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Dietary proteins that influence nonheme iron bioavailability, iron status, and plasma total antioxidant status

by

James Howard Swain

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Nutrition

Major Professor: Manju B. Reddy

Iowa State University

Ames, Iowa

2000

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For the Graduate College

TABLE OF CONTENTS

ACKNOWLEDGEMENTS vi

ABSTRACT vii

CHAPTER 1. GENERAL INTRODUCTION 1
- Dissertation Organization 1
- Introduction 2
  - Study I 2
  - Study II 3
- Review of Literature 5
  - Importance of Iron 5
  - Body Iron Distribution 6
  - Iron Requirements 7
  - Iron Deficiency Anemia 8
    - Prevalence of Iron Deficiency 8
    - Consequences of Iron Deficiency 9
  - Iron Excess 10
    - The Hfe Protein and Hereditary Hemochromatosis 11
    - Consequences of Excess Iron 12
    - Age-Related Iron Excess 12
    - Iron Excess and Oxidative Stress 13
  - Iron Status 16
    - Storage Iron Indices 16
    - Transport Iron Indices 18
    - Red Blood Cell Indices 18
    - Models Used in the Assessment of Iron Status 20
  - Iron Bioavailability 20
    - Heme and Nonheme Iron 21
    - Iron Absorption and Transport 23
  - Factors Affecting Nonheme Iron Bioavailability 25
    - Ascorbic Acid 25
    - Meat 26
    - Phenolic Compounds 28
    - Phytic Acid 29
    - Calcium 30
  - Methods of Assessing Nonheme Iron Bioavailability 31
- Literature Cited 33

CHAPTER 2. ISOLATION AND CHARACTERIZATION OF BEEF PROTEINS THAT ENHANCE NONHEME IRON BIOAVAILABILITY 49
- Abstract 49

Introduction 50
Materials and Methods 52
Sample Preparation 53
In Vitro Digestion 53
Ultrafiltration 54
Immobilized Metal Affinity Chromatography (IMAC) 54
Trace Metal Removal 55
Concentration and Desalting 55
Cell Culture 55
^{59}Fe Cell Uptake Procedures 57
Solubility Assays 58
Electrophoresis 58
Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) 59
Amino Acid Composition Analysis 59
Statistical Analysis 60
Results 60
Discussion 62
Acknowledgements 66
Literature Cited 67

CHAPTER 3. IRON INDICES AND TOTAL ANTIOXIDANT STATUS IN RESPONSE TO SOY PROTEIN INTAKE IN PERIMENOPAUSAL WOMEN 80
Abstract 80
Introduction 81
Subjects and Methods 83
Research design and treatment 83
Data collection 84
Statistical analyses 86
Results 87
Compliance 87
Baseline measures 87
Iron indices and antioxidant status 88
Discussion 89
References 93

CHAPTER 4. GENERAL CONCLUSIONS 105

APPENDIX A. ELECTRON MICROSCOPY 107

APPENDIX B. EFFECT OF TRITON X-100 ON THE YIELD OF SOLUBLE PROTEIN 112

APPENDIX C. SIZE-EXCLUSION CHROMATOGRAPHY 114

APPENDIX D. EFFECT OF 2-(4-MORPHOLINO)-ETHANE SULFONIC ACID (MES) ON RADIOIRON UPTAKE AND TRANSPORT BY CACO-2 CELLS 116

APPENDIX E. MATRIX ASSISTED LASER DESORPTION IONIZATION-TIME OF FLIGHT (MALDI-TOF) ANALYSIS: THE IMMOBILIZED METAL CHELATE AFFINITY (IMAC) WASH 118

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ABSTRACT

The objective of the first was to isolate and characterize beef muscle proteins that enhance nonheme iron bioavailability. Beef sirloin was cooked, lyophilized and reconstituted with water prior to in vitro digestion. Following centrifugation, the digest supernatant was sequentially ultrafiltered using 10K and 1K molecular weight cut-off membranes. Nonheme iron bioavailability was assessed by Caco-2 cell monolayer ^{59}Fe uptake using an extrinsic labeling method. Cell iron uptake was 45.2% and 56.5% higher ($p<0.05$) with the 1-kDa retentate (1KR) as compared to the 10-kDa retentate and 1-kDa filtrate, respectively, and 2- to 5-fold greater than the blank ($p<0.05$) and iron-nitrilotriacetic acid (NTA) ($p<0.001$), respectively. Thus, the 1KR was chosen for further analysis. Immobilized metal affinity chromatography of the 1KR yielded a series of four peaks: three distinct peaks (P1, P3, P4) and one with a few closely associated peaks (P2). Peptides in P1-P4 increased ($p<0.001$) iron solubility at pH 6 as compared to the blank; 2.1, 3.6, 4.7 and 4.4-fold, respectively. When compared to NTA, P1-P4 were found to enhance ^{59}Fe uptake by 3- to 5-fold ($p<0.01$). Iron uptake with P2 and P4 was also significantly greater than the blank ($p<0.05$). Gel electrophoresis illustrated that P1-P4 each contained many peptides ranging from 1- to 5-kDa. Matrix Assisted Laser Desorption Ionization-Time of Flight analysis confirmed these findings. Amino acid composition analysis revealed that histidine concentration increased progressively in P1-P4. Our results suggest that enhancement of nonheme iron absorption by beef may be due to peptides produced during gastrointestinal digestion and that histidine content may be important.

The objective of the second study was to determine the effect of soy protein isolate (SPI) intake on iron indices and plasma total antioxidant status (TAS), and determine the influence of other factors on TAS in perimenopausal women. Perimenopausal women (N=69) were randomly assigned (double-blind) to treatment: isoflavone-rich soy protein isolate (SPI+; n=24), isoflavone-poor soy protein isolate (SPI-; n=24), or whey protein (control; n=21). Each subject consumed 40 g soy or whey protein/d for 24 weeks. Plasma TAS, serum ferritin, serum iron, transferrin saturation, and hemoglobin were measured at baseline, week 12, and week 24. Treatment per se had no significant effect on serum ferritin, serum iron, and transferrin saturation. Time had an effect on serum ferritin ($P \leq 0.0001$) and TAS ($P \leq 0.0001$). At week 12, TAS was lower ($P=0.035$) in controls than SPI+, whereas no significant differences were found between SPI+ and SPI- at week 12 or 24. Multiple regression analysis revealed that at week 12, baseline TAS, alcohol intake, soy intake (soy versus control; $P=0.016$), plasma Lp(a), and dietary iron contributed to the variability (48%; $P \leq 0.0001$) in TAS. At week 24, 46% of the variability in TAS was contributed by baseline TAS, dietary meat, fish, and poultry (MFP) and zinc, serum ferritin and serum estrone. SPI consumption had no significant effect on iron status, but our results suggest that soy intake and decreased iron stores may protect from oxidative stress in perimenopausal women.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

Two papers are included in this dissertation. The first paper entitled "Isolation and characterization of beef proteins that enhance nonheme iron bioavailability" presents data suggesting that the enhancing effect of beef on nonheme iron absorption may be due to low-molecular weight peptides released during proteolytic digestion and that histidine may be an important amino acid. The second paper, "Iron indices and total antioxidant status in response to soy protein intake in perimenopausal women" was a subanalysis of a larger study designed to determine the effect of isoflavone-rich soy protein consumption on bone health of perimenopausal women. This study investigated whether consuming soy protein isolate over a period of six months altered the iron status and total antioxidant status of the perimenopausal women. The first paper will be submitted to the Journal of Nutrition and the second paper will be submitted to the American Journal of Clinical Nutrition for publication. Each paper presented in this dissertation follows the format requirements of its respective journal. Preceding the papers is a general introduction, which includes objectives, hypotheses, specific aims, and significance of the two proposed studies, followed by a review of pertinent literature. The papers are followed by a general conclusion, which summarizes findings from both studies. Literature cited in the general introduction and the review of literature are listed alphabetically and follow the review. Literature cited in the two papers is listed according to the format of the respective journal at the end of each paper.

Introduction

Study I

Hypothesis: The positive effect of beef on nonheme iron absorption is due to low molecular weight proteins released during proteolytic digestion, which increase the solubility of inorganic iron and enhance its uptake by the intestinal mucosa.

Objective and Specific Aims: The objective of this study was to isolate and characterize beef proteins that enhance nonheme iron bioavailability by:

1. Fractionating proteins in the digest supernatant using sequential ultrafiltration and immobilized metal affinity chromatography.
2. Assessing the influence of these protein fractions on radioiron solubility and uptake by Caco-2 cells
3. Determining the molecular weight and amino acid composition of these proteins.

Significance of Proposed Study: Unlike heme iron, the bioavailability of nonheme iron is influenced by various dietary components ingested simultaneously (Cook and Monsen 1977, Reddy et al. 1996). Meat is one of the known enhancing dietary factors on nonheme iron absorption. The positive influence of meat on nonheme iron absorption in humans was reported more than three decades ago (Layrisse et al. 1969, Bjorn-Rasmussen and Hallberg 1979). There is also evidence that the greater prevalence of iron deficiency in developing

countries is due to low meat intake, despite a high nonheme iron content of the diet (Hallberg and Rossander 1984, Gillespie and Johnston 1998). By identifying proteins in beef that enhance nonheme iron bioavailability, it may be possible to genetically engineer plant foods to contain these peptides to improve nonheme iron absorption and also to synthsize these peptides for use as food fortificants.

Study II

Hypothesis: Consumption of soy protein isolate during the menopausal transition attenuates menopause-associated increases in iron stores, thereby increasing antioxidant status in perimenopausal women.

Objective and Specific Aims: To determine the effect of soy protein intake on iron indices and plasma total antioxidant status and determine the influence of other factors on antioxidant status in perimenopausal women by assessing:

1. Changes in iron status indices, such as serum ferritin, serum iron, transferrin saturation, total iron binding capacity, hemoglobin, and hematocrit.
2. Changes in plasma total antioxidant status.

Significance of Proposed Study: Ample information is available on the iron status of pre- and postmenopausal women, but the change in iron status that occurs during the menopausal transition, which may last for several years, has not been studied. In addition,

the effect of changes in body iron stores on the total antioxidant status of women during the menopausal transition is also not known. Oxidative status typically decreases with age (Jayachandran et al. 1996), whereas body iron stores generally increase, especially in women after menopause (Berge et al. 1994). High iron stores have been hypothesized to induce oxidative stress due to the ability of iron to catalyze the Haber-Weiss reaction, which produces the hydroxyl radical. This radical is highly reactive with all biomolecules and is considered to be very toxic, causing structural damage to macromolecules, such as proteins and lipids and breakage of DNA strands (Santanam et al. 1998). Generation of this radical in excess may deplete antioxidants (McCord 1993). This disturbance in the equilibrium between prooxidants and antioxidants may contribute to diseases, such as heart disease and cancer. Since epidemiological evidence suggests that increased iron stores place one at risk for developing heart disease (Salonen 1993, Kiechl et al. 1994) and cancer (Nelson et al. 1994), postmenopausal women may be at greater risk, similar to men (Sullivan 1981). Antioxidant defense systems, which include enzymes, plasma proteins, and dietary factors (Thomas 1999), react with radicals and minimize their damage (Halliwell 1993). These defense systems may be compromised during and after menopause and the concomitant increase in iron stores may increase such oxidative damage (Berliner and Heinecke 1996, Regnström et al. 1994).

Soy protein isolate (SPI) is used extensively by the food industry, where it is incorporated into a variety of processed foods (Erdman and Fordyce 1989). Soy protein isolate is known to markedly inhibit nonheme iron absorption (Cook et al 1981, Gillooly et al. 1983) primarily because of its high phytic acid content (Hurrell et al. 1992) and the protein moeity (Lynch et al. 1994). Thus, diets poor in animal proteins that enhance iron

absorption and diets rich in soy may be inadequate for maintaining optimal iron status in populations at risk for developing iron deficiency, but beneficial to groups at risk of developing excess iron stores. In addition, naturally occurring isoflavones in soy may have antioxidant properties because of their ability to donate hydrogen atoms and/or electrons from their hydroxyl groups to free radicals, making them less reactive (Mitchell et al. 1998). However, the ability of these isoflavones in vivo to affect total antioxidant status during menopause has not been documented. Our findings may illustrate that the consumption of SPI during the menopausal transition attenuates menopause-associated increases in iron stores and thus, provides a strategy to optimize total antioxidant status in perimenopausal women.

Review of Literature

Importance of Iron

Iron is an essential mineral for human growth and development because it takes part in an array of biochemical processes vital in maintaining normal cellular function. Biochemical processes involving iron include electron transport (cytochromes, iron-sulfur proteins), handling of molecular oxygen (peroxidase, catalase), oxygen transport and storage (hemoglobin and myoglobin, respectively), porphyrin metabolism, collagen synthesis, lymphocyte and granulocyte function, and neurotransmitter anabolism and catabolism (Cammack et al. 1990, Pollitt and Leibel 1976).

Body iron commonly exists in two oxidative states, the divalent ferrous and the trivalent ferric. Because of it's oxidative states, heme iron functions in the respiratory

cascade as an electron carrier as part of cytochromes in mitochondria and as a ligand for O_2 and CO_2 transport between tissues in the form of hemoglobin and myoglobin. Iron is also part of myoglobin, which stores oxygen in the tissues. Aerobic metabolism requires iron in the Kreb's Cycle as a cofactor of most enzymes, such as aconitase and succinate dehydrogenase. The nonheme iron present in these enzymes is tightly bound to the sulfur atoms of cysteine residues. The complexes are often referred to as "iron-sulfur proteins" or "iron-sulfur clusters" if more than one such complex exists. Other nonheme iron-containing enzymes are ribonucleotide reductase, needed for deoxyribonucleic acid (DNA) synthesis, xanthine dehydrogenase, necessary for catabolism of the purine ring, and NADH dehydrogenase and coenzyme-Q reductase, required in the respiratory cascade (Brody 1994).

Body Iron Distribution

Total iron in the body is approximately 2 to 4 g and is distributed in several compartments (Figure 1). The amount of iron in the body varies with age, weight, and

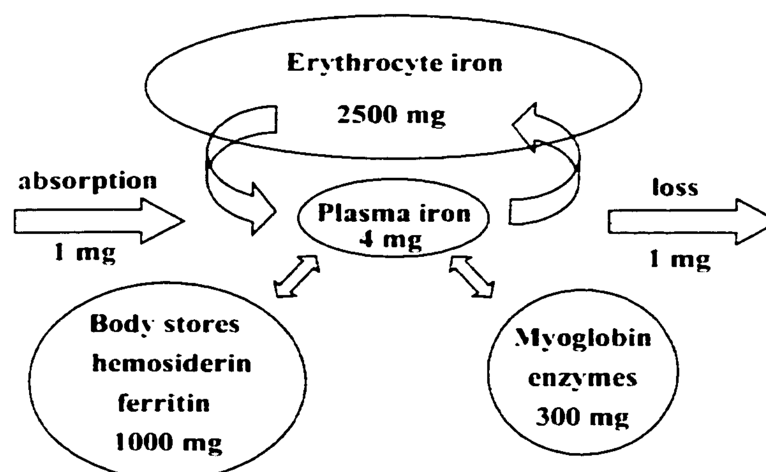


Figure 1. Iron distribution in the body.

gender. Although iron in the transport compartment accounts for a small proportion (0.8%) of the body's total reserve, 20 to 30 mg of iron cycles through this compartment each day bound to transferrin, the iron transport protein in the plasma. A large proportion (65%) of iron is present in circulating erythrocytes bound to hemoglobin. Tissue iron is primarily stored in myoglobin (10%), but a small fraction (2%) is present in a variety of iron-containing enzymes, such as aconitase (Dall 1990, Fairbanks 1998). Storage iron (20%) is contained in ferritin and hemosiderin in nonheme form. Some iron is in what is termed the "labile pool" (2.2%). This iron is constantly recycled and does not have a specific cellular or anatomical location within the body (Fairbanks 1998).

In healthy individuals, iron losses are limited. On average, obligatory daily total losses amount to approximately 1 mg in men and 1.5 mg in women (Cook et al. 1992). Iron losses primarily occur via desquamation of the gastrointestinal mucosa as ferritin-bound (0.1 mg) (Haurani et al. 1994), gastrointestinal blood losses passed via feces bound to hemoglobin (0.35 mg), in biliary secretions (0.2 mg), through the urinary tract (0.08 mg), with desquamation of the skin (0.2 mg), and smaller amounts in sweat. A significant amount of iron loss is also associated with menstruation (0.7 mg/43 mL flow/d) (Fairbanks 1998).

Iron Requirements

Approximately 1 mg/d of iron is required by adult males and females to replenish obligatory iron losses described above. A 65-kg premenopausal woman requires an additional 0.4 mg/d to cover iron lost due to menstruation (Monsen 1988). The recommended dietary allowances (RDA) for iron for males and postmenopausal women is set at 10 mg/d and 15 mg/d for females during childbearing years. The RDA is based on the

premise that approximately 10% of the total dietary iron ingested is actually absorbed (National Research Council 1989).

Iron Deficiency Anemia

Dietary factors influencing the development of iron deficiency anemia include low dietary iron intake and/or the intake of compounds that decrease the bioavailability of iron. Iron deficiency may also result from a low intake of other essential nutrients, such as vitamin A, E, B_{12}, and folate. Sufficient vitamin A is necessary for the mobilization of liver iron and its incorporation into red blood cell hemoglobin. Vitamin E plays a vital role in protecting the erythrocyte from hemolysis. Vitamin B_{12} and folate are involved in hemoglobin synthesis. Non-nutritional factors, such as multiparity, the presence of chronic infection, and blood loss (WHO 1988) may also cause iron deficiency, which is primarily seen in developing countries.

Prevalence of Iron Deficiency: Deficiency of iron and it's associated anemia affect more than 3.5 billion people worldwide, with greater prevalence in developing countries (WHO 1998). This understanding lead the World Health Organization (WHO) to classify iron deficiency anemia as one of its "Priority Action Areas" (WHO 1999). There are a number of population groups at risk for developing iron deficiency anemia. The groups include 1) infants and young children (6 mo to 4 yrs) because of rapid growth rates and insufficient reserves, 2) adolescents entering puberty due to rapidly growing tissues and an expanding blood supply, 3) women of childbearing age due to menstrual iron losses, and 4) pregnant women because of the needs of the fetus and it's rapidly expanding blood volume and additional losses to cover childbirth (Herbert 1987, National Research Council 1989;

IOM 1993). Data collected from the third National Health and Nutrition Examination Survey showed that in the United States 11% of women aged 20 - 49 years were deficient in iron and that 5% suffered from iron deficiency anemia (Looker et al. 1997). In addition, it was found that women of Hispanic or African American descent, those of lower socioeconomic status, and women with greater parity were more likely to have iron deficiency anemia (Figure 2). In the United States, factors contributing to the prevalence of iron deficiency in infants and children include age, socioeconomic status, participation in the Special Supplemental Food Program for Women, Infants, and Children (WIC), the extent and duration of breast-feeding, age when milk is introduced into the diet, and the extent and duration of feeding iron-fortified formulas (Oski 1993).

Consequences of Iron Deficiency: Iron deficient young children display behavioral abnormalities, such as reduced attention span and decreased cognitive performance (UNICEF/UNU/WHO/MI 1998). These abnormalities may be associated with alterations in dopamine synthesis and irreversible if not treated early using iron replacement therapy (Soemantri et al. 1985, Pollitt 1993, Dallman and Siimes 1979, Walker et al. 1996, Parks and Wharton 1989). A reduction in longitudinal growth rates and weight gain is also observed in iron deficient children. In adults, iron deficiency anemia impairs physical work capacity and places one at risk for non-hematologic ailments, such as abnormalities in neurologic response, such as memory deficits (Anezaki et al. 1992). Throughout the lifespan, iron deficiency impairs immune function. However, the correction of iron deficiency with iron supplementation may in some instances exacerbate bacterial and parasitic infections, especially in populations living in developing countries, since these parasitic microbes are

also dependent on iron for growth (Cook and Lynch 1986, Gillespie 1998, Bothwell and Charlton 1981).

Iron containing enzymes were once thought to be exempt from the effects of iron deficiency. However, extensive animal studies have shown that this is clearly not the case and that iron-containing enzymes are, in fact, sensitive in varying degrees to the depletion of body iron. Enzymes have been found to respond differentially in a tissue-specific manner to iron deficiency. For example, aconitase is easily depleted even with mild iron deficiency, but cytochrome oxidase and catalase are far less susceptible. Iron deficiency in rats is associated with a ~70% reduction in the activity of iron-sulfur enzymes in the mitochondria. Decreased work capacity is believed to be due to impaired tolerance brought on by lactic acidosis and alterations in the activity of the enzyme α-glycerophosphate dehydrogenase. In addition, impairment of temperature regulation and decreased cold tolerance in rats may be due to a secondary deficiency of thyroid hormone (Beard 1998).

Iron Excess

In the absence of malabsorption or genetic disorders, there is believed to be no risk of excess body iron or developing iron overload via absorption of iron from the diet even if many iron-fortified foods are consumed (Hallberg et al. 1997). However, once absorbed and then transported out of the mucosa and into the tissues, excess iron is deposited predominantly as hemosiderin in reticuloendothelial cells or in the parenchymal cells of specific tissues. Hemosiderosis has traditionally been used to describe the condition of increased iron storage without associated tissue damage. Hemochromatosis has been used to indicate that damage caused by excess iron is present, particularly in the liver, and also that

iron overload is generalized with the amount of body iron greatly increased, usually to 20 to 40 g (Fairbanks 1998).

Excess iron or iron overload may arise for many reasons, which include disease states, genetic disorders, and/or transfusions. Iron overload may be caused by malabsorption of iron or due to a condition termed hemochromatosis, a genetic disorder characterized by one's inability to regulate the absorption of dietary iron (Bothwell et al. 1979). Excessive iron absorption occurs in hereditary hemochromatosis, excessive intake as with prolonged oral therapy, chronic liver disease, alcholism, or pancreatic insufficiency. Iron overload may also occur in individuals having sickle-cell anemia or β–thalessemia when transfusions are given to treat iron deficiency. African Bantu siderosis is a condition that appears to have a genetic basis, but is primarily associated with chronically excessive intakes of iron, derived mainly from iron cookware (Fairbanks 1998). Frequency rates for hemochromatosis in African Americans appear to be consistent with the existence of a gene influencing iron metabolism (Gordeuk et al. 1998).

The Hfe Protein and Hereditary Hemochromatosis: The genetic basis of iron overload has been of great interest recently, because a gene involved in the development of hemochromatosis was identified (Feder et al. 1996). The gene encodes a protein, termed *Hfe*, which may regulate cellular iron homeostasis. The protein is similar to MHC class I molecules and includes a signal sequence, a peptide-binding region, an immunoglobulin-like domain, a transmembrane region, and a small cytoplasmic portion (Feder et al. 1996). Since the protein spans the cell membrane, it may be capable of sensing iron concentration in the cytosol and, thus, influence cellular iron homeostasis. Hereditary hemochromatosis is a relatively common inherited autosomal recessive disorder of iron metabolism. The

frequency in Caucasians is approximately 1:200-1:400 and a carrier rate of approximately 10-15% for Caucasians of northern European descent. Iron metabolism is disrupted due to loss of function of the *Hfe* protein resulting in greater deposition of iron in the tissues.

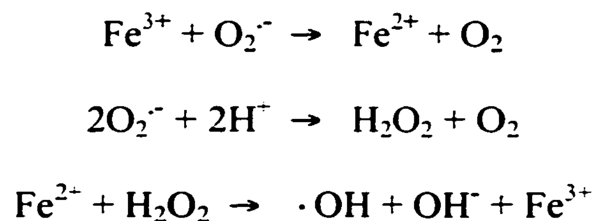
Consequences of Excess Iron: Accumulations of iron occur primarily in hepatic parenchymal cells, but also in the pancreas, heart, pituitary, and the gonads (Deugnier et al. 1993, Niederau et al. 1985). Symptoms include cirrhosis, diabetes mellitus, and cutaneous hyperpigmentation, but also fatigue, an increased risk of liver cancer, restrictive cardiomyopathy, hypothyroidism, arthropathy, and hypogonadism (Fairbanks 1998, Ascherio et al. 1994, Bacon and Britton 1990, Nichols and Bacon 1989, Piperno 1998). Individuals with advanced hemochromatosis may also experience aberations in mental function, including lethargy, semnolence, confusion, and disorientation (Fairbanks 1994).

Age-Related Iron Excess: Evidence suggests that iron stores increase with age and that there is a positive association between increased iron stores in men and postmenopausal women and cardiovascular disease (CVD) (Sullivan 1981). Elevated body iron and a high dietary intake of iron are associated with an increased risk of myocardial infarction (Salonen 1993). Upon resuscitation, reperfusional injury, thought to contribute to myocardial infarction, may result from hydroxyl radicals produced by the Fenton reaction, which involves the donation of one electron from ferrous iron to a molecule of hydrogen peroxide, generating ferric iron and $\cdot$OH (Brody 1994, McCord 1996). Ascherio et al. (1994) did not find a clear association between dietary iron and risk for coronary heart disease in men, but observed an increase in the risk of myocardial infarction among men with higher heme iron intakes. Investigations into whether high iron stores are a risk factor for advanced athersclerosis and coronary heart disease failed to show an association of body iron status

with these conditions (Rauramaa et al. 1994, Sempos et al. 1996). Nelson et al. (1994) found that body iron stores and dietary iron intake were positively associated with risk of colon cancer. However, a study investigating the risk of epithelial cancers in sites throughout the body found that transferrin saturation level, iron deficiency, and iron overload were not related to the incidence of cancer (Herriton et al. 1995).

Female sex hormones may be protective against CVD. Postmenopausal women are believed to be at greater risk of CVD because of the cessation of female hormones, increased ferritin, and the concomitant increase in total cholesterol, low-density lipoprotein (LDL) cholesterol (Berge et al. 1994, Sullivan 1981). A study investigating the hypothesis that high iron stores may be atherogenic and partially explain the sex difference in the incidence of ischemic heart disease found that transferrin, measured as total iron-binding capacity, was an independent negative risk factor for myocardial infarction, but not serum ferritin (Magnusson et al. 1994). Another study that same year provided strong evidence supporting the hypothesis that body iron stores have a significant influence on differences in the development of atherosclerosis in between men and women. Serum ferritin was found to be a strong indicator of cardiovascular disease in both sexes (Kiechl et al. 1994). This raises serious health concerns in postmenopausal women who typically experience a rapid increase in iron stores, along with other risk factors, such total cholesterol, LDL cholesterol, and triglycerides, which also increase after menopause (Jensen et al. 1990).

Iron Excess and Oxidative Stress: Normally, iron in the body is bound to storage or transport proteins and enzymes. However, in conditions where there is excess iron, free iron may interact with oxygen and its derivatives via the Haber-Weiss reaction (Figure 2), producing the free radicals (Halliwell 1993). Highly reactive molecules and radical



$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

$$2O_2^{\cdot -} + 2H^{+} \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^{-} + Fe^{3+}$$

Figure 2. The Haber-Weiss reaction.

species contribute to the oxidative stress of cells (Cheeseman and Slater 1993). The body's antioxidant defense system is intended to minimize production of these radicals and the subsequent damage they cause to biomolecules. Antioxidant defense is conferred by various components (Table 1), which include enzymes and dietary factors (Thomas 1999). These compounds can be collectively measured as total antioxidant status. The total antioxidant status of normal human plasma ranges from 1.3 to 1.8 mM (Miller et al. 1993). Lower antioxidant status indicates a decrease in enzymes and dietary factors described above that are involved in antioxidant reactions, which convert harmful substances into harmless compunds (Figure 3). Free radicals have been shown to initiate lipid peroxidation (McCord 1993). Low-density lipoprotein peroxidation is a marker for CVD (Berliner and Heinecke 1996, Regnström et al. 1994) because of the role oxidized LDL plays in atherogenesis (Jialal and Devaraj 1996). The transport of oxidized LDL is altered (Steinberg 1996), and is thought to facilitate formation of atherogenic plaques in the endothelia of smooth muscle cells.

Table 1. Antioxidant defense systems[1].

Enzymes:

superoxide dismutase – converts radicals to hydrogen peroxide

catalase – detoxifies hydrogen peroxide

glutathione peroxidase – detoxifies hydrogen peroxide and lipid peroxidases in the presence of reduced glutathione

Dietary:

alpha tocopherol – scavenges peroxyl radical intermediates

ascorbic acid – water soluble antioxidant; acts synergistically with tocopherol

beta-carotene – mechanism undefined

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[1] From Rice-Evans and Burdon 1993

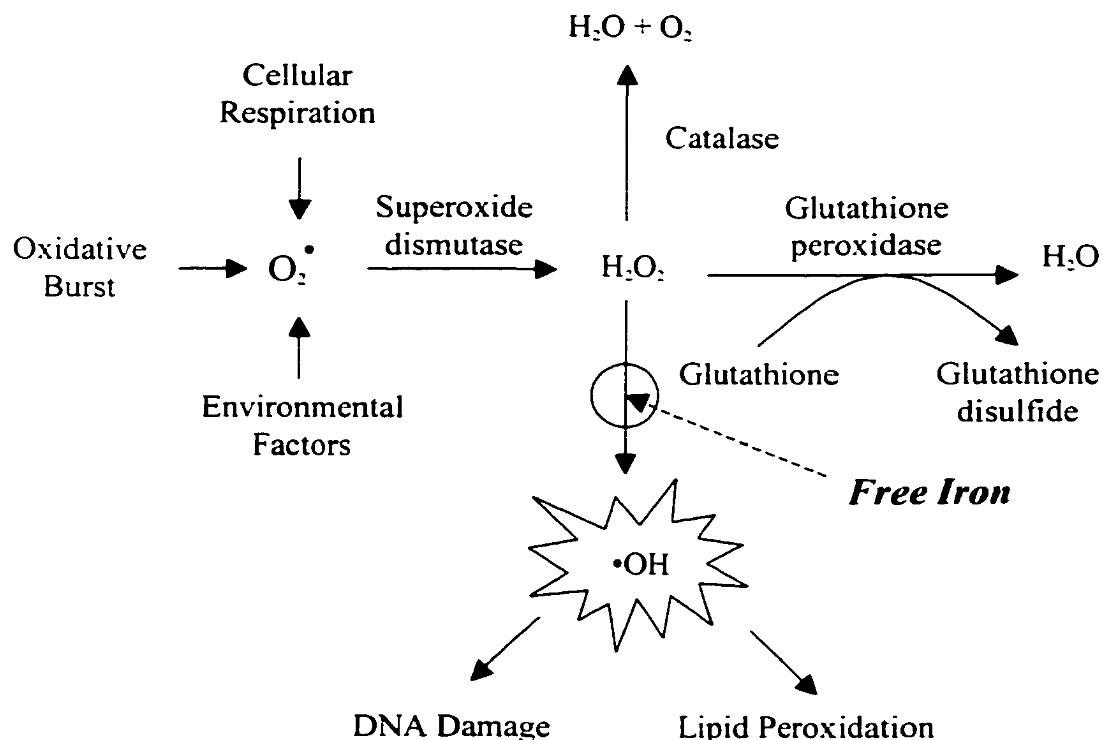


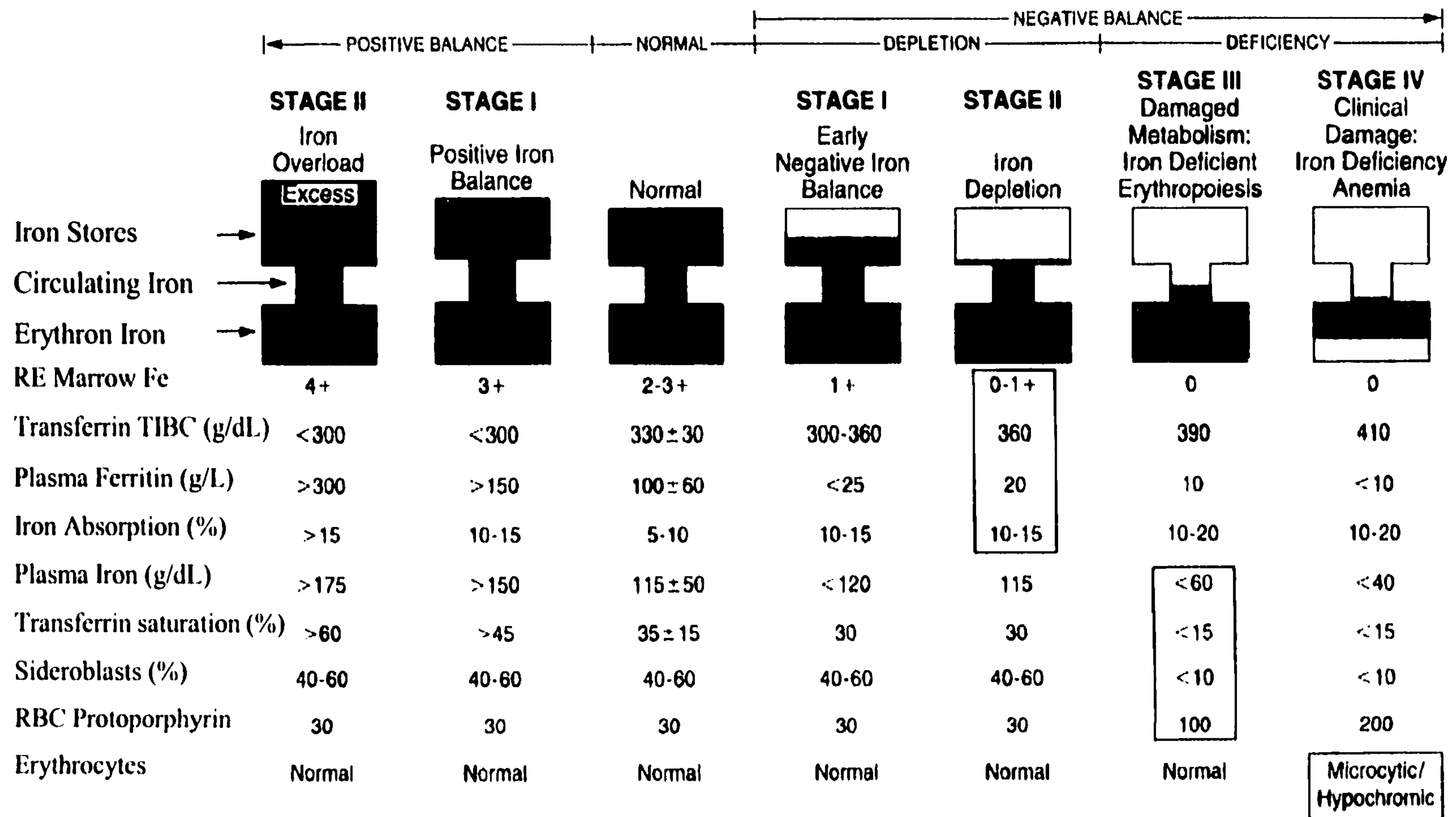
Figure 3. Oxidative stress: Role of antioxidants and free iron.

Iron Status

Figure 4 presents the sequential stages of iron status, iron indices, and values affected at each stage. Iron status may best be explained by discussing methods used in the assessment of iron status.

Storage Iron Indices: Serum ferritin is useful because it provides a precise quantitative measurement of the total iron in the storage compartment and it responds to changes in body iron sooner than other iron indices. However, this value only indicates the degree of iron deficiency, not iron deficiency anemia (Worwood 1979). Approximately 1µg/L serum ferritin corresponds to 8-10 mg of storage iron in the average adult. Some disadvantages of the serum ferritin indice are that it is influenced by age, sex, and the presence of chronic disease states, such as infection, inflammation, and certain neoplastic diseases. In pregnancy, serum ferritin concentrations may vary daily by 25% (Beard 1994) and may fall by 20 µg/L during the first 30 weeks of pregnancy (Kaufer and Casanueva 1990). Serum ferritin concentration typically remains lower in women of childbearing age than in men, but after menopause serum ferritin increases. This increase usually persists through the fifth and sixth decades of a woman's life, typically approaching that of males. Although results vary, medications and other prescriptions have been shown to influence serum ferritin concentration. Mooij et al. (1992) found that serum ferritin concentration decreased in response to routine oral contraceptive use. On the contrary, the duration of menses influenced serum ferritin, but that contraceptive use did not (Soustre et al. 1986).

To gauge the sufficiency of iron reserves and to determine the presence or iron deficiency anemia, serum ferritin concentration is often combined with other measurements, such as the transferrin receptor. The transferrin receptor assay is a more recent method,



	POSITIVE BALANCE: STAGE II Iron Overload (Excess)	POSITIVE BALANCE: STAGE I Positive Iron Balance	NORMAL: Normal	NEGATIVE BALANCE – DEPLETION: STAGE I Early Negative Iron Balance	NEGATIVE BALANCE – DEPLETION: STAGE II Iron Depletion	NEGATIVE BALANCE – DEFICIENCY: STAGE III Damaged Metabolism: Iron Deficient Erythropoiesis	NEGATIVE BALANCE – DEFICIENCY: STAGE IV Clinical Damage: Iron Deficiency Anemia
RE Marrow Fe	4+	3+	2-3+	1+	0-1+	0	0
Transferrin TIBC (g/dL)	<300	<300	330±30	300-360	360	390	410
Plasma Ferritin (g/L)	>300	>150	100±60	<25	20	10	<10
Iron Absorption (%)	>15	10-15	5-10	10-15	10-15	10-20	10-20
Plasma Iron (g/dL)	>175	>150	115±50	<120	115	<60	<40
Transferrin saturation (%)	>60	>45	35±15	30	30	<15	<15
Sideroblasts (%)	40-60	40-60	40-60	40-60	40-60	<10	<10
RBC Protoporphyrin	30	30	30	30	30	100	200
Erythrocytes	Normal	Normal	Normal	Normal	Normal	Normal	Microcytic/ Hypochromic

Figure 4. Sequential stages of iron status. From Herbert, V: Everyone should be tested for iron disorders (*J Amer Diet Assoc* 92:1502, 1992).

which reflects tissue iron sufficiency. The serum transferrin receptor concentration allows for the early detection of iron deficiency and to distinguish iron deficiency anemia from anemia caused by chronic disease states, such as from infection, inflammation, and malignancy (Ferguson et al. 1992, Cook et al. 1993, Baynes 1996). In healthy adults, the daily variation of the transferrin receptor fragment concentration is less than that of serum ferritin (Cooper and Zlotkin 1996). Carriaga et al. (1991) illustrated that by combining the serum transferrin receptor value with serum ferritin measurements, the entire spectrum of iron status can be assessed during pregnancy. When tissue iron needs are not being met synthesis of the transferrin receptor is upregulated. Skikne et al. (1990) found that serum receptor fragment levels remained normal during the period of storage depletion, but that once the serum ferritin concentration fell below 12 µg/L the serum receptor began to rise and continued to do so as deficiency persisted.

Transport Iron Indices: Serum iron is usually measured in tandem with its specific transport protein, transferrin. Transferrin saturation is measured via total iron-binding capacity (TIBC), which is the amount of iron that can be bound by plasma transferrin. A useful index of transport iron is plasma iron expressed as a percentage of TIBC; this is referred to as transferrin saturation. The major limitation with the transferrin saturation value is that there is wide diurnal variation; in healthy subjects the concentration may vary by 100% during a 24-h period (Nilson-Ehle 1990).

Red Blood Cell Indices: Measurement of hemoglobin concentration in whole blood is often done to screen for iron-deficiency anemia since a low hemoglobin concentration is associated with microcytic hypochromia, a characteristic of iron-deficiency anemia. Although hemoglobin concentration is useful as an index of iron status it is influenced by

many factors. Some factors that affect hemoglobin values are age, sex, race, the presence of chronic infection, inflammation, hemorrhage, protein-energy malnutrition, thalassemia minor, hemoglobinopathies, pregnancy, plasma expansion, and the deficiency of some vitamins, such as vitamin B_{12} or folate (Johnson-Spear and Yip 1994). Other factors affecting hemoglobin concentration are normal diurnal variation and cigarette smoking. Hemoglobin values decrease only after iron stores have been fully depleted. There is also considerable overlap in the hemoglobin values of normal non-anemic and iron-deficient individuals. When hemoglobin falls below 14 or 15.5 g/dL in women and men, respectively, iron deficiency anemia may be present.

The hematocrit value is determined by measuring the volume of packed red blood cells; usually the value is expressed as percent volume packed red blood cells or packed cell volume. Since the hematocrit is influenced by all of the factors that affect hemoglobin and is highly susceptible to technical errors (i.e. poorly packed red blood cells, improper mixing of the blood and tube components, and elevated white blood cell counts) the usefulness of the hematocrit is limited. Early in iron deficiency, a slightly reduced hemoglobin concentration may coexist with a near-normal hematocrit.

Once the hematocrit and other iron indices have been determined, the mean red blood cell volume (MCV). The MCV is a measure of the average size of the red blood cell. Since the red blood cell is unaffected by plasma dilution and because the anemia of iron deficiency is microcytic, the MCV is a relatively specific index for iron deficiency. Infection, chronic inflamation, thalessemia minor, and lead poisoning influence MCV and may, therefore, diminish its usefulness. The main limitation of MCV, as is the case with other iron status indices, is the period of time after onset of iron deficiency for the value to become abnormal.

In addition, the presence of concomitant vitamin B_{12} or folate deficiency should also be of concern, since a deficiency of either of these two nutrients may also result in anemia.

Models Used in the Assessment of Iron Status: A variety of laboratory techniques may be used to assess iron status when screening for iron deficiency and iron overload. Typically, to improve accuracy, screening involves the use of a combination of methods (Cook et al. 1976, Cook et al. 1992). For example, three models or techniques have often been used in the assessment of iron deficiency. The "ferritin model", uses serum ferritin, transferrin saturation, and erythrocyte protoporphyrin. Iron deficiency is confirmed if two of the three indices are abnormal. The "MCV model" uses the mean volume of the red blood cell instead of serum ferritin, but requires that two of the three indices be abnormal for iron deficiency to be confirmed. Use of the MCV model with other values, such as serum iron, transferrin receptor, hemoglobin, and hematocrit, allows one to more specifically define the stage and extent of iron deficiency and detect the presence of iron deficiency anemia (Gibson 1990, Cook et al. 1992, Cook et al. 1994). The MCV model incorporates serum ferritin, which is useful because it provides a precise quantitative measurement of the total iron in the storage compartment and it responds to changes in body iron sooner than other iron indices. The third technique, the "change in median hemoglobin concentration", is often used for final confirmation after first using one of the two models above (Expert Scientific Working Group 1985).

Iron Bioavailability

Iron balance is maintained primarily by regulation of heme and nonheme iron absorption and transport throughout the small intestine (Figure 5). Healthy individuals

absorb approximately 5 to 10% of dietary iron, whereas those who are iron deficient absorb from 10 to 20% (Fairbanks 1998). Iron absorption depends not only on an individual's iron status (Lynch et al. 1989) and the amount of iron ingested, but also by its availability (Hallberg 1981, Carpenter and Mahoney 1992). Iron absorption is influenced by various

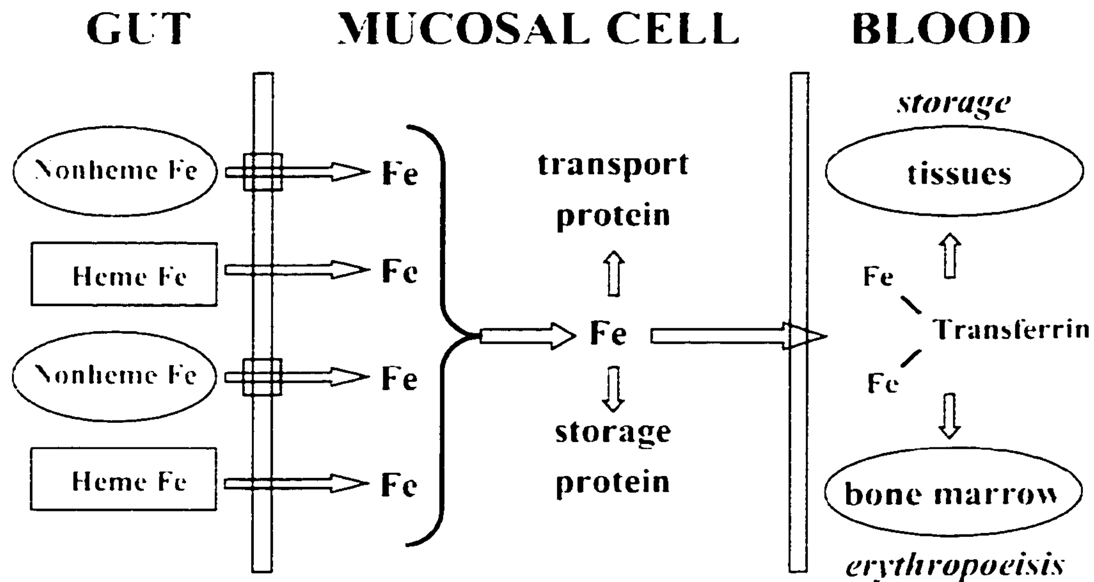


Figure 5. Overview of iron absorption and transport.

intraluminal factors, such as transit time, achlorhydria, steatorrhea, malabsorption syndromes, precipitation by alkalinization, phosphates, riboflavin deficiency, phytic acid, ingested alkaline clays, and antacid preparations (Fairweather-Tait et al. 1992, Groff et al. 1995). Impaired iron absorption in achlorhydric or gastrectomized subjects may be related to decreased solubilization and chelation of the ferric iron in food.

Heme and Nonheme Iron: Iron in foods exists in heme and nonheme forms. Heme is found primarily in animal tissue, whereas, nonheme is found in both animal and vegetable

foods (Fairbanks 1998, Kalpalathika et al. 1991) (Figure 6). The nonheme iron from a variety of vegetable and animal foods, once liberated from its food matrix, becomes part of

Heme Iron	Nonheme Iron
• found primarily in animal tissue	• Found primarily in grains and vegetables, but some in animal tissues
• 10-15% of total dietary iron	• 85-90% of total dietary iron
• ~ 25% absorption – ~1/3 of total iron absorbed	• ~ 10% absorption – ~2/3 of total iron absorbed
• not affected by dietary factors	• highly affected by dietary factors
• affected by iron status	• affected by iron status
	• $Fe^{2+} > Fe^{3+}$

Figure 6. Heme and nonheme iron absorption.

what is termed the "nonheme iron pool" in the gastrointestinal lumen. Heme iron comprises what is termed the "heme iron pool". In this discussion, heme iron or nonheme iron refers to iron coming from their respective pools.

Heme iron contributes only 10-15% of the total iron in Western diets (Hallberg and Rossander 1982), but because it is highly bioavailable it accounts for about one-third of the total iron absorbed per day (Bezkorovainy 1980). About 50 to 60% of the iron in meat, fish, and poultry is heme iron, which may be close to 30% available (Fairbanks 1994, Monsen 1988). Heme iron is absorbed by a mechanism that differs from that for nonheme iron. Most of the heme ingested is in the form of hemoglobin and myoglobin, which is absorbed directly by mucosal cells following the removal of the globin via proteolytic duodenal enzymes or the

protein portion may be removed within the mucosal epithelium (Fairbanks 1994). Once inside the cell, iron is liberated from heme by the enzyme hemeoxygenase before entering the plasma as Fe^{3+} bound to transferring (Uzel and Conrad 1998). Dietary heme iron absorption is relatively unaffected by enhancers and inhibitors, such as ascorbic acid and phytates, respectively.

Nonheme iron absorption is influenced by a variety of factors that affect its bioavailability (Narasinga-Rao 1981, Monsen 1988, Reddy et al. 1996, Fleming et al. 1998). Nearly all of the iron in plants is nonheme iron, but a large proportion of the iron in meat is also nonheme iron (Hallberg 1980). The availability of nonheme iron in plant foods such as beans, peas, corn, bread, and rice is relatively low, up to 10%. Furthermore, although nonheme iron is the major fraction of total dietary iron, its absorption rate may be only 1 to 7% from vegetable staples such as rice, wheat bran, and maize when consumed alone (Bothwell et al. 1979).

Iron Absorption and Transport: The divalent ferrous (Fe^{2+}) is more soluble than the trivalent ferric (Fe^{3+}) at the alkaline pH of intestinal environment and thus, Fe^{2+} is believed to cross the mucus layer more readily to reach the brush border of the intestinal epithelia. The absorption of iron generally occurs via mucin by one or more proteins on the luminal surface of the mucosal epithelium throughout the small intestine (Conrad et al. 1993), but absorption is most efficient in the duodenum. Two major recently identified iron transporters, independent of transferrin and TfR, are the H+-coupled divalent cation transporter-1 (DCT-1) and Nramp2, in the rat and human, respectively (Gunshin et al. 1997, Fleming et al. 1997, Fleming et al. 1998). Because of the similarity between these two transporters they are thought to be one in the same protein and have, together, been renamed as the divalent metal

transporter (DMT-1). The process by which DMT-1 modulates the rate of iron absorption by the intestinal mucosa has not yet been completely elucidated. Analysis of the expression of mRNA indicates that DMT-1 is produced in the proximal small intestine epithelia, but in other cells of the body as well. Transport mediated by DMT-1 is active and requires proton coupling, dependent on the membrane potential of the cell. A wide array of essential divalent metal ions can be transported into the cell by DMT-1. DMT-1 is abundant on the tips of villi of the duodenum and it is likely to be the primary transporter of dietary iron into the enterocyte (Gunshin et al. 1997, Fleming et al. 1999).

Iron is also believed to enter the mucosal cell as either an iron-Nramp2 or iron-integrin complex that is internalized and successively complexed with mobilferrin and then transferred to paraferritin, which functions as the cytosolic iron-transport protein that delivers iron to the serosal side of the cell (Umber et al. 1998). Once iron reaches the interior of the serosal wall the iron is released as Fe^{2+}, oxidized by ceruloplasmin to Fe^{3+}, taken up by transferrin in the subendothelial capillary network for transport to the liver and then to the rest of the body. The pathway for heme iron absorption differs from that for nonheme iron. Heme iron is absorbed by the mucosa and released from protoporphyrin by heme oxygenase before binding to paraferritin in the cytosol. Once bound to paraferritin, the iron from heme is transported as described above.

The following are a number of proteins believed to be involved in cellular acquisition of iron throughout the body: 1) an iron-binding protein which is a trimer of 54-kDa subunits, 2) a β_3-integrin of approximately 160-kDa that spans the cell membrane and consists of two subunits, 3) the *Hfe* protein that spans the cell membrane which is 44-kDa and functions

together with β_2-microglobulin, a small protein of approximately 11-kDa, and 4) natural resistance-associated macrophage protein 2 (Nramp2), a transmembrane protein.

Factors Affecting Nonheme Iron Bioavailability

Although absorption from the nonheme iron pool constitutes a major fraction of daily iron intake, unlike heme iron, its absorption depends on the presence of a variety of dietary components ingested simultaneously (Monsen 1988, Hallberg et al. 1989, Reddy et al. 1996, Fleming et al. 1998). Ascorbic acid (Cook and Monsen 1977, Hallberg et al. 1988, Hurrell et al. 1989, Lynch and Cook 1980) and muscle tissue from meat, fish, and poultry (Cook and Monsen 1976, Bjorn-Rasmussen and Hallberg 1979, Layrisse et al. 1969) enhance nonheme iron absorption, whereas phytic acid (Gillooly et al. 1983, Hallberg et al. 1987), polyphenols (Disler et al. 1975, Gillooly et al. 1983), and soy (Cook et al. 1981, Lynch et al. 1994) inhibit nonheme iron absorption. The mixture of foods eaten by humans and the interplay of nutrients and non-nutrients affects nonheme iron absorption in complex ways. In an effort to better understand this interplay, researchers have developed algorithms for calculating the bioavailability and absorption of nonheme iron (Reddy 2000, Hallberg and Hulthen 2000).

Ascorbic Acid: The ability of ascorbic acid to effectively increase nonheme iron absorption in humans is well documented (Cook and Monsen 1977, Hallberg and Rossander 1984, Hallberg et al. 1989). The ability of ascorbic acid to reduce iron and to form stable soluble complexes, even under alkaline conditions, are known to be responsible for its enhancing effect (Conrad and Schade 1968, Lynch and Cook 1980). Cook et al. (1972) found that the addition of 500-mg ascorbic acid to a maize meal resulted in a 6-fold increase in iron absorption. Concomitant fortification of iron and ascorbic acid has proven more

effective than iron alone in improving iron nutrition in countries where iron deficiency anemia was endemic (Sayers 1973, 1974a, 1974b). However, its susceptibility to oxidation in the presence of metal ions limits its use in foods to improve nonheme iron absorption (Monsen 1988, Cook and Monsen 1977, Hallberg et al. 1989, Lynch and Cook 1980). Although ascorbic acid is known to increase nonheme iron absorption, ascorbic acid supplementation with complex meals has only been shown to moderately improve serum ferritin concentrations after 10 to 16 weeks (Hunt et al. 1994, Cook et al. 1984).

Meat: Meat is an important component of a balanced diet and plays a substantial role in iron nutrition. Low intake of meat and meat products contributes to a higher incidence of iron deficiency in some populations despite a high iron intake (Hallberg and Rossander 1984, Gillespie and Johnston 1998). Meat offers not only highly absorbable heme iron, but also is known to enhance nonheme iron absorption. The positive influence of meat on nonheme iron absorption in humans has been reported by a number of researchers (Hallberg and Rossander 1984, Cook and Monsen 1976, Gordon and Godber 1989, Bjorn-Rasmussen and Hallberg 1979, Engelmann et al. 1998, Layrisse et al. 1969, Cook et al. 1982). Layrisse et al. (1968) initially reported that animal tissue enhanced nonheme iron absorption and in a later study (Martinez-Torres and Layrisse 1970) confirmed this observation, noting that the addition of fish muscle tissue enhanced nonheme iron absorption from a meal of black beans. This positive influence of animal tissue has been attributed to an unknown "factor" hypothesized to prevent iron from polymerizing and maintain iron in a stable soluble form suitable for absorption (Carpenter and Mahoney 1992). In vitro studies have shown that the factor(s) in meat may have a reducing capability that is similar to ascorbic acid, glutathione, and cysteine (Kapsokefalou and Miller 1991).

The effect of animal tissue on nonheme iron absorption was later found not to be due to differences in the quality and quantity of the proteins (Cook and Monsen 1976, Bjorn-Rassmussen and Hallberg 1979, Cook and Monsen 1975). High quality proteins were shown to have dissimilar effects on nonheme iron absorption. Animal tissues, such as pork, beef, chicken, liver, lamb, and fish had similar enhancing effects on iron absorption, whereas, egg, milk, cheese, and ovalbumin protein had no enhancing effect. Researchers have also suggested that the amino acid profile of proteins was responsible for the differences observed in iron absorption (Kroe et al. 1963, Van Campen and Gross 1969, Bjorn-Rassmussen and Hallberg 1979). Histidine and lysine were found to increase iron absorption when given concurrently with ^{59}Fe in rat intestinal segments (Van Campen and Gross 1969), which was surprising because of the known differences between human and rats (Layrisse et al. 1984). The decarboxylation of histidine and removal of the α-amino residue from lysine caused these amino acids to no longer enhance $FeCl_3$ absorption in rats (Van Campen 1973).

The influence of meat on nonheme iron absorption was also attributed to the stimulation of gastric acid secretion (Korman et al. 1971). However, the observation that nonheme iron absorption also increases immediately following beef consumption in achlorhydric patients did not support this hypothesis (Bjorn-Rassmussen and Hallberg 1979). Dietary saturated fat, such as stearic acid has also been found to enhance nonheme iron absorption and utilization (Johnson et al. 1992, Lukaski et al. 1993). Kapsokefalou and Miller (1993) suggested that the effect might involve an interaction between the lean and fat fractions in beef. The enhancement of nonheme iron absorption by meat was later hypothesized to involve many different mechanisms including both luminal pH and chelation (Zhang et al. 1990).

A controversy exists as to whether proteolytic digestion is necessary to release the component in meat responsible for enhancing nonheme iron absorption. Cellular meat proteins may possess nonheme iron absorption-enhancing capabilities because of certain amino acids and intermediary products, likely produced during gastrointestinal digestion. These substituents may chelate and solubilize iron, thereby facilitating nonheme iron absorption by the gut mucosa (Glahn and Van Campen 1997). Iron solubility has been shown to be influenced by the peptic and pancreatic digestion of meat (Slatkavitz and Clydesdale 1988). Cysteine and reduced N-terminal cysteinyl-peptides released during digestion have been hypothesized to be responsible for the positive effect on nonheme iron absorption (Glan and Van Campen 1997, Taylor et al. 1986, Garcia et al. 1996). On the contrary, findings indicate that the factor in meat responsible for enhanced nonheme iron absorption may not be released during gastrointesinal digestion (Au and Reddy 1997, Carpenter and Mahoney 1989). Since proteolytic digestion of meat was shown not to be necessary to increase dialyzable and soluble iron, this may indicate that meat contains enhancing factors, capable of solubilizing iron, that are independent of proteolytic digestion (Kane and Miller 1984, Carpenter and Mahoney 1989). A clear understanding of the positive effect of meat has not yet been achieved, but data suggest that proteins and/or their constituents in meat interact with nonheme iron and maintain its solubility.

Phenolic Compounds: Phenolic compounds in plant foods are known inhibitors of iron absorption (Disler et al. 1975, Rossander et al. 1979, Gillooly et al. 1983, Brune et al. 1989, Hurrell et al. 1999). Tea and coffee reduce iron absorption substantially in proportion to the amount ingested. One theory is that inhibition of nonheme iron absorption by tea is caused by temporary damage to the intestinal mucosa (Mitjavila et al. 1977). However,

South et al. (1997) demonstrated that the effect is primarily due to the chelation of metallic iron by tannins, resulting in the formation of insoluble complexes. Tea is more potent inhibitor of nonheme iron absorption than coffee and may reduce iron absorption by approximately 60%. A recent study by Hurrell et al. (1999) found that a number of polyphenol-containing beverages, such as herb teas, black tea, coffee, and cocoa are potent inhibitors of nonheme iron absorption.

Phytic Acid: Phytic acid (myoinositol hexaphosphate) is found in seeds, cereal grains, and legumes in which it functions as a major phosphorus storage compound. Phytic acid is present at 1 to 5% w/w of legumes, cereals, and nuts and has been identified as a major inhibitor of nonheme iron absorption by plant foods. The function of phytic acid may be to sequester metal ions needed later for plant growth and to prevent them from inducing oxidative spoilage. Phytic acid chelates multivalent metal ions, especially iron, calcium, and zinc, and forms insoluble salts that have poor bioavailability in the gastrointestinal tract (Zhou and Erdman 1995). McCance et al. (1943) found that phytic acid inhibited nonheme iron absorption. As little as 5 to 10 mg of phytic acid can reduce nonheme iron absorption by 50%. In addition, populations consuming diets of vegetable origin consisting mostly of legumes and cereal grains having appreciable quantities of phytic acid have a higher prevalence of iron deficiency anemia (Hallberg and Rossander 1984, Shaw et al. 1995). Of particular importance is the observation that the addition of meat or ascorbic acid to such a diet reverses the iron-chelating effect of phytate (Hallberg 1987).

Soybeans possess high amounts of phytic acid; soybeans contain approximately 1.4% w/w phytic acid (Graf and Eaton 1990). Hurrell et al. (1992) determined that there was an increase in nonheme iron absorption with a decrease in the phytic acid content of soy protein

isolates. The amount of phytic acid may have a significant effect on nonheme iron absorption; less than 0.3 mg phytic acid per gram soy protein isolate had a strong inhibitory affect on nonheme iron absorption in human subjects consuming a semipurified test meal (Hurrell et al. 1992). Lynch et al. (1994) determined that there are two major inhibitors of human nonheme iron absorption present in soy protein isolate, phytic acid and a protein-related moiety (7S conglycinin). They were able to demonstrate that purified and dephytinized 7S conglycinin had an inhibitory effect approximately equal to that of phytate.

Calcium: The effect of calcium on nonheme iron absorption is controversial. When calcium intake increased from 300-1,200 mg/day over a period of 5 days no significant affect on nonheme iron absorption was observed (Reddy and Cook 1997). However, the addition of both calcium and phosphate decreased the absorption of nonheme iron (Cook et al. 1991), but if calcium was provided alone, this inhibitory effect was not seen. The inhibitory effect of supplemental calcium was also demonstrated when milk and ferrous sulfate were ingested simultaneously (Hallberg et al. 1991). Sokoll and Dawson-Hughes (1992) studied the effect of supplemental calcium on the iron stores of healthy premenopausal women. They found that over a 12-week period 1 g supplemental calcium with meals did not influence plasma ferritin concentration. The effect may be due to the binding of iron by the insoluble calcium phosphate complex formed in the alkaline environment of the small intestine, resulting in a decrease in the bioavailability of iron (Morck and Cook 1981). However, the effect may also involve such variables as meal type (single or complex), normal calcium content of the meal, and the phytate content of the meal (Lynch 1997).

Methods of Assessing Nonheme Iron Bioavailability

The absorption of nonheme iron is influenced by many factors in the diet and, hence, the bioavailability of iron in the presence of a variety of factors is of particular importance to researchers in the field of iron nutrition. A relatively inexpensive and valid assay to measure the bioavailability of nonheme iron would advance understanding of the combined effect of dietary factors most influencing nonheme iron absorption.

Several methods have been used to assess nonheme iron bioavailability. Forbes et al. (1989) reviewed some of these methods and discussed their validity to human iron absorption. Rat models are appealing for in vivo studies, but no suitable small animal model is currently available for studying human nonheme iron absorption. In addition, extrapolation of iron absorption data from rats to humans is questionable since factors shown to influence nonheme iron absorption in rats do not in humans (Reddy and Cook 1991). In vitro studies are appealing because of the lower cost, decreased complexity, and less time consuming. The equilibrium dialysis method is the most common method used to assess iron bioavailability (Miller et al. 1981, Shen et al. 1994). Bioavailability as based on solubility or dialyzability alone has been shown to not accurately reflect uptake by the intestinal mucosa (Gangloff et al. 1996a, Miller and Berner 1989). Since foods are comprised of many different compounds, a nonheme iron absorption model that allows one to test many combinations of substances and individual compounds under more physiologic conditions would facilitate identification of factors responsible for enhancing nonheme iron absorption.

The Caco-2 cell line appears to be a good model to measure physiological iron absorption (Garcia et al. 1996, Glahn et al. 1996, Au and Reddy 2000). The Caco-2 cell is a human colon carcinoma cell, which undergoes spontaneous differentiation in cell culture to

form polarized epithelial cell monolayers with many of the characteristics of enterocytes (Pinto et al. 1983). Caco-2 cells have been shown to be useful in measuring nonheme iron absorption under a variety of conditions (Halleux and Schneider 1991, Alverez-Hernandez et al. 1991, Gangloff et al. 1996a and b, Glahn et al. 1996, Glahn and Van Campen 1997, Glahn et al. 1998, Glahn et al. 1999). Many factors that influence Caco-2 cell growth and nonheme iron uptake have been studied (Jumarie and Malo 1991, Halleux and Schneider 1994, Alverez-Hernandez et al. 1994, Han et al. 1994, Han. et al. 1995, Glahn et al. 1995, Gangloff et al. 1996b, Arredondo et al. 1997, Alverez-Hernandez et al. 1998). Use of the Caco-2 cell model has been shown to be a valid tool to study human nonheme iron bioavailability, including the enhancing effect of meat on nonheme iron absorption (Garcia 1996). In addition, iron absorption by Caco-2 cells correlates well to human iron absorption measurements (Au and Reddy 2000).

Use of the Caco-2 cell model allows one to determine the interaction among various food components individually and collectively, as mixtures of nutrients and non-nutrients, on nonheme iron absorption. One of the advantages of the Caco-2 cell model is that it can reasonably accommodate vast combinations of foods and components and a multitude of slight adjustments in concentration when individual compounds are studied. In addition, use of the Caco-2 cell model to study iron absorption is relatively inexpensive when compared to human studies or animal models.

Recent studies showing the validity of using the Caco-2 cell model to study human iron bioavailability, including the enhancement of meat on nonheme iron absorption (Garcia et al. 1996, Glahn et al. 1996, Glahn et al. 1999), prompted us to use this cell line to identify, and thereafter characterize, beef proteins that enhance nonheme iron bioavailability.

Literature Cited

Ahluwalia, N. (1998) Diagnostic utility of serum transferrin receptors measurement in assessing iron status. *Nutr. Revs.* 56;5:133-141.

Allen, L.H. (1993) Iron-deficiency anemia increases risk of preterm delivery. *Nutr. Revs.* 51(2):49-52.

Alvarez-Hernandez, X., Nichols, G.M. & Glass, J. (1991) Caco-2 cell line: A system for studying intestinal transport across epithelial cell monolayers. *Biochim. Biophys. Acta.* 1070:205-208.

Alverez-Hernandez, X., Smith, M. & Glass, J. (1994) Regulation of iron uptake and transport by transferrin in Caco-2 cell, an intestinal cell line. *Biochim. Biophy. Acta.* 1192:215-222.

Alverez-Hernandez, X., Smith, M. & Glass, J. (1998) The effect of apotransferrin on iron release from Caco-2 cells, an intestinal epithelial cell line. *Blood* 15;91(10):3974-3979.

Anezaki, T., Yanagisawa, K., Ibayashi, K. & Miyatake, T. (1992) A patient with severe iron-deficiency anemia and memory disturbance. *Internal Medicine* 31(11):1306-1309.

Arredondo, M., Orellana, A., Garate, M.A. & Nunez, M.T. (1997) Intracellular iron regulates iron absorption and IRP activity in intestinal epithelial (Caco-2) cells. *Am. J. Physiol.* 273:G275-G280.

Ascherio, A., Willett, W.C., Rimm, E.B., Giovannucci, E.L. & Stampfer, M.J. (1994) Dietary iron intake and risk of coronary disease among men. *Circulation* 89:969-974.

Au, A.P. & Reddy, M.B. (1997) Enzymatic digestion does not affect the iron uptake by the Caco-2 cells. *FASEB J.* 11(3):A607(3505).

Au, A.P. & Reddy, M.B. (2000) Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. *J. Nutr.* 130:1329-1334.

Bacon, B.R. & Britton, R.S. (1990) The pathology of hepatic iron overload: A free radical mediated process. *Hepatology* 11:127-137.

Baker, S.J. & Ramachandran, K. (1984) The design and analysis of iron supplementation trials. INACG, ILSI Press, pp. 5-8.

Baynes, R.D. (1996) Refining the assessment of body iron status. *Am. J. Clin. Nutr.* 64:793-794.

Baynes, R.D., Shih, Y.J. & Cook, J.D. (1994) Mechanism of production of the serum transferrin receptor. In: Progress in Iron Research. Ed. Hershko, C. et.al. Plenum Press, New York, pp. 61-67.

Beard, J. (1994) Iron deficiency: Assessment during preganancy and its importance in pregnant adolescents. *Am. J. Clin. Nutr.* 59(Suppl):502S-10S.

Beard, J.L., Brigham, D.E., Kelly, S.K. & Green, M.H. (1998) Plasma thyroid hormone kinetics are altered in iron-deficient rats. *J. Nutr.* 128:1401-1408.

Berge, L.N., Bonaa, K.H. & Norday, A. (1994) Serum ferritin, sex hormones, and cardiovascular risk factors in health women. *Arterioscler. Thromb.* 14:857-861.

Berliner, J.A. & Heinecke, J.W. (1996) The role of oxidized lipoproteins in atherogenesis. *Free Rad. Biol. Med.* 1996;20(5):707-27.

Berner, L.A. & Miller, D.D. (1985) Effects of dietary proteins on iron bioavailability - a review. *Food Chem.* 18:47-69.

Bezkorovainy, A. (1980) Biochemistry of nonheme iron. Plenum Press, New York, NY.

Bjorn-Rasmussen, E. & Hallberg, L. (1979) Effect of animal proteins on the absorption of food iron in man. *Nutr. Metab.* 23:192-202.

Bjorn-Rasmussen, E. & Hallberg, L., Isaksson, B. & Arvidsson, B. (1974) Food iron absorption in man: Applications of the two-pool extrinsic tag method to measure heme and nonheme iron absorption from the whole diet. *J. Clin. Invest.* 53:247-255.

Bothwell, T.H. & Charlton, R.W. (1981) Iron deficiency in women. Eds. Cook, J.D., Dallman, P.R., Hallberg, L. ILSI Press, The Nutrition Foundation, New York, pp. 4-9.

Bothwell, T.H., Charlton, R.W, Cook, J.D. & Finch,C.A. (1979) Iron metabolism in man, Oxford: Blackwell Scientific Publications, pp. 576.

Brody, T. (1994) Nutritional Biochemistry. Academic Press, Inc., New York, pp. 531-539.

Brune, M., Rossander-Hulten, L. & Hallberg, L. (1989) Iron absorption and phenolic compounds: The importance of different phenolic structures. *Eur. J. Clin. Nutr.* 43:547-558.

Cammack, R., Wrigglesworth, J.M. & Baum, H. (1990) Iron-dependent enzymes in mammalian systems. In Iron Transport and Storage. Ed. Ponka, P. et al. CRC Press, Inc., pp. 18-35.

Carpenter, C.E. & Mahoney, A.W. (1989) Proteolytic digestion of meat is not necessary for iron solubilization. J. Nutr. 119:1418-22.

Carpenter, C.E. & Mahoney, A.W. (1992) Contributions of heme and nonheme iron to human nutrition. *Crit. Revs. Food Sci. Nutr.* CRC Press, Inc., 31(4):333-67.

Carriaga, M.T., Skikne, B.S., Finley, B., Cutler, B. & Cook, J.D. (1991) Serum transferrin receptor for the detection of iron deficiency in pregnancy. *Am. J. Clin. Nutr.* 54:1077-1081.

Chen, O.S., Schalinske, K.L. & Eisenstein, R.S. (1997) Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver. *J. Nutr.* 127:238-248.

Conrad, M.E. & Schade, S. (1968) Ascorbic acid chelates in iron absorption: A role for hydrochloric acid and bile. *Gastroenterology* 55:35-45.

Conrad, M.E., Umbreit, J.N. & Moore, E.G. (1993) Regulation of iron absorption: Proteins involved in duodenal mucosal uptake and transport. *J. Am. Col. Nutr.* 12(6):720-728.

Cook, J.D., Baynes, R.D., & Skikne, B.S. (1992) Iron deficiency and the measurement of iron status. *Nutr. Res. Revs.* 5:189-202.

Cook, J.D., Baynes, R.D. & Skikne, B.S. (1994) The physiological significance of circulating transferrin receptors. In, Nutrient Regulation during Pregnancy, Lactation, and Infant Growth. Eds. Allen, L., King, J., Lönnerdal, B. Plenum Press, New York, pp. 119-126.

Cook, J.D., Dassenko, S.A. & Whittaker, P. (1991) Calcium supplementation: Effect on iron absorption. *Am. J. Clin. Nutr.* 53:106-111.

Cook, J.D. & Finch, C.A. (1979) Assessing iron status of a population. *Am. J. Clin. Nutr.* 32:2115-2119.

Cook, J.D., Finch, C.A. & Smith, N.J. (1976) Evaluation of the iron status of a population. *Blood* 48(3):449-455.

Cook, J.D., Layrisse, M., Martinez-Torres, C., Walker, L., Monsen, E. & Finch, C. (1972) Food iron absorption measured by an extrinsic tag. *J. Clin. Invest.* 51:805-815.

Cook, J.D. & Lynch, S.R. (1986) The liabilities of iron deficiency. *Blood* 68;4: 803-809.

Cook, J.D. & Monsen, E.R. (1975) Food iron absorption. I. Use of a semisynthetic diet to study absorption of nonheme iron. *Am. J. Clin. Nutr.* 28:1289-1295.

Cook, J.D. & Monsen, E.R. (1976) Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. *Am. J. Clin. Nutr.* 29:859-67.
Cook, J.D. & Monsen, E.R. (1977) Vitamin C, the common cold, and iron absorption. *Am. J. Clin. Nutr.* 30:235-41.

Cook, J.D., Morck, T.A. & Lynch, S.R. (1981) The inhibitory effect of soy products on nonheme iron absorption in man. *Am. J. Clin. Nutr.* 34:2622-2629.

Cook, J.D., Morck, T.A., Skikne, B.S. & Lynch, S.R. (1982) The importance of animal products in human iron nutrition. In Ch. 16, Academic Press, Inc., pp. 332-335.

Cook, J.D., Skikne, B.S. & Baynes, R.D. (1993) Serum transferrin receptor. *Ann. Rev. Med.* 44:63-74.

Cook, J.D., Skikne, B.S., Lynch, S.R., & Reusser, M.E. (1986) Estimates of iron sufficiency in the U.S. population. *Blood* 68(3):726-731.

Cook, J.D., Watson, S.S., Simpson, K.M., Lipschitz, D.A. & Skikne, B.S. (1984) The effect of high ascorbic acid supplementation on body iron stores. *Blood* 64:721-726.

Cooper, M.J. & Zlotkin, S.H. (1996) Day-to-day variation of transferrin receptor and ferritin in healthy men and women. *Am. J. Clin. Nutr.* 64:738-742.

Dall, P. (1990) Iron. In: Present Knowledge in Nutrition, 6th ed. (Brown, M. ed.), pp. 241-250. International Life Sciences Institute Nutrition Foundation, Washington, DC.

Dallman, P.R. (1986) Iron deficiency in the weanling: A nutritional problem on the way to resolution. *Acta. Paediatr. Scand.* Suppl;323:59.

Deugnier, Y.M., Guyader, D., Crantock, L., Lopez, J.M., Turlin, B., Yaouanq, J., Jouandlle, H., Campion, J.P., Launois, B., Halliday, J.W., Powell, L.W. & Brissot, P. (1993) Primary liver cancer in genetic hemochromatosis: A clinical, pathological and pathogenetic study of 54 cases. *Gastroenetrology* 99:1070-1091.

Disler, P., Lynch, S., Charlton, R., Torrance, J., Bothwell, T., Walker, R. & Mayet, R. (1975) The effect of tea on iron absorption. *Gut* 16:193-200.

Edwards, C.Q., Griffin, L.M., Goldgar, D., Drummond, C., Skolmik, M.H. & Kushner, J.P. (1988) Prevalence of hemochromatosis among 11,065 presumably healthy blood donors. *N. Engl. J. Med.* 318:1355-1362.

Eisenstein, R.S. & Blemings, K.P. (1998) Iron regulatory proteins, iron responsive elements and iron homeostasis. *J. Nutr.* 128:2295-2298.

Ellaurie, M. & Rubinstein, A. (1994) Ferritin levels in pediatric HIV-1 infection. *Acta. Pædiatr.* 83:1035-1037.

Engelmann, M.D., Davidson, L., Sandstrom, B., Walczyk, T., Hurrell, R.F. & Michaelsen, K.F. (1998) The influence of meat on nonheme iron absorption in infants. *Pediatr. Res.* 43:768-773.

Erdman, J.W. & Fordyce, E.J. (1989) Soy products and the human diet. *Am. J. Clin. Nutr.* 49:725-37.

Expert Scientific Working Group (ESWG) (1985) Summary of a report on assessment of the iron nutritional status of the United States population. *Am. J. Clin. Nutr.* 42:1318-1330.

Fairbanks, V.F. (1998) Iron in Medicine and Nutrition. In: Modern Nutrition In Health and Disease, 9th ed., Eds. Shils, ME, Olson, JA, and Shike, M. Lea and Febiger, Philadelphia, pp. 193-221.

Fairweather-Tait, S.J., Powers, H.J., Minski, M.J., Whitehead, J. & Downes, R. (1992) Riboflavin deficiency and iron absorption in adult Gambian men. *Ann. Nutr. Metab.* 36:34-40.

Feder, J.N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D.A., Basava, A., Dormishian, F., Domingo, R.J., Ellis, M.C., Fullan, A., Hinton, L.M., Jones, N.L., Kimmel, B.E., Kronmal, G.S., Lauer, P., Lee, V.K., Loeb, D.B., Mapa, F.A., McClelland, E., Meyer, N.C., Mintier, G.A., Moeller, N., Moore, T., Morikang, E., Prass, C.E., Quintana, L., Starnes, S.M., Schatzman, R.C., Brunke, K.J., Drayna, D.T., Risch, N.J., Bacon, B.R. & Wolff, R.K. (1996) A novel MHC class 1-like gene is mutated in patients with hereditary hemochromatosis. *Nat. Genet.* 13:399-408.

Feder, J.N., Penny, D.M., Irrinki, A., Lee, V.K., Lebron, J.A., Watson, N., Tsuchihashi, Z., Sigal, E., Bjorkman, P.J. & Schatzman, R.C. (1998) The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc. Natl. Acad. Sci.* 95:1472-1477.

Feder, J.N., Tsuchihashi, Z., Irrinki, A., Lee V.K., Mapa, F.A., Morikang, E., Prass, C.E., Starnes, S.M., Wolff, R.K., Parkkila, S., Sly, W.S. & Schatzman, R.C. (1997) The hemochromatosis founder mutation in HLA-H disrupts B2 microglobulin interaction and cell surface expression. *J. Biol. Chem.* 272:14025-14028.

Ferguson, B.J., Skikne, B.S., Simpson, K.M., Baynes, R.D. & Cook, J.D. (1992) Serum transferrin receptor distinguishes the anemia of chronic disease from iron deficiency anemia. *J. Lab. Clin. Med.* 119(4):385-390.

Fleming, D.J., Jacques, P.F., Dallal, G.E., Tucker, K.L., Wilson, P.W.F. & Wood, R.J. (1998) Dietary determinants of iron stores in a free-living elderly population: The Framingham Heart Study. *Am. J. Clin. Nutr.* 67:722-733.

Fleming, M.D., Romano, M.A., Su, M.A., Garrick, L.M., Garrick, M.D. & Andrews, N.C. (1998) Nramp2 is mutated in the anemic Belgrade (b) rat: Evidence of a role for Nramp2 in endosomal iron transport. *Proc. Natl. Acad. Sci.* 95:1148-1153.

Fleming, M.D., Trenor, C.C., Su, M.A., Foernzler, D., Beier, D.R., Dietrich, W.F. & Andrews, N.C. (1997) Microcytic anemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat. Genet.* 16:383-386.

Fleming, R.E., Migas, M.C., Zhou, X.Y., Jiang, J., Britton, R.S., Brunt. E.M., Tomatsu, S. Waheed, A., Bacon, B.R. & Sly, W.S. (1999) Mechanism of increased iron absorption in murine model of hereditary hemochromatosis: Increased duodenal expression of the iron transporter DMT1. *Proc. Natl. Acad. Sci.* 96:3143-3148.

Forbes, A.L., Adams, C.E., Arnaud, M.J., Chichester, C.O., Cook, J.D., Harrison, B.N., Hurrell, R.F., Kahn, S.G., Morris, E.R., Tanner, J.T. & Whittaker, P. (1989) Comparison of in vitro, animal, and clinical determinations of iron bioavailability: International Anemia Consultative Group Task Force report on iron bioavailability. *Am. J. Clin. Nutr.* 49:225-238.

Gangloff, M.B., Glahn, R.P., Miller, D.D., Wien, E.M., Kapsokelou, M. & Van Campen, D.R. (1996a) Assessment of iron availability using combined in vitro digestion and Caco-2 cell culture. *Nutr. Res.* 16:479-487.

Gangloff, M., Lai, M., Van Campen, D., Miller, D. & Glahn, R. (1996b) Caco-2 cell ferrous iron uptake but not transfer is down-regulated in cells grown in high iron serum-free medium. *J. Nutr.* 126:3118-3127.

Garcia, M.N., Martinez-Torres, C., Leets, I., Tropper, E., Ramirez, J. & Layrisse, M. (1996) Heat treatment on heme iron and iron-containing proteins in meat: Iron absorption in humans from diets containing cooked meat fractions. *Nutr. Biochem.* 7:49-54.

Garcia, M.N., Flowers, C. & Cook, J.D. (1996) The Caco-2 cell culture system can be used as a model to study food iron availability. *J. Nutr.* 251-8.

Gibson, R.S. (1990) Principles of Nutritional Assessment. Oxford Univ. Press, New York, pp. 350-351.

Gillespie, S. (1998) Major issues in the control of iron deficiency. Micronutrient Initiative/UNICEF, New York, pp. 6-9, 14-19, 22-29, & 44-51.

Gillespie, S. & Johnston, J.L. (1998) Expert consultation on anemia determinants and interventions: Proceedings. Micronutrient Initiative, Ottawa, Canada, pp. 3.

Gillooly, M., Bothwell, T., Torrance, J., MacPhail, A., Derman, D., Bezwoda, W., Mills, W., Charlton, R. & Mayet, F. (1983) The effect of organic acids, phytates and polyphenols on the absorption of iron from vegetables. *Br. J. Nutr.* 49:331-336.

Glahn, R.P., Gangloff, M.B., Van Campen, D.R., Miller, D.D., Wien, E.M. & Norvell, W.A. (1995) Bathophenanthrolene disulfonic acid and sodium dithionite effectively remove surface-bound iron from Caco-2 cell monolayers. *J. Nutr.* 125:1833-1840.

Glahn, R.P., Lee, O.A. & Miller, D.D. (1999) In vitro digestion/Caco-2 cell culture model to determine optimal ascorbic acid to Fe ratio in rice cereal. *J. Food Sci.* 64(5):925-928.

Glahn, R.P., Lee, O.A., Yeung, A., Goldman, M.I. & Miller, D.D. (1998) Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. *J. Nutr.* 128:1555-1561.

Glahn, R.P. & Van Campen, D.R. (1997) Iron uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl-glycine. *J. Nutr.* 642-7.

Glahn, R.P., Wien, E.M., Van Campen, D.R. & Milller, D.D. (1996) Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: use of a novel in vitro method for rapid estimation of iron bioavailability. *J. Nutr.* 126:332-339.

Goldenberg, H.A. (1997) Regulation of mammalian iron metabolism: Current state and need for further knowledge. *Crit. Revs. Clin. Lab. Sci.* 34(6):529-572.

Gordeuk, V.R., McLaren, C.E., Looker, A.C., Hasselblad, V. & Brittenham, G.M. (1998) Distribution of transferrin saturations in the African-American population. *Blood* 91(6):2175-2179.

Gordon, D.T. & Godber, J.S. (1989) The enhancement of nonheme iron bioavailability by beef protein in the rat. J Nutr 119:446-452.

Guindi, M.E., Skikne, B.S., Covell, A.M. & Cook, J.D. (1988) An immunoassay for human transferrin. *Am. J. Clin. Nutr.* 47:37-41.

Groff, J., Gropper, S. & Hunt, S. (1995) Advanced nutrition and human metabolism, 2nd ed., West Publ. Comp., St Paul, MN, pp. 352-366.

Graf, E. & Eaton, J.W. (1990) Antioxidant functions of phytic acid. *Free Rad. Biol. Med.* 8:61.

Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, M.F., Nussberger, S., Gollan, J.L. & Hediger, M.A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388:482-488.

Guo, L. & Reddy, M.B. (2000) The protective effect of phytic acid on reducing oxidative stress in β2-microglobulin-deficient mice. (In press.)

Hallberg, L. (1980) Food iron absorption. In: Iron, ed. Cook, JD, Livingstone-Churchill, New York, NY, pp. 116-133.

Hallberg, L. (1981) Bioavailability of dietary iron in man. *Ann. Rev. Nutr.* 1:123-147.

Hallberg, L (1998) Combating iron deficiency: daily administration of iron is far superior to weekly administration. *Am. J. Clin. Nutr.* 68:213-217.

Hallberg, L. (1994) Prevention of iron deficiency. *Bailliere's Clin. Haematol.* 7(4):805-814.

Hallberg, L., Brune, M., Erlandsson, M., Sandberg, A.S. & Rossander-Hulthen, L. (1991) Calcium: Effect of different amounts on nonheme- and heme-iron absorption in man. *Am. J. Clin. Nutr.* 53:112-119.

Hallberg, L., Brune, M. & Rossander, L. (1989) Iron absorption in man: Ascorbic acid and dose dependent inhibition by phytate. *Am. J. Clin. Nutr.* 49:140-4.

Hallberg, L., Brune, M. & Rossander, L. (1989) The role of vitamin C in iron absorption. *Int. J. Vit. Nutr. Res.* Suppl 30:103-108.

Hallberg, L., Rossander, L. & Skanberg, A. (1987) Phytate and the inhibitory effect of bran on iron absorption in man. *Am. J. Clin. Nutr.* 45:988-996.

Hallberg, L. & Hulthen, L. (2000) Prediction of dietary iron absorption: An algorithm for calculation absorption and bioavailability of dietary iron. *Am. J. Clin. Nutr.* 71:1147-1160.

Hallberg, L. & Hulthen, L. & Gramatkovski (1997) Iron absorption from the whole diet in men: How effective is the regulation of iron absorption? *Am. J. Clin. Nutr.* 66:347-356.

Hallberg, L. & Rossander, L. (1984) Improvement of iron nutrition in developing countries: Comparison of adding meat, soy protein, ascorbic acid, citric acid, and ferrous sulphate on iron absorption from a simple Latin American-type meal. *Am. J. Clin. Nutr.* 39:577-83.

Halleux, C. & Schneider, Y-J. (1991) Iron absorption by intestinal epithelial cells: Caco-2 cells cultivated in serum-free medium, on polyethyleneterephthalate microporous membranes, as an in vitro model. *In Vitro Cell Dev. Biol.* 27A:293-302.

Halleux, C. & Schneider, Y-J. (1994) Iron absorption by Caco-2 cells cultivated in serum-free medium as in vitro model of the human intestinal epithelial barrier. *J. Cell Physiol.* 158:17-28.

Halliwell B. (1993) The role of oxygen radicals in human disease, with particular reference to the vascular system. *Haemostasis* 1993;23 Suppl1:118-26.

Han, O., Failla, M.L., Hill, A.D., Morris, E.R. & Smith, J.C. Jr. (1994) Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line. *J. Nutr.* 124:580-587.

Han, O., Failla, M.L., Hill, A.D., Morris, E.R. & Smith, J.C. Jr. (1995) Reduction of Fe (III) is required for uptake of nonheme iron by Caco-2 cells. *J. Nutr.* 125:1291-1299

Haurani, F.I. & Marcolina, J. (1994) The mechanisms of gastrointestinal loss of iron. *Nutr. Res.* 14(12):1779-1787.

Herbert, V. (1987) Recommended dietary intakes (RDI) of iron in humans. *Am. J. Clin. Nutr.* 43:68-71.

Herbert, V., Jayatilleke, E., Shaw, S., Rosman, A.S., Giardini, P., Grady, R.W., Bowman, B. & Gunter, E.W. (1997) Serum ferritin iron, a new test, measures human body iron stores unconfounded by inflammation. *Stem Cells* 15:291-296.

Herrinton, L.J., Friedman, G.D., Baer, D. & Selby, J.V. (1995) Transferrin saturation and risk of cancer. *Am. J. Epidemiol.* 142:692-698.

Hunt, J.R., Gallagher, S.K. & Johnson, L.K. (1994) Effect of ascorbic acid on apparent iron absorption by women with low iron stores. *Am. J. Clin. Nutr.* 59:1381-1385.

Hurrell, R.F., Juillerat, M-A., Reddy, M.B., Lynch, S.R., Dasenko, S.A. & Cook, J.D. (1992) Soy protein, phytate, and iron absorption in humans. *Am. J. Clin. Nutr.* 56:573-578.

Hurrell, R., Lynch, S.R., Trinidad, T., Dassenko, S.S. & Cook, J.D. (1988) Iron absorption in humans: Bovine serum albumin compared with beef muscle and egg white. *Am. J. Clin. Nutr.* 47:102-107.

Hurrell, R.F., Reddy, M.B. & Cook, J.D. (1999) Inhibition of non-haem iron absorption in man by phenolic-containing beverages. *Br. J. Nutr.* 81:289-295.

INACG (1992) Combating iron deficiency anemia through food fortification technology: An action plan. The Nutrition Foundation, Washnigton, DC, pp. 3-4.

Institute of Medicine, Food and Nutrition Board (1993) Iron Deficiency Anemia: Recommended Guidelines for the Prevention, Detection, and Management Among U.S. Children and Women of Childbearing Age. National Academy Press, Washington, DC.

Jayachandran, M., Jayanthi, B., Sundaravadivel, B., Panneerselvan, C. (1996) Status oflipids, lipid peroxidation, and antioxidant systems with Vitamin C supplementation during aging in rats. *J. Nutr. Biochem.* 1996;7:270-5.

Jensen, J., Nilas, L. & Christiansen, C. (1990) Influence of menopause on serum lipids and lipoproteins. *Maturitas* 12:321-331.

Johnson, P., Lukaski, H. & Korynta, E. (1992) Effects of stearic acid and beef tallow on iron utilization by the rat. *P.D.E.B.M.* 200:480-486.

Johnson-Spear, M.A. & Yip, R. (1994) Hemoglobin difference between black and white women with comparable iron status: justification for race-specific anemia criteria. *Am. J. Clin. Nutr.* 60:117-121.

Jumarie, C. & Malo, C. (1991) Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation in vitro. *J. Cell Physiol.* 149:24-33.

Kalpalathika, P.V.M., Clark, E.M. & Mahoney, A.W. (1991) Heme iron content in selected ready-to-serve beef products. *J. Agric. Food Chem.* 39:1091-1093.

Kane, A.P. & Miller, D.D. (1984) In vitro estimation of the effects of selected proteins on iron bioavailability. *Am. J. Clin. Nutr.* 39:393-401.

Kapsokefalou, M. & Miller, D.D. (1991) Effects of meat and selected food components on the valence of nonheme iron during in vitro digestion. *J. Food Sci.* 56:352-358.

Kapsokefalou, M. & Miller, D. (1993) Lean beef and beef fat interact to enhance nonheme iron absorption in rats. *J. Nutr.* 123:1429-1434.

Kaufer, M. & Casanueva, E. (1990) Relation of pregnancy serum ferritin levels to hemoglobin levels throughout pregnancy. *Euro. J. Clin. Nutr.* 44:709-715.

Kiechl, S., Aichner, F., Gerstenbrand, F., Egger, G., Mair, A., Rungger, G., Spogler, F., Jarosch, E., Oberhollenzer, F. & Willeit, J. (1994) Body iron stores and presence of carotid atherosclerosis. *Arterioscler. Thromb.* 14:1625-1630.

Koller, B.H., Marrack, P., Kappler, J.W. & Smithies, O. (1990) Normal development of mice deficienct in β_2-microglobulin, MHC class 1 proteins, and $CD8^-$ T cells. *Science* 248:1227-1230.

Korman, M.G., Sovenly, C. & Hansky, J. (1971) Effect of food iron on serum gastrin evaluated by radioimmunoassay. *Gut* 12:619.

Kroe, D., Kinney, T.D., Kaufman, N. & Klavins, J.V. (1963) The influence of amino acids on iron absorption. *Blood* 21:546.

Kuvibidila, S., Yu, L., Warrier, R.P., Ode, D. & Mbele, V. (1994) Usefulness of serum ferritin levels in the assessment of iron status in non-pregnant Zairean women of childbearing age. *J. Trop. Med. Hyg.* 97:171-179.

Layrisse, M., Cook, J.D., Martinez-Torres, C., Roche, M., Kuhn, I., Walker, R. & Finch, C. (1969) Food iron absorption: A comparison of vegetable and animal foods. *Blood* 33:430-43.

Layrisse, M., Martinez-Torres, C., Leets, I., Taylor, P. & Ramirez, J. (1984) Effect of histidine, cysteine, glutathione or beef on iron absorption in humans. *J. Nutr.* 114:217-223.

Looker, A., Dallman, P., Carroll, M., Gunter, E. & Johnson, C. (1997) Prevalence of iron deficiency in the United States. *J. Am. Med. Assoc.* 227:973-976.

Lukaski, H., McLaren, G., Johnson, P. & Smith, M. (1993) Enhanced intestinal iron absorption by dietary stearic acid: Effect onmucosal iron kinetics. *FASEB J.* 7:A273.

Lynch, S.R. (1997) Interaction of iron with other nutrients. *Nutr. Rev.* 55(4):102-110.

Lynch, S.R. and Cook, J.D. (1980) Interaction of vitamin C and iron. *Ann. N.Y. Acad. Sci.* 355:32-44.

Lynch, S.R., Dassenko, S.A., Cook, J.D., Juillerat, M.A. & Hurell, R. (1994) Inhibitory effect of a soybean-protein-related moiety on iron absorption in humans. *Am. J. Clin. Nutr.* 60:567-572.

Lynch, S.R., Hurrell, R.F., Dassenko, S.A. & Cook, J.D. (1989a) The effect of dietary proteins on iron bioavailability in man. *Adv. Exp. Med. Biol.* 249:117-132.

Lynch, S.R., Skikne, B.S. & Cook, J.D. (1989b) Food iron absorption in ideopathic hemochromatosis. *Blood* 74,2187-2193.

Magnusson, M.K., Sigfusson, N., Sigvaldason, H., Johannesson, G.M., Magnusson, S., & Thorgeirsson, G. (1994) Low iron-binding capacity as a risk factor for myocardial infarction. *Circulation* 89:102-108.

Martinez-Torres, C., Romano, E. & Layrisse, M. (1981) Effect of cysteine on iron absorption in man. *Am. J. Clin. Nutr.* 34:322-327.

Mascotti, D.P., Rup, D. & Thach, R.E. (1995) Regulation of iron metabolism: Translational effects mediated by iron, heme, and cytokines. *Ann. Rev. Nutr.* 15:239-261.

McCance, J. (1943) Phytic acid and iron absorption. *Lancet* 2:126.

McCord, J.M. (1993) Human disease, free radicals, and the oxidant/antioxidant balance. *Clin Biochem* 26(5):351-7.

McCord, J.M. (1996) Effects of positive Iron Status at a Cellular Level. *Nutr. Rev.* 54(3):85-88.

Micronutrient Initiative and International Nutrition Foundation, UNICEF/UNU/WHO/MI Technical Workshop (1998) Preventing Iron Deficiency in Women and Children: Background and Consensus on Key Technical Issues and Resources for Advocacy, Planning

and Implementing National Programmes. Micronutrient Initiative Publ., Ottawa, ON, Canada.

Miller, D. (1996) Effects of cooking and food processing on the content of bioavailable iron in foods. In: Micronutrient interactions: Impact on child health and nutrition. ILSI Press, Washington, pp. 65.

Miller, D.D. & Berner, L.A. (1989) Is solubility in vitro a reliable predictor of iron bioavailability? *Biol. Trace Elem. Res.* 19:11-24.

Miller, D., Schricker, B., Rasmussen, R & Van Campen, D. (1981) An in vitro method for estimation of iron availability from meals. *Am. J. Clin. Nutr.* 34:2248-2256.

Mitchell, J.H., Gardner, P.T., McPhail, D.B., Morrice, P.C., Collins, A.R., Duthie, G.G. (1998) Antioxidant efficacy of phytoestrogens in chemical and biological model systems. *Arch. Biochem. Biophys.* 360(1):142-8.

Mitjavila, S., Lacombe, C., Carrera, G. & Derache, R. (1977) Tannic acid and oxidized tannic acid on the functional state of rat intestinal epithelium. *J. Nutr.* 107:2113-2121.

Monsen, E.R. (1988) Iron nutrition and absorption: Dietary factors which impact iron bioavailability. *J. Am. Diet. Assoc.* 7860-90.

Mooij, P.N.M., Thomas, C.M.G., Doesburg, W.H. & Eskes, T.K.A.B. (1992) The effects of oral contraceptives and multivitamin supplementation on serum ferritin and hematological parameters. *Intl. J. Clin. Pharm., Ther., Tox.* 30(2):57-62.

Morck, T.A. & Cook, J.D. (1981) Factors affecting the bioavailability of dietary iron. *Cereal Foods World* 26(12):667-672.

Narasinga Rao, B.S. (1981) Physiology of iron absorption and supplementation. *Brit. Med. Bulletin* 37:25-30.

National Research Council (1989) Recommended dietary allowances, 10th ed., National Academy Press, Washington, DC pp. 195-205.

Nelson, R.L., Davis, F.G., Sutter, E., Sobin, L.H., Kikendall, J.W. & Bowen, P. (1994) Body iron stores and risk of colonic neoplasia. *J. Nat. Cancer Inst.* 86(6):455-460.

Nichols, G.M. & Bacon, B.R. (1989) Hereditary hemochromatosis: Pathenogenesis and clinical features of common disease. *Am. J. Gastroenterology* 84:851-862.

Niederau, C. Fischer, R., Sonnenberg, W., Stremmel, W., Trampisch, H.J. & Strohmeyer, W. (1985) Survival and causes of death in cirrhitic and noncirrhitic patients with primary hemochromatosis. *N. Engl. J. Med.* 313:1256-1262.

Nilsson-Ehle, H. (1990) Iron deficiency. *Upsala J. Med. Sci.* 95:213-220.

Oski, FA. (1993) Iron deficiency in infancy and childhood: Current concepts, ed. Desforges, J.F. *N. Engl. J. Med.* 329;3:190-193.

Parks, Y.A. & Wharton, B.A. (1989) Iron deficiency and the brain. *Acta. Paediatr. Scand.* Suppl 361:71-77.

Parkkila, S., Waheed, A., Britton, R.S., Bacon, B.R., Zhou, X.Y., Tomatsu, S., Fleming, R.E. & Sly, W.S. (1997) Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. *Proc. Natl. Acad. Sci.* 94:13198-13202.

Pinto, M., Robine-Leon, S., Appay, M-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. & Zweibaum, A. (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* 47:323-330.

Piperno, A. (1998) Classification and diagnosis of iron overload. *Haematologica* 83:447-455.

Pollitt, E. (1993) Iron deficiency and cognitive function. *Ann. Rev. Nutr.* 13:521-37.

Pollitt, E. & Leibel, R.L. (1976) Iron deficiency and behavior. *J. Pediatr.* 88:372.

Punnonen, K., Irjala, K. & Rajamäki (1994) Iron-deficiency anemia is associated with high concentrations of transferrin receptor in serum. *Clin. Chem.* 40(5):774-776.

Rauramaa, R., Vaisanen, S., Mercuri, M., Rankinen, T., Penttila, I. & Bond, M.G. (1994) Association of risk factors and body iron status to carotid atherosclerosis in middle-aged Eastern Finnish men. *Euro. Heart J.* 15:1020-1027.

Reddy, M.B. & Cook, J.D. (1991) Assessment of dietary determinants of nonheme iron absorption in humans and rats. *Am. J. Clin. Nutr.* 54:723-728.

Reddy, M.B. & Cook, J.D. (1997) Effect of calcium intake on nonheme-iron absorption from a complete diet. *Am. J. Clin. Nutr.* 65:1820-1825.

Reddy, M.B., Hurrell, R.F. & Cook, J.D. (2000) Estimation of nonheme-iron bioavailability from meal composition. *Am. J. Clin. Nutr.* 71:937-943.

Reddy, M.B., Hurrell, R.F., Juillerat M.A. & Cook, J.D. (1996) The influence of different protein sources on phytate inhibition of nonheme-iron absorption in humans. *Am. J. Clin. Nutr.* 63:203-7.

Regnström, J., Walldius, G., Hådell, K., Johansson, J., Holme, I., Olsson, A.G., Nilsson, J. (1994) Influence of lipoprotein lipids, dietary fat and smoking on macrophage degradation of native and oxidized low density lipoprotein. *Atherosclerosis* 110:13-23.

Richardson, D.R. & Baker, E. (1992) Intermediate steps in cellular iron uptake from transferrin. *J. Biol. Chem.* 267(30):21384-21389.

Ross, J. & Horton, S. (1998) Economic consequences of iron deficiency. Micronutrient Initiative, Publ., Ottawa, Canada, pp. 34.

Rossander, L, Hallberg, L. & Bjorn-Rasmussen, E. (1979) Absorption of iron from breakfast meals. *Am. J. Clin. Nutr.* 32:2484-2489.

Roy, C.N., Penny, D.M., Feder, J.N. & Enns, C.A. (1999) The hereditary hemochromatosis protein, HFE, specifically regulates transferrin-mediated iron uptake in Hela cells. *J. Biol. Chem.* 274:9022-9028.

Salonen, J.T. (1993) The role of iron as a cardiovascular risk factor. *Curr. Opin. Lipidology* 4:277-282.

Santanam, N., Ramachandran, S., Parthasarathy, S. (1998) Oxygen radicals, antioxidants, and lipid peroxidation. *Semin. Reprod. Endocrinol.* 16(4) 275-80.

Santos, M., Schilham, M.W., Rademakers, L.H., Marx, J.J., de Sousa, M. & Clevers, H (1996) Defective iron homeostasis in β_2-microglobulin knockout mice recapitulates hereditary hemochromatosis in man. *J. Exp. Med.* 184:1975-1985.

Sayers, M. (1973) The effects of ascorbic acid supplementation on the absorption of iron in maize, wheat and soya. *Brit. J. Haematol.* 24:202-218.

Sayers, M. (1974a) Iron absorption from rice meals cooked with fortified salt containing ferrous sulphate and ascorbic acid. *Brit. J. Nutr.* 31:367-375.

Sayers, M. (1974b) The fortification of common salt with ascorbic acid and iron. *Brit. J. Haematol.* 28:483-495.

Schultink, W., Gross, R., Gliwitzki, M., Karyadi, D. & Matulessi, P. (1995) Effect of daily vs weekly iron supplementation in Indonesian preschool children with low iron stores. *Am. J. Clin. Nutr.* 61:111-5.

Sempos, C.T., Looker, A.C. & Gillum, R.F. (1996) Iron and heart disease: The epidemiological data. *Nutr. Rev.* 54(3):73-84.

Shaw, N-S., Chin, C-J. & Pan, W-H. (1995) A vegetarian diet rich in soybean products compromises iron status in young students. *J. Nutr.* 125:212-219.

Shen, L., Luten, J., Robberrecht, H., Bindels, J. & Deelstra, H. (1994) Modification of an in-vitro method for estimating the bioavailability of zinc and calcium from foods. *Z. Lebensm. Unters. Forsh.* 199:442-445.

Skikne, B.S., Flowers, C.H. & Cook, J.D. (1990) Serum transferrin receptor: A quantitative measure of tissue iron deficiency. *Blood* 75:1870-1876.

Slatkavitz, C.A. & Clydesdale, F.M. (1988) Solubility of inorganic iron as affected by proteolytic digestion. *J. Clin. Nutr.* 47:487-95.

Soemantri, A.G., Pollitt, E. & Kim, I. (1985) Iron deficiency anemia and educational achievement. *Am. J. Clin. Nutr.* 42:1221-1228.

Sokoll, L.J. & Dawson-Hughes, B. (1992) Calcium supplementation and plasma ferritin concentrations in premenopausal women. *Am. J. Clin. Nutr.* 56:1045-1048.

Soustre, Y., Dop, M.C., Galan, P. & Hercberg, S. (1986) Dietary determinants of the iron status in menstruating women. *Internat. J. Vit. Nutr. Res.* 56:281-286.

South, P., House, W. & Miller, D. (1997) Tea consumption does not affect iron absorption in rats unless tea and iron are consumed together. *Nutr. Res.* 17:1303-1310.

Stoltzfus, R.J. & Dreyfuss, M.L. (1998) Guidelines for the Use of Iron Supplements toPrevent and Treat Iron Deficiency Anemia. International Nutritional Anemia Consultative Group, World Health Organization, United Nation's Children's Fund, ILSI Press, pp. 4-11.

Sullivan, J.L. (1981) Iron and the sex difference in heart disease risk. *Lancet* June 13., pp. 1293-1294.

Taylor, P.G., Martinez-Torres, C., Romano, E.L. & Layrisse, M. (1986) The effect of cysteine-containing peptides released during meat digestion on iron absorption in humans. *Am. J. Clin. Nutr.* 43:68-71.

Thomas, J.A. (1999) Oxidative stress and oxidant defense. In: Shils ME, Olson JA, Shike M. Ross, A.C., eds. Modern Nutrition in Health and Disease. 9th ed. Baltimore, MD: Williams & Wilkins, pp. 751-60.

Toth, I. & Bridges, K.R. (1995) Ascorbic acid enhances ferritin mRNA translation by an IRP/aconitase switch. *J. Biol. Chem.* 270(33):19540-19544.

Umbreit, J.N., Conrad, M.E., Moore, E.G. & Latour, L.F. (1998) Iron absorption and cellular transport: The mobilferrin/paraferritin paradigm. *Sem. Hematol.* 35(1):13-26.

UNICEF/UNU/WHO/MI technical Workshop (1998) Preventing iron deficiency in women and children: Background and consensus on key technical issues and resources for advocacy,

planning and implementing national programmes. International Nutrition Foundation/Micronutrient Initiative, Boston/Ottawa, pp. 21-38.

Uzel, C. & Conrad, M.E. (1998) Absorption of heme iron. *Sem Hematol* 35(1):27-34.

Van Campen, D. (1972) Effect of histidine and ascorbic acid on the absorption and retention of ^{59}Fe by iron-depleted rats. *J. Nutr.* 102:165-170.

Van Campen, D. (1973) Enhancement of iron absorption from ligated segments of rat intestine by histidine, cysteine, and lysine: Effects of removing ionizing groups and of stereoisomerism. *J. Nutr.* 103:139-142.

Van Campen, D. & Gross, E. (1969) Effect of histidine and certain other amino acids on the absorption of ^{59}Fe by rats. *J. Nutr.* 99:68-74.

Viteri, F.E., Alvarez, E., Batres, R., Torun, B., Pineda, O., Mejia, L.A., & Sylvi, J. (1995) Fortification of sugar with iron sodium ethylenediaminotetraacetate (FeNaEDTA) improves iron status in semirural Guatemalan populations. *Am. J. Clin. Nutr.* 61:1153-1163.

Waheed, A., Parkkila, S., Saarnio, J., Fleming, R.E., Zhou, X.Y., Tomatsu, S., Britton, R.S., Bacon, B.R. & Sly, W.S. (1999) Association of HFE protein with transferrin receptor in crypt enterocytes of human duodenum. *Proc. Natl. Acad. Sci.* 96:1579-1584.

Walker, S.P., Grantham-McGregor, S.M., Himes, J.H., Powell, C.A. & Chang, S.M. (1996) Early childhood supplementation does not benefit the long-term growth of stunted children in Jamaica. *J. Nutr.* 126:3017-3024.

Walters, G.O., Miller, F.M. & Worwood, M. (1973) Serum ferritin concentration and iron stores in normal subjects. *J. Clin. Pathology* 26:770-772.

Williams, R.J.P. (1990) An introduction to the nature of iron transport and storage. In iron transport and storage. Ed. Ponka, P. et al. CRC Press, Inc., pp. 2-14.

Worwood, M. (1979) Serum ferritin. *Crit. Revs. Clin. Lab. Sci.* 10:171-204.

Yip, R., Johnson, C. & Dallman, P.R. (1984) Age-related changes in laboratory values used in the diagnosis of anemia and iron deficiency. *Am. J. Clin. Nutr.* 39:427-436.

Zhang, D., Carpenter, C.E. & Mahoney, AW. (1990) A mechanistic hypothesis for meat enhancement of nonheme iron absorption: Stimulation of gastric secretions and iron chelation. *Nutr. Res.* 10:929-935.

Zhou, J.R. & Erdman, J.W. (1995) Phytic acid in health and disease. *Crit. Revs. Food Sci. Nutr.* 35(6):495-508.

CHAPTER 2. ISOLATION AND CHARACTERIZATION OF BEEF PROTEINS THAT ENHANCE NONHEME IRON BIOAVAILABILITY

A paper to be submitted to Journal of Nutrition

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RUNNING TITLE: Beef proteins enhance nonheme iron bioavailability

ABSTRACT The objective was to isolate and characterize beef muscle proteins that enhance nonheme iron bioavailability. Beef sirloin was cooked, lyophilized and reconstituted with water prior to in vitro digestion. Following centrifugation, the digest supernatant was sequentially ultrafiltered using 10K and 1K molecular weight cut-off membranes. Nonheme iron bioavailability was assessed by Caco-2 cell monolayer ^{59}Fe uptake using an extrinsic labeling method. Cell iron uptake was 45.2% and 56.5% higher ($p<0.05$) with the 1-kDa retentate (1KR) as compared to the 10-kDa retentate and 1-kDa filtrate, respectively, and 2- to 5-fold greater than the blank ($p<0.05$) and iron-nitrilotriacetic acid (NTA) ($p<0.001$), respectively. Thus, the 1KR was chosen for further analysis. Immobilized metal affinity chromatography of the 1KR yielded a series of four peaks: three distinct peaks (P1, P3, P4) and one with a few closely associated peaks (P2). Peptides in P1-

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P4 increased ($p<0.001$) iron solubility at pH 6 as compared to the blank; 2.1, 3.6, 4.7 and 4.4-fold, respectively. When compared to NTA, P1-P4 were found to enhance ^{59}Fe uptake by 3- to 5-fold ($p<0.01$). Iron uptake with P2 and P4 was also significantly greater than the blank ($p<0.05$). Gel electrophoresis illustrated that P1-P4 each contained many peptides ranging from 1- to 5-kDa. Matrix Assisted Laser Desorption Ionization-Time of Flight analysis confirmed these findings. Amino acid composition analysis revealed that histidine concentration increased progressively in P1-P4. Our results suggest that enhancement of nonheme iron absorption by beef may be due to peptides produced during gastrointestinal digestion and that histidine content may be important.

KEY WORDS: • Beef proteins • nonheme iron • bioavailability • ultrafiltration • Caco-2 cells • histidine

INTRODUCTION

Iron absorption in humans depends on an individual's iron status (Lynch et al. 1989a and 1989b), dietary iron intake (Björn-Rasmussen et al. 1974), and its bioavailability (Hallberg 1981, Carpenter and Mahoney 1992). Nonheme iron typically constitutes the major fraction of daily iron intake, but unlike heme iron, its absorption is influenced by various dietary components (Layrisse et al. 1969, Narasinga-Rao et al. 1981, Cook and Monsen 1977, Monsen 1988, Reddy et al. 1996). Animal tissue (Layrisse et al. 1969, Cook and Monsen 1976, Hurrell et al. 1988) and ascorbic acid (Hallberg et al. 1989, Lynch and Cook 1980) enhance, whereas many plant factors, such as phytate (Gillooly et al. 1983, Hallberg et al. 1987, Reddy et al. 1996), polyphenols (Disler et al. 1975, Gillooly et al. 1983), and soy

protein (Cook et al. 1981, Lynch et al. 1994) inhibit nonheme iron absorption. Many researchers have reported the positive influence of meat on nonheme iron absorption in humans (Layrisse et al. 1969, Bjorn-Rasmussen and Hallberg 1979, Cook et al. 1982, Hallberg and Rossander 1984, Engelmann et al. 1998) and the greater prevalence of iron deficiency in developing countries has been attributed to a diet low in meat, despite a high nonheme iron content (Hallberg and Rossander 1984, Gillespie and Johnston, 1998).

The enhancing effect of meat is attributed to unknown factor(s), usually known as "meat factor(s)", that may maintain iron in a soluble form, thereby increasing its bioavailabilty (Carpenter and Mahoney 1992). Not all animal proteins have the same enhancing effect. For example, pork, beef, chicken, liver, lamb, and fish enhance nonheme iron absorption, but egg, milk, cheese, and ovalbumin protein do not (Layrisee et al. 1969; Cook and Monsen 1975, Cook and Monsen 1976, Bjorn-Rassmussen and Hallberg 1979). The influence of meat on nonheme iron absorption was initially attributed to the stimulation of gastric acid secretion (Korman et al. 1971). However, reducing components (Kapsokefalou and Miller 1991), stearic acid (Johnson et al. 1992, Lukaski et al., 1993), certain amino acids (Van Campen and Gross 1969, Marinez-Torres et al. 1981, Taylor et al. 1986, Glahn et al. 1997), and peptides released during proteolytic digestion (Kane and Miller 1984, Slatkavitz and Clydesdale 1988) have also been suggested to play a role in the enhancement of nonheme iron absorption.

Data on the factor(s) in meat responsible for the enhancement of nonheme iron absorption are inconclusive, possibly because of differences in the methodologies employed. In vivo, rat models have been used to study the influence of meat on nonheme iron absorption (Gordon and Godber 1989), but extrapolation of the data from rats to humans is

questionable since some factors shown to influence nonheme iron absorption in humans have been shown to have marginal or no effect in rats (Reddy and Cook 1991). An in vitro method, using equilibrium dialysis is the most common technique used to assess iron bioavailability (Miller et al. 1981; Shen et al. 1994). However, bioavailability based on solubility or dialyzability alone may not accurately reflect uptake by the intestinal mucosa (Miller and Berner 1989; Gangloff et al. 1996). The identity of the factor(s) in meat responsible for enhanced nonheme iron absorption remains poorly characterized.

Caco-2 cell iron uptake is of increasing interest in nutritional studies. Caco-2 cells, a human colon adenocarcinoma cell line, spontaneously differentiate into polarized monolayers with well-developed brush borders and exhibit many of the morphological and biochemical characteristics of enterocytes (Pinto et al. 1983). Caco-2 cell iron uptake is a physiological measure of iron absorption and shown to correlate well to human iron absorption when many dietary factors are tested (Au and Reddy 2000). Recent studies using this cell line to study iron bioavailability, including the enhancing effect of meat on nonheme iron uptake (Garcia et al. 1996, Glahn et al. 1996, Glahn and Van Campen 1997, Glahn et al. 1999), prompted us to use this in vitro model in an attempt to isolate and characterize beef proteins that enhance nonheme iron bioavailability.

MATERIALS AND METHODS

All of the enzymes and chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless noted otherwise. Deionized (DI) water was used throughout the study and reagents were prepared fresh before use. Beef samples were stored under nitrogen atmosphere at -80°C.

Sample Preparation. A total of 1.5 kg of lean beef, top sirloin steak, was obtained fresh from a local supermarket. All visible fat and connective tissue was removed and the remaining lean meat was cut into small thin pieces, placed in 450 mL boiling water, and simmered for 30 min. After chilling overnight, all visible fat was removed, additional water was added, and the sample homogenized in an Osterizer® blender (Sunbeam-Oster Household Products, Laurel, MS) for 5 min. The slurry was then rehomogenized using a Polytron PT-3000 Homogenizer for 5 min at 25,000 rpm with a PT-DA 3012/2S generator (Brinkmann Instruments, Westbury, NY). The slurry was lyophilized in ziploc bags and the resulting powder passed through a 40-mesh sieve. Total protein of the powder was determined by a micro-Kjeldahl procedure (AOAC 1996). Total iron was determined by dry ashing and the iron measured by flame atomic absorption spectrophotometry (Allied Analytical Systems, Waltham, MA). Nonheme iron in the meat powder was determined by the modified method of Torrance and Bothwell (1968) using ferrozine instead of bathophenanthroline disulfonic acid as the ferrous chromogen (Chidambaram et al., 1989).

In Vitro Digestion. Twenty grams of lyophilized beef powder was rehydrated with 110 mL water and the slurry was rehomogenized again using a Polytron Homogenizer for 45 s at 18,000 rpm with a PT-DA 3007/2 generator (Brinkmann Instruments, Westbury, NY). The pH of the homogenate was slowly adjusted to 2.0 with 5 mol/L HCl (Fisher Scientific, Fair Lawn, NJ) and 4 mL pepsin solution [0.08 g pepsin (2,800 U/mg protein) per mL 0.1 mol/L HCl] was added. The solution was then incubated for 1 h at 37°C in a shaking waterbath (Precision Scientific, Chicago, IL) at 90 rpm to simulate gastric digestion. Following incubation, the pH was slowly raised to 6.0 by the dropwise addition of $NaHCO_3$ (1 mol/L), and 20 mL pancreatin solution [0.004 g pancreatin (4 X U.S.P. activity) per mL 0.1 mol/L

$NaHCO_3$] was added. The sample was then incubated for 30 min at 37°C in a shaking water bath at 90 rpm to mimic duodenal digestion. The homogenate was then removed from the water bath, chilled on ice, and centrifuged at 4°C for 30 min at 10,200 x g (Beckman Instruments, Palo Alto, CA) to collect the supernatant. Protein concentration (Lowry et al. 1951) and nonheme iron concentration (Torrance and Bothwell 1968) of the digest supernatant was then measured.

Ultrafiltration. The digest supernatant was subjected to sequential ultrafiltration in an Amicon stirred-cell unit (Model 8200, SUC 200 ml; Amicon, Inc., Beverly, MA) above a magnetic stirrer using first 10K and then followed by 1K molecular weight cutoff (MWCO) membrane disks (Omega™, low protein-binding modified polyethersulfone; Pall/Gelman Sciences, Ann Arbor, MI) at 4°C under nitrogen at 55 psi. To achieve sufficient filtration, 150 mL nanopure analytical grade water, 18.0 MΩ-cm (Barnstead/Thermolyne, Dubuque, IA), was added to resuspend the remaining solution after 90% of the total volume of each solution had filtered. This was repeated three times per membrane. The three fractions collected were a 10-kDa retentate (10KR), 1-kDa retentate (1KR), and 1-kDa filtrate (1KF).

Immobilized Metal Affinity Chromatography (IMAC). The procedure is similar to that described by Lönnerdal et al. (1977) and briefly described below. A column bed (10 x 2.5 cm) of chelating sepharose consisting of highly cross-linked agarose beads coupled to imino-diacetic acid (Amersham Pharmacia Biotech, Piscataway, NJ) was prepared by packing 50 mL gel in a glass column and equilibrating with three column volumes water. The gel was then charged with approximately 20 mL of 0.1 mol/L $CuSO_4 \cdot 5H_2O$. The column was then washed with five column volumes of nanopure water prior to equilibration with five column volumes of start buffer (0.02 mol/L Na_2HPO_4 (Fisher Scientific, Pittsburgh, PA) and 1.0

mol/L NaCl at pH 8.1. Twenty mL of sample (1KR) solution (12.5 mg protein per mL start buffer) was applied to the column and washed with at least five column volumes of start buffer until the effluent had shown no absorbance at 280 nm. The wash was also collected to assure that no proteins of possible interest were lost. Adsorbed proteins were eluted by lowering the pH using elution buffer (0.02 mol/L Na_2HPO_4 and 0.5 mol/L NaCl) at pH 3.0. Solutions for use in IMAC and in each of the following steps were prepared using nanopure water.

Trace Metal Removal. The IMAC wash and eluents fractions were made metal-free with Chelex®-100 (200-400 mesh, sodium form; Bio-Rad Laboratories, Hercules, CA) resin treatment prior to amino acid composition analysis since even trace amounts of metals may interfere with the detection of some amino acids. Treatment was performed on an orbital platform shaker (Fisher Scientific, Fair Lawn, NJ) set at 120 rpm for 1 h. Fractions were then centrifuged at 4°C for 30 min at 10,200 x g (Beckman Instruments, Palo Alto, CA) to collect the supernatants.

Concentration and Desalting. Microsep™ 1-kDa MWCO centrifugal devices (Omega™, low protein-binding modified polyethersulfone; Pall/Gelman Sciences, Ann Arbor, MI) were used to simultaneously concentrate and desalt the IMAC wash and eluent fractions. Samples were centrifuged at 4°C for 30 min at 7,500 x g (Beckman Instruments, Palo Alto, CA). To achieve sufficient concentration and desalting of each sample, once 90% of the volume had filtered, nanopure water was used to resuspend the remaining solution, which was then recentrifuged. This process was repeated five times per sample.

Cell Culture. Caco-2 cells were purchased at passage 17 from American Type Culture Collection (Rockville, MD). Experiments were conducted at passages 33-36. Cells were

grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 1% nonessential amino acids (Gibco BRL, Grand Island, NY), and 1% antibiotic-antimycotic solution (Gibco BRL, Grand Island, NY). Cells were maintained at 37°C in an air incubator with 5% CO_2. Media was changed every 3 d. Once reaching 80-100% confluence, the cells were rinsed with Earle's Balanced Salt Solution (EBSS; Gibco BRL, Grand Island, NY), dissociated using trypsin-EDTA (Gibco BRL, Grand Island, NY), and centrifuged for 6 min at 22.6 x g (Fisher Scientific, Fair Lawn, NJ). Cells were then seeded in a 75-cm^2 culture flask (Corning Costar Corporation, Cambridge, MA) at a density of 5,600 cells/cm^2 for continued growth. For iron uptake experiments, the cells were seeded onto collagen-treated 24-well cluster plates with 15.5 mm diameter (Corning Costar Corporation, Cambridge, MA) at a density of 50,000 cells/cm^2. Collagen treatment of the 24 wells was performed by adding 40 µL of 3.76% NaCl to 1 mL of a purified collagen stock solution [40 µg collagen (Type I Rat Tail Collagen; Collaborative Biomedical Products, Bedford, MA) per mL sterile deionized water] per well (Methods in Enzymology, 1979). The 24-well plate was then placed in the air incubator with 5% CO_2 overnight (~18 h). After incubation, the collagen solution was removed and the plate was allowed to dry under sterile conditions immediately prior to use. Cells were used for iron uptake experiments at 14-15 d post-seeding (Glahn et al. 1997). To confirm that total cellular protein content in each of the 24 wells did not vary, cell monolayers were solubilized in 0.5 mol/L NaOH (Fisher Scientific, Fair Lawn, NJ) and sonicated using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA) at a setting of 2. The protein concentration of the sonicated solutions was then determined (Lowry et al., 1951).

^{59}Fe Cell Uptake Procedures. Uptake solutions were prepared in EBSS with 10 mmol MES (2-[4-morpholino]-ethane sulfonic acid; Fisher Scientific, Fair Lawn, NJ) buffer at pH 6.0 immediately prior to use. Uptake solutions containing 20 µmol/L Fe were prepared by adding a defined amount of ferric chloride ($FeCl_3 \cdot 6H_2O$, 1 mmol/L in 10 mmol/L HCl) to EBSS/MES with either ascorbic acid (ASC) (ferric:ascorbate; 1:20 molar ratio), nitrilotriacetic acid (NTA) (ferric:NTA; 1:5 molar ratio), no added ligand (blank), or test solutions (1 or 2 mg of beef protein per mL). The ASC was used as a positive control, NTA as a negative control, and $FeCl_3$ only as the blank. Test samples refer to the digestate supernatant (DS), 10KR, 1KR, 1KF, IMAC wash, or IMAC eluent fractions (peaks 1-4). Radioactive iron was purchased as $^{59}FeCl_3$ (DuPont/NEN, Boston, MA) and a trace amount (0.48 ng of Fe containing 277.5 Bq of ^{59}Fe in 10 mmol/L HCl) was added to each of the uptake solutions, which were then immediately vortexed and used in the cell uptake procedures.

Measurement of radioiron uptake was performed by modification of the method outlined by Glahn et al. (1997) with EBSS/MES at pH 6.0 instead of HBSS/PIPES (piperazine-N,N'-bis-[2-ethanesulfonic acid]) at pH 6.7. Fifteen days after seeding, growth medium was removed from each well and the cell monolayers rinsed twice with 500 µL of EBSS/MES at 37°C and pH 6.0. A 300 µL aliquot of ^{59}Fe cell uptake solution was then placed on the cell monolayer and another 300 µL aliquot was used to measure initial radioactivity. The monolayers were then placed in an air incubator at 37°C with 5% CO_2 for 1 h. After incubation, iron uptake was terminated by removing the uptake solution and immediately rinsing the monolayers three times with 500 µL of stop solution (140 mmol/L NaCl, 5 mmol/L KCl (Fisher Scientific, Fair Lawn, NJ), and 10 mmol/L MES) at 20°C and pH 6.0.

Nonspecifically bound iron was removed using the method described by Glahn et al. (1998). After the cells were rinsed again with stop solution, monolayers were solubilized in 1 mL of 0.5 mol/L NaOH and placed in scintillation tubes for ^{59}Fe counting using a gamma scintillation counter (Auto-Gamma™; Packard Instrument Company, Meriden, CT). Total radioactivity was measured in all of the solutions. Percent ^{59}Fe recovery was measured with respect to its initial radioactivity.

Solubility Assays. Iron solubility was also measured in the uptake solutions. After 300 µL was applied to the cell monolayer, 1.2 mL of each cell uptake solution containing ^{59}Fe was placed into a microcentrifuge tube and incubated for 30 min at ambient temperature. Samples were then centrifuged for 15 min at 15,000 x g using a microcentrifuge (Brinkmann Instruments, Westbury, NY). A 500 µL portion of the supernatant was used for radioisotope counting. Percent solubility was calculated with respect to iron (20 µmol/L $FeCl_3$ in 10 mmol/L HCl) solubility at pH 2.0.

Electrophoresis. Separation of proteins and peptides present in the digest supernatant, ultrafiltration fractions, and the IMAC wash and eluent fractions was performed by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Tris/Tricine/SDS buffer (Bio-Rad Laboratories, Hercules, CA). A precast gel consisting of 10-20% Tris-Tricine (NOVEX, San Diego, CA) was used for the digest supernatant and ultrafiltration fractions. A precast gel consisting of 16.5% Tris-Tricine (Bio-Rad Laboratories, Hercules, CA) was used for the 1KR and IMAC wash and eluent fractions to ensure detection and separation of the smaller molecular weight proteins. Low range (Bio-Rad Laboratories, Hercules, CA) and ultra-low range SDS-PAGE molecular weight markers were used for molecular weight estimations ranging from 1- to 100-kDa. Following electrophoresis, the

gels were rinsed for 10 min in DI water. Gels were then fixed in freshly prepared 5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) for 1 h and washed for 5 min three times using DI water. The gels were stained using 0.025% Coomassie Brilliant Blue G-250 (Eastman Kodak Company, Rochester, NY) in 10% acetic acid (Fisher Scientific, Fair Lawn, NJ) for 1 h. Destaining was done using 10% acetic acid (Fisher Scientific, Fair Lawn, NJ) overnight with several changes of this solution during the period.

Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Analysis. The MALDI-TOF mass analysis was performed to determine the molecular weight(s) of components in the IMAC wash and eluents. Test solutions were comprised of 1 μL sample solution (50 pmol protein per μL) mixed thoroughly with 24 μL of matrix solution (10 μg of α-cyano-4-hydroxycinnamic acid per mL). One μL of the test solution was then applied to the target region of the sample grid and allowed to crystallize. The MALDI-TOF analysis was performed using a Voyager - DE PRO (Perseptive Biosystems Inc./Perkin Elmer, Foster City, CA). Spectra were acquired at 50 shots/spectrum and a mass range of 0.5- to 20-kDa. Data Explorer software (version 3.4; Perseptive Biosystems, Inc.) was used to analyze the data.

Amino Acid Composition Analysis. The complete amino acid composition of the IMAC wash and eluents (P1-4) was determined at the University of Iowa (Molecular Analysis Facility, Iowa City, IA). Performic acid oxidation and hydrochloric acid hydrolysis were performed prior to anion-exchange analysis. Twenty μL of the IMAC wash or eluent sample solution (protein was 2 mg per mL for IMAC wash and 4 mg/mL for P1-4 in DI water) was used prior to evaporation. Norleucine was added to each sample as an internal standard. Samples were reconstituted with 100 μL of 6 N HCl and the dead air space purged with

argon gas to displace atmospheric oxygen before placing samples in an oven at 110°C for 24 h. Vacuum-evaporation was used to remove residual acid and the hydrolysates redissolved in sodium citrate diluent. Ion-exchange analysis (Beckman Instruments, Palo Alto, CA) was done with post-column derivatization using ninhydrin with separation on a 12 cm hydrolysate column using sodium citrate buffer in combination with a 3-step temperature program.

Statistical Analysis. Data were analyzed using one way Analysis of Variance (ANOVA) and the differences among group means tested using Tukey's multiple comparison test (Prism software, version 2.01; GraphPad Software Inc., San Diego, CA). The mean differences were considered statistically significant at $P \leq 0.05$. Unless otherwise noted, values are expressed as mean ± SEM.

RESULTS

The cooked, lyophilized beef powder contained 90.0 ± 0.2% (n=3) protein, 89.7 ± 3.4 ppm (n=2) total iron, and 32.7 ± 5.5 ppm (n=2) nonheme iron. Separation of the DS, 10KR, and 1KR by SDS-PAGE is shown in Figure 1. Preliminary results (data not shown) showed that the 1KF fraction was not detectable on the gel.

Figure 2A and **2B** show iron (^{59}Fe) solubility and uptake, respectively, for the blank, ASC and NTA controls, and test solutions (2 mg protein per mL) from the DS and ultrafiltration fractions. The addition of ASC or NTA maintained iron solubility (87.5% and 97.4%, respectively) at pH 6.0, whereas in the blank it was much less (8.7%). The presence of DS and the ultrafiltration fractions significantly ($P < 0.001$) improved iron solubility, 3 to 8-fold when compared to the blank. Of the ultrafiltration fractions, the 1KR maintained iron solubility at 43.8%, a significantly greater level than that of the 10KR ($P < 0.05$; 39.1%) or

1KF (29.1%; $P < 0.001$) fractions. Iron uptake by Caco-2 cells for the blank was low (8.6%). However, addition of ASC or NTA resulted in an increase (28.3%; $P < 0.001$) or decrease (3.1%; $P > 0.05$), respectively. In the presence of the 1KR fraction, iron uptake was 22.8%, 45.2%, and 56.5% greater than that of the DS, 10KR, and 1KF, respectively, and approximately two-fold higher than the blank ($P < 0.05$) and five-fold higher than NTA ($P < 0.001$).

Based on our iron solubility and uptake results, only the 1KR was chosen for further analysis by IMAC, which yielded a series of four peaks: three distinct peaks (P1, P3, P4) and one with a few closely associated peaks (P2) (**Fig. 3**). Iron solubility and uptake by Caco-2 cells in the presence of P1-P4 is shown in **Figure 4A** and **4B**. Due to low yield, 1 mg protein per mL was used in all test solutions. Iron solubility in the presence of DS was significantly ($P < 0.001$) than the blank, resulting in a 3-fold increase (from 8.7% to 27.3%). The IMAC wash and P1-P4 all conferred significantly higher iron solubility than the blank; from two- to five-fold ($P < 0.001$ for P2-P4; $P < 0.05$ for P1). The addition of ASC or NTA resulted in an increase (18.0%) or decrease (2.7%) in iron uptake when compared to the blank. Iron uptake in the presence of P2 and P4 increased significantly (47.7% and 40.1%, respectively) when compared to 8.5% for the blank ($P < 0.01$ and 0.05 for P2 and P4, respectively). When compared to NTA, P1-P4 were found to enhance iron uptake by three- to five-fold ($P < 0.001$). Although the IMAC wash increased iron solubility, it significantly ($P < 0.01$) decreased iron uptake by 60.5% when compared to the blank.

Electrophoretic separation of the 1KR and the IMAC wash and P1-P4 resulted in the detection of few distinct bands and revealed that each sample contained many peptides with molecular weights ranging from approximately 1- to 5-kDa (**Fig. 5**). Since the gel illustrated

that each sample was comprised of many peptides extremely close in size and shape, MALDI-TOF analysis was performed in an effort to distinguish the proteins. The MALDI-TOF analysis of the IMAC wash and P1-P4 (**Fig. 6A-D**) demonstrated that peptides in each sample differ minimally in size. Amino acid analysis (**Table 1**) revealed that histidine concentration was 14 to 32 times lower in the IMAC wash than in P2 to P4 and that it increased progressively from P1 to P4; ranging from 1.2 to 16.0 Mol%. **Figure 7** shows the relationship between histidine content and iron solubility and uptake in P1-P4. The content of cysteine was approximately three times greater in P1 (at 6.4 Mol%) than in the IMAC wash and P2-P4, whereas glutamine in the IMAC wash (at 20.1 Mol%) was approximately twice that of P1-P4. Lysine content of P1-P4 was 13 to 81% greater than the IMAC wash. Glycine content of the IMAC wash and P1-P4 was similar (mean 8.7 Mol%, ranging from 8-10 Mol%).

DISCUSSION

Identification of the factor(s) in meat that enhance nonheme iron absorption has been difficult since performing human studies for this type of investigation is often not an option due to the inconvenience and cost. Rat models are appealing for in vivo studies (Gordon and Godber 1989, Van Campen 1972), but extrapolation of iron absorption data from rats to humans is questionable since meat has shown no effect on nonheme iron absorption in rats as compared to humans (Reddy and Cook 1991). Other researchers have used equilibrium dialysis, as an in vitro method to assess iron bioavailability (Miller et al. 1981; Shen et al. 1994), but bioavailability as based on solubility or dialyzability alone has been shown to not accurately reflect uptake by the intestinal mucosa (Gangloff et al. 1996; Miller and Berner

1989). Iron uptake by Caco-2 cells offers a physiological means to measure iron absorption (Garcia et al. 1996; Glahn et al. 1996) and nonheme iron uptake by the cells has shown significant correlation to human iron absorption when many dietary factors are tested (Au and Reddy 2000). Based on the reliability of the Caco-2 cell system, we used this in vitro model to identify beef peptides that enhance nonheme iron bioavailability.

The enhancement of nonheme iron absorption by meat may involve chelation and solubilization (Zhang et al. 1990). Solubility was suggested to be a useful tool in predicting the enhancement of nonheme iron absorption (Slatkavitz and Clydesdale 1988, Carpenter and Mahoney 1989), but our data show that iron solubility does not reliably predict its uptake or bioavailability. For example, the solubility of iron in the presence of NTA was higher than ASC, but radioiron uptake by Caco-2 cells was much higher with ASC than NTA. Because NTA is a strong chelator of nonheme iron, it maintains the solubility of iron but does not permit its transfer to the mucosa, whereas ASC reduces and complexes with nonheme iron and allows for its exchange and subsequent uptake. Kapsokefalou and Miller (1991) proposed that components in meat released after digestion also have a reducing capability, similar to ascorbic acid based on an increase in the concentration of ferrous iron.

The influence of the IMAC wash and P2-P4 on nonheme iron solubility were not significantly different from one another or from DS. Our findings are in agreement with others who caution against the sole use of solubility as a predictor of iron absorption (Gangloff et al. 1996, Berner and Miller 1985). The affinity of a compound for iron and the type of complex formed may be equally important in determining the effect on nonheme iron absorption. If bound tightly, donation of iron to the mucosal cell may not occur, whereas if weakly bound, iron may dissociate and precipitate at the pH of the small intestine.

The effect of meat on nonheme iron absorption may be due to peptides released during proteolytic digestion, which are hypothesized to increase the solubility of inorganic iron (Kane and Miller 1984, Slatkavitz and Clydesdale 1988). On the contrary, others have found that the factor in meat responsible for enhanced nonheme iron absorption may not be related to gastrointesinal digestion (Au and Reddy 1997, Carpenter and Mahoney, 1989). Preliminary data in our lab (data not shown) suggested that, although undigested meat may possess factor(s) capable of solubilizing iron, the concentration of their counterparts in the digested sample is much greater. Therefore, we utilized proteolytic digestion to more closely simulate the physiologic condition and to increase the yield of proteins of interest. Although our data supports the findings of Slatkavitz and Clydesdale (1988), showing that proteolytic digestion of meat may result in the enhancement of nonheme iron absorption, it does not exclude the possibility that in the absence of proteolytic digestion, such factor(s) may exist.

We used copper as a ligand in IMAC based on the procedure described by Lönnerdal et al. (1977). Ferric iron forms polymers and precipitates within the optimal pH range that most proteins adsorb to the IMAC gel. Lönnerdal and Keen (1982) also used copper for purifying lactoferrin, an iron binding protein. Although use of IMAC did not produce separate peaks, it allowed us to separate peptides from the 1KR on the order of their affinity for copper. The solubility of iron increased from P1 to P4, matching copper binding affinity, indicating that IMAC using copper is a valid method.

Because proteins in the IMAC wash and P1-P4 were so similar in molecular weight (approximately 1- to 5-kDa), separation by gel electrophoresis and MALDI-TOF analysis could not be achieved. Since the sample was initially subjected to proteolytic digestion, this resulted in more closely associated protein fragments in the 1KR, perhaps differing by only

one amino acid. Since even a few metal atoms bound to any of the protein fragments present in the IMAC fractions would have added considerable mass and since the presence of metals may interfere with amino acid analysis, they were treated with Chelex-100 to ensure that they were essentially metal free.

Amino acid composition analysis shows that the IMAC wash and P1-P4 primarily differ in histidine, lysine, glutamine, and cysteine content. Of the amino acids that differed or showed a trend, histidine appears to be the best candidate for explaining the enhancement of iron solubility and radiorion uptake. Histidine increased progressively from P1 to P4. This result was not surprising since the affinity of these peptides to copper depends primarily on histidine. However, as histidine content of the fractions increased the percentage of soluble iron also increased, with a plateau after P3. Radioiron uptake was also enhanced with P2 and P4, which had greater histidine content than the IMAC wash and P1, which did not enhance uptake. Although no effect of histidine has been found on human iron absorption (Layrisse et al. 1984), our results support a study showing that histidine and lysine increase radioiron absorption when tested in rats (Van Campen and Gross 1969). Of the six amino acids tested (histidine, lysine, methionine, glutamic acid, glutamine, and glycine), only histidine and lysine significantly increased ^{59}Fe absorption. The same researchers later demonstrated that histidine increased ^{59}Fe retention, but only if ^{59}Fe and histidine were mixed together prior to administering them to the rats (Van Campen 1972) indicating the preformed complex plays a role in the enhancement iron absorption. In addition, decarboxylated histidine no longer enhances iron absorption in rats (Van Campen 1973), providing compelling evidence that histidine is involved in the enhancement of nonheme iron absorption. The biochemistry of histidine includes a pivitol role in the active binding sites of a number of iron-containing

enzymes and proteins of biological importance. Histidine often participates with other amino acids, such as lysine within these sites. The interaction between histidine and lysine has been shown to be responsible for iron binding in lactoferrin (Nicholson et al. 1997) and transferrin (He et al. 2000) and substitutions for these amino acids decreases the affinity of these proteins for iron.

Cysteine and reduced N-terminal cysteinyl-peptides have also been hypothesized to be responsible for the positive effect of meat on nonheme iron absorption (Taylor et al. 1986, Garcia et al. 1996; Glahn and Van Campen 1997). Taylor et al. (1986) found that oxidation of the sulfhydryl of cysteine and decreased human iron absorption. However, our data does not support the finding that cysteine enhances nonheme iron absorption. Methodological differences may be responsible for the differences among the results of other studies and ours.

In conclusion, our results suggest that the enhancing effect of beef on nonheme iron absorption may partially be due to low-molecular weight peptides produced during gastrointestinal digestion and that histidine content may be important. Further study to determine how histidine may enhance nonheme iron absorption and whether it's influence depends on the presence of other amino acids or in a specific sequence would be useful to understand the mechanism involved in the enhancement of nonheme iron absorption by meat.

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LITERATURE CITED

AOAC Official Methods of Analysis (1996) AOAC method 988.05: Protein (crude) in animal feed and pet food, $CuSO_4/TiO_2$ mixed catalyst kjeldahl method, and 960.52: Microchemical determination of nitrogen, micro-kjeldahl method. AOAC International Suppl. March Chapter 4 (p.13) and 12 (p.7).

Au, A. P. & Reddy, M. B. (2000) Caco-2 cells can be used to assess human iron bioavailability from a semipurified meal. J. Nutr. 130:1329-1334.

Bjorn-Rasmussen, E. & Hallberg, L. (1979) Effect of animal proteins on the absorption of food iron in man. Nutr. Metab. 23:192-202.

Bjorn-Rasmussen, E., Hallberg, L., Isaksson, B. & Arvidsson, B. (1974) Food iron absorption in man: Applications of the two-pool extrinsic tag method to measure heme and nonheme iron absorption from the whole diet. J. Clin. Invest. 53:247-255.

Carpenter, C. E. & Mahoney, A. W. (1992) Contributions of heme and nonheme iron to human nutrition. Crit. Reviews in Food Sci. and Nutr. 31(4):333-367.

Chidambaram, M., Reddy, M. B., Thompson, J. & Bates, G. W. (1989) In vitro studies of iron bioavailability: Probing the concentration and oxidation reduction reactivity of pinto bean iron with ferrous chromogens. Biol. Trace Elem. Res. 19:25-40.

Cook, J. D. & Monsen, E. R. (1975) Food iron absorption. I. Use of a semisynthetic diet to study absorption of nonheme iron. Am. J. Clin. Nutr. 28:1289-1295.

Cook, J. D. & Monsen, E. R. (1976) Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. Am. J. Clin. Nutr. 29:859-867.

Cook, J. D. & Monsen, E. R. (1977) Vitamin C, the common cold, and iron absorption. Am. J. Clin. Nutr. 30:235-241.

Cook, J. D., Morck, T. A. & Lynch, S. R. (1981) The inhibitory effect of soy products on nonheme iron absorption in man. Am. J. Clin. Nutr. 34:2622-2629.

Cook, J. D., Morck, T. A., Skikne, B. S. & Lynch, S. R. (1982) The importance of animal products in human iron nutrition. In Ch. 16, Academic Press, Inc., 332-335.

Disler, P., Lynch, S., Charlton, R., Torrance, J., Bothwell, T., Walker, R. & Mayet, R. (1975) The effect of tea on iron absorption. Gut 16:193-200.

Engelmann, M. D., Davidson, L., Sandstrom, B., Walczyk, T., Hurrell, R. F. & Michaelsen, K. F. (1998) The influence of meat on nonheme iron absorption in infants. Pediatr. Res. 43:768-773.

Gangloff, M. B., Glahn, R. P., Miller, D. D., Wien, E. M., Kapsokelou, M. & Van Campen, D. R. (1996) Assessment of iron availability using combined in vitro digestion and Caco-2 cell culture. Nutr. Res. 16:479-487.

Garcia M. N., Flowers C. & Cook J. D. The Caco-2 cell culture system can be used as a model to study food iron availability. J. Nutr. 1996:251-258.

Gillespie, S. & Johnston, J. L. (1998) Expert consultation on anemia determinants and interventions: proceedings. Micronutrient Initiative, Ottawa, Canada.

Gillooly, M., Bothwell, T., Torrance, J., MacPhail, A., Derman, D., Bezwoda, W., Mills, W., Charlton, R. & Mayet, F. (1983) The effect of organic acids, phytates and polyphenols on the absorption of iron from vegetables. Br. J. Nutr. 49:331-336.

Glahn, R. P., Lee, O. A. & Miller, D. D. (1999) In vitro digestion/Caco-2 cell culture model to determine optimal ascorbic acid to Fe ratio in rice cereal. J. Food Sci. 64(5):925-928.

Glahn, R. P. & Van Campen, D. R. (1997) Iron uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl-glycine. J. Nutr. :642-647.

Glahn, R. P., Wien, E. M., Van Campen, D. R. & Milller, D. D. (1996) Caco-2 cell iron uptake from meat and casein digests parallels in vivo studies: Use of a novel in vitro method for rapid estimation of iron bioavailability. J. Nutr. 126:332-339.

Gordon, D. T. & Godber, J. S. (1989) The enhancement of nonheme iron bioavailability by beef protein in the rat. J. Nutr. 119:446-452.

Hallberg, L. (1981) Bioavailability of dietary iron in man. Ann. Rev. Nutr. 1:123-147.

Hallberg, L., Brune, M. & Rossander, L. (1989) The role of vitamin C in iron absorption. Int. J. Vit. Nutr. Res., Suppl. 30:103-108.

Hallberg, L. & Rossander, L. (1984) Improvement of iron nutrition in developing countries: Comparison of adding meat, soy protein, ascorbic acid, citric acid, and ferrous sulphate on iron absorption from a simple Latin American-type meal. Am. J. Clin. Nutr. 39:577-583.

Hallberg, L., Rossander, L. & Skanberg, A. (1987) Phytate and the inhibitory effect of bran on iron absorption in man. Am. J. Clin. Nutr. 45:988-996.

He Q. Y., Mason A. B., Pakdaman R., Chasteen N. D., Dixon B. K., Tam B. M., Nguyen V., MacGillivray R. T., Woodworth R. C. (2000) Mutations at the histidine 249 ligand profoundly alter the spectral and iron-binding properties of human serum transferring N-lobe. Biochem. 15;39(6):1205-1210.

Hurrell, R., Lynch, S. R., Trinidad, T., Dassenko, S. S. & Cook, J. D. (1988) Iron absorption in humans: Bovine serum albumin compared with beef muscle and egg white. Am. J. Clin. Nutr. 47:102-107.

Johnson, P., Lukaski, H. & Korynta, E. (1992) Effects of stearic acid and beef tallow on iron utilization by the rat. P.D.E.B.M. 200:480-486.

Kane, A. P. & Miller, D. D. (1984) In vitro estimation of the effects of selected proteins on iron bioavailability. Am. J. Clin. Nutr. 39:393-401.

Kapsokefalou, M. & Miller, D. D. (1991) Effects of meat and selected food components on the valence of nonheme iron during in vitro digestion. J. Food Sci. 56:352-358.

Korman, M. G., Sovenly, C. & Hansky, J. (1971) Effect of food iron on serum gastrin evaluated by radioimmunoassay. Gut 12:619.

Layrisse, M., Cook, J. D., Martinez-Torres, C., Roche, M., Kuhn, I., Walker, R. & Finch, C. (1969) Food iron absorption: A comparison of vegetable and animal foods. Blood 33:430-443.

Lönnerdal, B., Carlsson, J. & Porath, J. (1977) Isolation of lactoferrin from human milk by metal-chelate affinity chromatography. FEBS Letters 75(1):89-92.

Lönnerdal, B. & Keen, C. L. (1982) Metal-chelate affinity chromatography of proteins. J. Appl. BioChem. 4:203-208.

Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Lukaski, H., McLaren, G., Johnson, P. & Smith, M. (1993) Enhanced intestinal iron absorption by dietary stearic acid: effect onmucosal iron kinetics. FASEB J. 7:A273.

Lynch, S. R., Hurrell, R. F., Dassenko, S. A., & Cook, J. D. (1989a) The effect of dietary proteins on iron bioavailability in man. Adv. Exp. Med. Biol. 249:117-132.

Lynch, S. R. & Cook, J. D. (1980) Interaction of vitamin C and iron. Ann. N.Y. Acad. Sci. 355:32-44.

Lynch, S. R., Dassenko, S. A., Cook, J. D., Juillerat, M. A. & Hurell, R. (1994) Inhibitory effect of a soybean-protein-related moiety on iron absorption in humans. Am. J. Clin. Nutr. 60:567-572.

Lynch, S. R., Skikne, B. S. & Cook, J. D. (1989b) Food iron absorption in ideopathic hemochromatosis. Blood 74,2187-2193.

Methods in Enzymology, Volume LVIII, (1979) Cell Culture. Eds. Jakoby, W.B. & Pastan, I. H. Academic Press, Inc., 526.

Miller, D. D. & Berner, L. A. (1989) Is solubility in vitro a reliable predictor of iron bioavailability? Biol. Trace Elem. Res. 19:11-24.

Miller, D., Schricker, B., Rasmussen, R. & Van campen, D. (1981) An in vitro method for estimation of iron availability from meals. Am. J. Clin. Nutr. 34:2248-2256.

Monsen, E. R. (1988) Iron nutrition and absorption: Dietary factors which impact iron bioavailability. J. Am. Diet. Assoc. 7860-7890.

Narasinga Rao, B. S. (1981) Physiology of iron absorption and supplementation. Brit. Med. Bull. 37:25-30.

Nicholson H., Anderson, B. F., Bland T., Shewry S. C., Tweedie J. W., Baker E. N. (1997) Mutagenesis of the histidine ligand in human lactoferrin: Iron bnding properties and crystal structure of the histidine-253 methionine mutant. Biochem. 14;34(2):341-346.

Pinto, M., Robine-Leon, S., Appay, M-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. & Zweibaum, A. (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol. Cell. 47:323-330.

Reddy, M. B., Hurrell, R. F., Juillerat M. A., & Cook, J. D. (1996) The influence of different protein sources on phytate inhibition of nonheme-iron absorption in humans. Am. J. Clin. Nutr. 63:203-207.

Shen, L., Luten, J., Robberrecht, H., Bindels, J. & Deelstra, H. (1994) Modification of an in-vitro method for estimating the bioavailability of zinc and calcium from foods. Z. Lebensm. Unters. Forsh. 199:442-445.

Slatkavitz, C. A. & Clydesdale, F. M. (1988) Solubility of inorganic iron as affected by proteolytic digestion. J. Clin. Nutr. 47:487-495.

Taylor, P. G., Martinez-Torres, M. A., Romano, E. L. & Layrisse, M. (1986) The effect of cysteine-containing peptides released during meat digestion on iron absorption in humans. Am J Clin Nutr 43:68-71.

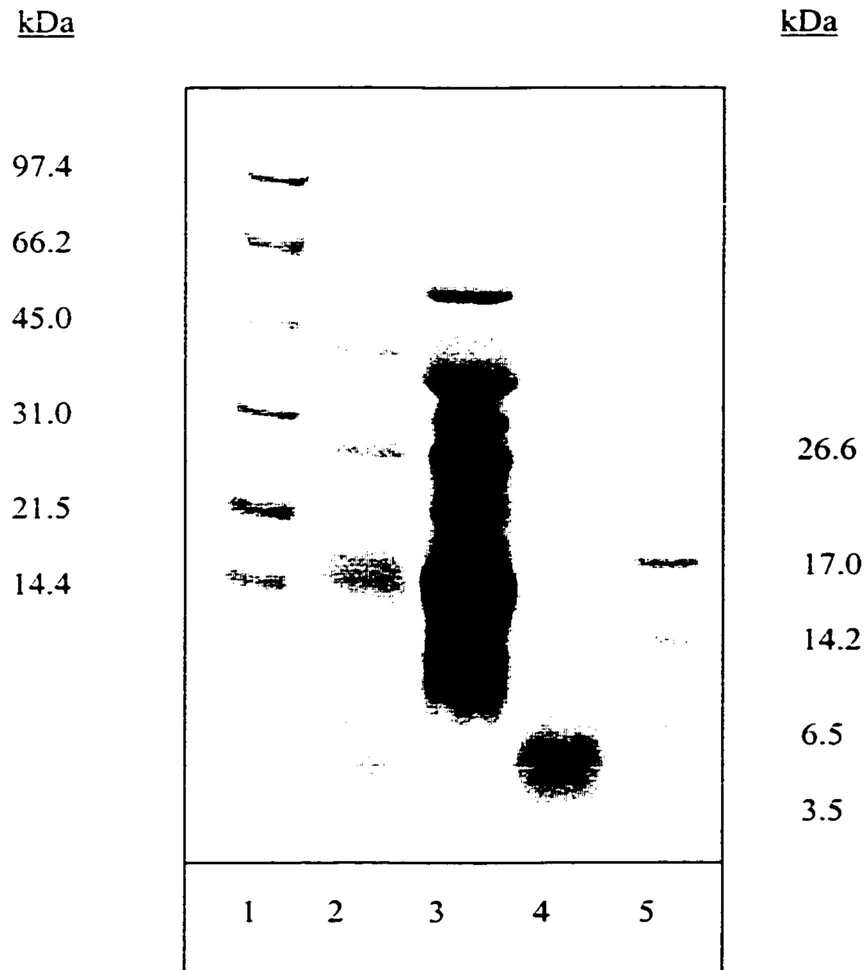
Torrance, J. D. & Bothwell, T. H. (1968) A simple technique for measuring storage iron concentrations in formalinized liver samples. S. Afr. J. Med. 33:9-11.

Van Campen, D. (1972) Effect of histidine and ascorbic acid on the absorption and retention of 59Fe by iron-depleted rats. J. Nutr. 102:165-170.

Van Campen, D. (1973) Enhancement of iron absorption from ligated segments of rat intestine by histidine, cysteine, and lysine: Effects of removing ionizing groups and of stereoisomerism. J. Nutr. 103:139-142.

Van Campen, D. & Gross, E. (1969) Effect of histidine and certain other amino acids on the absorption of ^{59}Fe by rats. J Nutr 99:68-74.

Zhang, D., Carpenter, C. E. & Mahoney, A W. (1990) A mechanistic hypothesis for meat enhancement of nonheme iron absorption: Stimulation of gastric secretions and iron chelation. Nutr. Res. 10:929-935.



Lane 1: Low range molecular weight standards
Lane 2: 22.5 μg digest supernatant
Lane 3: 22.5 μg 10KR
Lane 4: 22.5 μg 1KR
Lane 5: Ultra-low range molecular weight standards

Figure 1. SDS-PAGE (10-20 % Tris-Tricine) of digest supernatant and ultrafiltration fractions 10KR and 1KR.

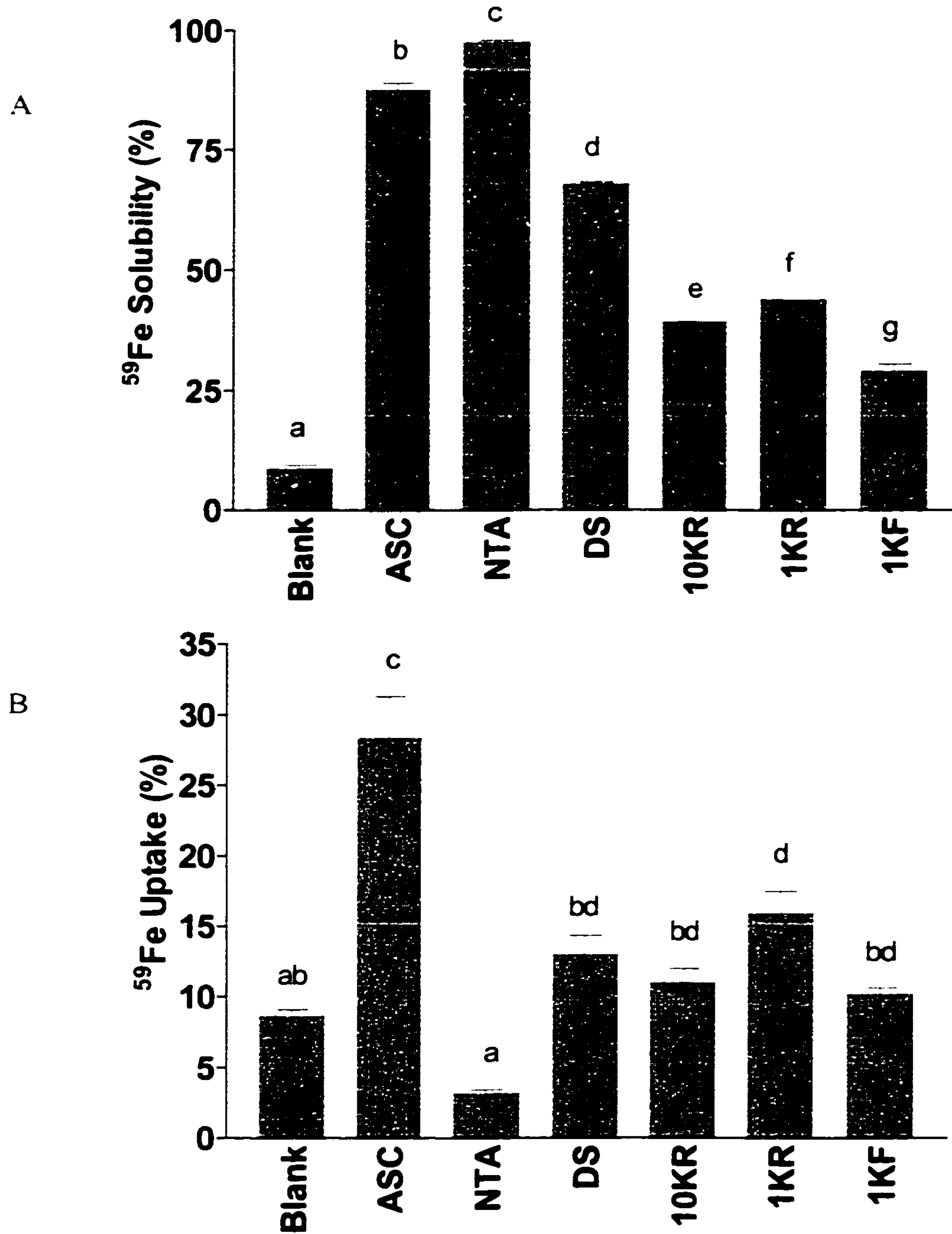


Figure 2. Iron solubility (A) and radioiron uptake by Caco-2 cell monolayers (B) in the presence of ferrous ascorbate (ASC) (Fe:ASC of 1:20 molar ratio), nitrilotriacetic acid (NTA) (Fe:NTA of 1:5 molar ratio), the digest supernatant (DS), and ultrafiltration fractions 10KR, 1KR, and 1KF. Blank consisted of $FeCl_3$ in EBSS/MES buffer alone (pH 6.0). Iron solubility was expressed as percent soluble iron compared to that at pH 2.0 using 0.01 N HCl. Each sample contained 20μM iron at intestinal pH (6.0) and 2 mg/ml protein. Bars (mean ± SEM, n=3 and n=6-9 for Fig. 2A and 2B, respectively) with different letters are significantly different at $p \leq 0.05$ when tested by ANOVA and Tukey's multiple comparison.

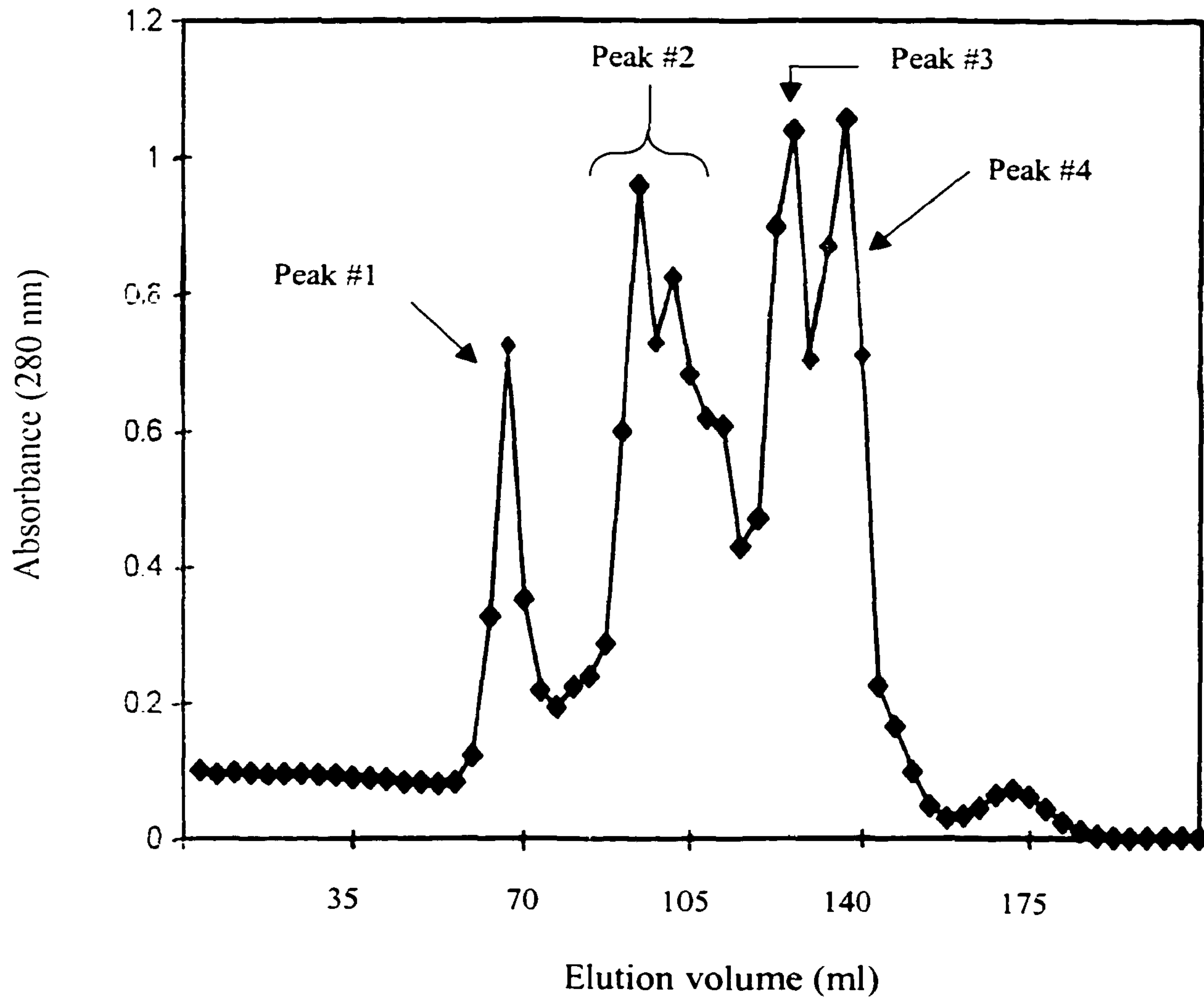


Figure 3. IMAC elution profile of 1KR showing collected fractions (3.5 mL/tube). Proteins were adsorbed at pH 8.1 and eluted by lowering the pH to 3.0 using phosphate buffer containing NaCl.

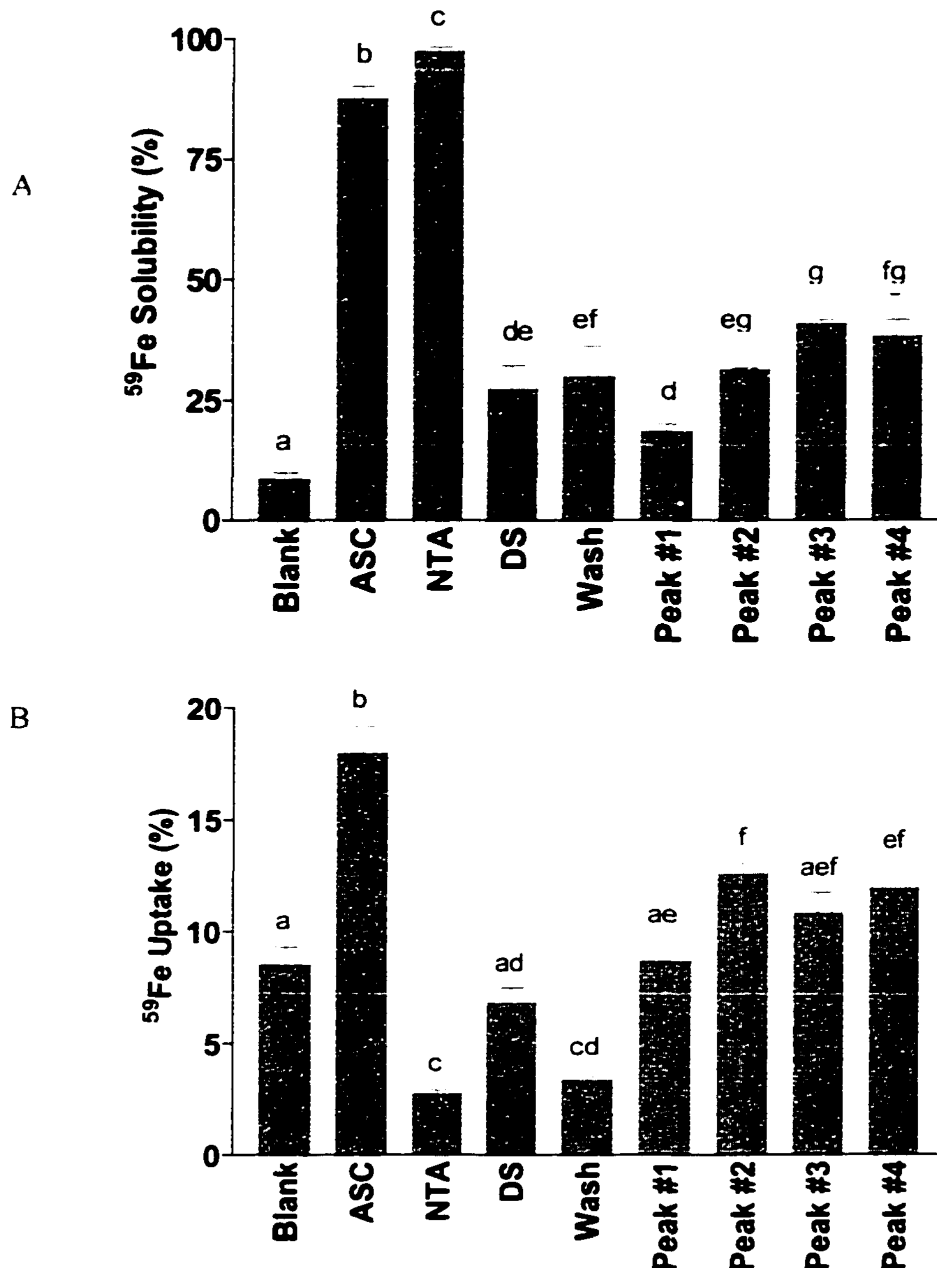
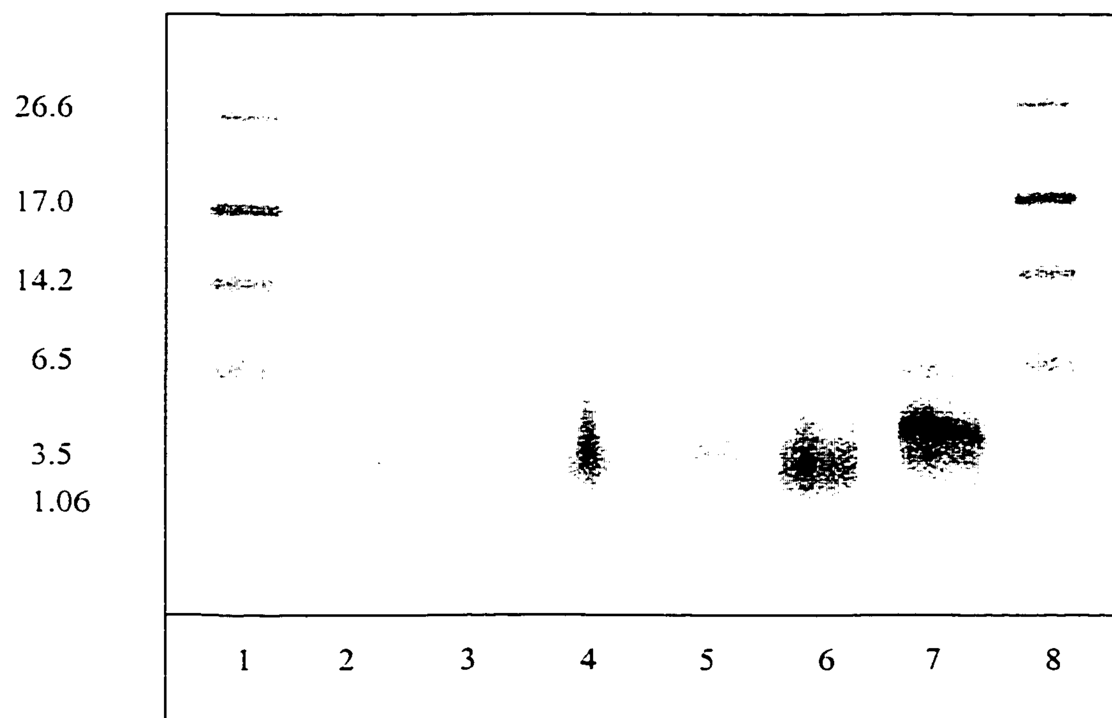
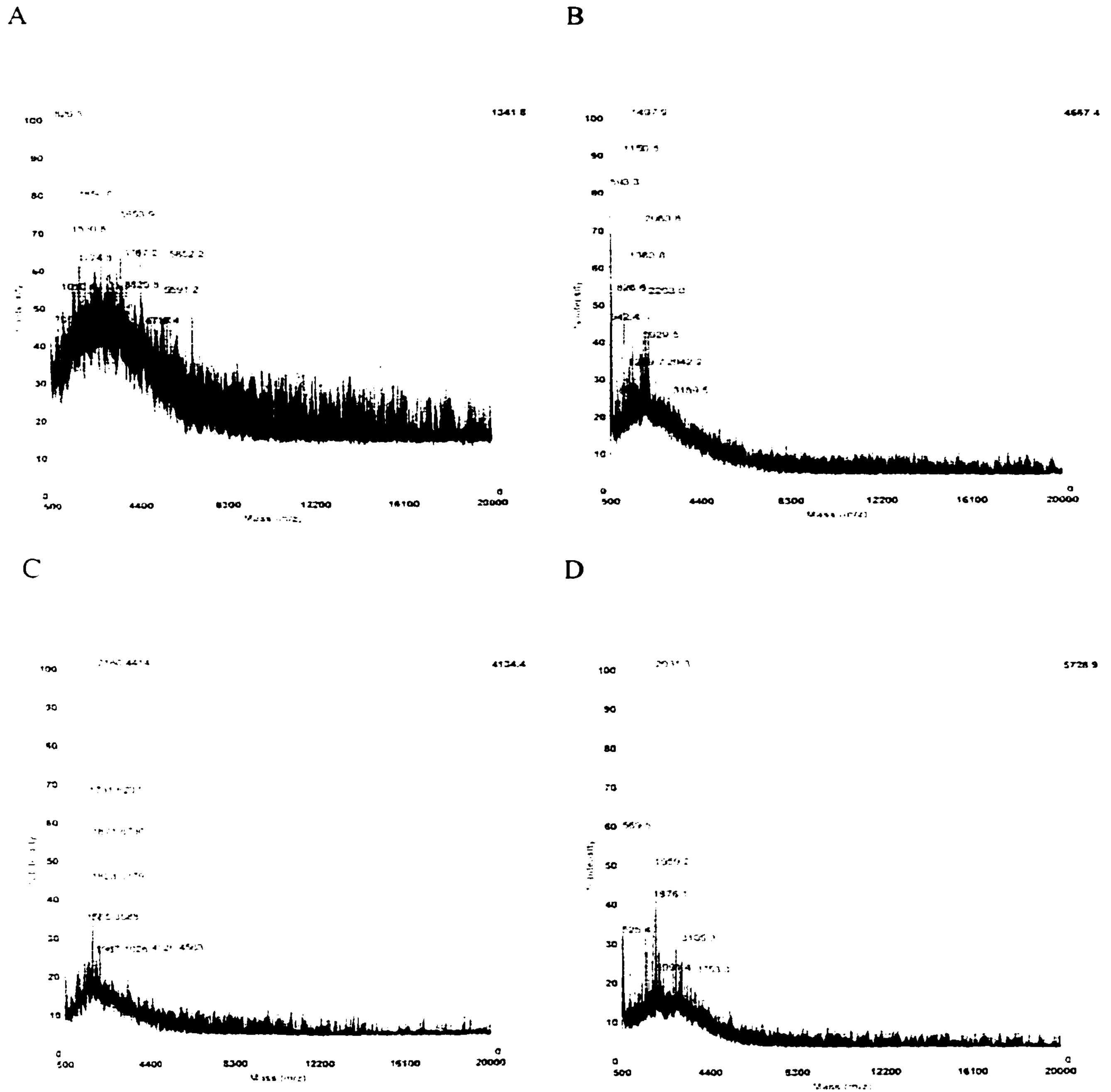


Figure 4. Iron solubility (A) and radioiron uptake by Caco-2 cell monolayers (B) in the presence of ferrous ascorbate (ASC) (Fe:ASC of 1:20 molar ratio), nitrilotriacetic acid (NTA) (Fe:NTA of 1:5 molar ratio), the digest supernatant (DS), and the IMAC wash and peaks #1-4 from the 1KR fraction. Blank consisted of $FeCl_3$ in EBSS/MES buffer alone (pH 6.0). Iron solubility was expressed as percent soluble iron as compared to that at pH 2.0 using HCl. Each sample contained 20μM iron at intestinal pH (6.0) and 1 mg/ml protein. Bars (mean ± SEM, n=3-4 and n=6-12 for Fig. 4A and 4B, respectively) with different letters are significantly different at $p \leq 0.05$ when tested by ANOVA and Tukey's multiple comparison.



Lanes 1& 8: Ultra-low range molecular weight standards
Lane 2: 4.5 μg 1KR
Lane 3: 7.5 μg IMAC wash (not detected)
Lane 4: 7.5 μg IMAC peak #1
Lane 5: 7.5 μg IMAC peak #2
Lane 6: 7.5 μg IMAC peak #3
Lane 7: 7.5 μg IMAC peak #4

Figure 5. SDS-PAGE (16.5 % Tris-Tricine) of 1KR and IMAC wash and peaks #1-4.



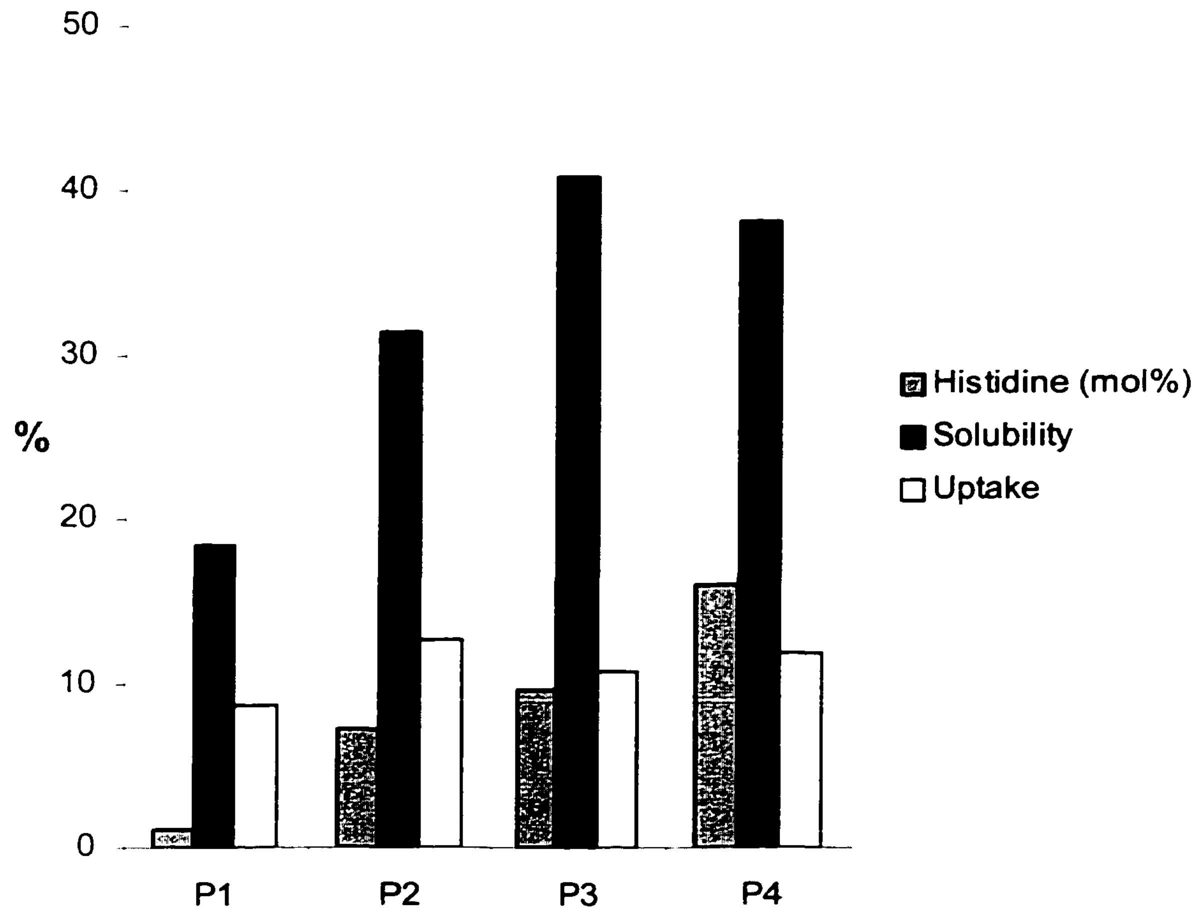
Figures 6. Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) analysis spectra of IMAC peaks #1-4 (A-D, respectively) from the 1KR fraction prepared using a α-cyano-4-hydroxycinnamic acid matrix. The IMAC wash spectrum was similar (data not shown, see Appendix E).

Table 1. Amino acid profile of IMAC wash and eluents from 1KR.

	Mol %				
Amino Acid	Wash	Peak #1	Peak #2	Peak #3	Peak #4
Cysteine[a]	1.7	6.4	1.7	2.7	1.6
Asx	13.7	11.4	10.0	10.0	11.1
Methionine[b]	1.9	1.4	1.7	1.8	1.1
Threonine	5.5	4.6	6.8	5.0	3.8
Serine	5.5	4.5	4.1	4.8	4.8
Glx	20.1	13.4	14.3	11.8	9.5
Proline	5.8	6.8	7.0	5.5	4.8
Glycine	9.0	10.0	8.7	8.0	8.0
Alanine	8.4	7.5	7.7	6.9	7.8
Valine	5.6	5.6	6.7	6.1	4.2
Isoleucine	3.9	4.3	4.7	3.5	2.8
Leucine	6.5	5.8	6.7	6.3	6.3
Tyrosine	1.5	1.3	1.2	2.4	1.6
Phenylalanine	1.4	1.4	1.3	2.8	2.5
Histidine	0.5	1.2	7.2	9.6	16.0
Lysine	6.9	11.5	7.8	9.8	12.5
Arginine	2.4	3.0	2.4	3.1	1.6

[a] Cysteic acid

[b] Methionine sulfoxide



Figures 7. Histidine (mol %), ^{59}Fe solubility, and ^{59}Fe uptake of IMAC P1-P4.

CHAPTER 3. IRON INDICES AND TOTAL ANTIOXIDANT STATUS IN RESPONSE TO SOY PROTEIN INTAKE IN PERIMENOPAUSAL WOMEN

A paper to be submitted to American Journal of Clinical Nutrition

James H. Swain[1], D. Lee Alekel[1], Sarah B. Dent[1], Charles T. Peterson[2], Manju B. Reddy[1]

Running Head: Total antioxidant and iron status during menopause

ABSTRACT

Background: Increased iron stores, oxidative stress, and estrogen deficiency place postmenopausal women at greater risk for heart disease and cancer.

Objective: To determine the effect of soy protein isolate (SPI) intake on iron indices and plasma total antioxidant status (TAS), and determine the influence of other factors on TAS in perimenopausal women.

Design: Perimenopausal women (N=69) were randomly assigned (double-blind) to treatment: isoflavone-rich soy protein isolate (SPI+; n=24), isoflavone-poor soy protein isolate (SPI-; n=24), or whey protein (control; n=21). Each subject consumed 40 g soy or whey protein/d for 24 weeks. Plasma TAS, serum ferritin, serum iron, transferrin saturation, and hemoglobin were measured at baseline, week 12, and week 24.

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Results: Treatment per se had no significant effect on serum ferritin, serum iron, and transferrin saturation. Time had an effect on serum ferritin ($P \leq 0.0001$) and TAS ($P \leq 0.0001$). At week 12, TAS was lower (P=0.035) in controls than SPI+, whereas no significant differences were found between SPI+ and SPI- at week 12 or 24. Multiple regression analysis revealed that at week 12, baseline TAS, alcohol intake, soy intake (soy versus control; P=0.016), plasma Lp(a), and dietary iron contributed to the variability (48%; $P \leq 0.0001$) in TAS. At week 24, 46% of the variability in TAS was contributed by baseline TAS, dietary meat, fish, and poultry (MFP) and zinc, serum ferritin and serum estrone.
Conclusions: SPI consumption had no significant effect on iron status, but our results suggest that soy intake and decreased iron stores may protect from oxidative stress in perimenopausal women.

KEY WORDS antioxidant status, ferritin, iron indices, lipoprotein(a), menopause, soy protein isolate

INTRODUCTION

Ample information is available on the iron status of pre- and postmenopausal women, but the change in iron status that occurs during the menopausal transition, which may last for several years, has not been studied. In addition, the effect of changes in body iron stores on the total antioxidant status of women during the menopausal transition is also not known. Oxidative status typically decreases with age (Jayachandran et al. 1996), whereas body iron stores generally increase, especially in women after menopause (Berge et al 1994). High iron stores have been hypothesized to induce oxidative stress due to the ability of iron to catalyze

the Haber-Weiss reaction, which produces the hydroxyl radical. This radical is highly reactive with all biomolecules and is considered to be very toxic, causing structural damage to macromolecules, such as proteins and lipids and breakage of DNA strands (Santanam et al. 1998). Generation of this radical in excess may deplete antioxidants (McCord 1993). This disturbance in the equilibrium between prooxidants and antioxidants may contribute to diseases, such as heart disease and cancer. Since epidemiological evidence suggests that increased iron stores place one at risk for developing heart disease (Salonen 1993; Kiechl et al. 1994) and cancer (Nelson et al. 1994), postmenopausal women may be at greater risk, similar to men (Sullivan 1981). Antioxidant defense systems, which include enzymes, plasma proteins, and dietary factors (Thomas 1999), react with radicals and minimize their damage (Halliwell 1993). These defense systems may be compromised during and after menopause and the concomitant increase in iron stores may increase such oxidative damage (Berliner and Heinecke 1996; Regnström et al. 1994).

Soy protein isolate (SPI) is used extensively by the food industry, where it is incorporated into a variety of processed foods (Erdman and Fordyce, 1989). Soy protein isolate is known to markedly inhibit nonheme iron absorption (Cook et al, 1981; Gillooly et al. 1983) primarily because of its high phytic acid content (Hurrell et al. 1992) and the protein moeity (Lynch et al. 1994). Thus, diets poor in animal proteins that enhance iron absorption and diets rich in soy may be inadequate for maintaining optimal iron status in populations at risk for developing iron deficiency, but beneficial to groups at risk of developing excess iron stores. In addition, naturally occurring isoflavones in soy may have antioxidant properties because of their ability to donate hydrogen atoms and/or electrons from their hydroxyl groups to free radicals, making them less reactive (Mitchell et al. 1998).

However, the ability of these isoflavones in vivo to affect TAS during menopause has not been documented. We hypothesized that consumption of SPI during the menopausal transition may reduce the rate of menopause-associated increases in iron stores, thereby increasing antioxidant status in perimenopausal women.

SUBJECTS AND METHODS

Research design and treatment

Perimenopausal women were recruited throughout the state of Iowa and bordering states. Subjects were included in the study if they met the following criteria: experiencing ≥ 10 hot flushes/wk, had irregular menses or menstrual period cessation, elevated follicle stimulating hormone (≥ 30 IU/L), a body mass index (BMI) between 20 to 31 (kg/m^2), were free from chronic disease, not excessively exercising (< 10.46 MJ expenditure/wk), had one or both ovaries remaining, able to participate for 24 weeks, and willing to be randomly assigned to treatment. Exclusion criteria included chronic disease and/or routine use of medications, smoking, alcohol abuse, estrogen or hormone replacement therapy during the prior 12 months, or a history of eating disorders. Women started the study in one of four waves or cohorts beginning in January 1997, May 1997, September 1997, or March 1998. The study protocol and consent forms were approved by the Iowa State University Human Subjects Review Committee (IRB# 01; Assurance ID# M1361).

In this double-blind 24-week study, sixty-nine women were randomly assigned to one of three treatment groups: isoflavone-rich soy protein (SPI+; n = 24; Protein Technologies International; St. Louis, MO), isoflavone-poor soy protein (SPI-; n = 24; Protein Technologies International; St. Louis, MO), or whey protein (n = 21; Ross Laboratories;

Columbus, OH) control. The women were free-living and supplied with a total of 40 g protein/d, with half of this protein incorporated into a muffin and the other half as a powder that subjects incorporated into food or beverage. Since the muffin and powder provided approximately 2.09 MJ (500 kcal)/d, subjects were instructed to consume these as a meal replacement and not a supplement. Muffins were prepared in the Human Metabolic Unit of the Center for Designing Foods to Improve Nutrition at Iowa State University. To control for variability in supplement intake among subjects, each participant was provided with a single daily over-the-counter vitamin and mineral supplement (Spring Valley Sentury-Vite™, Leiner Health Products Inc.; Carson, CA) and instructed not to take any other vitamin and/or supplement and to avoid food items throughout the study with soy containing isoflavones not provided.

Data collection

As described in Alekel et al. (2000), anthropometric measures, fasted blood draws, and 24-hour urine samples were collected for each subject at baseline, week 12, and week 24. Anthropometry data included measurement of height (using a stadiometer) and weight (using a balance beam scale [Health-o-meter, Inc.; Bridgeview, Illinois]). Dual-energy x-ray absorptiometry (QDR-2000+; Hologic, Inc.; Waltham, MA) was used to assess total body composition (lean and fat mass) by two trained researchers and the data analyzed using software (Version 7.10, 1992) provided by the manufacturer.

Dietary intake was assessed at baseline, week 12, and week 24 using five-d food records. To assist the subjects in quantifying portion sizes, two-dimensional food portion visual aids (Nutritional Consulting Enterprises, Morgan/Posner, 1981; Framingham, MA)

were provided. The food records were analyzed by trained nutrition students using the Nutritionist IV computerized nutrient database program (Version 4.1, 1995; San Bruno, CA).

Urine samples were aliquoted and frozen at -80°C for subsequent analysis of isoflavones (Fujicco, Inc.; Kobe, Japan) to monitor compliance. The serum and plasma from each blood draw was stored at -80°C until the serum ferritin, serum iron, transferrin saturation, lipids, serum reproductive hormones (follicle stimulating hormone, estrone, 17 β-estradiol), and plasma total antioxidant status (TAS) were determined. Hemoglobin concentration was determined in whole blood using the HemoCue® (HemoCue, Inc.; Mission Viejo, CA) system immediately following blood draws. Serum ferritin concentration was determined using an enzyme-linked immunoassay kit (RAMCO Laboratories; Houston, TX). Plasma TAS was determined using an assay kit according to the manufacturer's (Calbiochem-Novabiochem Corporation; La Jolla, CA) guidelines. Plasma TAS is measured colorimetrically and relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to •ABTS$^{®+}$ by metmyoglobin (a peroxidase). Quest Diagnostics (St. Louis, MO), a certified clinical laboratory, performed the 17 β-estradiol, estrone, total cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol measurements at each time point. LDL cholesterol concentrations were calculated using the Friedewald equation (Friedewald et al. 1972): (total cholesterol) – (HDL cholesterol) – (triacylglycerol/5). Plasma Lp(a) was analyzed at the University of Illinois at Chicago (Hematology and Coagulation Laboratory). Plasma Lp(a) [TintElize®Lp(a)] concentrations were measured at baseline, week 12, and week 24, using an immunoenzymetric method with affinity purified polyclonal antibodies against Lp(a),

according to the manufacturer's (Biopool, International; Ventura, CA) guidelines. Lp(a) concentrations were read using an automated microtiter plate reader (EL311sx, Bio-Tech Instruments, Inc.; Winooski, VT).

Statistical analyses

Statistical analyses were performed using SAS (Version 8.0, 1999; Cary, NC); results were considered statistically significant at $P<0.05$. Descriptive statistics include means for normally distributed data (age; body size and composition; serum iron and serum transferrin saturation) and medians for not normally distributed data (dietary intake of nutrients; serum ferritin; total serum cholesterol, triacylglycerol, LDL and HDL cholesterol, and Lp(a); estrone and 17 β-estradiol). Women with serum ferritin values of zero were assigned 0.5 μg/L for statistical purposes; exclusion of this data would have resulted in reduced statistical power. Repeated measures ANOVA was used to determine the effect of treatment on plasma TAS, serum ferritin, serum iron, transferrin saturation, and hemoglobin. Residual analysis indicated non-constancy of error variance (it increased with greater $\hat{Y}_i$ values) for the triacylglycerol, HDL cholesterol, and Lp(a) regression models. Thus, values for these measures were log-transformed for the repeated measures ANOVA and regression analyses. Log-transforming triacylglycerol, HDL cholesterol, and Lp(a) for the regression models markedly improved the residual plots. Stepwise multiple regression was used to determine the effect of contributors to plasma TAS at week 12 and week 24. Classes of variables in modeling plasma TAS at weeks 12 and 24 included baseline values for age, body size/composition (weight, lean mass, fat mass or percent body fat), dietary factors (total fat, polyunsaturated fat, alcohol, meat [beef, lamb, and pork], fish, and poultry (MFP), vitamin A, vitamin C, vitamin E or alpha-tocopherol, iron, and zinc), iron indices (serum iron and

serum ferritin), and serum estrogens (17 β-estradiol and estrone). Violation of model assumptions was not evident, since residual analyses indicated that the model assumptions of independence of residuals, normality of error terms, and homogeneity of residual variance were satisfied for these regression models. No notable multicollinearities emerged among the independent variables, as indicated by the low variance inflation factors in the regression analyses.

RESULTS

Compliance

Compliance was based on reported intake by the women and corroborated by the urinary excretion of isoflavones (Alekel et al. 2000). Eighty seven percent of the women (60 of 69 women) reported consuming 100% of the muffins and 84% (58 of 69) reported consuming 100% of the powder throughout the study. One woman's data from the control group was removed from all analysis because her urinary excretion of isoflavones during the treatment period was similar to that of subjects in the isoflavone-rich soy group.

Baseline measures

Baseline characteristics of the women are presented in **Table 1**, which includes anthropometric measures, dietary intake of selected nutrients, and serum measures. Since the characteristics at baseline were not significantly different among the three treatment groups, reported mean or median values represent all the women in the study. Alcohol intake and serum estrogens varied widely among subjects (Table 1).

Iron indices and antioxidant status

Serum iron and transferrin saturation declined from baseline to week 24 in the SPI+ and SPI- groups, whereas these iron indices increased in the control group (**Table 2**). The mean serum iron concentration was 14.7 μmol/L in the control group as compared to the groups consuming soy (SPI+ μmol/L = 16.4 and SPI- = 16.9 μmol/L), but the three groups had similar mean concentrations at the end of the study (14.6 to 15.0 μmol/L). By week 24, serum iron concentration decreased by approximately 11% from baseline in the two groups consuming SPI compared to a small increase of 2% in the control group. Similarly, transferrin saturation decreased by 2.6 to 7.2% from baseline in the two groups consuming SPI, whereas an increase of 9.5% was observed in the control group.

Treatment also did not affect hemoglobin, serum ferritin, and plasma TAS (**Figure 1**). Plasma TAS and hemoglobin values are presented as mean ± SEM, whereas serum ferritin values were not normally distributed and thus are presented as median, with the 25th and 75th percentile bars indicating variability. Figure 1 illustrates that time had a significant effect on hemoglobin (P=0.004), serum ferritin ($P \leq 0.0001$), and plasma TAS ($P \leq 0.0001$). Although the control group had a lower mean hemoglobin value than the groups consuming SPI, the differences were not significantly different and at week 24 the groups had similar values. Hemoglobin increased only in controls, possibly because of the marginally lower (albeit NS; P=0.052) values these subjects had at baseline. The serum ferritin value for each group generally increased by week 24, but there were no significant differences among the groups during the study. At week 12, plasma TAS was significantly lower (P=0.035) in controls than SPI+, but these differences did not remain significant at week 24 (P=0.057). No significant differences were found between SPI+ and SPI- either at week 12 or 24.

Since TAS was influenced significantly by treatment at week 12, we further explored the relationship between the factors in Table 1 and iron indices with plasma TAS. Since both soy treatments (SPI+ and SPI-) were not significantly different, these groups were combined for the regression analysis using contrast coding. In this context, "soy" refers to SPI+ and SPI- contrasted with the control group. Factors contributing to plasma TAS at week 12 and week 24 are presented in **Table 3**. In the regression model for week 12, the only significant treatment contrast (soy versus control; P=0.016) indicated that plasma TAS was affected positively by soy protein rather than by isoflavones. Other contrasts were not significant. At week 12, baseline plasma TAS, alcohol intake, soy intake, plasma Lp(a), and dietary iron accounted for 48% of the variability ($P \leq 0.0001$) in TAS. However, at week 24, baseline plasma TAS, dietary MFP and zinc, serum ferritin and serum estrone accounted for 46% of the variability in plasma TAS. A key finding was that at both weeks 12 and 24, baseline TAS was an important ($P \leq 0.0001$) contributor to plasma TAS.

DISCUSSION

At baseline, 13% of all the women were anemic (hemoglobin < 12 g/L), compared to 7% by week 24, suggesting that the incidence of anemia was reduced during the menopausal transition. This is particularly true in the control group (Figure 1). The increase in hemoglobin observed among control subjects may have been attributable to their lower baseline values compared to the other two groups. Regardless of treatment, there was a trend toward increased serum ferritin values, consistent with other studies (Berge et al., 1994; Penckofer and Schwertz 2000), showing age- and/or menopause-associated increases in serum ferritin. The increase in serum ferritin may have been due to decreased iron losses via

menstruation secondary to hormonal changes (Milman et al., 1992). Alternatively, the increase may have been influenced by the overall lower mean serum ferritin values observed in our study at baseline (28.6 ng/mL) compared to values reported by other researchers (Pilch and Senti 1984, Penckofer and Schwertz 2000). In our study, serum iron and transferrin saturation decreased with soy consumption, regardless of its isoflavone content, suggesting that the decrease in iron status may have been due to the inhibitory effect of phytic acid (Reddy et al. 1996) or the protein moiety itself (Lynch et al. 1994) on nonheme iron absorption. However, the decrease in these iron indices was not significant, possibly due to a relatively small sample size.

Oxidative status depends on the balance between prooxidants, such as iron, and antioxidants, such as enzymes, plasma proteins, and dietary factors (Rice-Evans and Burdon 1993). Plasma TAS is an overall indicator of oxidative status. Women in our study had baseline plasma TAS values ranging from 0.53 to 0.68 mM, apparently lower than values previously reported (1.3 to 1.8 mM) in humans (Miller et al. 1993). Because of the difference between the SPI+ and control group in plasma TAS at week 12, we performed a multiple regression analysis at weeks 12 and 24 to determine factors that may have influenced plasma TAS. Baseline plasma TAS, soy consumption, and alcohol intake favorably contributed to plasma TAS at week 12. The finding that baseline TAS had such a favorable association illustrates that women who began the study with higher plasma TAS maintained this status throughout the study. Soy intake, irrespective of isoflavone content, had a beneficial effect on TAS, perhaps in part because phytic acid is an antioxidant (Graf and Eaton 1990; Ko and Godin 1990) and/or because both phytic acid (Reddy et al. 1996) and the protein moiety (Lynch et al. 1994) reduce nonheme iron absorption, possibly

attenuating iron stores. The positive effect of alcohol on plasma TAS may have been due to the polyphenol content of alcoholic beverages. Phenolic compounds have been shown to have an antioxidant effect, as illustrated by their ability to inhibit the oxidation of human LDL in vitro (Frankel et al. 1995). The positive relationship of dietary MFP to TAS at week 24 may be related to the antioxidant properties of conjugated linoleic acid, the only known antioxidant associated primarily with animal foods (Pariza et al. 2000). Conjugated linoleic acid has been shown to inhibit free radical generation (Steinhart 1996), thereby providing a plausible explanation for the favorable effect we found of MFP on plasma TAS.

Plasma lipoprotein(a) and dietary iron were negatively associated with TAS at week 12, but not at week 24. Lipoprotein(a) is of increasing interest since it is considered both a marker of thrombotic and of atherosclerotic risk (Dobroski and Loscalzo 1996). Hormonal changes that occur during menopause may elevate lipoprotein(a) (Mijatovic et al. 1997). Elevated lipoprotein(a) concentrations are associated with an increased risk of atherosclerotic cardiovascular disease, a disease known to be associated with lipid peroxidation. This association may exist because greater lipoprotein(a) concentrations may provide more abundant substrate for reaction with radicals, which in turn may burden the antioxidant system, thereby decreasing plasma TAS. The inverse relationship between dietary iron and TAS is suggestive of the role of iron as a prooxidant, shown to promote lipid peroxidation in vitro (McCord 1993). Serum ferritin, serum estrone, and dietary zinc were negatively related to plasma TAS only at week 24. Our data support epidemiological studies showing an inverse relationship between serum ferritin and oxidative status and that lower iron stores may protect from oxidative stress (Berge et al. 1994; Keichl et al. 1994). The negative relationship between serum ferritin and plasma TAS may have reflected the overall increase

in iron stores by week 24 in these perimenopausal women, which may have been age- and/or menopause-related. Although we could not distinguish between an age- or menopause-related effect on iron stores, this latter idea is strengthened by the negative relationship between serum estrone and TAS in the regression model, since serum estrone may influence iron stores, and by finding that serum estrone increased ($P \leq 0.0001$) overall during the course of the study. The observation of increased serum estrone during our study may have been due to their relatively high fat mass (~37%) throughout the study. Adipose tissue is a primary site for peripheral aromatization of adrostenedione to estrone (Nordin 1981). Thus, we speculate that the increase in serum estrone may have reflected adipose tissue conversion from adrostenedione, and that the inverse relationship between estrone and TAS simply mirrored changes due to the menopausal transition. Although physiological doses of 17 β–estradiol have been shown to inhibit lipoprotein oxidation (Sack et al. 1994), estrone has not been shown to have antioxidant properties.

In contrast, it was surprising to observe an inverse relationship between dietary zinc and plasma TAS because zinc is an essential cofactor for many antioxidant enzymes, such as superoxide dismutase. Since zinc also binds to phytic acid (Zhou and Erdman 1995), dietary zinc competes intraluminally with iron (Solomons et al. 1983) for binding sites on phytic acid, which may have resulted in a greater proportion of iron available for absorption. Thus, as dietary zinc increases it may allow for greater nonheme iron absorption, thereby increasing serum ferritin and decreasing TAS. This hypothesis is consistent with the inverse relationship between dietary iron and TAS at week 12 and serum ferritin and TAS at week 24.

In conclusion, treatment had no significant influence on iron indices during the 24-week study period, but soy intake had a beneficial effect on TAS at week 12 in these perimenopausal women. Our data suggest that lower iron stores and soy protein isolate, regardless of its isoflavone content, may enhance antioxidant status. However, there may also be other factors that we did not assess, but influence iron status and play a major role in determining TAS in perimenopausal women. Our data also indicate that some perimenopausal women had low serum ferritin and may not yet be at risk of compromised oxidative status associated with higher iron stores typically found in postmenopausal women. In addition, women entering menopause with greater TAS were more likely to maintain this status throughout the study. These subjects were a self-selected group of mid-life Iowa women seeking an alternative to traditional hormone replacement therapy. Thus, we must be cautious in applying the results of this small-scale study to perimenopausal women in general. Future studies are needed to explore preventative measures aimed at controlling menopause- and age-related increases in body iron, thereby maintaining optimum TAS.

REFERENCES

Alekel DL, St. Germain A, Peterson CT, Hanson KB, Stewart JW, Toda T. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of postmenopausal women. Am J Clin Nutr 2000;72(3):844-52.

Berge LN, Bonaa KH, Norday A. Serum ferritin, sex hormones, and cardiovascular risk factors in healthy women. Arterioscler Thromb 1994;14:857-61.

Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. Free Rad Biol Med 1996;20(5):707-27.

Cook JD, Morck TA, Lynch SR. The inhibitory effect of soy products on nonheme iron absorption in man. Am J Clin Nutr 1981;34:2622-9.

Dobroski DR, Loscalzo J. Thrombotic risk factors for atherosclerosis. Coronary Artery Disease 1996;7:919-32.

Erdman JW, Fordyce EJ. Soy products and the human diet. Am J Clin Nutr 1989;49:725-37.

Frankel E, Waterhouse A, Teissedre P. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. J Agric Food Chem 1995;43:890-4.

Gillooly M, Bothwell T, Torrance J, MacPhail A, Derman D, Bezwoda W, Mills W, Charlton R, Mayet F. The effect of organic acids, phytates and polyphenols on the absorption of iron from vegetables. Br J Nutr 1983;49:331-6.

Graf E, Eaton JW. Antioxidant functions of phytic acid. Free Radical Biol. Med. 1990;8:61-9.

Halliwell B. The role of oxygen radicals in human disease, with particular reference to the vascular system. Haemostasis 1993;23 Suppl1:118-26.

Hurrell RF, Juillerat M-A, Reddy MB, Lynch SR, Dasenko SA, Cook JD. Soy protein, phytate, and iron absorption in humans. Am J Clin Nutr 1992;56:573-8.

Jayachandran M, Jayanthi B, Sundaravadivel B, Panneerselvan C. Status oflipids, lipid peroxidation, and antioxidant systems with Vitamin C supplementation during aging in rats. J Nutr Biochem 1996;7:270-5.

Kiechl S, Aichner F, Gerstenbrand F, Egger G, Mair A, Rungger G, Spogler F, Jarosch E, Oberhollenzer F, Willeit J. Body iron stores and presence of carotid atherosclerosis. Arterioscler Thromb 1994;14:1625-30.

Ko KM, Godin DV. Ferric ion-induced lipid peroxidation in erythrocyte membrane: effects of phytic acid and butylated hydroxytoluene. Molec Cell Biochem 1990;95:125-31.

Lynch SR, Dassenko SA, Cook JD, Juillerat MA, Hurell R. Inhibitory effect of a soybean-protein-related moiety on iron absorption in humans. Am J Clin Nutr 1994;60:567-72.

McCord JM. Human disease, free radicals, and the oxidant/antioxidant balance. Clin Biochem 1993;26(5):351-7.

Mijatovic V, Kenemans P, Netelenbos JC, Peters-Muller ER, van Kamp GJ, Voetberg GA, van de Weijer PH, van der Mooren MJU. Oral 17 beta-estradiol continuously combined with dydrogesterone lowers serum lipoprotein(a) concentrations in health postmenopausal women. J Clin Endocrinol Metab 1997;82(11):3543-7.

Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for

measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sce (Colch) 1993;84(4):407-12.

Milman N, Kirchoff M, Jorgensen T. Iron status markers, serum ferritin and hemoglobin in 1359 Danish women in relation to menstruation, hormonal contraception, parity, and postmenopausal hormone treatment. Ann Hematol 1992;65:96-102.

Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, Duthie GG. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch Biochem Biophys 1998;360(1):142-8.

Nelson RL, Davis FG, Sutter E, Sobin LH, Kikendall JW, Bowen P. Body iron stores and risk of colonic neoplasia. J Nat Cancer Inst 1994;86(6):455-60.

Nordin BE, Crilly RG, Marshall DH, Barkworth SA Oestrogens, the menopause and the andrenopause. J Endocrinol 1981;89 Suppl:131P-43P.

Pariza MW, Park Y, Cook ME. Mechanisms of action of conjugated linoleic acid: evidence and speculation. Proc Soc Exp Biol Med 2000;223:8–13.

Penckofer S, Schwertz D. Improved iron status parameters may be a benefit of hormone replacement therapy. J Women Health Gen Med 2000;9(2):141-51.

Pilch SM, Senti FR, eds. Assessment of the iron nutritional status of the US population based on data collected in the second National Health and Nutrition Examination Survey, 1976-1980. Rockville, MD: Life Sciences Research Office, FASEB J 1984.

Reddy MB, Hurrell RF, Juillerat MA, Cook JD. The influence of different protein sources on phytate inhibition of nonheme-iron absorption in humans. Am J Clin Nutr 1996;63:203-7.

Regnström J, Walldius G, Hådell K, Johansson J, Holme I, Olsson AG, Nilsson J. Influence of lipoprotein lipids, dietary fat and smoking on macrophage degradation of native and oxidized low density lipoprotein. Atherosclerosis 1994;110:13-23.

Rice-Evans CA, Burdon R. Free radical-lipid interactions and their pathological consequences. Prog Lipid Res 1993;32(1):71-110.

Sack MN, Rader DJ, Cannon RO 3rd. Oestrogen and inhibition of oxidation of low-density lipoproteins in postmenopausal women. Lancet 1994;343:269-70.

Salonen JT. The role of iron as a cardiovascular risk factor. Curr Opin Lipidol 1993;4:277-82.

Santanam N, Ramachandran S, Parthasarathy S. Oxygen radicals, antioxidants, and lipid peroxidation. Semin Reprod Endocrinol 1998;16(4) 275-80.

SAS Institute Inc. SAS/STAT users guide. Version 8.0. Cary, NC: SAS Institute Inc, 1999.

Solomons NW, Pineda O, Viteri F, Sanstead HH. Studies on the bioavailability of zinc in humans: Mechanism of the intestinal interaction of nonheme iron and zinc. J Nutr 1983;113:337-49.

Steinhart C. Conjugated linoleic acid - the good news about animal fat. J Chem Ed 1996;73:A302-3.

Sullivan JL. Iron and the sex difference in heart disease risk. Lancet 1981;1:1293-4.

Thomas JA. Oxidative stress and oxidant defense. In: Shils ME, Olson JA, Shike M, Ross AC, eds. Modern Nutrition in Health and Disease. 9th ed. Baltimore, MD: Williams & Wilkins, 1999:751-60.

Zhou JR, Erdman JW Jr. Phytic acid in health and disease. Crit Revs Food Sci Nutr 1995;35(6):495-508.

Figure Legend

FIGURE 1. Concentrations of plasma total antioxidant status (TAS; mean ± SEM), serum ferritin (median; error bars indicate 25th and 75th percentiles since values are not normally distributed), and hemoglobin (mean ± SEM) are presented from baseline to week 24 in each of the three treatment groups of perimenopausal women: isoflavone-rich soy (SPI+ = ■; n = 24), isoflavone-poor soy (SPI- ◆; n = 24), and whey (control ●; n = 21) protein. Values were not significantly different among treatment groups at baseline. Repeated measures ANOVA indicated that there was a treatment effect only for hemoglobin (P=0.052) and a significant time effect on concentrations of hemoglobin (P=0.004). serum ferritin ($P \leq 0.0001$), and plasma TAS ($P \leq 0.0001$).

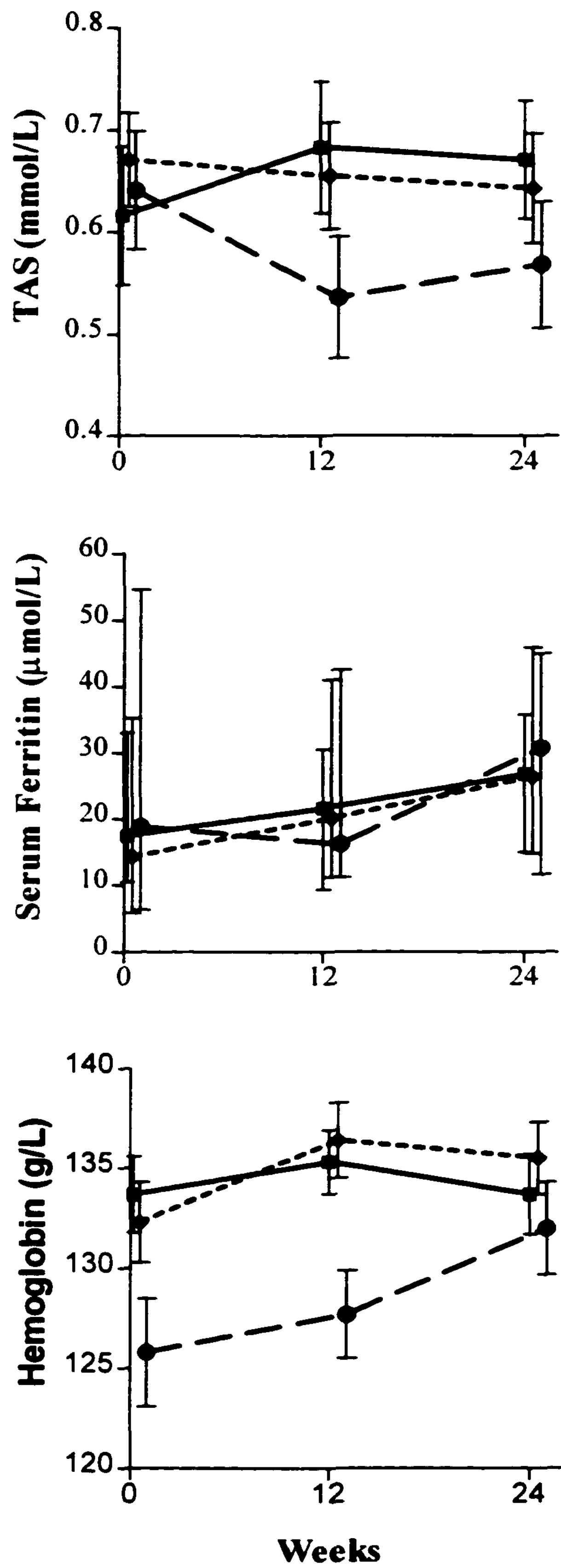

FIGURE 1.

TABLE 1

Baseline characteristics[1] in perimenopausal women

Measurement (n = 69)	**Mean±SD** (min-max)
Age (years)	50.2±3.6 (41.9-61.6)
Height (cm)	164.8±5.0 (151.6-177.5)
Weight (kg)	65.3±9.0 (48.5-98.1)
BMI (kg/m^2)	24.1±3.2 (17.4-33.7)
Lean mass (kg)[2]	39.8±6.8 (31.4-48.6)
Body fat (%)[2]	37.2±6.3 (18.4-53.1)
Dietary intake/d [3,4]	**Median** (min-max)
Vitamin E (mg)	10.7 (1.5-29.7)
Alcohol intake (g)[5]	162.1 (0.8-1131.3)
Iron (mg)	13.5 (5.8-25.7)

TABLE 1 (continued)

Zinc (mg)	8.4
	(2.8-43.9)
Dietary MFP (serving)[6]	1.2
	(0-2.5)
Serum[3]	**Median**
	(min-max)
Total cholesterol (mmol/L)	5.4
	(3.6-8.0)
Triacylglycerol (mmol/L)	1.0
	(0.5-4.5)
LDL cholesterol (mmol/L)	3.3
	(1.9-5.9)
HDL cholesterol (mmol/L)	1.5
	(0.9-2.9)
Lipoprotein(a) (nmol/L)	21.2
	(3.1-209.3)
Estrone (pmol/L)	122.1
	(37.0-447.6)
17 β-estradiol (pmol/L)	150.5
	(73.4-785.6)

[1] These characteristics at baseline were not significantly different among the three treatment groups

[2] Assessed by dual energy x-ray absorptiometry

[3] Median values are reported for serum analytes and dietary intake, since most were not normally distributed

TABLE 1 (continued)

[4] Dietary intake was assessed from a 5-d food diary collected prior to baseline testing

[5] Refers to 33 women who reported that they consumed alcohol while recording their food intake

[6] Refers to the collective contribution of meat (beef, lamb, and pork), fish, and poultry; 1 serving = 3 oz/d

TABLE 2

Transport iron indices of perimenopausal women at baseline, week 12, and week 24

Serum Measure[1]	Time (wk)	SPI+[2] (*n*=24)	SPI-[2] (*n*=24)	Control[2] (*n*=21)	Treatment	Time
					***P* value[3]**	
Iron (µmol/L)	0	16.9±6.2	16.4±6.2	14.7±5.2		
	12	15.3±5.3	15.1±6.9	14.7±4.8		
	24	15.0±4.3	14.6±6.1	15.0±5.8	0.75	0.39
% change[4]		-11.3	-11.0	+2.0		
Transferrin saturation (%)	0	29.2±10.6	27.0±11.6	26.2±10.1		
	12	26.4±10.0	25.6±12.2	26.7±9.2		
	24	27.1±7.7	26.3±10.6	28.7±9.4	0.94	0.64
% change[4]		-7.2	-2.6	+9.5		

[1] Mean ± SEM

[2] SPI+ = isoflavone-rich soy protein isolate; SPI- = isoflavone-poor soy protein isolate; control = whey protein

[3] *P* values based on repeated measures ANOVA

[4] % change = week 0 – week 24/week 0

TABLE 3

Regression analyses: Contributors to plasma total antioxidant status at week 12 and week 24

Total Antioxidant Status at Week 12

Overall Model R^2=48.3% (Adj. R^2=41.3%); $F(8, 67) = 6.88$; ($P \leq 0.0001$)

Independent Variable	Parameter Estimate	Percentage Variance[1]	*P* value[2]	Variance Inflation[3]
Intercept	0.5638		0.0005	
Total antioxidant status - baseline	0.5412	23.6	≤0.0001	1.15
Alcohol intake[4]	0.0125	5.7	0.013	1.21
Soy vs. control[5]	0.0961	5.4	0.016	1.37
Plasma lipoprotein(a)[4]	-0.0014	5.1	0.019	1.15
Dietary iron[4]	-0.0130	2.6	0.091	1.63

Total Antioxidant Status at Week 24

Overall Model R^2=45.7% (Adj. R^2=38.4%); $F(8, 68) = 6.30$; ($P \leq 0.0001$)

Independent Variable	Parameter Estimate	Percentage Variance[1]	*P* value[2]	Variance Inflation[3]
Intercept	0.5634		≤0.0001	
Total antioxidant status - baseline	0.4460	16.3	≤0.0001	1.20
Dietary MFP[6,7]	0.2234	10.3	0.001	1.29
Serum ferritin[7]	-0.0024	8.7	0.003	1.10
Serum estrone[7]	-0.0007	7.0	0.008	1.21

TABLE 3 (continued)

Dietary zinc[6]	-0.0253	4.9	0.024	1.13

[1] Squared semi-partial Type II correlation coefficient; accounts for shared variance among variables

[2] Variables left in models are significant at $P \leq 0.05$ level, except for dietary iron at $P \leq 0.1$

[3] Measures inflation in the variances of parameter estimates due to multicollinearities among regressors

[4] These independent variables reflect measures at week 12

[5] SPI+ plus SPI- vs. control

[6] Refers to the collective contribution of meat (beef, lamb, and pork), fish, and poultry; 1 serving = 3 oz/d

[7] These independent variables reflect measures at week 24

CHAPTER 4. GENERAL CONCLUSIONS

The objective of the first study was to isolate and characterize beef muscle proteins that enhance nonheme iron bioavailability. Our results suggest that the enhancing effect of beef on nonheme iron absorption may partially be due to low-molecular weight peptides produced during gastrointestinal digestion and that histidine content may be important. Further study to determine how histidine may enhance nonheme iron absorption and whether it's influence depends on the presence of other amino acids or in a specific sequence would be useful to understand the mechanism involved in the enhancement of nonheme iron absorption by meat.

The objective of the second study was to determine the effect of soy protein isolate (SPI) intake on iron indices and plasma total antioxidant status (TAS), while accounting for the effect of other factors on TAS in perimenopausal women. Treatment had no significant influence on iron indices during the 24-week study period, but soy intake had a beneficial effect on TAS at week 12 in these perimenopausal women. Our data suggest that lower iron stores and soy protein isolate, regardless of its isoflavone content, may enhance antioxidant status. However, there may also be other factors that we did not assess, but influence iron status and play a major role in determining TAS in perimenopausal women. Our data also indicate that some perimenopausal women had low serum ferritin and may not yet be at risk of compromised oxidative status associated with higher iron stores typically found in postmenopausal women. In addition, women entering menopause with greater TAS were more likely to maintain this status throughout the study. These subjects were a self-selected group of mid-life Iowa women seeking an alternative to traditional hormone replacement

therapy. Thus, we must be cautious in applying the results of this small-scale study to perimenopausal women in general. Future studies are needed to explore preventative measures aimed at controlling menopause- and age-related increases in body iron, thereby maintaining optimum TAS.

APPENDIX A. ELECTRON MICROSCOPY

Scanning Electron Microscopy (SEM): Caco-2 cell monolayer sections were fixed initially in 3% paraformaldehyde and 2%glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2) for 24 hrs 4°C. Monolayer sections were then subjected to secondary fixation using 1% osmium tetroxide in 0.05 M sodium phosphate buffer (pH 7.2) for 1 hr at room temperature. The sections were then washed with nanopure water, dehydrated in a graded ethanol series, and dried using a Denton DCP-1 critical point drying apparatus. Sections were then mounted on aluminum stubs and sputter coated using a Polaron SEM coating unit ES100 with a 60/40 palladium/gold target. Monolayer sections were viewed using a JEOL 5800LV scanning electron microscope. Digital images were captured and stored using ARC58 software (JEOL, Japan) (**Fig. 1A & B**).

Transmission Electron Microscopy (TEM): To facilitate sectioning, Caco-2 cell monolayer sections were fixed initially in 3% paraformaldehyde and 2%glutaraldehyde in cell culture media (10% fetal bovine serum, 0.82% amino acid solution, 0.82% antibiotic/antimycotic, 88.36% minimum essential media) (pH 7.4) for 24 hrs at 4°C. Monolayer sections were then subjected to secondary fixation using 1% osmium tetroxide in the cell media as described previously (pH 7.4) for 1 hr at room temperature. The sections were then washed with nanopure water, dehydrated in a graded ethanol series, and cleared with pure acetone prior to infiltration using EPON polyapoxy resin (Electron Microscopy Sciences, Fort Washington, PA). Sections 1 µm thick were prepared using a Reichert Ultracut-S and stained in 1% toluidine blue. Sections 50-70 nm were then collected on 200

mesh copper grids and stained for 20 min using 8% methanolic uranylacetate followed by SATO's lead stain. TEM images were captured using a JEOL 1200EX dual SEM/TEM (JEOL, Japan) (**Fig. 3 & 4**).

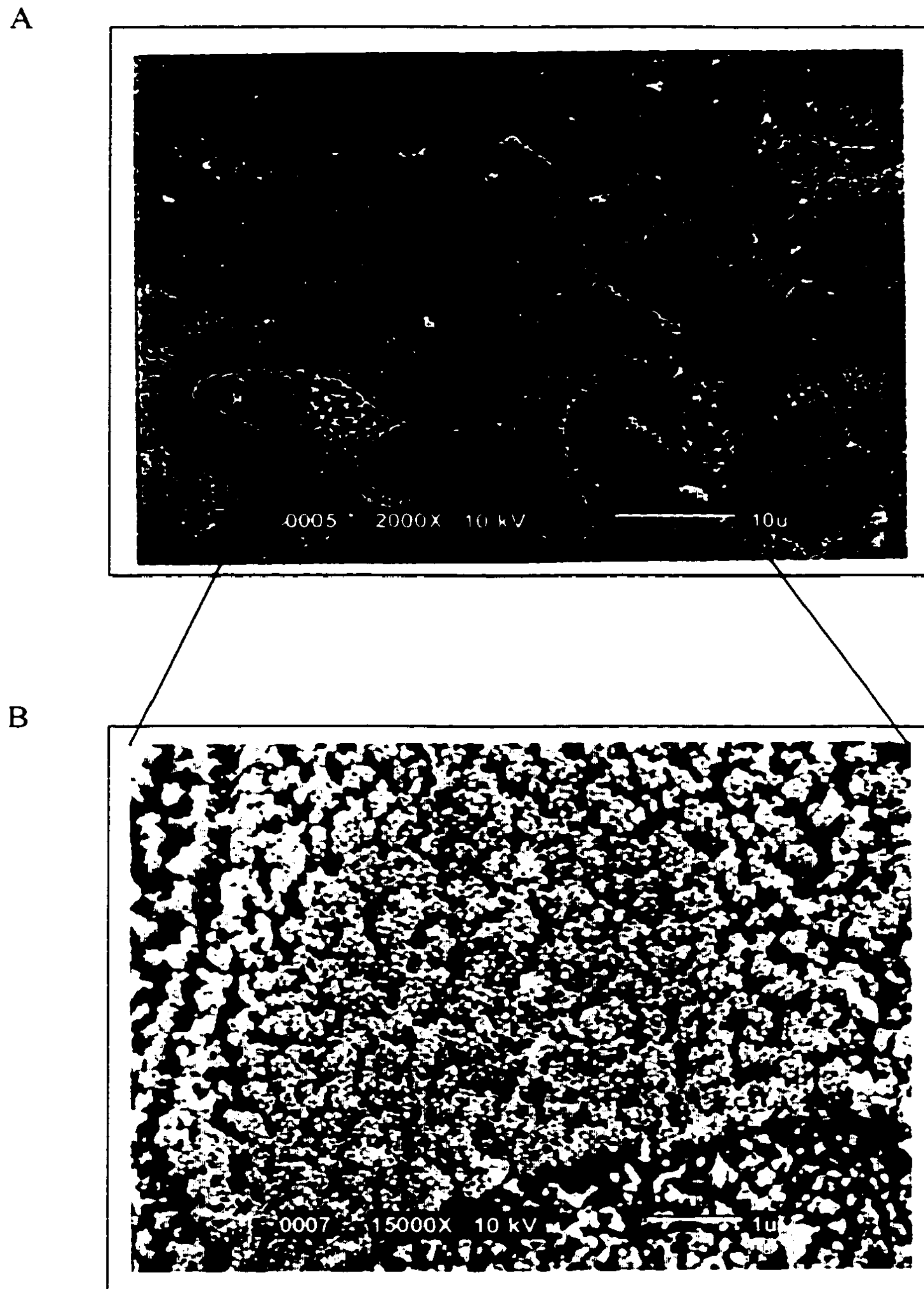

Figure 1A & 1B. Scanning electron micrographs showing monolayer of confluent differentiated Caco-2 cells with dense surface villi at 2K and 15K x magnification.

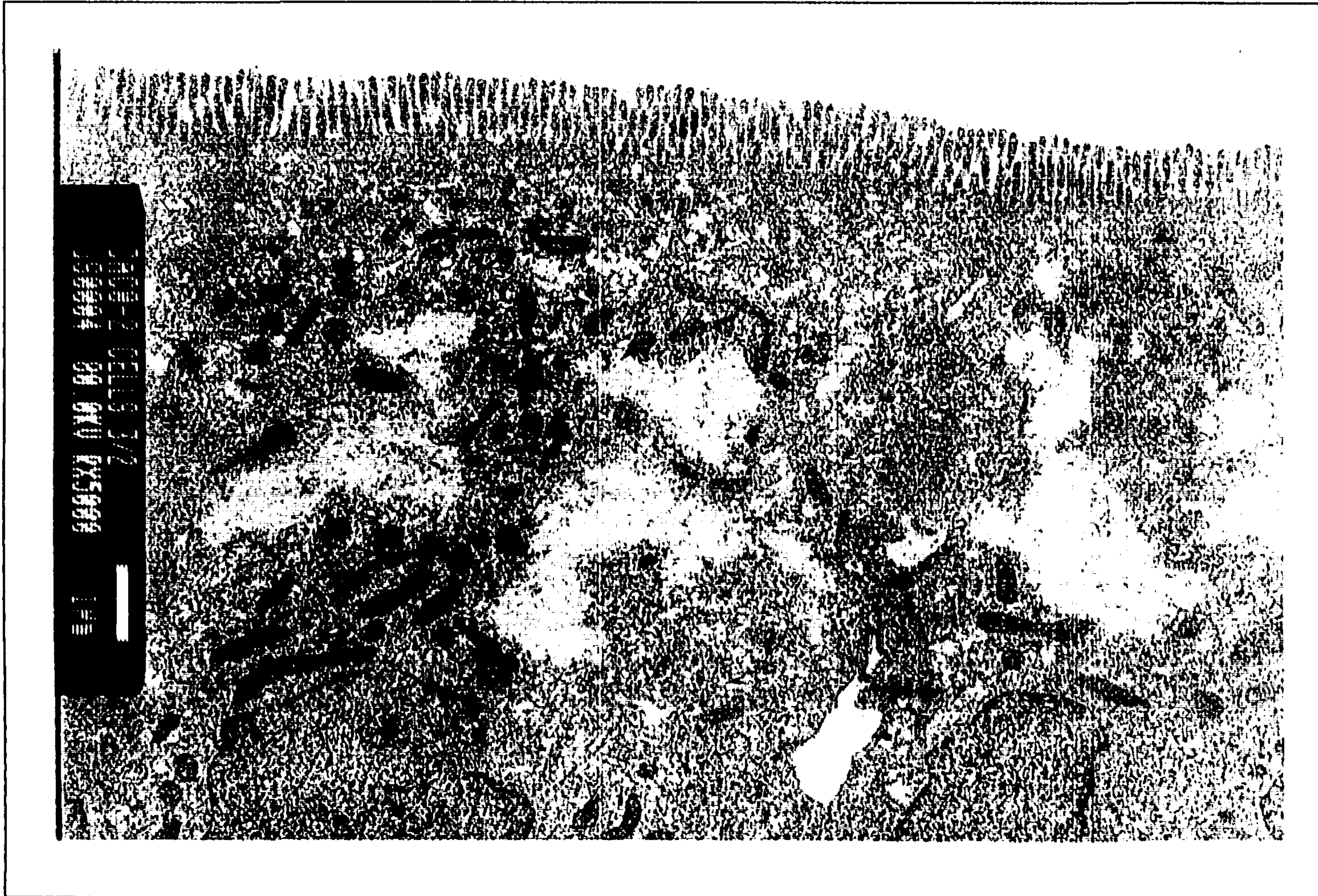

Figure 2. Transmission electron micrograph of Caco-2 cell monolayer at 5K x magnification.

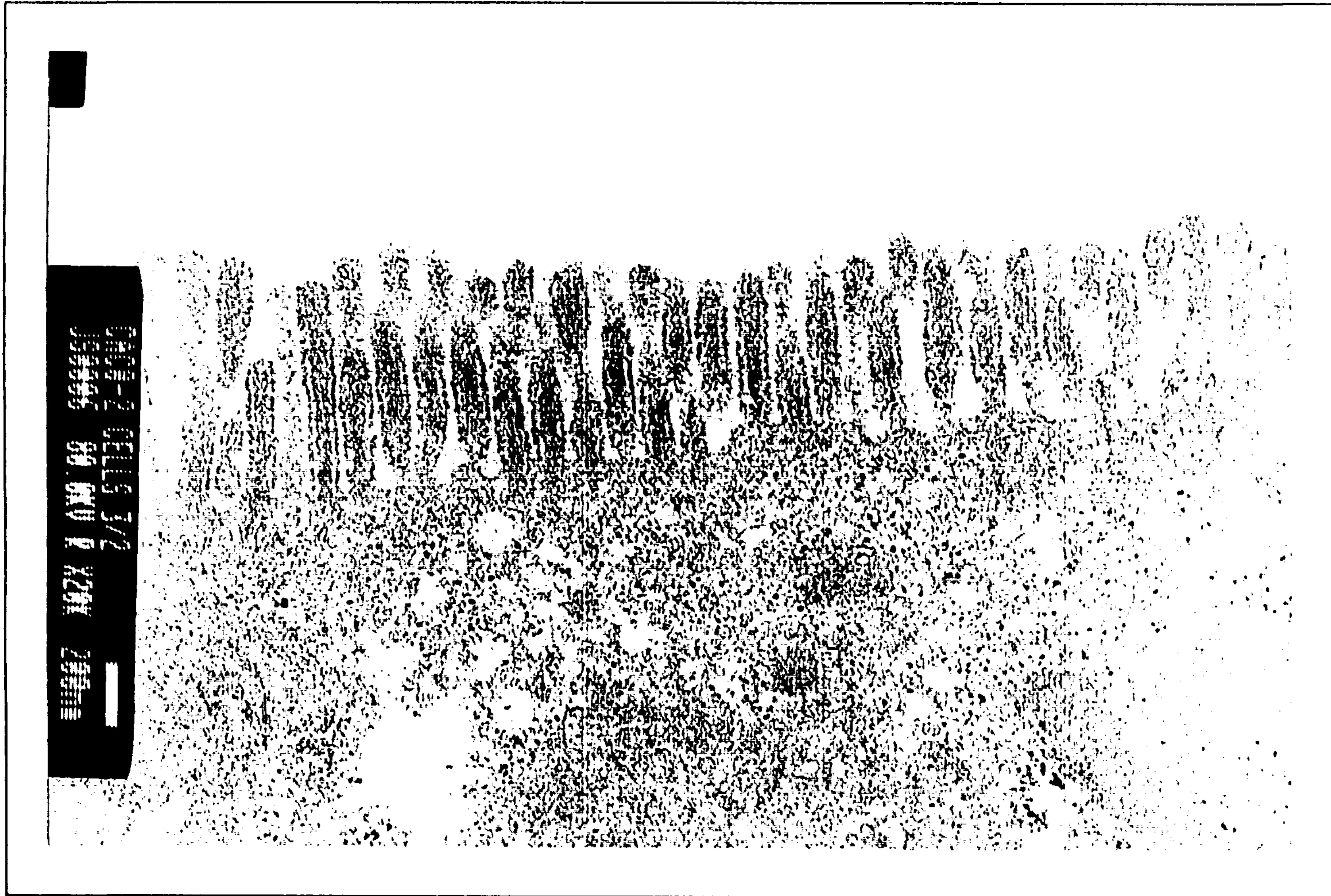

Figure 3. Transmission electron micrograph of Caco-2 cell monolayer at 20K x magnification.

APPENDIX B. EFFECT OF TRITON X-100 ON THE YIELD OF SOLUBLE PROTEIN

The cooked beef water extract supernatant and digest supernatant were both found to possess soluble proteins, which bound iron and enhanced iron uptake. However, we decided to use the digest supernatant because protein yield was greater. To determine if the yield of soluble iron-binding proteins could be increased, we subjected two grams of the initial sediment to resuspension using detergent (25 mL of 4% Triton X-100; Sigma Chemical Company, St. Louis, MO). The slurry was then vortexed, allowed to incubate at ambient temperature for 20 min, and centrifuged at 1380 x g for 25 min. Radioiron (^{59}Fe) was added during the incubation period and measured in the supernatant to assess the yield of soluble iron-binding proteins. The addition of 2 or 4% Triton X-100 resulted in the solubilization of 9.7 and 15.7% of the total radioiron, respectively **(Fig. 1)**. Findings show that addition of 2 or 4% Triton X-100 to the initial sediment increased, slightly, the yield of soluble iron-binding proteins, but not enough to warrant the routine use of the detergent. In addition, it would have been necessary to remove the detergent prior to use of the sample during the subsequent separation and uptake procedures.

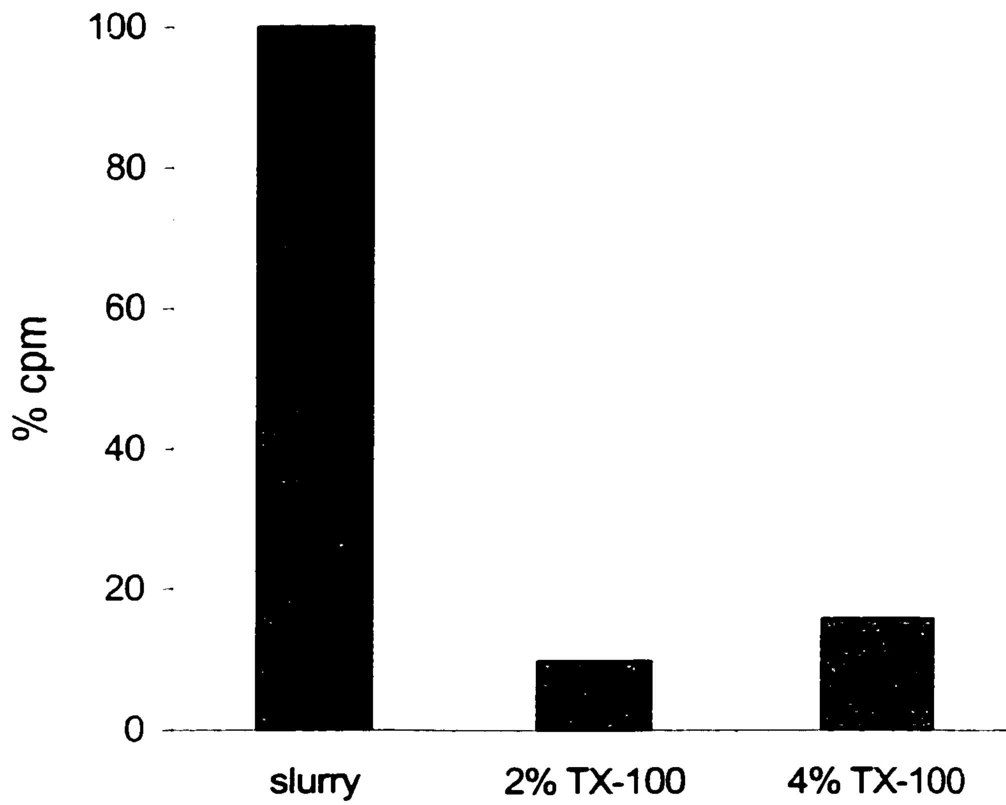

Figure 1. Effect of resuspension of the initial beef sediment using 2% and 4% Triton X-100 on recovery of ^{59}Fe.

APPENDIX C. SIZE-EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography of beef proteins precipitated at 80% saturated ammonium sulfate (NH_3SO_4) solution was performed using polyacrylamide gel beads (Biogel P60; BioRad Laboratories, Hercules, CA) having a nominal fractionation range of 3.5- to 60-kDa. Chromatographic separation, using 20 mM 2-(4-morpholino)-ethane sulfonic acid (MES; Fisher Scientific, Fair Lawn, NJ) buffer at pH 5.4 and 5.5°C, revealed two distinct peaks (**Fig. 1**). Electrophoresis revealed that both peaks were comprised of many proteins ranging of varying molecular weight (data not shown). It was also found that salt was partially co-eluting with the later portion of the second peak. Since this procedure resulted in minimal separation of the components present in the 80% saturated NH_3SO_4 fraction and because salt was found to partially co-elute with the second, more dominant peak, we decided to investigate other techniques to better separate the proteins.

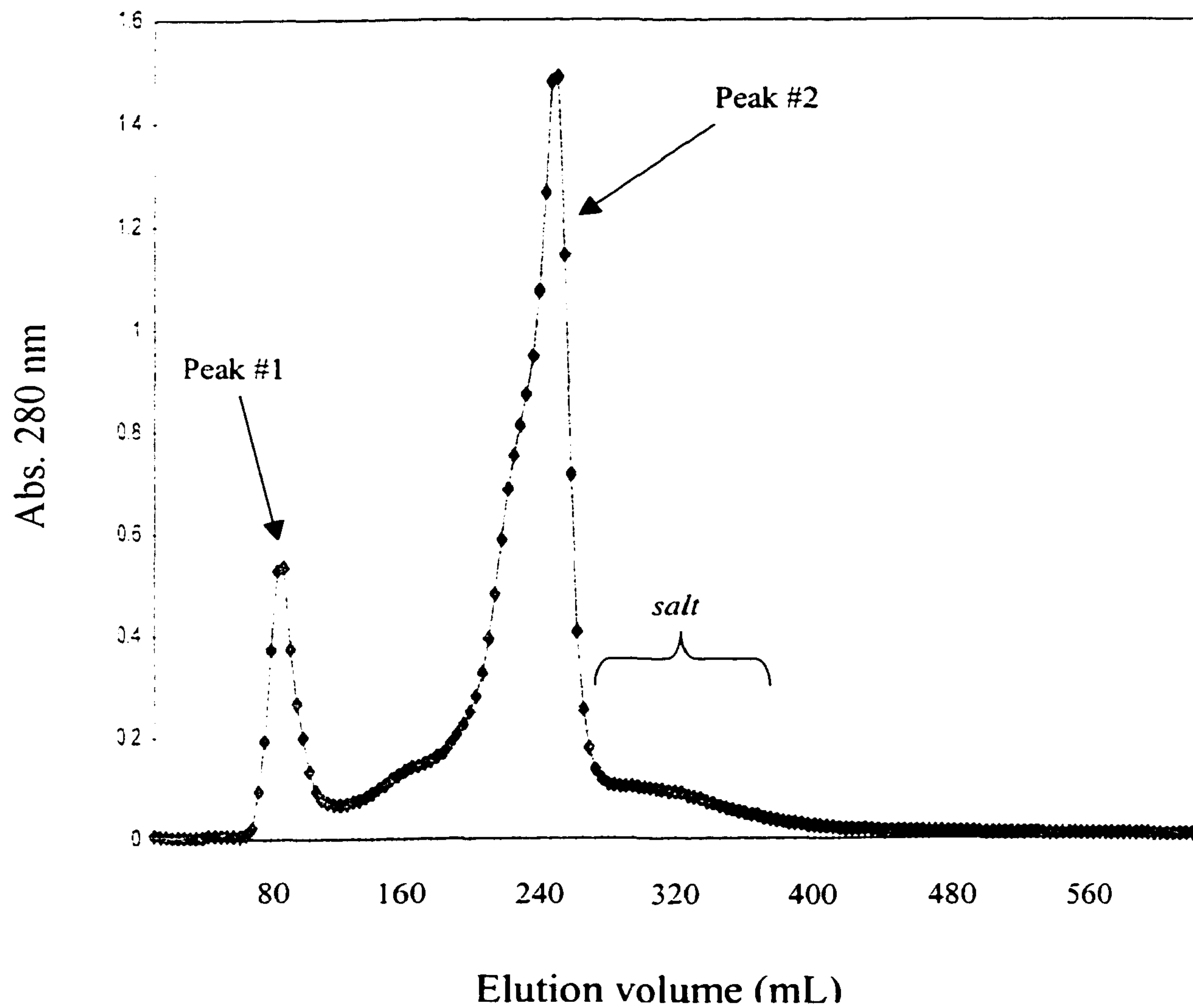

Figure 1. Size exclusion chromatography of beef proteins precipitated at 80% saturated NH_3SO_4 solution performed using polyacrylamide gel beads with a nominal fractionation range of 3.5- to 60-kDa. Chromatographic separation was done using 20 mM 2-(4-morpholino)-ethane sulfonic acid (MES) buffer at 5.5°C and pH 5.4.

APPENDIX D. EFFECT OF 2-(4-MORPHOLINO)-ETHANE SULFONIC ACID (MES) ON RADIOIRON UPTAKE AND TRANSPORT BY CACO-2 CELLS

Radioiron uptake and transport by Caco-2 cells were found to be positively associated with MES concentration. The characteristics of MES are a pKa of 5.96 at 37°C and buffer range of pH 5.5 to 6.5. MES is considered to be the most useful buffer among 3,3-dimethylglutamic acid, tricarballyic acid, trans-aconitic acid and HEPES in the pH range 5.0 to 6.0 due to its biological inertness, buffering capacity, the minimal requirement for excess base to adjust pH, and its minimal metal complexing ability. The influence of MES was first observed when pooled size-exclusion chromatography fractions were lyophilized in order to obtain reasonable working protein concentrations and then used in an experiment designed to measure radioiron uptake and transport by Caco-2 cells grown on collagen treated membranes forming bicameral (apical and basal) chambers. To confirm the existence of the effect, an experiment was designed where only the MES concentration was varied in the presence of iron and nitrilotriacetic acid (NTA) (ferric:NTA; 1:5 molar ratio) with no beef sample. The MES concentrations used in this experiment matched those resulting from the earlier mentioned lyophilization process. A sample of 10 mM MES was also included. Results show that when the concentration of MES increases from 10 mM to 27 mM or 43 mM Caco-2 cell radioiron uptake and transport increase (**Fig. 1**). The MES concentration in our uptake solutions was meticulously controlled for (see Chapter 2, ^{59}Fe Cell Uptake Procedures).

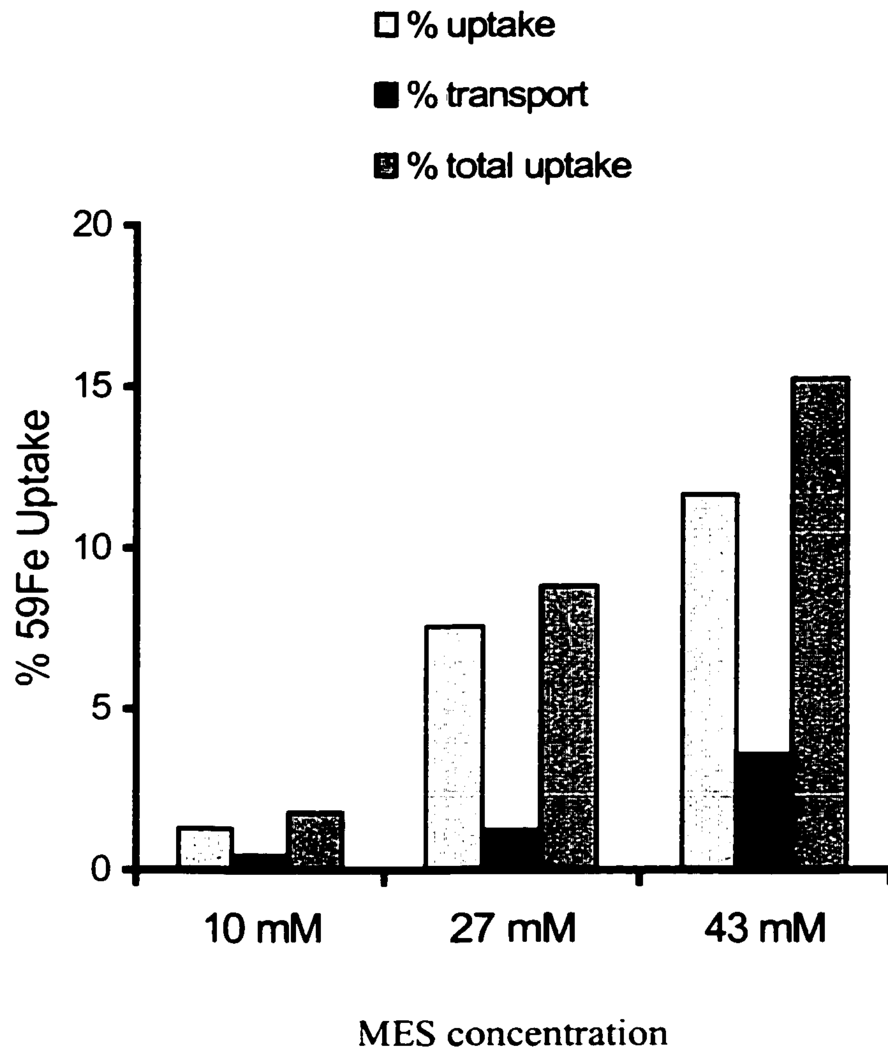

Figure 1. Effect of 2-(4-morpholino)-ethane sulfonic acid (MES) on radioiron uptake and transport by Caco-2 cells grown on collagen treated membranes forming bicameral chambers.

APPENDIX E. MATRIX ASSISTED LASER DESORPTION IONIZATION-TIME OF FLIGHT (MALDI-TOF) ANALYSIS: THE IMMOBILIZED METAL CHELATE AFFINITY (IMAC) WASH

The MALDI-TOF analysis of the IMAC wash was performed as described earlier (see Chapter 2, Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Analysis). The IMAC wash spectrum was similar to the spectra of each eluent peak (P1-P4) (**Fig. 1**).

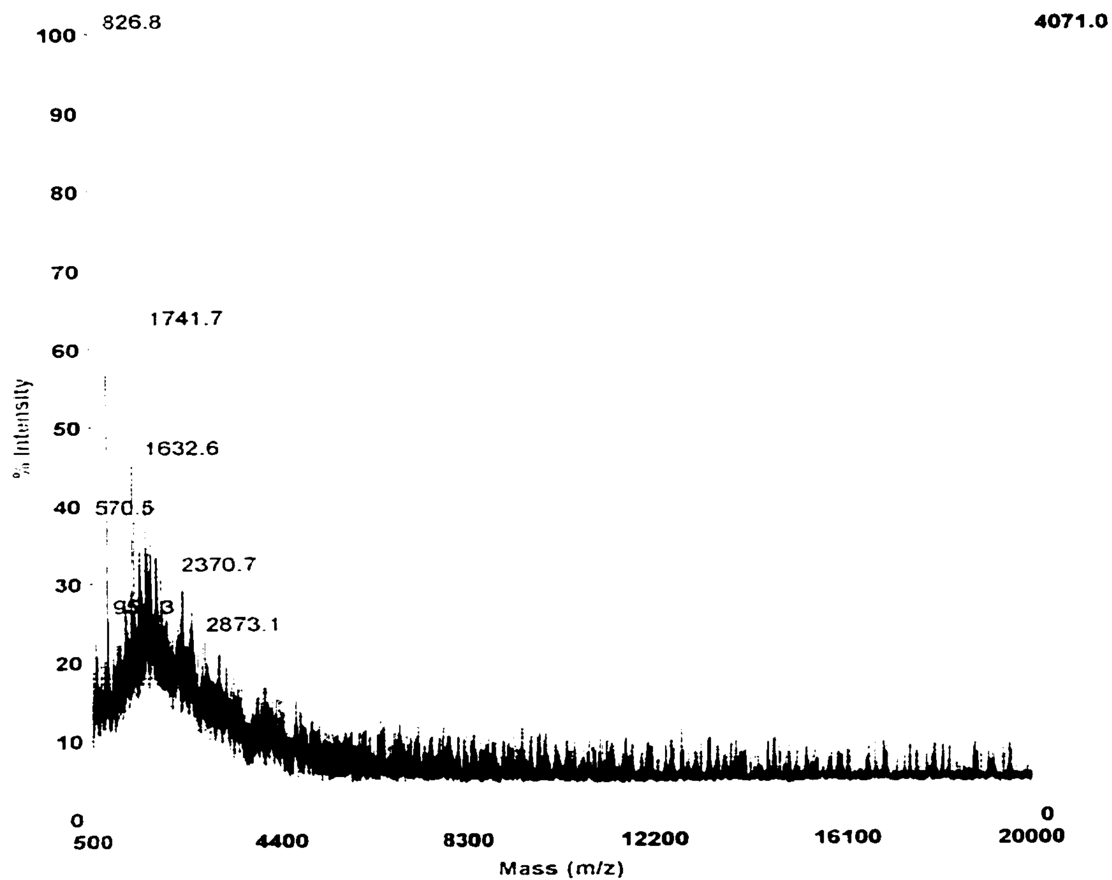

Figure 1. Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) analysis spectrum of the IMAC wash from the 1KR fraction prepared using a α-cyano-4-hydroxycinnamic acid matrix.