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EFFECT OF VARYING LEVELS OF DIETARY IRON ON TISSUE CONCENTRATIONS OF IRON, COPPER, AND ZINC IN FEMALE RATS

By

MARY JOSEPHINE M. FISHER

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Thesis Approved:

Andrew B Arguett

Thesis Advisor

E Gabrielle A Olsen

Barbara J. Stoecker

Timothy J. Pettitone

Dean of the Graduate College

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Abbreviations

AAS	Atomic absorption spectrophotometer
AI	Adequate Intake
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
DRI	Dietary Reference Intakes
DTH	Delayed type hypersensitivity
DXA	Dual energy x-ray Absorptiometry
EAR	Estimated Average Requirement
FDA	Food and Drug Administration
HCl	Hydrochloric acid
HCT	Hematocrit
HLA	Human leukocyte antigen
MCV	Mean corpuscular volume
MFP	Meat factor; content of meat, fish, and poultry
N	Nitrogen
NaCl	Sodium chloride
NHANES	National Health and Nutrition Examination Survey

PBW	Percent body weight
RBC	Red blood cell
RDA	Recommended Daily Allowance
RTC	Reticulocyte
TIDC	Total iron binding capacity
UL	Tolerable Upper Intake Level
USDA	United States Department of Agriculture
WBC	White blood cell

CHAPTER ONE

INTRODUCTION

Problem and Importance

Iron status is a dichotomous nutritional dilemma. Iron deficiency anemia is the most common nutritional deficiency disease worldwide, while increased food fortification and supplement use have raised concern regarding the potential for a higher incidence of iron overload (Roughead, et al. 1999).

Anemia affects approximately 4.1 million Americans, and of these cases, approximately 60% of those affected are less than 45 years of age. Anemia is more prevalent in female populations. In 1995, anemia affected 55 women out of every 1000 less than 64 years of age, whereas only 9 per 1000 men of the same age group were affected (Vital and Health Statistics Series 10, No. 199, 1995). Anemia has been found consistently in the NHANES studies from 1983 through the present. Meyers et al (1983) examined NHANES I data to determine the prevalence of anemia in women in the United States. They found iron deficiency to be the primary cause of anemia and that the disease predominates in African American women versus Caucasian. Using a hemoglobin "cut off" value of 12 g/dL to classify anemia, they estimated that approximately 4% of Caucasian study participants and 20% African American study participants were anemic (Meyers, et al. 1983). Dallman et al (1984) examined NHANES II data to estimate the prevalence of anemia in that study population. Classification of anemia was based on hemoglobin values below the 95th percentile reference ranges. In women aged 18-64, this value was 11.7 mg/dL, and the greatest prevalence of anemia was found in women aged

25-44 (5.8%). They concluded, as did Meyers and his colleagues, that iron deficiency was the primary cause of anemia (Dallman, et al. 1984). Analysis of NHANES III data found that dietary iron intake was related to iron status and that low iron stores were found in approximately 8% and 17% of non-Hispanic white (NHW) women and Mexican American (MA) women, respectively (Ramakrishanan et al 2002). NHANES III data was also analyzed to estimate the prevalence of iron deficiency, anemia, and iron deficiency anemia in 12-39 year old NHW and MA females. Mexican American females had much higher rates of iron deficiency, anemia, and iron deficiency anemia (16.6%, 10.1%, and 6.2%, respectively) than the NHW females (6.1%, 6.6%, and 2.3%, respectively). Collectively, over one third of the females with iron deficiency concomitantly had anemia (Frith-Terhune et al, 2000). A comparison of NHANES I, II and III data regarding the prevalence of anemia in Caucasian females shows a gradual rise from 1971 to 1994. The approximate prevalence percentages increased from 4% in NHANES I to 5.8% in NHANES II to 6.6% in NHANES III (Meyers et al 1983, Dallman, et al. 1984, Frith-Terhune et al, 2000).

The Food and Nutrition Board of the Institute of Medicine published the Dietary Reference Intakes (DRIs), which is a comprehensive report of reference intake values for essential nutrients and dietary components. The DRIs are composed of reference values for the Recommended Daily Allowances (RDA), Estimated Average Requirements (EAR), Adequate Intakes (AI), and Tolerable Upper Intake Levels (UL) (IOM 2001). The reference values published in this document are useful for comparing with estimates of nutrient intakes of specific populations to assess the adequacy of that populations' intake. Alaimo et al (1994) found that the average daily iron intake of females 12-49

years of age was approximately 12.40 mg, which is consistently below the RDA of 15 mg/day for females 14-18 years and 18 mg/day for females 19-50 years (IOM, 2001). Cusatis et al (2000) examined stability versus variability in nutrient intakes of adolescent females 12-18 years of age. A cohort of 81 females was divided into quartiles based on weight, and for each variable, the quartiles were compared. At the beginning of the study period, mean dietary iron intake ranged from 9.4-16.6 mg, which is well above the RDA of 8 mg/day for adolescents 9-13 years of age. However, longitudinal linear analyses revealed a significant decrease ($p=0.0411$) in iron intake over time in the 4th quartile, and that the mean iron intake of females in the 2nd and 3rd quartiles did not change significantly from 10.4 and 12.5 mg/day, respectively, both of which are below the RDA for females 14-18 years (Cusatis et al 2000, IOM 2001). Dietary iron intake, as it is affected by dieting, was examined by Mulvihill et al (2002). Sixty-four females 14-18 years of age were classified at low, medium, or high levels of dietary restraint, and were found to have average dietary iron intakes of 9.59, 9.46, and 9.46 mg/day, respectively. The differences in dietary iron intakes among groups were not significant but failed to meet the RDA of 15 mg/day (IOM 2001, Mulvihill et al 2002). The RDAs are set at levels that will ensure the nutrient needs of 98% of the healthy population are met. Conversely, the EARs are estimates of average requirements resulting in meeting the approximate nutrient needs of 50% of the healthy population. The EAR of iron is 5.7mg/d for adolescent females 9-13 years, 7.9 mg/d for women 14-18 years, 8.1 mg/d for women 31-50 years, and 5 mg/d for females over age 50 (IOM 2001). When compared to the EARs, the findings of Alaimo et al (1994), Cusatis et al (2000), and Mulvilhill et al (2002), meet or exceed the EAR. Dietary iron needs decrease for women

over 51 years of age (RDA=8 mg/day and EAR=5 mg/day (IOM 2001)). The average daily dietary iron intake of this population was found by Alaimo et al (1994) to be 12.35 mg/day. Ervin and Kennedy-Stephenson (2002) assessed the iron intakes of elderly adults (>60 years of age) who participated in NHANES III. Female participants whose iron was supplied solely from diet had mean iron intakes of 11.4 mg/day (Ervin and Kennedy-Stephenson 2002). The findings of both Alaimo et al (1994) and Ervin and Kennedy-Stephenson (2002) indicate that the iron intakes of females greater than 50 years of age well exceed both the RDA and EAR for this population (IOM 2001).

Supplement use is a popular habit among Americans, with usage rates being greater in women than men (Bender et al 1992, Messerer et al 2001, Stewart et al 1985, Subar and Block 1990). Bender et al (1992) examined rates of supplement usage and perceptions about the health benefits of supplements and found that supplement usage correlated with perceptions of health and number of self-reported health conditions. They found usage to be highest among survey respondents with greater than three self-reported health conditions as well as among those who perceived their health to be "very good" or "excellent." Blendon et al (2001) also examined Americans' views on supplements and found that some survey respondents would continue to take dietary supplements even if clinical research found that they were of no health benefit.

Popularity of dietary supplements has increased considerably over the past two decades, as evidenced most notably by the increase in sales. In 1981, sales were estimated at 1.7 billion (Stewart et al 1985), in 1988 2.7 billion (Bender et al 1992), in 1992 3.7 billion (Slesinski et al 1995), and 11.75 billion in 1997 (Naylor and Gleich, 1999). As the supplement market continues to grow, the percentage of the population

that consumes dietary supplements on a daily basis may also grow. Currently it is estimated that 42% of Americans consume dietary supplements on a daily basis (Liebman and Schardt, 2001). Ervin and Kennedy-Stephenson (2002) observed a similar percentage of supplement use in females over age 60 who participated in NHANES III. Mean daily iron intakes of these supplement users was determined to be 23.6 mg/day, which far exceeds the RDA and EAR, but is less than the tolerable upper limit of 45 mg/day (Ervin and Kennedy-Stephenson 2002, IOM 2001). However, the increasing popularity of supplements coupled with regular consumption of iron-fortified foods may increase the incidence of iron overload, especially in the elderly population.

The trace elements iron, copper, and zinc are known to interact resulting in alterations in absorption. Hill and Matrone (1970) attribute trace element interactions to "similarities in the physicochemical properties of their ions, and that ions with similar valence shell electronic structures are likely to be antagonistic." However, the specific interactions among these trace minerals when any or all are consumed in varying amounts have yet to be clearly delineated (Rodriquez-Matas, et al. 1998). Therefore, not only do excessive and insufficient iron intakes have serious consequences in and of themselves, but they may also adversely affect zinc and copper utilization.

Yokoi et al (1991) examined the effects of an iron deficient diet on tissue stores of minerals in rats. The iron deficient experimental group consumed a diet providing approximately 5.9 μg Fe/g of diet for three weeks which resulted in decreased concentration of iron and increased concentration of copper in the liver and spleen, with no effect on zinc concentrations in either tissue. In a study examining the effects of excessive and deficient dietary iron on copper status, Yu and his colleagues (1994) found

that high dietary iron (389 mg Fe/kg diet) significantly increased concentrations of iron in the liver and the spleen as compared to the tissue concentrations of the animals fed the normal iron diet (40 mg Fe/kg diet). High dietary iron intake also resulted in significantly lower copper concentrations in the kidney and heart as compared to the tissue concentrations of the normal iron controls. Low dietary iron intake (7 mg Fe/kg diet) produced the opposite effects, specifically that iron concentrations in the liver, spleen, kidney and heart were significantly lower than that of the animals in the normal iron group and that the liver copper concentration was significantly greater than that of the animals in the normal iron group (Yu, et al. 1994). Storey and Greger (1987) assessed the interactions between iron, copper, and zinc in rats consuming adequate and excess amounts of iron and zinc and they found that the animals that consumed excess dietary iron exhibited lower zinc concentrations in their kidneys when compared to the pair fed control animals. Furthermore, tissue copper concentrations were also influenced by excessive dietary iron with the animals consuming excessive dietary iron exhibiting significantly lower concentrations of copper in their kidneys and liver than the pair-fed controls. The results described illustrate the interactions that occur with insufficient or excessive intakes of dietary iron and the ultimate effects on the tissue concentrations of iron, copper, and zinc in rats.

Larsen and Sandström (1992) examined trace mineral tissue stores as an indicator of trace mineral absorption in male rats fed varying levels of calcium, copper, and zinc. Dietary iron content was constant in all experimental diets (99 mg/kg dry matter). Their findings are based on the statistical probability that absorbed trace minerals affect the trace mineral content of tissues. They found that zinc absorption was inversely correlated

with iron deposition and that increased zinc absorption contributed to greater zinc concentration of the spleen, kidney, heart and liver. They concluded that the inverse relationship between zinc absorption and iron deposition in tissues was the result of an interaction between the two minerals before uptake by the intestinal mucosal cells, and that "increased zinc absorption also inhibits iron storage via a post-absorptive action" (Larsen and Sandström 1992). With respect to zinc and copper, Larson and Sandström (1992) found that increased copper and zinc absorption increased kidney copper concentration, while kidney zinc concentrations were inversely related to copper absorption. They concluded that the inverse relationship between zinc concentration of the kidneys and copper absorption indicated the interactions between these two minerals. The results of Larsen and Sandström's research serve as an illustration of both the relationship between trace mineral tissue storage and absorption and the interactions that occur between iron, copper, and zinc.

Alterations in the absorption of trace minerals, specifically iron, copper, and zinc, due to antagonistic interactions can subsequently hinder the normal physiological processes requiring these nutrients. Trace element interactions hindering the absorption of iron may impede the oxygen-carrying capacity of hemoglobin, may lead to the development of iron-deficiency anemia (Sherman 1992; Abdel-Mageed and Oehme 1990), may compromise immune function, may cause aberrant serum lipid levels, and may hinder both the physical and mental maturity of children (Castillo-Duran and Cassorla, 1999). Clinical studies examining iron overload diseases such as hemochromatosis have found that increased susceptibility to infection, cirrhosis, cancer, and excessive iron deposition in the tissues were common manifestations of such

diseases. Consequently, trace mineral interactions that may induce excessive iron absorption may also result in the aforementioned consequences (Abdel-Mageed and Oehme, 1990; Sherman, 1992). Interactions inducing copper deficiency may alter the immune response, affect the functioning of copper metalloenzymes, lead to anemia secondary to reduced levels of ceruloplasmin, cause skeletal abnormalities, and induce hypercholesterolemia. Research examining copper toxicity in animals found that excessive copper may increase copper deposition in tissues as well as induce a number of maladies including renal tubular and liver necrosis, jaundice, and hemosiderosis (Abdel-Mageed and Oehme, 1990). Trace mineral interactions affecting zinc absorption may cause growth retardation, delay puberty and psychomotor development, suppress immune function, and impair protein, glucose, and insulin metabolism (Abdel-Mageed and Oehme, 1990; Castillo-Duran and Cassorla, 1999; Sherman, 1992). Anemia and growth retardation have been found to occur in rats consuming excessive dietary zinc (Abdel-Mageed and Oehme, 1990). In humans, both acute and chronic zinc toxicity resulted in gastrointestinal complications (Abdel-Mageed and Oehme, 1990). The severity of the potential consequences described resulting from iron, copper, and zinc deficiencies and toxicities necessitate the need to determine the interactions among these nutrients.

Purpose

The purpose of this study was to determine the effect of varying intakes of dietary iron on the tissue concentrations of iron, zinc, and copper and to determine if tissue concentrations differed in young mature and sham-operated or ovariectomized female rats.

Two experiments were performed to address the question, and, overall, there were eight experimental diets and three treatment groups. The diets were prepared according to AIN-93 guidelines for growth and maintenance of rodents. In the growth and maintenance diet formulations there were four iron concentrations each. The calculated concentrations of iron in these experimental diets were from very low to high (6, 12, 35, and 150 mg/kg diet). The three treatment groups included young mature, sham-operated, and ovariectomized animals. The young mature animals did not undergo any surgical treatments. In order to control for the stress of the ovariectomy surgery, the sham animals had their ovaries exposed outside the body and then replaced.

Experiment one involved the young mature rats only and these animals consumed the experimental diets for approximately 15 weeks (7 weeks growth and 8 weeks maintenance) and were then killed. Their tissues were harvested and trace mineral analyses were performed on the liver, kidney, spleen, and heart using atomic absorption spectroscopy. Experiment two involved the sham-operated and ovariectomized rats, and these animals consumed the experimental diets for approximately 27 weeks (7 weeks growth and 20 weeks maintenance) and then were killed. As in the young mature rats, their tissues were harvested and trace mineral analyses were performed on the liver, kidney, spleen, and heart using atomic absorption spectroscopy.

The rationale for experiment one was the prevalence of iron deficiency anemia in female adolescent and pre-menopausal human populations. The rationale for experiment two was the increased potential for iron overload in female post-menopausal human populations due to reduced iron needs and a tendency for increased supplement use.

It is currently not known if storage of trace minerals varies during different stages of the female lifespan, or if it is affected by the presence or absence of ovarian hormones. The tissue mineral analyses of the animals in both experiments were compared to determine if differences occurred in trace mineral storage at different stages of maturity and/or during ovarian hormone deficiency.

Hypotheses

The aim of this study was to discern what effects, if any, dietary iron would have on the tissue content of iron, copper, and zinc in female rats across the life span.

The following hypotheses were developed for this study:

1. There will be no statistically significant differences in the tissue iron concentrations of young mature rats due to varying levels of dietary iron.
2. There will be no statistically significant differences in tissue copper concentrations of young mature rats due to varying levels of dietary iron.
3. There will be no statistically significant differences in tissue zinc concentrations of young mature rats due to varying levels of dietary iron.
4. There will be no statistically significant interactions among iron, copper, and zinc in young mature rats due to varying levels of dietary iron.
5. There will be no statistically significant differences in the tissue iron concentrations of mature rats due to varying levels of dietary iron.
    a. There will be no statistically significant differences in the tissue iron concentrations of the sham-operated animals due to varying levels of dietary iron.

b. There will be no statistically significant differences in the tissue iron concentrations of the ovariectomized animals due to varying levels of dietary iron.

c. There will be no statistically significant differences in tissue iron concentrations between the sham-operated and ovariectomized animals.

6. There will be no statistically significant difference in tissue copper concentrations of mature rats due to varying levels of dietary iron.

d. There will be no statistically significant differences in the tissue copper concentrations of the sham-operated animals due to varying levels of dietary iron.

e. There will be no statistically significant differences in the tissue copper concentrations of the ovariectomized animals due to varying levels of dietary iron.

f. There will be no statistically significant differences in tissue copper concentrations between the sham-operated and ovariectomized animals.

7. There will be no statistically significant differences in the tissue zinc concentrations of mature rats due to varying levels of dietary iron.

g. There will be no statistically significant differences in the tissue zinc concentrations of the sham-operated animals due to varying levels of dietary iron.

h. There will be no statistically significant differences in the tissue zinc concentrations of the ovariectomized animals due to varying levels of dietary iron.

i. There will be no statistically significant differences in tissue zinc concentrations between the sham-operated and ovariectomized animals.

8. There will be no statistically significant interactions among iron, copper, and zinc in mature rats due to varying levels of dietary iron.

j. There will be no statistically significant interactions among iron, copper, and zinc in sham-operated rats due to varying levels of dietary iron.

k. There will be no statistically significant interactions among iron, copper, and zinc in ovariectomized rats due to varying levels of dietary iron.

l. There will be no statistically significant interactions among iron, copper, and zinc between sham-operated and ovariectomized rats due to varying levels of dietary iron.

Assumptions

1. The housing conditions (i.e. light cycle, humidity, temperature, etc) were identical in both rooms and, therefore, did not affect eating patterns of the animals.
2. The procedure of pair feeding to the lowest weight gain did not result in growth retardation due to insufficient nutrient intake.
3. The deionized water provided ad libitum to the animals was not contaminated with trace minerals.
4. The extremes in dietary iron intake will not be enough to significantly affect body or organ weight.

Limitations

Although the ovariectomized rat model is considered acceptable for studies regarding menopause, the extrapolation of these findings to humans will be limited due to physiological and dietary differences between the two species.

CHAPTER TWO

LITERATURE REVIEW

Iron

Physiological Roles

The essentiality of iron is apparent by its prominent presence in living matter and its significant physiological functions in the human body (Morris, 1987; Prasad, 1978). Within the body there exist two iron compartments, the primary being the functional compartment and the secondary being the storage compartment (Cook 1990). The functional compartment accounts for 75% of the total amount of iron in the body (Lynch 1984). It consists of protein bound iron complexes, or functional compounds, that perform vital physiological functions throughout the body. Iron binds to proteins and forms these functional compounds because of its ability to exist in more than one oxidation state. When these compounds are formed, iron becomes a component of the prosthetic group at its active site; therefore, iron is not only vital to structure, but function as well (Prasad, 1978).

The iron-protein complexes are classified as either heme- or non-heme compounds. The heme compounds include hemoglobin, myoglobin, and the cytochromes; whereas transferrin, ferritin, and hemosiderin constitute the non-heme protein complexes (Morris, 1987). A small percentage, less than 1%, of the body's iron are contained in enzymes and these enzymes fall into one of three categories: heme protein enzymes, iron-flavoproteins, and enzymes requiring iron as a cofactor (Morris

1987, Prasad 1978). Although enzymatic iron is the smallest faction of the body's total iron pool, it is quite significant as the iron containing enzymes are vital to energy production and other physiological processes (Prasad, 1978).

The remaining 25% of the body's iron is in the storage compartment (Lynch 1987). Stored iron is found throughout the body, with the primary storage sites being the reticuloendothelial cells in the bone marrow, the liver, and the spleen (Cook 1990). The kidney, heart, skeletal muscles, pancreas, and brain also serve as iron storage sites, but to a much lesser extent. Combined, these organs store one-half to one-tenth the amount of iron found in the liver and spleen (Prasad, 1978). Iron is stored in one of two forms: ferritin or hemosiderin (Morgan and Walters, 1963). Overall, a greater proportion of iron is stored as ferritin, but most sites contain a combination of both forms (Morris, 1987; Prasad, 1978). Stored iron functions as a reserve and it is mobilized when the iron levels in the functional compartment become depleted (Cook 1990).

Functional Compounds. The oxygen-requiring production of energy, the citric acid cycle as it known today, evolved with the generation of oxygen by photosynthesis. Aerobic generation of energy required substances that could reversibly bind with oxygen, and so emerged the specialized iron-protein complexes, the cytochromes. Iron's ability to serve as both an electron donor and acceptor allows the cytochromes, in conjunction with the copper-dependent enzyme cytochrome c oxidase, to transport electrons, and thus generate energy, in the citric acid cycle. Iron plays multiple roles in the citric acid cycle, as 24 enzymes that facilitate oxidative phosphorylation require iron as a cofactor or it is present in their active sites (Morris, 1987; Prasad, 1978).

With the evolution of the aerobic production of energy, a need for molecules to carry oxygen to the cells evolved as well. The iron-protein complexes, hemoglobin and myoglobin, met this need (Prasad 1978). Hemoglobin is composed of a globin protein and four ferroprotoporphyrin, or heme, compounds. The molecule is generated in the bone marrow during the last stages of red cell development. The function of hemoglobin is to transport oxygen and carbon dioxide in the blood. It is capable of performing this function because the structure of the hemoglobin molecule permits the reversible binding of both molecules (Morris 1987). Blood hemoglobin levels vary throughout the life span and between sexes. These levels are also affected by a number of other factors, including diet, disease, and altitude (Morris 1987, Prasad 1978). Myoglobin is the less prominent heme protein and, makes up only 8% of total body iron whereas hemoglobin makes up approximately 60% of total body iron (Lynch 1984). The structure of myoglobin is less complex than hemoglobin but very similar. It is made up of one globin protein and only one ferroprotoporphyrin, or heme, compound. The role of myoglobin in oxygen transport is limited to the muscles. It stores oxygen in the muscle until it is needed, at which point myoglobin will mobilize the oxygen needed for muscle contractions (Morris 1987, Prasad 1978).

The formation of hemoglobin and myoglobin in the bone marrow requires that molecules of iron be transported to the bone marrow. Transferrin or siderophilin, a non-heme iron-protein complex, serves as the primary iron transport protein. There are two forms of transferrin involved in iron transport: mucosal and apomucosal transferrin. Mucosal transferrin is involved in transmucosal iron transport and apomucosal transferrin transports iron between cells and the brush border of the small intestine. Transferrin is

composed of a glycoprotein with two iron-binding sites each capable of carrying one atom of ferric iron. Given its role in iron transport, transferrin functions in iron metabolism. For instance, transferrin carries ferric iron to the bone marrow where it is reduced to the ferrous form. It then is detached from the transferrin molecule and attached to a protoporphyrin molecule, thus making ferroprotoporphyrin, the heme compound of hemoglobin. The body's immune system also depends on transferrin for efficient functioning (Morris 1987). Transferrin plays a role in the formation of lymphocytes, B cells, and antibodies as well as the efficient functioning of natural killer and phagocytic cells (Farthing 1989).

Total iron binding capacity (TIBC) refers to the body's full iron transport capabilities. In normal, healthy adults, only 30-40% of transferrin is "saturated" or actively transporting iron. The remaining transferrin is collectively referred to as the body's latent iron binding capacity. Total and latent iron binding capacity as well as the percent of saturated transferrin varies with stage of life and disease (Morris 1987, Prasad 1978). Lactoferrin is another, although less predominant, iron binding transport protein (Prasad 1978). As its name implies, lactoferrin is found in milk, but it is also found in other body fluids including saliva and sweat. Iron binds similarly to transferrin and lactoferrin, but these two molecules differ with respect to their amino acid and peptide make-up, immunological functions, and mechanism of mobility (Morris 1987).

There are a number of iron-proteins that perform functions that are not intimately related to either energy production or iron transport and storage. As with the functional compounds discussed, these, too, are vital to the efficient functioning of the body, but in a different manner. Hemopexin and haptoglobin are iron-containing glycoproteins that

function in iron conservation. They are secreted by the liver due to a receptor-mediated signal induced by the presence of free hemoglobin and heme in the blood following hemolysis. The free heme and hemoglobin are bound by the hemopexin and haptoglobin, respectively, and delivered to the liver for recycling (Morris 1987). Catalase and peroxidase are two heme enzymes responsible for protecting the cells from damage by hydrogen peroxide. These enzymes function by breaking down the hydrogen peroxide compound. Peroxidase digests hydrogen peroxide into water and a dehydrogenated product, whereas the end products of catalase activity are water and oxygen (Lynch 1984). Collagen proline hydroxylase is not an iron-containing enzyme, but it requires iron, as well as ascorbic acid and alpha-ketogluturate, to function. Collagen synthesis requires the hydroxylation of proline and lysine into hydroxyproline and hydroxylysine and the enzyme collagen proline hydroxylase mediates this conversion (Prasad 1978).

Storage Compounds. The body's demands for iron are great, and when there is insufficient iron available for incorporation into the aforementioned functional compounds, the body's iron reserves are mobilized. The liver, bone marrow, and spleen are the predominant iron storage sites, but other tissues, including the heart and kidney, store iron as well (Morris 1987, Prasad 1978). Ferritin and hemosiderin are the two forms of storage iron, and although they perform the same function, they are very different molecules. The ferritin molecule is water-soluble, can contain up to 20% iron, and is found in both the serum and the tissues. Hemosiderin is an insoluble molecule formed from ferritin that can contain up to 35% iron and is found only in tissues (Richter 1984, Morris 1987). Both ferritin and hemosiderin are available for use when the need for mobilization of iron stores arises (Morgan 1961a, 1961b 1962).

The factors that have been found to be determinants of iron storage as ferritin or hemosiderin are the concentration of iron stored, the rate of administration, and the form of iron that is administered (Morris 1987, Prasad 1978, Shoden and Sturgen 1958,1960, 1962). Morgan and Walters (1963) found in their examination of iron storage in disease states, that a greater percentage of iron is stored in the ferritin form when iron stores are less than 500 μg/g tissue whereas, the hemosiderin form predominated when tissue iron concentrations exceeded 1000 μg/g tissue. Furthermore, they found that the percentage of iron stored as ferritin and hemosiderin were similar when iron stores were between 500-1000 μg/g of tissue (Morgan and Walters 1963). They also observed that hemosiderin storage will dominate when iron is administered at a high velocity or if it is in a form of iron that is easily removed from the serum, such as saccharated iron. Conversely, iron stored in the ferritin form will dominate when iron is infused slowly or if it is in a form that stays in the serum for an extended time, such as iron dextran or dextrose (Morris 1987, Shoden and Sturgen 1960, 1962). Aside from tissue stores of iron, ferritin is present in the serum, and the levels of serum ferritin have been positively correlated with the body's stored iron reserves (Cook et al 1974).

This brief discussion of the physiological roles of iron illustrates its vitality to life It also illustrates the significant and far reaching effects of iron deficiency and excess. In iron deficiency states, tissue stores will be exhausted to meet the iron demands of the functional compounds, which aid in the maintenance of normal physiology. Conversely, the presence of excessive iron may overload the tissue storage sites, which may adversely affect tissue functioning and overall physiology.

Absorption

The human body's need for iron will dictate absorption because excretion of iron is limited (Kinney et al 1949). Normal iron excretion rates, determined using radioiron studies, range from 0.2-0.5 mg/day. Iron deficiency or overload alters excretion with rates seen as low as 0.03-0.06 mg/day and as high as 6.5 mg/day (Morris 1987). Iron requirements to meet the physiological demands of age, gender, and disease states influence iron absorption. Iron stores have also been shown to affect iron absorption, but the exact mechanism of how these are related remains controversial (Gavin et al 1994, Conrad et al 1994). Dietary composition, specifically the presence of certain food components at the time of iron consumption, influences iron absorption by either enhancing or inhibiting iron absorption. The form of iron ingested also influences absorption, as some forms are more readily absorbed than others (Prasad 1978, Morris 1987).

Estimated Adequate Requirements of Iron.

The amount of iron estimated to meet the body's needs throughout the lifespan and between the sexes significantly influences iron absorption. The Dietary Reference Intakes, published by the Institute of Medicine, define requirement as "the lowest continuing intake level of a nutrient that will maintain a defined level of nutriture in an individual (IOM, 2001, p 29)." The DRIs are a compilation of the RDAs, EARs, ULs, and AIs (included only when a nutrient does not have an RDA), which delineates vitamin and mineral intake ranges to address the nutrient needs of the healthy population, such that the adverse effects of deficiency or excess are prevented (IOM, 2001,p 2-7, 29-30).

The iron requirements of women are greater than those of men due primarily to losses from menstruation and to meet the demands of pregnancy. Throughout the life span of healthy adult males (age 19+), the RDA for iron is 8 mg/day. In healthy adult females aged 19-50, the RDA for iron is significantly greater than that for males at 18 mg/day. After age 50, the iron RDA for women decreases to that of adult males. The EARs are lower than the RDAs, as they are designed to address the iron requirements of 50% of the healthy population for a given age and gender. The EAR of iron for females 14-18 years and 19-50 years are 7.9 and 8.1 mg/day, respectively. For the remainder of the female lifespan, the EAR is 5mg/day. The UL of iron for all adults is 45 mg/day (IOM, 2001, p18-19, 375).

There is no difference in the RDA for iron between the sexes up through 13 years of age, whereas the EAR is the same in both sexes only through 8 years of age. Infants and children have significant iron requirements relative to their body size in order to meet the demands of growth. The RDA for iron for infants 7-12 months is 11 mg/d, for children 1-3 years is 7mg/day, and for children 4-8 years, 10 mg/d. During early adolescence (9-13 years), the RDA of iron decreases to 8 mg/day, as growth rates have slowed. The EARs for iron for the same age groups through 8 years of age are 6.9 mg, 3.0 mg, and 4.1mg per day, respectively. The EAR for iron in adolescent males and females 9-13 years are 5.9 mg and 5.7 mg/day, respectively. The divergence in the RDA for iron between sexes is seen at 14 years, at which point it increases to 11 mg/day for males and 15 mg/day for females. The EAR for iron for males and females in the same age group is 7.7 and 7.9 mg/day, respectively. The UL for iron for infants and children up to 13 years of age is 40 mg/day and that for adolescents 14-18 years is 45 mg/day

(IOM, 2001, p 18-19, 376). The iron requirements of females will continue to exceed that of males until age 50, at which point, the RDA becomes the same for both sexes at 8mg/day and the EAR is 6mg/day for males and 5mg/day for females. Pregnancy, like infancy and childhood, dramatically increases the body's iron requirements. The RDA for pregnant women of all ages is 27 mg/day. The EARs for pregnant women 14-18 years and 19-50 years are 23 and 22 mg/day, respectively. Iron requirements during lactation are significantly less that that of pregnancy and are even less than that of non-lactating women. The RDAs for lactating women 14-18 and 19-50 years are 10 and 9 mg/day, respectively. The EARs for lactating women 14-18 and 19-50 years are 7 and 6.5 mg/day, respectively. The UL for both pregnant and lactating women is 45 mg/day, which is the same as that for all adults (IOM, 2001, p 18-19, 375).

Absorption Process.

The amount of iron absorbed is dictated by physiological need, storage reserves, and gastrointestinal factors, the latter of which involves regulation at the absorption site, namely the small intestine. The mechanism of the regulation of iron absorption in the small intestine has been researched extensively, but has yet to be clearly determined (Conrad et al, 1994, Gavin et al, 1994, Hoglund and Reizenstein, 1969)

The most active site of iron absorption is the duodenum (Brown and Justus 1958). Iron is also absorbed in the jejunum, ileum, stomach and colon but to a much lesser extent. Iron is absorbed and enters circulation via a two-step process. The initial rapid phase involves the absorption of iron from the intestinal lumen by the mucosal cells. Subsequently, the iron is transported through the cells and across the cell membrane into

circulation (Carpenter and Ummandi 1995, Rhodes et al 1968, Hahn et al 1945, Wheby 1966, Chraisiri and Izak 1966, Wheby et al 1964, Manis and Schachter 1962).

Availability of Dietary Iron for Absorption

Iron needs, dictated by age, sex, disease, and iron status, will influence the amount of iron absorbed from food. When iron stores are maximized and iron status is not compromised, between 5-10% of the iron available in the food supply will be absorbed; however, when iron status is compromised and stores are depleted, absorption increases to 10-20% of available dietary iron (Prasad 1978). There are two main types of iron found in food sources: heme and non-heme (Monsen et al 1978). Hemoglobin and myoglobin supply the highly available heme-iron to the diet, and foods of animal origin, specifically meats, are the primary sources of this form of iron. In western countries, in which meat is a more prominent constituent of the typical diet, heme iron makes up 10-15% of dietary iron, which provides the average healthy adult with nearly one-third of their iron requirement (Cook 1990, Björn-Rasmussen et al 1974). Heme iron does not require modification to be absorbed and as such the mucosal cells assimilate it as an intact molecule. After absorption, heme oxygenase liberates the iron from the porphyrin complex and it subsequently becomes part of the available iron pool (Cook 1990, Raffin et al 1974). Dietary heme iron is highly available because its porphyrin "shell" protects the iron molecule from the components of the diet that inhibit absorption (Cook 1990, Björn-Rasmussen et al, 1974). Non-heme iron is the most abundant form of dietary iron, and it is found primarily in foods of plant origin, which are the foundation of most diets around the world. Non-heme iron is in the ferric (Fe^{+3}) form and is not readily

assimilated by the mucosal cells; therefore, to be absorbed non-heme iron must be reduced to the ferrous (Fe^{+2}) form (Fritz et al 1970, Hallberg and Solvell 1967, Jacobs and Miles 1969). This reduction is facilitated by gastric juices and takes place in the duodenum, as this is a more acidic region of the intestines, and in this locale, iron is at its greatest solubility (Cook 1990). Absorption of non-heme iron is quite variable, as simultaneous intake of certain dietary components will either enhance or inhibit its absorption (Layrisse et al 1968).

Dietary Inhibitors of Absorption. The dietary components that inhibit iron absorption are numerous and, in countries where these inhibitors are staples of the native diet, the effect on the iron status of the population is detrimental. Phytates, a phosphorus storage compound in plants, are potent inhibitors of iron absorption. Specific phytate-rich foods observed to inhibit absorption include wheat germ and bran, beans, brown and green lentils, and nuts (Cook 1990, Gillooly et al 1983). The phytates bind iron to form an insoluble complex that is not available for absorption (Hussain and Patwardhan, 1959). The decreased iron absorption seen in humans when phytate-rich foods or sodium phytate are added to a meal is not seen in rats (Cowan et al 1966, Sharpe et al, 1950).

Coffee and tea, which are customarily consumed with meals throughout the world, inhibit iron absorption with tea being the more significant inhibitor of the two (Morck et al 1983, Disler et al 1974). The adverse effects of tea on iron absorption are attributed to the tannins in tea, which form a complex with iron rendering it insoluble and, thus, unavailable for absorption (Disler et al 1974). Disler et al (1974) examined the effect of tea on the absorption of non-heme and hemoglobin iron and found that tea significantly inhibited non-heme iron absorption, as well as absorption of hemoglobin

iron, but only if the food source (i.e. meat) was uncooked. It was postulated that tannins inhibit uncooked heme iron absorption by "tanning" the globin protein, which hinders the digestive proteolytic enzymes from breaking down the protein and, hence, liberating the iron for subsequent absorption. Meat is almost exclusively consumed cooked; therefore, this inhibitory effect of tea on the absorption of uncooked heme iron is not as relevant to iron status as is its inhibitory effect on non-heme iron absorption (Disler et al 1974). Morck et al (1983) examined coffee's effect on iron and found that it has an inhibitory effect but to a lesser extent than that of tea. Specific variables of their study were coffee concentration and time of coffee consumption relative to meal intake. They found that the concentration of the coffee was inversely related to iron absorption and that coffee consumed up to one hour after a meal inhibited absorption to the same degree as coffee consumed with the meal (Morck et al 1983). Phosphorus, both alone and in conjunction with calcium has been linked to decreased iron absorption. Phosphorus is thought to be such a strong inhibitor that it may prevent iron absorption almost completely; however, studies have shown that the combined presence of calcium and phosphorus in a meal hinders iron absorption to a greater degree than either mineral alone (Morris 1987). High or low calcium diets supplemented with 1000 mg phosphorus resulted in increased fecal loss of iron. This suggests that phosphorus, like the other absorption inhibitors previously mentioned, binds with iron making it unavailable for absorption (Morris 1987).

Dietary Enhancers of Absorption. The dietary components that have been shown to enhance iron absorption include meat, vitamin C, and certain organic acids, amino acids, and carbohydrates. The meat content of a meal, also known as the "meat factor" or MFP, has a positive influence on the amount of iron absorbed from a meal of both heme

and non-heme iron food sources. The mechanics of the absorption enhancing effects of meat have not been concretely determined, but it is thought that meat or the amino acids in meat facilitate the transport or act as the transporter of iron across the mucosal cell membrane. Furthermore, the enhanced absorption of iron from vegetable foods consumed with meat in the presence of know absorption inhibitors has led researchers to postulate that meat prevents the binding of iron to compounds that would prevent its absorption (Layrisse et al 1984, Björn-Rasmussen and Hallberg 1979, Morris 1987).

Studies utilizing radio-iron labeled foods have found that the amount of iron absorbed from vegetables is greater when the vegetables are consumed with meat then if they are consumed alone. Layrisse et al (1968) conducted one of the earliest studies of iron absorption in foods of animal and plant origin consumed alone and together in a meal, and they found that the amount of iron absorbed from corn and black beans increased significantly when these foods were consumed with veal ($p<0.01$ and $p<0.001$, respectively). They also found that iron absorption from black beans increased significantly ($p<0.001$) when consumed with a solution of amino acids and 1mg ferrous sulfate (Layrisse et al, 1968). Layrisse and colleagues (1984) later examined the effects of amino acids on iron absorption from vegetables and compared it with the absorption enhancing effects of beef. Although not statistically significant, they observed a similar trend in percent of iron absorbed from corn consumed with cysteine, reduced glutathione, or beef. They concluded that the absorption enhancing effects of meat may be attributable to cysteine, and that the end products of protein breakdown prevent the complexing of iron such that it is rendered insoluble (Layrisse et al 1984). In their studies examining the effects of animal proteins on iron absorption, Björn-Rasmussen

and Hallberg (1979) found that beef, chicken, and fish increased iron absorption from an iron-fortified maize porridge, whereas egg protein failed to produce such absorption enhancing effects. They also found that beef consumed concomitantly with solutions of inorganic iron salts or inorganic iron salts plus sodium phytate enhanced the absorption of iron from these solutions (Björn-Rasmussen and Hallberg, 1979). They speculated that meat facilitates the uptake of iron by the mucosal cell, as well as blocking the action of compounds that inhibit iron absorption.

Organic acids are another meal component that is associated with increased iron absorption. The most notable absorption enhancing organic acid is ascorbic acid or vitamin C (Gillooly et al, 1983, Cook and Reddy, 2001). Ascorbic acid is able to chelate iron as well as reduce ferric iron into the ferrous form, both of which facilitate absorption of iron by the mucosal cells. It is also thought that ascorbic acid facilitates iron absorption by keeping iron soluble when the pH in the duodenum rises. In order for ascorbic acid to be effective, it must be consumed with the meal and be supplied by fruits and vegetables or a vitamin C supplement in crystalline form. The absorption enhancing effect of ascorbic acid is diminished when it is exposed to heat for an extended period of time because the acid is destroyed by heat. Therefore, the longer a meal is exposed to heat, the less ascorbic acid is present to enhance iron absorption (Morris 1987). The other organic acids that have been shown to facilitate iron absorption include lactic, citric, malic, pyruvic, tartaric and succinic acids, which are founds in certain vegetables. In their examination of the effects of organic acids on iron absorption, Gillooly et al (1983) found that the bioavailability of iron was higher in vegetables rich in ascorbic, malic, and/or citric acids. They also observed that the amount of iron absorbed from a

typically low iron meal of rice was increased with the addition of ascorbic, citric, L-malic, or tartaric acid to the meal (Gillooly et al, 1983).

Carbohydrates, in various forms, have been shown to exert a positive effect on iron absorption (Morris 1987). Lactose, sucrose, glucose and starch were found to enhance iron absorption with lactose exhibiting the greatest effect and starch the least (Amine and Hegstead 1971). Garretson and Conrad (1967) came to the same conclusion with respect to lactose and glucose, but not starch and sucrose. Research has also found that sorbitol and fructose enhance iron absorption (Morris 1987). One such study examined the effect of ferric fructose, a complex of ferric iron and fructose, on iron absorption and retention. Ferric fructose enhanced iron absorption and retention, and it was found to be more effective than iron sulfate, which is the most common form of supplemental iron (Bates et al 1972).

Iron Absorption in Disease.

Disease and deficiency states have been shown both to increase and decrease iron absorption. Increased absorption is usually seen in disease and deficiency as the body is either trying to meet the iron demands induced by the disease or it is trying to correct the nutritional deficiency (Morris 1987).

The disease that will increase iron absorption to the greatest extent is idiopathic hemochromatosis. This disease is caused by a genetic abnormality and it is characterized by unregulated iron absorption that leads to excessive iron deposition in the tissues (Lynch 1984). Diseases that cause blood loss, such as hookworm or schistosomiasis infections and malignancies, will increase iron absorption in attempts to replenish the lost

iron (Lynch 1984, Morgan and Walters 1963). Aplastic and hemolytic anemia, two types of anemia not associated with a nutritional deficiency, have been shown to stimulate dietary iron absorption as well (Morris 1987). Morgan and Walters (1963) examined iron storage in disease states and found that total storage iron concentrations were increased in aplastic anemia, multiple myeloma, and leukemia, and they attributed this in part to increased dietary iron absorption. Diseases associated with decreased iron absorption include polycythemia vera and transfusional polycythemia (Morris 1987). Morgan and Walters (1963) found that their subjects with polycythemia vera had diminished iron stores, as did those with rheumatic heart disease and myelofibrosis. These results suggest that the latter two diseases may decrease iron absorption similarly to polycythemia vera.

The most prominent nutritional deficiency disease associated with increased iron absorption from food is iron deficiency anemia. Standard absorption of iron from food in normal, healthy adults ranges from 5-15%. In iron deficiency states, this absorption percentage range increases to 20-60%, depending on the severity of the deficiency. Pernicious anemia, which is caused by reduced absorption of vitamin B_{12} due to lack of intrinsic factor, and pyridoxine deficiency are two other nutritional deficiencies that have also been associated with increased iron absorption (Morris 1987).

This brief overview of iron absorption and the factors that influence it either positively or negatively illustrates the complexity of the process, which helps explain why iron deficiency anemia is such a prevalent nutritional disease throughout the world.

Adequacy of Dietary Iron Intake of Females

The DRIs are the standards by which adequacy of dietary intake is measured. Until recently, the Recommended Dietary Allowances (RDA) were commonly used to assess adequacy of intake of populations as well as individuals. These guidelines are set to meet the needs of 97% of healthy individuals. The RDAs have been the guidelines by which adequacy of nutrient intakes has been measured since 1943 and they are periodically re-evaluated and updated based on research. The most recent revision of the RDA guidelines was released in 2001. In the 2001 edition, the iron recommendations for infants, children, and female adolescents either stayed the same or decreased slightly. However, the iron recommended for females age 19-50 increased significantly from 15mg/day to 18mg/day (IOM 2001, National Research Council, 1989). The RDA values are, in part, based on the assumption that 10% of dietary iron is absorbed (Raper et al 1984). Therefore, in order to meet the RDA for iron, it is recommended that the diet include 30-90g of meat, which provides the readily available heme iron, or that 25-75 mg ascorbic acid be consumed with non-heme iron sources to enhance the iron absorption from such foods (Food and Nutrition Board 1989, p 6). Research examining the total iron content and percentage of available iron of diets of the US population have found that children and women typically consume less iron than the amount necessary to meet the RDA. Raper et al (1984) utilized the 1977-1978 Nationwide Food Consumption Survey data to quantify the average dietary iron intake of approximately 9500 US citizens one year of age and older and then compared this intake data to the 1980 RDA values. They found that children 1-2 years and 3-5 years consumed, on average, only 51% and

77% of the RDA for iron, respectively. The average intakes of adolescent girls and women of childbearing age were also found to be inadequate with intakes ranging from only 55-61% of the RDA. Raper et al (1984) also examined the percentage of iron available in the foods consumed, and it was determined that the percent of iron available for absorption was less than the 10% needed to meet the RDA. In children ages 1-8 years, 6.5-7.5% of iron was found to be available, and in all females age 9 years and older, only 7.4-8.2% of iron was found to be available (Raper et al 1984). The Total Diet Study is conducted by the Food and Drug Administration and the Department of Agriculture to assess the core diet of the United States population to estimate the average intake of eleven major nutrients, including iron, and to determine if there are changes in intake of these nutrients over time. Specifically, the core foods determined by the USDA's Nationwide Food Consumption Survey are used for the FDA's Total Diet Study, which estimates nutrient availability for eight age-sex groups (Pennington et al 1989, Pennington and Young 1991, Pennington and Schoen 1996). Yearly examinations of adequacy of nutrient intakes from 1982-1991 have found that children and females consistently consumed less than the 80% of the RDA for iron. The specific age groups affected and their average intake expressed as percent RDA from 1982-1991 are as follows: 2 yrs (57%), 14-16 yrs (61%), and 25-30 yrs (56%). In the 1982-91 Total Diet Study, the average iron intakes were compared to the 9th Edition RDA values. The results of the 1991-1996 Total Diet Study were compared to the 10th edition RDA values that were published in 1989. This examination of the population's nutrient intake also found that adolescent females and women of childbearing age had inadequate intakes of iron (Egan et al, 2002). There was a slight improvement in percent RDA intake from the

1982-91 findings, but average intake in mg/day decreased in girls 14-16 years and women 25-30 years (Egan et al 2002, Pennington and Schoen 1996). The improvement seen in percent RDA intake was due to the changes in the RDA values. The average intakes of 14-16 year old girls, 25-30 and 40-45 year old women were 73%, 61%, and 60% of the RDA, respectively. The most significant change seen in average intakes from the 1982-91 Total Diet Study to the 1991-96 was in the infants 6-11 months old. From 1982-91, this population group consumed an average of 11.9 mg iron per day, whereas from 1991-96, their average intake decreased to 7.9 mg/day (Egan et al 2002, Pennington and Schoen 1996). Egan et al (2002) failed to give an explanation for the decrease in average iron intakes.

The Total Diet Study also assessed the percentage of a nutrient provided by each of twelve food groups for each age group. In both of the Total Diet Studies discussed, grain products provided between 35-65% of the daily iron intakes of children, adolescent females, and women of childbearing age. Meat, fish, and poultry, which are the richest sources of the highly available heme iron, provided only 9-23% of average daily iron intake (Egan et al 2002, Pennington and Young 1991). The chronically insufficient iron intakes of children, teenage girls, and women of childbearing age coupled with the primary contribution of non-heme iron to total dietary iron intake, may help to explain the high rates of iron deficiency anemia in these age groups (Pennington and Schoen 1996, Pennington et al 1989, Expert Scientific Working Group 1985).

Iron Deficiency

Iron deficiency is a prevalent nutrition problem throughout the world (CDC 2002). It is estimated that approximately 30% of the world's population is anemic and that nutritional iron deficiency is the primary cause (Baynes and Bothwell 1990). Children, women of childbearing age, and persons living in third world countries are most affected, but this nutritional deficiency is a problem for men and persons living in developed countries as well (Holst and Lozoff 1998, Finch and Cook 1984).

Iron deficiency does not occur all at once, but rather in three stages. The first two stages, iron depletion and latent iron deficiency (iron deficient erythropoiesis) do not result in anemia as does the third, overt iron deficiency (Herbert 1987, Holst and Lozoff 1998). Each iron deficiency stage is characterized by changes in hematological parameters. Persons with depleted iron stores will exhibit only decreased plasma ferritin levels. Latent iron deficiency will cause an increase of transferrin iron binding capacity (TIBC) out of the normal range, a greater decrease in plasma ferritin from that seen in iron depletion, a significant decrease in plasma iron and transferrin saturation, and an increase in red cell protoporphyrin. The decreased transferrin saturation levels found in this stage are consistent with a diminished supply of iron to the bone marrow for erythropoiesis which helps explain the increased levels of erythrocyte protoporphyrin as it replaces iron in the erythrocyte (Finch and Cook 1984, Cook 1990, Herbert 1987). In both iron depletion and latent iron deficiency, the erythrocytes are not affected. Overt iron deficiency, which results in microcytic hypochromic anemia, is characterized by elevated TIBC and red cell protoporphyrin as well as depressed plasma ferritin, plasma iron, and transferrin saturation. At this stage of deficiency, the erythrocytes become

small and pale in color, hence the name for this type of anemia (Herbert 1987, Cook 1990).

Diagnosis of iron deficient erythropoiesis and iron deficiency anemia are based on change in the aforementioned parameters in addition to changes in hemoglobin and hematocrit. Iron sufficient status is characterized by normal hemoglobin levels and up to three of the following four iron parameters within normal limits: serum ferritin, transferrin saturation, erythrocyte protoporphyrin, and mean corpuscular volume. Normal hemoglobin levels concomitant with aberrant values of three of the four iron parameters previously listed characterize iron deficient erythropoiesis. The diagnostic criteria for iron deficiency anemia are the same as that for iron deficient erythropoiesis except that instead of normal hemoglobin, iron deficiency anemia is accompanied by hemoglobin or hematocrit levels more than two standard deviations below the reference range (Holst and Lozoff 1998, Finch and Cook, 1984).

Iron plays a prominent role in a number of physiological processes, and, as such, the physiological manifestations of iron deficiency anemia are numerous and far reaching. Some of the initial overt signs and symptoms include weakness, fatigue, and impaired tolerance to cold temperatures (Morris 1987, Sato 1991). The physical signs associated with, but not exclusive to, latent and overt iron deficiency include dry brittle hair, hair loss, koilonychia (thin, flat, convex nails), lesions in the mouth and on the tongue, generalized itching, and blue sclera (Sato 1991). Chronic iron deficiency has also been shown to adversely affect immunity, metabolism, and enzyme function, as well as behavioral and physical development in children (Cook 1990, Finch and Cook 1984, Holst and Lozoff 1998, Lozoff et al 1991). Iron deficiency has not been shown to affect

the function of the immune system in its entirety, but rather only individual components. Cell mediated immunity is one facet of the immune system hindered by iron deficiency. T cell numbers, delayed type hypersensitivity (DTH), and lymphocyte proliferation have all been shown to be depressed in iron deficient subjects (Baynes and Bothwell 1990, Cook 1990, Farthing 1989, Finch and Cook 1984). The phagocytic capabilities of neutrophils and macrophages, which are considered components of the "non-specific" immune system, have also been shown to be impaired by iron deficiency (Farthing 1989, Baynes and Bothwell 1990, Cook 1990). With respect to metabolism and enzyme function, iron deficiency has been shown to impair energy metabolism, manifested by decreased work capacity, and this was found to be related to a decrease in mitochondrial enzymes (Finch and Cook, 1984). Siimes et al (1980) found that iron deficiency decreased cytochrome c concentrations in rat muscle and intestinal cells, and it was postulated that this could hinder mitochondrial functions dependent on the cytochrome enzymes.

Iron deficiency occurs concomitantly with a number of diseases as well as conditions associated with chronic inflammation. However, the disease or the inflammation is typically the cause of the iron deficiency, not vice versa. This is not the case with pica, a disease characterized by cravings for substances not considered food. This disease is seen most often in children and pregnant women, two populations in which iron deficiency is rampant. It is estimated that 50% of persons with iron deficiency exhibit pica, but it is not known why this disorder occurs in some persons with iron deficiency and not others (Sato 1991, Prasad 1978).

Iron Overload

Iron overload is a much less common disease than iron deficiency, but its physiological consequences are very detrimental. Furthermore, iron overload is typically seen in persons with accelerated iron absorption or persons with diseases that cause accelerated iron absorption, as opposed to iron deficiency, which is primarily caused by nutritional deficiency (Lynch 1995). The literature describes two main classes of iron overload, primary, which is a genetically linked disorder, and secondary, which includes those instances of iron overload that occur as a result of another disease (Franks and Marks 1998, Pippard 1997, Britton et al 1994). There is one form of documented nutritionally related iron overload that occurs in African males who regularly consume a homemade brew that is rich in a highly absorbable form of iron (Lynch 1995, Gordeuk et al 1992).

Primary iron overload, or hereditary hemochromatosis, is an autosomal recessive disease linked to the HLA-A locus on chromosome 6. This genetic disease causes iron to be absorbed uninhibitedly from the diet, and, due to the body's poor excretion capabilities, massive quantities of iron are deposited in tissues throughout the body (Chua-Anusorn 1999, Lynch 1995). There are both homozygous and heterozygous expressions of this disease, with the former having more serious clinical manifestations (Lynch 1995, Pippard 1997). Secondary hemochromatosis is the occurrence of iron overload induced by or as a manifestation of another disease. Persons with sideroblastic anemia, aplastic anemia, hemolytic anemia, or thalassaemia typically exhibit this secondary hemochromatosis. In aplastic anemia, iron overload is a result of the repeated blood transfusions required for treatment of this form of anemia. In thalassaemia,

sideroblastic anemia, and hemolytic anemia, iron overload is a result of both repeated blood transfusions and increased dietary absorption induced by impaired red blood cell formation (Chua-Anusorn et al 1999, Halliday 1989). Moirand et al (1997) reported a new form of iron overload that they found was not attributable to genetics or another form of anemia. They concluded that this form of iron overload is not linked to the HLA-A locus on chromosome 6, and is characterized by normal transferrin saturation, high serum ferritin, and liver iron concentrations greater than the upper limit of normal (> 36μ mol/g dry liver weight). The authors also postulated that this form of iron overload might be associated with excessive weight (BMI > 25) and/or metabolic disorders including hyperlipidemia, hypertension, and impaired glucose metabolism (Moirand et al 1997). The only documented form of iron overload due to excessive dietary iron intake is termed Sub-Saharan dietary iron overload because it is only exhibited by South African and Zimbabwean populations. These populations regularly consume a brew that is prepared and stored in steel drums, which makes it a rich source of dietary iron. It is estimated that one liter contains 80 mg of iron, and observation of these populations revealed that several liters may bee consumed at any given point in time. Excessive consumption causes iron overload, and the clinical manifestations are the same as those seen in hemochromatosis (Lynch 1995, Gordeuk et al 1992). Due to the similarities in symptoms between hemochromatosis and the symptoms in these African populations, it was postulated that genetics were playing a role; however, research by Gordeuk et al (1992) did not find conclusive evidence that such a link was at work in these populations.

There are specific hematological changes associated with hereditary hemochromatosis and the minor forms of iron overload. Transferrin saturation is one

such parameter. Normal transferrin saturation levels are approximately 35% (Herbert 1987). Subsequent fasting transferrin saturation levels in excess of 50% in women and 60% in men are indicative of hemochromatosis and upon detection, further evaluation of the disease is initiated (Pippard 1997, Lynch 1995). Plasma ferritin, which is indicative of iron stores, is also evaluated, but usually only after transferrin saturation is found to be elevated because plasma ferritin levels are subject to influences other than iron stores (Lynch 1995). Plasma ferritin levels in excess of 300 μg/mL coupled with transferrin saturation greater than 60% are potent indicators for hemochromatosis and subsequent to these findings, a liver biopsy is typically performed for definitive diagnosis (Finch and Huebers 1986, Lynch 1995, Herbert 1987). In hemochromatosis, staining of the liver sample will show an increase in hepatocyte hemosiderin with concentrations reaching up to 50-100 times normal (Lynch 1995, Britton et al 1994). Conversely, in the dietary form of iron overload seen in African males, iron loading occurs preferentially in the reticuloendothelial system, although there definitely is an increase in liver iron content as well (Lynch 1995). The normal liver iron concentration in humans is less than 36 micromoles of iron per gram of dry liver weight (Moirand et al 1997). In heterozygous hemochromatosis, liver iron concentrations are approximately 54 micromoles of iron per gram of dry liver weight. Both of these parameters correspond to a hepatic iron index (μ mol Fe/g dry weight/age in years) less than two. In homozygous hemochromatosis, the hepatic iron index is greater than two. This index is used in evaluating liver biopsy results because iron content increases with age; therefore, in diagnosing hemochromatosis or another disease that causes tissue iron overload, age must be considered (Lynch 1995).

The average functional iron pool is comprised of approximately 40 mg of iron per kg of body weight, and the storage pool contains approximately 1 gram in men and 300 mg in women. The signs and symptoms of iron overload begin to appear when total body iron content becomes 5-10 times higher than normal (Lynch 1995). The physiological manifestations of iron overload are systemic, but the primary organ affected is the liver, as it is a major iron storage site; however, excess iron is also stored in the parenchymal cells of the heart, pancreas, and spleen, and the function of these organs is adversely affected as well (Britton et al 1994, Halliday 1989). As the disease progresses and iron concentrations of the tissues increase, fibrogenesis occurs which causes organ dysfunction (Britton et al 1994). In the latter stages of the disease cirrhosis develops, and in approximately 25% of these patients liver cancer will develop (Lynch 1995). Excessive iron deposition also causes dysfunction of the endocrine and cardiac system. Endocrine system dysfunction can cause diabetes, while arrhythmias and heart failure can ensue from failure of the cardiac system (Lynch 1995, Halliday 1989). Osteoporosis, increased susceptibility to infections, increased lipid peroxidation, and mitochondrial damage have also been attributed to iron overload (Lynch 1995, Halliday 1989, Oppenheimer 1989, Walter et al 2002). Complications from diabetes and cirrhosis, as well as arrhythmias, heart failure, and cancer are the common causes of death in patients with iron overload (Lynch 1985).

Typical treatment methods for iron overload include repeated phlebotomy and iron chelation therapy, although the former is much more effective (Halliday 1989). The most effective treatment regimen is consistent phlebotomy treatments such that serum iron and serum ferritin concentrations are maintained at the low-normal level, or

approximately 65 μg/dL and 40 μg/mL, respectively (Halliday 1989, Herbert 1987). The diagnosis and prompt treatment of iron overload prior to the development of cirrhosis is associated with improved cardiac and endocrine function as well as overall improved prognosis (Lynch 1995).

Iron Deficiency and Overload in the Rat. Similarities in iron metabolism between humans and the rat allow for use of the rat model for studies of iron metabolism as it is not ethically feasible to perform the invasive procedures necessary for such studies on humans. However, differences in physiology exist, which preclude complete extrapolation of the results of animal studies to humans (Dallman et al 1982, Aggett and Davies 1980).

Rats fed iron deficient diets and who subsequently developed iron deficiency anemia, exhibited poor rates of growth. Beard et al (1995) conducted a study to examine the effects of prolonged dietary iron deficiency and food restriction on growth. Forty-six male Sprague-Dawley rats were divided into three groups each receiving one of three dietary treatments. For six weeks, the animals were fed either an iron deficient diet (5 ppm), an iron adequate diet (50 ppm), or the iron adequate diet in amounts 5-15% less than what was consumed by the iron deficient group on the previous day. Prolonged feeding of an iron deficient diet did affect hematological parameters, as the iron deficient group had hemoglobin and hematocrit values (4.3 g/dl and 20%, respectively) significantly less than that of the food restricted and control groups ($p \leq 0.05$). Both the iron deficient diet and food restriction were found to affect body composition with the body weight and body fat percent of both the groups being significantly less than that of the control group ($p \leq 0.05$). An interesting finding was that in the 4^{th} and 5^{th} week of the

experiment, the food intake of the iron deficient animals was significantly greater than the intake of the ad libitum control animals ($p \leq 0.05$). Furthermore, metabolic rate of the iron deficient anemic rats was found to be significantly greater than that of the food-restricted and ad libitum control groups ($p \leq 0.05$). The authors concluded that the increased metabolic rate was likely a compensatory mechanism to maintain core body temperature, as these animals had low body fat, which reduces heat insulation. The increase in metabolic rate may also partially explain the depressed weight gain of the iron deficient animals in the face of increased food intake during the last two weeks of the study (Beard et al 1995).

Schwartz et al (1973) restricted food intake of male and female albino rats to examine the effects on weight, hematology, and clinical chemistry. The experimental diet was iron adequate, but by virtue of restricting intake, the animals were supplied with less than adequate dietary iron. There were four male and four female experimental groups, each consisting of 25 rats. The control group was fed ad libitum and the remaining three groups were provided the same diet in amounts equal to 50, 75, or 87.5% of the amount consumed by the control group; therefore, the nutrient intake of the restricted animals was 50, 75, and 87.5% of that of the control group. The female rats in the restricted groups gained significantly less weight ($p<0.05$) than the female control group, and the percent reduction in weight, as compared to the control, corresponded to the percent of dietary restriction. Food restriction affected hematological parameters in the female rats with significant differences observed primarily between the 50% restricted and control group ($p<0.05$). Red blood cell counts were significantly greater in the 50% group at 90 and 210 days, and in the 75% group at 210 days. Surprisingly,

hemoglobin and hematocrit were significantly greater in the restricted groups versus the controls. Specifically, HCT percentages and HGB levels were greater in the 50% group at 21, 90, and 210 days. In the 75% restricted group, HCT was greater at 210 days whereas HGB was greater at 21 days, and the latter was greater in the 87.5% restricted group at 90 days. The authors attributed the aforementioned differences to hemoconcentration rather than inadequate nutrient intakes. There were no significant differences observed in BUN and SGPT between the female restricted groups and the female control group; however, glucose in the female 75 and 87.5% groups was significantly greater than the female control at the end of 42 days, and alkaline phosphatase was significantly greater in the female controls versus the female 75% group at 90 days and all female groups at 210 days ($p<0.05$). The authors postulated that the significant differences in alkaline phosphatase observed were due to metabolic, as opposed to pathologic, alterations in the liver. Food restriction was observed to produce significant differences among the females in kidney and liver weights as a percentage of body weight (PBW). Kidney percentages were significantly greater in the 50 and 87.5% groups after 90 days of restriction ($p<0.005$ and $p<0.01$, respectively). Liver percent body weight (PBW) was significantly greater in the 50% group at 210 days ($p<0.005$), 75% group at 90 days ($p<0.005$), and the 87.5% group at 90 days ($p<0.01$). The authors observed increased fat and iron-laden macrophages in the liver, which may partially explain the greater weight of the livers of the restricted animals versus the controls. Iron laden macrophages were also found in the spleens, but there were no significant differences in spleen PBW between the restricted groups and the controls, and there were

no described changes in the kidneys to explain the greater weights of the kidneys in the restricted animals versus the controls (Schwartz et al 1973).

Dallman and his colleagues (1982) examined the progression of iron deficiency by feeding iron replete rats iron deficient diets. For the first two weeks of the study, the weanling rats consumed a 100 ppm iron diet with the goal of maximizing iron stores. Subsequently, the animals were randomly assigned to receive 2, 6, or 50 ppm iron diet for the remainder of the study (54 days). Liver iron concentrations in the control group (50 ppm) varied little throughout the study, whereas, spleen iron concentrations increased from 40 μg/g at day 0 of the experimental period to 426 μg/g at day 54 of the study period. The authors concluded that the dramatic increase in spleen iron observed was a manifestation of the mechanism of iron storage. During the early stages of development, there is insufficient iron available for storage as the iron demands of growth are great, and the RBC, which contribute iron when they are removed from circulation and broken down by the spleen, have not reached the end of their life cycle, which has been estimated at 60 days (Dallman et al 1982). In the 2 and 6 ppm groups, both liver and spleen iron concentrations decreased significantly with the passage of time and were significantly less than that of the control group ($p<0.05$). Hematocrit values in the control group increased gradually from 35.7% at initiation of the experimental feedings to 43.6% at the end of the study, and the authors attributed this change to the growth and development of the animals (Dallman et al 1982). Significant differences in hematocrit values between the control group and the 2 and 6 ppm groups were first observed on the 6^{th} day of the experimental feeding period and continued to the end of the study ($p<0.05$). Feeding iron deficient diets to iron replete rats also produced significant differences

($p<0.05$) in serum iron and transferrin saturation between the control group and both deficient groups. The changes in laboratory parameters indicative of the various stages of iron deficiency were observed, however, the changes did not occur in the order that corresponded to passage from one stage to the next. Specifically, the stages overlapped, giving a less clear picture of the progression of the disease than indicated elsewhere (Herbert 1987, Dallman et al 1982).

The objective of Dallman et al (1982) was to determine the stages of iron deficiency development in iron-replete rats fed iron deficient diets. Conversely, Siimes et al (1980) fed weanling male Sprague-Dawley rats diets of deficient, marginal, adequate and excessive iron content to determine the level of dietary iron intakes that would result in iron deficiency as indicated by changes in hemoglobin, myoglobin, hematocrit, transferrin saturation, cytochrome c, and liver iron. The animals were maintained on one of eleven experimental diets with iron concentrations ranging from 7 ppm to 500 ppm for three weeks. Hematocrit levels were determined at different time points over the course of the study, and there was a net decrease in the iron deficient groups (7, 10, 13, and 17 ppm) with the lowest levels exhibited by the 7 and 10 ppm groups. The remaining diet groups (25, 40, 50, 75, 125, 250, and 500 ppm) exhibited a net increase in hematocrit levels with no differences detected among these groups. Hemoglobin, myoglobin, cytochrome c, and transferrin saturation increased with increasing dietary iron concentration from 7 ppm up through 17 ppm, and subsequently exhibited a plateau effect from 25 ppm-500 ppm. Significant differences in hemoglobin ($p<0.001$) and cytochrome c ($p<0.05$) were detected between all iron deficient animals and those on marginal, adequate, or excessive iron diets. Myoglobin levels in the two lowest dietary

iron groups (7 and 10 ppm) were significantly different ($p<0.001$ and $p<0.05$, respectively) than the myoglobin levels of the animals receiving the 25-500 ppm diets. Liver iron concentrations did not increase in a pattern comparable to that observed in hemoglobin, myoglobin, and cytochrome c, but rather the animals receiving the iron deficient diets (7-17 ppm) had similar liver iron concentrations, while from 25-500 ppm, liver iron concentrations increased at each level of increasing dietary iron concentration (Siimes et al 1980). The authors concluded that their findings support the concept that iron deficiency first results in depleted iron stores, which reduces transferrin saturation because there is less iron available to be transported. Decreased iron availability subsequently hinders production of hemoglobin, as there is insufficient iron to be incorporated into the hemoglobin molecule. They also postulated that symptoms of iron deficiency, including depressed production of hemoglobin, myoglobin, and cytochrome c, occur with regular consumption of diets providing less than 25 mg Fe/kg diet, and that decreased cytochrome c levels suggest that maintenance on iron deficient diets during periods of rapid growth may hinder energy production in cell mitochondria (Siimes et al 1980).

Iron overload research in rats frequently examines deposition of iron in the tissues; one of the manifestations of overload is excessive tissue deposition, which affects organ function. Similarly to studies of iron deficiency, the effects of iron excess on hematology, weight, and plasma proteins are evaluated as well.

Kimura and Yokoi (1996) evaluated an experimental hemochromatosis model by feeding male Wistar rats diets adequate or deficient in magnesium and deficient, adequate, or excessive in iron for twenty days. The iron concentrations of the adequate

and iron excess diets were 120 and 240 ppm, respectively. There were no significant differences in hematology, weight gain, food intake and efficiency, and tissue iron concentrations of the animals receiving the magnesium adequate-iron adequate and magnesium adequate-iron excess diet (Kimura and Yokoi 1996). Although not significant, there were differences in the aforementioned variables between iron adequate and iron excess groups. The rats consuming excess iron gained more weight, had higher feed efficiency, and consumed more diet than the animals on the iron deficient diet. Hematocrit was higher in the iron excess group, but hemoglobin and mean corpuscular hemoglobin concentrations were less. As would be expected, liver, heart, spleen, and kidney iron concentrations were greater in the excess group. The only significant difference in plasma parameters between adequate and excess groups was in amylase, with levels in the excess group being significantly higher than that of the adequate group ($p<0.05$). Albumin, SGOT, SGPT, and alkaline phosphatase were higher in the excess group, whereas urea nitrogen was lower (Kimura and Yokoi 1996). The lack of significant differences observed between the iron adequate and iron excess group may be due in part to the short study duration.

A similar study to that just described, but with a longer experimental period, was conducted by Shah and Belonje (1991). Fifty female weanling Sprague-Dawley rats were randomly assigned to one of five experimental diet groups (25, 47, 150, 270 (chow) or 1260 ppm), and at 6 and 12 weeks, the rats were killed for analyses. Body weights were not significantly different among the different diet groups. Hemoglobin levels were higher in all diet groups at six weeks versus 12, and at six weeks the highest hemoglobin levels were seen in the 150 and 1260 ppm diet groups ($p<0.05$). An interesting finding

was that the animals receiving the chow had the lowest hemoglobin levels at both 6 and 12 weeks, which the authors attributed to the effects of compounds such as phytates that were in the chow, but not the purified diet (Shah and Belonje 1991). However, this potential inhibition of iron absorption was not exhibited in the tissues, as the animals fed the chow did not have the lowest liver or spleen iron concentrations. Actually, they had the second highest liver and spleen iron concentrations, preceded only by the 1260 ppm diet group, and followed by the 150 ppm group. The authors addressed this as well and concluded that dietary iron concentration affects tissue iron concentrations, but does not exert an effect on hemoglobin levels (Shah and Belonje 1991). This conclusion is in conflict with that of Siimes et al (1980) and Kimura and Yokoi (1996) who attributed differences in hemoglobin concentrations among animals fed diets of varying iron concentrations to dietary iron intake. Furthermore, hemoglobin is often used in the diagnosis of iron deficiency anemia, which results from inadequate dietary iron intake (Holst and Lozoff 1998, Finch and Cook, 1984). Analysis of liver and spleen iron concentrations in the female rats revealed that the concentration of iron in these tissues increased with increasing dietary iron concentration and with the passage of time. At both 6 and 12 weeks, the 25 and 1260 ppm groups had the lowest and highest liver iron concentrations, respectively, but the differences in liver iron concentrations among all the diet groups, particularly 47, 150, and 270 ppm, were less pronounced at 12 weeks versus 6. Furthermore, the 25 and 47 ppm groups experienced the greatest increase in liver iron, whereas the 1260 ppm group experienced a slight decrease. At 6 and 12 weeks, liver iron concentrations in the 1260 ppm diet group were significantly greater than that of all the other animals ($p<0.05$). At 6 weeks the 150 ppm diet group exhibited liver iron

concentrations significantly greater than the animals receiving the 25 ppm diet, but this significance was not detected at 12 weeks. In the spleen, significant differences in iron concentrations were only detected at 12 weeks between the animals receiving the 1260 ppm diet and those receiving the 25, 47, and 150 ppm diets ($p<0.05$). The liver and the bone marrow are the two primary iron storage sites, yet in this study, the iron concentrations in the spleen far exceeded those of the liver, except in the 1260 ppm group at 6 weeks, for at this time the concentration of iron in the liver exceeded that of the spleen. Furthermore, the magnitude of change in spleen iron concentrations from 6 to 12 weeks was also much greater than what was observed in the liver. In the liver, there was a doubling of liver iron concentrations in the 25 and 47 ppm groups, which was the greatest increase observed among all of the diet groups. Conversely, the spleen iron concentrations increased over four-fold in the 25 and 150 ppm groups and 5-fold in the 47, 270 (chow), and 1260 ppm groups. Also, the difference in spleen iron concentrations among all diet groups at 6 weeks was less pronounced than what was observed at twelve weeks, which is the opposite of what occurred in the liver. The large difference in the spleen and liver iron concentrations raises the question of adequate perfusion of the spleen at the time of necropsy. The spleen functions in both the destruction of old red blood cells and the storage of blood for incorporation into circulation in the event of acute injury; therefore, inadequate perfusion could cause analyzed spleen iron concentrations to be higher than what would have actually been due to iron deposition alone (Cohen and Wood 2000). Unfortunately, the researchers failed to describe whether or not perfusion of the tissues was a component of the methodology. Shah and Belonje

(1991) ultimately postulated that iron absorption is more efficient with prolonged periods of insufficient iron intake.

Iron deficiency in humans is treated with iron supplementation, and to examine the effects of iron excess via supplementation, Knutson et al (2000) fed 48 weanling male Sprague-Dawley rats iron deficient (0 μg iron/d) or iron adequate diets (800 μg iron/d) for twelve days followed by a 22-day period during which half of the rats were maintained on their original diet, and the other half were provided 8000 μg provided by their diet. The body weight of the rats provided the adequate diet plus the supplements was significantly greater than both the deficient and deficient plus supplement groups ($p<0.05$), but it was not significantly different than the adequate diet only group. Hemoglobin levels were not significantly different between the adequate and adequate plus supplement group, but liver iron concentrations were significantly greater in the supplemented group ($p<0.05$). The authors described their rats as having "subacute, minor iron overload" that increased liver iron deposition as well as lipid peroxidation in the animals (Knutson et al 2000).

Chua and Morgan (1996) observed different effects of iron overload on animal weight than described thus far. The effect of iron overload and manganese loading on tissue deposition of both minerals was examined. Twelve pregnant Wistar rats were maintained on either a 20 g Fe/kg diet (iron loaded) or a 0.5 g Fe/kg diet (control) beginning at day 18-19 of pregnancy and through weaning of the rat pups. At 15 days of age, half of the rat pups were killed for analyses, while the remaining animals were maintained on their respective diets until 63 days of age. The body weight of the iron-overloaded rat pups at both 15 and 63 days were significantly less ($p<0.05$) than the body

weight of the control animals (Chua and Morgan 1996). Similar to the aforementioned studies, Chua and Morgan (1996) found that the liver and kidney iron concentrations of the iron- overloaded rats were significantly greater than the animals receiving the control diet ($p<0.05$).

The research discussed here illustrates the effects of iron deficiency and iron overload on body weight and composition, hematology, and tissue iron concentrations in rats. The effects described were not always significant, but that does not disprove the occurrence of adverse effects of either iron deficiency or overload. In some of the cases discussed, the duration of the experimental period was relatively short, which raises the question of whether or not differences would become significant with the passage of time.

Zinc

Physiological Roles

Zinc has a number of vital functions throughout the body and as such it is essential for life. Adequate zinc nutriture is required for normal growth and development, wound healing, efficient immune function, and enzyme activity (Prasad 1988, Baer et al 1985). Protein-calorie malnutrition that often occurs in the elderly and persons of low socioeconomic status has been identified as a causative factor in the development of zinc deficiency (Prasad 1988). Other causes include the poor bioavailability of zinc in the grain-based diets that are prominent throughout the world, namely in underdeveloped countries, diseases inducing malabsorption, and highly

catabolic states that cause excessive urinary zinc losses. The effects of zinc deficiency have been examined with respect to all of the aforementioned functions.

Sandstead et al (1967) examined the characteristics of zinc deficiency with particular reference to growth and sexual maturation in 40 Egyptian males ages 12-20 years. Zinc deficiency was confirmed by studies of zinc metabolism, which included assessment of the zinc concentration of the urine, plasma, and sweat. In those subjects not infected with parasites, inadequate dietary zinc intake was the primary causative factor of the disease. Diet history revealed that typical intake was lacking in zinc and iron. Animal proteins were an insignificant part of normal intake, and the primary food sources were wheat or corn bread, rice, and vegetables, which varied depending on the season. Growth retardation was a prominent symptom of zinc deficiency and was characterized primarily by depressed linear and bone growth. Other clinical manifestations of zinc deficiency included hypogonadism, hypopituitarism, dry skin, hyperpigmentation, abnormal alkaline phosphatase levels reflecting the inflammatory changes in the liver determined by biopsy, and impaired glucose absorption. Iron deficiency and pellagra commonly occurred concomitantly with zinc deficiency, which was not surprising given the poor nutrient availability of typical Egyptian fare. The authors concluded that inadequate nutrient intakes coupled with excessive losses via sweat and due to bleeding from parasites were primary determinants of the development of zinc deficiency. Zinc supplementation facilitated reversal of zinc deficiency symptoms, namely growth and sexual maturation (Sandstead et al 1967).

Carter et al (1969) also examined the role of zinc in growth and development in humans, and like Sandstead et al (1967), their study population consisted of adolescent

Egyptian boys. At the time of this preliminary research on zinc and physical development (1969), children in Middle Eastern countries, such as Egypt, Jordan, Iran, Turkey, Libya and Lebanon, commonly exhibited growth retardation. Such was the reasoning behind the selection of Egyptian children for this study, and an adolescent age group was utilized because adolescence is a period of rapid growth and developmental changes. The objective of this year-long study was to determine if zinc or iron supplementation would exert positive effects on the growth and development of adolescent boys. Two hundred seventy-nine boys age 11-18 years composed the study population, and were randomly assigned to receive one of three dietary supplements (placebo, 300 mg iron sulfate, or 60 mg zinc sulfate) for 6 days a week over the course of 5 ½ months. A sub-sample of 90 boys exhibited growth retardation as defined by the researchers as "heights greater than 2 standard deviations below the Iowa mean for height (Carter et al 1969)." This sub-population of boys received the same dietary supplements, but they were observed as a separate entity in order to assess the effects, if any, of supplementation on correcting growth retardation. The control group for this sub-sample was randomly selected from the remaining 189 boys and consisted of 30 boys with heights less than 2 standard deviations below the Iowa mean for height. Nutrient analyses of typical intake revealed that overall, the nutrient intake of the study population was inadequate to meet the demands of growth and development during adolescence. Interestingly, average dietary intake of zinc was not inadequate at 14 mg /day, but the authors postulated that zinc absorption may be inhibited by other dietary components, including phytate. All boys exhibited a number of physical characteristics suggestive of nutrient deficiencies with the most prevalent being angular lesions, cheilosis, papillary

atrophy, eyelid scarring, hepatomegaly, and splenomegaly. There were no significant differences in the occurrence of the aforementioned clinical characteristics, as well as all others, among the study participants. Serum iron and zinc levels were below normal (less than 100μg and 90μg/ 100mL) in all study participants at the initial evaluation. Serum iron and zinc improved to normal levels with respective supplementation, but when supplementation was discontinued, both serum indices returned to sub-normal levels. Height, bone age, and sexual development improved by the end of the study, but there were no significant differences among supplemental treatment groups (Carter et al 1969). Ultimately, the authors observed improvements in serum zinc with zinc supplementation, but positive effects on growth and sexual maturation were not greater than what was observed in the iron supplementation and placebo groups. One possible explanation for the lack of significant differences observed among supplemental groups and between the growth retarded and remaining boys may be the passage of time between the end of the supplementation period and the final physical examination. The authors observed improvements in serum zinc with zinc supplementation, but noted that the improvements were lost when the supplementation ceased; therefore, there may have been insufficient time for the additional zinc to positively affect growth. The boys receiving zinc supplementation did experience decreased incidences of papillary atrophy, dry/scaling skin, glossitis, eye-lid scarring, corneal scarring, parotid enlargement, hepatomegaly, and splenomegaly. Another possible explanation for the lack of positive effects on growth and development is the level of zinc supplementation utilized in the study, which may have been insufficient to correct the long-term effects of zinc deficiency on growth and development.

The function of zinc in wound healing is thought to be related to its role in growth, specifically growth on the cellular level, and enzymatic activity (Halsted and Smith 1970, Hallböök and Hedelin 1977). However, wounds, from surgery, burns, or ulcers, have been shown to increase urinary zinc losses, which may deplete zinc stores such that wound healing is compromised (Prasad 1988, Hallbook and Hedelin 1977). To examine the effects of surgery on zinc status, Hallbook and Hedelin (1977) measured serum zinc and 24-hr urinary zinc excretion pre-operatively and each day for five days post-operatively. Thirty-eight adults composed the study population, and all patient had pre-op serum zinc values below the standard of 91 μg/100mL. The difference between the standard serum zinc value and average pre-op serum zinc values of the patients less than 60 years of age (86±8 μg/100mL) and those over 60 years of age (75±15 μg/100mL) was significant at $p<0.01$ and $p<0.005$, respectively. All patients experienced a decrease in serum zinc after surgery, with the lowest levels observed on post-op day two. Subsequently an increase in serum zinc was observed from post-op day two through post-op day five, but at the 5th day, serum zinc levels had not returned to pre-op levels. An overall inverse relationship between serum zinc and urinary zinc excretion was observed with the greatest excretion rates observed between pre-op and post-op day one, and between post-op days four and five. The authors concluded that the adverse effects of surgery on serum zinc levels warrant the use of zinc supplements post-operatively to ensure zinc status is maintained such that wound healing is facilitated rather than delayed (Hallböök and Hedelin 1977).

Halsted and Smith (1970) observed depressed (58 μg/100mL) serum zinc levels in persons with leg ulcers, which led Hallböök and Lanner (1972) to examine the effects of

zinc supplementation on the healing of venous ulcers. Twenty-seven patients with leg ulcers between 100-1000 sq mm were first divided into two groups, those with initial serum zinc levels <100μg/100mL (Group 1) and those with initial serum zinc levels >110 μg/100mL (Group 2). In a double-blind randomized design, the subjects within each group were assigned to receive either a zinc supplement (600 mg/day) or a placebo for 18-weeks. In Group 1, the subjects receiving the zinc supplement exhibited a healing rate significantly greater than the controls ($p<0.02$), but in Group 2, there were no significant differences in healing rate between the zinc supplement and placebo groups. Furthermore, significant differences were observed between the two placebo groups, with the subjects in Group 1 exhibiting a healing rate significantly slower than exhibited in Group 2 ($p<0.02$). At the end of the study, the greatest healing rate was observed in those receiving the placebo in Group 2, followed by those receiving the zinc supplement in Group 1. The Group 1 placebo subjects experienced a net decrease in serum zinc at the end of the study period, which supports the findings of Halsted and Smith (1970) that the presence of leg ulcers adversely affects serum zinc levels. The findings of Hallböök and Lanner (1972) also suggest that zinc supplementation improves wound healing in persons with serum zinc levels below the normal range.

Depressed immune function has been observed in persons with zinc deficiency, and the essentiality of this nutrient for immune function has been revealed by research showing improved immune function with zinc supplementation. Characteristics of impaired immune function observed in zinc deficiency that have been corrected with zinc supplementation include thymic atrophy, impaired cell mediated immunity and delayed hypersensitivity, lymphopenia, depressed helper T cell activity, increased suppressor T

cell activity, and decreased natural killer cell activity (Keen and Gershwin 1990).

Prasad (1988) assessed the zinc status of 23 elderly subjects, and one-third exhibited signs and symptoms of mild zinc deficiency. The zinc concentrations of the granulocytes and platelets of the zinc deficient group were significantly less than what was observed in the controls. Furthermore, the subjects with zinc deficiency exhibited decreased reactivity to antigens, as well as significantly depressed IL-2 activity of the helper T cells ($p<0.001$).

Baer et al (1985) examined the effects of zinc depletion and subsequent repletion on indices of zinc functions in males. The study population consisted of six men housed in a metabolic unit for a 10-11 weeks. During the first week, they were maintained on a baseline diet that provided 15.7 mg zinc per day. Subsequently, the subjects were placed on an zinc-depleted diet that provided 0.28 mg zinc per day for 30-63 days. The repleted subjects received diets providing 6, 23.3, or 46.3 mg zinc per day for two weeks (2 subjects) or five weeks (1 subject). Assessment of the effects of zinc depletion on immune function included measurements of monocyte and neutrophil chemotaxis. Monocyte chemotaxis was observed to be normal, but neutrophil chemotaxis was impaired after 8 weeks of depletion. The lower end laboratory value indicative of normal neutrophil chemotaxis is 4%, and two of the depleted subjects exhibited chemotaxis values of 1.2 and 1.3%. During the depletion period, all subjects experienced sore throats, increased acne, and/or apthous stomatitis, all of which are indicative of impaired immune function. Furthermore, these symptoms disappeared during the repletion period. The authors concluded that the aforementioned findings suggest that resistance to infection is impaired in persons with zinc deficiency (Baer et al 1985).

Over one hundred enzymes, the majority of which are metalloenzymes, are dependant on zinc for maintenance of structure and function. The zinc-dependent enzymes function in DNA synthesis, the dehydration of bicarbonate and hydration of carbon dioxide, oxidation and reduction reactions, and hydrolysis of peptide bonds and phosphate esters (Abdel-Mageed and Oehme 1990, Baer et al 1985).

In their study examining the effects of zinc depletion, Baer et al (1985) also assessed its effects on the levels of zinc dependent enzymes. They observed a significant decrease ($p<0.0005$) in serum alkaline phosphatase (AP) levels at the end of the depletion period in the group designated for repletion, and after two weeks of repletion serum AP levels improved significantly ($p<0.05$). Conversely, leukocyte alkaline phosphatase (LAP) levels increased in excess of the normal range during the depletion period, but the change from baseline was not significant. The group designated for repletion subsequently experienced a significant drop in LAP ($p<0.05$) to a level within the normal range. Lactic dehydrogenase levels decreased from baseline after the depletion period, but the changes were not significant and the levels of this enzyme did not respond to zinc repletion. Erythrocyte delta-aminolevulinic acid dehydratase levels decreased significantly ($p<0.05$) during the depletion period, and subsequently rose with repletion, although this change was not significant. An overall increase in plasma ribonuclease was observed in all subjects, but was not significantly greater than baseline; however, ribonuclease levels in the repletion group increased significantly ($p<0.05$) during the repletion period. Despite the inconsistent changes in the aforementioned enzyme levels during a relatively short period of depletion, the authors concluded that some zinc

dependent enzymes are adversely affected by insufficient dietary zinc intake (Baer et al 1985).

Iron and Zinc Interactions in the Rat

Hill and Matrone (1970) proposed that the antagonistic interactions among transition metals are attributable to similar physical and chemical characteristics of these metals. Iron and zinc are two such minerals, and elucidating the nature of the interaction between them has been the objective of trace mineral research in man and animals (Solomons 1986). The ensuing discussion will focus on the results of research involving the rat, as that was the experimental model used in our study.

Bougle et al (1999) assessed the effects of dietary iron and zinc on growth and absorption, as reflected by metabolic balance studies and tissue concentrations. Thirty-two weanling male Sprague-Dawley rats were assigned to receive one of four experimental diets varying in iron and zinc concentration for two months. The iron and zinc concentrations were neither below or above the recommended intake range for rats, and the four diet combinations were as follows: 300 ppm Fe/45 ppm Zn (Fe+/Zn+), 300 ppm Fe/14.2 ppm Zn (Fe+/Zn-), 44.1 ppm Fe/45 ppm Zn (Fe-/Zn+), 44.1 ppm Fe/14.2 ppm Zn (Fe-/Zn-). The metabolic balance studies revealed that iron and zinc absorption and retention were significantly ($p<0.0001$) greater in the animals provided the higher diet concentrations of these minerals (300 ppm Fe and 45 ppm Zn, respectively). In the first metabolic balance study, iron absorption was significantly affected by dietary iron concentration ($p<0.0001$), but not dietary zinc concentration ($p=0.578$). Zinc absorption and retention were significantly affected by both dietary iron ($p=0.015$ and $p=0.046$,

respectively) and zinc concentrations ($p<0.001$). The second balance study, which was conducted during the last week of the experimental period, revealed the same relationships between iron and zinc intakes and absorption as the first, except that a significant effect of dietary zinc on iron absorption was detected ($p=0.015$). The liver iron concentrations of the animals receiving the 44.1 ppm iron diet (Fe -) were significantly less than the liver iron concentrations of the animals receiving the 300 ppm iron diet ($p<0.05$). The greatest liver zinc concentration was observed in the group receiving the Fe-/Zn+ diet (44.1 ppm Fe/45 ppm Zn), and it was significantly greater ($p<0.05$) than the liver zinc concentrations of the animals receiving the Fe+/ Zn+ (300 ppm Fe/45 ppm Zn) and Fe+/Zn- (300 ppm Fe/14.2 ppm Zn) diets. These findings suggest that there is an inverse relationship between dietary iron intake and zinc absorption. Specifically, when iron intakes were at the lower end of the recommended intake range (44.1 ppm), liver zinc concentrations were the greatest, and when dietary iron intake was at the upper end of the recommended intake range, liver zinc concentrations were the lowest. Weight gain among the diet groups was not significantly different ($p=0.06$), but there was a trend toward significance, which is not surprising given the role of zinc in physical growth and development. The authors concluded that there is a negative relationship between iron and zinc, owing to the similarities in their physical and chemical characteristics that result in competition at the absorption site such that zinc absorption is depressed by a greater proportion of dietary iron (Bougle et al 1999).

Dursun and Aydogan (1995) also examined the relationship between iron and zinc with an emphasis on the effects of variable dietary iron intake on zinc absorption.

Twenty male Swiss albino rats were obtained at 6 weeks of age and randomly assigned to one of four dietary treatment groups: control (150 ppm Fe), low iron (10.44 ppm), high iron (388 ppm), and very high iron (827 ppm). Following a 7-day acclimatization period during which time the animals were fed standard rat diet, the animals were provided 15 g of their respective diets every day for two weeks. The rats were injected with radio-labeled zinc (2 μCi Zn-65) and metabolic balance studies were performed to assess the effects of dietary iron on absorption, retention, and excretion of zinc. The animals receiving the control and low iron diets gained weight, whereas the animals receiving the high and very high iron diets lost weight; however, significance was only detected between the control group and the very high iron group ($p<0.05$). Fecal losses of zinc were the greatest in the high and very high iron groups, and the low iron group experienced fecal Zn losses less than that experienced by the controls. An inverse relationship between dietary iron and zinc absorption and retention was observed. Specifically, zinc absorption and retention in the very high iron group was significantly lower than that of the controls throughout the examination period post Zn-65 administration ($p<0.01$). Conversely, the low iron group exhibited zinc absorption significantly greater than the controls at 24 and 48 hours post Zn-65 administration ($p<0.01$). Furthermore, zinc retention in the low iron group was significantly greater than the controls at 48, 72, and 96 hours after Zn-65 administration ($p<0.05$). The inverse relationship between dietary iron and zinc was reflected in the tissue concentrations of zinc as well. Liver zinc concentration (μg Zn/g dry tissue) was significantly greater in the low iron diet group versus the control group ($p<0.001$), whereas, liver zinc concentrations of the very high iron group were significantly lower than the controls

($p<0.01$). The authors concluded that the inverse relationship between iron and zinc that they observed was in accord with the theory that the interaction between these two elements is a function of their similar absorptive pathways (Dursun and Aydogan 1995).

The role of zinc in a number of life requiring physiological processes cements its position as an "essential" trace element. The observed inverse relationship between dietary iron concentration and zinc absorption and retention may have serious consequences for zinc nutriture in persons given iron supplementation to correct iron deficiency anemia.

Copper

Physiological Roles

Copper, like iron and zinc, is a nutrient essential for life, and many of the functions of these minerals are similar. Copper plays a role in immune function and growth, as does zinc, and it and iron are components of the cytochrome oxidases, which function in energy production (Linder and Hazegh-Asam 1996, Olivares and Uauy 1996). One of the primary similarities among these minerals is that all serve as functional components of enzymes, and many of the functions of copper throughout the body, including anti-oxidation and iron metabolism, are related to its enzyme involvement). Copper is also a component of proteins that function in the metabolism, transport, and storage of minerals (Abdel-Mageed and Oehme 1990, Linder and Hazegh-Asam 1996, Olivares and Uauy 1996).

The functions of the copper-containing proteins, namely enzymes, throughout the body are numerous and diverse. Metallothionein is one such protein, and it is involved in the metabolism, transport, and storage of minerals such as copper, zinc, and cadmium (Linder and Hazegh-Azam 1996). Superoxide dismutase (SOD) is an enzyme with copper at its active site, and it functions in the dismutation of superoxide radicals and as such, is involved in antioxidant activity (McCord and Fridovich 1969, Solomons1979, Linder and Hazegh-Azam 1996). Lysyl oxidase, another copper-containing enzyme, is involved in the cross-linking of collagen and elastin necessary for bone and tissue development (Abdel-Mageed and Oehme 1990). Ceruloplasmin is one of the primary copper-containing enzymes, as it is intimately related to iron metabolism and energy production, with ceruloplasmin's role in the latter related to another copper-containing enzyme, cytochrome c oxidase (Osaki et al 1966, Roeser et al, 1970, Hsieh and Frieden 1975).

Holmberg and Laurell (1948) performed precipitation studies on human and pig serum that resulted in their identification of a "blue protein" different in nature from hemocuprein that they termed 'ceruloplasmin.' Their subsequent research on ceruloplasmin identified it as an enzyme with copper in its active site (Holmberg and Laurell 1951). Ceruloplasmin functions as a copper transport protein, and its enzymatic capabilities are required for iron metabolism, and as such it is referred to as a 'ferroxidase' (Roeser et al 1970, Hsieh and Frieden 1975, Solomons 1979).

Osaki et al (1966) assessed the role of ceruloplasmin in iron metabolism which led them to conclude that ceruloplasmin exhibited ferroxidase properties. Nineteen samples of human blood were collected for use in evaluating Fe (II) oxidation in the both

the presence and absence of ceruloplasmin, and the formation of Fe (III) transferrin under approximate physiological conditions. The presence of ceruloplasmin was associated with an accelerated rate of Fe (II) oxidation as compared to oxidation of Fe (II) without ceruloplasmin present. The conversion of Fe (II)-transferrin to Fe (III)-transferrin was also observed to be greater in the presence of ceruloplasmin. The significance of these findings lies in the fact that increased rates of Fe (II) oxidation and formation of Fe (III)-transferrin in the presence of ceruloplasmin translates to greater amounts of iron transported to the bone marrow for erythrocyte formation (Osaki et al 1966). Osaki et al (1966) ultimately suggested that ceruloplasmin be classified as a ferroxidase, as it was observed to exhibit enzymatic properties, specifically by stimulating oxidation reactions in iron metabolism.

Roeser et al (1970) fed 5-6 day old pigs a copper and iron deficient diet to examine the effects of copper deficiency on iron status as it related to ceruloplasmin activity. The control animals received the original copper and iron deficient diet preparation that had been supplemented with copper sulfate, whereas the diet of the piglets in the experimental group was not supplemented. All animals consumed the diet for 73 days, and all were given intramuscular iron dextran injections during the first 3-4 weeks of the study. Assays of plasma ceruloplasmin levels revealed that control pigs had greater ceruloplasmin levels than the copper deficient animals, which illustrates the necessity of copper for ceruloplasmin formation. In the copper deficient pigs, plasma ceruloplasmin levels decreased continually with the passage of time, and hypoferremia developed between the 8^{th} and 9^{th} week of the study with the lowest plasma iron level observed between the 11^{th} and 12^{th} week of the study. Administration of copper and

ceruloplasmin improved plasma iron levels, but the ceruloplasmin exhibited a more positive effect on plasma iron than the administration of copper alone. The authors also assessed the role of ceruloplasmin on iron metabolism by administering intravenous injections of ferric and ferrous iron to both the control and copper deficient animals. In the control animals, the form of iron injected did not significantly affect plasma iron concentrations ($p>0.05$). However, in the copper deficient animals, there were significant differences in plasma iron concentrations as a result of the ferric and ferrous iron injections, with the increase in plasma iron stimulated by the ferric injections being significantly greater than that produced by the ferrous injections ($P<0.01$). Furthermore, increases in plasma iron concentrations due to the ferrous iron injection were significantly different between the control and copper deficient animals, with the increase in the control animals being significantly greater than that observed in the copper deficient animals. The aforementioned findings led the authors to conclude that copper facilitates iron metabolism by enabling the production of ceruloplasmin, which is required for efficient iron metabolism (Roeser et al 1970).

Hsieh and Frieden (1975) concluded that ceruloplasmin functions in copper transport based on the findings of their research that examined the roles of ceruloplasmin relative to cytochrome c oxidase activity. Sprague-Dawley rats of weanling age were maintained on a copper deficient diet for 8-weeks. The control and copper deficient groups received the same diet, but the water provided the control group was supplemented with copper (10 ppm). Two sub-experiments were included in this study that involved the injection of copper compounds, including ceruloplasmin, into the copper deficient rats to determine the effects on cytochrome c oxidase activity in

different tissues. In both sub-experiments, the ceruloplasmin injections had the most profound effects on cytochrome c oxidase activity in all tissues examined (spleen, liver, heart, lung, pancreas, and kidney). The greatest differences in cytochrome c oxidase activity were observed between the copper deficient animals injected with saline and those injected with rat or human ceruloplasmin in the spleen, heart, and liver (Hsieh and Frieden 1975). The aforementioned findings led Hsieh and Frieden (1975) to conclude that ceruloplasmin facilitates the production of cytochrome c oxidase more so than other copper compounds including copper chloride ($CuCl_2$), copper-albumin, and copper-histidine by virtue of its role in copper transport. Furthermore, the role of ceruloplasmin in the formation of cytochrome c oxidase has implications for energy production throughout the body, as it is the final enzyme of the electron transport chain (Linder and Hasegh-Azam 1996).

Iron and Copper Interactions in the Rat

The interaction between iron and copper, like that of iron and zinc, can be attributed to the similarities in the physical and chemical properties of these two minerals (Hill and Matrone 1970). Furthermore, they are intimately related by virtue of the role of copper in iron metabolism (Ozcelik et al 2002).

Ozcelik et al (2002) examined the relationship between iron and copper as it related to hematology. For nine weeks, two groups of seven Wistar rats were fed the same diet, with one group (experimental) receiving copper supplemented water. At the end of nine weeks, blood was drawn and analyzed for erythrocyte deformability, viscosity, hematocrit, hemoglobin, and red cell count. The experimental group exhibited

significantly greater hematocrit, erythrocyte count, and blood viscosity levels than the control group ($p<0.05$). Conversely, erythrocyte deformability and hemoglobin were lower in the experimental group, with the hemoglobin levels between the two groups being significantly different ($p<0.05$). The authors concluded the observed depression of hemoglobin despite increased erythrocyte counts and hematocrit in the experimental group was due to adverse effects of copper on the enzymes involved in hemoglobin production. They also postulated that excessive copper interferes with membrane proteins and as such affected erythrocyte deformity and blood viscosity (Ozcelik et al 2002).

Crow and Morgan (1996) examined the effects of excessive iron and copper on deposition of both minerals in tissues. Twelve pregnant Wistar rats were provided iron and copper loaded diets starting on day 20 of their pregnancy. Litters contained 6-8 pups, and all were maintained on the same diet as their dams. At 15, 21, and 63 days of age, rat pups from each group were killed for analyses. The control diet contained 16 ppm copper and 70 ppm iron. The experimental groups were provided with either copper supplemented water (Cu+ group), diet supplemented with 20 g/kg carbonyl iron (Fe+), or both (Cu+, Fe+). An inverse relationship was observed between dietary copper and iron deposition in the liver. Specifically, the animals fed the copper loaded diet had liver and kidney iron concentrations significantly lower than the controls ($p<0.05$). The authors concluded that copper loading can have an inhibitory effect on iron deposition in organs (Crowe and Morgan 1996).

Trace Mineral Interactions Among Iron, Zinc, Copper

Interactions among iron, zinc, and copper can have serious consequences stemming from impaired or enhanced absorption that subsequently affects availability of these nutrients to perform their physiological functions (Sherman and Tissue 1981). Gomez-Ayala et al (1997) examined the effects of iron deficiency on the absorption of iron, zinc, and copper in rats. Their research involved 131 male Wistar rats that were maintained on either an iron adequate or iron deficient diet for forty days, and at the end of the study, a portion of their duodenum was perfused with a mineral solution containing different forms of iron. They observed decreased absorption of heme iron, copper, and zinc in the iron deficient animals, and subsequently concluded that ferropoenic anemia hinders the absorption of minerals that require active processes (Gomez-Ayala et al 1997).

The risk of negative interaction effects occurring among iron, zinc, and copper is greater when one or all of these minerals are consumed in amounts greater than the amount normally consumed in a typical meal. Primary examples of such situations are nutritional programs aimed at correcting mineral deficiencies, namely iron deficiency, or regular consumption of supplements and highly fortified foods (Sandström 2001). The latter situation is not as likely to lead to adverse interactions as the former, but increased supplement use coupled with prevalence of iron fortified foods on the market does pose the threat of excessive consumption of one nutrient such that the others are affected (Roughead et al 1999). Iron deficiency due to poor nutrient intake or malnutrition can be accompanied by zinc or copper deficiency, and the supplemental iron doses utilized to correct the iron deficiency may exacerbate concomitant mineral deficiencies or contribute

to their development. The reverse may also be true specifically that copper or zinc supplementation can adversely affect iron status (Castillo-Duran and Cassorla 1999, Sherman and Tissue 1981). Interactions among iron, zinc, and copper have been the focus of animal research involving the manipulation of dietary iron, zinc, and/or copper, with examination of the subsequent effects on body weight, tissue mineral concentrations, and, in cases that included iron deficiency or overload, hematology. Analyses of tissue mineral concentrations are often a component of trace mineral interaction research because tissue mineral concentrations are thought to be indicative of absorption as well as reserves of the minerals (Larson and Sandström 1992).

Roughead et al (1999) conducted a surface response study to examine iron, copper, and zinc interactions using female Sprague-Dawley weanling rats and 15 experimental diets of varying iron, copper, and zinc concentrations. The iron concentrations ranged from deficient (7 ppm) to excessive (300 ppm), with the latter selected because it was deemed to be comparable to excessive levels that could be reasonably achieved by humans. They found that the various dietary treatments did not significantly affect mean weight gain of the animals, which ranged from 123-127 grams over the six-week experimental period. Hemoglobin, hematocrit, serum ferritin, and liver iron were evaluated to determine iron status in the animals. Dietary iron, but not copper or zinc, was observed to have a significant effect on hemoglobin and hematocrit levels ($p<0.0001$), with the lowest levels of both observed in the animals who received the 7 ppm iron diet (10.4 and 32%, respectively). Both dietary iron and copper affected serum ferritin level, which is evaluated as an indicator of iron stores. The highest serum ferritin level (578 μg/L) was observed in the animals receiving the diet highest in copper

(20ppm) and moderate in iron and zinc content (45.8 ppm Fe and Zn). Excessive dietary iron intake (300 ppm) coupled with moderate zinc and copper intakes (45.8 and 3.2 ppm, respectively) also resulted in high serum ferritin (554 μg/L). These results led the authors to speculate that dietary copper and zinc may have an effect on serum ferritin, which they further stated has implications for the evaluation of serum ferritin levels relative to chronic disease (Roughead et al 1999). Liver iron concentrations were affected primarily by dietary iron, as the lowest concentration (0.4 μmol/g) was observed in the animals receiving the iron deficient diet and the highest concentration (11.8 μmol/g) was observed in the animals receiving the two highest iron diets (135.6 and 300 ppm). The authors concluded that dietary iron was the key determinant of iron status and that the effects of zinc and copper, while evident, were minimal (Roughead et al, 1999).

The worldwide prevalence of iron deficiency anemia has fueled research examining the clinical manifestations of this disorder. Furthermore, such studies include determination of the effects of iron deficiency on tissue mineral concentrations, namely iron, zinc, and copper, because interactions are know to occur among these minerals, although the exact mechanism has yet to be clearly elucidated.

Rodriguez-Matas et al (1998) examined the progression of iron deficiency induced by prolonged feeding of an iron deficient diet as well as the effects of iron deficiency on tissue concentrations of iron, zinc, and copper. Ninety-four male Wistar rats were randomly assigned to one of nine experimental groups. One group of animals served as baseline and was killed at the beginning of the study without having received an experimental diet. Half of the remaining groups were maintained on an iron deficient diet (14.1 ppm) while the other half consumed an iron adequate diet (44.6 ppm). The

experimental diets differed only in iron content and were provided ad libitum to the animals. At the end of 10, 20, 30, and 40 days, one group of iron deficient and one group of control animals were killed for analyses. Differences in final body weight were only observed between the control and iron deficient rats at 40 days, with the control rats weighing significantly more than the iron deficient rats ($p<0.001$). The baseline control animals (0 days) did not have hemoglobin, hematocrit, RBC, or WBC values significantly different than the iron-deficient animals. However, the control animals had hemoglobin, hematocrit, RBC, and WBC values significantly greater ($p<0.05$) than the iron-deficient animals at all subsequent time points (10, 20, 30, 40 days). Dietary iron deficiency was also observed to exert an effect on tissue mineral concentrations (Rodriguez-Matas et al 1997). Liver and spleen iron concentrations were significantly greater in the control animals at 20, 30, and 40 days ($p<0.05$). Liver iron concentrations decreased in the iron deficient animals from baseline through 30 days, and then between 30 and 40 days, there was a slight increase. In the control animals, liver iron decreased from baseline to day 10, increased from day 10 through day 30, and then decreased from day 30 to day 40. Spleen iron in the controls decreased from baseline to day 10 and subsequently increased through the end of the study. In the iron deficient animals, spleen iron decreased from baseline through day 20, at which point it increased through the end of the study. Conversely to the observations for tissue iron, tissue zinc concentrations were lower in the control animals versus those on the iron deficient diet. Specifically, liver zinc concentrations in the control animals were significantly lower than the iron deficient animals at each time point ($p<0.01$). In the spleen, significant differences were only observed between the two groups at 10 days ($p<0.01$) and 40 days ($p<0.001$). Liver

copper concentrations were not as affected by dietary iron as liver iron and zinc, with only the control animals at day 10 having liver copper concentrations significantly less than the iron deficient animals at the same time. Such was not the case with spleen copper, as the concentrations in the control animals were significantly less than observed in the iron deficient animals ($p<0.001$). The authors concluded that dietary iron deficiency persisting longer than 30 days depletes iron stores such that there is inadequate iron available for both the iron dependant enzymes and the production of heme proteins, resulting in diminished production of hemoglobin (Rodriguez-Matas et al 1997). Hemoglobin functions as an oxygen transporter in the blood, and they postulated that the reduced hemoglobin levels induced by iron deficiency lead to reduced metabolic rates (Rodriguez-Matas et al 1998). This is in contrast to the findings of Beard et al (1995) who observed increased metabolic rates in rats with iron deficiency. Rodriguez-Matas et al (1998) also examined the effects of iron deficiency on tissue concentrations of zinc and copper. Their results suggest an inverse relationship between dietary iron and the concentration of zinc and copper. The inverse relationship was evident in liver zinc and spleen copper as both were significantly lower in the control animals at all time points. However, the authors concluded that iron deficiency has a more profound effect on copper metabolism due to the greater number of significant differences in the copper concentrations in tissues and bones observed between the control and deficient animals (Rodriguez-Matas et al 1998). Their conclusions regarding zinc were in contrast to those of Larson and Sandström (1992), who examined tissue mineral concentrations as indicators of absorption and observed an inverse relationship between iron and zinc that was most evident in the liver.

Fifteen male Wistar rats, weighing approximately 100 g at the start of the study, were fed either an iron deficient (5.9 ppm) or iron adequate (128 ppm) diet for 3 weeks to determine the effect of iron deficiency on tissue mineral concentrations (Yokoi et al 1991). The initial and final weights of the iron deficient animals were less than the control animals, but these differences were not significant. Interestingly, food intake during the study period was significantly lower in the iron deficient group ($p<0.05$), but this was not reflected in weight differences. Liver, spleen, and kidney weight expressed as a percentage of body weight were not significantly different between diet groups, but the weight of the heart was significantly greater in the iron deficient group than the control group ($p<0.05$). As would be expected, hemoglobin and hematocrit concentrations were significantly lower in the iron deficient group than the control group ($p<0.01$), and the levels observed were below the reference range for these parameters (Yokoi et al 1991, Hrapkiewicz et al 1998). The authors concluded that the rats maintained on the iron deficient diet were anemic based on the hematological data and physical characteristics indicative of anemia, including pale tissue and conjunctiva color, abnormal incisors, and noticeably diminished physical activity. The iron deficient rats exhibited liver, heart, spleen, and kidney iron concentrations significantly less than those of the control animals ($p<0.01$). An inverse relationship between dietary iron and tissue copper was evident in the liver and spleen, as the tissue concentrations in both organs were greater in the deficient group ($p<0.05$). Copper concentrations in the heart and kidneys and zinc concentrations in all of the organs studied were not significantly different between diet groups (Yokoi et al 1991). The findings of Yokoi et al (1991) are

consistent with those of Rodriguez-Matas, et al (1998), and both authors concluded that dietary iron has a more profound effect on tissue copper than tissue zinc.

The research of Shukla et al (1990) was similar in purpose to that of Rodriguez-Matas et al (1998) and Yokoi et al (1991), yet it involved female rats that were maintained on the experimental diets for a longer duration than what was described by the previous researchers. Weanling rats of the C-F strain were randomly assigned to receive either a low iron (18-20 ppm) or iron-adequate (390 ppm) diet for eight weeks. At the end of the experimental period, the rats were killed, and their liver, spleen, and kidneys were analyzed for trace mineral content. The primary difference in tissue mineral concentrations between the deficient and control groups was observed in the liver. There was a direct relationship between dietary iron and liver iron concentration, with the iron deficient group having liver iron concentrations significantly less than the control animals ($p<0.01$). Liver zinc and copper concentrations were observed to be inversely related to dietary iron, with the iron deficient group having significantly greater zinc and copper concentrations than the control animals ($p<0.02$ and $p<0.01$, respectively) (Shukla et al 1990). The direct relationship between dietary iron and liver iron, as well as dietary iron and liver zinc were also observed by Rodriguez-Matas (1998) and Larsen and Sandström (1992), respectively. In the kidney and spleen, iron concentrations were significantly lower in the iron deficient group ($p<0.05$), and the inverse relationship observed in the liver between dietary iron and copper concentration was also observed in the spleen ($p<0.05$). The authors postulated that the increased mineral concentrations in the animals maintained on iron deficient diets could be attributed to enhanced absorption of these minerals in the face of iron deficiency. They

further stated that the increased absorption of these minerals could be explained by the fact that transferrin functions as a transport protein for other minerals, and when less iron is available, there are more transport sites available for heavy minerals. A portion of this study involved feeding half of the iron deficient animals the iron adequate diet for two weeks following the original 8-week experimental period. The authors found that two weeks of iron repletion corrected the tissue mineral concentrations such that they were comparable to the concentrations observed in the control animals. They concluded that the increased tissue mineral concentrations observed in iron deficiency are due to interactions between iron and other minerals at the absorption and transport sites, and the fact that transferrin functions in the transport of many minerals, not just iron (Shukla 1990).

Sherman and Tissue (1981) also examined the effects of iron deficiency on tissue mineral concentrations, with an emphasis on iron nutriture during pregnancy and its effect on female offspring. Sprague-Dawley rats were randomly assigned to either an iron deficient (5 ppm) or control (307 ppm) diet upon determination of pregnancy and maintained on this diet through lactation. At 21 days of age, the female rat pups were randomly assigned to receive either the iron deficient or control diet, which resulted in a total of four experimental groups: deficient during gestation and weaning (DD), deficient during gestation and control after weaning (DC), control during gestation and weaning (CC), control during gestation and deficient during weaning (CD). Rat pups were killed for analyses at two days of age to assess gestational effects of iron deficiency, at 21 days to assess lactation effects, and then post weaning at 30, 60, and 90 days. In the 2-day old pups, there were no significant differences in body weight, but the concentration of iron

was significantly greater in the liver of the control pups, while liver copper was significantly greater in the deficient pups ($p<0.02$). Liver zinc concentrations were not significantly different between the two groups. The 21-day old rat pups of dams on the iron deficient diet weighed significantly less and had significantly lower hemoglobin and hematocrit levels than their counterparts ($p<0.0001$). Similar to what was observed in the 2-day old rat pups, liver iron was significantly greater ($p<0.001$) in the control groups, liver copper was significantly greater ($p<0.001$) in the deficient pups, and liver zinc concentrations were not significantly different between groups. In the spleen, copper concentrations were significantly greater in the deficient animals ($p<0.005$), and in the kidney iron concentrations were significantly greater in the control group ($p<0.05$). The DD rat pups had significantly lower body weights at both 30 and 60 days than the other animals. At 90 days, the lowest body weight was observed in the CD pups, with significant differences in body weight only observed between the CD and DC pups ($p<0.001$). Hemoglobin and hematocrit levels were significantly lower in the CD and DD rat pups compared to the DC and CC pups at 30, 60, and 90 days ($p<0.001$). In the liver, iron concentrations at 30, 60, and 90 days were significantly greater in pups weaned to control diets than those weaned to deficient diets ($p<0.001$) regardless of their gestation diet. At 30 and 60 days, liver copper concentrations were significantly greater in the DD pups ($p<0.001$), whereas at 90 days, they were the greatest in the pups weaned to the deficient diet (CD) ($p<0.001$). As with the 2 and 21-day old pups, liver zinc concentrations were not significantly different among diet groups. The rat pups weaned to the deficient diet (CD) exhibited spleen iron concentrations significantly lower than the other animals at 30 days, while both groups weaned to deficient diets (CD and DD)

exhibited the lowest spleen iron concentrations at 60 and 90 days ($p<0.005$). Spleen copper and zinc concentrations were only observed to be significantly different among diet groups at 60 days, with the greatest concentrations of both minerals observed in the DD group ($p<0.005$). The pups weaned to the deficient diets exhibited kidney iron concentrations significantly less than the kidney iron concentrations of the control pups, with significant differences observed at 30, 60, and 90 days ($p<0.05$). Kidney copper concentrations at all time points as well as kidney zinc concentrations at 30 days were not significantly different among diet groups. Significant differences in kidney zinc concentrations were observed at 60 and 90 days with the pups weaned to deficient diets having lower kidney zinc concentrations than the pups weaned to the control diets (Sherman and Tissue 1981). As would be expected, there was a direct relationship between dietary iron concentration and body weight, hematology, and tissue iron concentrations with obvious differences in these parameters observed between the iron deficient and control animals at all ages. Furthermore, the observed detrimental effects of maternal iron deficiency on the iron status of offspring illustrated the importance of adequate iron nutriture during pregnancy. The findings of Sherman and Tissue (1981) suggest that dietary iron concentration is inversely related to tissue copper concentration, and that this relationship is more evident in the liver than the spleen or kidneys. The relationship between dietary iron and tissue zinc concentrations is much less clear, as significant differences were not observed in the liver, the findings in the spleen at 60 days suggest an inverse relationship, and at both 60 and 90 days the kidney zinc concentrations suggest a direct relationship.

Iron plays a key role in energy metabolism, and given the prominent problem of iron deficiency throughout the world, Stangl and Kirchgebner (1998) examined the effects of deficient and adequate dietary iron intakes on the citric acid cycle enzymes and cytochrome oxidase. They also observed the effects of iron deficiency on the liver enzymes affected by liver cell damage (AST, ALT, AP), and the tissue stores of iron, zinc, and copper, as tissue concentrations have been deemed indicative of trace mineral status (Stangl and Kirchgebner 1998, Larsen and Sandström 1992). Eighty-four weanling male Sprague-Dawley rats were equally divided into seven groups, and fed diets of varying iron concentrations for five weeks. There were three iron deficient diet groups (9, 13, 18 ppm), three groups of animals provided the iron adequate diet (50 ppm) but pair-fed to each of the three deficient groups, and one group that received the iron adequate diet (50 ppm) ad libitum. The authors found that the animals receiving the lowest dietary iron (9 ppm) and their pair-fed controls consumed significantly less food per day and gained significantly less weight than all of the other experimental animals ($p<0.05$). Hematological analyses revealed that the animals receiving the 9 ppm diet had significantly lower hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin concentration than all other animals ($p<0.05$). Red blood cell counts were also significantly affected by dietary iron intake, with the lowest count observed in the 9 ppm group. Interestingly, the RBC counts in the 9 ppm group were significantly less than all of the diet groups except the 50 ppm ad libitum group, as the RBC counts in these two groups were not significantly different from each other (Stangl and Kirchgebner 1998). Of note, hemoglobin and hematocrit levels in the 9 and 13 ppm diet groups and RBC counts in the 9 ppm diet and ad libitum group were lower than the

reference range for the aforementioned hematological parameter (Hrapkiewicz et al 1998). This led the authors to conclude that iron deficiency was achieved after a mere 5 weeks of sustenance on an iron deficient diet. Liver enzymes were also observed to be different among dietary iron groups. Aspartate aminotransferase (AST) levels were significantly greater in the 9 ppm group than all of the other diet groups ($p<0.05$), and the average AST level observed in this group exceeded the upper limit of the AST reference range. The AST levels in the 13 ppm diet group also exceeded the upper limit of the reference range, but the AST level observed in this group was only significantly greater than the 13 ppm pair fed controls (Stangl and Kirchgebner 1998, Hrapkiewicz et al 1998). The 9 ppm diet group exhibited the highest alanine aminotransferase (ALT) levels, and they were significantly greater than all groups except the 13 ppm and 13 ppm pair fed groups (Stangl and Kirchgebner 1998). Despite the significant differences, the ALT levels in all animals were within the reference range (Hrapkiewicz et al 1998). Alkaline phosphatase (AP) levels were elevated in all of the experimental animals (Hrapkiewicz et al 1998); however, significant differences were observed. Specifically, the 9 ppm animals had the highest AP levels that were significantly greater than the AP levels observed in the 9 ppm pair fed, 13 ppm pair fed, and 18 ppm pair fed groups. The AP levels of the 50 ppm ad libitum group were not significantly different than the AP levels of any other groups. The authors did not speculate on the latter results, but they did conclude that iron deficiency affects serum enzymes and that the elevations in liver enzymes that they observed may be suggestive of liver cell damage (Stangl and Kirchgebner 1998). Analyses of tissue mineral concentrations revealed that differences in dietary iron intake were reflected in the iron concentration of the liver and that dietary

iron influenced copper deposition in the liver as well. Liver iron concentrations were significantly lower in all three iron deficient groups compared to the pair-fed controls and the ad libitum group ($p<0.05$), but they were not significantly different from each other. An interesting observation was that the 9 ppm pair-fed group had liver iron concentrations significantly greater than the 13 and 18 ppm pair-fed groups and the ad libitum group ($p<0.05$). These findings are unusual given that the 9 ppm and 9 ppm pair-fed groups consumed significantly less feed per day than all of the other groups ($p<0.05$) (Stangl and Kirchgebner 1998). The authors did not speculate on this, but a possible explanation is that the body absorbs more iron in the face of insufficient food intake, a concept that was suggested by Shah and Belonje (1991) in response to the findings of their research on the effects of dietary iron on tissue trace element levels. The observed phenomenon was likely not due to the increased iron absorption sometimes seen in iron deficiency because the 9 ppm pair fed animals were likely not iron deficient, as indicated by their normal RBC counts, HGB, and HCT levels (Cook 1990, Gavin et al 1994, Hrapkiewicz et al 1998). Liver copper concentrations were inversely related to dietary iron concentration, and the animals in the 9 ppm iron deficient group had liver copper concentrations significantly greater than that of all other animals ($p<0.05$). The authors speculated that iron deficiency impairs the body's use of copper and, as such, this leads to increased deposition in the tissues (Stangl and Kirchgebner 1998). The pair fed groups and the ad libitum group all consumed 50 ppm diet in varying amounts, and their liver copper concentrations were not significantly different from each other. The authors also analyzed liver zinc concentrations, but failed to observe significant differences among any of the experimental groups (Stangl and Kirchgebner 1998). Overall, the authors

concluded that the early stages of iron deficiency affect hematological parameters, liver enzymes, and liver iron and copper concentrations, but that the citric acid cycle and the activity of cytochrome oxidase in the liver are not affected early in the development of iron deficiency (Stangl and Kirchgebner 1998).

The aforementioned research on the effects of dietary iron on tissue iron, zinc, and copper concentrations supports the concept of a direct relationship between dietary iron and tissue iron concentrations, particularly in studies comparing the effects of deficient and adequate dietary iron intake. These results also support the inverse relationship between dietary iron and tissue copper, particularly in the liver and spleen. Tissue zinc concentrations were observed to be less affected by dietary iron concentrations than copper, as evidenced most notably by the lack of consistency in the results of the aforementioned studies. Specifically, direct, indirect, and lack of relationships between tissue zinc and dietary iron concentrations were described, which prevents the elucidation of the relationship, or lack thereof, between dietary iron and tissue zinc.

Trace Elements and Ovarian Hormones

The effect of ovarian hormones and trace mineral deposition in tissues has not been the focus of much research, but relationships among trace minerals and ovarian hormones have been described. Bureau et al (2002) compared the trace mineral status of post-menopausal women receiving hormone replacement therapy to those who were not to examine the nature of the relationship between ovarian hormones and trace mineral status. A total of forty-four post-menopausal women between the ages of 50-60 were

randomly assigned to either the HRT (18) or non-HRT group and were followed for two years. Zinc, copper, chromium, selenium, magnesium, and calcium levels in the serum, plasma, and/or urine were analyzed, as was erythrocyte copper, to assess trace mineral status. Estradiol levels were significantly different between the two groups ($p<0.001$), and the levels observed in the HRT group were similar to what would be observed in pre-menopausal women. There were no significant differences between groups with respect to body weight, duration of menopause, BMI, alcohol use, and number of smokers and exercisers. Significant differences in plasma copper, serum and urine chromium, urine zinc, and urine magnesium were observed between the two groups with urine analyses being lower in the HRT group, and serum and plasma analyses being significantly greater in the HRT group ($p<0.05$). Metalloenzyme activity in the erythrocyte was also examined, but significant differences between groups were not detected. A positive relationship between Cu-Zn SOD and erythrocyte copper was observed, but such a relationship was not observed between this metalloenzyme and plasma copper. Furthermore, plasma copper was significantly greater in the HRT group, but there was not a significant difference between groups in erythrocyte copper levels (Bureau et al 2002). Conclusions were not made regarding the effects of ovarian hormones on copper status, as the results of this facet of the study were conflicting. A percentage of both groups exhibited plasma zinc levels (<10.7 μmol/L) that were below the reference range, with the greater percentage of women with low plasma zinc observed in the HRT group (33% versus 11%). However, the opposite was true with urine zinc, as the non-HRT had urine zinc levels significantly greater than the HRT group. The authors postulated that the decreased plasma zinc may be due to an adverse effect of HRT on the binding

capacity of zinc with plasma proteins, and that the decreased urinary excretion of zinc, as well as magnesium and calcium was a reflection of the positive effects of HRT in the prevention of osteoporosis. Selenium was not overtly influenced by HRT, but a positive effect on chromium status was observed. Overall, the authors concluded that the changes in ovarian hormone levels that occur in menopause affect trace mineral metabolism, but that more research must be performed to elucidate this relationship (Bureau et al 2002).

Kanias and Kouri (1996) examined the trace mineral content of human ovaries to determine if a relationship exists between trace elements and both the ovary as an organ and the ovarian hormone phases (reproductive and menopausal). A total of 40 human ovary samples were collected from women at both reproductive phases, and 7 samples were collected from stillborn fetuses for trace mineral analyses. Statistical comparison of ovarian trace mineral concentrations at the different ovarian stages (fetal, reproductive, and menopausal) reveal significant differences in ovarian iron and zinc concentration at these stages ($p<0.05$). An interesting observation in the data collected by Kanias and Kouri (1996) was that trace mineral content of the ovaries decreased with progression from one ovarian stage to the next, with the greatest iron and zinc concentrations observed in the fetal ovaries, and the lowest concentrations observed in the menopausal ovaries. Unfortunately, the authors failed to describe any relationships between ovarian hormone stages and any possible effects on trace mineral deposition in other tissues.

CHAPTER THREE

METHODS

Iron, copper, and zinc competitively interact resulting in depressed or enhanced absorption of any or all of these trace nutrients. However, the specific mechanism of these trace mineral interactions has yet to be determined (Rodriquez-Matas, et al. 1998).

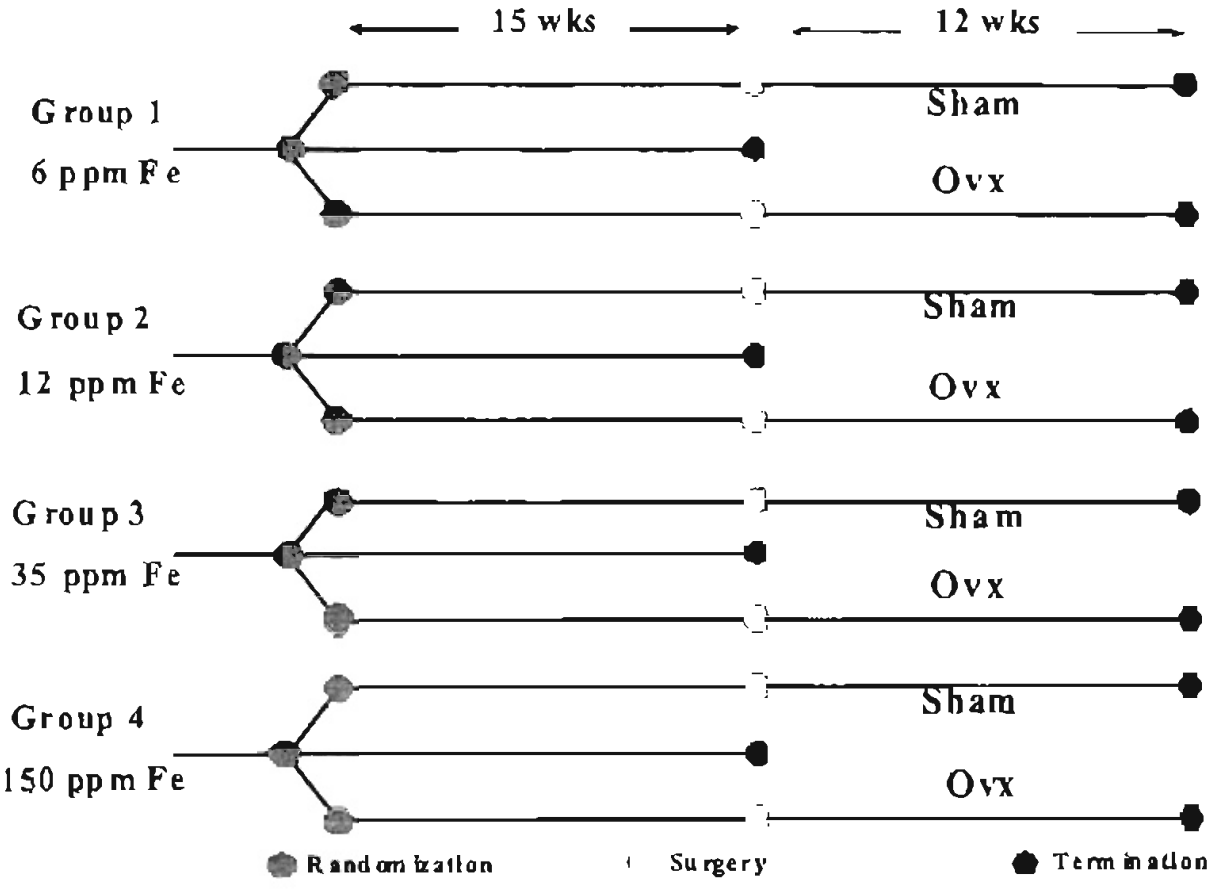
The purpose of this study was to examine the effects of varying levels of dietary iron on tissue stores of iron, copper, and zinc as an indication of competitive interactions among these trace nutrients. Also, differences in tissue storage between young mature and mature rats that were sham-operated or ovariectomized rats were examined to determine if age or ovarian hormones influence tissue storage of iron, copper, and zinc. The study was approved by the Oklahoma State University Animal Care and Use committee (Appendix A).

Experimental Design

This study consisted of two experiments (Figure 1). Experiment one was a completely randomized design with experimental units of 40 young mature female rats. Experiment two was a blocked, completely randomized 2 x 4 factorial design using 42 sham-operated and 42 ovariectomized female rats as experimental units.

The dietary treatment groups were designated for 6, 12, 35, and 150 ppm iron. Both the 6 and 12 ppm diets were considered inadequate, the 35 ppm was considered adequate based on the recommendations of Reeves et al (1993), and the 150 ppm diet was considered high.

Figure 1. Research Design for Experiments One and Two Combined



Animals

One hundred twenty-four weanling female Sprague Dawley rats were obtained from Harlan Sprague-Dawley, Indianapolis, IN. This study was part of a larger study designed to investigate the effects of various levels of dietary iron on bone during growth and the effects of dietary iron and ovarian hormone status on bone in sham-operated and ovariectomized rats. The rat model was used because it has been identified as a useful model for studying post menopausal bone loss. Specifically, ovariectomized rats experience bone loss similarly to post-menopausal women (Kalu 1991).

The animals arrived one week apart in two groups of 62 for surgery and necropsy scheduling purposes. Throughout the study, rats 1-62 were collectively labeled 'Group 1' and rats 63-124 were collectively labeled 'Group 2.'

Housing

The groups were housed in separate rooms at the Laboratory Animal Resource building at Oklahoma State University in Stillwater, Oklahoma until the fifteenth week of the study, at which point the remaining animals were housed in the same room. The experiment was designed for rooms kept at a temperature and humidity level appropriate for the animals and the lighting was such that the room was illuminated for 12 hours and dark for 12 hours.

Upon arrival, the animals were individually housed in clear plastic shoebox cages with raised floor grids and stainless steel lids. Initially, ground corncobs served as the bedding in the cages. After 3 days, plastic grating attached to plastic pipe was placed on top of the ground corncob in the bottom of the cages. The purpose was to prevent the

animals from consuming their feces and/or urine as both contain minerals, and our aim was for the diet to serve as the sole mineral source.

The cages, water bottles, and food bowls were cleaned and sanitized once a week. The animals were provided with deionized water ad libitum and fed a specific amount of food once a day. The animals were also provided with a one-inch piece of wooden dowel for chewing. The purpose was to prevent the animal's teeth from growing too long, which would hinder the animal's ability to eat.

Randomization

After the animals were placed in their individual cages and assigned a number (1-124), they were randomly assigned to ovarian hormone status and to diet groups. The animals were randomized within their respective group: Group 1, rats 1-62 and Group 2, rats 62-124.

The random assignment to ovarian hormone status and diet group started with the first rat in each group (rat 1 and rat 63, respectively) and continued in numerical order to the last rat in each group (rat 62 and rat 124, respectively). A random drawing was performed to assign each animal to the experimental groups. The overall distribution of animals in the ovarian hormone status group was as follows: 40 young mature, 42 mature sham, and 42 mature ovx. The overall distribution of animals in each diet group was as follows: 32 animals received the 6 ppm iron diet, 30 animals received the 12 ppm iron diet, 30 animals received the 35 ppm iron diet, and 32 animals received the 150 ppm iron diet.

Diet

A total of eight different diets were used in this study: growth 6 ppm iron diet, growth 12 ppm iron diet, growth 35 ppm iron diet, growth 150 ppm iron diet, maintenance 6 ppm iron diet, maintenance 12 ppm iron diet, maintenance 35 ppm iron diet, and maintenance 150 ppm iron diet. The diets within each of the main groups (growth and maintenance) only differed in the iron content.

The diets were prepared according to the AIN-93 methodology for growth and maintenance diets at the Oklahoma State University Nutritional Sciences Department diet-mixing lab, Stillwater, Oklahoma (Reeves, et al. 1993). The diet composition for the growth and maintenance diets is listed in Table 1.

The mineral mix was prepared according to the AIN-93 methodology at the Oklahoma State University Nutritional Sciences diet mixing lab, Stillwater, OK (Reeves, et al. 1993). The ingredients and the amount of each per kilogram of growth and maintenance mineral mix are listed in Table 2.

All mineral mix components were combined for five hours in a ceramic ball mixer and the final product was stored in plastic bags at room temperature. Lab coats and mineral free gloves were worn during preparation to prevent mineral contamination.

TABLE 1

DIET COMPOSITION

Component	AIN-93G g/kg	AIN-93M g/kg
Corn starch[1]	397.5	582.1
Casein[2]	200.0	140.0
Dextrinized corn starch[3]	132.0	155.0
Sucrose[4]	100.0	100.0
Soybean oil[5]	70.0	40.0
Cellulose[6]	50.0	50.0
Mineral mix (AIN-93G/93M-MX)[7]	35.0	35.0
Vitamin mix (AIN-93G/93M-VX)[8]	10.0	10.0
L-Cystine[9]	3.0	1.8
Choline[10]	2.5	2.5

[1] Harlan Teklad, Madison, WI. Lot # 98302
[2] Harlan Teklad, Madison, WI. Lot # 98308
[3] Clo-Dex 10-Maltodextrin Lot # 98227
[4] Great Value Extra Fine Granulated Sugar
[5] Crisco All Natural Pure Vegetable Oil
[6] Harlan Teklad, Madison, WI. Lot # 98197
[7] Mineral mix was prepared in the lab
[8] Harlan Teklad, Madison, WI. RX 892395
[9] Harlan Teklad, Madison, WI. Lot # 98295
[10] Harlan Teklad, Madison, WI. Lot # 98101

TABLE 2

MINERAL MIXES

Component	AIN93-G g/kg[1]	AIN93-M g/kg[1]
Calcium Carbonate	357.0	357.0
Potassium Phosphate	196.0	250.0
Potassium Citrate	70.78	28.0
NaCl	73.275	73.275
Potassium Sulfate	46.6	46.6
Magnesium Oxide	24.0	24.0
Zinc Carbonate	1.65	1.65
Manganous Carbonate	0.63	0.63
Cupric Carbonate	0.30	0.30
Potassium iodate	0.01	0.01
Sodium selenate	0.01025	0.01025
Ammonium paramolybdate	0.00795	0.00795
Sodium meta-silicate	1.45	1.45
Chromium potassium sulfate	0.275	0.275
Lithium chloride	0.0174	0.0174
Boric acid	0.0815	0.0815
Sodium Fluoride	0.0635	0.0635
Nickel carbonate	0.0318	0.0318
Ammonium vanadate	0.0066	0.0066

Levels of dietary iron for AIN93-G and AIN93-M
(corrected for amount of iron in cellulose)

	6 ppm	12 ppm	35 ppm	150 ppm
Ferric Citrate g/kg	0.88	1.86555	5.8883	25.8126

Powdered sucrose corrected for amount in titrated minerals for
AIN93-G/AIN93-M

Sucrose g/kg	217.37/ 206.15	216.38/ 205.16	212.36/ 201.14	191.98/ 181.21

[1]g/kg mineral mix

Diet Preparation

The diet was prepared in 5-kilogram batches, stored in plastic bags, and kept refrigerated. Diet was prepared as needed throughout the study using the same lot of ingredients. The diet was formulated according to Reeves and colleagues, including all potentially beneficial trace elements (Reeves et al, 1993). The Oklahoma State University Nutritional Sciences laboratory standardized procedures for diet preparation were utilized (Appendix B).

Feeding

Throughout the study, the animals consumed deionized water ad libitum and were fed in the late afternoon to coincide with their nighttime feeding pattern. The animals consumed standard rat chow for the first three days of experimentation to allow for acclimatization to their surroundings. Initially, all animals were provided with 15 grams of diet per day to allow adequate access to the diet. Subsequently, the amount of diet provided the animals was based on feed consumption of the animals that gained the least weight in order to match body weight among all diet groups. The animals and the food remaining in their food dishes were weighed twice a week. This data was used to calculate the appropriate amount of food to be provided the following week.

In experiment one, the amount of feed provided was based on the diet group gaining the least weight and in experiment two, it was based on the diet and ovarian hormone status group gaining the least weight. The animals that gained the least amount of weight, received the most food. The goal was to provide these animals with sufficient

food to meet their needs. The remaining animals received the same amount or slightly less than that consumed by the animals that gained the least weight.

The young mature animals consumed the growth diet for 7 weeks and the maintenance diet for 8 weeks. They were necropsied after this 15-week dietary intervention period. The mature sham-operated and ovariectomized animals consumed the growth diet for 7 weeks and the maintenance diet for 20 weeks. They underwent surgery during the 15th dietary intervention week, and they were necropsied after the 27th week of dietary intervention.

Surgery

At 15 weeks of age, the animals randomized to the sham or ovariectomy group underwent their respective surgery. The procedure for the sham and ovariectomy surgeries was the same except that the sham-operated animals did not have their ovaries excised. They were simply lifted from the body cavity and replaced prior to closure. The sham operation was performed to control for the effects of surgery.

The animals were initially anesthetized via box induction with halothane. The 2% halothane anesthetic was continued during surgery via a mask. After being anesthetized, the animals were shaved 2 centimeters on either side of their dorsal midline from the scapula to the pelvis. This area was cleaned for surgery with chlorhexidine scrub. The animals were positioned with the dorsal surgery area exposed. This was covered with an impervious fenestrated drape with an opening centered over the cranial to lumbar vertebrae. A one-centimeter incision was made in the skin over L1-L4. A second 3-millimeter incision was made in the paralumbar abdominal musculature. The left ovary

was extracted with a traumatic forceps, isolated with mosquito forceps, and then removed. The ovarian pedicle and uterine horn were replaced and the muscle incision was closed. This procedure was then repeated on the right ovary. The skin incision was then closed with Vetbond adhesive.

Necropsy

The animals were housed in plastic metabolic cages and provided only with deionized water during the 12-hour period prior to necropsy. Metabolic cages were used for the purpose of collecting the animal's feces and urine. At the end of the 12-hour period, each animal's feces were weighed, stored in sterile plastic sample bags, and frozen for analysis in another study. The urine volume was recorded and it was subsequently centrifuged. The supernatant was separated from the precipitate via pipette, stored in a 15 mL Falcon test tube, and frozen for later analysis for another study.

The animals were anesthetized with 50 mg of ketamine and 2.5 mg of xylazine per kilogram of the most recent body weight obtained at the Laboratory Animal Resource building. The animal's final body weight was recorded after administration of the anesthesia. A total body scan was performed by dual energy X-ray absorptiometry (DXA), (Hologic QDR 4500A, Waltham, MA) to determine the animal's body composition.

Blood Collection, Organ and Bone Harvest

With the animal in a supine position, an incision was made through the skin and musculature at the frontal midline extending from the inferior pelvic region to the

sternum. The intestines were moved out of the abdominal cavity to expose the abdominal aorta. The blood was collected via abdominal aortic puncture. EDTA (1.6mg/mL, Sarstedt, Newton, NC) treated syringes were used to collect the blood for hematological analysis. Syringes not treated with anticoagulant were used to collect the blood for serum measures. This blood was clotted on ice and subsequently centrifuged. The serum was removed from the precipitant via pipette, distributed for various analyses, or frozen until analyses could be performed.

After the blood collection was complete, the tissues were perfused with physiological saline via peristaltic pump inserted at the aortic puncture site until the liver and kidneys became pale. The liver, kidneys, heart, spleen, and uterus were harvested from the body. Each organ was trimmed of superficial fat and/or connective tissue, weighed, and stored in sterile plastic sample bags. The kidneys, heart, spleen, uterus, and the largest lobe of the liver were frozen at -20°C. All tissues except the uterus were analyzed for mineral content. Liver lobes 1 and 2 were frozen at -70°C for enzyme assay.

The right and left tibia and femur were separated from the hind leg and collected for analyses. All clinging tissue was carefully removed from the bones to prevent damaging the periosteum using scissors, forceps, deionized water, and Kim-wipes. The entire vertebral column was immediately frozen after necropsy at -20°C in a 50 mL Falcon centrifuge tube. Later, it was cleaned, as were the other bones, to expose the vertebrae. Lumbar vertebrae 3, 4, and 5 were separated from the column and individually stored at -20°C in sterile plastic sample bags.

Lab coats and latex gloves were worn during bone and organ harvest to prevent mineral contamination.

Analyses

Nutritional Status

Whole blood was analyzed using the ABX Pentra 120 Retic (ABX Diagnostics, Irvine, CA). The complete blood count, five-part differential leukocyte count, reticulocyte, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, and platelet count were determined immediately after the blood was collected.

The serum was analyzed for serum albumin, urea nitrogen, glucose, alkaline phosphatase, creatinine, and uric acid. The COBAS Fara II clinical analyzer (Roche Diagnostic Systems, Nutley NJ) was used to perform these analyses.

Enzymatic assays of ALT and AST were performed using the COBAS Fara II clinical analyzer. This analysis was performed to determine if the liver had been damaged by insufficient or excessive iron intake.

Mineral Analysis of Diet

Four one-gram samples of each experimental diet were taken from different bags of prepared diet for mineral analyses. The diet was dried and subsequently ashed using a modification of the procedures of Hill et al (1986) (Appendix C). The iron, zinc, copper, manganese, calcium and magnesium concentrations of the diet were determined using a Perkin Elmer 5100PC Atomic Absorption Spectrophotometer with deuterium and Zeman background correction as appropriate. The phosphorus content of the diet was determined using the COBAS Fara II clinical analyzer and the appropriate reagents from Roche

Diagnostics (Roche Diagnostic Systems, Indianapolis, IN). The analyzed mineral content of the growth and maintenance diets is listed in Table 3.

TABLE 3

AVERAGE MINERAL CONTENT PER KILOGRAM OF GROWTH AND MAINTENANCE DIETS[1]

Mineral (mg/kg diet)	6 ppm	12 ppm	35 ppm	150 ppm
Growth				
Iron	10.8±4.8	14.6±4.8	36.0±4.8	166.1±4.8
Zinc	37.9±0.7	38.3±0.7	36.6±0.7	35.7±0.7
Copper	4.90±0.12	5.09±0.12	5.12±0.12	4.62±0.12
Manganese	9.01±0.15	8.40±0.15	8.67±0.15	8.88±0.15
Calcium	3613±90	3840±90	3986±90	3943±90
Magnesium	397.8±12.6	393.7±12.6	425.9±12.6	408.3±12.6
Phosphorus	2846±61.6	2732±61.6	2749±61.6	2674±61.6
Maintenance				
Iron	9.0±4.8	18.3±4.8	40.3±4.8	150.8±4.8
Zinc	32.4±0.7	33.0±0.7	33.2±0.7	32.1±0.7
Copper	4.61±0.12	4.50±0.12	4.41±0.12	4.26±0.12
Manganese	8.12±0.15	8.53±0.15	8.11±0.15	8.42±0.15
Calcium	3457±90	3460±90	3494±90	3182±90
Magnesium	337.7±12.6	340.1±12.6	340±12.6	274.3±12.6
Phosphorus	2324±61.6	2474±61.6	2327±61.6	2061±61.6

[1]LS Means ± SE

Mineral Concentrations of Tissues

All materials used in the sampling of organs (i.e. test tubes, beakers, forceps, etc) were soaked for 24 hours in 10% hydrochloric acid, rinsed with deionized water, and dried at 50°C in a drying oven (Lindberg 847, Watertown, WI). Tissues were prepared for mineral analyses using a modification of the procedures of Hill et al (Hill AD, et al. 1986) (Appendix D). The organs of each diet treatment group were sampled with separate utensils that were labeled according to diet designation and stored separately. All utensils were soaked, rinsed, and dried as previously described between sampling of different organs. Lab coats and mineral free gloves were worn during all procedures. These measures were employed to prevent mineral contamination.

Two samples of each organ were analyzed for iron, copper, and zinc content using a Perkin Elmer 5100PC Atomic Absorption Spectrophotometer with deuterium or Zeman background correction as appropriate. All tissue iron and zinc concentrations as well as kidney calcium were analyzed via the flame method. Tissue copper concentrations were analyzed via the flame and furnace methods. Furnace analyses required determination of recoveries, which are set at the 85^{th} to 115^{th} percentile in our lab; therefore, those recoveries in the 85^{th} to 115^{th} percentile were deemed acceptable. However, there were instances when an 85-115% recovery was not attainable with multiple sample analyses. In such instances, the analyzed value with the percent recovery closest to the ideal range was used; if all analyzed values were similar, the average was calculated and used as the result for that particular sample. The flame and furnace parameters are detailed in Appendix E.

Change in Tissue Mineral Concentrations from Surgery to Necropsy

The mean of each tissue mineral concentration by diet in the young mature group was subtracted from the mean tissue mineral concentration by diet in both treatment groups. For example, the change in liver iron concentration of the sham-operated animals receiving the 6 ppm diet was calculated by subtracting the mean 6 ppm young mature liver iron concentration from the mean 6 ppm sham-operated liver iron concentration.

Sampling Methodology

The test tubes were doubly labeled with a heat-resistant wax pencil and weighed prior to sampling. Each beaker contained a maximum of thirty-six organ samples and 3 blank (empty) test tubes. The sampling utensils (forceps, scalpels, glass knives, glass stirring rods, watch glasses) were rinsed with Type I water (millipore < 18.2Ω) and dried with Kim-wipes between samples and prior to storage.

Liver A 0.1-0.3 g section of the largest lobe of the liver (tissue bag 3) was excised using a scalpel and forceps. The sample was inserted into the test tube using forceps and weighed. The weight was recorded and the test tube was placed in a beaker. The samples were dried for 48 hours at 100°C and the dry weight of each sample was recorded.

Kidney, Spleen, and Heart. Each organ was halved using a glass knife and forceps at a point perpendicular to the midline. The sample was inserted into the test tube with forceps and a glass-stirring rod and weighed. The weight was recorded and the test

tube was placed in a beaker. The samples were dried for 24 hours at 100°C and the dry weight of each sample was recorded.

Ashing

The samples were completely ashed (all organic material dissipated) prior to mineral analyses. The ashing procedure used was a modification of the Hill method (Hill AD, et al. 1986) (Appendix D). Type I water, double distilled ultra pure nitric acid (GFS Chemical, Powell OH), and ultrex ultra pure 30% hydrogen peroxide (J.T. Baker, Phillipsburg, NJ, catalog # 5155-01) were used to dissolve the organic materials. Trace mineral free pipet tips were used for all reagent additions. A new pipet tip was used each time a different reagent was added or if the pipet tip touched the test tube.

After the samples had been dried and weighed, the test tubes were placed in a heating block and type I water, nitric acid, and hydrogen peroxide were added to the samples and heat was applied. The water, acid, and peroxide were repeatedly added until the solution in the test tube became clear. When the test tubes were completely dry, the samples were placed in a beaker and dry ashed in a Lindberg 847 ashing oven on the appropriate program. The wet and dry ashing cycles continued until the ash in the test tube became white, which indicated all organic material had been destroyed. The mineral ash was weighed and the samples were prepared for trace mineral analyses.

Duplicate samples were analyzed for each tissue in each animal. When an analyzed tissue concentration was greater than 3 SD of the mean for that diet and treatment group, that value was dropped from all analyses.

Statistical Analyses

All data were analyzed using PC SAS version 8.2 (SAS Institute Cary, NC). The variability of the tissue mineral data required use of the Levene's Test to test for equal variances. Equal variances could not be supported; therefore, SAS proc mixed with the slice option was used for analysis of variance. The Tukey-Kramer test was used to determine significance among variances instead of Least Square Means as it is a more conservative test for significance. Specifically, the Tukey procedure compares the means of all possible diet (Experiment One) or diet and treatment (Experiment Two) combinations such that the probability of detecting differences where there are none is low. Quadratic and cubic analyses were performed to determine the non-linear line fit using the General Linear Model. For variables other than tissue minerals, the general linear model was used for analysis of variance. Significance level was set at $p \leq 0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

One hundred twenty-four female Sprague Dawley rats were obtained at 3 weeks of age and were randomly assigned to one of three experimental treatment groups (young mature, sham-operated, or ovariectomized) and one of four experimental diet groups (6, 12, 35, or 150 ppm iron). This research on the effects of varying levels of dietary iron on tissue minerals entailed two experiments. Experiment 1 was completed to determine the effects of dietary iron on tissue minerals of young mature rats. Experiment 2 was completed to determine the effects of dietary iron on tissue minerals of both mature rats and mature rats with ovarian hormone deficiency.

Experiment One: Young Mature Animals

Weight Gain and Food Intake

Forty rats comprised the experiment, with 10 animals per dietary treatment. The animals weighed an average of 72.5 grams at the beginning and 230 grams at the end of the study, with no significant differences in either weight among diet groups (Table 4). Our findings were also similar to those of Shah and Belonje (1991) who examined the effects of both marginal and excessive dietary iron on body weight and found no significant differences in body weight among diet groups at the end of both the six-week and 12 week feeding periods. However, our results indicate a trend toward significant differences in weight gain and final weight among the diet groups ($p=0.0576$ and $p=0.0737$, respectively). Although not significant, the rats in the 150 ppm group weighed

the most and gained the most weight during the study, whereas the iron deficient groups weighed the least and gained the least weight during the study. The latter findings are similar to those of Beard et al (1995) and Stangl and Kirchgebner (1998) who observed lower body weights in male rats maintained on iron deficient diets. Beard et al (1995) found that the animals receiving the very low iron diet (<5 ppm) for 6 weeks gained less weight and had lower final body weights than the controls who were maintained on an iron adequate diet (50 ppm) for the same duration. Stangl and Kirchgebner (1998) also observed the lowest final weights in their animals maintained on an iron deficient diet (9 ppm) for five weeks.

Feed efficiency was not significantly different among the diet groups, but the 6 ppm group gained the least weight per gram of diet consumed whereas the 35 and 150 ppm groups gained the most (Table 4). Beard et al (1995) and Stangl and Kirchgebner (1998) also examined weight gain relative to dietary intake and found, as we did, that the iron deficient animals gained the least amount of weight relative to intake. Beard and his colleagues (1995) concluded that the minimal weight gain observed in the iron deficient rats in the face of increased food consumption was due to increased metabolic rate in these animals (Beard et al 1995). It is feasible that our iron deficient animals had increased metabolic rates, but this cannot be concluded because we did not measure metabolic rate, and our animals were fed to the amount of the group that gained the least amount of weight, whereas those in the experiment by Beard et al (1995) were fed ad libitum. Furthermore, our lowest iron group had the overall highest body fat percentage (Table 4), which does not reflect an increase in metabolic rate as was observed by Beard et al (1995). Therefore, the differences in weights among our diet groups could be due to

TABLE 4

EFFECT OF DIETARY IRON ON WEIGHT GAIN AND BODY COMPOSITION IN YOUNG MATURE RATS[1,2]

Weight and Body Composition Variables	6 ppm	12 ppm	35 ppm	150 ppm	
n	10	10	10	10	Significance
Initial Weight (g)	74.2±2.9	74.8±2.9	73.5±2.9	70.7±2.9	p=0.8245
Final Weight (g)	225.4±4.6	223.1±4.6	231.2±4.6	239.5±4.6	p=0.0737
Weight Gain (g)	151.2±5.0	151.3±5.0	157.7±5.0	168.8±5.0	p=0.0576
Gain to Fed (mg/g diet)	1.20±0.05	1.25±0.05	1.3±0.05	1.3±0.05	p=0.7157
Lean Body Mass (g)	203.9±5.1	203.9±4.8	208.5±5.1	216.9±4.8	p=0.2117
Percent Fat (%)	12.6±1.2	10.8±1.1	11.3±1.2	10.6±1.1	p=0.6054
Liver pbw (%)	0.026 ± 0.001^{ab}	0.025 ± 0.001^{a}	0.028 ± 0.001^{bc}	0.029 ± 0.001^{c}	p=0.0183
Spleen pbw (%)	0.0026 ± 0.0001^{a}	0.0022 ± 0.0001^{b}	0.0024 ± 0.0001^{ab}	0.0024 ± 0.0001^{ab}	p=0.0459
Kidney pbw (%)	0.0069±0.0002	0.0070±0.0002	0.0072±0.0002	0.0072±0.0002	p=0.6737
Heart pbw (%)	0.0040±0.0001	0.0039±0.0001	0.0038±0.0001	0.0037±0.0001	p=0.3586

[1]LS Means ± SE

[2]Variables in rows with different character superscripts are significantly different ($p<0.05$).

feeding methodology. Schwartz et al (1973) examined the effects of food restriction in rats and found that food restriction did reduce the weight gain in the restricted animals versus those not restricted.

Assessment of body composition of the young mature rats showed that there were no significant differences in lean body mass or percent body fat among diet groups. Although not significant, the animals receiving the 150 ppm diet were the leanest with the highest lean body mass and the lowest percentage of body fat (216.93 grams and 10.63%, respectively). Conversely, the animals receiving the 6 ppm diet had the highest percent body fat (12.64%), and those receiving the 12 ppm diet had the lowest lean body mass (203.87 grams) (Table 4). Our results are in conflict with those of Beard et al (1995) who observed the lowest body fat percentages in the iron deficient and food restricted animals. They postulated that iron deficiency and food restriction led to poor growth and low body fat percentages, the latter of which stimulated an increase in metabolic rate to maintain core body temperature as there was insufficient fat for heat insulation (Beard et al 1995).

Liver and spleen weight as a percentage of body weight were significantly different among diet groups, whereas the weights of the kidney and heart were not (Table 4). The liver of the 150 ppm group was the largest, and weighed significantly more than the livers of the animals receiving the 6 or 12 ppm diet. Conversely, the spleens of the 6 ppm group were the largest and weighed significantly more than the spleens of the 12 ppm group, which were the smallest. The observations of Schwartz et al (1973) regarding the effects of food restriction on organ weights suggest that food restriction, which would to a certain extent decrease mineral intake, does affect organ weight as the

animals provided 50% less diet than the control group had lower kidney, liver, spleen, and heart weights ($p<0.001$) (Schwartz et al 1973). Our findings were similar only with respect to the liver, as we too observed lower liver weights in our deficient animals. The lack of more similarities in findings between our study and that of Schwartz and colleagues (1973) is that the caloric intake of our animals was not restricted, but only the iron intakes. Even so, it cannot be concluded that the reduced organ weights of the restricted animals were not due at least in part to reduced mineral intake.

Diet and Trace Mineral Intake

The young mature rats consumed the AIN-93 diet formulated for growth for approximately 7 weeks followed by an 8-week period during which they consumed the maintenance formulation of the AIN-93 rodent diet. The average daily iron intake was significantly different among diet groups during both the growth and maintenance feeding periods (Table 5). Conversely, the average daily intakes of the growth and maintenance diets as well as copper and zinc during both feeding periods were not significantly different among the diet groups (Table 5). The lack of significant differences in diet, copper, and zinc intakes indicate that they were adequately controlled among the diet groups such that any differences in tissue mineral concentrations among the diet groups could not be attributed to differences in these variables.

TABLE 5

AVERAGE DAILY DIET AND TRACE MINERAL INTAKES OF YOUNG MATURE RATS[1,2]

Diet and Respective Trace Minerals	6 ppm	12 ppm	35 ppm	150 ppm	Significance
Growth Diet (g)	12.15±0.29	11.82±0.29	12.15±0.29	12.28±0.29	p=0.7054
Iron (mg)	0.13±0.02[a]	0.17±0.02[a]	0.44±0.02[b]	2.04±0.02[c]	p<0.0001
Zinc (mg)	0.46±0.01	0.45±0.01	0.45±0.01	0.44±0.01	p=0.4830
Copper (mg)	0.059±0.001	0.060±0.001	0.062±0.001	0.057±0.001	p=0.0653
Maintenance Diet (g)	13.72±0.29	13.48±0.29	13.69±0.29	13.64±0.29	p=0.9395
Iron (mg)	0.12±0.01[a]	0.25±0.01[b]	0.55±0.01[c]	2.06±0.01[d]	p<0.0001
Zinc (mg)	0.44±0.01	0.44±0.01	0.46±0.01	0.44±0.01	p=0.6408
Copper (mg)	0.063±0.001	0.061±0.001	0.060±0.001	0.058±0.001	p=0.0715

[1]LS Means ± SE

[2]Variables in rows with different letters are significantly different (p<0.0001)

Hematology, Nutritional, and Liver Function Analyses

Very low iron diet formulations were included in this study with the goal of inducing iron deficiency because iron deficiency anemia is the most common nutritional deficiency disease throughout the world (CDC 2002). All hematological analyses, except leukocyte count, were significantly different among the diet groups (Table 6). The average hemoglobin (HGB) level observed in the 6 ppm diet group was borderline low and was significantly less than that exhibited by the other animals, which had hemoglobin levels well within the normal range (Hrapkiewicz et al 1998) (Table 6). Furthermore, mean corpuscular volume (MCV) and hematocrit (HCT) in the 6 ppm animals were significantly lower than that of the other animals, with HCT levels in the 6ppm animals below the reference range (Hrapkiewicz et al 1998) (Table 6). Despite their depressed HGB, HCT, and MCV levels, the 6 ppm animals exhibited red blood cell and reticulocyte counts significantly greater than that of the remaining diet groups (Table 6). These results suggest that the animals receiving the 6 ppm diet were iron deficient. Specifically, they exhibited greater reticulocyte (RTC) and erythrocyte (RBC) counts than the other diet groups, which suggests that the bone marrow is releasing immature and mature RBC at a higher rate to improve the diminished oxygen carrying capacity of the blood, as reflected by the depressed hemoglobin levels. There were no differences in leukocyte counts among the diet groups (Table 6), but all animals exhibited leukocyte (WBC) counts lower than the reference range (Hrapkiewicz et al 1998, Young 1998). Our findings are similar to those of Stangl and Kirchgebner (1998) in all of the aforementioned parameters, except RBC levels, as we observed the greatest RBC counts in our lowest dietary iron group whereas Stangl and Kirchgebner (1998) observed the

TABLE 6

THE EFFECT OF DIETARY IRON ON HEMATOLOGY VALUES IN YOUNG MATURE RATS[1,2]

Serum Analyses	6 ppm	12 ppm	35 ppm	150 ppm	Ref Range	Significance
RBC (10^3/mm^3)	9.0±0.3[a]	7.6±0.3[b]	7.2±0.3[b]	7.4±0.2[b]	7-10[3,4]	p=0.0002
WBC (10^6/mm^3)	2.9±0.7	2.6±0.7	3.0±0.7	3.3±0.6	6-17[3,4]	p=0.8504
Hgb (g/dL)	11.2±0.3[a]	13.8±0.4[b]	14.3±0.4[b]	14.7±0.3[b]	11-18[3]	p<0.0001
HCT (%)	29.8±0.8[a]	36.4±0.8[b]	37.4±0.8[bc]	38.9±0.8[c]	36-48[3]	p<0.0001
MCV (μg/m^3)	32.8±1.4[a]	48.2±1.4[b]	51.8±1.4[b]	41.4±1.3[b]		p<0.0001
Reticulocyte Count (10^6/mm^3)	0.40±0.03[a]	0.20±0.04[b]	0.16±0.04[b]	0.20±0.03[b]		p=0.0005

[1]LS Means ±SE

[2] Variables in rows with different character superscripts are different (p<0.05).

[3]Hrapkiewicz , et al (1998)

[4]Young, 1998

lowest RBC counts in their lowest dietary iron group. The differences in feeding duration (15 weeks versus 5) and dietary iron concentration (6 ppm versus 9 ppm) may partially explain this difference in RBC levels. In their research examining dietary iron deficiency, Dallman et al (1982) evaluated differences in hematocrit and Siimes et al (1980) evaluated differences in hemoglobin and hematocrit among their dietary iron groups. Both researchers observed that male Sprague-Dawley rats maintained on very low iron diets (2 and 6 ppm and 7 ppm, respectively) exhibited depressed hemoglobin and hematocrit values (Dallman et al 1982, Siimes et al 1980). Our findings were similar despite the differences in study duration and gender of the animals used in the study.

Serum analyses were performed to assess nutritional status and liver function. Glucose, creatinine, urea nitrogen, albumin, and alkaline phosphatase were not significantly different among the diet groups (Table 7). Furthermore, albumin, creatinine, and alkaline phosphatase levels were within normal limits for rats (Young 1998). Although not significantly different among groups, glucose levels were higher and urea nitrogen and albumin levels were lower than the normal levels for rats (Young, 1998). The elevation in glucose may have been induced by stress, as the animals were in a new environment for approximately 12 hours prior to killing and had been injected with anesthesia. The minimal depression of albumin levels may have been due in part to the feeding methodology employed. Pair feeding all animals according to the group that consumed the least amount of diet was done to control weight gain among the diet groups. Therefore, some of the animals may have been routinely receiving less food than they needed which may explain the lower albumin levels. The depressed albumin levels

TABLE 7

THE EFFECT OF DIETARY IRON ON LIVER FUNCTION AND NUTRITIONAL INDICATORS IN YOUNG MATURE RATS[1,2]

Serum Analyses	6 ppm	12 ppm	35 ppm	150 ppm	Ref Range[3,4]	Significance
Glucose (mmol/L)	10.1±1.5	9.7±2.5	13.4±1.6	10.8±2.1	2.8-7.5	p=0.4395
Albumin (g/L)	37±1.3	35±1.3	38±1.3	37±1.3	38-48	p=0.4589
Creatinine (µmol/L)	48.9±3.3	52.5±3.3	55.0±3.3	52.1±3.3	18-70	p=0.6396
Urea N (mmol/L)	0.5±0.04	0.7±0.07	0.6±0.04	0.6±0.06	5.4-7.5	p=0.0568
ALP (uKat/L)	1.2±0.1	1.0±0.1	0.7±0.1	0.9±0.1	0.3-2.1	p=0.0679
ALT (uKat/L)	0.41 ± 0.03^{a}	0.33 ± 0.03^{b}	0.36 ± 0.03^{ab}	0.30 ± 0.03^{b}	0.3-1.5	p=0.0361
AST (uKat/L)	1.55 ± 0.11^{a}	1.34 ± 0.11^{ab}	1.14 ± 0.11^{b}	1.07 ± 0.11^{b}	3.2-4.4	p=0.0138

[1]LS Means ± SE

[2] Variables in rows with different character superscripts are significantly different

[3]Hrapkiewicz , et al (1998)

[4]Young, 1998

observed may also be due in part to decreased production of albumin by the liver (Tietz 1990). Inadequate, marginal, and excessive dietary iron intakes may have impacted the liver such that production of albumin was compromised. Urea nitrogen levels are depressed in liver dysfunction and negative nitrogen balance, the latter of which can occur in malnutrition (Pagana and Pagana 2003). As with the albumin levels, it is possible that pair feeding caused a certain level of malnutrition in these animals, or the inadequate, marginal, and excessive dietary iron intakes adversely affected liver function. However, it must be noted that two of the liver function tests, ALP and ALT, were within normal limits for rats and, therefore, fail to support liver dysfunction in these animals (Young 1998).

The liver function tests, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly different among the diet groups (Table 7). ALT levels in the 6 ppm diet group were greater than the levels observed in the animals receiving the 12 and 150 ppm diets ($p=0.0361$). The animals receiving the 6 ppm diet had AST levels significantly greater than those of the animals receiving the 35 and 150 ppm diets. Aspartate aminotrasferase and ALT are enzymes found in the liver, and when liver function is impaired, the blood levels of these enzymes are increased (Pagana and Pagana 2003). However, our animals exhibited ALT and AST levels at the low end of normal and below normal (Young 1998), respectively, and levels were higher in the lowest dietary iron group (6 ppm). Our findings were different than those observed by Stangl and Kirchgebner (1998) who fed iron deficient diets (9, 13, 18 ppm) to male Sprague Dawley rats for five weeks. They observed greater liver enzyme levels in their animals fed the iron deficient diets as compared to pair fed controls. The most profound

differences were observed between the 9 ppm group and the other animals, as the 9 ppm group typically exhibited significantly greater liver enzyme levels than their counterparts, which the authors postulated was suggestive of liver cell damage (Stangl and Kirchgebner 1998). The similarity between our findings and those of Stangl and Kirchgebner (1998) was that we both observed the highest liver enzyme levels in the animals receiving the lowest dietary iron concentration.

The liver functions as a primary iron storage site, second only to the bone marrow, and liver function may be adversely affected by liver iron concentration. Stangl and Kirchgebner (1998) found that iron deficiency was related to an increase in liver enzyme levels, which are suggestive of liver cell damage (Pagana and Pagana 2003). Excessive dietary iron may affect liver function as well, but in a different manner such that liver enzyme levels are decreased. In their evaluation of characteristics of iron overload, Moirand et al (1997) assessed AST and ALT levels in persons diagnosed with homozygous genetic hemochromatosis and found that these enzyme levels were within normal limits, but were at the lower end of the normal range. The ALT levels in our animals exhibited this pattern, whereas AST levels in all animals were below the reference range with the lowest levels observed by the 150 ppm animals. Moirand and colleagues (1997) did not discuss the results of the liver function tests of their subjects.

Tissue Minerals

The purpose of this study was to examine the effects of dietary iron on tissue mineral concentrations. The young mature animals consumed diets of varying iron

content for approximately 15 weeks, and the iron, zinc, and copper concentrations in the liver, kidney, heart, and spleen of these animals were analyzed (Table 8).

Liver

Dietary iron had an overall effect on liver iron concentrations ($p<0.0001$). The animals receiving the 6 and 12 ppm iron diets had lower liver iron concentrations than those animals receiving the 35 and 150 ppm iron diets ($p<0.0001$) (Table 8). Further testing utilizing the Tukey procedure found no difference between the 6 and 12 ppm diet groups ($p=0.2371$). Comparison between the 35 and 150 ppm diet groups was not significant, but the analyses suggested a trend toward significance ($p=0.0691$). Regression analyses indicate a quadratic relationship ($p<0.0001$) between dietary iron concentration and its effect on liver iron concentration. The distribution of liver iron concentrations was linear up to the 35 ppm concentration, but from 35 to 150 ppm there was a tendency toward a plateau. Our study design was similar to that of Shah and Belonje (1991) in that both studies involved female Sprague-Dawley rats fed diets of varying dietary iron concentrations for a similar duration (15 and 12 weeks, respectively). The dietary iron concentrations in the two studies were different, as we examined the effects of deficient (6 and 12 ppm), marginal (35 ppm), and excessive (150 ppm) dietary iron intakes while Shah and Belonje (1991) examined marginal (25 ppm), adequate (47 ppm), and excessive (150 and 1260 ppm) dietary iron intakes. Our findings were similar, as we both observed increased liver iron concentrations with increasing dietary iron concentrations.

TABLE 8

EFFECT OF VARYING LEVELS OF DIETARY IRON ON TISSUE MINERAL CONCENTRATIONS IN YOUNG MATURE RATS[1,2]

Mineral (μg/g)[3]	6 ppm	12 ppm	35 ppm	150 ppm	Significance[4]
Liver					
Iron	178.6±157.7[a]	572.0±157.7[a]	1777.3±157.7[b]	2299.5±157.7[b]	p<0.0001
Zinc	86.8±12.4	89.5±12.4	101.1±12.4	91.9±12.4	p=0.7223
Copper	56.0±10.1	36.2±10.1	24.7±10.1	20.0±10.1	p=0.0747
Spleen					
Iron	998.3±632.4	1812.9±632.4	6212.0±666.6	8503.6±666.6	p=0.1328
Zinc	70.6±2.7	66.4±2.7	67.8±2.7	73.5±2.8	p=0.2174
Copper	5.5±0.9	4.4±0.9	4.9±0.9	5.0±0.9	p=0.5064
Kidney					
Iron	123.3±128.1[a]	298.3±121.5[a]	324.4±121.5[ab]	760.0±121.5[b]	p=0.0065
Zinc	89.1±17.8	88.3±17.8	81.8±17.8	89.4±17.8	p=0.7458
Copper	44.6±17.4	48.4±17.4	32.2±17.4	38.2±17.4	p=0.7054
Calcium	215.8±12.2	195.9±12.2	217.2±12.2	193.9±12.9	p=0.3963
Heart					
Iron	199.3±24.3[a]	239.6±24.3[ab]	269.8±24.3[b]	287.8±24.3[b]	p=0.0019
Zinc	59.4±3.5	57.3±3.5	54.1±3.5	59.8±3.5	p=0.5786
Copper	20.1±1.0	19.8±1.0	19.4±1.0	20.3±1.0	p=0.7835

[1] Tukey-Kramer adjusted LS Means ± SE

[2] Variables in the same row not sharing the same superscript are significantly different

[3] Per dry tissue weight

[4] Type 3 Test of Fixed Effects

However, a notable difference was that the liver iron concentrations observed in our animals fed the 35 and 150 ppm diets were much greater than the liver iron concentrations of the animals fed 25, 47, and 150 ppm diets in the study by Shah and Belonje (1991). We both fed our animals 150 ppm iron diets, yet our animals exhibited liver iron concentrations almost one and a half times greater than the animals in the aforementioned study. Furthermore, the animals fed the 1260 ppm iron diet in the study by Shah and Belonje (1991) had liver iron concentrations less than our 150 ppm animals ($1940\pm340\mu g/g$ versus $2299\pm157.7\mu g/g$). These differences are likely not significant due to the large standard error in both studies; however, the observed disparity in the liver iron concentrations resulting from maintenance on diets of very different iron concentrations (150 ppm versus 1260 ppm) may be due in part to the difference in study duration (12 versus 15 weeks) and/or the difference in feeding methodology. The animals in the study by Shah and Belonje (1991) were fed ad libitum, whereas our animals were fed to control weight gain among the groups. Significant differences in final weight and weight gain were not observed in our animals, but a trend toward significance was detected ($p<0.0737$ and $p<0.0576$, respectively). The animals in the 6 and 12 ppm groups gained the least amount of weight overall and gained the least amount of weight per gram of diet consumed; therefore, the food intake of the 35 and 150 ppm animals was restricted, which may have stimulated increased iron absorption despite the concentration of iron in the diet, as was suggested by Shah and Belonje (1991). Another difference in our study and that of Shah and Belonje (1991) was the form of iron used in the diets. We supplemented our diets with ferric citrate, whereas they used ferrous sulphate heptahydrate. Ferrous iron is better absorbed than ferric; therefore greater

absorption and, hence, greater liver iron concentrations would be expected, but this was not the case. Our analyses detected a significant quadratic relationship between dietary iron and its effect on liver iron concentrations, which may be reflected in the findings of Shah and Belonje (1991) compared with our findings. Specifically, rats fed a 150 ppm iron diet had liver iron concentrations comparable to animals fed a 1260 ppm iron diet prepared from a highly absorbable form of iron (ferrous).

In addition to examining the effects of excessive dietary iron on tissue concentrations, we examined the effects of deficient dietary iron. This facet of our study was similar to that of Shukla et al (1991) who examined the effects of dietary iron deficiency on tissue mineral concentrations in weanling female rats. Their animals were maintained on either an iron deficient diet (18-20 ppm) or iron adequate diet (390 ppm) for eight weeks. They observed, as did we, that inadequate dietary iron intake results in depressed liver iron concentrations. Specifically, their iron deficient animals exhibited liver iron concentrations less ($p<0.01$) than those observed in the control animals (Shukla et al 1991). We observed liver iron concentrations in both of our iron-deficient groups (6 and 12 ppm) to be significantly less than observed in our animals maintained on a marginal iron diet (35 ppm). The difference in study durations (8 weeks versus 15 weeks) is reflected in the actual liver iron concentrations of the animals in both studies. The iron deficient and control animals in the study by Shukla et al (1991) had liver iron concentrations (86.25 and 159.7 μg/g, respectively) less than that exhibited by our 6 ppm animals (178.6 μg/g). Despite the differences in average liver iron concentrations between the two studies, the conclusion of both is similar: iron deficiency results in decreased liver iron concentrations.

Liver zinc concentrations were not different among diet groups ($p=0.7223$) (Table 8), but regression analyses indicated a trend toward a quadratic relationship between dietary iron concentration and its effect on liver zinc ($p=0.0905$). Liver zinc concentrations increased slightly from the 6 to 35 ppm diet concentrations and then decreased between the 35 and 150 ppm, and, although not significant, this distribution of data is suggestive of a quadratic relationship. Our findings were similar to those of Shah and Belonje (1991) who also failed to observe significant differences in liver zinc concentrations due to marginal or excessive dietary iron intakes. A facet of the research of Sherman and Tissue (1981) involved feeding pregnant Sprague-Dawley rats an iron deficient diet (5 ppm) throughout their pregnancy, and then maintaining twenty rat pups on the deficient diet after weaning. At 21, 30, 60, and 90 days, rats from this group were killed for analyses. They observed, as did we, that dietary iron deficiency did not have a significant effect on liver zinc concentrations (Sherman and Tissue 1981). However, our results were in contrast to those of Shukla et al (1990), who found that liver zinc concentrations were inversely related to dietary iron intake as their iron deficient rats exhibited liver zinc concentrations greater than that of the control animals ($p<0.02$). Although not significant, the greatest liver zinc concentration was observed in our animals maintained on the iron-adequate (35 ppm) diet, which does not coincide with the findings of Shukla et al (1990). A possible explanation for this disparity in results is that the effects of iron deficiency on liver zinc deposition are dissipated with time, as our study was almost twice as long as that of Shukla et al (1990). The findings of Sherman and Tissue (1981) do not support this conclusion, as they failed to observed significant differences in liver zinc concentrations of their rats at all time points.

The concentration of copper in the liver was not significantly different among diet groups, but analyses suggested a trend toward significance between dietary iron and liver copper ($p=0.0747$) (Table 8). The Tukey procedure indicated a trend in differences between the liver copper concentrations of the 6 and 150 ppm diet groups ($p=0.0753$). Regression analyses suggested a trend toward a linear relationship between dietary iron and liver copper concentrations ($p=0.0585$). The concentration of copper in the liver decreased between the 6 and 12 ppm diet groups and continued to decrease with higher dietary iron, but much less severely than between 6 and 12 ppm (Table 8). Our results were similar to those of Shah and Belonje (1991), who failed to observe a significant effect of marginal or excessive dietary iron intakes on liver copper concentrations. However, our results conflicted with the findings of Sherman and Tissue (1981) and Shukla et al (1990), as they both observed that deficient iron intake increased liver copper concentrations ($p<0.001$ and $p<0.01$, respectively). These differences can not be explained by the passage of time because Shah and Belonje (1991) did not observe significant differences in liver copper concentrations at six or twelve weeks, and Sherman and Tissue (1981) observed significant differences at 21, 30, and 60 days. The form of iron used in the diets may partially explain the differences in findings, as the two studies that observed significant differences in liver copper concentrations used iron sulfate ($FeSO_4 \cdot 7H_2O$) whereas our study used ferric citrate and Shah and Belonje (1991) also used ferrous sulphate heptahydrate.

Spleen

Spleen iron concentrations increased with increasing dietary iron intake, but were not different among diet groups ($p=0.1328$) (Table 8). The lack of significance among spleen iron concentrations can be attributed to the variability of the data, which may be a reflection of inadequate perfusion of the spleen. Despite the lack of significant differences among spleen iron concentrations per the Tukey procedure, testing for non-linear fit revealed a quadratic relationship between dietary iron and its effect on spleen iron concentrations ($p<0.0001$). Similar to liver iron, the distribution of spleen iron concentrations was linear in nature up to the 35 ppm, and between 35 and 150 ppm, there was a tendency toward a plateau. Our findings were similar to those of Shah and Belonje (1991) in that we both observed increasing spleen iron concentrations as dietary iron increased. Furthermore, the spleen iron concentrations observed in our animals receiving the 35 and 150 ppm iron diets were comparable to the spleen iron concentrations observed by Shah and Belonje (1991) in their animals receiving 47 and 150 ppm and 1260 ppm iron diets, respectively. As was the case with liver iron, our 150 ppm animals exhibited tissue iron storage patterns similar to the 1260 ppm animals in the study by Shah and Belonje (1991), suggesting that dietary iron does not continue to be deposited in the spleen. As we did not measure fecal iron, the actual amount of iron absorbed may not have increased between the 35 and 150 ppm groups. In our animals iron deficiency did not significantly affect spleen iron concentrations, but as stated, the lack of significance was likely due to the variability of the data. Although not significantly different from the other animals, spleen iron concentrations were the lowest in the animals maintained on the iron deficient diet. Shukla et al (1990) and Sherman and

Tissue (1981) both observed lower spleen iron concentrations in their iron deficient animals than in the controls ($p<0.05$ and $p<0.005$, respectively).

Spleen zinc and copper concentrations were not different among diet groups ($p=0.2174$ and $p=0.5064$, respectively) (Table 8). Furthermore, regression analyses failed to support a linear, cubic, or quadratic relationship between dietary iron and spleen zinc or copper concentrations ($p=0.2170$ and $p=0.4459$, respectively). Shah and Belonje (1991) also failed to observe significant differences in spleen zinc concentrations among their animals maintained on diets of marginal or excessive dietary iron concentration. Furthermore, our findings were similar to Shukla et al (1990) in that we both failed to observe a significant difference in spleen zinc concentrations among our dietary treatment groups. However, they did observe greater spleen copper concentrations ($p<0.05$) in their iron deficient animals, whereas we failed to make such an observation (Shukla et al 1990). Sherman and Tissue (1981) observed significant differences in both spleen zinc and copper concentrations. Specifically, the 60-day old animals maintained on the iron deficient diet during gestation, lactation, and post-weaning had spleen zinc concentrations greater than all other 60-day old animals ($p<0.005$). Spleen copper concentrations were greater in the weanling pups of the iron deficient dams, and in the 60-day old rats of the iron deficient dams who were weaned to the iron deficient diet ($p<0.05$) (Sherman and Tissue 1981). The findings of Shukla et al (1990) and Sherman and Tissue (1981) suggest that there is an inverse relationship between dietary iron and spleen copper concentrations that may be due to maturation, as both observed significant differences at 60 days However, at 90 days, Sherman and Tissue (1981) failed to observe significant differences in spleen copper concentrations. This possible effect of

maturation may explain why we did not detect significance, as our animals were maintained on experimental diets for 15 weeks.

Kidney

Dietary iron concentration had an overall effect on kidney iron concentrations ($p=0.0065$) (Table 8). The Tukey procedure identified that the kidney iron concentration of the animals receiving the 150 ppm diet was greater than that of the animals receiving the 6 and 12 ppm diets ($p=0.0051$ and $p=0.05$, respectively). Differences in kidney iron concentrations were not observed among the 6 and 12 and 35 ppm animals ($p>0.60$); however, a trend toward differences in kidney iron was observed between the animals receiving the 35 and 150 ppm diets ($p=0.0719$). Kidney iron concentrations increased with increasing dietary iron, and regression analyses suggested a liner relationship between dietary iron and its effect on kidney iron concentrations ($p=0.0006$). Our findings are in accord with Shukla et al (1990) and Sherman and Tissue (1981) who also observed that dietary iron had a significant effect on kidney iron concentrations. In the study by Shukla et al (1990), the iron deficient animals had kidney iron concentrations less than that of the control animals ($p<0.05$). Sherman and Tissue (1981) observed lower kidney iron concentrations in their weanling pups of iron deficient dams and in their 60- and 90-day old animals maintained on the iron deficient diet after weanling ($p<0.005$). A facet of the study of Kimura and Yokoi (1996) utilized an experimental hemochromatosis model to examine the effects of excessive dietary iron (240 ppm) on tissue iron deposition. Our studies were different in that they used male Wistar rats maintained on an experimental diet for 20 days, whereas we used female Sprague-

Dawley rats and fed them an experimental diet for 105 days. However, they examined the effects of excessive dietary iron on kidney iron concentrations, which was not evaluated in the other studies discussed thus far. Their findings were similar to ours in that they observed kidney iron concentration of the animals on the high iron diet to be greater than those of the animals on the iron deficient diet ($p<0.05$), but not significantly different than those of the animals on the iron-adequate diet (Kimura and Yokoi 1996).

Kidney calcium was analyzed to determine if differences in iron concentrations in the kidney could be influenced by calcification of the kidney. Kidney calcium concentrations were not different among diet groups ($p=0.3963$) (Table 8), and regression analyses did not suggest a linear or non-linear relationship between dietary iron and kidney calcium ($p=0.3693$). Given the lack of significant differences among kidney calcium concentrations, the significant differences in kidney iron concentrations do not suggest kidney damage. Differences in kidney zinc and copper concentrations were not observed among the diet groups ($p=0.7458$ and $p=0.7054$)) (Table 8). Regression analyses also failed to suggest a linear, quadratic, or cubic relationship between dietary iron and kidney zinc or copper concentrations ($p=0.8446$ and $p=0.6773$, respectively). Our kidney zinc findings were not similar to those of Sherman and Tissue (1981) who observed kidney zinc concentrations significantly less in their 60- and 90-day old rats on the iron deficient diet than those on the control diet. However, we both failed to observe significant differences in kidney copper concentrations among our diet groups (Sherman and Tissue 1981). Yokoi et al (1991) fed male Wistar rats an iron deficient (5.9 ppm) or iron adequate (128ppm) diet for three weeks, and like us, failed to observe significant differences in kidney zinc or copper concentrations due to iron deficiency. These results

cannot be totally extrapolated to ours, given the difference in study duration and sex of the animals used, but they support the suggestion of an effect of maturation on tissue mineral deposition.

Heart

Heart iron concentrations were affected by dietary iron concentration ($p=0.0019$) (Table 8). According to Tukey analyses, the heart iron concentrations of the 6 ppm animals were less than the heart iron concentrations of the animals receiving the 35 and 150 ppm diets ($p=0.0155$ and $p=0.0017$, respectively). Differences in heart iron concentrations were not observed between the 6 and 12 ppm animals ($p=0.2819$), or among the animals receiving the 12, 35, and 150 ppm iron diets ($p>0.10$). Heart iron concentration increased with increasing dietary iron; however, the increase in heart iron was not proportional to the increase in dietary iron. Regression analyses suggested a quadratic relationship between dietary iron and its effect on heart iron concentrations ($p=0.0323$). Our findings were similar to those of Kimura and Yokoi (1996) and Yokoi et al (1991) who both observed significant effects of dietary iron on heart iron concentrations. The iron deficient animals in the study of Kimura and Yokoi (1996) exhibited heart iron concentrations significantly less than that exhibited by the adequate or excessive dietary iron groups, and the heart iron concentrations of the animals in the latter two groups were not significantly different from each other. Yokoi et al (1991) also observed lower heart iron concentrations in their iron deficient animals as compared to the controls ($p<0.01$). As with the other three tissues analyzed, heart zinc and copper concentrations were not different among diet groups ($p=0.5786$ and $p=0.7835$,

respectively) (Table 8), and regression analyses failed to reveal a relationship between dietary iron and heart zinc and copper concentrations ($p=0.5864$ and $p=0.8107$, respectively). Yokoi et al (1991) also failed to observe significant differences in heart zinc and copper concentrations due to dietary iron deficiency.

Tissue iron concentrations were affected by dietary iron; however, significant effects on tissue zinc or copper concentrations were not identified. Our animals were maintained on experimental diets for 15 weeks, which is longer in duration than any of the studies discussed that did observe differences in tissue zinc and copper concentrations in animals maintained on diets of varying iron content (Shukla et al 1990, Sherman and Tissue 1981). If dietary iron affected tissue zinc and copper concentrations during any stage of development, this effect was lost by longer maturation. Perhaps iron deposition occurred early in development, and as such, inhibited absorption of the other minerals. As the animals matured, iron absorption decreased in response to maximized tissue stores, which facilitated the absorption and tissue deposition of the other minerals. However, without serial fecal or serum mineral analyses, we are unable to verify this hypothesis.

Experiment Two: Sham-Operated and Ovariectomized Rats

Weight Gain and Food Intake

Eighty-four female Sprague-Dawley rats comprised the experiment, with forty-two animals per surgical treatment. Randomization to diet and treatment groups was done upon animal arrival. Within each surgical treatment, there were 11 animals in the 6 ppm and 150 ppm groups and 10 animals in the 12 and 35 ppm groups. At the start of the experimental period, the animals weighed between 68-75 grams, with no differences observed due to diet ($p=0.9209$), treatment ($p=0.6954$), or an interaction effect ($p=0.9801$) (Table 9). Weight gain during the experimental period and final weight were different between treatment groups ($p<0.0001$) (Table 9). The sham-operated animals gained an average of 181 grams during the study and weighed an average of 253 grams at its conclusion, whereas the ovariectomized rats gained an average of 200 grams during the study and weighed an average of 272 grams at the end of the study. Diet or an interaction between diet and treatment did not have a significant effect on the weight gain or final weight of the animals (Table 9). Although dietary iron did not have a significant effect on weight gain or final weight, differences in these parameters were noted within each treatment group (Table 9). In the sham-operated group, the animals receiving the 150 ppm diet gained the least amount of weight, and in the ovariectomized animals, the lowest weight gain and final weight were observed in the 6 ppm diet group. The differences in weight gain in the ovariectomized animals among diet groups were similar to the findings of Stangl and Kirchgebner (1998) who observed the lowest weight gain in the lowest dietary iron group. Daily weight gain per gram of

TABLE 9

EFFECT OF DIETARY IRON ON WEIGHT GAIN AND BODY COMPOSITION IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1,2]

	Initial Weight,g	Final Weight,g	Weight Gain,g	Gain-Feed,mg/g	Lean Mass,g	Percent Fat
Sham-operated						
6 ppm	70.5±2.1	257.0±5.1	186.5±5.4	0.42±0.01[a]	236.2±6.2	10.6±1.3
12 ppm	72.5±2.1	253.2±5.1	180.7±5.4	0.42±0.01[a]	228.6±6.2	12.1±1.3
35 ppm	72.0±2.2	257.9±5.4	185.8±5.7	0.43±0.01[a]	234.6±6.5	11.4±1.4
150 ppm	72.3±2.0	244.0±4.8	171.7±5.2	0.37±0.01[b]	212.8±5.9	15.4±1.2
Ovariectomized						
6 ppm	72.0±2.0	270.1±4.8	198.1±5.2	0.44±0.01[a]	249.3±5.9	9.9±1.2
12 ppm	72.3±2.1	271.5±5.1	199.2±5.4	0.47±0.01[ab]	246.2±6.2	11.5±1.3
35 ppm	72.8±2.1	272.2±5.1	199.4±5.4	0.48±0.01[b]	246.7±6.2	11.8±1.3
150 ppm	72.7±2.2	275.9±5.4	203.2±5.7	0.46±0.01[ab]	254.5±6.5	10.0±1.4
Diet	p=0.9209	p=0.7883	p=0.7623	p=0.0135	p=0.4793	p=0.3007
Trt	p=0.6954	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p=0.0955
Diet*Trt	p=0.9801	p=0.2405	p=0.2615	p=0.0745	p=0.0637	p=0.1380

[1] LS Means ± SE

[2] Variables in the same column within a treatment group not sharing the same letter are significantly different

diet consumed was different among diet groups ($p=0.0135$) and between treatment groups ($p<0.0001$), but only a tendency toward an interaction effect was observed ($p=0.0745$) (Table 9). The 150 ppm sham-operated animals gained the least amount of weight per gram of diet consumed with significant differences observed between the 150ppm animals and all other diet groups. Within the ovariectomized group, the animals fed 6ppm gained the lease per gram fed, but significant differences were found only between the 6 and 35 ppm groups. The animals in both treatment groups receiving the 35 ppm diet gained more weight per gram of diet consumed than the other animals within each treatment group (Table 9).

The body composition measures of lean body mass and percent body fat were not influenced by dietary iron concentration ($p=0.4793$ and $p=0.3007$, respectively). Lean body mass was different between treatment groups ($p<0.0001$), with a trend suggestive of an interaction observed as well. In a pattern similar to that of weight gain, the 150 ppm sham-operated animals had the lowest lean body mass, and the 150 ppm ovariectomized animals had the highest lean body mass (Table 9). Body fat percentages were not different due to an interaction between diet and treatment ($p=0.1380$); however, statistical analyses suggest a trend toward differences due to treatment ($p=0.0955$) (Table 9). Although significant differences were not detected, it is worthwhile to note that the 150 ppm sham-operated animals, which were the smallest animals in the study, had the highest body fat percentage (Table 9). The difference in body fat percent between the sham-operated and ovariectomized animals may be due to the loss of ovarian hormones, or a reflection of the feeding methodology employed. Specifically, the food intake of the ovariectomized animals was restricted in attempts to match weight gain among the

animals. The ovariectomized animals continued to gain weight throughout the study, but because their food intake was restricted, they may not have been receiving food in excess of their needs such that fat storage was increased. Another possible explanation is that the sham-operated animals were provided food in excess of their needs and as such they experienced greater fat storage. We also observed the lowest body fat percentage in the 6 ppm ovariectomized animals, which is similar to the findings of Beard et al (1995) who found that dietary iron deficiency was related to low body fat percentages.

Liver and spleen weight as a percentage of body weight were different due to diet ($p=0.0374$ and $p=0.0020$, respectively), but not treatment ($p=0.4279$ and $p=0.9864$, respectively). Conversely, kidney and heart weight as a percentage of body weight were different due to treatment ($p<0.0001$ and $p=0.0307$, respectively), but not diet ($p=0.1570$ and $p=0.5784$, respectively). There were no interaction effects on organ weight as a percentage of body weight ($p>0.10$) (Table 10). Differences in liver percent body weight (PBW) tended to increase with increasing dietary iron concentration as the sham animals receiving the 12 ppm diet had significantly lower liver PBW than the animals receiving the 150 ppm diet. In the ovariectomized animals, those receiving the 150 ppm diet had significantly greater liver PBW than the other groups. Furthermore, the spleens of all animals receiving the 12 ppm diet were significantly lower PBW than the spleens of all the other animals (Table 10). The differences in liver and spleen PBW among diet groups may be a reflection of the effects of iron on tissue growth. The sham-operated animals had kidney and heart PBW ($p<0.0001$ and $p=0.0307$, respectively) than the ovariectomized

TABLE 10

EFFECT OF DIETARY IRON ON ORGAN WEIGHT EXPRESSED AS PERCENT BODY WEIGHT IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1,2]

	Liver pbw	Spleen pbw	Kidney pbw	Heart pbw
Sham-operated				
6 ppm	0.025±0.001[ab]	0.0024±0.0001[a]	0.0068±0.0002	0.0042±0.0001
12 ppm	0.024±0.001[a]	0.0020±0.0001[b]	0.0070±0.0002	0.0040±0.0001
35 ppm	0.027±0.001[b]	0.0024±0.0001[a]	0.0071±0.0002	0.0041±0.0001
150 ppm	0.026±0.0009[b]	0.0022±0.0001[a]	0.0067±0.0002	0.0039±0.0001
Ovariectomized				
6 ppm	0.025±0.001[a]	0.0024±0.0001[a]	0.0058±0.0002	0.0039±0.0001
12 ppm	0.025±0.001[a]	0.0021±0.0001[b]	0.0062±0.0002	0.0039±0.0001
35 ppm	0.026±0.001[a]	0.0022±0.0001[a]	0.0060±0.0002	0.0038±0.0001
150 ppm	0.028±0.001[b]	0.0023±0.0001[a]	0.0059±0.0002	0.0038±0.0001
Diet	p=0.0374	p=0.0020	p=0.1570	p=0.5784
Trt	p=0.4279	p=0.9864	p<0.0001	p=0.0307
Diet*Trt	p=0.1573	p=0.4193	p=0.8599	p=0.8972

[1] LS Means ± SE

[2] Variables in the same column within a treatment group not sharing the same letter are significantly different

animals (Table 10). This may be a reflection of the lower final body weights of the sham-operated animals versus the ovariectomized as the sham animals weighed less than the ovariectomized animals ($p<0.0001$), and therefore, their organs constituted a greater percentage of their body weight. Our findings are similar to Schwartz et al (1973) as they too observed the greatest percent organ weights in animals with the lowest final body weight.

Diet and Trace Mineral Intake

The sham-operated and ovariectomized animals consumed the AIN-93 diet formulated for growth for approximately 7 weeks followed by a 20-week period during which they consumed the maintenance formulation of the AIN-93 rodent diet. Growth diet intake was not significantly different due to diet, treatment, or an interaction effect (Table 11). During the maintenance period, however, diet intake was different between treatment groups ($p<0.0001$). We attempted to control diet intake to prevent differences in weight between the treatment groups by providing a greater amount of feed to the animals in the diet and ovarian hormone status group gaining the least weight. The significant differences observed are a result of the 150 ppm sham animals continuously receiving the largest quantity of diet because they gained the least amount of weight throughout the study (Tables 9 and 11).

TABLE 11

AVERAGE DAILY GROWTH AND MAINTENANCE DIET INTAKES OF SHAM-OPERATED AND OVARIECTOMIZED RATS[1]

	Growth (g)	Maintenance (g)
Sham-operated		
6 ppm	12.1±0.3	13.7±0.2
12 ppm	12.0±0.3	13.5±0.2
35 ppm	11.8±0.3	13.6±0.2
150 ppm	11.9±0.3	14.1±0.1
Ovariectomized		
6 ppm	12.3±0.3	13.5±0.1
12 ppm	11.8±0.3	13.3±0.2
35 ppm	11.5±0.3	13.1±0.2
150 ppm	12.1+0.3	13.3±0.2
diet	p=0.2329	p=0.1590
trt	p=0.8365	p=0.0001
diet*trt	p=0.6952	p=0.1197

[1]LS Means ± SE

The average daily trace mineral intakes during the growth period were significantly different due to diet, but not treatment or interaction (Table 12). Dietary iron intakes of the animals in the 150 ppm diet group were significantly greater than that of the animals in the other three diet groups. Furthermore, the animals receiving the 35 ppm diet had iron intakes significantly greater than the animals in the 6 and 12 ppm groups, whose intakes did not differ significantly (Table 12). Overall, average daily zinc intakes were inversely related to dietary iron concentration, with the animals receiving the 6 and 12 ppm diets consuming significantly more zinc than the animals receiving the 150 ppm diet. The difference in zinc intake due to diet effects cannot be attributable to differences in zinc concentration of the diet, nor diet intake as differences in both were not observed (Tables 3 and 11, respectively). Whether or not the difference in zinc intake was due to

TABLE 12

AVERAGE DAILY TRACE MINERAL INTAKES IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1]

	G-Fe (mg)[2]	G-Zn (mg)[2]	G-Cu (mg)[2]	M-Fe (mg)[3]	M-Zn (mg)	M-Cu (mg)[2]
Sham-operated						
6 ppm	0.13±0.02[a]	0.46±0.01[a]	0.056±0.002[ab]	0.12±0.02[a]	0.444±0.005	0.063±0.001[a]
12 ppm	0.17±0.02[a]	0.46±0.01[a]	0.061±0.002[a]	0.25±0.02[b]	0.445±0.005	0.061±0.001[b]
35 ppm	0.43±0.02[b]	0.43±0.01[ac]	0.061±0.002[a]	0.55±0.02[c]	0.453±0.005	0.060±0.001[b]
150 ppm	1.97±0.02[c]	0.42±0.01[bc]	0.055±0.002[b]	2.13±0.01[d]	0.454±0.005	0.060±0.001[b]
Ovariectomized						
6 ppm	0.13±0.02[a]	0.47±0.01[a]	0.060±0.002[ab]	0.12±0.01[a]	0.436±0.005	0.062±0.001[a]
12 ppm	0.17±0.02[a]	0.45±0.01[a]	0.060±0.002[a]	0.24±0.02[b]	0.440±0.005	0.060±0.001[b]
35 ppm	0.41±0.02[b]	0.42±0.01[b]	0.059±0.002[a]	0.53±0.02[c]	0.436±0.005	0.058±0.001[c]
150 ppm	2.00±0.02[c]	0.43±0.01[b]	0.056±0.002[b]	2.00±0.02[c]	0.426±0.005	0.057±0.001[c]
diet	p<0.0001	p=0.0004	p=0.0263	p<0.0001	p=0.7760	p<0.0001
trt	p=0.7594	p=0.8337	p=0.6069	p=0.0006	p=0.0001	p=0.0001
diet*trt	p=0.7451	p=0.7039	p=0.3002	p=0.0001	p=0.1298	p=0.1508

[1] LS Means + SE

[2] Variables in the same column within a treatment group not sharing the same letter superscript are significantly different ($p<0.05$).

[3] Variables in the same column not sharing the same letter superscript are significantly different.

G-Fe (growth iron), G-Zn (growth zinc), G-Cu (growth copper)

M-Fe (maintenance iron), M-Zn (maintenance zinc), M-Cu (maintenance copper)

an interaction between iron and zinc is not quantifiable based on the data collected in this study.

Dietary copper intakes exhibited an inverse relationship to dietary iron concentration as well, but only to a certain extent. Specifically, the animals receiving the 150 ppm diet had significantly lower copper intakes than the animals receiving the 12 and 35 ppm diets; however, there were no significant differences in copper intakes between the animals receiving the 6 and 150 ppm diets and the 6, 12, or 35 ppm diets (Table 12). As was the case with dietary zinc intake, the differences observed cannot be attributable to differences in the copper concentration of the diet nor differences in diet intake among the diet groups (Tables 3 and 11). Also, as was the case with growth zinc intakes, we cannot conclude that the observed difference in copper intakes in the different dietary iron groups was due to an interaction between these two minerals.

Differences in trace mineral intakes during the maintenance period were attributable to diet, treatment, and interaction effects in the case of iron (Table 12). Dietary iron intakes were found to be different due to an interaction effect ($p=0.0001$). Specifically, iron intakes among diet groups within each surgical treatment group were significantly different, whereas iron intakes of all animals within the 6, 12, and 35 ppm groups were not significantly different. The interaction effect was apparent between the sham-operated and ovariectomized animals receiving the 150 ppm diet, as the iron intakes of these animals were significantly different from each other and all of the other animals (Table 12). Specifically, the 150 ppm sham animals consumed significantly more iron per day than the 150ppm ovariectomized animals, and both 150 ppm iron groups consumed significantly more iron per day than all other diet groups. Average zinc

intakes were different between treatment groups ($p=0.0001$) (Table 12). In the sham-operated animals, zinc intakes were directly related to dietary iron concentration, while the zinc intakes in the ovariectomized animals were lowest in 150 ppm diet group and highest in the 12 ppm diet group. Differences in copper intakes were due to both a diet ($p<0.0001$) and treatment ($p=0.0001$) effect, but statistical analyses did not indicate occurrence of an interaction ($p=0.1508$). An inverse relationship between copper intakes and dietary iron concentration was observed, with the ovariectomized animals receiving the 35 and 150 ppm diets consuming significantly less copper than the animals on the 6 or 12 ppm diets. The animals receiving the 6 ppm diet had the highest copper intakes in both treatment groups, which were significantly greater than the copper intakes of the animals receiving the 35 or 150 ppm diet (Table 12). The differences in maintenance zinc and copper intakes were not due to differences in the zinc and copper content of the maintenance diet (Table 3), but rather differences ($p=0.0001$) in maintenance diet intake between the two treatment groups (Table 11).

Hematology, Nutritional, and Liver Function Analyses

Prolonged maintenance on an iron deficient diet, as well as the surgical treatment, had a significant effect on the hematological parameters of both the sham-operated and ovariectomized rats (Table 13). Although not outside the reference range (Hrapkiewicz et al 1998), hemoglobin levels were lower in the 6 ppm animals ($p=0.0019$), and there was a significant difference in these values between treatment groups ($p=0.0020$), with the lower hemoglobin values observed in the sham-operated animals. The diet and dietary iron intake of the sham-operated animals was greater than the intakes of the

ovariectomized animals (Table 11); therefore, the difference in hemoglobin concentrations between the two treatment groups are likely not due to differences in iron intake, but rather differences in ovarian hormone status. Iron deficiency anemia is more common in pre-menopausal women partially due to menstrual losses (IOM 2002, pgs 18-19, 375) and such may be the case rats. Red blood cell counts, reticulocyte counts, and mean corpuscular volume were affected by dietary iron intake (Table 13). The animals receiving the 6 ppm diet had greater RBC and reticulocyte counts than the animals receiving the 12, 35, or 150 ppm diets ($p=0.0017$ and $p=0.0001$), while MCV values were lower in the 6 ppm animals ($p=0.0002$). Hematocrit levels were not different among diet groups or between treatments, but a trend toward a treatment effect was observed ($p=0.0759$). Furthermore, the sham-operated animals receiving the 6 ppm diet had the lowest hematocrit levels overall (Table 13). Leukocyte counts were not affected by diet, but differences were observed between treatment groups ($p<0.0001$), with the lower values exhibited by the sham-operated animals (Table 13). Furthermore, leukocyte counts in both treatment groups were below the reference range (Hrapkiewicz, et al 1998). Although differences in leukocyte counts were observed between treatment groups, the fact that the leukocyte levels of all animals were depressed raises the question of effects of housing environment on the immune system. Unfortunately, concrete assessment of this speculation is beyond the scope of this study.

Our findings regarding the effects of dietary iron concentration on hematological parameters are similar to some of the findings of other researchers. Stangl and Kirchgebner (1998), who examined the effects of deficient and marginal dietary iron intakes found, as we did, that the animals maintained on iron deficient diets had lower

THE EFFECT OF DIETARY IRON ON HEMATOLOGY VALUES IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1,2]

	RBC $10^3/mm^3$	WBC $10^6/mm^3$	Hgb g/dL	HCT %	MCV $\mu g/m^3$	RTC $10^6/mm^3$
Reference Range	*7-10*[3,4]	*6-17*[3,4]	*11-18*[3]	*36-48*[3]		
Sham-operated						
6 ppm	7.8±0.2[a]	2.9±0.5	13.4±0.2[a]	33.4±1.4	47.3±1.3[a]	0.330+0.025[a]
12 ppm	7.5±0.2[b]	2.6±0.5	14.3±0.2[b]	38.4±1.4	51.7±1.3[b]	0.198+0.025[b]
35 ppm	7.4±0.2[b]	3.6±0.6	14.5±0.3[b]	39.2±1.6	53.1±1.5[b]	0.225+0.030[b]
150 ppm	7.5±0.2[b]	2.9±0.5	14.2±0.2[b]	38.6±1.4	51.6±1.3[b]	0.203+0.028[b]
Ovariectomized						
6 ppm	8.4±0.2[a]	4.6±0.5	14.1±0.2[a]	38.7±1.5	46.9±1.4[a]	0.294+0.028[a]
12 ppm	7.6±0.2[b]	4.4±0.5	15.0±0.3[b]	40.0±1.6	52.4±1.5[b]	0.194+0.028[b]
35 ppm	7.3±0.2[b]	5.2±0.5	14.6±0.3[b]	38.6±1.4	52.7±1.5[b]	0.180+0.028[b]
150 ppm	7.6±0.2[b]	4.5±0.5	14.9±0.3[b]	40.2±1.6	53.1+1.5[b]	0.211+0.028[b]
diet	p=0.0017	p=0.3761	p=0.0019	p=0.1030	p=0.0002	p=0.0001
trt	p=0.1637	p<0.0001	p=0.0020	p=0.0759	p=0.7218	p=0.3380
diet*trt	p=0.3035	p=0.9982	p=0.5536	p=0.2797	p=0.8804	p=0.7445

[1] LS Means + SE

[2] Variables in columns within a treatment group with different character superscripts are significantly different

[3] Hrapkiewicz, et al (1998)

[4] Young 1998

hemoglobin, hematocrit, and mean corpuscular volume than those animals maintained on iron-adequate diets. Our results also concur with Stangl and Kirchgebner (1998) in that neither we nor they observed differences in WBC counts among dietary iron groups. Shah and Belonje (1991) fed male and female rats diets containing marginal (25 ppm), adequate (47 ppm), high (150 ppm), or excessive (1260 ppm) iron for 6 and 12 weeks. They found, as did we, that at the end of both experimental periods the lowest hemoglobin levels were observed in the animals receiving the lowest dietary iron concentration (Shah and Belonje 1991); however, the hemoglobin concentrations of all animals were within normal limits (Hrapkiewicz 1998).

Nutritional status and liver function were primarily affected by treatment, as opposed to diet or an interaction, but the latter two effects did occur. Creatinine and AST were the only parameters not affected by diet, surgical treatment, or an interaction between diet and treatment (Table 14), and they were within the reference range for rats (Hrapkiewicz, et al 1998). Glucose and urea nitrogen were different due to an interaction effect ($p=0.0398$ and $p=0.0114$, respectively), and both were outside the reference ranges for rats (Hrapkiewicz et al 1998). All animals had glucose levels higher than the reference value, which may have been due to stress, as animals were moved approximately 12 hours prior to necropsy and due to the administration of anesthesia prior to blood collection. Urea nitrogen levels were lower than the reference range, which can be a sign of liver dysfunction, as efficient protein metabolism results in the production of urea by the liver. Low urea nitrogen levels are also seen in malnutrition as a response to reduced protein intake (Pagana and Pagana 2003).

TABLE 14

THE EFFECT OF DIETARY IRON ON LIVER FUNCTION AND NUTRITIONAL INDICATORS IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1]

	Glucose[2] mmol/L	Albumin g/L	Creatinine µmol/L	Urea N[2] mmol/L	ALP uKat/L	ALT uKat/L	AST uKat/L
Reference Range[3]	*2.8-7.5*	*38-48*	*18-70*	*5.4-7.5*[4]	*0.3-2.1*	*0.3-1.5*	*3.2-4.4*
Sham-operated							
6 ppm	12.9±0.9^{abe}	40.7±0.8	51.6±3.1	0.50±0.03^{a}	0.9±0.1	0.32±0.03	1.20±0.09
12 ppm	14.4±0.9^{b}	41.9±0.8	51.0±3.1	0.61±0.03^{b}	0.7±0.1	0.29±0.03	1.09±0.09
35 ppm	14.9±0.9^{adc}	41.4±0.8	49.6±3.2	0.48±0.03^{a}	1.0±0.1	0.33±0.03	1.07±0.10
150 ppm	11.8±0.8^{be}	43.1±0.7	49.2±2.9	0.62±0.03^{b}	0.6±0.1	0.31±0.03	1.01±0.09
Ovariectomized							
6 ppm	16.8±0.9^{cdf}	38.3±0.7	47.6±2.9	0.47±0.03^{a}	1.4±0.1	0.36±0.03	1.22±0.09
12 ppm	17.9±0.9^{f}	38.3±0.8	55.0±3.1	0.47±0.03^{a}	1.2±0.1	0.34±0.03	1.10±0.09
35 ppm	16.1±0.9^{cdf}	37.2±0.8	47.7±3.1	0.48±0.03^{a}	1.1±0.1	0.37±0.03	1.08±0.09
150 ppm	16.5±0.9^{cf}	38.1±0.8	47.4±3.2	0.43±0.03^{a}	1.1±0.1	0.46±0.03	1.34±0.10
diet	p=0.5236	p=0.3646	p=0.4169	p=0.1411	p=0.0543	p=0.1649	p=0.4005
trt	p<0.0001	p<0.0001	p=0.6744	p=0.0002	p<0.0001	p=0.0022	p=0.1852
diet*trt	p=0.0398	p=0.4240	p=0.5979	p=0.0114	p=0.5077	p=0.2419	p=0.2353

[1] LS Means + SE

[2] Variables not sharing the same character superscript are significantly different

[3] Hrapkiewicz, et al (1998)

[4] Young 1998

Liver dysfunction and malnutrition also can cause depressed albumin levels by affecting the liver's ability to produce this serum protein (Tietz 1990). Albumin levels in the ovariectomized rats were below the reference range (Hrapkiewicz et al 1998), and there was a difference in albumin levels between treatments ($p<0.0001$). Our animals were not overtly malnourished; however, the food intake of the ovariectomized animals was typically restricted per the feeding protocol described earlier. Alkaline phosphatase, ALT, and AST are all found in the liver, and aberrant levels can indicate liver dysfunction (Pagana and Pagana 2003). Alkaline phosphatase levels were normal (Hrapkiewicz et al 1998), but different among diet groups ($p=0.0543$) and between treatments ($p<0.0001$) (Table 14). ALT levels were marginal in all animals (Hrapkiewicz et al 1998), but there were differences between treatments ($p=0.0022$). AST levels were not significantly different, but all animals had AST levels much lower than the reference range (Pagana and Pagana 2003). Stangl and Kirchgebner (1998) also examined the effects of varying dietary iron concentrations on liver enzymes, and although their study involved male Sprague-Dawley rats and iron deficient and iron adequate diets only, there were some similarities to our findings. Specifically, when comparing our two lowest dietary iron groups we consistently found that the animals receiving the 6 ppm diets had greater liver enzyme levels than the animals receiving the 12 ppm diet, which is similar to the overall greater liver enzyme levels observed by Stangl and Kirchgebner (1998) in their lowest dietary iron group (9 ppm).

The results presented do not provide conclusive evidence to suggest liver dysfunction occurred in our animals, and we cannot make conclusive statements regarding liver damage because we do not have histological analyses of liver tissue that

would clarify if structural changes had occurred. However, the prevalence of significant differences in the aforementioned clinical analyses due to treatment does suggest some difference in these animals that may be attributable to ovarian hormone status.

Tissue Mineral Analyses

Sham-operated and ovariectomized female rats were fed diets of varying iron concentrations for a total of 27 weeks to examine the effects on tissue mineral concentrations. The iron, copper, and zinc concentrations of the liver, spleen, and heart were determined, as were the iron, copper, zinc, and calcium concentrations of the kidneys. Kidney calcium concentrations were determined to, ideally, eliminate calcification of the kidneys as a possible explanation of any differences observed in the trace mineral concentrations of the kidney.

Liver

Liver iron concentrations were different among the diet groups ($p=0.0002$), but treatment ($p=0.2850$) and interaction effects ($p=0.4571$) were not evident (Table 15), and the lack of interaction was further supported by the slice option of proc mixed analyses. The Tukey procedure supported the findings of significant differences in the liver iron concentrations of the animals receiving the 6 and 12 ppm iron diets and those receiving the 35 and 150 ppm iron diets. Regression analyses suggest a quadratic relationship between dietary iron and its effect on liver iron concentrations ($p<0.0001$). Liver iron concentrations increased as dietary iron increased, with the change linear up to 35 ppm, and then from 35 ppm to 150 ppm there was a tendency toward a plateau.

TABLE 15

EFFECTS OF DIETARY IRON ON LIVER MINERAL CONCENTRATIONS IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1,2,3]

	Iron (μg/g)	Zinc (μg/g)[4]	Copper (μg/g)
Sham-operated			
6 ppm	223.4±261.8[a]	92.9±8.4[abc]	45.8±4.6[a]
12 ppm	886.2±261.8[a]	93.1±8.4[abc]	26.8±4.6[b]
35 ppm	1963.3±266.7[b]	81.4±8.5[ac]	20.7±4.8[b]
150 ppm	2805.8±258.2[b]	103.3±8.3[b]	35.3±4.5[ab]
Ovariectomized			
6 ppm	233.0±258.2[a]	89.0±8.3[abc]	26.4±4.5[a]
12 ppm	909.3±266.7[a]	79.1±8.4[c]	20.3±4.6[b]
35 ppm	1829.6±261.8[b]	87.0±8.4[abc]	20.0±4.6[b]
150 ppm	2105.9±266.7[b]	81.7±8.5[ac]	20.3±4.7[ab]
diet	p=0.0002	p=0.1512	p=0.0129
trt	p=0.2850	p=0.0046	p=0.0041
diet*trt	p=0.4571	p=0.0096	p=0.1162

[1] Mineral concentrations expressed per gram dry tissue
[2] Proc Mixed with Tukey-Kramer Adjustment
[3] Variables in the same column within a treatment group not sharing the same letter superscript are significantly different.
[4] Variables in the same column not sharing the same letter superscript are significantly different.

Liver zinc concentrations were different among diet and treatment groups due to an interaction effect (p=0.0096) (Table 15). The sham-operated animals receiving the 150 ppm diet had significantly greater liver zinc concentrations than the sham animals receiving the 35 ppm diet and the ovariectomized animals receiving the 12 and 150 ppm diets (Table 15). Regression analyses were performed separately for the sham-operated and ovariectomized rats, per the results of proc mixed slice analyses that suggested interaction. A quadratic relationship between liver zinc concentrations and dietary iron was observed in the sham-operated animals (p=0.0329), whereas regression analyses of the liver zinc concentrations of the ovariectomized were not significant. The observed

differences in regression results between treatment groups further supports the occurrence of an interaction effect on liver zinc concentrations.

Liver copper concentrations were affected by diet and treatment ($p=0.0129$ and $p=0.0041$, respectively) (Table 15). The highest liver copper concentrations were observed in the lowest dietary iron groups in both the sham-operated and ovariectomized animals. The 6 ppm animals exhibited liver copper concentrations significantly greater than those of the animals receiving the 12 and 35 ppm diets, but not those receiving the 150 ppm diet. Significant differences in liver copper concentrations were observed between treatment groups as well, with concentrations in the sham-operated animals being greater than those of the ovariectomized animals. Proc mixed analyses did not support an interaction until employment of the slice test, which required that regression analyses be performed separately for each treatment. These results suggest a cubic relationship ($p<0.04$) between dietary iron concentrations and its effect on liver copper concentrations in both sham-operated and ovariectomized rats.

Spleen

Proc mixed analyses failed to identify differences in spleen iron concentrations due to diet, treatment, or interaction effects ($p>0.10$) (Table 16). Spleen iron concentrations increased as dietary iron concentration increased, but the wide range of spleen iron concentrations observed in each diet group prevented detection of statistical significance. A function of the spleen is to recycle red blood cells (Cohen and Wood 2000), and inadequate perfusion of the spleen at necropsy may explain the

TABLE 16

EFFECTS OF DIETARY IRON ON SPLEEN MINERAL CONCENTRATIONS IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1,2]

	Iron (μg/g)	Zinc (μg/g)	Copper (μg/g)
Sham-operated			
6 ppm	1270.6±998.4	70.1±3.9	5.7±0.9
12 ppm	3543.6±1193.3	68.1±3.9	4.4±0.9
35 ppm	8434.7±1288.9	72.4±4.0	4.5±0.9
150 ppm	11047.0±1052.4	69.2±3.8	4.5±0.9
Ovariectomized			
6 ppm	1239.3±951.9	71.3±3.8	4.7±0.9
12 ppm	3594.3±998.4	73.7±3.9	5.0±0.9
35 ppm	5695.5±1193.3	75.8±3.9	4.8±0.9
150 ppm	9296.4±1052.4	73.4±4.1	4.8±0.9
diet	p=0.1361	p=0.5954	p=0.3266
trt	p=0.3862	p=0.0974	p=0.8714
diet*trt	p=0.6743	p=0.8698	p=0.1310

[1] Proc Mixed with Tukey-Kramer Adjustment
[2] Mineral concentrations expressed per gram dry tissue

variation in spleen iron concentrations we observed. Testing for non-linear fit suggests a quadratic relationship between dietary iron and spleen iron concentrations, which is reflected in the change in spleen iron concentration with increasing dietary iron. Similar to liver iron, but only in the sham-operated animals, spleen iron concentrations increased in a linear fashion up through the 35 ppm diet, and then exhibited a tendency to plateau between 35 and 150 ppm (Table 16). In the ovariectomized animals, the increase in spleen iron concentrations was gradual from the 6 ppm diet up through the 35 ppm diet, and from 35 to 150 ppm, the increase in spleen iron was more pronounced (Table 16).

Spleen zinc concentrations were not different among diet groups (p=0.5954), nor did proc mixed analyses suggest an interaction (p=0.8698) (Table 16). Treatment did not

have an effect on spleen zinc as well, but there was a tendency toward a treatment effect ($p=0.0974$), which was reflected in the subtle difference in values between treatment groups (Table 16). Regression analyses failed to identify a linear, quadratic, or cubic relationship between dietary iron and spleen zinc concentrations ($p=0.5180$).

Proc mixed analyses failed to identify differences in spleen copper concentrations due to diet ($p=0.3266$), treatment ($p=0.8714$), or interaction effects ($p=0.1310$) (Table 16). However, proc mixed analyses including slice testing did suggest an interaction; therefore, separate regression analyses were performed for each treatment, and they failed to identify a relationship between dietary iron concentration and spleen copper. Although not significant, the overall highest spleen copper concentration (5.7μg/g) was observed in the 6 ppm sham animals and the lowest (4.4μg/g) was in the 12 ppm sham animals.

Kidney

Kidney iron concentrations were different among diet groups ($p=0.0208$) and a trend toward a treatment effect was observed as well ($p=0.0893$) (Table 17). The animals receiving the 6 and 12 ppm diets had significantly lower kidney iron concentrations than the animals receiving the 35 and 150 ppm diets (Table 17). Proc mixed analyses with slice testing suggested an interaction effect, which required individual regression analyses be performed on each treatment group. Neither a linear nor a non-linear relationship between kidney iron and dietary iron concentrations was identified in the sham-operated animals, but a quadratic relationship was found in the ovariectomized rats ($p=0.0158$), with the pattern of change in kidney iron concentrations resembling that of the liver and spleen (Tables 15 and 16). The difference in regression analyses of the

TABLE 17

EFFECTS OF DIETARY IRON ON KIDNEY MINERAL CONCENTRATIONS IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1,2,3]

	Iron (μg/g)	Zinc (μg/g)	Copper (μg/g)	Calcium (μg/g)
Sham-operated				
6 ppm	210.4±290.2[a]	95.6±10.8	50.0±15.0	203.4±19.3
12 ppm	746.7±290.2[a]	91.0±10.8	56.2±15.0	177.2±19.3
35 ppm	1280.7±305.9[b]	83.2±11.0	42.4±15.3	174.9±20.3
150 ppm	1178.4±276.7[b]	95.2±10.6	32.9±14.7	181.6±18.4
Ovariectomized				
6 ppm	203.9±290.2[a]	86.2±10.8	30.3±14.7	180.1±18.4
12 ppm	284.0±290.2[a]	77.6±10.8	33.5±15.0	195.8±19.3
35 ppm	731.5±290.2[b]	84.5±10.8	33.3±15.0	195.1±19.3
150 ppm	770.9±305.9[b]	99.7±11.0	37.1±15.3	248.2±20.3
diet	p=0.0208	p=0.1157	p=0.8503	p=0.3886
trt	p=0.0893	p=0.3451	p=0.1864	p=0.1382
diet*trt	p=0.7969	p=0.4372	p=0.6533	p=0.1467

[1] Mineral concentrations are expressed per gram dry tissue
[2] Proc Mixed with Tukey-Kramer Adjustment
[3] Variables in the same column within a treatment group not sharing the same letter superscript are significantly different.

sham-operated and ovariectomized animals further supports the occurrence of an interaction effect between diet and treatment that subsequently affected kidney iron concentrations.

Dietary iron, ovarian hormone status, or an interaction between diet and treatment failed to have a significant effect on kidney zinc and copper concentrations ($p>0.10$) (Table 17). In both the sham-operated and ovariectomized animals, kidney zinc concentrations were greatest in the animals at the extremes of the dietary iron spectrum. Kidney copper concentrations, although not significantly different were inversely related to dietary iron concentration in the sham-operated animals, as the two iron deficient

groups had kidney copper concentrations greater than that of the animals receiving the adequate and high dietary iron. Conversely, the greatest kidney copper concentrations in the ovariectomized animals were exhibited by the 150 ppm group, followed by the 12 ppm group (Table 17). Regression analyses were not significant for either kidney zinc or copper, but there was a tendency toward a linear relationship between dietary iron and its effect on kidney zinc concentrations ($p=0.0835$, per proc GLM). Proc mixed analyses of kidney calcium failed to identify differences due to diet ($p=0.3886$) or treatment ($p=0.1382$) effects (Table 17). Interaction effects were not identified by proc mixed alone, but with the employment of slice testing, an interaction was suggested. However, regression analyses failed to identify a relationship, but a trend toward a quadratic relationship in the sham-operated animals ($p=0.0602$), and a linear relationship in the ovariectomized animals ($p=0.0644$) was observed. The lack of significant differences in kidney calcium concentrations among diet and treatment groups suggests that differences in kidney iron concentration may not be attributed to calcification of the kidney.

Heart

Heart iron concentrations were different among diet groups ($p<0.0001$) with the 6 ppm animals in both treatment groups exhibiting significantly lower heart iron concentrations than the animals receiving the 12, 35, and 150 ppm diets (Table 18). Proc mixed analyses failed to identify treatment ($p=0.9258$) or interaction effects ($p=0.5523$). Testing for non-linear fit produced results similar to those observed in the liver, spleen and kidney, specifically that a significant quadratic relationship was identified between dietary iron and heart iron concentrations. In a pattern similar to that identified in the

TABLE 18

EFFECTS OF DIETARY IRON ON HEART MINERAL CONCENTRATIONS IN SHAM-OPERATED AND OVARIECTOMIZED RATS[1,2,3]

	Iron (μg/g)	Zinc (μg/g)	Copper (μg/g)
Sham-operated			
6 ppm	229.1±18.6[a]	56.8±3.6	20.0±1.3
12 ppm	252.6±18.6[b]	55.5±3.6	19.2±1.3
35 ppm	279.1±19.3[bc]	51.2±3.8	18.4±1.3
150 ppm	317.4±18.0[c]	54.4±3.5	19.9±1.3
Ovariectomized			
6 ppm	211.1±18.0[a]	51.7±3.5	18.7±1.3
12 ppm	266.9±18.6[b]	54.8±3.6	19.3±1.3
35 ppm	298.2±18.6[bc]	54.8±3.6	19.0±1.3
150 ppm	306.1±20.1[c]	53.3±3.8	19.8±1.3
diet	p<0.0001	p=0.9354	p=0.5427
trt	p=0.9258	p=0.7308	p=0.7233
diet*trt	p=0.5523	p=0.6302	p=0.6427

[1] Mineral concentrations are expressed per gram dry tissue
[2] Proc Mixed with Tukey-Kramer Adjustment
[3] Variables in the same column within a treatment group not sharing the same letter superscripts are significantly different.

liver, spleen, and kidney, heart iron concentrations increased in a linear trend up through the 35 ppm diet concentration, and from the 35 to 150 ppm concentrations, a plateau effect was observed. This trend in the magnitude of change with increasing dietary iron concentration was more pronounced in the ovariectomized animals (Table 18).

Heart zinc and copper concentrations were not different among diet or treatment groups ($p>0.50$), and proc mixed analyses with slice testing failed to indicate an interaction (Table18). Regression analyses were not significant for a linear, quadratic, or cubic relationship between dietary iron concentration and heart zinc or copper concentrations.

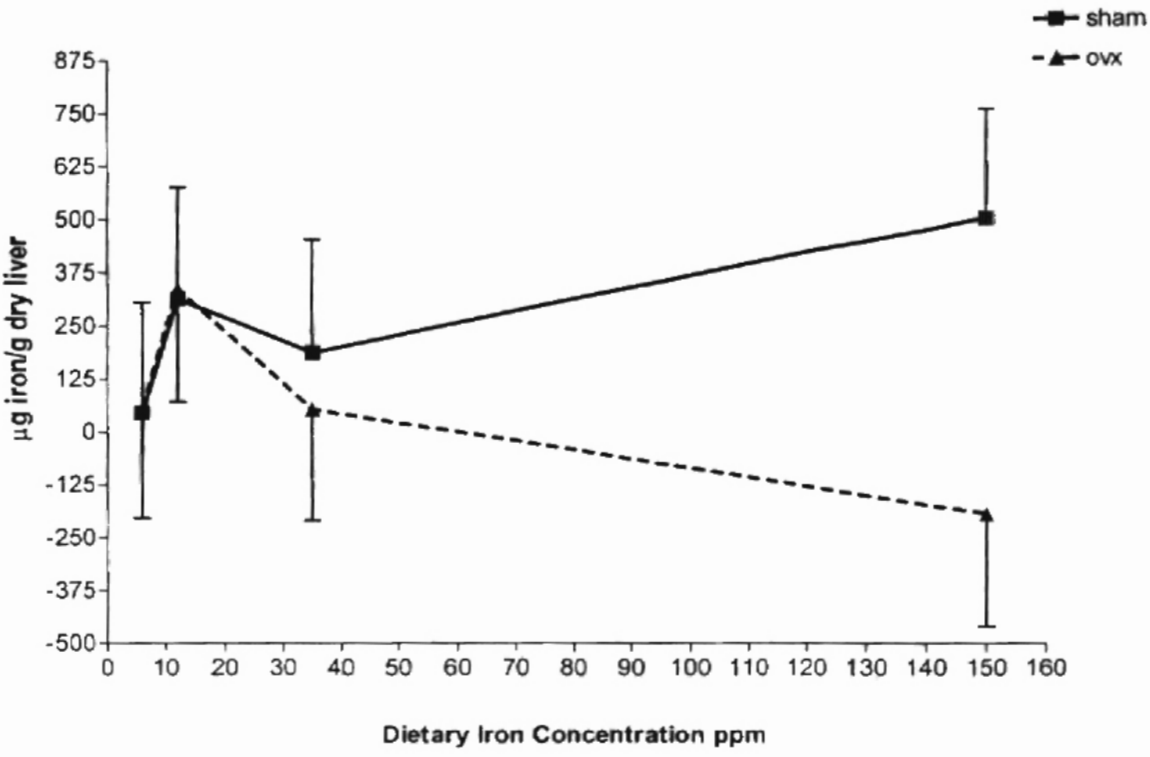
Change in Tissue Mineral Concentrations from Surgery to Necropsy

Analysis of the change in tissue mineral concentrations from surgery to necropsy was performed to evaluate the effects of time and ovarian hormone deficiency.

Liver

Iron. Liver iron concentrations did not change from surgery to necropsy due to diet ($p=0.7172$), treatment ($p=0.2850$), or interaction effects ($p=0.4571$), and regression analyses failed to identify a linear, quadratic, or cubic relationship between change in liver iron concentration and dietary iron concentration ($p=0.3991$). Although not significant, the pattern of change between treatment groups began to differ as dietary iron concentration increased between 12 and 35 ppm and up through 150 ppm. Specifically, the ovariectomized animals receiving the 150 ppm diet lost liver iron (-193.5μg/g dry tissue), whereas the concentration of iron in the livers of the sham-operated animals increased (+506.4 μg/g dry tissue) (Figure 2, Appendix F, Table 1). Dallman et al (1982) examined the changes in tissue iron concentrations that occurred as a result of prolonged feeding of iron deficient diets in male Sprague-Dawley rats. The animals were obtained at weaning and fed a 100 ppm iron diet for 15 days, the

Figure 2. Change in Liver Iron Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Non-Significant Changes in Liver Iron Concentrations.

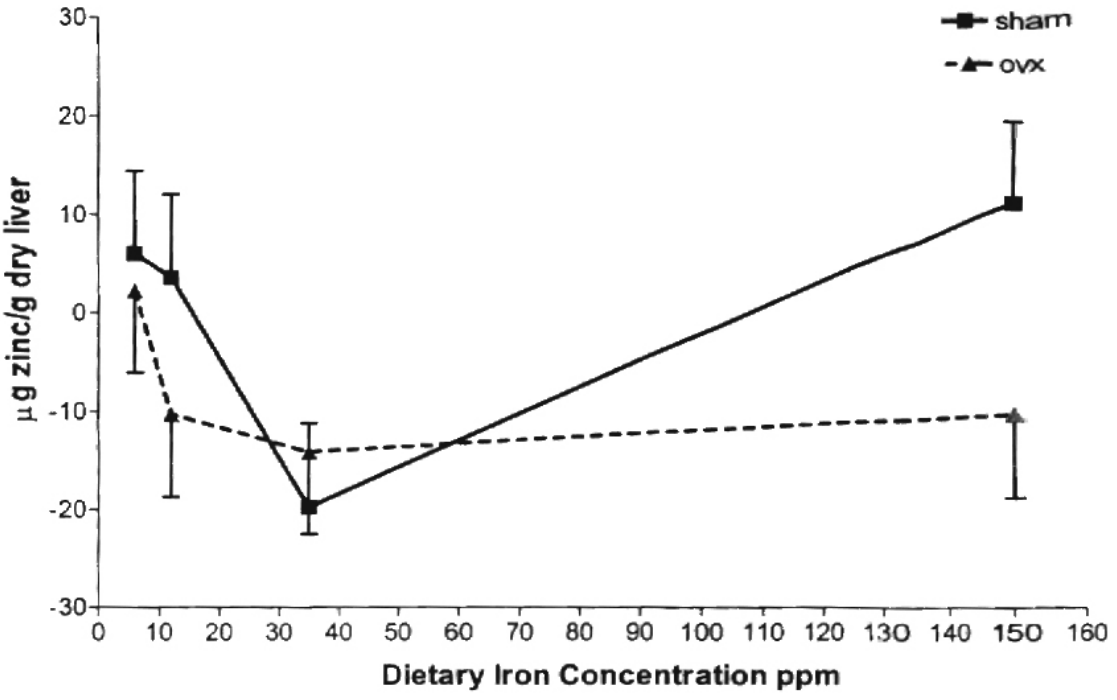


purpose of which was to maximize iron stores prior to being placed on an iron deficient diet. Subsequently, the animals were randomly assigned to the control group (50 ppm) or one of two deficient groups (2 or 6 ppm), and sacrificed at intervals during the following 54-day feeding period. At the outset of the experimental feeding period, 6-8 rats were killed and the iron content of the liver and spleen was analyzed for baseline values. Liver iron concentrations increased in all three groups from baseline to 3 days, but from day 3 through day 24, liver iron concentrations decreased. In the iron deficient groups, the drop in liver iron was most dramatic from day 3 to day 6, whereas in the control group, the most dramatic decrease was observed between days 10 and 14. Surprisingly, there was a consistent increase in liver iron concentrations in the animals receiving the 2 and 6 ppm

diets from day 24 to the end of the experimental feeding period (day 54). E the overall change in liver iron concentrations from day three to day 54 (ap weeks) reveals an overall decrease in liver iron concentrations in all anima would be expected but was not observed in our iron deficient animals. Ho not monitor the changes in liver iron concentrations at different stages, but and given that the animals in the study by Dallman et al (1982) began to e liver iron stores toward the end of the experimental feeding period raises t whether or not they too would have experienced a net increase in liver iro in liver iron concentrations in our animals, and the tendency toward an inc passage of time in the study by Dallman et al (1982) is a reflection of the presented by Shah and Belonje (1991) that iron absorption is more efficie prolonged maintenance on an iron deficient diet due.

Zinc. Changes in liver zinc concentrations were due to an interact ($p=0.0096$), and slice testing further supported the occurrence of an intera ovariectomized animals receiving the 12, 35, and 150 ppm diets, liver zin decreased between 10-14.5 µg/g dry tissue from surgery through necrops greatest decrease observed in the 35 ppm group. Conversely, the only sh experience a decrease in liver zinc were those receiving the 35 ppm diet, magnitude of change observed in this group was the greatest overall (-19 tissue) (Figure 3; Appendix F, Table 1). In the remaining animals, the ir zinc ranged from 2.2-11.4µg/g dry tissue, with the greatest increase obse animals receiving the 150 ppm diet. Regression analyses of each treatm

Figure 3.Change in Liver Zinc Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized rats. Diet x Treatment, p<0.001; Quadratic Fit Sham-Operated Rats p=0.0002, Quadratic Fit Ovariectomized Rats p=0.0360

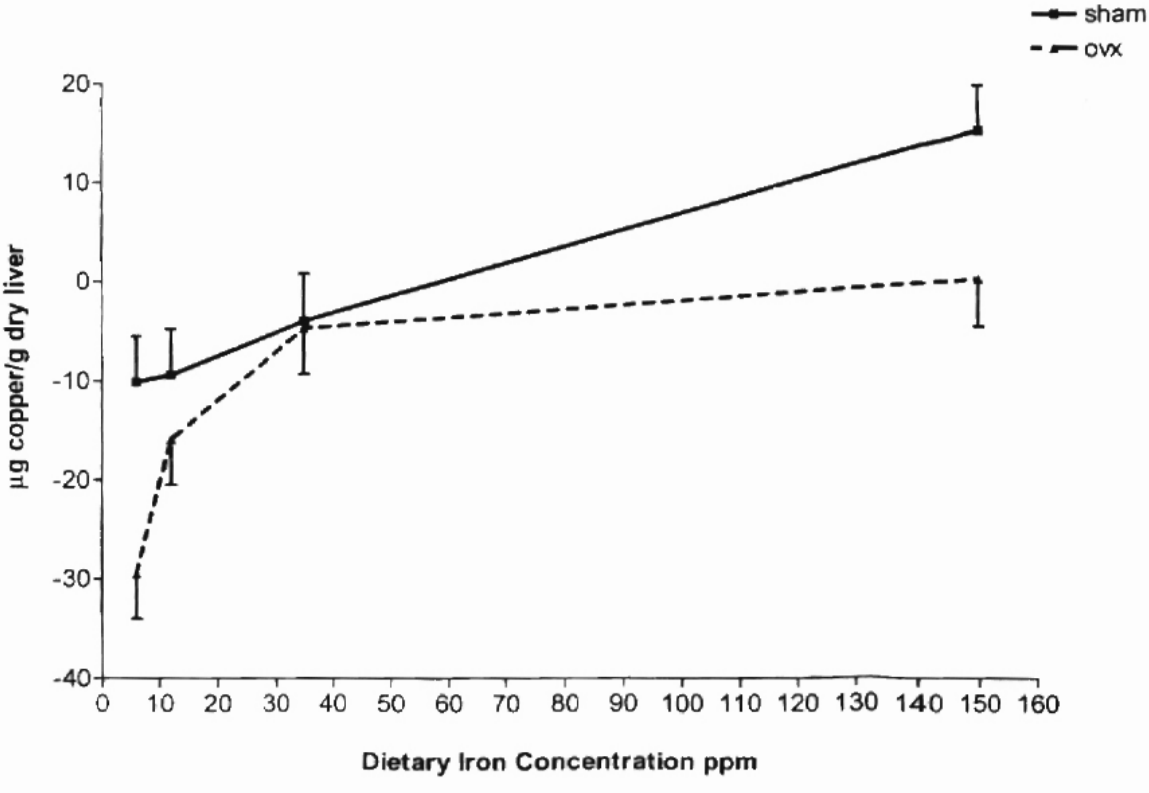


identified a quadratic relationship between the change in liver zinc and dietary iron in the sham-operated (p=0.0002) and ovariectomized (p=0.0360) animals.

Copper. The change in liver copper concentration from surgery to necropsy was different among diet (p=0.0005) and between treatment (p=0.0041) groups, and proc mixed analyses with slice testing suggested the presence of an interaction. There was a direct, inverse relationship exhibited between dietary iron concentration and change in liver copper concentrations. Specifically, the animals in both 6 ppm diet groups experienced the greatest decrease in liver copper concentrations, with the sham-operated animals losing approximately 10µg/g dry tissue and the ovariectomized animals losing approximately 29.5 µg/g dry tissue. Furthermore, as dietary iron increased, the negative

change in liver copper was minimized up through the 35 ppm diet. A positive change in liver copper occurred from the 35 to 150 ppm diet concentration, with the greatest increase exhibited by the sham-operated animals receiving the 150 ppm diet (+15.30µg/g dry tissue) (Figure 4). Figure 4 depicts the disparity in the change in liver copper concentrations between the sham-operated and ovariectomized animals. Regression analyses describe the relationship between dietary iron concentration and its effect on change in liver copper as linear in the sham-operated animals ($p<0.0001$) and quadratic in the ovariectomized ($p<0.0001$), which further supports the occurrence of an interaction.

Figure 4. Change in Liver Copper Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Diet $p<0.0005$, Treatment $p<0.0041$; Linear Fit Sham-Operated $p<0.0001$, Quadratic Fit Ovariectomized $p<0.0011$



In their study examining the effects of marginal and excessive dietary iron on tissue mineral concentrations, Shah and Belonje (1991) evaluated the difference in liver iron, zinc, and copper concentrations from 6 weeks of experimental feeding to 12 weeks. They observed increased liver iron concentrations in all female rats except those receiving the 1260 ppm diet, which exhibited a decrease in liver iron. Our sham-operated animals receiving the 150 ppm diet also experienced an increase in liver iron, but the 150 ppm ovariectomized animals lost iron. This may be explained by the greater diet intake of the sham-operated animals, or by the loss of ovarian hormones. The changes in liver zinc concentrations observed by Shah and Belonje (1991) were primarily different from our findings as they observed increased liver zinc concentrations in all females consuming diets with iron concentration similar to those used in our study. Changes in liver copper concentrations were quite different in our study versus those observed by Shah and Belonje (1991). We observed decreased liver copper concentrations in all animals except those receiving the highest dietary iron concentration, whereas the only animals exhibiting decreased liver copper concentrations in the study by Shah and Belonje (1991) were those receiving the chow (270 ppm iron) and the highest dietary iron concentration (1260 ppm).

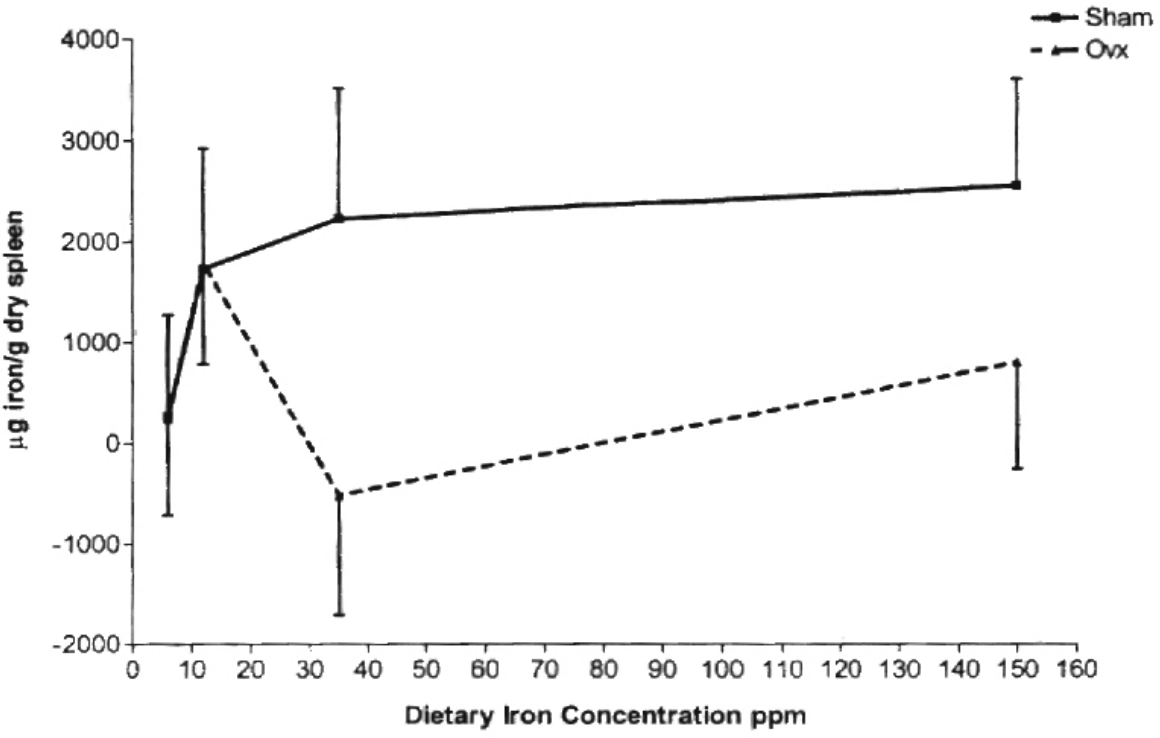
The greatest overall changes in liver iron, zinc, and copper concentrations were consistently experienced by the sham-operated animals receiving the 150 ppm diet, as compared to their ovariectomized counterparts. This could be a reflection of the greater diet intake of the sham animals or an effect of ovarian hormone deficiency on tissue mineral deposition. Furthermore, the similarity in the change in liver iron and zinc concentrations in our ovariectomized animals on the 150 ppm diet and the 1260 ppm

animals in the study by Shah and Belonje (1991) suggests a possible adverse effect of both excessive dietary iron and/or ovarian hormone deficiency on deposition of minerals in the liver. Specifically, the ovariectomized animals in our study experienced loss of liver minerals, as did the 1260 ppm female rats in the study of Shah and Belonje (1991).

Spleen

Iron. The changes in spleen iron concentrations were not identified as different due to diet, treatment, or interaction effects ($p>0.30$), and testing for non-linear fit failed to identify a relationship between changes in spleen iron and dietary iron concentrations ($p>0.10$). These findings are similar to the liver iron change data, and may be attributable to the variability of the data. In the sham-operated animals, spleen iron concentrations increased relative to dietary iron concentration from surgery to necropsy, with the greatest increase experienced by the 150 ppm group (+2543.8 μg/g dry tissue) (Figure 5). Such was not the case in the ovariectomized animals, as the 35 ppm diet group experienced a dramatic decrease in spleen iron concentration from surgery to necropsy (-516.5μg/g dry tissue), and the greatest increase was observed in the 12 ppm group (1781.5μg/g dry tissue). Furthermore, the increase in spleen iron from surgery to necropsy exhibited by the sham-operated 150 ppm group was three times greater than that experienced by the ovariectomized 150 ppm animals (+2543.8 versus +792.9 μg/g dry tissue, respectively). Our findings are not entirely consistent with those of Dallman et al (1982) who only observed a net increase in spleen iron in their control animals (50 ppm), whereas we observed a net increase in spleen iron in all of our animals except the ovariectomized animals receiving the 35 ppm diet. The

Figure 5. Change in Spleen Iron Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Non-Significant Changes in Spleen Iron Concentrations.

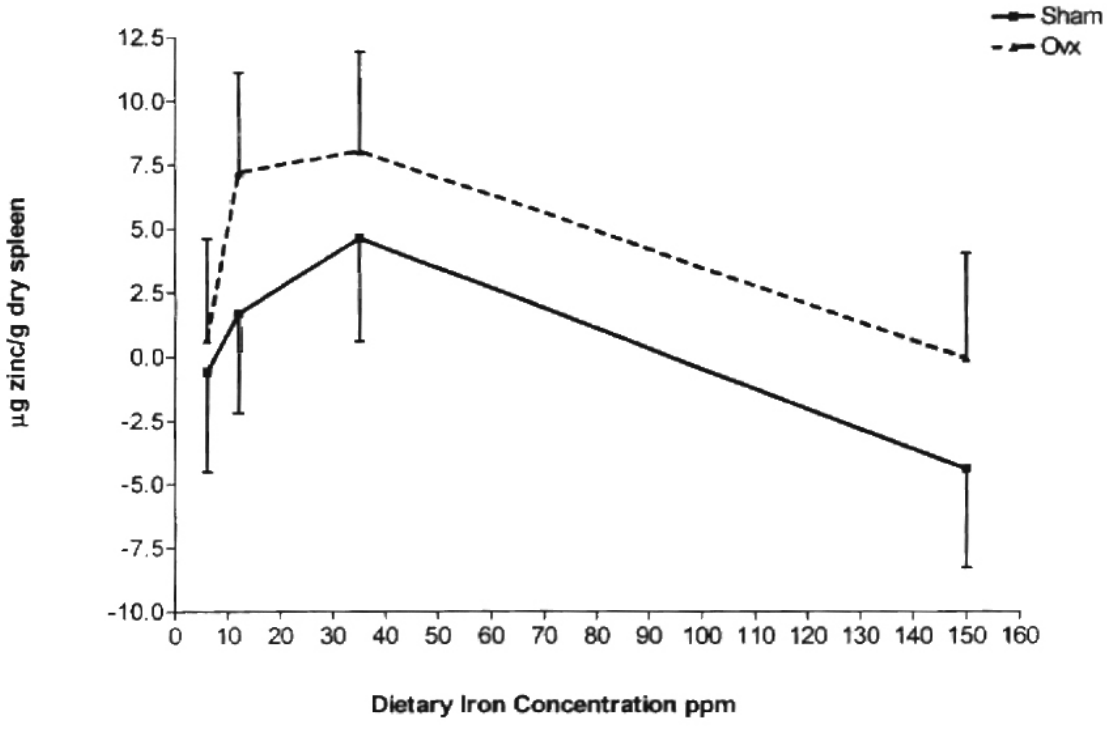


pattern of change in spleen iron concentrations observed by Dallman et al (1982) in their iron deficient animals was similar to their observations in the liver. Specifically, they observed a decrease in spleen iron concentrations up through day 14, but from day 14 through day 54, spleen iron concentrations increased (Dallman et al 1982). The disparity in the change in spleen iron concentrations observed in our iron deficient animals compared with that observed by Dallman et al (1982) may be partially due to the 5-week difference in study duration. Furthermore, as was suggested previously in the discussion of changes in liver iron, our observations may reflect more efficient iron absorption due to chronic insufficient dietary iron intake. Shah and Belonje (1991) observed that spleen iron concentrations increased with increasing dietary iron intake and with the passage of

time, which is in accord with our findings in the sham-operated animals. Such was not entirely the case in our ovariectomized animals as spleen iron concentrations decreased from the 12 ppm to the 35 ppm diet groups followed by an increase from 35 to 150 ppm. The difference in the pattern of change in spleen iron concentrations in the sham-operated and ovariectomized animals may be a reflection of the effects of ovarian hormone deficiency on spleen iron deposition. Of note, the amount of iron gained from 35 to 150 ppm in the ovariectomized animals was greater than that gained by the sham-operated animals (+1309.4 μg/g versus +311.1 μg/g dry tissue, respectively). It is interesting that the magnitude of change in spleen iron concentrations from 6 to 12 weeks in the study by Shah and Belonje (1991) was much greater than that observed in our animals from surgery to necropsy, despite the fact that our animals were maintained on the experimental diets for much longer duration. We failed to observe a significant quadratic or cubic relationship between spleen iron and dietary iron concentration that may have helped explain this difference; however, our spleen iron data was variable which may have precluded detection of a significant quadratic or cubic relationship. The disparity in magnitude of change in spleen iron concentrations between our study and that of Shah and Belonje (1991) may be due to duration in that a storage threshold may be reached and, barring a metabolic disorder (hemochromatosis), the net increase in spleen iron may be lessened with time.

Zinc. Diet did not have a significant effect on the change is spleen zinc concentrations from surgery to necropsy (Figure 6). Treatment and interaction effects were not significant, and slice testing supported the lack of an interaction effect.

Figure 6. Change in Spleen Zinc Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Diet p=0.05; Quadratic Fit p=0.0105

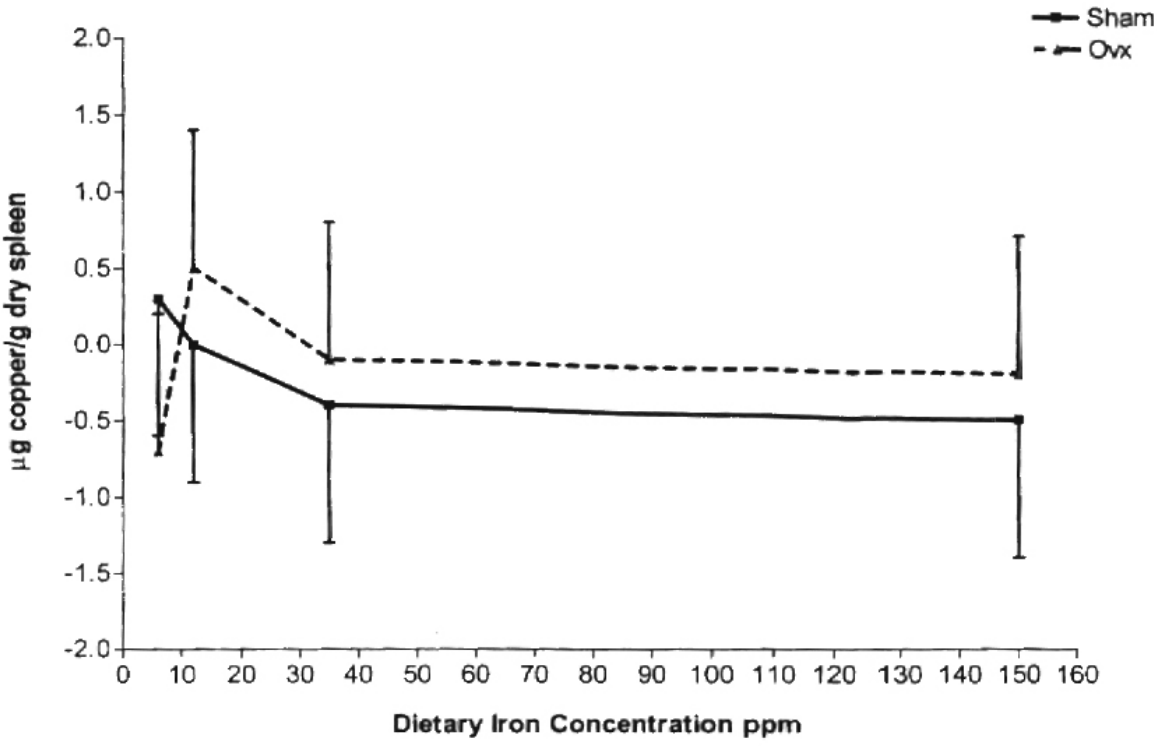


In both the sham-operated and ovariectomized animals, the greatest decrease in spleen zinc was observed in the 150 ppm diet group, while the greatest increase was observed in the 35 ppm diet group (Figure 6; Appendix F, Table 1). There were obvious differences in the magnitude of change observed between treatment groups, as the amount of zinc lost from surgery to necropsy was almost six times greater in the sham-operated group, and the gain in spleen zinc was approximately 40 percent greater in the ovariectomized group. The findings of Shah and Belonje (1991) were primarily not in accord with our findings, as they observed a decrease in spleen zinc concentrations with the passage of time in all animals except those receiving the 47 ppm diet, whereas the majority of our animals experienced an increase in spleen zinc. The similarity between our studies was

that the animals in both studies receiving 150 ppm iron diet experienced decreased spleen zinc concentrations with the passage of time. Regression analyses identified a quadratic relationship ($p<0.0105$) between dietary iron concentration and the change in spleen zinc from surgery to necropsy.

Copper. Change in spleen copper was not different among diet ($p=0.3632$) or between treatment ($p=0.8714$) groups; however, the occurrence of an interaction was suggested by proc mixed with slice testing. Therefore, regression analyses were completed on the treatment groups individually, which failed to identify a non-linear trend in the sham-operated animals, but there was a cubic relationship ($p=0.0098$) between dietary iron concentration and the changes in spleen copper from surgery to necropsy only in the ovariectomized animals. At each dietary iron concentration, there was an evident disparity in the magnitude of change in spleen copper that occurred in the sham-operated and ovariectomized animals (Figure 7). However, the greatest losses and gains of copper in the spleen were observed in the 6 and 12 ppm ovariectomized animals, respectively, while there was little change in the 35 and 150 ppm groups.

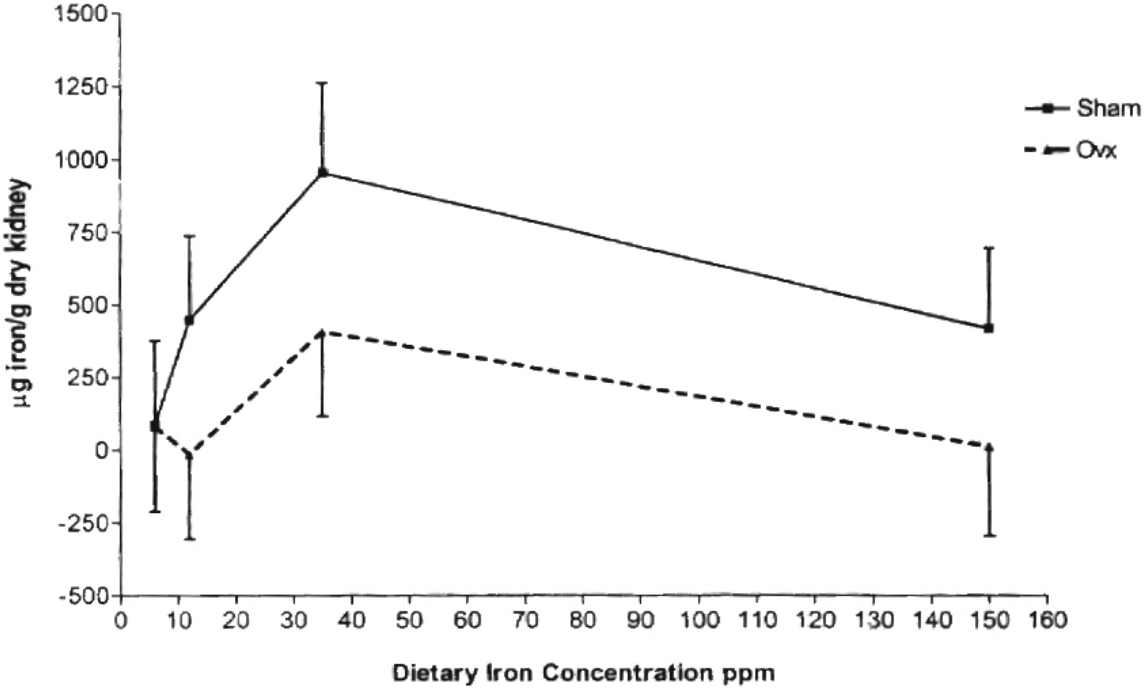
Figure 7. Change in Spleen Copper Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Ovariectomized Rats Cubic Fit p=0.0098.



Kidney

Iron. Kidney iron concentrations did not change among diet groups (p=0.2036) or due to an interaction effect (p=0.7939); however, a trend toward a treatment effect was observed (p=0.0893). This is reflected in the differences in the change data between the sham-operated and ovariectomized animals (Figure 8). In the 12, 35, and150 ppm diet groups, the gain of kidney iron was an average of 460 µg/gram dry tissue greater in the sham-operated animals than in the ovariectomized rats. Furthermore, there was a loss of kidney iron in the 12 ppm ovariectomized animals, although it was not as severe as

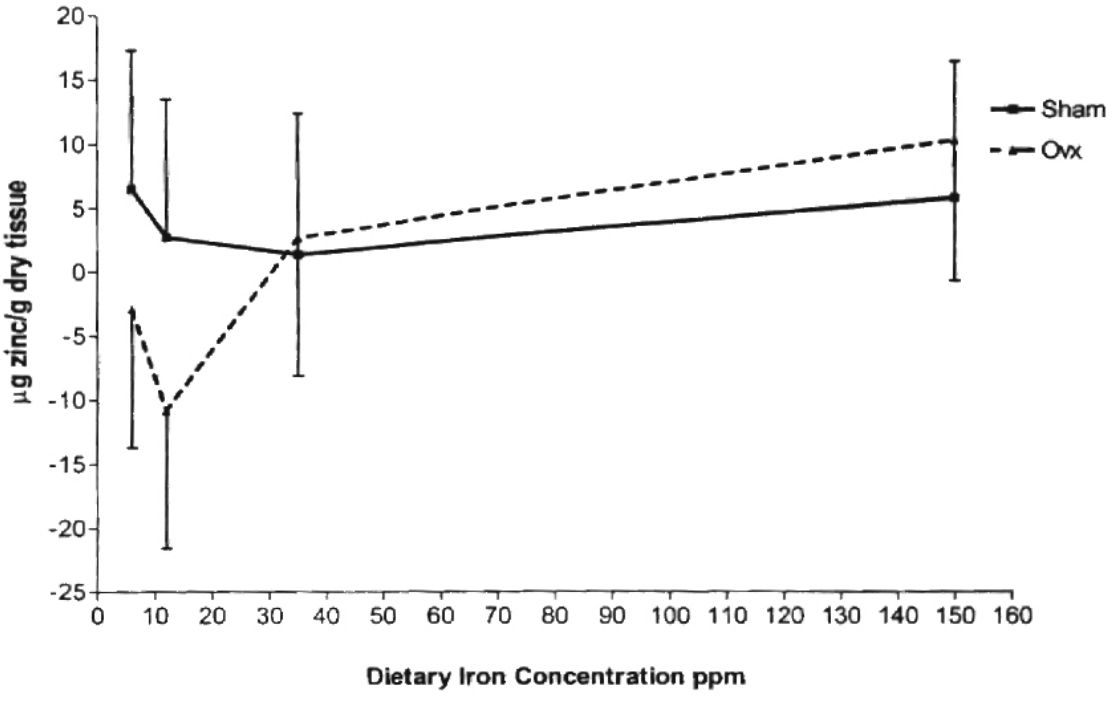
Figure 8. Change in Kidney Iron Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Quadratic Fit p=0.0377



that lost in the liver of the 150 ppm ovariectomized animals and the spleen of the 35 ppm ovariectomized animals. Despite this variability, regression analyses described a quadratic relationship (p=0.0377) between dietary iron and the change in kidney iron from surgery to necropsy in both treatment groups (Figure 8).

Zinc. Proc mixed analyses failed to detect differences in kidney zinc concentrations due to diet, treatment, or interaction effects, and testing for non-linear fit did not detect a relationship between dietary iron and kidney zinc concentrations (p>0.30). However, there was a disparity in the changes experienced by the sham-operated and ovariectomized animals. Specifically, the kidney zinc concentrations

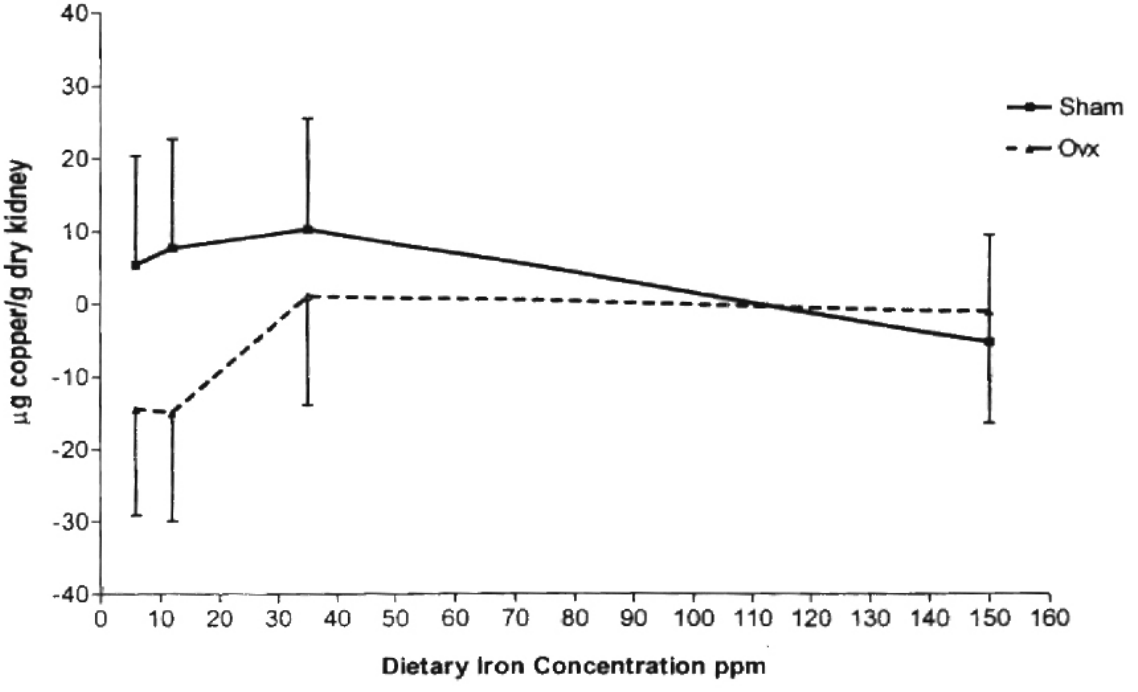
Figure 9. Change in Kidney Zinc Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Non-Significant Changes in Kidney Zinc Concentrations.



increased in all sham-operated animals and in the ovariectomized animals receiving the 35 and 150 ppm diets, whereas the ovariectomized animals receiving the 6 and 12 ppm diets lost zinc. The greatest gain and loss of kidney zinc occurred in the ovariectomized animals receiving the 150 ppm and 12 ppm diets (+10.3 and –10.8µg/g dry tissue, respectively) (Figure 9).

Copper. Differences in the change in kidney copper concentrations were not detected by proc mixed analyses ($p>0.10$), and regression analyses failed to suggest a linear, quadratic, or cubic relationship between the change in kidney copper from surgery to necropsy and dietary iron concentration ($p=0.7348$). In the ovariectomized animals, only the 35 ppm group did not lose kidney copper from surgery to necropsy (Figure 10, Appendix F, Table 2). The overall greatest decrease in kidney copper occurred in the

Figure 10. Change in Kidney Copper Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Non-Significant Changes in Kidney Copper Concentrations.



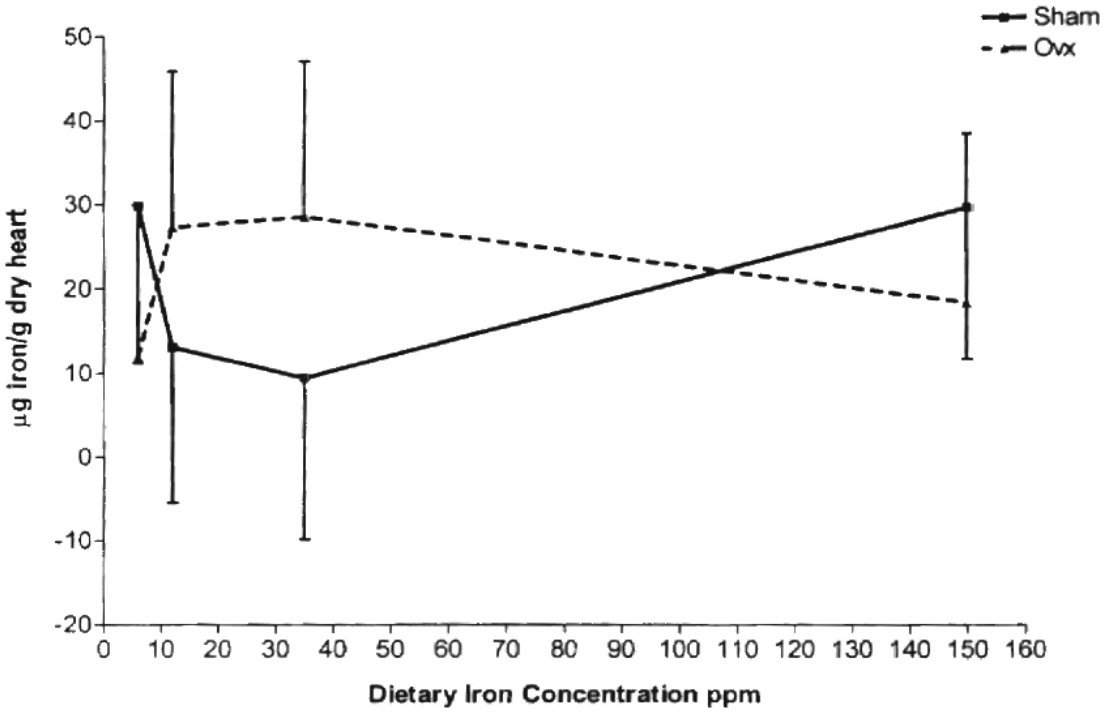
12 ppm ovariectomized animals (-14.9µg/g dry tissue) followed by the 6 ppm ovariectomized animals (-14.4µg/g dry tissue), which is the same pattern of change observed in the kidney zinc concentrations. The pattern of kidney copper gain was not similar to that of kidney zinc, as the greatest increase in kidney copper occurred in the sham-operated animals receiving the 35 ppm diet (+10.3µg/g dry tissue), followed by the shams receiving the 12 ppm diet (7.7µg/g dry tissue) (Figure 10). In the sham animals, the pattern of change appeared to be different although the change was not statistically significant. That is, all diet groups except the 150 ppm group accumulated additional copper in the kidney whereas 150 ppm sham animals lost copper. This change in kidney copper apparently was not due to calcification of the kidney as the 150 ppm sham animals lost kidney calcium (Appendix F, Table 2).

Calcium. The change in kidney calcium concentrations was different among diet groups ($p=0.0405$), and proc mixed analyses with slice testing suggested the presence of an interaction between diet and treatments. Regression analyses failed to identify a relationship between the change in kidney calcium and dietary iron in the sham animals ($p=0.4478$), but a linear relationship was detected in the ovariectomized animals ($p=0.0231$). This difference in regression findings for each treatment further supports the occurrence of an interaction. Specifically, the greatest decrease in kidney calcium from surgery to necropsy occurred in the sham-operated animals receiving 35 ppm diet (-42.3μ g/g) whereas the greatest increase was observed in the ovariectomized animals receiving the 150 ppm diet (+54.4 μg/g). The fact that the extremes of the results (highest versus lowest) occurred in different diet and treatment groups is suggestive of an interaction.

Heart

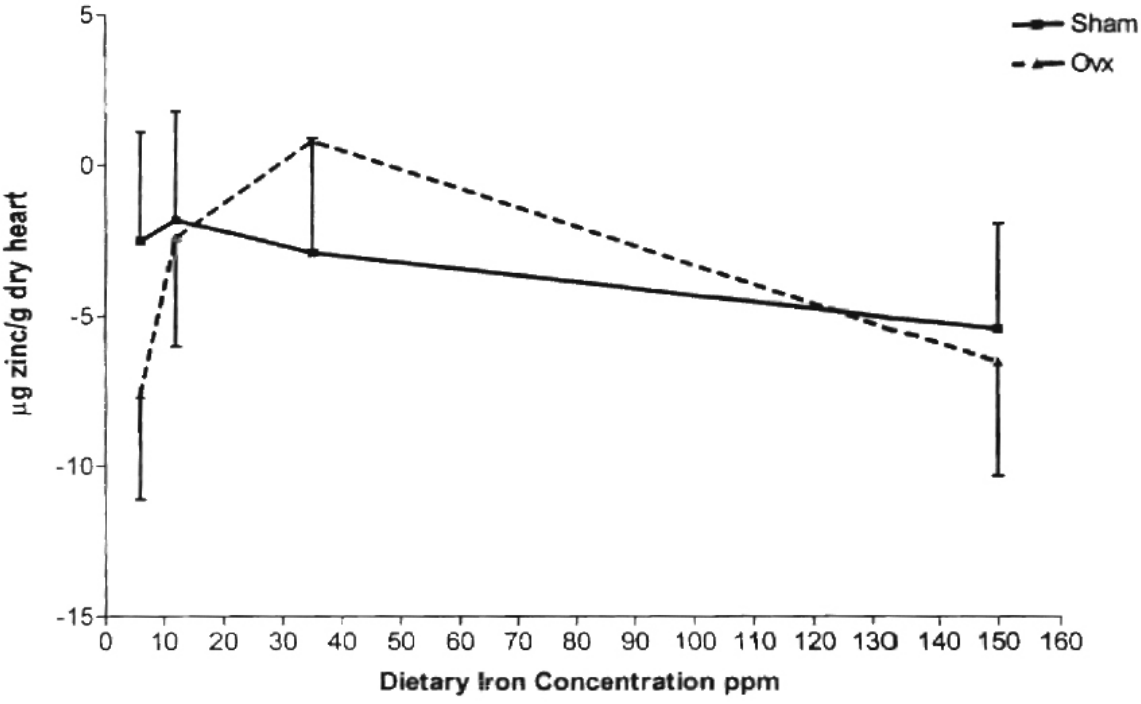
Iron. Heart tissue mineral concentrations did not change from surgery to necropsy per proc mixed analyses ($p>0.40$), and regression analyses failed to identify a linear, quadratic, or cubic relationship between the change in heart mineral concentrations and dietary iron concentration ($p>0.30$) (Appendix F; Table 2). Heart iron concentrations increased in all animals, with the greatest increase observed in the sham-operated animals receiving the 6 ppm diet (+29.8μg/g dry tissue) (Figure 11).

Figure 11. Change in Heart Iron Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Non-Significant Changes in Heart Iron Concentrations.



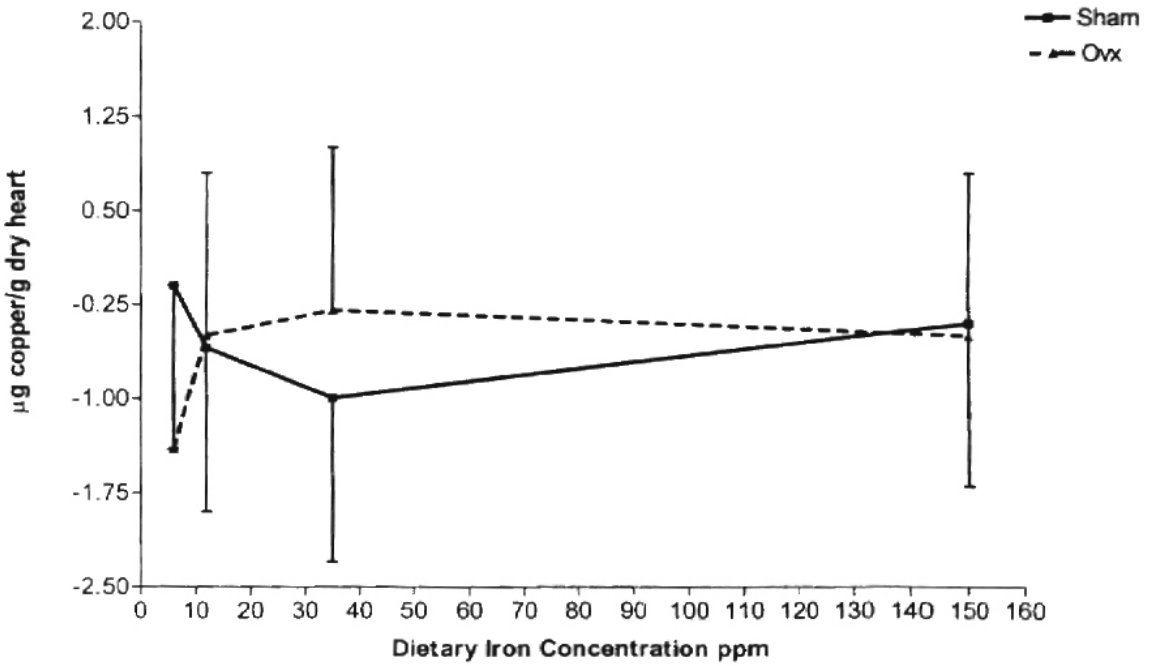
Zinc. Heart zinc concentrations decreased in all animals except the ovariectomized animals receiving the 35 ppm diet that experienced a slight increase (Figure 12). The greatest decrease in heart zinc concentrations from surgery to necropsy occurred in the ovariectomized animals receiving the 6 ppm diet (-7.6 µg/g dry tissue).

Figure 12. Change in Heart Zinc Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Non-Significant Changes in Heart Zinc Concentrations.



Copper. Heart copper concentrations decreased in all animals with the greatest loss occurring in the ovariectomized animals receiving the 6 ppm diet (-1.4µg/g dry tissue) (Figure 13). Although the changes in heart mineral concentrations were not significant, the results as a whole are suggestive of the interaction between iron, copper and zinc. Specifically, heart iron concentrations increased in all animals and the majority experienced a concomitant decrease in heart zinc and copper concentrations.

Figure 13. Change in Heart Copper Concentrations from Surgery to Necropsy in Sham-Operated and Ovariectomized Rats. Non-Significant Changes in Heart Copper Concentrations.



CHAPTER 5

SUMMARY

One hundred and twenty-four female Sprague Dawley rats were fed diets of varying levels of dietary iron to examine the effect of iron on tissue mineral concentrations at different stages of maturity. In the young mature animals, dietary iron concentration affected tissue iron concentrations, with significant differences observed in all tissues except the spleen. The concentration of iron in the spleen increased with increasing dietary iron; however, significance was not detected due to the variability of the data. Tissue copper and zinc concentrations were not significantly affected by dietary iron concentration. In comparing our study with others of similar design and purpose, we found that our study was typically longer by at least three weeks. Furthermore, these studies observed significant differences in tissue zinc and copper concentrations, whereas we did not. Our failure to observe significant differences in tissue copper and zinc concentrations due to deficient or high dietary iron intake may be a reflection of the concentrations of iron used or the effects of maturity on tissue mineral deposition.

In the sham-operated and ovariectomized animals, the most profound effects of dietary iron and ovarian hormone status were observed in the liver. As was observed in the young mature animals, dietary iron also had a significant effect on all tissue iron concentrations, except the spleen, in the sham-operated and ovariectomized animals. In fact, there were no significant differences in spleen iron, zinc, or copper concentrations due to diet, treatment, or interaction effects, and, as was the case in the young mature animals; this was likely due to the variability of the data. A facet of the purpose of

experiment two was to determine if interactions would occur between dietary iron concentrations and ovarian hormone status such that tissue mineral concentrations were affected. The only significant interaction effect was observed in liver zinc concentrations. The ovariectomized animals exhibited liver zinc concentrations less than that of the sham-operated animals, and the 150 ppm sham group exhibited the highest liver zinc concentration. The relationship between dietary iron and liver zinc concentrations in the two treatment groups were not similar. Specifically, the highest liver zinc concentration was observed in the 150 ppm sham animals, and the lowest was observed in the 12 ppm ovariectomized animals. Testing for non-linear fit described a quadratic relationship as opposed to linear between dietary iron and liver zinc concentrations, and this is reflected in the data presented in Table 15. Liver copper concentrations were significantly different due to diet and treatment effects, but an interaction was not observed. In both treatment groups, the 6 ppm diet group exhibited the greatest liver copper concentrations, but the sham-operated animals exhibited liver copper concentrations greater than that of the ovariectomized animals. In the kidney and heart, significant differences in zinc or copper concentrations were not observed, which reflects their relatively minor role in storage of these minerals. The liver is a primary iron storage site, second only to the bone marrow and followed by the spleen. It is fitting then that the primary effects of dietary iron on tissue mineral deposition were observed in the liver, and had it not been for the variability of the spleen data, significant differences in mineral concentrations may have been observed in that tissue as well.

The changes in tissue mineral concentrations from surgery to necropsy were analyzed to further assess the effects of maturity as well as ovarian hormone status. The

majority of the groups experienced increases in tissue iron concentrations from surgery to necropsy, but these changes were not significantly different due to diet, treatment, or interaction effects ($p>0.05$). These findings support our earlier suggestion that tissue iron deposition primarily occurs early in development. Significant changes in zinc and copper concentrations were only observed in the liver, with the effects of diet, treatment and interactions the same as our previous observations. The change in liver zinc concentration was different among the diet and treatment groups due to an interaction between dietary iron concentration and ovarian hormone status ($p=0.0096$). The changes in liver copper concentrations were different due to a diet and ovarian hormone status effect ($p=0.0005$ and $p=0.0041$, respectively), but an interaction was not observed. The ovariectomized animals experienced more liver zinc and copper losses than the sham-operated animals, which suggest that ovarian hormones may contribute to tissue mineral deposition.

Results of Hypothesis Testing

The following section presents the results of hypothesis testing.

1. There will be no statistically significant differences in the tissue iron concentrations of young mature rats due to varying levels of dietary iron.

Hypothesis 1 was rejected because AAS analyses showed that the iron concentration in the liver, kidney, and heart was significantly different among diet groups (Table 8). Mean liver iron concentrations of animals in the 6 and 12 ppm diet groups were lower than the mean liver iron concentrations of the animals in the 35 and 150 ppm diet groups ($p<0.0001$). Animals receiving the 6 and 12 ppm diets had mean kidney iron

concentrations lower than the animals receiving the 150 ppm diet ($p<0.005$ and $p<0.05$, respectively). Mean iron concentrations in the heart tissue of animals in the 6 ppm diet group were lower than that of the animals in the 35 and 150 ppm diet groups ($p<0.02$ and $p<0.001$, respectively).

2. There will be no statistically significant differences in tissue copper concentrations of young mature rats due to varying levels of dietary iron.

Hypothesis 2 was not rejected because AAS analyses showed that liver copper ($p<0.07$), kidney copper ($p<0.71$), heart copper ($p<0.78$), and spleen copper ($p<0.51$) were not different among diet groups (Table 8).

3. There will be no statistically significant differences in tissue zinc concentrations of young mature rats due to varying levels of dietary iron.

Hypothesis 3 was not rejected because AAS analyses showed that liver zinc ($p<0.72$), kidney zinc ($p<0.75$), heart zinc ($p<0.58$), and spleen zinc ($p<0.22$) were not different among diet groups (Table 8).

4. There will be no statistically significant interactions among iron, copper, and zinc in young mature rats due to varying levels of dietary iron.

Hypothesis 4 was not rejected because AAS analyses showed that there were no differences in tissue zinc and copper concentrations ($p>0.05$) concomitant with variable dietary iron intake (Table 8).

5. There will be no statistically significant differences in the tissue iron concentrations of mature rats due to varying levels of dietary iron.

Hypothesis 5 was rejected because AAS analyses showed that iron concentrations

of the liver, kidney, and heart were different among diet groups ($p=0.0002$, $p=0.0208$, and $p<0.0001$, respectively) (Tables 15, 17-18). Furthermore, had the spleen data been less variable, significant differences in spleen iron concentrations may have been detected as well. The liver and kidney iron concentrations of the animals receiving the 6 and 12 ppm diets were significantly less than that of the animals receiving the 35 and 150 ppm diets. The heart iron concentrations in the animals receiving the 6 ppm diet were significantly less than that of the other animals.

a. There will be no statistically significant differences in the tissue iron concentrations of the sham-operated animals due to varying levels of dietary iron.

Hypothesis 5a was rejected because AAS analyses showed that the iron concentrations of the liver, kidney, and heart of the sham-operated animals were significantly different among diet groups.

b. There will be no statistically significant differences in the tissue iron concentrations of the ovariectomized animals due to varying levels of dietary iron.

Hypothesis 5b was rejected because AAS analyses showed that the iron concentrations of the liver, kidney, and heart of the ovariectomized animals were significantly different among diet groups.

c. There will be no statistically significant differences in tissue iron concentrations between the sham-operated and ovariectomized animals.

Hypothesis 5c was not rejected because AAS analyses failed to

detect significant differences in tissue iron concentrations between the ovariectomized and sham-operated animals, which suggest that ovarian hormones, or lack thereof, do not influence tissue iron deposition.

6. There will be no statistically significant difference in tissue copper concentrations of mature rats due to varying levels of dietary iron.

Hypothesis 6 was rejected because AAS analyses showed that liver copper concentrations were different among diet groups ($p<0.0129$) (Table 15). The animals receiving the 6 ppm iron diet exhibited liver copper concentrations significantly greater than that of the animals receiving the 12 and 35 ppm diets. Significant differences in copper concentrations were not detected in the remaining tissues.

a. There will be no statistically significant differences in the tissue copper concentrations of the sham-operated animals due to varying levels of dietary iron.

Hypothesis 6a was rejected because AAS analyses showed that the liver copper concentrations of the sham-operated animals were significantly different due to dietary iron. Specifically, the animals receiving the 6 ppm iron diet exhibited liver copper concentrations significantly greater than the animals receiving the 12 and 35 ppm iron diets.

b. There will be no statistically significant differences in the tissue copper concentrations of the ovariectomized animals due to varying levels of dietary iron.

Hypothesis 6b was rejected because AAS analyses showed that the liver

copper concentrations of the ovariectomized animals were significantly different due to dietary iron. Specifically, the animals receiving the 6 ppm iron diet exhibited liver copper concentrations significantly greater than the animals receiving the 12 and 35 ppm iron diets.

c. There will be no statistically significant differences in tissue copper concentrations between the sham-operated and ovariectomized animals.

Hypothesis 6c was rejected because AAS analyses showed that the liver copper concentrations were different between treatment groups ($p=0.0041$).

7. There will be no statistically significant differences in the tissue zinc concentrations of mature rats due to varying levels of dietary iron.

Hypothesis 7 was not rejected because AAS analyses failed to detect significant differences in tissue zinc concentrations among diet groups (Tables 15-18).

a. There will be no statistically significant differences in the tissue zinc concentrations of the sham-operated animals due to varying levels of dietary iron.

Hypothesis 7a was not rejected because AAS analyses failed to detect significant differences in tissue zinc concentrations of the sham-operated animals due to dietary iron intake.

b. There will be no statistically significant differences in the tissue zinc concentrations of the ovariectomized animals due to varying levels of dietary iron.

Hypothesis 7b was not rejected because AAS analyses failed to detect

significant differences in tissue zinc concentrations of the ovariectomized animals due to dietary iron intake.

c. There will be no statistically significant differences in tissue zinc concentrations between the sham-operated and ovariectomized animals.

Hypothesis 7c was rejected because AAS analyses showed that the liver zinc concentrations of the sham-operated animals were different than that of the ovariectomized animals ($p=0.0046$) (Table 15).

8. There will be no statistically significant interactions among iron, copper, and zinc in mature rats due to varying levels of dietary iron.

a. There will be no statistically significant interactions among iron, copper, and zinc in sham-operated rats due to varying levels of dietary iron.

b. There will be no statistically significant interactions among iron, copper, and zinc in ovariectomized rats due to varying levels of dietary iron.

Hypotheses 8, 8a, and 8b were rejected because liver copper concentrations were significantly different among diet groups. Furthermore, the fact that the lowest dietary iron group exhibited the highest liver copper concentration suggests an inverse relationship between deficient dietary iron intake and liver deposition of copper. Such cannot be said for excessive dietary iron intake and liver copper deposition because the liver copper concentration of the animals receiving the 150 ppm diet was not significantly different than that of any of the other dietary treatments.

c. There will be no statistically significant interactions among iron, copper, and zinc between sham-operated and ovariectomized rats due to varying levels of dietary iron.

Hypothesis 8c was rejected because AAS analyses detected a diet and treatment interaction that affected liver zinc concentrations ($p=0.0096$).

Conclusions

Dietary iron affects tissue iron concentrations in young mature rats as well as sham-operated and ovariectomized mature rats. However, maturity and ovarian hormone status appeared to affect tissue iron accumulation. The mature rats had greater tissue iron concentrations than the young mature rats, but the amount of iron accumulated in the tissues during the last 12-weeks of the study was not as great as that accumulated during the first 15weeks. Furthermore, the tissue mineral concentrations of the sham-operated rats exceeded that of the ovariectomized rats suggesting that ovarian hormones may play a role in tissue mineral deposition.

Dietary iron had less effect on tissue zinc and copper concentrations in the young mature rats perhaps due to adaptation with the passage of time. In the mature animals, dietary iron and ovarian hormone status affected zinc and copper concentrations, but these effects were only seen in the liver, which may be attributable to its role as a primary iron storage site.

Recommendations

Recommendations for further research include the following changes in the experiment. Due to the difficulties encountered with weight gain in the sham-operated animals, the feeding methodology should be changed such that a portion of

the animals are pair-fed to control weight gain, and another portion is fed ad libitum to assess the effects of iron deficiency or overload on eating habits and growth.

Our assessment of trace mineral status was limited by tissue mineral concentrations, which are not the only means of quantifying trace mineral status. Therefore, measurement of serum mineral concentrations should be performed to further assess trace mineral status and absorption. Furthermore, zinc and copper status can be assessed by ceruloplasmin, superoxide dismutase, and metallothionein levels, and therefore such analyses should be included to examine possible effects of dietary iron on zinc and copper status. Measurement of fecal mineral concentrations would also aid in the assessment of trace mineral status by quantifying mineral excretion relative to dietary intake.

Our results suggested an effect of maturity on tissue mineral deposition, and to further examine these effects, measurements of tissue, serum, and fecal mineral concentrations should be collected during early and later stages of development. The bone marrow serves as a primary iron storage site, and analysis of the trace mineral concentration of the femur would illustrate the effects of deficient and excessive dietary iron intake on iron storage as well as interactions among iron and other minerals at the storage sites.

The results of animal testing have limited extrapolation to humans. Therefore, human studies should be performed to examine iron, copper, and zinc indices in pre- and post-menopausal women, or post menopausal women on hormone replacement therapy versus those who are not, to assess the role of ovarian hormones in the metabolism of these minerals.

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APPENDIX A
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL

OKLAHOMA STATE UNIVERSITY

OSU

College of Veterinary Medicine
Laboratory Animal Resources Unit
Stillwater, Oklahoma 74078-2007
405-744-7631

Memorandum

DATE: February 3, 1998

TO: Dr. Andrea Arquitt
Nutritional Sciences

FROM: Dr. K. Vargas
IACUC Veterinarian

SUBJECT: Protocol Approval

Your protocol, #709, entitled "Effects of Iron on Bone in Growing and in Mature Rats", has been approved for 108 rats by the Institutional Animal Care and Use Committee. The protocol is approved through January 31, 2001.

A modification must be submitted to the committee for approval prior to any changes in the protocol.

Institutional Assurance number A3722-01

The Campaign for OSU
Bringing Dreams to Life

OKLAHOMA STATE UNIVERSITY

OSU

College of Veterinary Medicine
Laboratory Animal Resources Unit
Stillwater, Oklahoma 74078-2002
405-744-7631

Memorandum

DATE: August 21, 1998

TO: Dr. Andrea Arquitt
Nutritional Sciences

FROM: Dr. Archie Clutter
IACUC Chairman

SUBJECT: Modification Approval

The modification to protocol, #709, entitled "Effects of Iron on Bone in Growing and in Mature Rats", for addition of 12 rats has been approved by the Institutional Animal Care and Use Committee.

dgm

Bringing Dreams to Life
The Campaign for OSU

APPENDIX B

DIET PREPARATION

A lab coat and mineral free gloves were worn during all stages of diet preparation to prevent environmental mineral contamination. The diet was prepared in five-kilogram batches. Prior to preparation, the cornstarch, casein, maltodextrin, sucrose, cellulose, L-cystine, and choline were weighed in the amounts appropriate for five-kilograms of diet and stored in plastic bags. The weighed cornstarch, casein, cellulose, maltodextrin, and sucrose were stored in sealed cardboard tubs at room temperature. The weighed L-Cystine and Choline were stored in sealed plastic bags and refrigerated. The soybean oil, vitamin mix, and mineral mix were weighed as each batch of diet was prepared. The soybean oil was stored in the freezer, the vitamin mix was refrigerated, and, as previously stated, the mineral mix was stored at room temperature.

The utensils used in preparation of the diet were soaked in 10 % HCl for 24 hours, rinsed with deionized water, and dried with Kim-wipes prior to use. The mixing bowls were cleaned and rinsed with deionized water and allowed to air-dry in the diet mixing laboratory prior to use.

The lowest iron diet was prepared first, followed by the 12ppm, 35ppm, and 150ppm iron diets, in that order. The mixing bowls and utensils were cleaned with deionized water and Kim-wipes between preparations of the different diets.

The procedure for preparing the diet is as follows:

Using a small bowl:

1. Thoroughly combine the vitamin mix, L-cystine, choline, sugar, approximately half of the maltodextrin, and approximately 1 cup of casein manually in a small mixing bowl.

2. Form a well in the dry mixture and add two tablespoons of soybean oil. Cover the oil with a spoonful of the dry mixture. This will aid in even distribution of the oil throughout the dry mixture.
3. Cover the mixer with a plastic bag to prevent contamination and minimize loss. Set the mixer at the lowest speed for approximately five minutes.
4. Manually fold the mineral mix into this mixture and combine again at the lowest speed for another five minutes.

Using a large bowl:

1. Manually combine the cornstarch, fiber, casein, and remaining maltodextrin in a large mixing bowl.
2. Form a well in the dry ingredients and add approximately half of the remaining soybean oil. Cover the oil with a spoonful of the dry mixture. This will aid in even distribution of oil throughout the dry mixture.
3. Combine in a covered mixer on the lowest speed for five minutes.
4. Form a well in the mixture and add the remaining oil. Cover the oil, as in step 6, and mix on the lowest setting for five minutes.

Final mixing:

1. Add the vitamin-mineral mixture from the small mixing bowl to the cornstarch mixture in the large mixing bowl. Mix the diet at a moderate speed until it is of a uniform consistency (approximately15-20 minutes).
2. Store the final product in labeled plastic bags in the refrigerator.

APPENDIX C

ASHING PROCEDURES FOR DIET

Type I (millipore) water, double distilled ultra pure nitric acid (GFS Chemical) and 30% ultrex hydrogen peroxide (Baker) were the reagents used to digest the organic material of all the diet samples. Every cycle of wet ashing was followed by a cycle of dry ashing. All diet samples were dry ashed in a Lindberg 847 ashing oven. The specific program used for the diet is detailed in the appropriate section.

Wet Ashing

1. Insert the test tubes into the wells of the heating block.
2. Add 500µL (.5mL) each of Type 1 water, hydrogen peroxide, and nitric acid, in that sequence, to the test tubes. Set the temperature at 80°C.
3. After 15 minutes, increase temperature to 90°C.
4. After 15 minutes, add 500µL of hydrogen peroxide and increase temperature to 100°C.
5. After 30 minutes, add 500µL of hydrogen peroxide and increase temperature to 105°C.
6. After 15 minutes, add 500µL hydrogen peroxide.
7. Twenty-four hours later, add 500µL hydrogen peroxide.
8. After 30 minutes, add 500µL hydrogen peroxide.
9. After 30 minutes, add 500µL hydrogen peroxide.
10. Forty-eight hours later, add 500µL each of Type 1 water, hydrogen peroxide, and nitric acid.
11. Continue adding hydrogen peroxide until samples appeared white. When completely dry, place test tubes in acid washed beaker for dry ashing.

Dry Ash Cycle

The program used to dry ash the diet samples was as follows:

Parameter	**Setting**
Prog	idle
SP	100
Tune	off

Ramp Program Values

Parameter	Setting
LC	1
Ramp#1 (r1-°C/min)	1
First Soak temp (L1-°C)	375
First Soak Time (d1-minutes)	2880
Ramp#2 (r2-°C/min)	5
Second Soak Temp (L2-°C)	100
Second Soak Time (d2-minutes)	0
Holdback (Hb)	25

Prop	20
Int.t	120
DEr.t	30

The oven was programmed as detailed above and then tuned. Once the oven was tuned, the "Prog" parameter was adjusted to "run".

Continue wet/dry ash cycle until diet ash completely white. Weigh test tubes, cover with parafilm, and store in sealed plastic bag until time for min

APPENDIX D

ASHING PROCEDURES FOR LIVER, KIDNEY, SPLEEN, AND HEART

Type I (millipore) water, double distilled ultra pure nitric acid (GFS Chemical) and 30% ultrex hydrogen peroxide (Baker) were the reagents used to digest the organic material of all tissue samples. Every cycle of wet ashing was followed by a cycle of dry ashing. All tissue samples were dry ashed in a Lindberg 847 dry ashing oven. The specific programs used for each tissue are detailed in the appropriate section.

Liver

After the liver samples had been dried for 24 hours at 100°C and the dry weight recorded, the following procedures were used to ash the samples.

Wet Ash – Initial Cycle

1. Insert the test tubes into the wells of the heating block.
2. Add 50 µL each of Type I water, hydrogen peroxide, and nitric acid, in that sequence, to the test tubes. Set the temperature at 80°C.
3. After 15 minutes, increase the temperature to 90°C.
4. After 15 minutes, add 50µL of hydrogen peroxide and increase the temperature to 100°C.
5. After 30 minutes, add 50 µL of hydrogen peroxide and increase the temperature to 105°C.
6. After 15 minutes, add 50 µL of hydrogen peroxide.
7. After 30 minutes, add 50 µL hydrogen peroxide.
8. After 30 minutes, add 50 µL hydrogen peroxide and allow the samples to dry.
9. When all samples are completely dry, place the test tubes in an acid washed beaker for dry ashing.

Dry Ash Cycle

The program used to dry ash the liver samples was as follows:

Parameter	Setting
Prog	idle
SP	100
Tune	off

Ramp Program Values

Parameter	Setting
LC	1
Ramp#1 (r1-°C/min)	0.25
First Soak temp (L1-°C)	275
First Soak Time (d1-minutes)	600
Ramp#2 (r2-°C/min)	0.5
Second Soak Temp (L2-°C)	375
Second Soak Time (d2-minutes)	1440
Holdback (Hb)	25

Prop	20
Int.t	120
DEr.t	30

The oven was programmed as detailed above and then tuned. Once the oven was tuned, the "Prog" parameter was adjusted to "run".

Wet Ash - Subsequent Cycles

1. Insert the test tubes into the wells of the heating block.
2. Add 100 µL of Type I water, hydrogen peroxide, and nitric acid, in that sequence, and set the temperature at 50°C.
3. After 15 minutes, increase temperature to 70°C.

4. After 15 minutes, add 100 μL each Type I water, hydrogen peroxide, and nitric acid and increase temperature to 90°C. Repeat this step, but increase the temperature to 105°C.
5. Periodically thereafter, continue adding 100 μL each of Type I water, hydrogen peroxide, and nitric acid until the solution in the test tube becomes clear. When the solution becomes clear, allow the samples to dry.
6. When the samples are completely dry, repeat the dry ashing procedure described previously.
7. Continue the wet and dry ashing cycles until the ash in the test tubes becomes white.
8. When the samples are completely ashed, record the ash weights, cap the tubes, and place them in a test tube rack. Store the rack in a labeled, sealed plastic bag until analysis.

Kidney

After the kidney samples had been dried for 24 hours at 100°C and the dry weight recorded, the following procedures were used to ash the samples.

Wet Ash – Initial Cycle

1. Insert the test tubes into the well of the heating block.
2. Add 50 μL each Type I water, hydrogen peroxide, and nitric acid, in that sequence, to the test tubes. Set the temperature at 65°C.
3. After a minimum of 30 minutes, add 50 μL each of Type I water, hydrogen peroxide, and nitric acid.

4. After a minimum of 30 minutes, add 50 μL each Type I water, hydrogen peroxide, and nitric acid. Increase the temperature to 75°C. Allow the samples to sit overnight.
5. Add 100 μL each Type I water, hydrogen peroxide, and nitric acid. Increase the temperature to 85°C.
6. After a minimum of 30 minutes, add 100 μL hydrogen peroxide. Increase temperature to 95°C. Repeat this step, but increase the temperature to 105°C after adding the hydrogen peroxide.
7. Continue adding 100 μL hydrogen peroxide periodically until the solution in the test tube becomes clear. Then allow the samples to dry. If, after a significant amount of time has elapsed between additions and the samples dry before becoming clear, add 100 μL each of Type I water, hydrogen peroxide, and nitric acid. Then continue with the periodic additions of 100 μL of hydrogen peroxide.
8. After the samples have completely dried, place the test tubes in an acid washed beaker for dry ashing.

Dry Ash Cycle

The program used to dry ash the kidney samples was as follows:

Parameter	**Setting**
Prog	idle
SP	100
Tune	off

Ramp Program Values

Parameter	Setting
LC	1
Ramp#1 (r1-°C/min)	1
First Soak temp (L1-°C)	375
First Soak Time (d1-minutes)	2880
Ramp#2 (r2-°C/min)	5
Second Soak Temp (L2-°C)	100
Second Soak Time (d2-minutes)	0
Holdback (Hb)	25

Prop	20
Int.t	120
DEr.t	30

The oven was programmed as detailed above and then tuned. Once the oven was tuned, the "Prog" parameter was adjusted to "run".

Wet Ash - Subsequent Cycles

1. Insert the test tubes into the wells of the heating block.
2. Add 100 µL each Type I water, hydrogen peroxide, and nitric acid. Set temperature at 85°C.
3. After a one-hour, add 100 µL hydrogen peroxide and increase temperature to 95°C. Repeat this step, but increase the temperature to 105°C after adding the hydrogen peroxide.

4. Continue adding 100 μL hydrogen peroxide periodically until the solution in the test tube becomes clear. Then allow the samples to dry. If, after a significant amount of time has elapsed between additions and the samples dry before becoming clear, add 100 μL each of Type I water, hydrogen peroxide, and nitric acid. Then continue with the periodic additions of 100 μL of hydrogen peroxide.
5. After the samples have completely dried, place the test tubes in an acid washed beaker for dry ashing. Repeat the dry ashing procedure described previously.
6. Continue the wet and dry ashing cycles until the ash in the test tubes becomes white.
7. When the samples are completely ashed, record the ash weights, place the test tubes in a rack, and cover with parafilm. Store the rack in a labeled, sealed plastic bag until all the kidney samples are ashed.

Spleen

After the spleen samples had been dried for 24 hours at 100°C and the dry weight recorded, the following procedures were used to ash the samples.

Wet Ash – Initial Cycle

1. Insert the test tubes into the well of the heating block.
2. Add 50 μL each Type I water, hydrogen peroxide, and nitric acid, in that sequence, to the test tubes. Set the temperature at 65°C.
3. After a minimum of 30 minutes, add 50 μL each of Type I water, hydrogen peroxide, and nitric acid.

4. After a minimum of 30 minutes, add 50 μL each Type I water, hydrogen peroxide, and nitric acid. Increase the temperature to 75°C. Allow the samples to sit overnight.
5. Add 100 μL each Type I water, hydrogen peroxide, and nitric acid. Increase the temperature to 85°C.
6. After a minimum of 30 minutes, add 100 μL hydrogen peroxide. Increase temperature to 95°C. Repeat this step, but increase the temperature to 105°C after adding the hydrogen peroxide.
7. Continue adding 100 μL hydrogen peroxide periodically until the solution in the test tube becomes clear. Then allow the samples to dry. If, after a significant amount of time has elapsed between additions and the samples dry before becoming clear, add 100 μL each of Type I water, hydrogen peroxide, and nitric acid. Then continue with the periodic additions of 100 μL of hydrogen peroxide.
8. After the samples have completely dried, place the test tubes in an acid washed beaker for dry ashing.

Dry Ash Cycle

The program used to dry ash the spleen was the same as that used to dry as the kidney samples.

Wet Ash - Subsequent Cycles

1. Insert the test tubes into the wells of the heating block.
2. Add 50 μL each of Type I water, hydrogen peroxide, and nitric acid. Set the temperature at 85°C.
3. After one hour, add 50 μL each Type I water, hydrogen peroxide, and nitric acid and increase the temperature to 95°C.
4. After a minimum of one hour, add 100 μL each Type I water, hydrogen peroxide, and nitric acid and increase the temperature to 105°C.
5. Continue adding 100 μL hydrogen peroxide periodically until the solution in the test tube becomes clear. Then allow the samples to dry. If a significant amount of time has elapsed between additions and the samples dry before becoming clear, add 100 μL each of Type I water, hydrogen peroxide, and nitric acid. Then continue with the periodic additions of 100 μL of hydrogen peroxide.
6. After the samples have completely dried, place the test tubes in an acid washed beaker for dry ashing. Repeat the dry ashing procedure described previously.
7. Continue the wet and dry ashing cycles until the ash in the test tubes becomes white.
8. When the samples are completely ashed, record the ash weights, place the test tubes in a rack, and cover with parafilm. Store the rack in a labeled, sealed plastic bag until analysis

Heart

After the heart samples had been dried for 24 hours at 100°C and the dry weight recorded, the following procedures were used to ash the samples.

Wet Ash – Initial Cycle

1. Insert the test tubes into the wells of the heating block.
2. Add 50 μL each Type I water, hydrogen peroxide, and nitric acid. Set the temperature at 65°C.
3. After a minimum of one hour, add 50 μL each Type I water, hydrogen peroxide, and nitric acid.
4. After a minimum of one hour, add 50 μL each Type I water, hydrogen peroxide, and nitric acid. Increase the temperature to 75°C.
5. After 45 minutes, add 100 μL each Type I water, hydrogen peroxide, and nitric acid. Increase the temperature to 85°C.
6. After a minimum of one hour, add 100 μL Type I water, hydrogen peroxide, and nitric acid. Increase the temperature to 95°C. Repeat this step, but increase the temperature to 105°C.
7. After a minimum of one hour, add 100 μL hydrogen peroxide. Repeat. Allow the samples to sit overnight.
8. Add 100 μL each Type I water, hydrogen peroxide, and nitric acid.
9. After one hour, add 100 μL hydrogen peroxide. Allow the samples to sit overnight.
10. Add 100 μL each Type I water, hydrogen peroxide, and nitric acid.
11. After a minimum of 20 minutes, add 100 μL hydrogen peroxide. Allow the samples to sit overnight.

12. Add 100 µL hydrogen peroxide. Allow the samples to dry. When the samples are completely dry, place the test tubes in an acid washed beaker and proceed with dry ashing.

Dry Ash Cycle

The program used to dry ash the heart was the same as that used to dry ash the kidney and spleen samples.

Wet Ash – Subsequent Cycles

1. Insert the test tubes into the wells of a cold heating block. Add 100µL nitric acid. Allow the samples to sit overnight at room temperature.
2. Set the temperature at 55°C.
3. After a minimum of 30 minutes, add 100µL nitric acid and increase the temperature to 60°C.
4. After a minimum of one hour, increase the temperature to 65°C.
5. After a minimum of one hour, increase the temperature to 70°C.
6. After a minimum of one hour, add 100µL nitric acid and increase the temperature to 75°C.
7. Periodically thereafter (approximately every 30-60 minutes) increase the temperature in 5°C increments until reaching a final temperature of 105°C. Then allow the samples to dry. Repeat the dry ashing procedure.
8. Continue the wet and dry ashing cycles until the ash in the test tubes becomes white.

9. When the samples are completely ashed, record the ash weights, place the test tubes in a rack, and cover with parafilm. Store the rack in a labeled, sealed plastic bag until analysis.

APPENDIX E

FLAME AND FURNACE SETTINGS FOR TRACE MINERAL ANALYSES

FLAME SETTINGS FOR TRACE MINERAL ANALYSIS[1,2]
NON-LINEAR mg/L

	Iron	Zinc	Copper	Calcium
Wavelength (nm)	248.3	213.9	324.8	422.7
Slit Width	0.2	0.7	0.7	0.7
Fuel Flow (L/min)	2.0	2.0	2.0	3.8
Calib. Std. 1	1.00	0.25	0.50	1.0
Calib. Std. 2	2.50	0.50	1.00	2.5
Calib. Std. 3	5.00	1.00	2.00	5.0
Calib. Std. 4	NA	2.0	4.00	10.0
Calib. Std. 5	NA	4.00	NA	NA

[1]Flame Type: Air/C2H2
[2]Oxidant Flow (L/min): 10.0

FURNACE SETTING FOR COPPER ANALYSIS
NON-LINEAR µg/L

Wavelength (nm)	324.8
Signal Type	Zeeman AA
Calib. Std. 1	10 ppb
Calib. Std. 2	20 ppb

APPENDIX F

CHANGE IN TISSUE MINERAL CONCENTRATIONS FROM SURGERY TO NECROPSY

TABLE 1

THE CHANGE IN LIVER AND SPLEEN MINERAL CONCENTRATIONS FROM SURGERY TO NECROPSY

	Liver Fe	Liver Zn	Liver Cu	Spleen Fe	Spleen Zn	Spleen Cu
Sham-operated						
6 ppm	44.8±261.8[a,c]	6.0±8.4	-10.1±4.6[a]	272.3±998.4	-0.6±3.9	0.3±0.9
12 ppm	314.2±261.8[a,c]	3.6±8.4	-9.4±4.6[a]	1730.7±1193.3	1.7±3.9	-0.005±0.9
35 ppm	186.0±266.7[b,c]	-19.7±8.5	-4.0±4.8[b]	222.7±1288.9	4.6±4.0	-0.4±0.9
150 ppm	506.4±258.2[a,b]	11.4±8.3	15.3±4.5[c]	2543.8±1052.4	-4.4±3.9	-0.5±0.9
Ovariectomized						
6 ppm	54.4±258.2[c]	2.2±8.3	-29.5±4.5[a]	241.0±951.9	0.7±3.9	-0.7±0.9
12 ppm	337.3±266.7[c]	-10.3±8.4	-15.9±4.6[a]	1781.5±998.4	7.2±3.9	0.5±0.9
35 ppm	52.3±261.8[c]	-14.1±8.4	-4.7±4.6[b]	-516.5±1193.3	8.0±3.9	-0.1±0.9
150 ppm	-193.5±266.8[c]	-10.2±8.5	0.2±4.8[c]	792.9±1052.4	-0.08±4.4	-0.2±0.9
Diet	p=0.7172	p<0.0001	p=0.0005	p=0.6182	p=0.0519	p=0.3632
Trt	p=0.2850	p=0.0046	p=0.0041	p=0.3862	p=0.0974	p=0.8714
Diet*Trt	p=0.4571	p=0.0096	p=0.1162	p=0.6743	p=0.8698	p=0.1310

[1] Tukey-Kramer adjusted LS Means ± SE

TABLE 2

THE CHANGE IN KIDNEY AND HEART MINERAL CONCENTRATIONS FROM SURGERY TO NECROPSY

	Kidney Fe	Kidney Zn	Kidney Cu	Kidney Ca	Heart Fe	Heart Zn	Heart Cu
Sham-operated							
6 ppm	87.0 ± 290.2	6.5 ± 10.8	5.4 ± 15.0	-12.3 ± 19.3[a]	29.8 ± 18.6	-2.5 ± 3.6	-0.1 ± 1.3
12 ppm	448.3 ± 290.2	2.7 ± 10.8	7.7 ± 15.0	-18.6 ± 19.3[a]	13.1 ± 18.6	-1.8 ± 3.6	-0.6 ± 1.3
35 ppm	956.3 ± 305.9	1.4 ± 11.0	10.3 + 15.3	-42.3 ± 20.3[a]	9.4 + 19.3	-2.9 ± 3.8	-1.0 ± 1.3
150 ppm	418.5 ± 276.7	5.8 ± 10.6	-5.3 ± 14.7	-12.2 ± 18.4[a]	29.6 ± 18	-5.4 ± 3.5	-0.4 ± 1.3
Ovariectomized							
6 ppm	80.5 ± 290.2	-2.9 ± 10.8	-14.4 ± 14.7	-35.7 ± 18.4[a]	11.9 ± 18	-7.6 ± 3.5	-1.4 ± 1.3
12 ppm	-14.3 ± 290.2	-10.8 ± 10.8	-14.9 ± 15.0	-0.1 ± 19.[3a,b]	27.3 ± 18.6	-2.4 ± 3.6	-0.5 ± 1.3
35 ppm	407.1 ± 290.2	2.7 ± 10.8	1.1 ± 15.0	-22.1 ± 19.[3a,b]	28.5 ± 18.6	0.8 ± 3.6	-3.0 ± 1.3
150 ppm	10.9 ± 305.9	10.3 ± 11.0	-1.1 ± 15.3	54.4 ± 20.3[b]	18.3 ± 20.1	-6.5 ± 3.8	-0.5 ± 1.3
Diet	p=0.2036	p=0.3105	p=0.7964	p=0.0405	p=0.9907	p=0.4104	p=0.9772
Trt	p=0.0893	p=0.3451	p=0.1864	p=0.1382	p=0.9258	p=0.7308	p=0.7233
Diet*Trt	p=0.7969	p=0.4372	p=0.6533	p=0.1467	p=0.5523	p=0.6302	p=0.6427

[1] Tukey-Kramer adjusted LS Means ± SE

VITA

Mary Josephine M. Fisher

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF VARYING LEVELS OF DIETARY IRON ON TISSUE CONCENTRATIONS OF IRON, COPPER, AND ZINC IN FEMALE RATS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Pottsville, Pennsylvania, On January 23, 1976, the daughter of Robert and Carole Fisher.

Education: Graduated from Muskogee High School, Muskogee, Oklahoma in May 1994; received Bachelor of Science degree in Human Environmental Sciences from Oklahoma State University, Stillwater, Oklahoma in May 1994. Completed the requirements for the Master of Science degree with a major in Human Nutrition at Oklahoma State University in July, 2003.

Experience: Employed by Oklahoma State University, Department of Nutritional Sciences as a undergraduate research assistant; Oklahoma State University, Department of Nutritional Sciences, January 1995 to January 1997 and June 1998 to May 1999. Employed by Oklahoma State University, Department of Nutritional Sciences as a graduate research assistant; Oklahoma State University, Department of Nutritional Sciences, August 1999 to January 2001. Employed by Oklahoma State University, Department of Nutritional Sciences as a graduate teaching assistant; Oklahoma State University, Department of Nutritional Sciences, August 2000 to December 2000. Employed by Scott & White Hospital, Temple, Texas as a renal dietitian; Scott & White Hospital, Departments of Nephrology and Transplant Surgery, April 2001 to present.