm 3.3 \mu\text{g/g}$  (Figure 31A). Although the ZR group had kidney zinc concentration significantly higher than the ZD group, it was significantly lower than the PF, PFR and C groups ( $93.4 \pm 2.6 \mu\text{g/g}$  as compared with  $106.6 \pm 2.2 \mu\text{g/g}$ ,  $102.2 \pm 1.3 \mu\text{g/g}$  and  $105.0 \pm 1.4 \mu\text{g/g}$ , respectively). There were no significant differences among PF, PFR and C groups.

### ***Metallothionein Concentration***

When measured as  $\mu\text{g}$  per  $\text{g}$  kidney, MT concentration was highest in the kidney of ZR, PF and PFR groups and these groups were not significantly different from each other (Figure 31B). The C group had an intermediate MT concentration that was significantly lower than the MT concentrations of ZR, PF and PFR groups ( $33.1 \pm 3.1 \mu\text{g/g}$  vs.  $43.6 \pm 3.4 \mu\text{g/g}$ ,  $47.6 \pm 4.3 \mu\text{g/g}$  and  $46.7 \pm 3.8 \mu\text{g/g}$ , respectively) but it was significantly higher than the ZD group ( $33.1 \pm 3.1 \mu\text{g/g}$  vs.  $21.8 \pm 2.7 \mu\text{g/g}$ ). Similarly, when measured as  $\mu\text{g}$  per  $\text{mg}$  protein, kidney MT was significantly lower in the ZD group ( $0.087 \pm 0.009 \mu\text{g/mg}$  protein) than all other groups, and it was significantly higher in the PF group than in the C group ( $0.213 \pm 0.024 \mu\text{g/mg}$  protein vs.  $0.160 \pm 0.012 \mu\text{g/mg}$  protein) as shown in Figure 31C. In contrast to the concentrations of MT measured as  $\mu\text{g}$  per  $\text{g}$  kidney, the mean MT concentrations of ZR, PFR and C groups measured as  $\mu\text{g}$  MT per  $\text{mg}$  protein were not significantly different from each other. To examine the relationship between MT concentration and zinc status in rat kidney, Pearson's correlation analysis was performed. A significant positive correlation was

found between renal zinc concentration and MT concentration measured as  $\mu\text{g}$  MT per g kidney ( $r = 0.57$ ,  $p = 0.0001$ ) (Figure 32) and between zinc concentration and MT concentration measured as  $\mu\text{g}$  MT per mg protein ( $r = 0.66$ ,  $p = 0.0001$ ).

### ***Metallothionein Immunostaining***

Results of the immunoperoxidase staining using mouse monoclonal anti-MT as primary antibody on rat kidney are presented in Figures 33 and 34. The staining was specific for MT since the negative control procedures gave no staining (Figure 33). In kidney of AL, PF and PFR groups, epithelial cells of renal convoluted tubules showed strong MT staining as opposed to moderate and very weak staining in ZR and ZD groups, respectively (Figure 33). The staining was present predominantly in epithelia of proximal convoluted tubules as opposed to distal convoluted tubules, and was detected in both the cytoplasm and nuclei of epithelial cells. In AL, PF and PFR groups, the staining was localized to the entire cortex, and some staining was also present in the medulla. In contrast, ZD and ZR groups showed staining only in the outer rim of the cortex and no staining in the medulla. The results of MT staining in the medulla are not shown. All groups, except the ZD group, had some MT staining in the lumina of renal tubules (Figure 33). In all groups, no MT staining was detected in glomeruli (Figure 33). Interestingly, epithelia of large collecting ducts at the base of renal pelvis stained with varying intensity in kidney of rats from all treatment groups. Figure 34 presents the MT staining of collecting ducts in the kidney of the ZD, PF and PFR groups. Morphologically, there were less collecting ducts per surface area in the kidney of ZD and PF groups as compared with the PFR group, and the collecting ducts of ZD and PF

groups appeared dilated. In addition, the kidneys of ZD and PF groups may be infiltrated with immune cells. These morphological differences appear interesting but they were not pursued further for this thesis project.

Differentiation of proximal tubules from distal tubules was aided by the comparison of MT stained sections with hematoxylin and eosin stained sections under high magnification. Figure 35 shows the two types of tubules under 25x and 40x magnification. Hematoxylin and eosin staining allows for discrimination of structural differences between the two types of tubules. Proximal tubules have a distinct brush border that is lacking in distal tubules, large cells, and basally placed nuclei (Rhoolin, 1975; Gartner and Hiatt, 1997). In distal tubules, cells are narrower and nuclei are apically located (Rhoolin, 1975; Gartner and Hiatt, 1997). Since the cells of distal tubules are narrower than the cells of proximal tubules, there are more nuclei per cross-section of distal tubules as opposed to proximal tubules (Gartner and Hiatt, 1997). In addition, in paraffin sections, the lumen of proximal tubules usually appears occluded in contrast to the widely open lumen of distal tubules (Gartner and Hiatt, 1997).

### ***Ki-67 Immunostaining***

Figures 36, 37 and 38 show results of the immunoperoxidase staining for Ki-67 nuclear antigen in rat kidney. The staining was specific for Ki-67 since negative procedures gave no staining (Figure 37). In the kidney cortex, nuclei of the majority of epithelial cells of renal convoluted tubules stained for Ki-67 with moderate to high intensity in all treatment groups (Figure 36 and 37). No staining was detected in glomeruli. In the cortex of ZD, ZR, PF and PFR groups, most of the nuclei stained with

moderate intensity and only a few showed dark staining. In contrast, there were many more darkly stained nuclei in the cortex of AL group as compared with the other groups. In all groups, Ki-67 staining diminished toward the medulla. In the medulla, there were apparent differences between groups in the number of stained nuclei and the intensity of the staining (Figure 38). In ZD and ZR groups, the staining was mostly light and limited to only a few cells, and there were no or very few darkly stained nuclei present. PF and PFR groups had more darkly stained nuclei than ZD and ZR groups, and AL group had the greatest number of darkly stained nuclei among all groups. For comparison, PCNA staining was performed on one kidney section from ZD and C groups. The results of Ki-67 staining and PCNA staining in the kidney of C group are compared in Figure 39. The pattern of PCNA staining and the number of stained cells differed from that observed for KI-67 staining. Strong PCNA staining was detected in both medulla and cortex of ZD and C groups. However, less cells stained for PCNA than for Ki-67. Further, there were no differences between ZD and C groups in the number of PCNA stained cells or the intensity of staining.

## Key to Figures

The following letter codes were used to identify the different treatment groups:

**ZD** = Zinc deficient

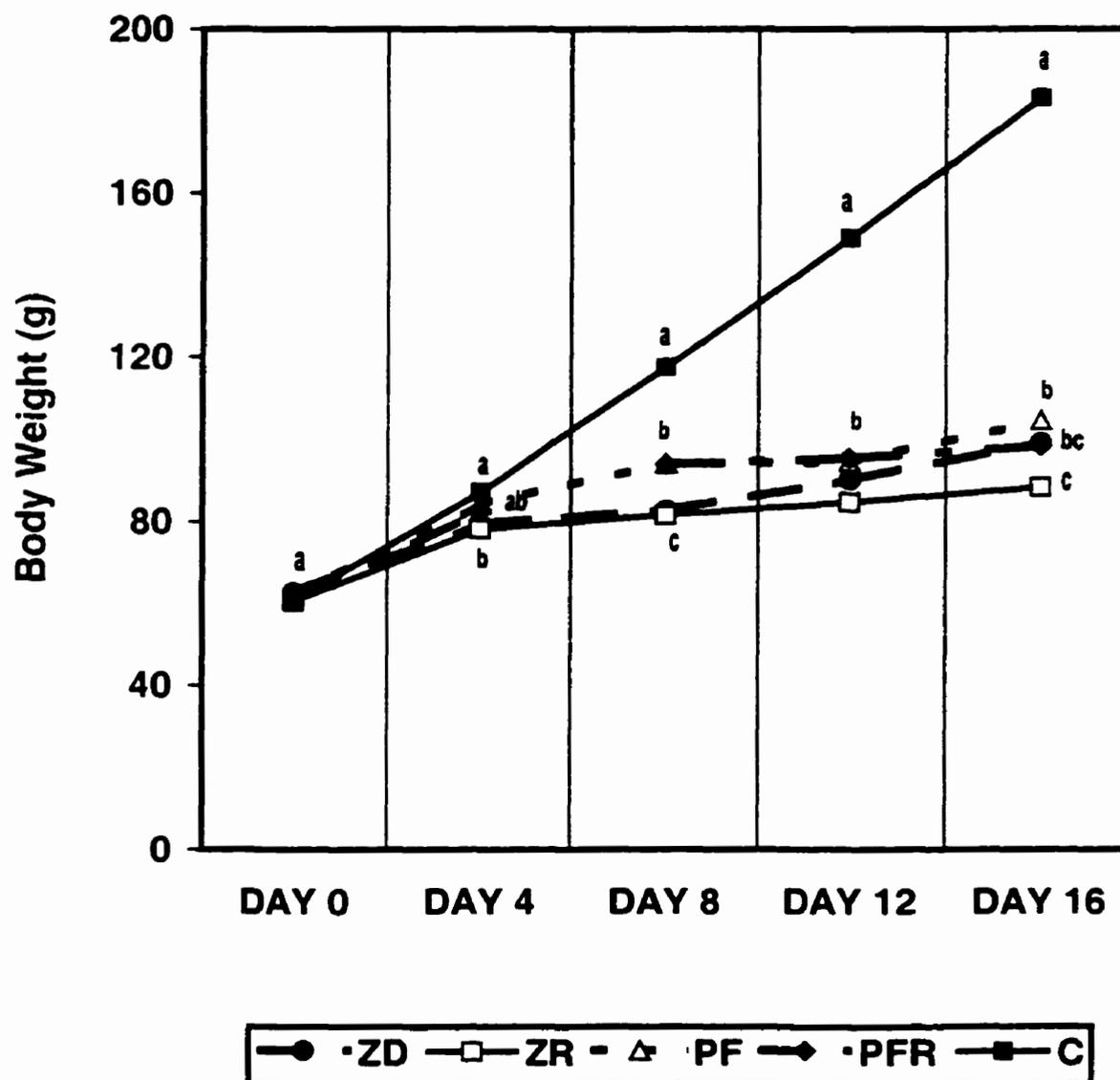
**ZR** = Zinc repleted

**PF** = Pair-fed

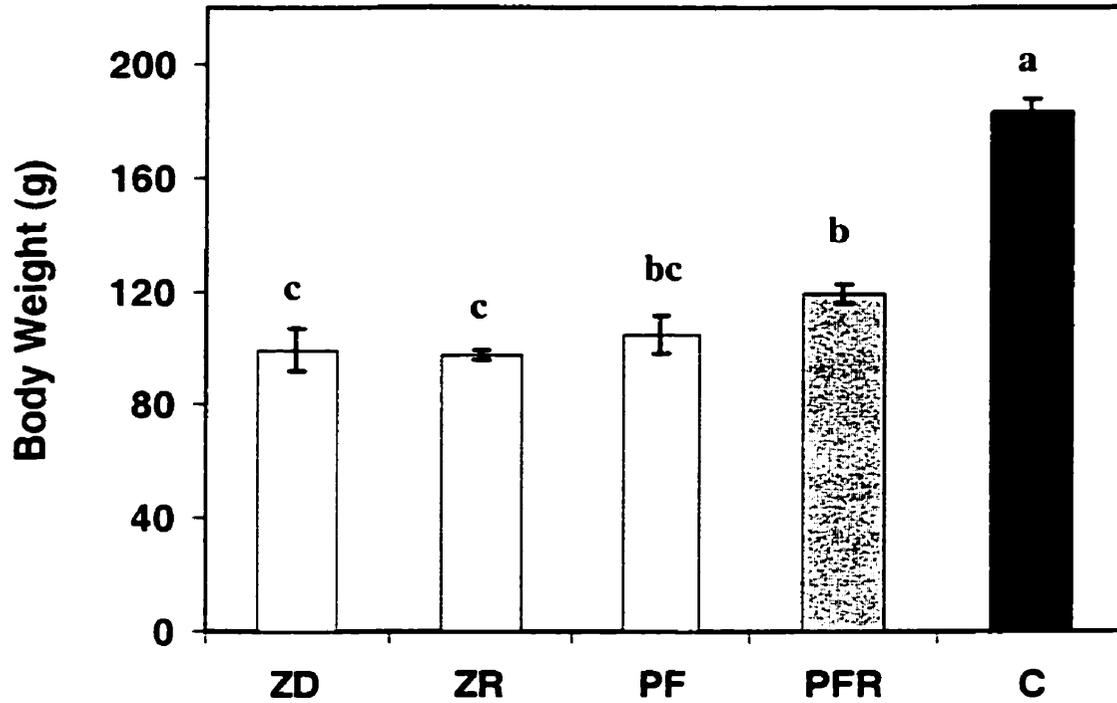
**PFR** = Calorie repleted

**C** = Control

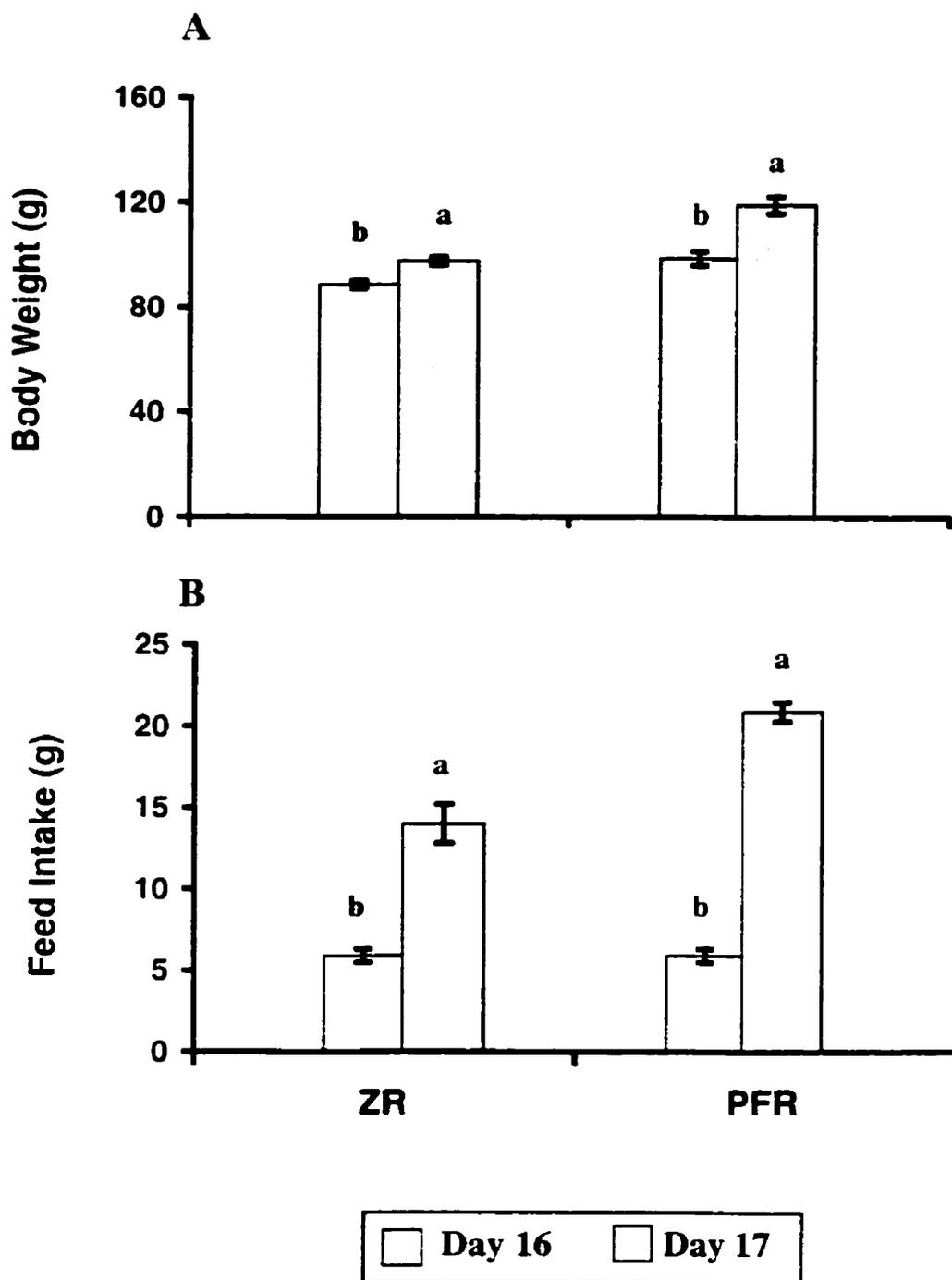
In addition, the data for the different treatment groups were presented in different colors to aid in their comparison.



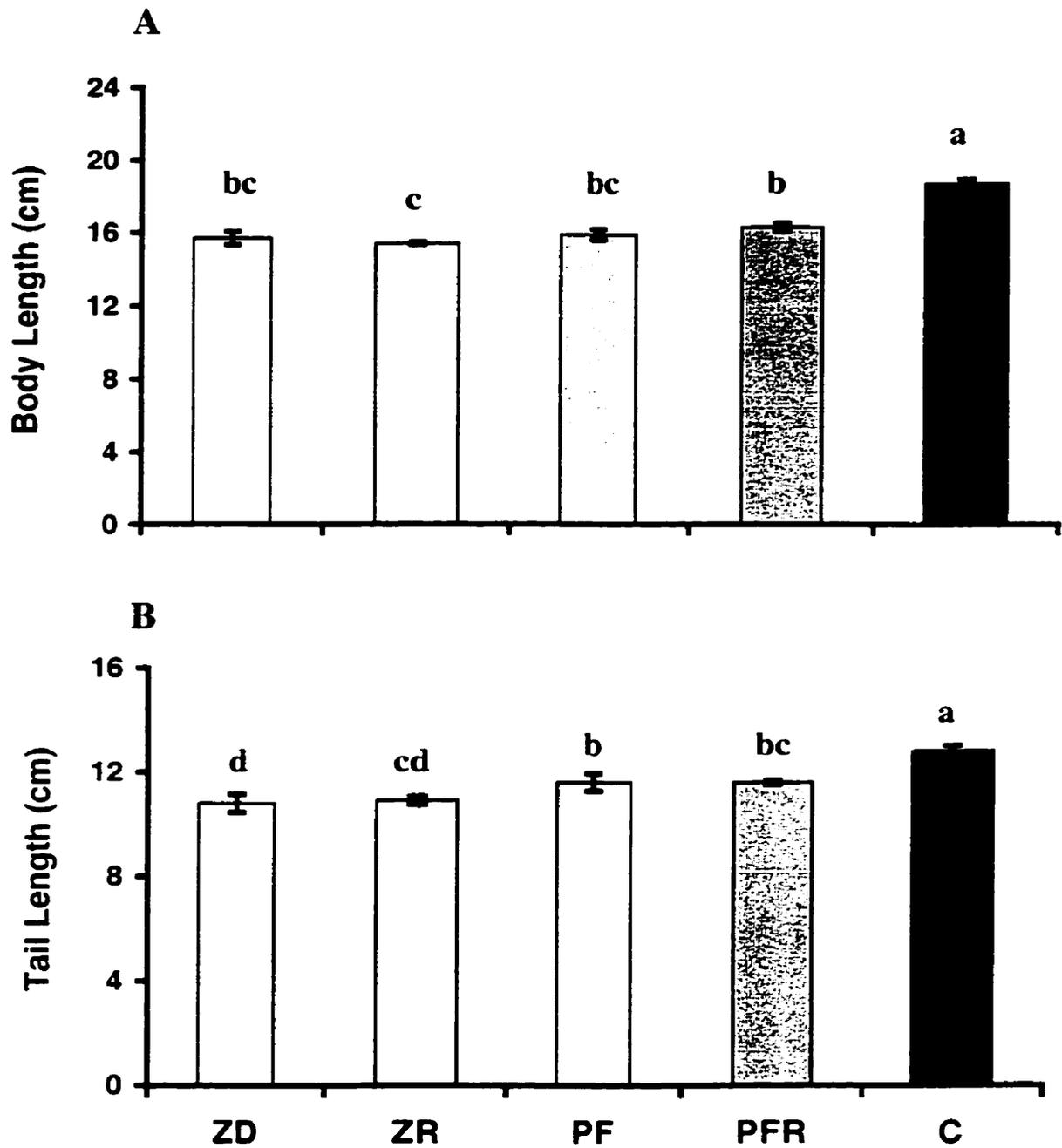
**Figure 5.** Effect of zinc deficiency on body weight at days 0, 4, 8, 12 and 16 of the study. Data points represent means  $\pm$  SEM for  $n = 8$ . Data points with different lower case letters are significantly different within the same week as determined by repeated measures testing. For data points that overlap, the same letter of significance was used to indicate that the data points were not significantly different from each other.



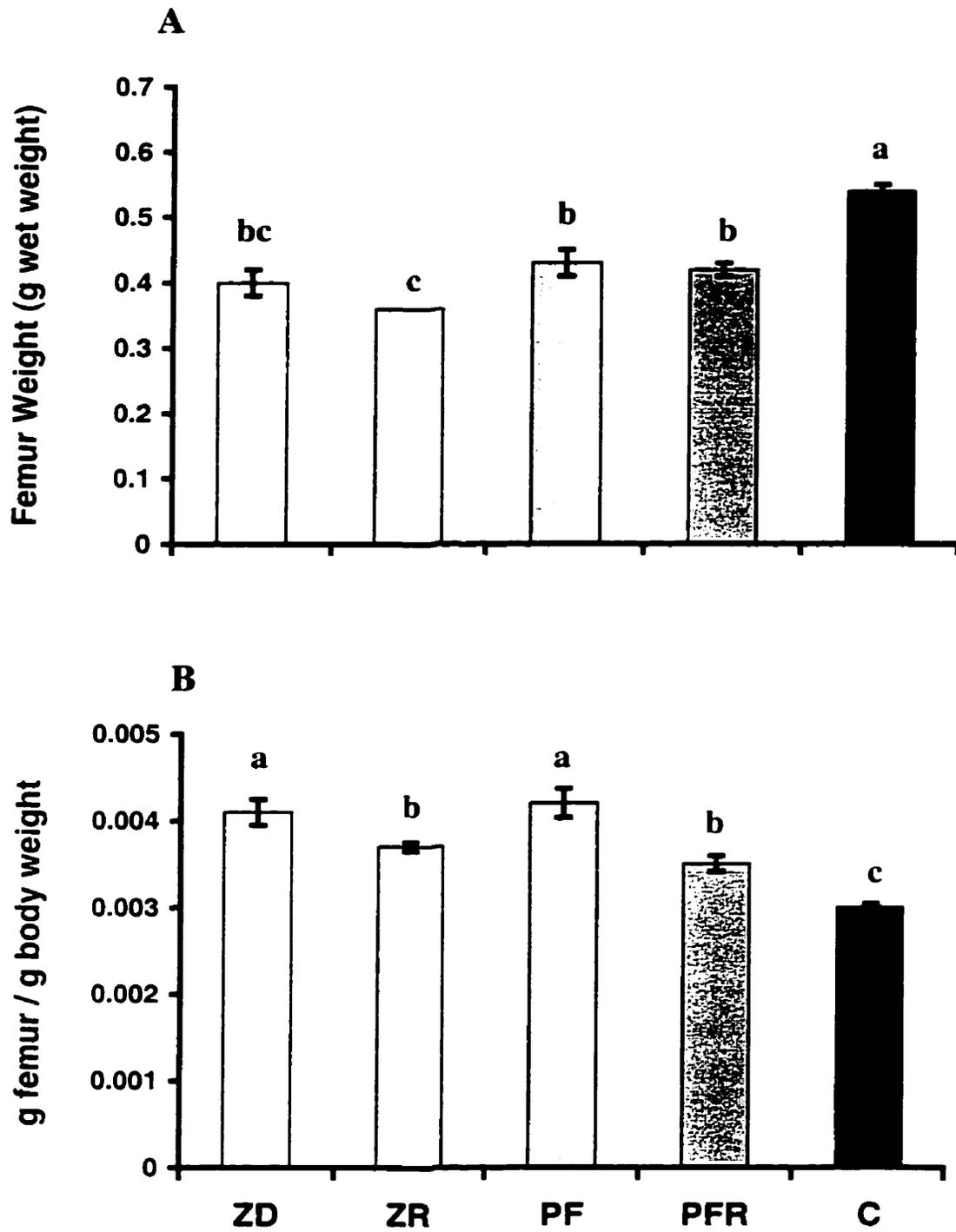
**Figure 6.** Effect of zinc deficiency (ZD, PF, C at day 16 of the study) and zinc repletion (ZR and PFR at day 17 of the study) on body weight. Columns represent group means  $\pm$  SEM for  $n = 8$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



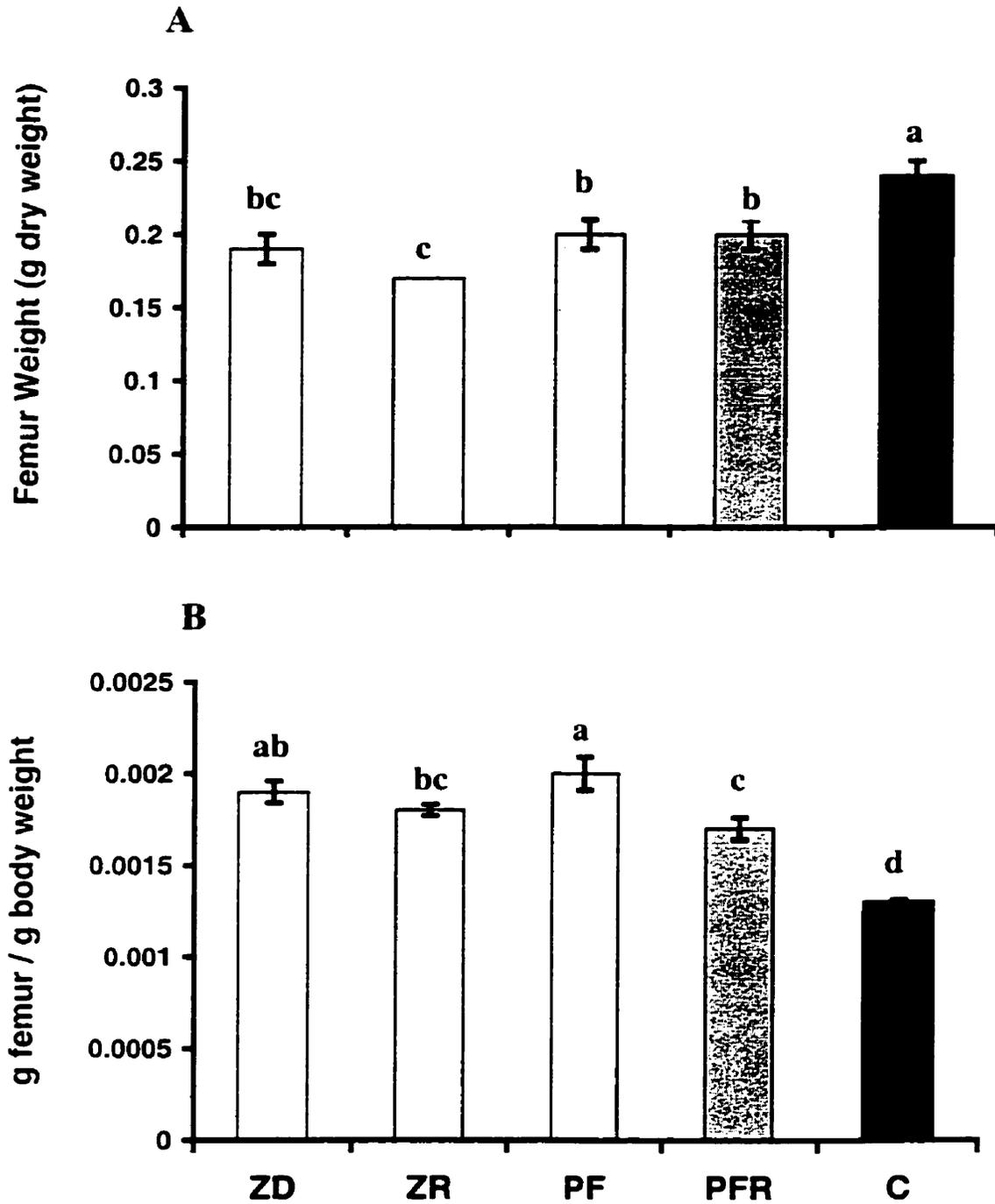
**Figure 7.** Body weight and feed intake of ZR and PFR groups before zinc repletion (Day 16 of the study) and after zinc repletion (Day 17 of the study). Columns represent group means  $\pm$  SEM for  $n = 8$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



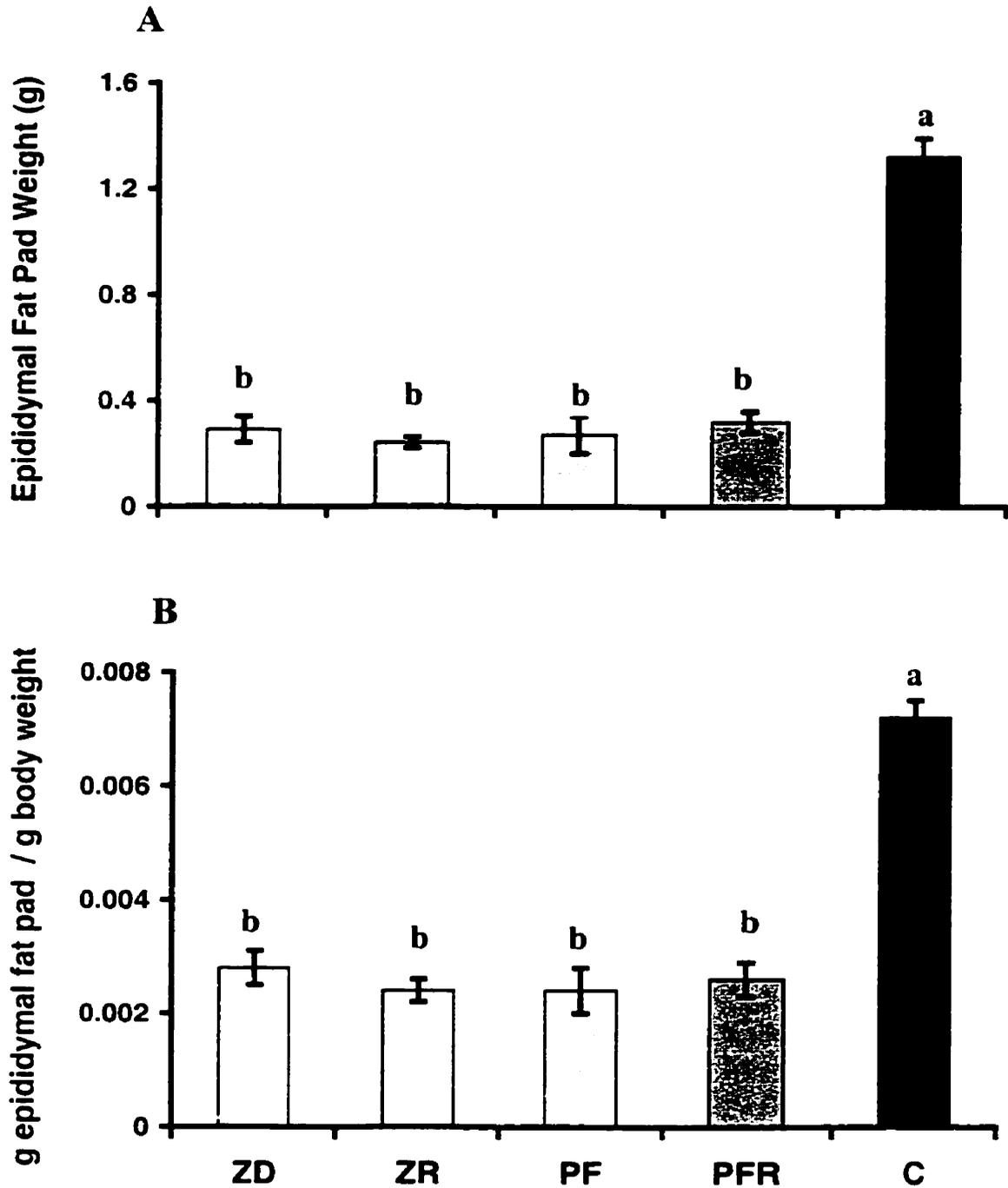
**Figure 8.** Effect of zinc deficiency and repletion on body length and tail length. Columns represent means  $\pm$  SEM for  $n = 8$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



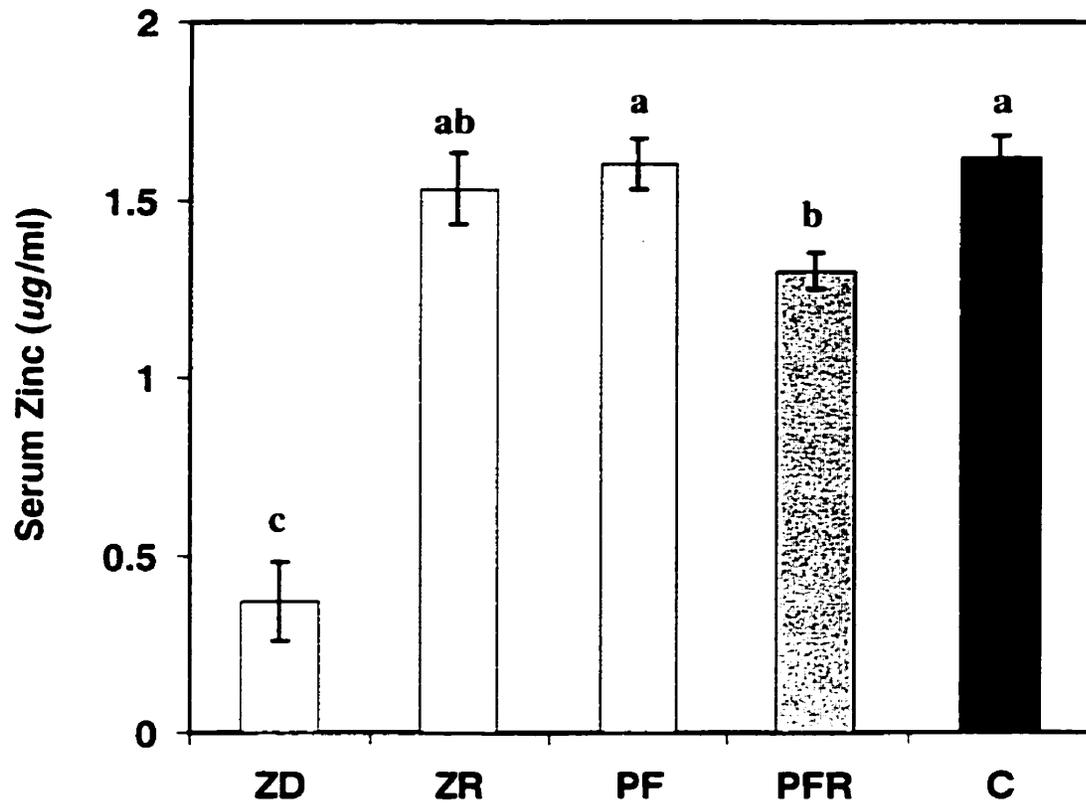
**Figure 9.** Effect of zinc deficiency and repletion on femur wet weight (A) and femur wet weight to body weight ratio (B). Columns represent group means  $\pm$  SEM for  $n = 8$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



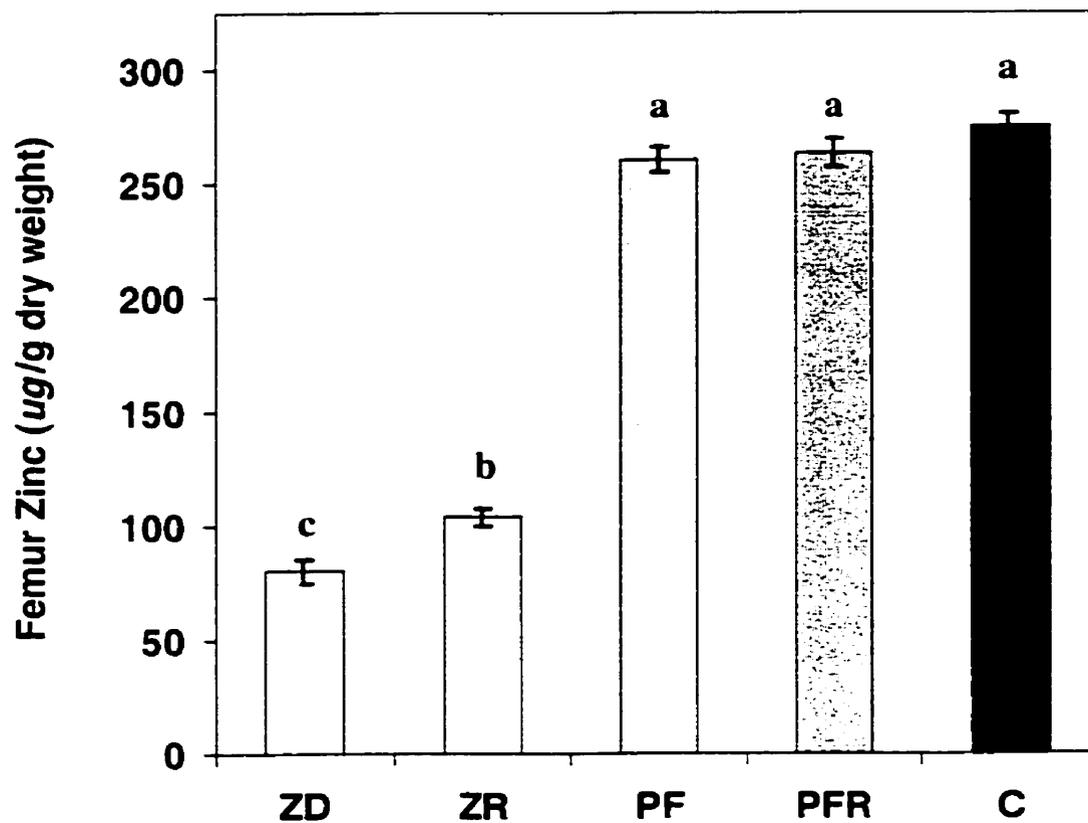
**Figure 10.** Effect of zinc deficiency and repletion on femur dry weight (A) and femur dry weight to body weight ratio (B). Columns represent group means  $\pm$  SEM for  $n = 8$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



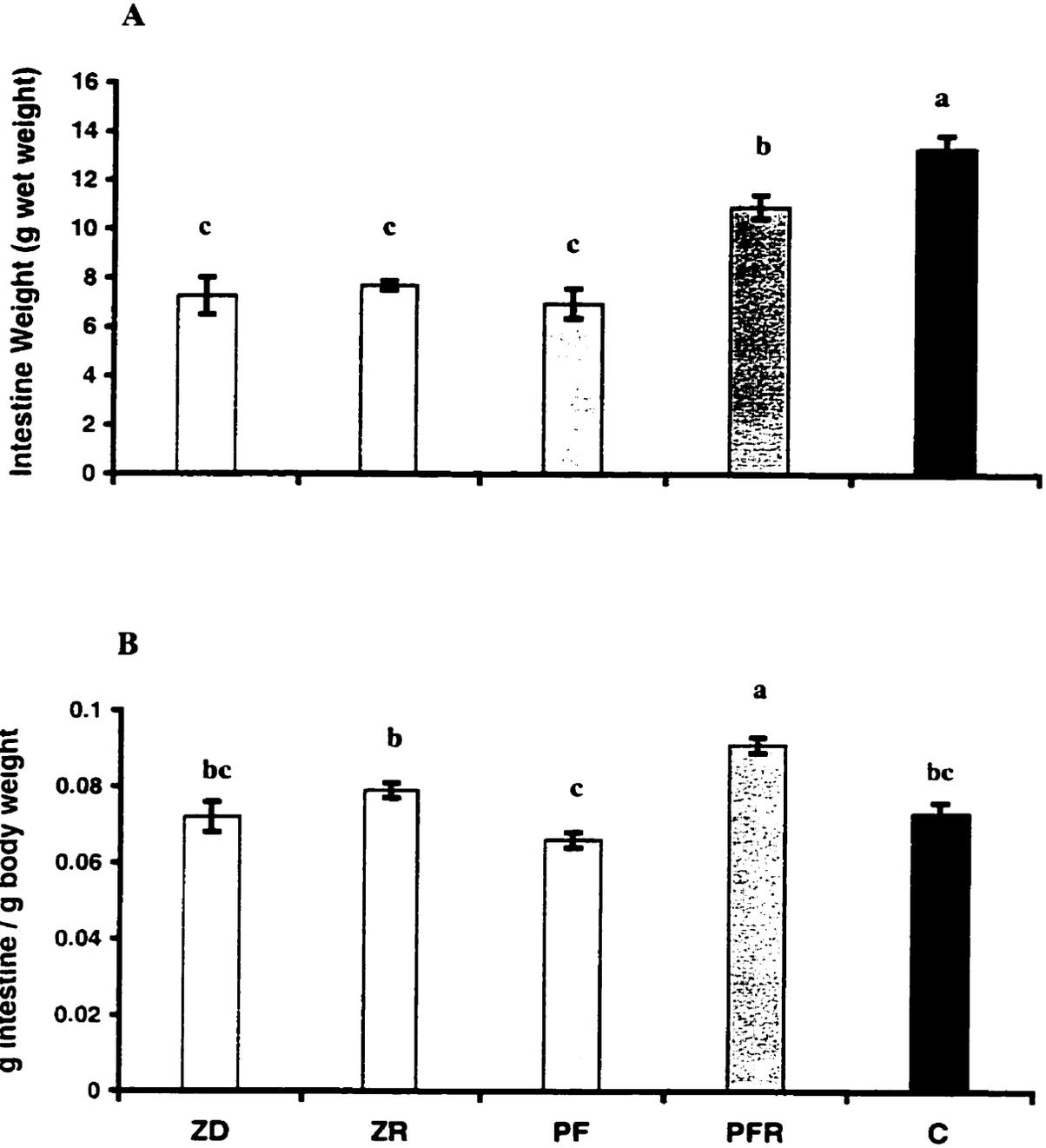
**Figure 11.** Effect of zinc deficiency and repletion on epididymal fat pad weight (A) and epididymal fat pad weight to body weight ratio (B). Columns represent group means  $\pm$  SEM for  $n = 8$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



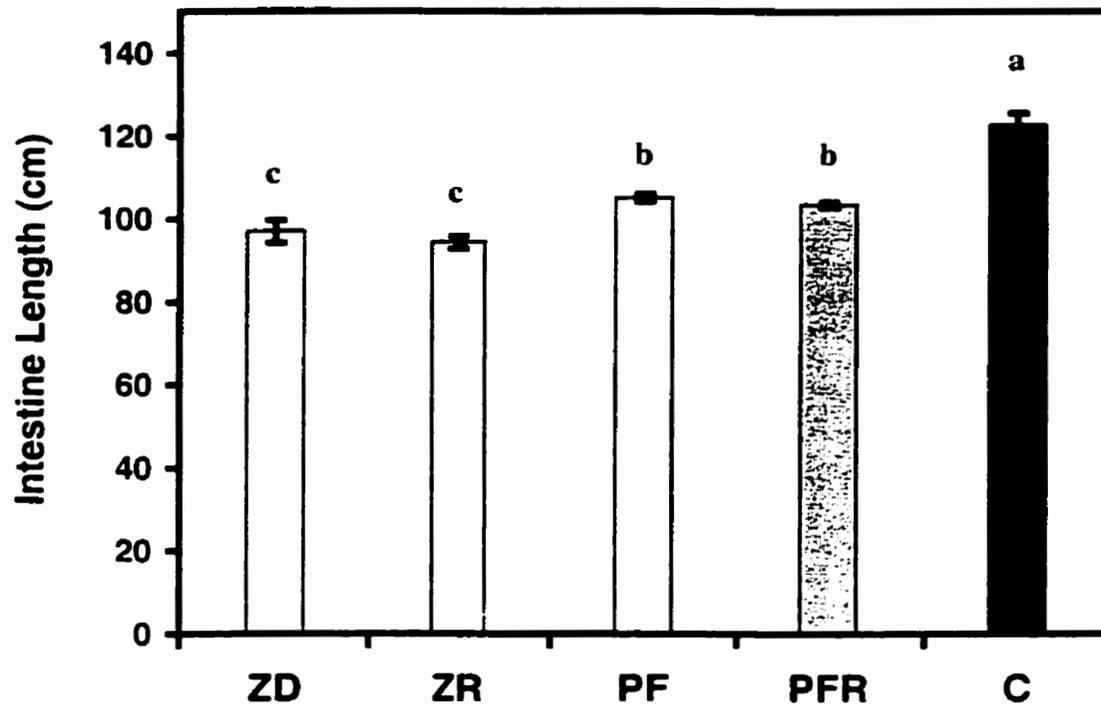
**Figure 12.** Effect of zinc deficiency and repletion on serum zinc concentration. Columns represent group means  $\pm$  SEM,  $n = 8$  except for ZD group where  $n = 7$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



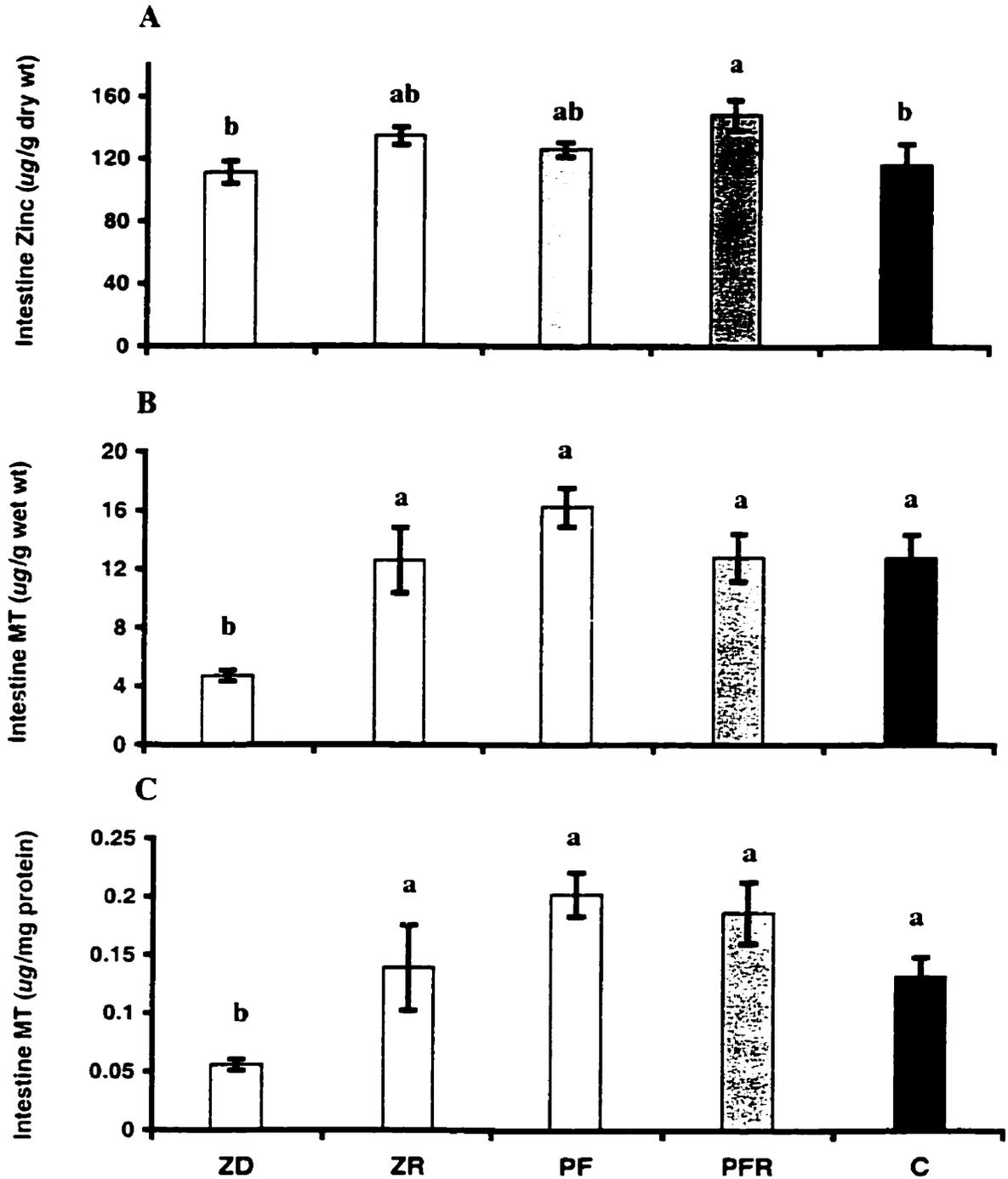
**Figure 13.** Effect of zinc deficiency and repletion on femur zinc concentration. Columns represent means  $\pm$  SEM for  $n = 8$ . Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



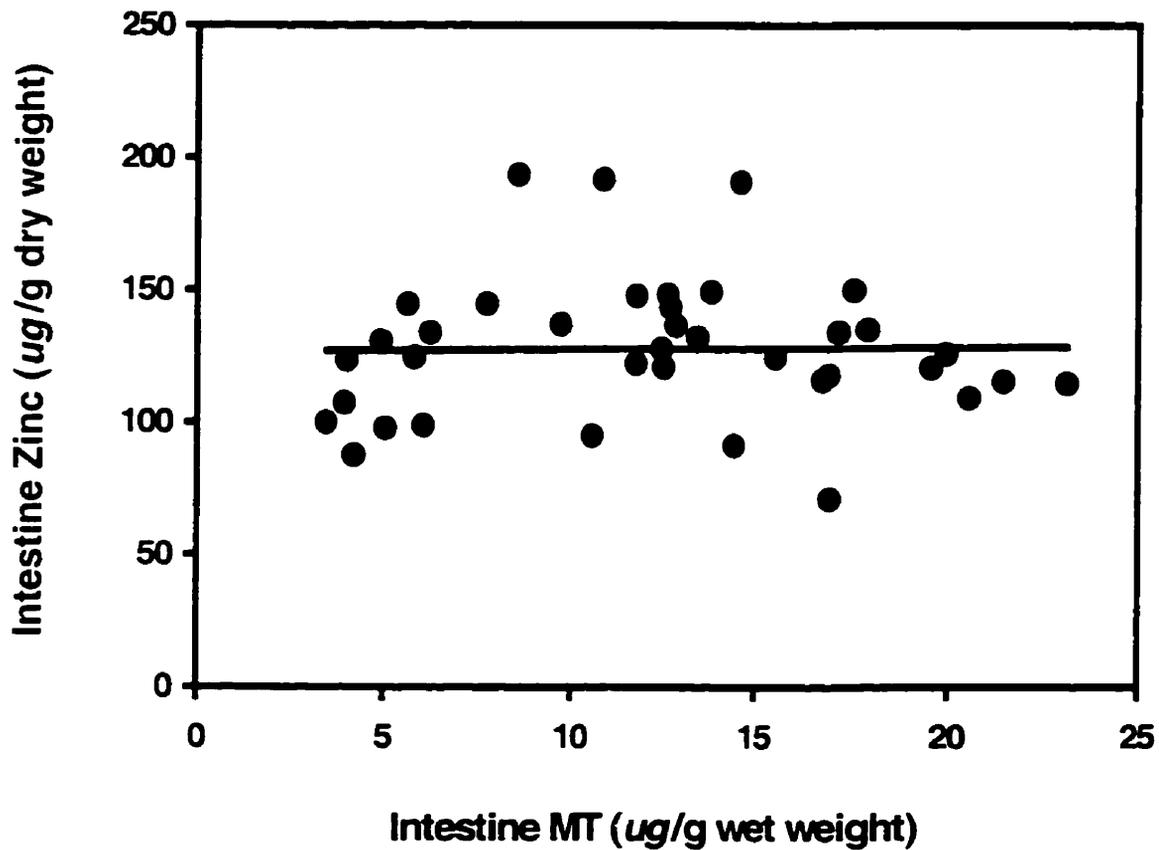
**Figure 14.** Effect of zinc deficiency and repletion on intestine weight (A) and intestine weight to body weight ratio (B). Columns represent means  $\pm$  SEM.  $n = 8$  except  $n = 7$  for ZD group. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



**Figure 15.** Effect of zinc deficiency and repletion on intestine length. Columns represent means  $\pm$  SEM,  $n = 8$  except  $n = 7$  for ZD group. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



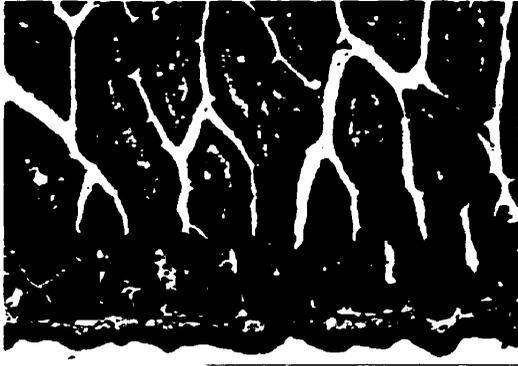
**Figure 16.** Effect of zinc deficiency and repletion on the concentrations of zinc (A), metallothionein per g tissue (B), and metallothionein per mg protein (C) in small intestine. Columns represent means  $\pm$  SEM,  $n = 8$  except  $n = 6$  for intestine zinc for ZD, and intestine metallothionein per mg protein for ZD and PFR, and  $n = 7$  for intestine metallothionein per g of tissue for ZD. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



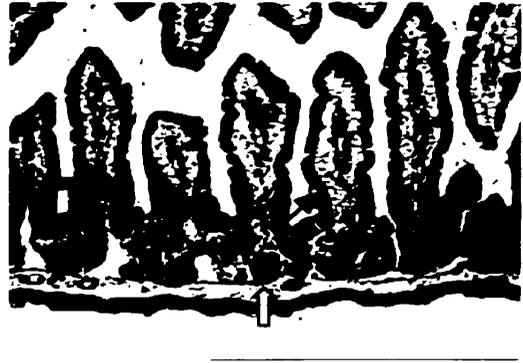
**Figure 17.** Scatter plot of small intestine zinc concentration vs. small intestine metallothionein (MT) concentration. Data points are values obtained from individual rats,  $n = 38$ . No significant correlation was found ( $r = 0.02$ ,  $p = 0.916$ ) by Pearson's correlation coefficient analysis.

**Figure 18.** Immunohistological staining for metallothionein in rat small intestine (mid-section). There was strong nuclear and cytoplasmic metallothionein staining in Paneth cells (white block arrow) and surface epithelial cells (black block arrow) in all treatment groups except the ZD group, and no metallothionein staining in goblet cells (arrow) and lamina propria (lp) in all treatment groups. Scale bars shown at the bottom left corner equal 100  $\mu\text{m}$ .

**ZD**



**ZR**



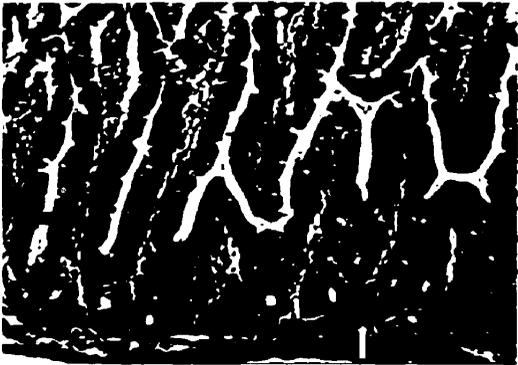
**PF**



**PFR**



**C**

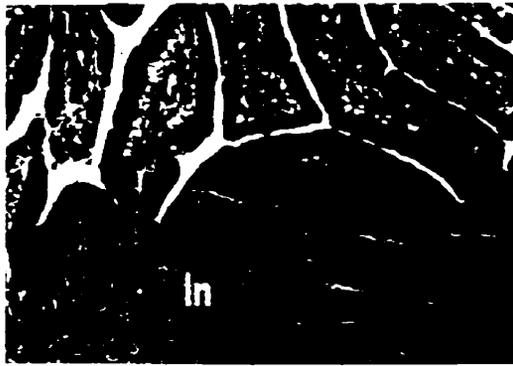


**Negative Control**

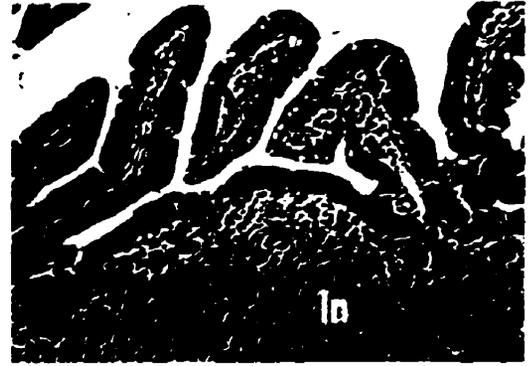


**Figure 19.** Immunohistological staining for metallothionein in lymphoid nodules (ln) of rat small intestine (mid-section). There was strong metallothionein staining in M-cells (arrow) in all treatment groups (ZR and PFR not shown). Scale bars shown at the bottom left corner equal 100  $\mu\text{m}$ .

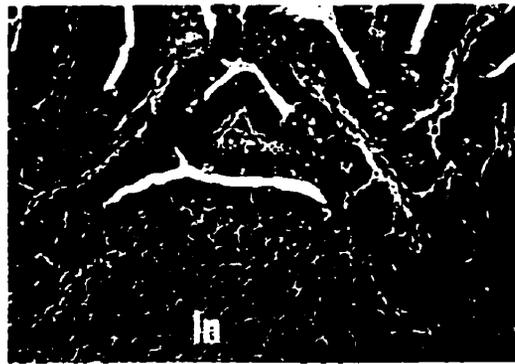
**ZD**



**PF**



**C**



**Figure 20.** Immunohistological staining for Ki-67 in rat small intestine (mid-section).

There was strong Ki-67 staining in the proliferative region of villi at the base of the crypts of Lieberkuhn in all treatment groups. Scale bars shown at the bottom left corner equal 160 $\mu$ m.

**ZD**



**ZR**



**PF**



**PFR**



**C**



**Negative Control**

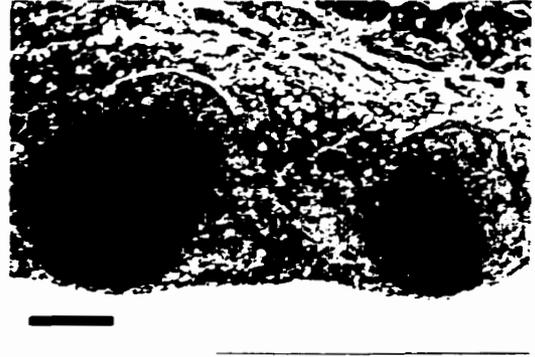


**Figure 21.** Immunohistological staining for Ki-67 in lymphoid nodules of rat small intestine (mid-section). There was strong Ki-67 staining in lymphoid cells in germinal centers (gn) in all treatment groups (ZR and PF not shown). Scale bars shown at the bottom left corner equal 160 $\mu$ m.

**ZD**



**PFR**

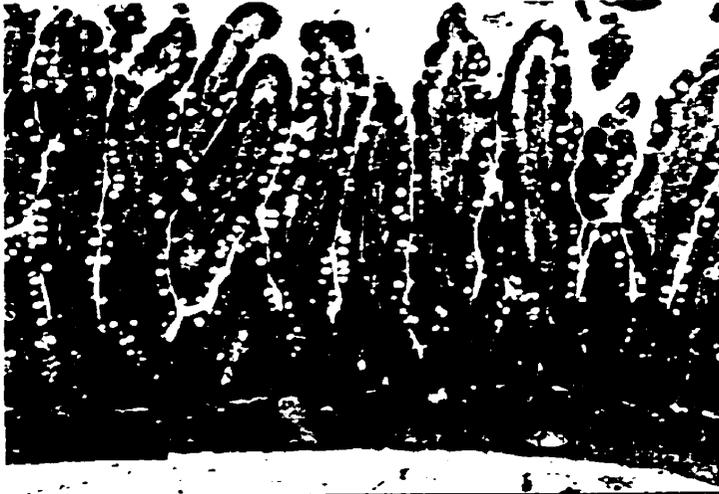


**C**



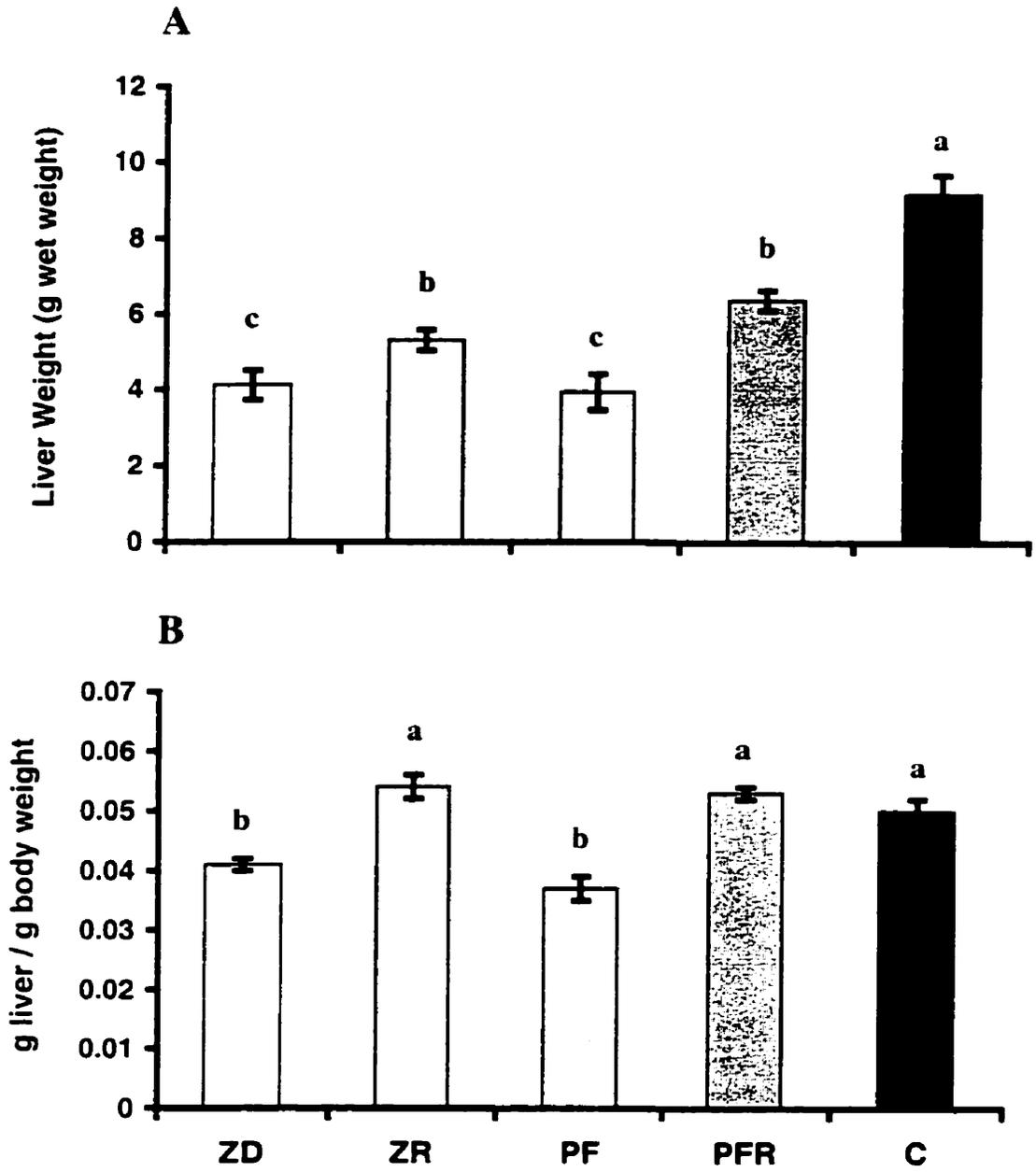
**Figure 22.** Immunohistochemical staining for Ki-67 and PCNA in small intestine of a control rat (mid-section). There was strong Ki-67 staining (A) and PCNA staining (B) in the proliferative region of villi at the base of the crypts of Lieberkuhn. Scale bars shown at the bottom left corner equal 160 $\mu$ m.

**A**

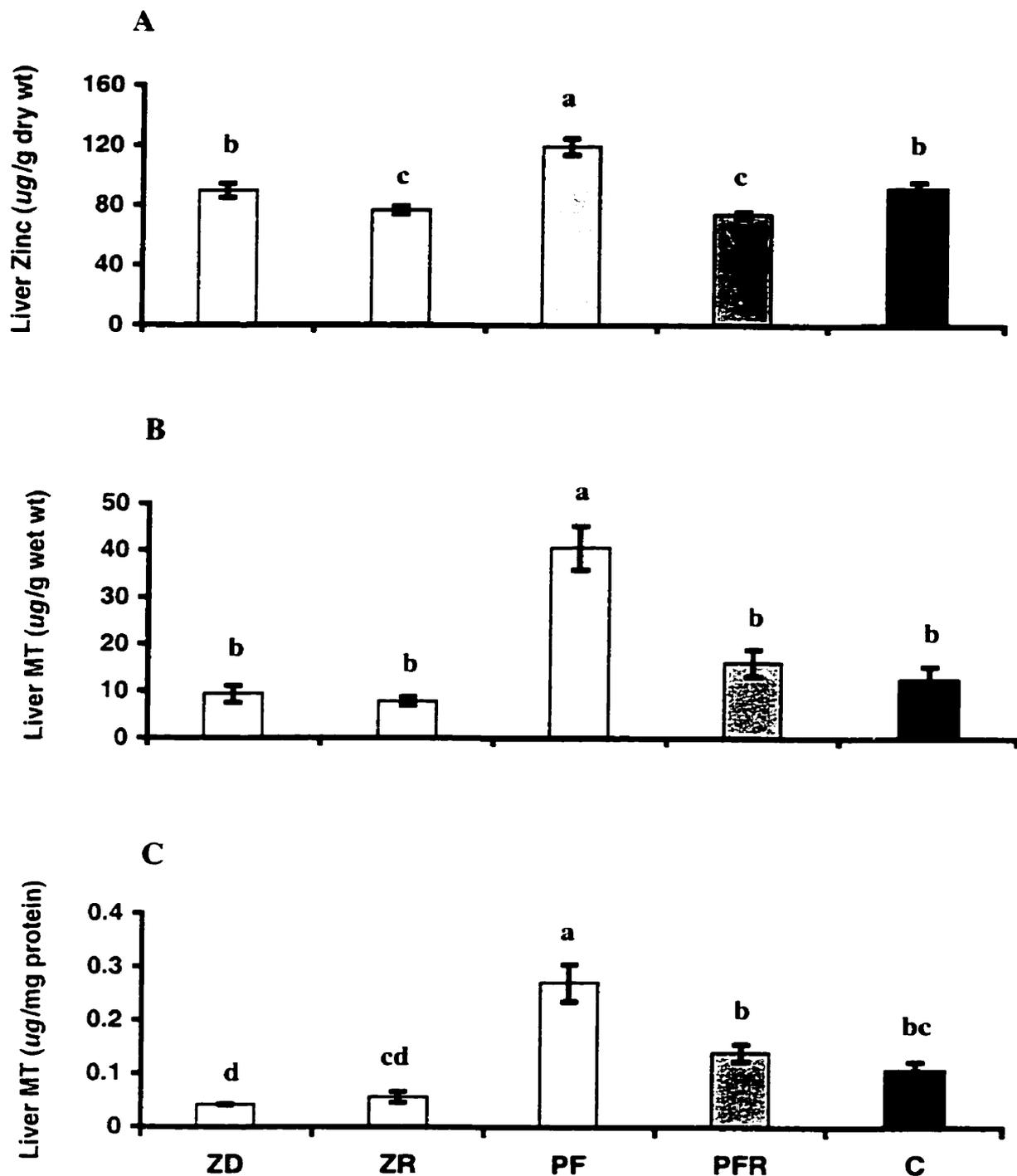


**B**

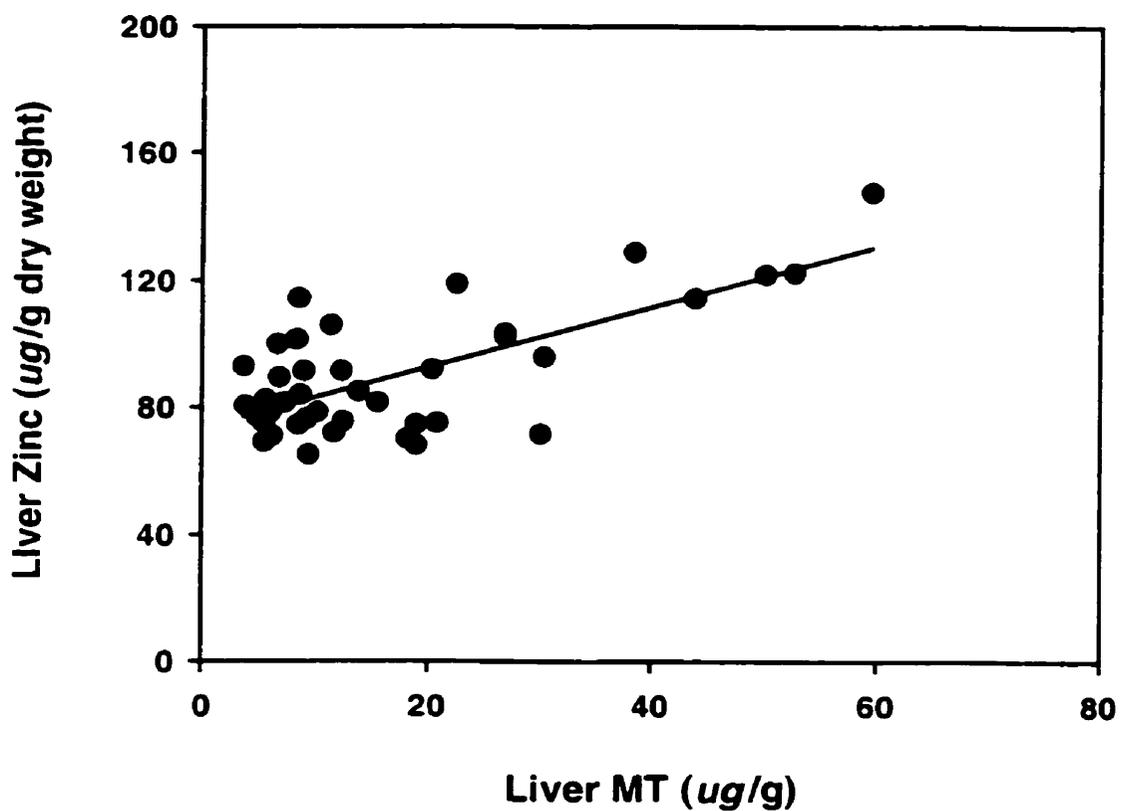




**Figure 23.** Effect of zinc deficiency and repletion on liver weight (A) and liver weight to body weight ratio (B). Columns represent group means  $\pm$  SEM for n = 8. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



**Figure 24.** Effect of zinc deficiency and repletion on the concentrations of liver zinc (A), liver metallothionein per g liver (B), and liver metallothionein per mg protein (C). Columns represent group means  $\pm$  SEM, n = 8. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



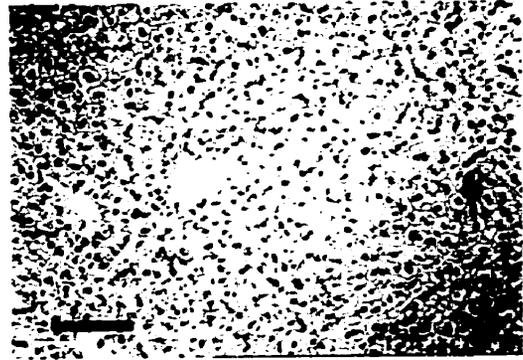
**Figure 25.** Scatter plot of liver zinc concentration vs. liver metallothionein concentration. Data points are values obtained from individual rat,  $n = 40$ . A significant positive correlation was found ( $r = 0.70$ ,  $P = 0.0001$ ) by Pearson's correlation coefficient analysis.

**Figure 26.** Immunohistological staining for metallothionein in rat liver. In the ZD and ZR, metallothionein staining was present in only a few cells. Strong and moderate metallothionein staining was localized around central veins (V) in the PF and PFR, respectively. Metallothionein staining was scattered in the C. Scale bars at the bottom left corner equal 100  $\mu\text{m}$  with the exception of the scale bar on the Negative Control image which equals 50  $\mu\text{m}$ .

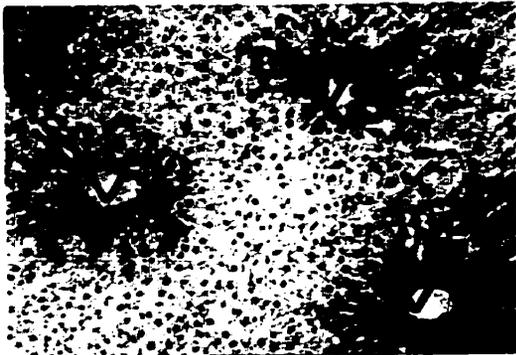
**ZD**



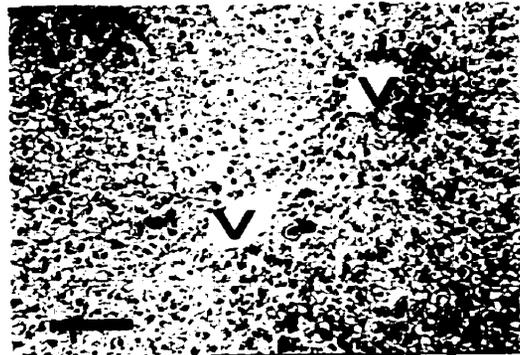
**ZR**



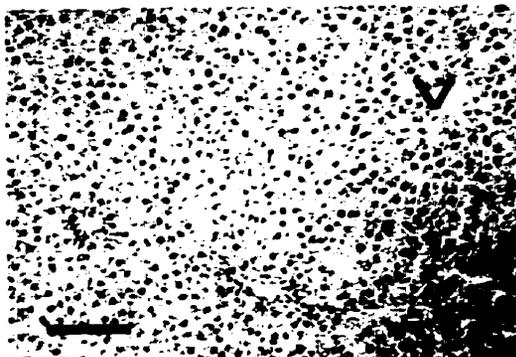
**PF**



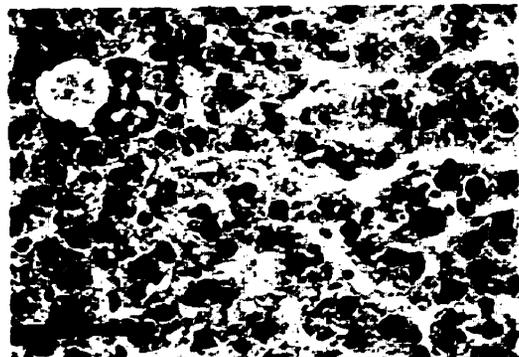
**PFR**



**C**

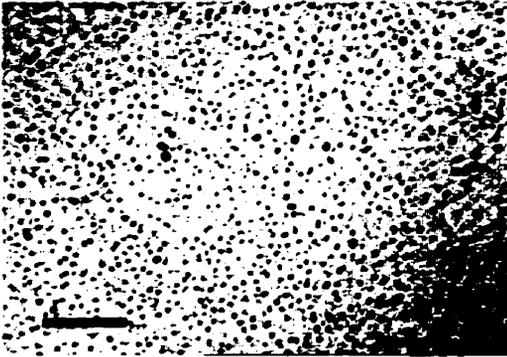


**Negative Control**

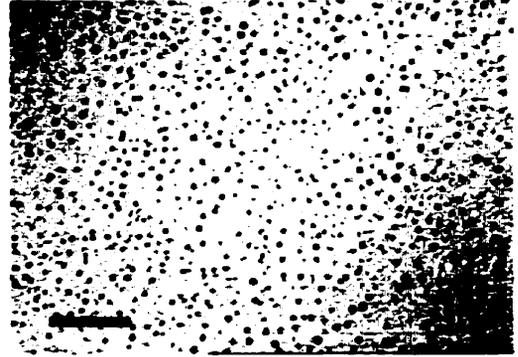


**Figure 27.** Immunohistological staining for Ki-67 in rat liver. Note fewer darkly stained nuclei in the ZD, ZR, PF and PFR as compared with the C. Scale bars at the bottom left corner equal 100  $\mu\text{m}$ .

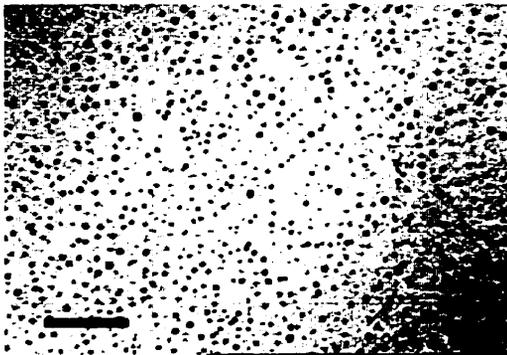
**ZD**



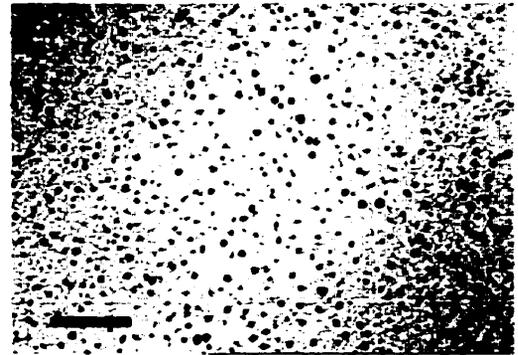
**ZR**



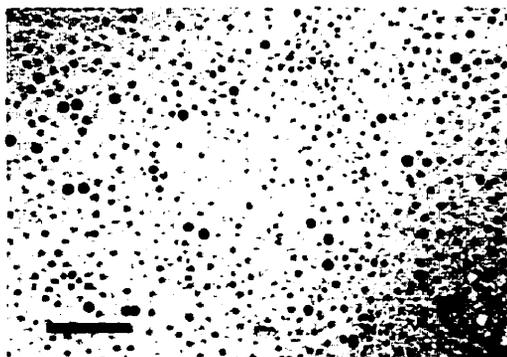
**PF**



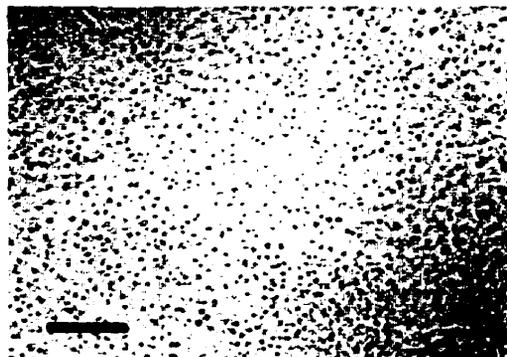
**PFR**



**C**



**Negative Control**

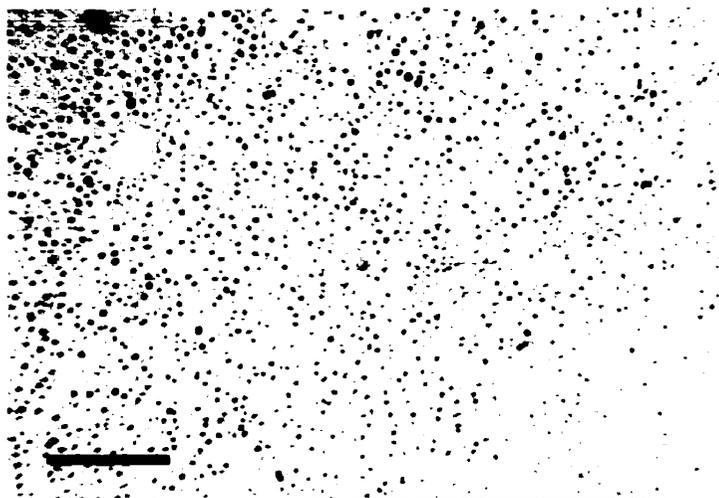


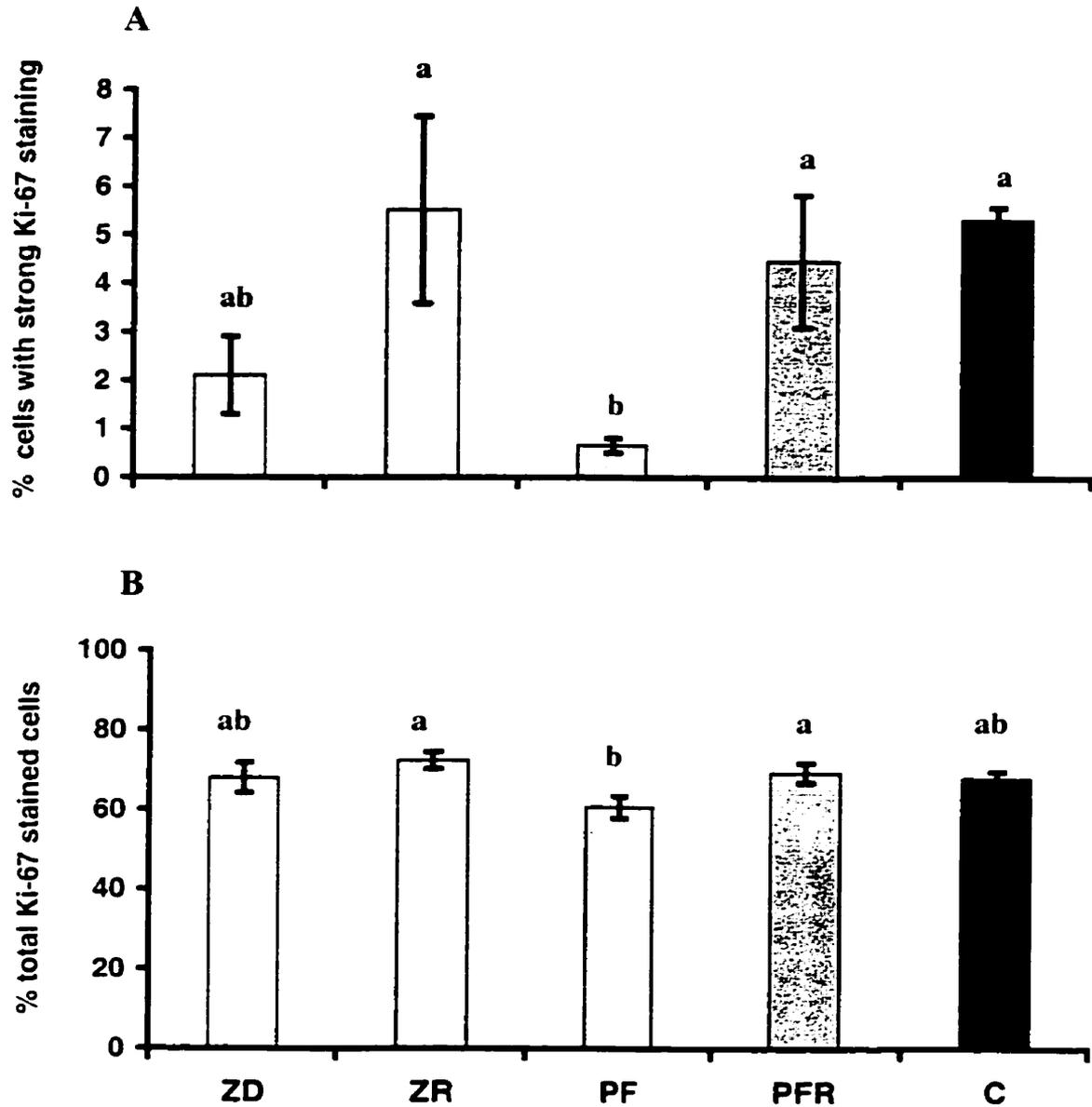
**Figure 28.** Immunohistological staining for Ki-67 (A) and PCNA (B) in liver of a control rat. Scale bar at the bottom left corner of (A) equals 100  $\mu\text{m}$ . Scale bar at the bottom left corner of (B) equals 160  $\mu\text{m}$ .

**A**

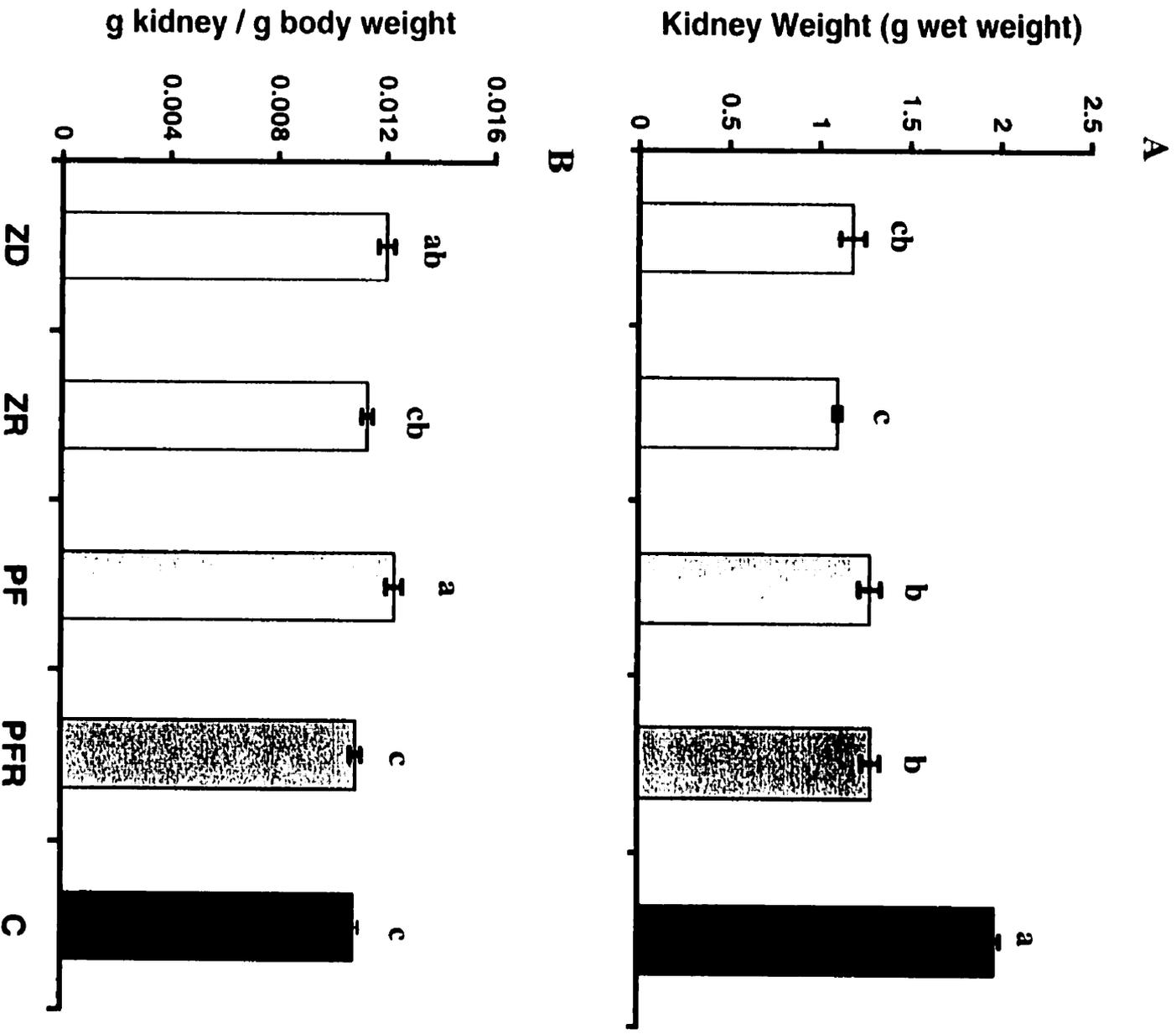


**B**

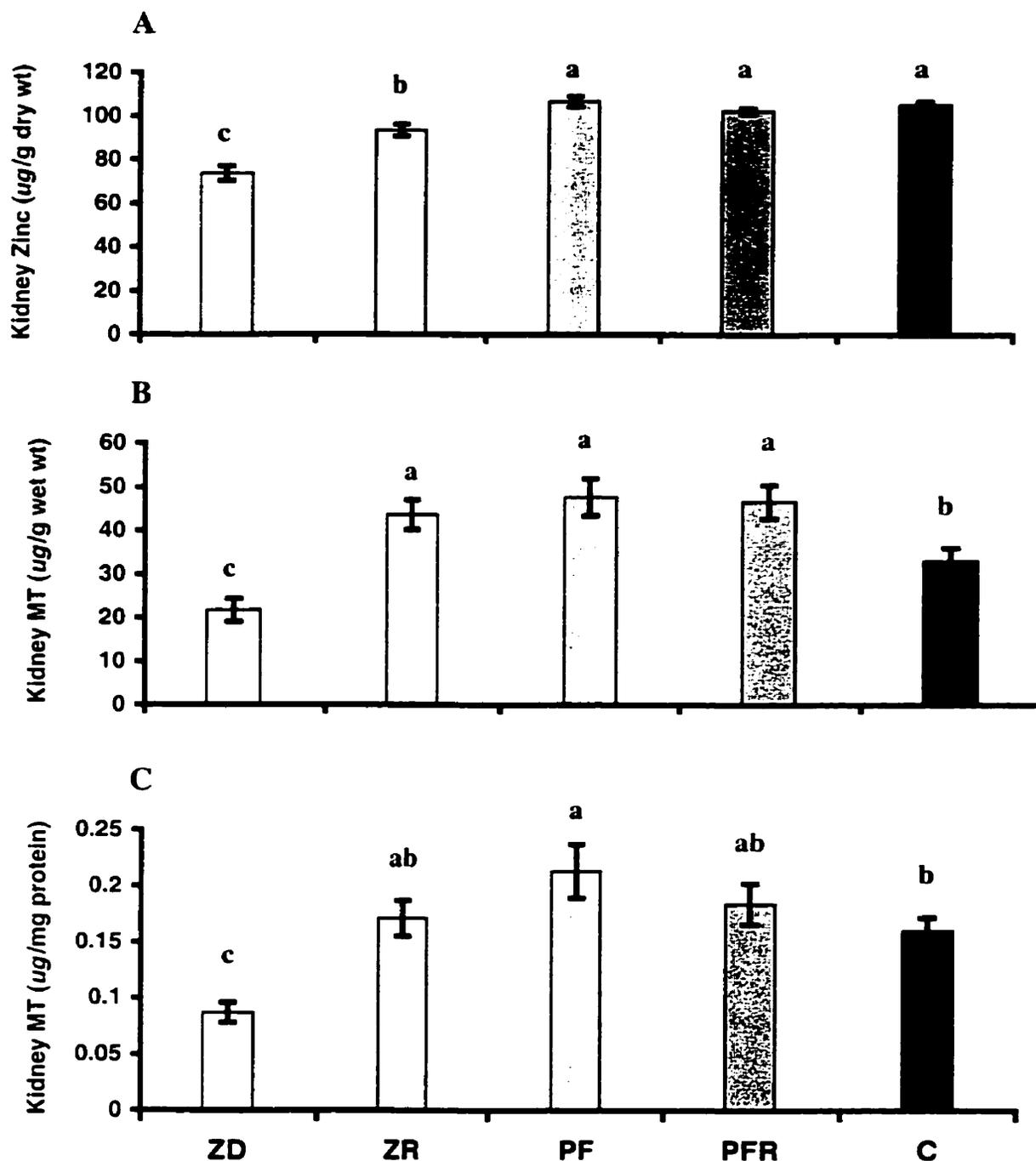




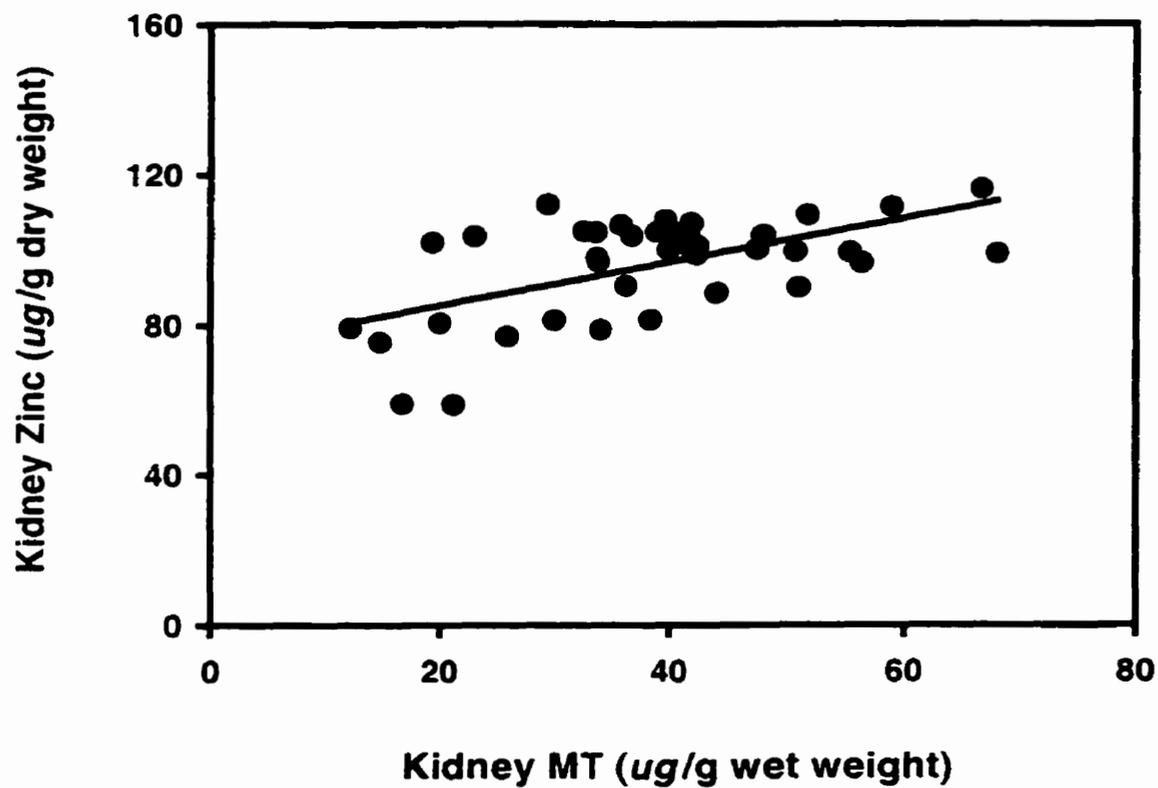
**Figure 29.** Effect of zinc deficiency and repletion on % Ki67 immunostaining. Number of cells with strong Ki-67 staining (A) and total number of Ki-67 stained cells (B) are expressed as percent of total nucleated cells. Columns represent group means  $\pm$  SEM for  $n = 8$ . Each  $n$  is a mean of a number of cells counted in three areas of a liver section. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



**Figure 30.** Effect of zinc deficiency and repletion on kidney wet weight (A) and kidney wet weight to body weight ratio (B). Columns represent means  $\pm$  SEM for n = 8. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



**Figure 31.** Effect of zinc deficiency and repletion on the concentrations of kidney zinc (A), kidney metallothionein per g kidney (B), and kidney metallothionein per mg protein (C). Columns represent means  $\pm$  SEM,  $n = 8$  except  $n = 7$  for kidney metallothionein for PF group. Columns with different lower case letters are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.



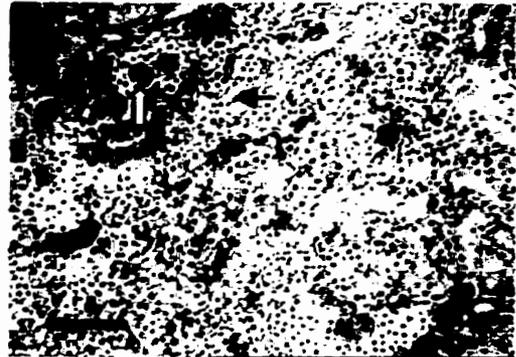
**Figure 32.** Scatter plot of kidney zinc concentration vs. kidney metallothionein concentration. Data points are values obtained from individual rats,  $n = 39$ . A significant positive correlation between the two variables was found ( $r = 0.57$ ,  $P = 0.0001$ ) by Pearson's correlation coefficient analysis.

**Figure 33.** Immunohistological staining for metallothionein in the cortex of rat kidney. Metallothionein staining was localized predominantly in proximal convoluted tubules (white block arrow), staining was weak or absent in distal convoluted tubules (arrow), and no staining was present in glomeruli (g). Note strong metallothionein staining in the PF, PFR and C groups, moderate staining in the ZR group, and weak staining in the ZD group. Scale bars at the bottom left corner equal 100  $\mu\text{m}$ .

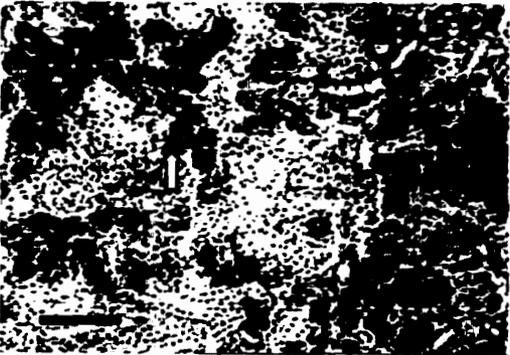
**ZD**



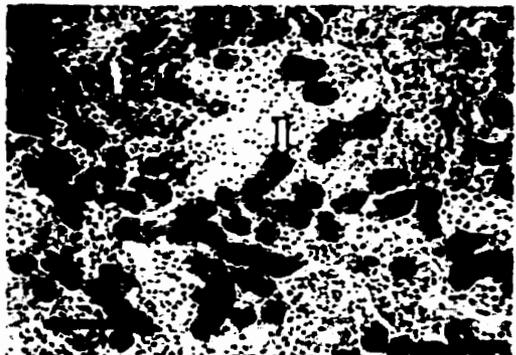
**ZR**



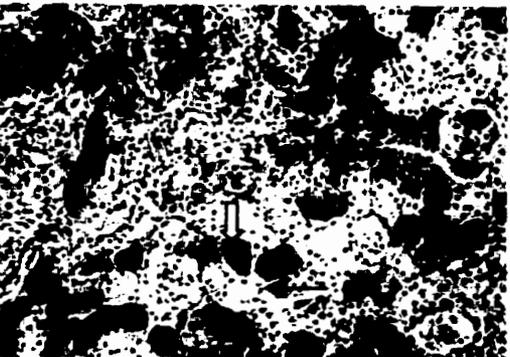
**PF**



**PFR**



**C**

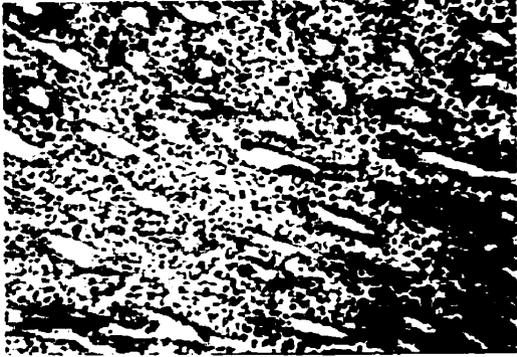


**Negative Control**

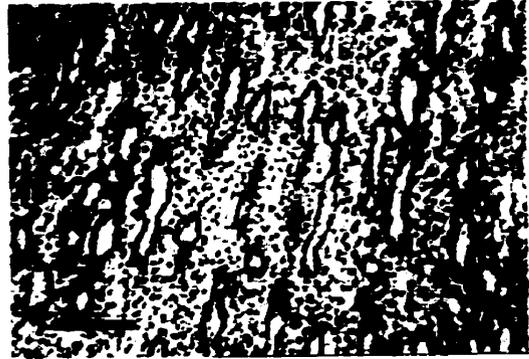


**Figure 34.** Immunohistological staining for metallothionein in the pelvis of rat kidney. There was strong metallothionein staining in the epithelia of large collecting ducts in all treatment groups (ZR, and C not shown). Scale bars at the bottom left corner equal 100  $\mu\text{m}$ .

**ZD**



**PF**

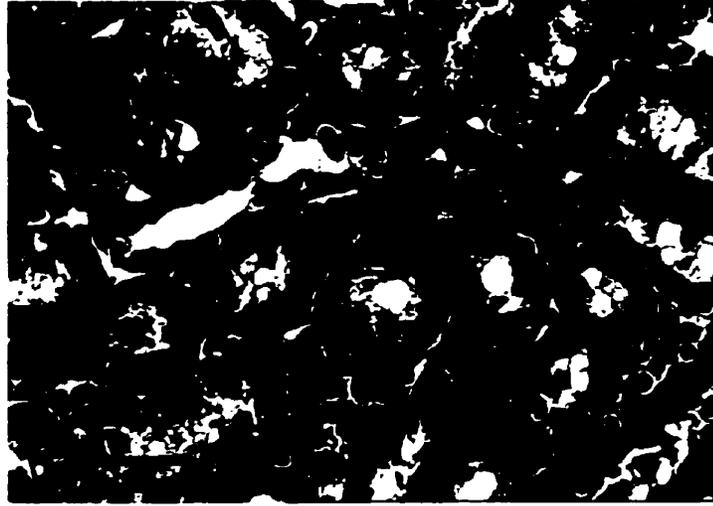


**PFR**

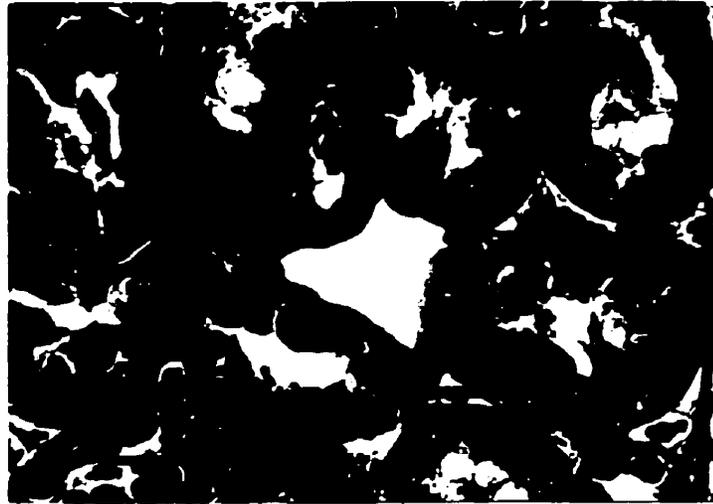


**Figure 35.** Hematoxylin and eosin staining of the cortex of rat kidney. Note the distinct brush border, basally placed nuclei, and the occluded lumen of distal convoluted tubules (arrow) vs. absent brush border, apically placed nuclei, and widely open lumen of distal convoluted tubules (black block arrow). Note also the greater number of nuclei per cross-section of distal convoluted tubules (black block arrow) as opposed to proximal convoluted tubules (arrow). Scale bar at the bottom left corner of (A) equals 50  $\mu\text{m}$ . Scale bar at the bottom left corner of (B) equals 25  $\mu\text{m}$ .

**A**

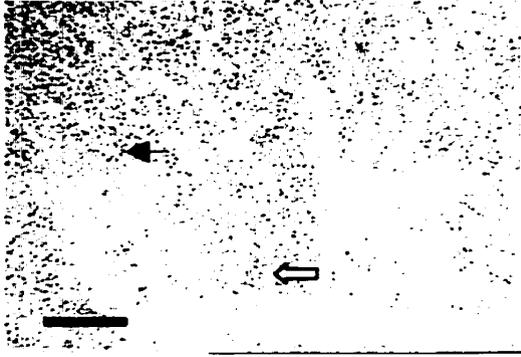


**B**

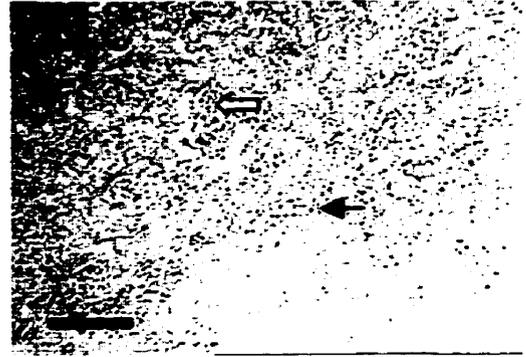


**Figure 36.** Immunohistological staining for Ki-67 in the cortex of rat kidney. Moderate to strong nuclear staining was present in tubular epithelial cells (arrow) of all treatment groups. No staining was present in glomeruli (white block arrow). Note the greater number of darkly stained nuclei in the C group as opposed to the other groups. Scale bars at the bottom left corner equal 100  $\mu\text{m}$ .

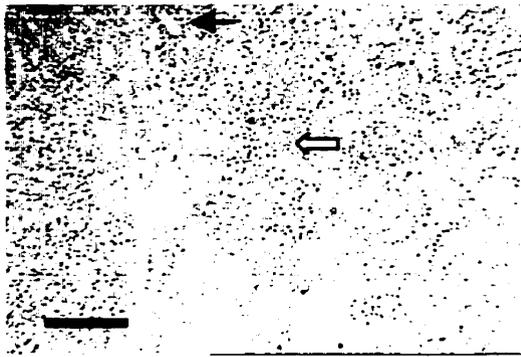
**ZD**



**ZR**



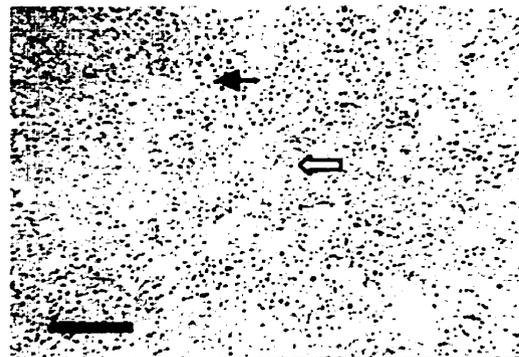
**PF**



**PFR**

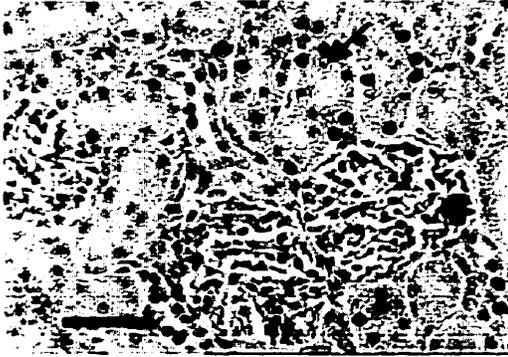


**C**



**Figure 37.** Immunohistological staining for Ki-67 in the cortex of rat kidney. Nuclear staining was present in tubular epithelial cells (arrow) of all treatment groups, and no staining was present in glomeruli (g). Scale bars at the bottom left corner equal 50  $\mu\text{m}$ .

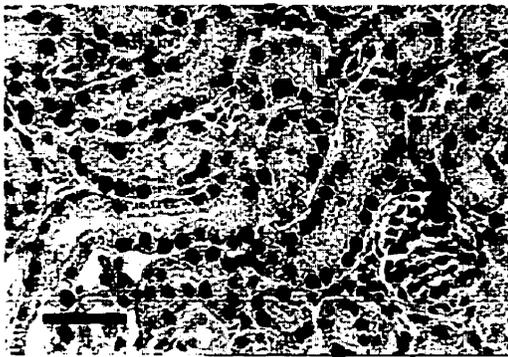
**ZD**



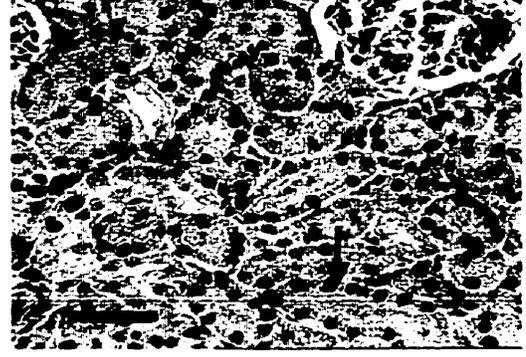
**ZR**



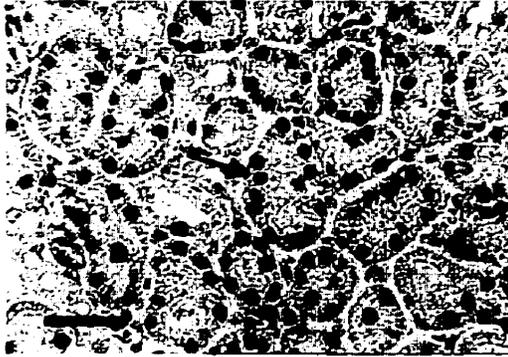
**PF**



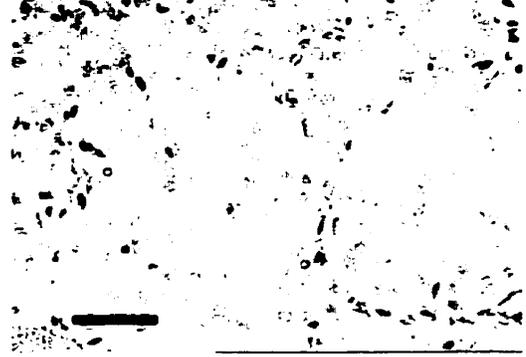
**PFR**



**C**

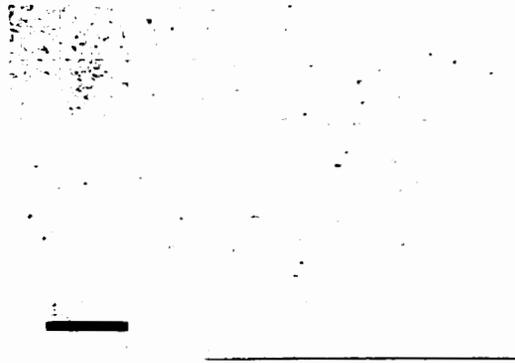


**Negative Control**

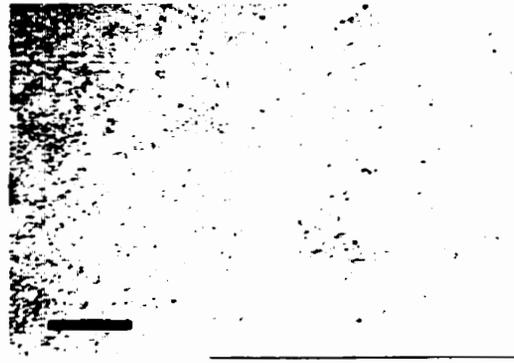


**Figure 38.** Immunohistological staining for Ki-67 in the medulla of rat kidney. Nuclear staining was present in tubular epithelial cells. Note the greater number of darkly stained nuclei in the C group, followed by the PF and PFR groups, as compared with the ZD and ZR groups. Scale bars equal 160  $\mu\text{m}$ .

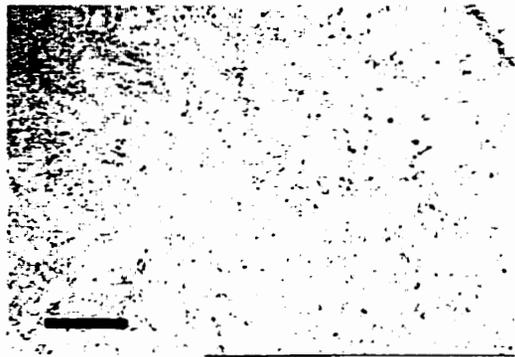
**ZD**



**ZR**



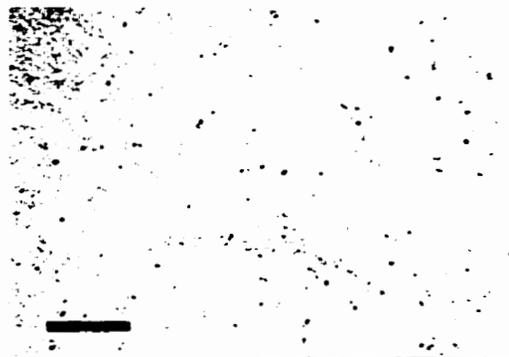
**PF**



**PFR**

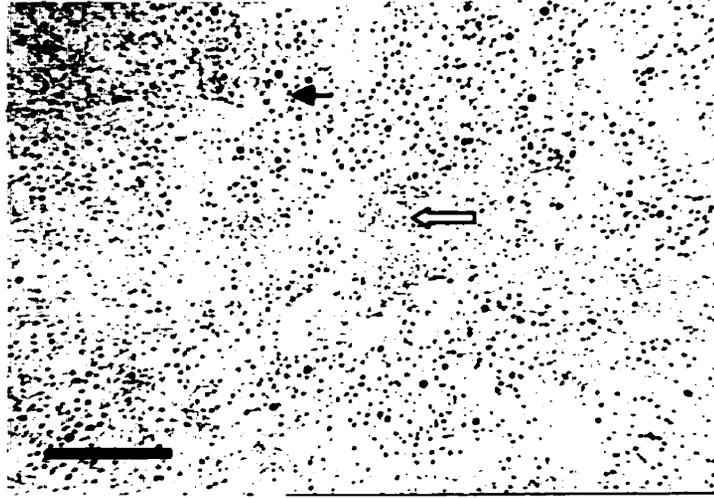


**C**

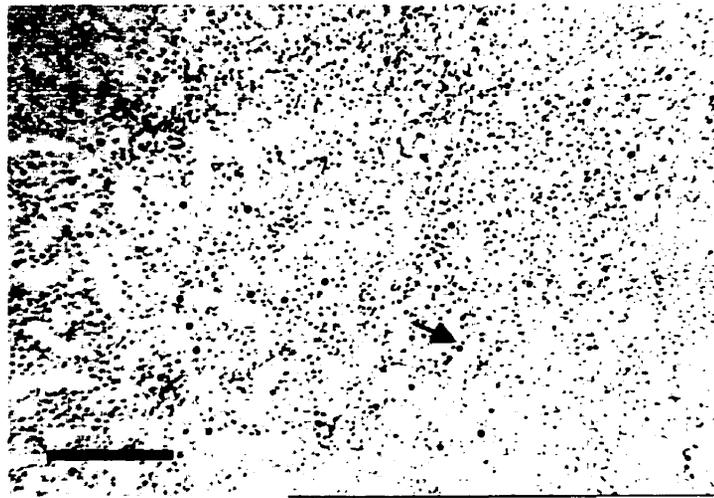


**Figure 39.** Immunohistological staining for Ki-67 and PCNA in kidney of a control rat. Ki-67 staining (A) and PCNA staining (B) was present in tubular epithelial cells (arrow), and staining was absent in glomeruli (white block arrow). Note the greater number of positive nuclei in the Ki-67 stained section as compared with the PCNA stained section. Scale bars equal 160  $\mu\text{m}$ .

**A**



**B**



## V. Discussion

This study was undertaken with the purpose of examining the concentration and immunohistochemical localization of MT in rat tissues in response to zinc deficiency and short-term zinc repletion and comparing these variables with changes in growth and cell cycle activity. The study extended the present knowledge about the interactions between dietary zinc and MT by evaluating the effect of dietary zinc manipulations on MT tissue distribution. It also provided new information about the relationship of zinc and MT status with cell cycle activity and growth. The new clone of anti-Ki-67 antibody, MM1, was used in this study to assess cell cycle activity. To the best of our knowledge, at time of this experiment, no other investigations of Ki-67 immunoreactivity in rat tissue have employed this novel clone. Furthermore, there have not been any studies that examined immunohistochemical localization of Ki-67 in relation to zinc nutrition.

### Growth

During the course of the study, the adequately nourished C group underwent rapid growth and development as evidenced by greater body weight (Figure 5), body and tail length (Figure 8A and B), organ weights (Figure 14A, 23A, 30A), femur weight (Figure 9A and 10A) and epididymal fat pad weight (Figure 11A) than the other groups. In contrast, the deficient groups experienced growth retardation. The difference in body weight between ZR and C groups was already apparent on Day 4 of the study, and on Day 8 of the study, all deficient groups weighed significantly less than the C group (Figure 5). It is interesting to note that on Day 8 of the study, PF and PFR groups still

weighed more than zinc deficient groups. The slower weight gain in zinc deficient rats was probably due to the immediate reduction in feed intake that was observed in ZD and ZR groups within first few days of zinc deficiency. The difference in body weight between zinc deficient and pair-fed rats disappeared on Day 12 of the study, and throughout the rest of the experiment they had similar body weight.

In addition to decreased body weight, zinc deficient and pair-fed groups had reduced body and tail length, and organ, femur and epididymal fat pad weights as compared with the C group (Figures 8A and B, 14A, 23A, 30A, 9A, 10A and 11A) indicating depressed growth. There were no differences between ZD and PF groups for all these parameters with the exception of tail length (Figure 8B) and intestine length (Figure 15) which were greater for the PF group. The body weight, body length, tail length, femur dry weight, and epididymal fat pad, liver, intestine and kidney weights of the ZD group were 54%, 84%, 83%, 78%, 22%, 45%, 55% and 60%, respectively, of the C group. Consistent with lower liver and epididymal fat pad weights, liver and epididymal fat pad weight to body weight ratios of ZD and PF groups were less than the C group (Figure 23A and B). In contrast, although femur and kidney weights of ZD and PF groups were less than the C group (Figure 9A, 10A and 30A), the proportions of body mass taken by these organs were greater in ZD and PF groups (Figure 9B, 10B and 30B). Further, the ZD group had an intestine weight that was less than the C group but its intestine weight to body weight ratio was not different from the C group (Figure 14A and B). The PF group had both intestine weight and intestine weight to body weight ratio that were less than the C group (Figure 14A and B). These findings indicate that zinc deficiency and calorie restriction may influence the growth of liver but not the growth of

femur and kidney. They also suggest that calorie restriction may suppress the growth of intestine to greater degree than zinc deficiency. The reduced epididymal fat pad weight and epididymal fat pad weight to body weight ratio of ZD and PF groups is indicative of energy malnutrition.

In summary, in this study, both zinc deficiency and calorie restriction inhibited the growth of the young rats. Further, both ZD and PF groups experienced the loss of adipose tissue which is characteristic of energy malnutrition. Growth inhibition is a major manifestation of zinc deficiency in weanling rodents and is characteristic of rats and children suffering from nutritional deficiencies (Gallant and Cherian, 1987; Dorub et al. 1991; Ninh et al, 1996; Prescod, 1998). Prescod (1998) fed weanling rats a 1 ppm zinc diet for 3 weeks and found a similar degree of growth suppression to that observed in our study. Also in agreement with our findings, Prescod (1998) observed the loss of adipose tissue in zinc deficient and energy restricted rats, and did not find differences between the two groups in body weight and length, and organ weights. In contrast to our study, Prescod (1998) found no significant differences in tail length between zinc deficient and pair-fed rats. The shorter tail and intestine observed in this study in the ZD group as compared with PF group may suggest an independent effect of zinc deficiency on these parameters. In general, this study and the Prescod (1998) study indicate that the growth suppression in zinc deficient rats results from the combination of zinc deficiency and calorie malnutrition, and zinc status per se does not affect growth. However, both studies used absolute pair-feeding to control for the decreased feed intake of zinc deficient rats. This type of pair-feeding does not account for the differences in growth rates and the feeding patterns of zinc deficient and pair-fed animals making it difficult to

separate the effects of zinc deficiency on growth from that of reduced food intake. A more appropriate type of pair-feeding is relative-pair feeding which involves feeding pair-fed animals the same amount of feed as is consumed by zinc deficient animals on a body weight basis. Using relative pair-feeding, Dorub et al (1991) found that the body weight of pair-fed rats was significantly greater than the zinc deficient rats and was similar to the *ad libitum* controls indicating that the zinc deficiency per se has limiting effect on growth. Another example of the independent effect of zinc on growth is the force-feeding study by Park et al (1985). Force-feeding controls not only for the reduction in food intake but also for different feeding patterns of zinc deficient and pair-fed animals. The study by Park and his colleagues demonstrated that rats force-fed zinc deficient diet for 8 days had significantly reduced body weight as compared with the force-fed and *ad libitum* fed control groups.

ZD and ZR groups, and PF and PFR groups experienced a similar degree of growth retardation during the zinc deficiency phase of the study as evidenced by their similar body weights on Day 16 (Figure 5). The body weights of ZR and PFR groups on Day 17 of the study were not different from the body weights of ZD and PF groups, respectively, on Day 16 of the study (Figure 6) indicating that 24 hour zinc and calorie repletion had no significant effect on body weight. However, when body weights of ZR and PFR groups on Day 16 of the study (before zinc and calorie repletion) were compared with their body weights on Day 17 of the study (after zinc and calorie repletion), a 9% and 17% increase in body weight was observed, respectively (Figure 7A). Similarly, the feed intake of ZR and PFR groups increased from Day 16 to Day 17 of the study by 58% and 72%, respectively (Figure 7B). Comparison of the same groups

over time gives a better indication of the changes due to the dietary treatments as it is more sensitive to the effects of the treatments and eliminates error variance. From this standpoint, the 24 hour zinc and calorie repletion resulted in a rapid increase in feed intake, and a consequent increase in body weight. Body length, tail length, intestine length, and femur, kidney and epididymal fat pad weights of ZD and ZR groups, and of PF and PFR groups were not different from each other (Figures 8A and B, 15, 9A, 10A, 30A and 11A) indicating that the 24 hour zinc and calorie repletion had no effect on these parameters. On the other hand, liver weight and liver weight to body weight ratio of ZR and PFR groups were greater than the ZD and PF groups, respectively (Figure 23A and B). The PFR group also had greater intestine weight and intestine weight to body weight ratio as compared with the PF group (Figure 14A and B). In addition, the PFR group had kidney and femur dry weight to body weight ratios lower than the PF group (Figure 30B and 10B).

Zinc repletion is known to cause a rapid increase in feed intake, and consequently, a rapid increase in body weight of zinc deficient rats. Prescod (1998) observed an increase in growth rate of zinc deficient rats in the first week of zinc repletion. Dorub et al (1991) reported an immediate increase in body weight and growth rate of zinc deficient rats upon zinc repletion but the authors did not provide actual numbers and statistics. In our study, 24 hour zinc repletion did not influence linear growth and most of the other parameters measured as a part of growth assessment. These results are not surprising considering very short repletion time. However, the increases in body weight and feed intake of ZR and PFR groups on Day 16 as compared with Day 17 of the study suggest that 24 hour zinc and calorie repletion had an immediate effect on feed consumption and

growth. The greater liver weight and liver weight to body weight ratio following zinc and calorie repletion, and the greater intestine weight and intestine weight to body weight ratio after calorie repletion may reflect a rapid response of these organs of absorption and storage to changes in feed intake. The lower kidney and femur dry weight to body weight ratios of the PFR group may be a reflection of increased body weight in response to calorie repletion.

## Zinc Status

Serum and femur zinc concentrations were used in this study to assess zinc status of the rats. Femur zinc concentration was measured since serum zinc can be affected by fluctuations in zinc intake and by stress-producing conditions such as starvation and infection (Vallee and Falchuk, 1983; Cousins, 1996), and thus, may not be an accurate reflection of zinc status. In rats, bone zinc represents a metabolically active pool of zinc, and therefore, it is sensitive to changes in dietary zinc levels (Cousins, 1996). The dramatic reduction in both serum and femur zinc concentrations of the ZD group compared to C and PF groups (Figures 12 and 13) confirmed that the experimental diets produced zinc deficiency. The ZD group had serum zinc and femur zinc concentrations that were 23% and 29%, respectively, of the C group. Calorie restriction did not have a significant effect on serum or femur zinc concentrations. The degree of reduction in serum zinc concentration observed in the zinc deficient rats in this study is in agreement with previous studies conducted in this laboratory by Prescod (1998) and Taylor et al (1988). In the Prescod (1998) and Taylor et al (1988) studies, serum zinc concentrations of rats fed zinc deficient diet (< 1ppm zinc) for 3 weeks were 22% and 19% of *ad libitum*

fed controls, respectively. Consistent with the results obtained in our laboratory, Dorub et al (1991) reported that rats fed zinc deficient diet (1.8 ppm zinc) for 2 weeks had serum zinc concentrations that were 19% of *ad libitum* fed controls. While this study and the studies by Taylor et al (1988) and Dorub et al (1991) found no differences in serum zinc concentration between pair-fed and *ad libitum* fed controls, Prescod (1998) reported that the energy restricted group had serum zinc concentrations significantly higher than *ad libitum* fed controls. The author indicated that the high serum zinc concentration of energy restricted group could have been a result of dehydration. The reduction in femur zinc concentration observed in this study was greater than that found in the Prescod study where femur zinc concentration of zinc deficient rats was 35% of the controls. This difference is surprising considering the longer duration of zinc deficiency (3 weeks) in the Prescod (1998) study. Similar to the Prescod study (1998), Wallwork et al (1980) observed a 65% decline in femur zinc concentration of rats fed zinc deficient diet (1 ppm zinc) for 3 weeks. At Day 15 of the Wallwork study, femur zinc concentration of zinc deficient rats decreased by only 56%. The decrease in femur zinc concentration observed in this and other studies may be due to increased release of femur zinc for use by other tissues or decreased zinc uptake by femur due to low plasma zinc (King et al , 2000).

Zinc repletion for 24 hours restored serum zinc concentration but not femur zinc concentration (Figures 12 and 13). Although the femur zinc concentration of the ZR group was lower than the C group, it was significantly higher (23%) than the ZD group. It is noteworthy that as short as 24 hour zinc repletion produced a significant increase in femur zinc concentration. Interestingly, 24 hour calorie repletion resulted in a significant decrease (19%) in serum zinc concentration (Figure 12). This may represent the normal

postprandial decrease in serum zinc (Cousins, 1996), or it is possible that as more calories are supplied zinc is rapidly transported to tissues for growth and repair processes resulting in a transient decrease in serum zinc. The rapid response of serum zinc to the change in zinc content of the diet is consistent with other studies. Richards and Cousins (1976) found that changing the level of dietary zinc from 0.8 ppm to 150 ppm on alternating days caused significant fluctuations in serum zinc of rats. In a study by Dorub et al (1991), normalization of serum zinc concentration was observed within 3 days of zinc repletion. No studies of the effect of short-term zinc repletion on femur zinc concentration are available.

## **Intestine Growth**

The effect of zinc deficiency on cell division should be most obvious in rapidly proliferating tissues. Therefore, the relationship between zinc and intestinal growth was investigated in this study by examining the effect of dietary zinc on intestinal length and weight, and the morphology of intestinal mucosa.

Both zinc deficiency and calorie restriction resulted in impaired growth of the intestine as evidenced by reduced intestinal weight and length in ZD and PF groups as compared with the C group (Figure 14A, 15 and 14B). Consistent with intestine weight, the intestine weight to body weight ratio of the PF group was less than the C group (Figure 14B). In contrast to intestine weight, the intestine weight to body weight ratio of the ZD group was not different from the C group (Figure 14B). These findings may indicate that calorie restriction but not zinc deficiency affects intestinal growth. On the other hand, although ZD and PF groups had a similar intestinal weight, the intestine

length of ZD group was significantly shorter than the PF group (Figure 15) suggesting that zinc deficiency may have a more severe effect on intestinal growth than calorie restriction. In addition to reduced intestinal length and weight, zinc deficient and pair-fed groups had flattened and scarcer villi, and thinner submucosa, muscularis and serosa than *ad libitum* fed controls. These morphological abnormalities are characteristic of atrophic mucosa (Kelly et al, 1976; Tomkins et al, 1993). The appearance of the small intestine sections presented in Figure 18 and 20 in the Results Chapter of these thesis does not reflect true histological differences among the treatment groups since not all of the sections are complete longitudinal sections. These sections were selected with the purpose of demonstrating MT and Ki-67 immunohistochemical localization.

Interrelationships of zinc with intestinal mucosal turnover and function has been previously demonstrated. Experimental animals fed zinc deficient diet exhibit a reduction in jejunal weight, atrophy of jejunal mucosa and impairment of mucosal function (Tomkins et al, 1993). As a consequence they develop watery diarrhea. Zinc supplementation of zinc deficient animals results in a rapid increase in the weight of intestinal mucosa and cessation of diarrhea (Tomkins et al. 1993). Similarly, zinc supplementation has been shown to improve intestinal function and to reduce the severity and duration of diarrhea in undernourished children with persistent and acute diarrhea syndrome (Roy et al, 1992). Increased epithelial regeneration and repair of ultrastructural abnormalities, and significant improvements in diarrhea are also observed in patients with *Acrodermatitis Enteropathica* following treatment with zinc (Moynahan, 1974; Kelly et al, 1976; Vallee and Falchuk, 1993).

The changes in intestinal growth and function observed in zinc deficient animals are also evident in pair-fed controls and undernourished children that do not respond to zinc supplementation (Roy et al, 1992; Bates et al, 1993; Tomkins et al, 1993) making it difficult to separate the effects of zinc deficiency from that of calorie deprivation. In addition, it is known that intestinal mucosal turnover is affected not only by the nutritional and caloric content of a diet but also by the frequency of food intake (Park et al, 1985). Therefore, the use of pair-fed animals in zinc deficiency studies may not be adequate since these animals develop meal-like eating pattern as opposed to nibbling observed in zinc deficient animals. On the other hand, in the study by Park et al (1985), isolated zinc deficiency produced by force-feeding rats zinc deficient diet resulted in decreased mucosal DNA and protein content, and decreased activity of several intestinal enzymes. This indicates that zinc deficiency per se without a decrease in food intake and changes in eating patterns may impair intestinal growth and function.

Consistent with previous studies, zinc deficiency and feed restriction in this study resulted in atrophy of intestinal mucosa as evidenced by morphological abnormalities and reduced intestine weight and length. In contrast to other studies, the zinc deficient rats in this study did not develop diarrhea. Further, zinc repletion in this study did not improve intestine weight or length. It is possible that a 24 hour repletion was not enough time to observe an increase in intestinal growth. In their review, Tomkins et al (1993) reported that the weight of intestinal mucosa in zinc deficient animals increased after 48 hour zinc repletion.

In the present study, calorie repletion increased intestine weight but did not restore it to the weight of C rats (Figure 14A). The increase in intestine weight observed

after calorie repletion, but not after zinc repletion, may indicate that zinc deficiency caused more severe mucosal damage than calorie deprivation and the damage requires more time for repair. However, it is possible that the increased intestine weight of PFR group was the result of increased feed intake, and thus, the increased weight of intestinal contents rather than actual growth of the intestine.

The findings of this study together with the previous studies suggest that zinc may play important role in mucosal repair and gut immunity. However, the results of this study have to be interpreted with caution. The intestinal weight and length were taken before intestinal contents were cleaned, and are measurements of the whole intestine from pylorus to rectum, and thus, may not be accurate. The measurements of small intestine cleaned of luminal contents and taken from pylorus to caecum are more accurate. Ideally, the atrophy of intestinal mucosa should be quantitated by measuring mucosal weight as mg/mm intestine or mucosal mass as DNA/mg/mm intestine (Tomkins et al, 1993).

### **Metallothionein and Zinc Concentrations**

The number of studies that have investigated the relationship between dietary zinc and MT status are limited making the interpretation of our results difficult. Further, the comparison of our results with those of others is complicated by the use of different experimental designs and techniques. While some techniques allow for estimation of specific MT isoforms, others, including  $^{109}\text{Cd}$ -binding assay that was used in this study, measure total MT. Finally, it is known that MT can be affected by environmental

conditions, the age and even the strain of animals making comparisons between studies difficult.

### ***Intestine***

Zinc deficiency had a significant effect on MT concentration (Figure 16B and C) but not on zinc concentration (Figure 16A) in small intestine. Consistent with this observation, no significant correlation was found between intestinal zinc and MT concentrations (Figure 17). Both zinc concentration and MT concentration were not influenced by calorie restriction (Figure 16A, B and C). Intestinal MT concentration of the ZD group was 2.7-fold lower than the C group when measured as ug MT/g tissue, and 2.3-fold lower when measured as ug MT/mg protein. Twenty four hour zinc repletion restored intestinal MT concentration (Figure 16B and C).

A few studies demonstrated similar effects of dietary zinc on intestinal MT as those observed in this study. In the study by Richards and Cousins (1976), feeding rats a 0.8 ppm zinc diet for 24 hours resulted in a reduction of intestinal MT-bound zinc, and repletion with a 150 ppm zinc diet for the next 24 hours significantly increased MT-bound zinc. It is important to notice that in our study, the 24 hour repletion with only 30 ppm zinc in the diet produced a significant increase in MT concentration. Other studies have demonstrated an effect of zinc status on MT gene expression in rat intestine. Cousins and Lee-Ambrose (1992) found that MT mRNA increased in a dose-dependent manner in the intestine of rats fed diets containing 5, 50 and 180 ppm zinc for only 2 hours.

The unchanged intestinal zinc concentration observed in the ZD group (Figure 16A) is in agreement with the study by Reinstein et al (1984) that showed no differences in intestinal zinc concentrations in pregnant rats fed diets containing 1 ppm, 4.5 ppm, 10 ppm and 100 ppm zinc for 21 days. Similarly, in the study by Gallant and Cherian (1987), the intestinal zinc concentrations of rats that were suckled from zinc deficient dams were not different from control rats at day 22 postpartum. The unchanged intestinal zinc concentration of zinc deficient rats may be a consequence of an adaptive increase in zinc absorption and/or a decrease in endogenous fecal zinc losses during zinc deficiency. Enhanced zinc absorption and increased reabsorption of endogenous zinc has been previously demonstrated in zinc deficient rats and humans (Richards and Cousins, 1976; Flanagan et al. 1983; King et al, 2000). In addition, Richards and Cousins (1976) observed an inverse relationship between intestinal MT and  $^{65}\text{Zn}$  absorption. This finding explains the changes in MT concentration observed in our study in response to zinc deficiency and repletion. Richards and Cousins (1976) also found that  $^{65}\text{Zn}$  tracer bound predominantly to MT when rats were fed 150 ppm zinc diet, but the majority of  $^{65}\text{Zn}$  bound to high MW proteins when a 0.8 ppm zinc diet was fed. Our findings together with the previous studies suggest that MT is not involved in zinc absorption but rather plays a role as transient zinc storage when excessive amounts of intestinal zinc are present. These findings also indicate the possible existence of other proteins involved in the absorption and/or transport of zinc. Further support for this idea comes from the recent study by Reeves (1998). Using the MT-null mouse, Reeves (1998) demonstrated that the induction of intestinal MT is not required for the development of copper deficiency in mice fed a high zinc diet. He proposed that the mechanism for copper

inhibition by zinc may involve inhibition or modulation of a copper transporter protein by zinc. Thus, it is possible that zinc and copper share the same mucosal transporters.

In addition to the biochemical measurement of MT, the immunohistochemical localization of MT (discussed in the Metallothionein Immunostaining section of this Chapter) was investigated in this study. While MT in the intestine of ZD group could be quantitated by  $^{109}\text{Cd}$ -binding assay, it was not detected by immunohistochemical techniques. The discrepancy between the results obtained by the two methods may be due to the limitations of Cd-binding assay. The accuracy of Cd-binding assay may be affected by the presence of low molecular weight thiols in a sample. Like MT, low molecular weight thiols are heat stable, and thus, can bind to cadmium in a sample giving an overestimation of MT concentration (Eaton and Cherian, 1991). This idea was supported in our study by detection of MT in the intestine of MT-1 and MT-2 null mouse by Cd-binding assay but not by immunohistochemistry. It is possible that unknown sulfur containing proteins are induced in a MT null mouse giving an overestimation of true MT concentration. Ideally, immunoassays such as radioimmunoassay or enzyme-linked immunosorbant assay are preferable techniques for the estimation of MT concentration. They offer more accuracy and sensitivity, and allow for the determination of specific MT isoforms. These techniques are capable of measuring picomolar concentrations of MT, thus, making possible the determination of MT in plasma and other body fluids (Summer and Klein, 1991).

## *Liver*

Liver zinc concentration was not affected by zinc deficiency but it was significantly influenced by calorie restriction (Figure 24A). The liver zinc concentration of the PF group was significantly higher than other groups and 23% higher than the C group (Figure 24A). Zinc repletion and calorie repletion resulted in a significant reduction in liver zinc concentration (Figure 24A). In the ZR group, liver zinc concentration decreased by 16%, and in the PFR group, it decreased by 19% as compared with the C group (Figure 24A). Similar to liver zinc concentration, liver MT concentration was dramatically influenced by calorie restriction and repletion (Figure 24B and C). The PF group had MT concentrations that were 69% greater than the C group when measured as  $\mu\text{g/g}$  (Figure 24B) and 61% greater than the C group when measured as  $\mu\text{g/mg}$  protein (Figure 24C). Twenty four hour calorie repletion resulted in liver MT concentrations that were similar to the C group (Figure 24B and C). Zinc deficiency did not change liver MT concentration measured as  $\mu\text{g/g}$  (Figure 24B). However, when measured as  $\mu\text{g/mg}$  protein, liver MT concentration of the ZD group was only 39% of the C group and it was restored by 24 hour zinc repletion (Figure 24C). Although the liver MT concentration of ZR group was not significantly different from the C group, it was still only 52% of the C.

In agreement with this study, Wallwork et al (1981) observed unchanged liver zinc concentration in zinc deficient weanling rats fed < 1ppm zinc diet for 15 days, and a 50% increase in zinc concentration in pair-fed controls as compared with *ad libitum* fed controls. Similarly, Sato et al (1984) found slight but not significant decreases in liver zinc concentration in weanling rats fed < 1ppm zinc diet for 14 days. It has been

demonstrated by Wallwork et al (1980) that plasma and liver zinc of zinc deficient rats fluctuates with the feeding cycle and stabilizes with the duration of zinc deficiency. Therefore, it is evident that liver zinc concentration responds to immediate changes in dietary zinc levels but it is not affected by long term zinc deficiency in a young growing rat.

In the Sato et al (1984) study, liver MT-1 concentration of zinc deficient rats decreased to nondetectable levels and there was 10-fold increase in liver MT concentration in the pair-fed rats as compared with *ad libitum* fed controls. These findings are similar to the results of this study, however, we were still able to detect MT in the liver of ZD group, and the magnitude of MT increase in the PF group observed in our study was much less than the results of the Sato et al (1984) study. The differences between our findings and the Sato results may be due to the use of different techniques for MT quantification. Sato et al (1984) determined MT concentration by radioimmunoassay which is a more sensitive and accurate method than the  $^{109}\text{Cd}$ -binding assay used in this study. As described earlier in the Intestine section, the Cd-binding assay may overestimate MT concentration. In addition, Sato et al (1984) measured only the MT-1 isoform while the Cd-binding assay is not isoform specific.

Consistent with the present study and the study by Sato et al (1984), Gallant and Cherian (1987) found that postpartum zinc deficiency resulted in a significant decrease in liver MT of 22 day old rats as determined by  $^{109}\text{Cd}$ -binding assay. In contrast to this study and previous studies, in the Gallant and Cherian (1987) study, the reduction in hepatic MT was paralleled by a decrease in liver zinc concentration. Thus, it appears that

hepatic zinc is affected by zinc deficiency during neonatal period but not when rats are weaned.

The decrease in hepatic MT concentration observed in zinc deficient rats in this and previous studies is also in agreement with the studies that demonstrated a dose-dependent relationship between dietary zinc level and MT mRNA expression (Blalock et al, 1988; Cousins and Lee-Ambrose, 1994). Cousins and Lee-Ambrose (1994) and Blalock et al (1988) observed that MT mRNA increased in proportion to dietary zinc level in rats fed diets containing 5, 30 and 180 ppm zinc for 14 days. In addition, Cousins and Lee-Ambrose (1994) found that feeding the same diets for only 2 hours resulted in a similar dose-dependent relationship between dietary zinc and hepatic MT mRNA, and  $^{65}\text{Zn}$  uptake by liver. The authors observed a significant correlation between dietary zinc level and  $^{65}\text{Zn}$  recovered in liver.

Unchanged hepatic zinc and decreased femur zinc concentration (discussed in Zinc Status section of this Chapter) observed in zinc deficient rats in this and previous studies indicate that during zinc deficiency liver zinc is stored very efficiently at the expense of femur zinc. The stored hepatic zinc may be dispensed by liver for maintenance of basic physiological processes. Further, the reduction in hepatic MT concentration without changes in hepatic zinc concentration during zinc deficiency suggest that at low dietary zinc levels hepatic zinc is not bound by MT. It was reported previously that zinc accumulated by liver is associated with MT only in response to very high dietary zinc levels (> 500 ppm) (Chen et al, 1977)

The increased liver MT concentration accompanied by a reduction in liver zinc concentration observed in this study in response to 24 hour zinc repletion is puzzling and

in contrast with the study by Richards and Cousins (1976) who observed significant increase in hepatic zinc concentration and MT-bound zinc after feeding zinc deficient rats a diet containing 180 ppm zinc for 24 hours. It can be speculated that after short-term zinc repletion with normal levels of zinc, there may be an increase in MT-bound zinc as suggested by increased MT concentration observed in this study, but total hepatic zinc decreases as it is rapidly transported to other tissues for repair and growth processes. On the other hand, total hepatic zinc increases when zinc deficient rats are fed high supplementary levels of zinc.

The high concentration of MT and zinc in the liver of pair-fed rats observed in this and previous studies may be related to feed restriction. It is known that physical stress including starvation results in the induction of hepatic MT synthesis that is believed to be mediated via a stress hormone response (Cousins et al. 1986; Sato et al, 1984). In calorie-restricted animals, it is possible that zinc released by tissue catabolism is sequestered by liver and stored bound to MT. When calories are repleted, zinc is rapidly dispensed from the liver to be utilized in repair and growth processes, and consequently hepatic MT concentration decreases as was demonstrated in this study. Alternatively, the increased hepatic MT concentration in the PF group may be due to decreased turnover rate of the protein during energy restriction. Thus, the observed lowering of elevated MT concentration following calorie repletion may suggest increased MT turnover. Since calorie repletion also resulted in increased body, intestine and liver weights (see the Growth section of this Chapter), the potential increase in MT turnover could be related to the initiation of compensatory growth. The concurrent changes in hepatic zinc and MT concentrations in response to calorie restriction and depletion

support the role of MT in hepatic storage of zinc. This idea is further supported by the significant positive correlation found in this study between liver MT and zinc concentration (Figure 25).

In summary, liver MT is affected by zinc status but liver zinc concentration is not a determinant of hepatic MT concentration at deficient and normal nonsupplemented levels of dietary zinc. Changes in liver MT concentration in response to dietary zinc deficiency and repletion support the idea of a MT function in hepatic zinc storage and/or transport. The concurrent changes in hepatic MT and zinc concentration in response to calorie restriction and repletion further support the role of MT in hepatic zinc storage, and indicate the involvement of MT in stress-induced responses.

### ***Kidney***

In this study, zinc deficiency had a significant effect on both zinc and MT concentrations in rat kidney (Figure 31A, B and C). The ZD group had a renal zinc concentration that was 70% of the C group. The decrease in zinc was paralleled by a decrease in MT (Figure 31B and C). Renal MT concentration of the ZD group was 66% of C when measured as  $\mu\text{g/g}$  and 54% of C when measured as  $\mu\text{g/mg}$  protein. Zinc repletion restored renal MT but not renal zinc concentration (Figure 31A, B and C). Although zinc concentration was not restored upon zinc repletion, the ZR group had a zinc concentration that was significantly greater than the ZD group (Figure 31A). This may be an important finding considering the very short time of zinc repletion. Calorie restriction did not influence renal zinc concentration but it had a significant effect on MT concentration (Figure 31B and C). The PF group had a MT concentration that was 30%

and 25% greater than the C group when measured as  $\mu\text{g/g}$  and  $\mu\text{g/mg}$  protein, respectively. Following calorie repletion, the MT concentration was similar to the C group (Figure 31B and C).

Similar effects of zinc deficiency on renal zinc and MT were observed by Gallant and Cherian (1987) in the kidney of rat pups made zinc deficient by suckling them from zinc deficient dams. At Day 22 postpartum, the zinc deficient pups had renal zinc and MT concentrations that were 60% and 67% of control pups. Thus, the magnitude of decrease in renal MT concentration was almost identical to that found in our study when MT was measured as  $\mu\text{g/g}$  tissue. The greater decrease in renal zinc concentration in the Gallant and Cherian (1987) study may reflect developmental differences between the rats in that study and those used in our study. The younger pups in the Gallant and Cherian (1987) study are expected to be more vulnerable to zinc deficiency due to their rapid growth and development. The results of the study by Blalock et al (1988) are also consistent with our study. These authors found a decrease in renal MT concentration after feeding rats a diet containing 5 ppm zinc for 14 days. The degree of decrease was less than observed in our study. In the Blalock et al (1988) study, zinc deficient rats had a renal MT concentration that was 76% of controls. This difference may be due to a higher level of zinc in the diet and the age of rats. Blalock et al (1988) used adult rats as opposed to weanling rats that were used in our study. Since weanling rats are rapidly growing they may be affected by zinc deficiency more severely. In addition to the decrease in MT concentration, Blalock et al (1988) also observed a 4-fold decrease in MT-1 and -2 mRNA in the kidney of zinc deficient rats as compared with controls. Similarly, Cousins and Lee-Ambrose (1992) found a dose-dependent relationship

between dietary zinc and renal MT-1 and MT-2 mRNA after feeding weanling rats diets containing 5, 30 and 180 ppm zinc for 2 weeks. Feeding the same diets for only 2 hours resulted in a similar differential response in MT mRNA synthesis. Other authors have demonstrated significant decreases in zinc concentration in the kidney of zinc deficient rats as compared with *ad libitum* or pair-fed controls (Bergman and Wing, 1974; Boeckner and Kies, 1986).

The decrease in renal zinc concentration observed in zinc deficient rats in this and previous studies may indicate decreased excretion of zinc by kidney that occurs as a result of adaptation to zinc deficiency. Decreased urinary zinc losses have been previously observed in animals and humans during severe zinc deficiency (King et al, 2000). Consequently, the increase in renal zinc and MT concentrations observed in this study after zinc repletion may be due to increased availability of zinc for excretion. Alternatively, it may reflect increased zinc retention. Similarly, increased MT concentration found in this study in the kidney of PF rats may indicate that zinc released during tissue catabolism is stored by renal MT or is eliminated by renal excretion. In future studies, zinc concentration in urine should be measured to see if urinary zinc losses are affected by zinc or calorie restriction.

Our findings together with previous studies indicate that renal zinc and MT are influenced by dietary zinc intake. Changes in renal zinc concentration in response to alterations in dietary zinc levels are paralleled by changes in renal MT concentration and its mRNA, suggesting that renal MT is synthesized in response to dietary zinc. The relationship between zinc status and MT status was further strengthened in this study by the finding of significant positive correlation between the two parameters (Figure 25).

Based on our results and those of others, it is plausible that MT plays role in renal zinc metabolism by excretion and/or detoxification. By binding zinc, MT may control urinary zinc loss or may prevent damage to the urinary tract when excess zinc is present.

In summary, the findings of this study indicate that during zinc deficiency, as an adaptive mechanism, absorption of zinc in small intestine may be increased while excretion by kidney may decrease, and upon zinc repletion these processes are reversed. Our results also suggest that in liver, during zinc deprivation, zinc is stored very efficiently at the expense of femur zinc, possibly to be used for basic metabolic processes. After zinc repletion, hepatic zinc may be dispensed by liver to other organs for repair and growth related functions. Further, this study has demonstrated that MT concentration in small intestine, liver and kidney is sensitive to changes in dietary zinc and calorie intake, supporting the role of MT in zinc storage and elimination, and the involvement of MT in stressed induced responses. The decline in hepatic MT following calorie repletion accompanied by the increase in body weight and liver and intestine weights may imply MT involvement in growth processes.

### **Metallothionein Immunostaining**

Previous immunohistochemical studies of MT have either focused on developmental or injury related changes in MT tissue localization and distribution, or have investigated MT localization in tissues from cadmium-treated rats (Nishimura et al, 1989; Nartey et al, 1987; Danielson, 1982). This study is the first to examine MT immunolocalization in response to dietary zinc. Therefore, the comparison of our

findings with other studies is limited, and the interpretation of results is based mostly on speculations. However, the cell type specific MT localization and the pattern of its distribution observed in this study in the small intestine, liver and kidney of control rats is, in general, consistent with the results of previous immunohistochemical studies.

### ***Intestine***

In this study, zinc deficiency resulted in the absence of MT staining in small intestine, but MT staining was detected following 24 hour zinc repletion (Figure 18). Calorie restriction did not affect MT staining (Figure 18). Thus, strong nuclear and cytoplasmic MT staining of Paneth cells was observed in small intestine of all treatment groups with the exception of zinc deficient group (Figure 18). Similarly, cytoplasm and nuclei of surface epithelial cells stained in all groups but the ZD group (Figure 18). MT staining of epithelial cells was localized to the proliferative region of villi at the base of intestinal crypts. In addition to MT localization in epithelial and Paneth cells, MT was detected in M-cells of lymphoid nodules (Figure 19). The MT staining in M-cells was not affected by dietary treatments.

The cell-type specific localization of MT observed in the present study in rat small intestine is in agreement with other studies (Nishimura, 1989; Danielson, 1982) with the exception of MT detection in M-cells. Previous studies did not report MT localization in M-cells.

MT has been implicated to function in the regulation of zinc metabolism, heavy metal detoxification and protection against oxidative and chemical stress (Bremner, 1991; Templeton and Cherian, 1991). Further, MT was proposed to be involved in cellular

defense processes and to play a role in cell proliferation (Kägi, 1991; Nishimura, 1989). Similarly, zinc is known to be essential for cell proliferation and proper functioning of immune system (Vallee and Falchuk, 1993; Keen and Gershwin, 1990), and its role as an antioxidant is well established (Bray and Bettger, 1990). The changes in MT staining of surface epithelial cells observed in this study in response to dietary zinc deficiency and repletion support the role of MT in zinc storage, and the function of MT and zinc in toxin or heavy metal protection. The pattern of MT distribution in epithelial cells where it was predominantly localized to the proliferative region of villi as opposed to the tip of villi does not favor the idea of MT function in zinc absorption, but may rather imply the role of MT and zinc in cell proliferation and mucosal turnover. The observed changes in MT staining of Paneth cells in response to dietary zinc manipulations further support the role of MT and zinc in the heavy metal protection, and also suggest the involvement of zinc and MT in gut immunity. Paneth cells are known to accumulate Cu (Bloom and Fawcett, 1994) and they may play important role in the elimination and storage of heavy metals. Paneth cells have also been implicated in the regulation of microbial gut flora. They control intestinal pathogens by continuously secreting lysozyme (Bloom and Fawcett, 1994), and they have been observed to phagocytize and digest certain bacteria and protozoa (Bloom and Fawcett, 1994). Some Paneth cells contain IgA, which suggests their cooperation with antibody producing immune cells of lamina propria (Bloom and Fawcett, 1994). In addition, patients with *Acrodermatitis Enteropathica* show structural abnormalities in Paneth cells that disappear following zinc supplementation (Kelly et al, 1976). The detection of MT staining in M-cells of lymphoid nodules further supports the role of zinc and MT in gut immunity. M-cells are specialized epithelial cells covering

lymphoid nodules in the intestine and take part in antigen processing and its transport to immune cells in lymphoid nodules (Bloom and Fawcett, 1994).

The immunohistochemical localization of MT in the intestine of all groups but the ZD group reflected the MT quantification by Cd-binding assay. Similar to immunohistochemical results, intestine MT concentration increased after zinc repletion and was not affected by calorie restriction. In contrast to immunohistochemical results, although intestine MT concentration decreased in response to zinc deficiency, it was still detectable by the Cd-binding assay. This difference is likely due to the overestimation of MT concentration by the Cd-binding assay as discussed in the Metallothionein and Zinc Concentrations section of this Chapter.

### *Liver*

In this study, liver MT immunolocalization was affected by zinc deficiency but was not influenced by 24 hour zinc repletion as evidenced by the lack of MT staining in hepatocytes of the ZD and ZR groups as compared with weak staining in the C group (Figure 26). Further, the intensity of MT staining and the pattern of its distribution were influenced by calorie restriction and repletion. MT staining was strong in hepatocytes of PF group and moderate in the hepatocytes of PFR group (Figure 26). In addition, while MT staining had a scatter pattern in the liver of the C group, it was concentrated around central veins in the PF and PFR groups (Figure 26).

The weak MT staining in hepatocytes of the C rats indicates low basal level of MT in adult liver and is consistent with other immunohistochemical studies (Nishimura, 1989; Nartey, 1987). The absence of MT staining in the liver of ZD rats suggests that

MT synthesis decreases in response to low dietary zinc levels and this is in agreement with the study by Sato et al (1984) that did not detect MT in the liver of zinc deficient rats using radioimmunoassay. In this and other studies that measured hepatic MT concentration in zinc deficient rats by Cd-binding assay, MT concentration was reduced but still detectable (Gallant and Cherian, 1987). This again points to the limitations of Cd-binding assay, as discussed earlier. The strong MT staining in the liver of PF rats reflects the results of Cd-binding assay obtained in this study and is consistent with the study by Sato et al (1984) that demonstrated increased MT concentration in pair-fed and starved rats. The increased hepatic immunostaining and concentration of MT during calorie deprivation indicates a stress induced response in MT synthesis (as discussed in the Metallothionein and Zinc Concentration section of this Chapter). Further, the changes in MT immunostaining in response to calorie restriction and depletion support the role of MT in hepatic storage of zinc. The localization of MT around the central veins in the livers of PF and PFR groups may be suggestive of a zinc transport or excretory function for MT. The distribution around central veins may also reflect functional aspects of the zonation of hepatic lobules. Hepatocytes in the area surrounding central veins are specialized in drug and lipid metabolism and glycolysis (Bloom and Fawcett, 1994). Therefore, the localization of MT around the central veins may be linked to detoxification processes or energy metabolism. Strong MT staining was demonstrated previously in the livers of fetal and neonatal rats, and cadmium-treated rats (Nishimura, 1989; Nartey, 1987; Danielson, 1982). However, the specific distribution around central veins that was observed in this study in the PF and PFR rats was not observed in other studies.

## ***Kidney***

Immunohistochemical localization of MT in rat kidney demonstrated strong staining in epithelial cells of renal convoluted tubules in the AL, PF and PFR groups, (Figure 33). In contrast, in the ZD and ZR groups, the staining was weak and moderate, respectively (Figure 33). In all groups, the staining was present predominantly in the epithelia of proximal convoluted tubules as opposed to distal convoluted tubules (Figure 33). In addition to the differences in the intensity of MT staining, changes in MT distribution were observed in response to dietary zinc manipulations. In AL, PF and PFR rats, the staining was localized in the entire cortex and some staining was also present in the medulla (results not shown). In contrast, the ZD and PFR groups showed staining only in the outer rim of the cortex and no staining in the medulla (results not shown). Further, all groups with the exception of ZD group had some staining in the lumina of renal tubules (Figure 33) and all groups showed strong staining in the epithelia of large collecting ducts in the lower portion of renal pelvis (Figure 34).

MT localization in renal epithelial tubular cells, in the epithelia of collecting ducts and in the lumina of tubules indicates involvement of MT in metal excretion/detoxification. Similar to our study, strong MT staining in renal epithelial tubular cells and in the lumen of some tubules was previously observed in rats treated with CdCl<sub>2</sub> (Danielson et al, 1982). The reason for the presence of strong MT staining in the collecting ducts of all treatment groups is not clear and was not reported in previous studies (Nishimura, 1989; Nartey, 1987; Danielson et al, 1982). The significance of morphological abnormalities in the structure of collecting ducts observed in this study in

ZD and PF groups but not in the PFR group (see the Kidney section of Chapter IV and Figure 34) is not known.

The changes observed in the present study in the intensity of MT staining and pattern of its distribution in response to dietary zinc indicate that renal MT synthesis is affected by zinc status and support the role of MT in zinc storage and/or excretion. The localization of MT predominantly in proximal tubules further supports this idea since zinc is known to be excreted mostly through secretion from proximal tubules (Vallee and Falchuk, 1993). The weak staining observed in the renal tubules of zinc deficient rats localized only to the cortex as opposed to strong staining in the kidney of the control rats that was found both in the cortex and medulla may indicate decreased zinc excretion during zinc deficiency. Consequently, the increase in the intensity of staining after zinc repletion may suggest increased renal zinc excretion or increased zinc retention.

The changes in the intensity of MT immunostaining were paralleled by similar changes in renal MT concentration (determined in this study by  $^{109}\text{Cd}$ -binding assay) further supporting the involvement of MT in zinc metabolism. However, the immunostaining demonstrated differences between ZR and C groups with the ZR group showing less intense staining than the AL group. On the other hand, the MT quantification by the  $^{109}\text{Cd}$  -binding assay did not reveal significant differences in renal MT concentration between those two groups. The difference may be due to the overestimation of MT concentration by the  $^{109}\text{Cd}$ -binding assay. On the other hand, calorie restriction resulted in the increased renal MT concentration as compared with the C group, but no differences were observed between the PF and C groups in MT

immunostaining. This discrepancy may be due to the subjective assessment of MT immunostaining that does not allow for differentiation of subtle differences by eye.

In summary, MT immunolocalization and its tissue distribution in small intestine, liver and kidney are affected by zinc status. In addition, calorie restriction influences MT immunoexpression and its distribution in liver and kidney. The presence of MT in hepatocytes, intestinal epithelia and renal tubular epithelia, and the changes in MT immunolocalization in response to dietary zinc support the role of MT in zinc storage, excretion and possibly transport. In addition, the differential distribution of MT in the small intestine in response to dietary zinc suggest that zinc and MT may be involved in the protection against toxins/heavy metals, gut immunity and intestinal mucosal turnover.

### **Ki-67 Immunostaining**

The use of antibodies to Ki-67 is mostly limited to histopathology, particularly cancer research and studies of hypertrophic conditions (Grigioni et al, 1989; Hall et al, 1988; Schwartz et al, 1989; Shepherd et al, 1988). The findings of these studies show large variability in Ki-67 staining between different tissue types, different pathologies and stages of disease, and even within the same tissue. Reports of the use of Ki-67 in normal healthy tissues are scarce. Until recently, only antibodies to human Ki-67 were available, and their use was limited to frozen tissue sections. The anti-Ki-67 antibody (clone MM1) used in this study has been shown to cross-react with rat tissue (personal communication with a technical representative at Novocastra company) and can be used on paraffin sections after antigen retrieval treatment. However, there are no published

reports to our knowledge on the use of the clone MM1 on rat tissue. Further, this study is the first one that investigates Ki-67 immunolocalization under different dietary treatments. For all these reasons, it is difficult to compare our study with previous studies and make interpretations of our results. Therefore, the following discussion is mostly based on speculations.

### *Intestine*

In small intestine, the pattern of Ki-67 immunostaining was consistent with the organization of functional compartments of intestinal epithelium. As expected, strong nuclear staining was observed in surface epithelial cells in proliferative region of villi (Figure 20) and in lymphoid cells in germinal centers of lymphoid nodules (Figure 21) reflecting high proliferative activity of these cells (Bloom and Fawcett, 1994). Only a few Paneth cells stained for Ki-67, the observation consistent with the nature of these long-lived cells (Bloom and Fawcett, 1994). However, there were no differences between treatment groups in the intensity or the pattern of Ki-67 staining indicating that the zinc and calorie content of experimental diets had no effect on Ki-67 expression in this organ. For comparison, selected intestine sections were also stained for PCNA. The pattern and intensity of the PCNA staining was similar to that observed for Ki-67 (Figure 22) confirming the specificity of anti-Ki-67 antibody used in this study.

### *Liver*

In contrast to the observations for small intestine, the detection of Ki-67 staining in the majority of hepatocytes in livers of all treatment groups (Figure 27) was

unexpected. Hepatocytes have a long life span and are presumed to have low proliferative activity under normal conditions (Bloom and Fawcett, 1994). In this study, the quantification of Ki-67 positivity by computer-aided nuclear counting demonstrated that the percentage of cells with strong Ki-67 staining ranged from 0.66% in PF group to 5.52% in ZR group, and was 5.31% in C group (Figure 29B). These findings are similar to the results obtained by Grigioni et al (1989) for human liver. In the Grigioni study (1989), 5% hepatocytes stained for Ki-67 in normal human liver and in human liver with benign lesions. However, when the total number of Ki-67 positive hepatocytes were determined in the present study, the staining ranged from approximately 60-70 % (Figure 29A), well above the % staining reported by Grigioni and his colleagues for normal human liver. The percentage of total Ki-67 positive cells that was obtained in this study was even higher than the % staining reported by Grigioni et al (1989) in liver with hepatocellular carcinoma. In hepatocellular carcinomas, the percentage of labeled cells ranged from 15-50%. However, Grigioni et al (1989) studied Ki-67 immunorexpression in livers from adult humans as opposed to the tissues from young growing rats that were used in our study. Therefore, it is possible that the abundant Ki-67 staining observed in this study in livers of young rats reflects developmental immaturity.

There was a discrepancy in our study between the subjective assessment of Ki-67 immunostaining by light microscopy and the computer-aided determination of the percentage of Ki-67 stained cells that further complicates the interpretation of the results. Upon microscopic examination, ZD, ZR, PF and PFR groups showed similar light to moderate nuclear Ki-67 staining in the majority of hepatocytes, and these groups had a few hepatocytes with darkly stained nuclei. In contrast, the AL group had numerous

hepatocytes with darkly stained nuclei (Figure 27). On the other hand, the percentage of Ki-67 positive cells with strongly stained nuclei by computer-aided determination was lowest in PF group, followed by ZD, PFR, C and ZR groups, but statistically, only the PF group was significantly different from ZR, PFR and C groups (Figure 29B). Similarly, the PF group had the lowest percentage of total Ki-67 positive cells, and it was significantly different from ZR and PFR groups (Figure 29A). Therefore, the subjective assessment of Ki-67 staining by microscopic examination indicates that zinc deficiency and calorie restriction may have a negative effect on cell cycle activity of hepatocytes resulting in slower cycling cells while there is no effect of 24-hour zinc or calorie repletion on the cell cycle activity. On the other hand, the results of computer-aided nuclear counting of Ki-67 positive cells indicate that only calorie restriction may result in slower cycling hepatocytes, and that calorie repletion may increase the cell cycle activity. However, although the percentage of Ki-67 positive cells with strong nuclear staining in the ZD group was not significantly different from the C and ZR groups, it was only 40% and 38% of the C and ZR groups, respectively. This difference, although statistically nonsignificant, may have biological significance.

Both methods that were used in this study to assess Ki-67 staining (the subjective examination by light microscopy and the computer-aided nuclear counting) have their limitations. The microscopic examination by eye does not allow for the discrimination of subtle differences between treatment groups, and the computer-aided nuclear counting is a subject to subjective threshold setting by an evaluator, and thus, can potentially introduce human error within a given threshold setting. To increase reliability and

accuracy of results, the quantification of Ki-67 positive cells can be performed by more than one evaluator with inter-observer variation assessment.

### *Kidney*

In kidney of all treatment groups, nuclei of the majority of renal tubular epithelial cells of the cortex stained for Ki-67 with varying intensity (Figure 36 and 37), and some staining was also observed in the medulla (Figure 38). No Ki-67 staining was present in glomeruli (Figure 36 and 37). Since renal tubular cells have a long life span (Bloom and Fawcett, 1994), the extensive staining observed in this study is puzzling and in contrast with the study by Hall et al (1988) who did not find any Ki-67 positive nuclei in the cortex or medulla of normal or atrophic human kidney. The authors detected positive Ki-67 staining only in the diseased kidney, especially in biopsies with tubulo-interstitial diseases. The Ki-67 positivity correlated with the probability of tubular regeneration indicating that Ki-67 is expressed at times of active proliferation. Similar to our findings in rat kidney, the staining was localized in nuclei of tubular epithelial cells, and no staining was present in glomeruli. However, the % of Ki-67 stained cells ranged from only 1-9%. Although the % of Ki-67 stained cells was not determined in this study, it was evident upon microscopic examination that the number of Ki-67 positive cells was much more than that determined by Hall and his colleagues. In addition, Hall et al (1988) investigated Ki-67 immunoexpression in adult human kidney. Therefore, the abundant Ki-67 staining observed in this study in kidney of young growing rats may be due to developmental immaturity.

On the other hand, *in vitro* studies have shown that nutritional deprivation can arrest cells in any phase of the cell cycle, and that zinc is required in early G<sub>1</sub> and G<sub>1</sub>/S transition (Hunt and Murray, 1993; Chesters et al, 1990; Chesters et al, 1989). Consistent with cell cycle experiments, our study showed differences in the intensity and pattern of Ki-67 staining in rat kidney in response to dietary treatments. In kidney cortex, the C group had more darkly stained nuclei in renal tubular epithelia than zinc deficient and pair-fed groups (Figure 36), suggesting greater cell cycle activity in renal epithelia of nutritionally adequate rats as opposed to deficient rats. In the medulla, the intensity of staining and the number of stained nuclei were lowest in ZD and ZR groups, moderate in PF and PFR groups and highest in the C group (Figure 38), indicating again greater cell cycle activity in renal epithelia of the C group, followed by pair-fed groups and zinc deficient groups. However, zinc and calorie repletion had no apparent effect on the intensity or the pattern of Ki-67 staining (Figure 38). It is likely that 24-hour repletion was not an adequate time to cause changes in cell cycle activity of renal tubular cells.

The PCNA staining was performed in this study on selected kidney sections. Although less extensive than the Ki-67 staining, the PCNA staining was positive confirming specificity of the anti-Ki-67 antibody used in this study (Figure 39).

These findings indicate that the cell cycle activity of renal tubular cells may be affected by zinc deficiency and calorie restriction. It is also possible that zinc deficiency has a greater negative effect on cell cycle activity in the tubular epithelial cells of medulla than calorie depletion.

In summary, this study demonstrated extensive Ki-67 staining in rat small intestine, liver and kidney. Ki-67 immunolocalization in the small intestine was not affected by dietary treatments. In liver and kidney, different intensities and patterns of Ki-67 staining were observed in zinc deficient and pair-fed groups as compared with the control group. This finding may indicate alterations in the cell cycle activity in response to zinc deficiency and calorie restriction. Since Ki-67 expression peaks at M and G<sub>2</sub>, it can be further speculated that the numerous darkly stained nuclei found in the liver and kidney of control rats may indicate high G<sub>2</sub> and M activity. On the other hand, moderate Ki-67 staining in majority of nuclei, with few darkly stained nuclei, in the liver and kidney of zinc deficient and pair-fed rats, may indicate slowly cycling cells staying longer in G<sub>1</sub> or arrested in G<sub>1</sub>.

However, the findings of this study have to be interpreted with caution.

Firstly, it is possible that the results of this study provided an overestimation of Ki-67 expression. The heat pretreatment used for the antigen unmasking could have been too long resulting in overstaining. On the other hand, the unexpected abundant Ki-67 staining may be a result of very sensitive secondary antibody system used in this study, and may represent true Ki-67 antigen expression in developmentally immature rat tissues.

Secondly, several in vitro studies demonstrated that in nutritionally deprived cells Ki-67 staining does not correlate with other indices of cell proliferation. For example, Verheijen et al (1989) showed the absence of Ki-67 staining in nutritionally deprived leukaemic cells that were in S, M and G<sub>2</sub> as assessed by flow cytometry. On the other hand, Baisch and Gerdes (1987) found that the Ki-67 staining overestimated the growth

fraction of nutritionally deprived cells as compared with stathmokinetic measurements with colchicine and bromodioxuridine labeling.

Finally, a more reliable and complete assessment of the cell cycle activity should employ double labeling with another cell cycle associated marker in addition to Ki-67. However, that was not feasible given the time constraints for this project.

## **VI. Summary and Conclusions**

### **Growth during zinc and calorie deficiencies and after zinc and calorie repletion**

- Zinc deficiency and calorie restriction resulted in growth retardation and less adipose tissue.
- 24 hour zinc and calorie repletion produced a marked increase in feed intake, and an increase in body weight as evidenced by the results of ZR and PFR groups on Day 16 of the study as compared with Day 17.
- Zinc and calorie deficiencies impaired the growth of small intestine as evidenced by reduced weight and length as compared with the C group, and the atrophy of intestinal mucosa. Zinc deficiency had a more severe effect on intestinal length.
- Calorie repletion for 24 hours, but not zinc repletion, resulted in an increased weight of the intestine suggesting a more severe effect of zinc deficiency on intestinal growth than calorie restriction.
- Zinc and calorie deficiencies resulted in lower liver weights as compared with the C group. Liver weight increased following 24 hour zinc and calorie repletion indicating a rapid response of liver to changes in feed intake and calorie intake.

### **Zinc Status**

- The zinc deficient diet (< 1ppm zinc) was associated with reduced serum and femur zinc concentrations compared to the C group indicating zinc deficiency.
- Zinc repletion for 24 hours increased but did not restore femur zinc concentration, and restored serum zinc concentration.

- Calorie restriction had no effect on serum or femur zinc concentration.

### **Tissue MT and zinc concentrations**

#### *Zinc deficiency and repletion*

- Zinc deficiency resulted in the reduction of intestinal, hepatic and renal MT concentrations, and decreased renal zinc concentration as compared with the C group. It did not change intestinal and hepatic zinc concentrations.
- Zinc repletion for 24 hours restored intestinal, hepatic and renal MT concentrations, increased renal zinc concentration, and reduced hepatic zinc concentration.

These findings indicate that during zinc deficiency, absorption of zinc in the small intestine increases while excretion by the kidney decreases, and upon zinc repletion those processes are reversed. They also suggest that during zinc deficiency, liver zinc is stored very efficiently, and following zinc repletion it is rapidly dispensed, possibly to be used by other tissues for growth and repair. The lower intestinal MT and unchanged intestinal zinc concentrations in response to zinc deficiency indicate that MT is not involved in zinc absorption, and that intestinal zinc is not associated with MT at low dietary zinc levels. Similarly, the reduction in hepatic MT concentration without changes in hepatic zinc concentration in response to zinc deficiency suggest that at low dietary zinc levels, hepatic zinc is not associated with MT.

### *Calorie restriction and repletion*

- Calorie restriction did not influence zinc or MT concentration in small intestine.
- Calorie restriction resulted in higher MT concentration in kidney, and markedly greater MT and zinc concentrations in liver as compared with the C group. These increases were reversed by 24 hour calorie repletion.

The elevations in liver zinc and MT concentrations in response to calorie restriction may reflect a stress induced response in MT synthesis and hepatic zinc uptake, and support the role of MT in stress induced processes. Alternatively, the changes in hepatic MT concentration during calorie restriction and repletion may suggest alterations in the MT turnover rate. A possible increase in MT turnover following calorie repletion, as suggested by the decreased MT concentration, accompanied by increases in body, liver and intestine weights may imply a relationship between MT and growth.

The results of this study support the role of MT in zinc storage, excretion, and possibly transport.

### **Metallothionein Immunolocalization**

This study is the first to report differential MT localization in rat tissue in response to dietary manipulations.

### *Zinc deficiency and repletion*

- Zinc deficiency resulted in the absence of MT staining in small intestine, and the absence of staining or weak staining in liver and kidney.
- Following zinc repletion, strong MT staining was present in Paneth cells and some staining was also observed in surface epithelial cells.
- After zinc repletion, moderate MT staining was demonstrated in renal tubular epithelial cells.
- Zinc repletion did not affect MT staining in liver.

The differential MT staining in small intestine, liver and kidney in response to dietary zinc manipulations supports the function of MT in zinc metabolism, and indicates a role for zinc and MT in gut immunity and mucosal turnover.

### *Calorie restriction and repletion*

- Calorie restriction and repletion had no effect on MT staining in small intestine and kidney.
- In liver, calorie restriction resulted in strong MT staining that was localized around portal veins. The staining diminished after calorie repletion.

The differential MT staining in liver in response to calorie restriction and repletion support the involvement of MT in stressed induced responses, and the role of MT in hepatic zinc storage and disposition.

Independent of dietary treatment, MT staining was detected in M-cells of lymphoid nodules in small intestine, and in epithelia of collecting ducts in kidney. The detection of MT in M-cells gives further support for the role of zinc and MT in gut immunity. The interpretation for presence of MT staining in the epithelia of collecting ducts of all treatment groups is unclear. MT immunolocalization in M-cells and the epithelia of collecting ducts has not been previously reported.

### **KI-67 Immunolocalization**

This study is the first to investigate Ki-67 immunolocalization in relation to zinc nutrition.

- In small intestine, strong Ki-67 staining was present in the proliferative region of villi and in germinal centers of lymphoid nodules independent of dietary treatments.
- In liver, zinc deficiency and calorie restriction resulted in fewer darkly stained nuclei compared with the C group as determined by microscopic examination, and there was no effect of zinc or calorie repletion on the Ki-67 expression. The results of nuclear counting indicate that only calorie restriction resulted in a lower % of darkly stained nuclei, and following calorie repletion, the % of darkly stained nuclei was similar to the C group.
- In kidney, zinc deficiency and calorie restriction resulted in fewer darkly stained nuclei in the cortex, and zinc deficiency also resulted in less darkly stained nuclei in the medulla. Zinc and calorie repletion did not affect Ki-67 immunostaining.

These findings may indicate that zinc and calorie deficiencies have no effect on the cell cycle activity in small intestine but may cause cell cycle disturbances in liver and kidney.

### **Future Research**

- Effects of dietary zinc manipulations on the cell cycle activity need to be further examined by other cell cycle associated markers in addition to Ki-67, and/or by other indices of cell proliferation.
- More studies are required to investigate effects of zinc status on the time course for the response of tissue MT concentration and distribution to zinc deficiency and repletion.
- Mechanisms that maintain hepatic and intestinal zinc concentrations during zinc deficiency need to be elucidated.
- Studies are warranted that would examine the effects of protein deficiency and combined zinc and protein deficiency on MT status.

## VII. References

- Alison, M.R. (1995) Assessing cellular proliferation: What's worth measuring? *Human Exp. Toxicol.* 14:935-944.
- Baisch, H. & Gerdes, J. (1987) Simultaneous staining of exponentially growing versus plateau phase cells with the proliferation-associated antibody Ki-67 and propidium iodide: Analysis by flow cytometry. *Cell Tissue Kinetics* 20:387-391.
- Bates, C.J., Evans, P.H., Dardenne, M., Prentice, A., Lunn, P.G., Northrop-Clewes, C.A., Hoare, S., Cole, T.J., Horan, S.J., Longman, S.C., Stirling, D. & Aggett, P.J. (1993) A trial of zinc supplementation in young rural Gambian children. *Br. J. Nutr.* 69:243-255.
- Bergman, B. & Wing, K.R. (1974) The turnover of Zn in rats fed a zinc-deficient diet. *Acta Physiol. Scand.* 92:451-464.
- Bettger, W.J. & O'Dell, B.L. (1993) Physiological roles of zinc in the plasma membrane of mammalian cells. *J. Nutr. Biochem.* 4:194-207.
- Blalock, T.L., Dunn, M.A. & Cousins, R.J. (1988) Metallothionein gene expression in rats: Tissue-specific regulation by dietary copper and zinc. *J. Nutr.* 118:222-228.
- Bloom & Fawcett. (1994) Intestines. In: *A Textbook of Histology*. New York: Chapman & Hall. pp.617-635.
- Bloom & Fawcett. (1994) The liver and gall bladder. In: *A Textbook of Histology*. New York: Chapman & Hall. pp.652--677.
- Boeckner, L.S. & Kies, C. (1986) Zinc content of selected tissues and taste perception in zinc deficient and zinc adequate rats. *Nutr. Rep. Int.* 34(6): 921-930.
- Bremner, I. (1991) Nutritional and physiologic significance of metallothionein. *Methods Enzymol.* 205:25-35.
- Brown, D.C. & Gatter, K.C. (1990) Monoclonal antibody Ki-67: Its use in histopathology. *Histopathol.* 17:489-503.
- Canadian Council on Animal Care Guide (1993) Guide to the care and use of experimental animals. Volume 1 & 2 (Olfert, E.D., Cross, B.M. & McWilliam, ed).
- Chen, R.W., Vasey, E.J. & Whanger, P.D. (1977) Accumulation and depletion of zinc in rat liver and kidney metallothioneins. *J. Nutr.* 107:805-813.

- Cherian, M.G. (1994) The significance of the nuclear and cytoplasmic localization of metallothionein in human liver and tumor cells. *Environ. Health Persp.* 102:131-135.
- Chesters, J.K. & Boyne, R. (1991) Nature of the Zn<sup>2+</sup> requirements for DNA synthesis by 3T3 cells. *Exp. Cell Res.* 192:631-634.
- Chesters, J.K., Petrie, L. & Lipson, K.E. (1993) Two zinc-dependent steps during G<sub>1</sub> to S phase transition. *J. Cell. Physiol.* 155:445-451.
- Chesters, J.K., Petrie, L. & Travis, A. J. (1990) A requirement for Zn<sup>2+</sup> for the induction of thymidine kinase but not ornithine decarboxylase in 3T3 cells stimulated from quiescence. *Biochem. J.* 272:525-527.
- Chesters, J.K., Petrie, L. & Vint, H. (1989) Specificity and timing of the Zn<sup>2+</sup> requirement for DNA synthesis by 3T3 cells. *Exp. Cell Res.* 184:499-508.
- Coleman, J.E. (1992) Zinc proteins: Enzymes, storage proteins, transcription factors, and replication proteins. *Annu. Rev. Biochem.* 61:897-946.
- Cousins, R.J. (1996) Zinc. In: *Present Knowledge in Nutrition.* (Ziegler, E.E. & Filer, L.J., ed). pp.293-306. International Life Sciences Institute, Washington, DC.
- Cousins, R.J., Dunn, M.A., Leinart, A.S., Yedinak, K.C. & DiSilvestro R.A. (1986) Coordinate regulation of zinc metabolism and metallothionein gene expression in rats. *Am. J. Physiol.* 251:E688-E694.
- Cousins, R.J. & Lee-Ambrose, L.M. (1992) Nuclear zinc uptake and interactions and metallothionein gene expression are influenced by dietary zinc in rats. *J. Nutr.* 122:56-64.
- Dalton, T., Fu, K., Palmiter, R.D. & Andrews, G.K. Transgenic mice that overexpress metallothionein-1 resist dietary zinc deficiency. *J. Nutr.* 126:825-833.
- Danielson, K.G., Ohi, S. & Huang, P.C. (1982) Immunochemical detection of metallothionein in specific epithelial cells of rat organs. *Cell Biol.* 79:2301-2304.
- Dorup, I., Flyvbjerg, A., Everts, M.E. & Clausen T. (1991) Role of insulin-like growth factor-1 and growth hormone in growth inhibition induced by magnesium and zinc deficiencies. *Br. J. Nutr.* 66:505-521.
- Dunn, M.A., Blalock, T.L. & Cousins R.J. (1987) Metallothionein. *Proc. Soc. Exp. Biol. Med.* 185:107-119.
- Eaton, D.L. & Cherian, M.G. (1991) Determination of metallothionein in tissues by cadmium-hemoglobin affinity assay. *Methods Enzymol.* 205:83-88.

- Eaton, D.L. & Toal, B.F. (1982) Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. *Toxicol. Appl. Pharmacol.* 66:134-142.
- Flanagan, P.R., Haist, J. & Valberg, L.S. (1983) Zinc absorption, intraluminal zinc and intestinal metallothionein levels in zinc-deficient and zinc-replete rodents. *J. Nutr.* 113:962-972.
- Gallant, K.R. & Cherian, M.G. (1987) Changes in dietary zinc result in specific alterations of metallothionein concentrations in newborn rat liver. *J. Nutr.* 117:709-716.
- Gartner, L.P. & Hiatt, J.L. (1997) Urinary system. In: *Color Textbook of Histology*. Philadelphia: W.S. Saunders Company. pp.367-370.
- Grigioni, W.F., D'Errico, A., Bacci, F., Gaudio, M., Mazziotti, A., Gozzetti, G. & Mancini, A.M. (1989) Primary liver neoplasms: Evaluation of proliferative index using MoAb Ki 67. *J. Pathol.* 158:23-29.
- Hall, P.A., Greenwood, R.A., d'Ardenne, A.J. & Levison, D.A. (1988) In situ demonstration of renal tubular regeneration using the monoclonal antibody Ki67. *Nephron* 49:122-125.
- Hall, P.A. & Levison, D.A. (1990) Assessment of cell proliferation in histological material. *J. Clin. Pathol.* 43:184-192.
- Heilmaier, H.E. & Summer, K.H. (1985) Metallothionein content and zinc status in various tissues of rats treated with iodoacetic acid and zinc. *Arch. Toxicol.* 56:247-251.
- Iszard, M.B., Liu, J., Liu, Y., Dalton, T., Andrews, G.K., Palmiter, R.D. & Klaassen, C.D. (1995) Characterization of metallothionein-1-transgenic mice. *Toxicol. Appl. Pharmacol.* 133:305-312.
- Jacob, C., Maret, W. & Vallee, B.L. (1998) Control of zinc transfer between thionein, metallothionein, and zinc proteins. *Biochem.* 95:3489-3494.
- Jasani, B. & Schmid, K.W. (1997) Significance of metallothionein overexpression in human tumours. *Histopathol.* 31:211-214.
- Jiang, L.J., Maret, W. & Vallee, B.L. (1998) The ATP-metallothionein complex. *Biochem.* 95:9146-9149.
- Kägi, J.H.R. (1991) Overview of metallothionein. *Methods Enzymol.* 205:613-626.

- Kägi, J.H.R. & Schäffer, A. (1988) Biochemistry of metallothionein. *Biochem.* 27(23):8509-8515.
- Keen, C.L. & Gershwin, M.E. (1990) Zinc deficiency and immune function. *Annu. Rev. Nutr.* 10:415-431.
- Keen, C.L., Taubeneck, M.W., Daston, G.P., Rogers, J.M. & Gershwin, M.E. (1993) Primary and secondary zinc deficiency as factors underlying abnormal CNS development. *Ann. N. Y. Acad. Sci.* 678:37-47.
- Kelly, R., Davidson, G.P., Townly, R.R.W. & Campbell, P.C. (1976) Reversible intestinal mucosal abnormality in acrodermatitis enteropathica. *Arch. Dis. Child.* 51:219-227.
- King, J.C., Shames, D.M. and Woodhouse, L.R. (2000) Zinc homeostasis in humans. *J. Nutr.* 130:1360S-1366S.
- Knoepfel, L., Steinkuhler, C., Carri, M.T. & Rotilio, G. (1994) Role of zinc-coordination and of the glutathione redox couple in the redox susceptibility of human transcription factor Sp1. *Biochem. Biophys. Res. Commun.* 201:871-877.
- Lau, J.C. & Cherian, G.M. (1998) Developmental changes in hepatic metallothionein, zinc, and copper levels in genetically altered mice. *Biochem. Cell. Biol.* 76:615-623.
- Maret, W. (1994) Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. *Biochem.* 91:237-241.
- Maret, W. (1995) Metallothionein/disulfide interactions, oxidative stress, and the mobilization of cellular zinc. *Neurochem. Int.* 27(1):111-117.
- Morgan, P.N., Keen, C.L., Calvert, C.C. & Lonnerdal, B. (1988) Effect of varying dietary zinc intake of weanling mouse pups during recovery from early undernutrition on growth, body composition and composition of gain. *J. Nutr.* 118:690-698.
- Morrison, S.J., Shah, N.M. & Anderson, D.J. (1997) Regulatory mechanisms in stem cell biology. *Cell* 88:287-298.
- Moynahan, E.J. (1974) Acrodermatitis enteropathica: A lethal inherited human zinc-deficiency disorder. *Lancet* 2:399-400.
- Murray, A. & Hunt, T. (1993) Cells and the cell cycle. In: *The Cell Cycle: An Introduction*. New York: W.H. Freeman & Company. pp.3-15

- Nagel, W.W. & Vallee, B.L. (1995) Cell cycle regulation of metallothionein in human colonic cancer cells. *Cell Biol.* 92:579-583.
- Nartey, N.O., Banerjee, D. & Cherian, M.G. (1987) Immunohistochemical localization of metallothionein in cell nucleus and cytoplasm of fetal human liver and kidney and its changes during development. *Pathol.* 19:233-238.
- Ninh, N.X., Thissen, J.P., Collette, L., Gerard, G., Khoi, H.H. & Ketelslegers, J.M. (1996) Zinc supplementation increases growth and circulating insulin-like growth factor I (IGF-I) in growth-retarded Vietnamese children. *Am. J. Clin. Nutr.* 63:514-519.
- Nishimura, H., Nishimura, N. & Tohyama, C. (1989) Immunohistochemical localization of metallothionein in developing rat tissues. *J. Histochem. Cytochem.* 37:715-722.
- O'Dell, B.L. & Reeves, P.G. (1989) Zinc status and food intake. In: *Zinc in Human Biology*. (Mills, C.F., ed) pp.173-181. Springer Verlag, NY.
- Park, J.H.Y., Grandjean, C.J., Antonson, D.L. & Vanderhoof, J.A. (1985) Effects of short-term isolated zinc deficiency on intestinal growth and activities of several brush border enzymes in weanling rats. *Pediatr. Res.* 19:1333-1336.
- Prasad, A.S. (1984) Discovery and importance of zinc in human nutrition. *FASEB* 43:2829-2834.
- Prescod, A. (1998) Divergent effects of zinc, protein and energy deficiencies on skeletal muscle mass, muscle fiber diameter and serum insulin-like growth factor-1 concentration in growing rats. MSc. Thesis, University of Manitoba.
- Reddy, G.P.V. (1994) Cell cycle: Regulatory events in  $G_1 \rightarrow S$  transition of mammalian cells. *J. Cell. Biochem.* 54:379-386.
- Reeves, P.G. (1998) Copper metabolism in metallothionein-null mice fed a high-zinc diet. *J. Nutr. Biochem.* 9:598-601.
- Reeves, P.G., Nielsen, F.H. & Fahey, G.C. (1993) AIN-93 purified diets for laboratory rodents: final report of the american institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* 123:1939-1951.
- Reinstein, N.H., Lonnerdal, B.O., Keen, C.L. & Hurley, L.S. (1984) Zinc-copper interactions in the pregnant rat: Fetal outcome and maternal and fetal zinc, copper and iron. *J. Nutr.* 114:1266-1279.

- Rhodin, Y.A.G. (1975) The urinary system. In: An Atlas of Histology. Oxford University Press, New York, NY. pp.370-376 & 422-425.
- Richards, M.P. (1989) Recent developments in trace element metabolism and function: Role of metallothionein in copper and zinc metabolism. *J. Nutr.* 119:1062-1070.
- Richards, M.P. & Cousins, R.J. (1976) Metallothionein and its relationship to the metabolism of dietary zinc in rats. *J. Nutr.* 106:1591-1599.
- Rogers, J.M., Weldon-Taubeneck, M., Daston, G.P., Sulik, K.K., Zucker, R.M., Elstein, K.H., Jankowski, M.A. & Keen, C.L. (1995) Zinc deficiency causes apoptosis but not cell cycle alterations in organogenesis-stage rat embryos: Effect of varying durations of deficiency. *Teratology* 52:149-159.
- Roy, S.K., Behrens, R.H., Haider, R., Akramuzzaman, S.M., Mahalanabis, D., Wahed, M.A. & Tomkins, A.M. (1992) Impact of zinc supplementation on intestinal permeability in Bangladeshi children with acute diarrhoea and persistent diarrhoea syndrome. *J. Pediatr. Gastroenterol. Nutr.* 15:289-296.
- Sato, M., Mehra, R.K. & Bremner, I. (1984) Measurement of plasma metallothionein-I in the assessment of the zinc status of zinc-deficient and stressed rats. *J. Nutr.* 114:1683-1689.
- Schwartz, B.R., Pinkus, G., Bacus, S., Toder, M. & Weinberg, D.S. (1989) Cell proliferation in non-hodgkin's lymphomas: Digital image analysis of Ki-67 antibody staining. *Am. J. Pathol.* 134(2):327-336.
- Shepherd, N.A., Richman, P.I. & England, J. (1988) Ki-67 derived proliferative activity in colorectal adenocarcinoma with prognostic correlations. *J. Pathol.* 155:213-219.
- Shi, Y. & Berg, J.M. (1996) DNA unwinding induced by zinc finger protein binding. *Biochem.* 35:3845-3848.
- Summer, K.H. & Klein, D. (1991) Determination of metallothionein in biological materials. *Methods Enzymol.* 205:57-60.
- Taylor, C.G., Bettger, W.J. & Bray, T.M. (1988) Effect of dietary zinc and copper deficiency on the primary free radical defense system in rats. *J. Nutr.* 118:613-621.
- Templeton, D.M. & Cherian, G.M. (1991) Toxicological significance of metallothionein. *Methods Enzymol.* 205:11-24.
- Tomkins, A., Behrens, R. & Roy, S. (1993) The role of zinc and vitamin A deficiency in diarrhoeal syndromes in developing countries. *Proc. Nutr. Soc.* 52:131-142.

- Tsujikawa, K., Imai, T., Kakutani, M., Kayamori, Y., Mimura, T., Otaki, N., Kimura, M., Fukuyama, R. & Shimizu, N. (1991) Localization of metallothionein in nuclei of growing primary cultured adult rat hepatocytes. *Fed. Eur. Biochem. Soc.* 283:239-242.
- Vallee, B.L. (1991) Introduction to metallothionein. *Methods Enzymol.* 205:3-19.
- Vallee, B.L. & Auld, D.S. (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochem.* 29:5647-5659.
- Vallee, B.L., Coleman, J.E. & Auld, D.S. (1991) Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Biochem.* 88:999-1003.
- Vallee, B.L. & Falchuk, K.H. (1993) The biochemical basis of zinc physiology. *Physiol. Rev.* 73:79-118.
- Verheijen, R., Kuijpers, H.J.H., Driel, R.V., Beck, J.L.M., Dierendonck, J.H.V., Brakenhoff, G.J. & Ramaekers, F.C.S. (1989) Ki-67 detects a nuclear matrix-associated proliferation-related antigen II. Localization in mitotic cells and association with chromosomes. *J. Cell Sci.* 92:531-540.
- Waalkes, M.P. & Klaassen, C.D. (1985) Concentration of metallothionein in major organs of rats after administration of various metals. *Fund. Appl. Toxicol.* 5:473-477.
- Wallwork, J.C., Fosmire, G.J. & Sandstead, H.H. (1981) Effect of zinc deficiency on appetite and plasma amino acid concentrations in the rat. *J. Nutr.* 45:127.
- Williams, R.B. & Chesters, J.K. (1970) The effects of early zinc deficiency on DNA and protein synthesis in the rat. *Br. J. Nutr.* 24:1053-1059.
- Wu, F.Y.H. & Wu, C.W. (1987) Zinc in DNA replication and transcription. *Annu. Rev. Nutr.* 7:251-272.
- Yu, C.C.W., Woods, A.L. & Levison, D.A. (1992) The assessment of cellular proliferation by immunohistochemistry: A review of currently available methods and their applications. *Histochem. J.* 24:121-131.
- Zaia, J., Fabris, D., Wei, D., Karpel, R.L. & Fenselau, C. (1998) Monitoring metal ion flux in reactions of metallothionein and drug-modified metallothionein by electrospray mass spectrometry. *Protein Sci.* 7:2398-2404.
- Zeng, J., Heuchel, R., Schäffner, W. & Kägi, J.H.R. (1991) Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Spl. *Fed. Eur. Biochem. Soc.* 279:310-312.

Zeng, J., Vallee, B.L. & Kägi, J.H.R. (1991) Zinc transfer from transcription factor IIIA fingers to thionein clusters. *Biochem.* 88:9984-9988.

## VIII. Appendix

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## Key to tables

The following letter code was used to identify the different treatment groups:

**ZD** = zinc deficient

**ZR** = zinc repleted

**PF** = pair-fed

**PFR** = calorie repleted

**C** = control

The data (mean + SEM) were obtained at Day 16 of the study for **ZD**, **PF** and **C**, and at Day 17 of the study for **ZR** and **PFR**, unless otherwise specified.

**Table 1.** Body weights at day 0, 4, 8, 12 and 16 of the study.<sup>1,2</sup>

	<b>Treatment Group</b>				
	<b>ZD</b>	<b>ZR</b>	<b>PF</b>	<b>PFR</b>	<b>C</b>
Body Weight Day 0 (g)	62.6 <sup>A</sup> ±2.2	60.5 <sup>A</sup> ±1.3	62.0 <sup>A</sup> ±2.0	60.5 <sup>A</sup> ±1.6	60.6 <sup>A</sup> ±2.1
Body Weight Day 4 (g)	79.4 <sup>AB</sup> ±4.1	78.1 <sup>B</sup> ±1.4	84.2 <sup>AB</sup> ±2.4	82.8 <sup>AB</sup> ±1.7	87.1 <sup>A</sup> ±2.7
Body Weight Day 8 (g)	82.7 <sup>C</sup> ±4.6	81.6 <sup>C</sup> ±1.5	94.1 <sup>B</sup> ±3.1	93.9 <sup>B</sup> ±2.1	117.4 <sup>A</sup> ±3.5
Body Weight Day 12 (g)	90.2 <sup>B</sup> ±4.8	84.8 <sup>B</sup> ±1.7	94.5 <sup>B</sup> ±3.8	95.5 <sup>B</sup> ±2.3	149.1 <sup>A</sup> ±4.1
Body Weight Day 16 (g)	99.3 <sup>BC</sup> ±7.5	88.5 <sup>C</sup> ±1.5	104.7 <sup>B</sup> ±6.9	98.5 <sup>BC</sup> ±2.7	183.3 <sup>A</sup> ±4.5

<sup>1</sup> Values are means ± SEM for n = 8. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test.

<sup>2</sup> Body weight for the various timepoints was analyzed by repeated measures and there were significant main effects for diet, time and diet\*time interaction (p < 0.0001).

**Table 2.** Body weight before zinc repletion (ZD, PF and C at Day 16 of the study) and after zinc repletion (ZR and PFR at Day 17 of the study), and body length and tail length at termination. <sup>1</sup>

	<b>Treatment Group</b>				
	<b>ZD</b>	<b>ZR</b>	<b>PF</b>	<b>PFR</b>	<b>C</b>
Body Weight (g)	99.3 <sup>BC</sup> ±7.5	97.5 <sup>C</sup> ±1.5	104.7 <sup>B</sup> ±6.9	119.1 <sup>BC</sup> ±3.2	183.3 <sup>A</sup> ±4.5
Body Length (cm)	15.7 <sup>BC</sup> ±0.4	15.4 <sup>C</sup> ±0.1	15.9 <sup>BC</sup> ±0.3	16.3 <sup>B</sup> ±0.2	18.7 <sup>A</sup> ±0.2
Tail Length (cm)	10.8 <sup>D</sup> ±0.4	10.9 <sup>DC</sup> ±0.2	11.6 <sup>B</sup> ±0.3	11.6 <sup>BC</sup> ±0.1	12.8 <sup>A</sup> ±0.2

<sup>1</sup> Values are means ± SEM for n = 8. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test.

**Table 3.** Body weight and feed intake of ZR and PFR groups on Day 16 (before zinc repletion) and Day 17 of the study (after zinc repletion).<sup>1</sup>

<b>Treatment Group</b>		
	<b>ZR</b>	<b>PFR</b>
Body Weight Day 16 (g)	88.5 <sup>B</sup> ±1.5	98.5 <sup>B</sup> ±2.7
Body Weight Day 17 (g)	97.5 <sup>A</sup> ±1.5	119.1 <sup>A</sup> ±3.2
Feed Intake Day 16 (g)	5.9 <sup>B</sup> ±0.4	5.9 <sup>B</sup> ±0.4
Feed Intake Day 17 (g)	14.0 <sup>A</sup> +1.2	20.9 <sup>A</sup> ±0.6

<sup>1</sup> Values are means ± SEM for n = 8. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test.

**Table 4.** Effect of zinc deficiency and repletion on femur wet weight and femur wet weight to body weight ratio, and femur dry weight and femur dry weight to body weight ratio.<sup>1</sup>

	<b>Treatment Group</b>				
	<b>ZD</b>	<b>ZR</b>	<b>PF</b>	<b>PFR</b>	<b>C</b>
Wet Weight (g)	0.40 <sup>BC</sup> ±0.02	0.36 <sup>C</sup> ±0.00	0.43 <sup>B</sup> ±0.02	0.42 <sup>B</sup> ±0.01	0.54 <sup>A</sup> ±0.01
Wet weight / Body Weight	0.0041 <sup>A</sup> ±0.0001	0.0037 <sup>B</sup> ±0.00005	0.0042 <sup>A</sup> ±0.0002	0.0035 <sup>B</sup> ±0.0001	0.0030 <sup>C</sup> ±0.00004
Dry Weight (g)	0.19 <sup>BC</sup> ±0.01	0.17 <sup>C</sup> ±0.00	0.20 <sup>B</sup> ±0.01	0.20 <sup>B</sup> ±0.01	0.24 <sup>A</sup> ±0.01
Dry weight / Body Weight	0.00189 <sup>AB</sup> ±0.00006	0.00179 <sup>CB</sup> ±0.00003	0.00198 <sup>A</sup> ±0.00009	0.00167 <sup>C</sup> ±0.00004	0.00133 <sup>D</sup> ±0.00001

<sup>1</sup> Values are means ± SEM for n = 8. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test.

**Table 5.** Effect of zinc deficiency and repletion on epididymal fat pad weight and epididymal fat pad weight to body weight ratio. <sup>1</sup>

	<b>Treatment Group</b>				
	<b>ZD</b>	<b>ZR</b>	<b>PF</b>	<b>PFR</b>	<b>C</b>
Weight (g wet weight)	0.29 <sup>B</sup> ±0.05	0.24 <sup>B</sup> ±0.02	0.27 <sup>B</sup> ±0.07	0.32 <sup>B</sup> ±0.04	1.32 <sup>A</sup> ±0.07
Weight / Body Weight	0.0028 <sup>B</sup> ±0.0003	0.0024 <sup>B</sup> ±0.0002	0.0024 <sup>B</sup> ±0.0004	0.0026 <sup>B</sup> ±0.0003	0.0072 <sup>A</sup> ±0.0003

<sup>1</sup> Values are means ± SEM for n = 8. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test.

**Table 6.** Effect of zinc deficiency and repletion on serum zinc and femur zinc concentrations.<sup>1</sup>

	Treatment Group				
	ZD	ZR	PF	PFR	C
Serum Zinc ( $\mu\text{g/ml}$ )	0.37 <sup>C</sup> $\pm 0.11$	1.53 <sup>AB</sup> $\pm 0.10$	1.60 <sup>A</sup> $\pm 0.07$	1.30 <sup>B</sup> $\pm 0.05$	1.62 <sup>A</sup> $\pm 0.06$
Femur Zinc ( $\mu\text{g/g}$ dry wt.)	80.0 <sup>C</sup> $\pm 5.2$	103.8 <sup>B</sup> $\pm 3.8$	260.5 <sup>A</sup> $\pm 5.3$	263.2 <sup>A</sup> $\pm 6.3$	275.1 <sup>A</sup> $\pm 5.3$

<sup>1</sup> Values are means  $\pm$  SEM for n = 8, except n = 7 for serum zinc for ZD group. Means within rows with different superscripts are significantly different ( $p < 0.05$ ) as determined by Duncan's multiple range test.

**Table 7.** Effect of zinc deficiency and repletion on intestine weight and intestine weight to body weight ratio, intestine length, and concentrations of zinc and metallothionein in small intestine.<sup>1</sup>

	Treatment Group				
	ZD	ZR	PF	PFR	C
Weight (g wet wt.)	7.26 <sup>C</sup> ±0.75	7.69 <sup>C</sup> ±0.19	6.96 <sup>C</sup> ±0.62	10.92 <sup>B</sup> ±0.47	13.30 <sup>A</sup> ±0.56
Weight / Body Weight	0.072 <sup>BC</sup> ±0.004	0.079 <sup>B</sup> ±0.002	0.066 <sup>C</sup> ±0.002	0.091 <sup>A</sup> ±0.002	0.073 <sup>B<sup>C</sup></sup> ±0.003
Length (cm)	96.9 <sup>C</sup> ±2.7	94.3 <sup>C</sup> ±1.5	105.0 <sup>B</sup> ±0.9	103.5 <sup>B</sup> ±0.6	122.6 <sup>A</sup> ±3.0
Zinc (µg/g dry wt.)	110.9 <sup>B</sup> ±7.2	134.3 <sup>AB</sup> ±5.7	125.9 <sup>AB</sup> ±4.5	148.1 <sup>A</sup> ±9.7	115.7 <sup>B</sup> ±13.5
MT (µg/g wet wt.)	4.70 <sup>B</sup> ±0.36	12.57 <sup>A</sup> ±2.24	16.23 <sup>A</sup> ±1.33	12.82 <sup>A</sup> ±1.61	12.75 <sup>A</sup> ±1.64
MT (µg/mg protein)	0.056 <sup>B</sup> ±0.005	0.139 <sup>A</sup> ±0.037	0.202 <sup>A</sup> ±0.019	0.186 <sup>A</sup> ±0.027	0.131 <sup>A</sup> ±0.017

<sup>1</sup> Values are means ± SEM for n=8, except n = 6 and n = 7 for zinc concentration for ZD group, and for metallothionein concentration (µg/g) for ZD group and metallothionein concentration (µg/mg protein) for ZD and PFR groups, respectively. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test.

**Table 8.** Effect of zinc deficiency and repletion on liver weight and liver weight to body weight ratio, and concentrations of zinc and metallothionein, and % Ki67 immunostaining in liver.<sup>1</sup>

	Treatment Group				
	ZD	ZR	PF	PFR	C
Weight (g wet wt.)	4.13 <sup>C</sup> ±0.39	5.31 <sup>B</sup> ±0.27	3.95 <sup>C</sup> ±0.47	6.38 <sup>B</sup> ±0.74	9.16 <sup>A</sup> ±0.52
Weight / Body Weight	0.041 <sup>B</sup> ±0.001	0.054 <sup>A</sup> ±0.002	0.037 <sup>B</sup> ±0.002	0.053 <sup>A</sup> ±0.001	0.050 <sup>A</sup> ±0.002
Zinc (µg/g dry wt.)	89.7 <sup>B</sup> ±4.6	76.5 <sup>C</sup> ±2.6	119.2 <sup>A</sup> ±5.5	73.7 <sup>C</sup> ±1.9	91.5 <sup>B</sup> ±4.0
MT (µg/g wet wt.)	9.39 <sup>B</sup> ±1.82	7.86 <sup>B</sup> ±0.88	40.62 <sup>A</sup> ±4.66	16.07 <sup>B</sup> ±2.81	12.60 <sup>B</sup> ±2.63
MT (µg/mg protein)	0.042 <sup>D</sup> ±0.010	0.056 <sup>DC</sup> ±0.010	0.271 <sup>A</sup> ±0.035	0.140 <sup>B</sup> ±0.016	0.107 <sup>BC</sup> ±0.015
% cells with strong Ki-67 staining	2.10 <sup>AB</sup> ±0.80	5.52 <sup>A</sup> ±1.93	0.66 <sup>B</sup> ±0.15	4.47 <sup>A</sup> ±1.36	5.31 <sup>A</sup> ±0.26
% total Ki-67 stained cells	67.9 <sup>AB</sup> ±3.8	72.2 <sup>A</sup> ±2.1	60.6 <sup>B</sup> ±2.7	69.2 <sup>A</sup> ±2.5	67.8 <sup>AB</sup> ±1.8

<sup>1</sup> Values are means ± SEM for n = 40. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test

**Table 9.** Effect of zinc deficiency and repletion on kidney weight and kidney weight to body weight ratio, and concentrations of zinc and metallothionein in kidney.<sup>1</sup>

	Treatment Group				
	ZD	ZR	PF	PFR	C
Weight (g wet wt.)	1.18 <sup>BC</sup> ±0.07	1.10 <sup>C</sup> ±0.02	1.28 <sup>B</sup> ±0.06	1.29 <sup>B</sup> ±0.05	1.97 <sup>A</sup> ±0.01
Weight / Body Weight	0.0120 <sup>AB</sup> ±0.0003	0.0113 <sup>CB</sup> ±0.0002	0.0123 <sup>A</sup> ±0.0003	0.0109 <sup>C</sup> ±0.0002	0.0108 <sup>C</sup> ±0.0002
Zinc (µg/g dry wt.)	73.7 <sup>C</sup> ±3.3	93.4 <sup>B</sup> ±2.6	106.6 <sup>A</sup> ±2.2	102.2 <sup>A</sup> ±1.3	105.0 <sup>A</sup> ±1.4
MT (µg/g wet wt.)	21.8 <sup>C</sup> ±2.7	43.6 <sup>A</sup> ±3.4	47.6 <sup>A</sup> ±4.3	46.7 <sup>A</sup> ±3.8	33.1 <sup>B</sup> ±3.1
MT (µg/mg protein)	0.087 <sup>C</sup> ±0.009	0.171 <sup>AB</sup> ±0.016	0.213 <sup>A</sup> ±0.024	0.184 <sup>AB</sup> ±0.018	0.160 <sup>B</sup> ±0.012

<sup>1</sup> Values are means ± SEM for n = 8, except n = 7 for metallothionein concentration (µg/g and µg/mg protein) for PF group. Means within rows with different superscripts are significantly different (p < 0.05) as determined by Duncan's multiple range test.