This is to certify that the

thesis entitled

Bioavailability of Copper in Kidney and Garbanzo Beans

presented by

Yin-Chieh Li

has been accepted towards fulfillment
of the requirements for

Masters degree in Human Nutrition

Sanda Clemente
Major professor

Date November 16, 1994

BIOAVAILABILITY OF COPPER IN KIDNEY AND GARBANZO BEANS

By

Yin-Chieh Li

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1994

ABSTRACT

BIOAVAILABILITY OF COPPER IN KIDNEY AND GARBANZO BEANS

By

Yin-Chieh Li

In the first of two experiments to evaluate effects of phytate in legumes on copper and zinc bioavailability, rats were fed diets containing 2 μg/g Cu supplied by garbanzo beans, kidney beans or copper carbonate. The garbanzo bean and kidney bean diets contained 0.13 and 0.19% phytate, respectively. Ceruloplasmin activity and serum zinc at the end of 3 wk were significantly different among groups: control > garbanzo bean > kidney bean. Serum copper was significantly higher in the control group. In the second experiment, all rats were fed a copper deficient diet (0.12 μg Cu/g) for 4 wk. At the end of a 7 day repletion period (2 μg Cu/g), ceruloplasmin activity and serum copper were significantly higher in rats fed the diets without phytate (control and phytase-treated kidney bean diets) than in those containing phytate (0.14% in kidney bean and control+phytate diets). In both experiments, tissue concentrations of copper and zinc were similar in all groups. The overall results indicate that copper is less bioavailable in diets containing phytate. The apparent failure of phytate to inhibit zinc bioavailability may have been related to the low phytate : zinc molar ratios of the diets.

To my family

ACKNOWLEDGMENTS

I wish to express my gratitude to my advisor, Dr. Wanda L. Chenoweth for her generous assistance and valuable advice throughout the entire study. Sincere appreciation are extended to my committee members, Dr. Maurice R. Bennink and Dr. Dale R. Romsos for being on my committee and providing any guidance and help they could.

I would also like to thank Ritu Ummadi, Mei-Chuan Chen and Yongsoo Chung for their friendship and support through the best and worst of times. I want to thank each member of my family for their understanding and support.

Last but not least, this thesis is dedicated to my husband, Kuo-Ding, for his patience, encouragement and love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Recognition of the biological importance of copper is of recent origin. Copper is an essential component of metalloenzymes such as lysyl oxidase, cytochrome c oxidase and superoxide dismutase. Activities of these enzymes are decreased by copper deficiency. Although copper is widely distributed in foodstuffs, its absorption and utilization may be affected by various dietary factors.

Consumption of legumes is increasing as people become aware of their potential nutritional benefits (Morrow, 1991). Legumes contain high amounts of complex carbohydrate, dietary fiber, protein, certain vitamins and minerals including copper and zinc. However, legumes also contain antinutritional factors such as phytate, which accounts for up to 85% of total phosphorus in many cereals and legumes. Concern about the presence of phytate in cereals and legumes arises from the evidence that it decreases the bioavailability of essential minerals such as zinc, calcium and iron by forming insoluble complexes and rendering them unavailable for intestinal absorption. This interference with intestinal absorption of minerals may lead to mineral deficiencies in humans and animals.

It is known that zinc in beans is poorly available for animals and humans. Phytate is one of factors that contributes to this low bioavailability. The effect of phytate on copper bioavailability, however, is

still controversial. Based on animal studies, some researchers claimed phytic acid had no effect on copper bioavailability, whereas others reported that it enhanced copper bioavailability (Lo et al., 1984; Lee et al., 1988). Although extensive work has been done on the effect of phytic acid on zinc bioavailability, little is known about the bioavailability of copper in various kind of beans which contain variable amounts of phytic acid.

REVIEW OF LITERATURE

Copper metabolism and functions

Although copper can be absorbed from all portions of the small intestine as well as from stomach, the upper portion of the small intestine is the site of maximal absorption of copper. The precise biochemical mechanism for absorption of copper across the brush border surface of the small intestine is not known. It is believed that copper absorption involves mechanisms other than simple diffusion. Bronner and Yost (1985) reported that at least two mechanisms were involved in copper absorption. One was saturable suggesting an active transport process; the other was unsaturable indicating passive diffusion. Like transport systems for other nutrients, when dietary copper concentration is low, copper is primarily absorbed via the active transport pathway, whereas passive transport occurs when the dietary copper concentration is high.

In the blood stream, copper is distributed among three major plasma constituents: the protein ceruloplasmin, albumin, and free amino acids. Over 90% of the total plasma copper is tightly bound to ceruloplasmin. Most of the remainder is less tightly bound to albumin, and 1-2% of the nonceruloplasmin bound copper is loosely attached to amino acids (DiSilvestro and Cousins, 1983). Albumin and amino acids are the ligands that transport copper in the portal circulation following its

intestinal absorption and that deliver the metal to the liver. Once in the liver, copper is incorporated into ceruloplasmin which is released into the blood where it transports copper to extra hepatic tissue for synthesis of cuproenzymes. Copper is excreted primarily via the gastrointestinal tract. Bile contributes the major portion of endogenous fecal copper, especially in humans. A small amount of copper is lost in the urine. Figure 1 summarizes the basic aspects of mammalian copper metabolism.

Like other essential trace metals, the essentiality of copper is due to its presence as part of the structure of essential enzymes. For example, copper is required in collagen synthesis (lysyl oxidase), energy production via oxidative phosphorylation (cytochrome c oxidase), and disposal of peroxides in tissues (superoxide dismutase). Copper also plays a key role in the metabolism of other elements such as iron which requires ceruloplasmin (ferroxidase) to oxidize iron to ferric iron for attachment to transferrin for transport and distribution. As interest in copper's role in metabolism has grown so has interest in the factors which might affect its absorption or utilization by the body.

Dietary factors affecting copper utilization in humans and animals

The absorption and utilization of copper in food may be influenced by a large number of dietary factors such as ascorbic acid, fructose, sucrose and possibly phytate. These dietary components can reduce copper absorption. In addition, other trace minerals, if present in sufficiently high concentrations, have an antagonistic effect on copper absorption and the antagonistic effect of zinc on copper bioavailability is perhaps of greatest practical significance (O'Dell, 1990).

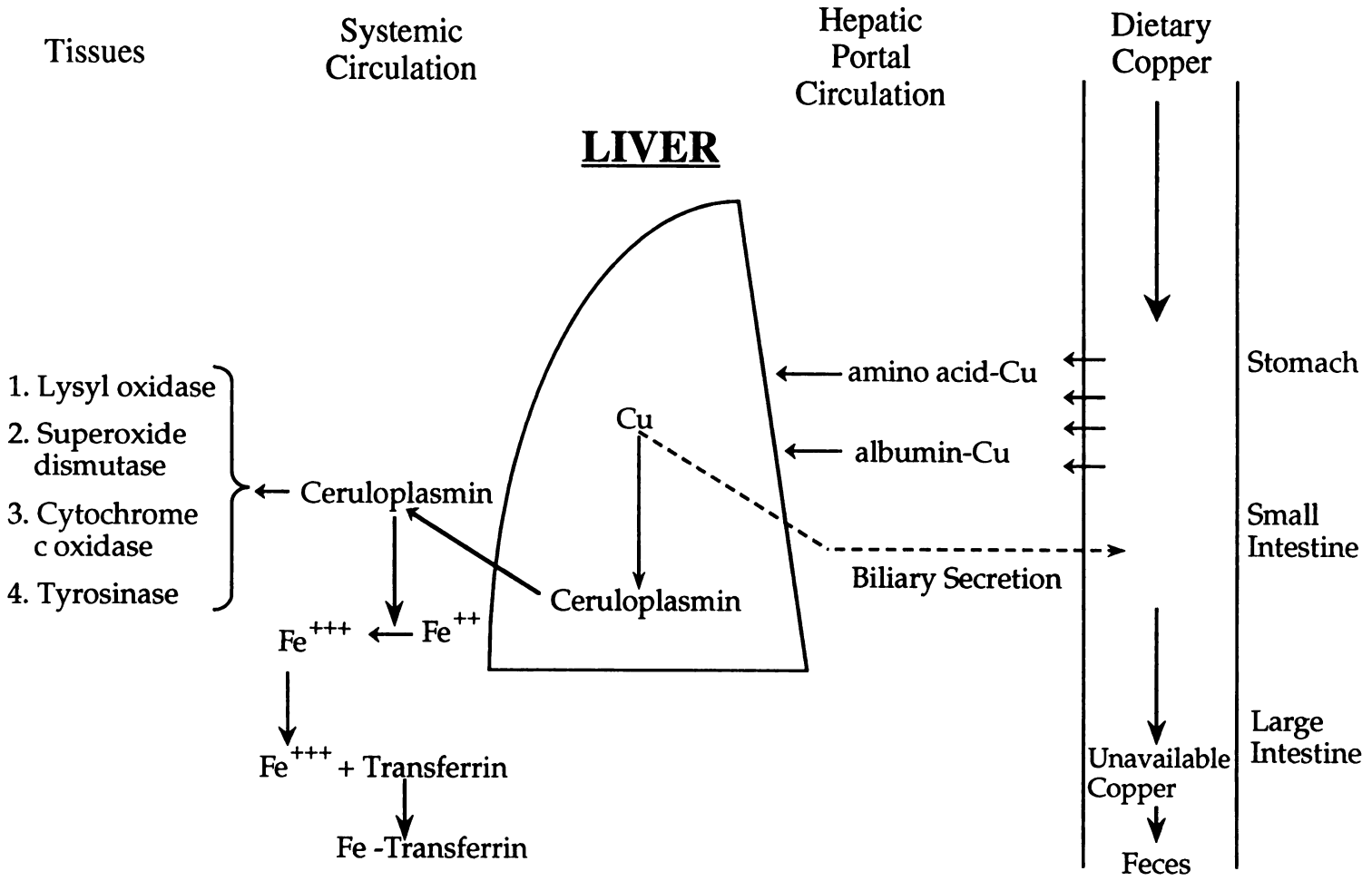

Figure 1. Basic aspects of mammalian copper metabolism

Ascorbic acid-copper interactions. A high dietary intake of ascorbic acid is likely to decrease copper utilization in several species. Rats fed copper deficient diets supplemented with 1-5% ascorbic acid had higher mortality rates, increased cardiac and/or vascular abnormalities, lower body weights, and lower hematocrit and hemoglobin levels than control animals (Johnson, 1986; Johnson and Murphy, 1988). In a short term study using orally administered radiolabeled copper to measure copper status, Van Campen and Gross (1968) found that high level of ascorbic acid decreased the absorption of copper from ligated intestinal segments, and decreased the retention of copper in the liver and whole body of rats. From these studies, it was proposed that ascorbic acid impairs copper utilization through decreased copper absorption or through increased turnover of copper. In human studies, Finley and Cerklewski (1983) investigated the influence of ascorbic acid supplementation on the copper status of young men. Results showed that ingestion of 1500 mg ascorbic acid daily for 60 days significantly reduced serum ceruloplasmin activity by 26% and lowered serum copper concentrations. Serum copper was significantly increased 20 days after the ascorbic acid supplements were discontinued. This study confirmed that a high ascorbic acid intake is antagonistic to the copper status of men supporting results found in laboratory animals.

Carbohydrates-copper interactions. It has been reported that the severity and rate of development of copper deficiency in rats are related to the carbohydrate component of the diet (Fields et al., 1984; Fields et al., 1986). These effects could be caused by a difference in bioavailability of

copper in the presence of different carbohydrate, or by differences in excretion of copper when different carbohydrates are fed. Studies with radiolabeled copper were conducted by Fields et al. (1986) who investigated the effect of different dietary carbohydrates (fructose and starch) on the absorption, tissue distribution and excretion of copper with copper deficient or copper supplemented rats. Forty eight and 96 hours after intubation of ^{67}Cu-labeled diets, there was a significantly higher retention of ^{67}Cu in the gastrointestinal tract accompanied by decreased whole body retention and urinary excretion in copper deficient rats fed the fructose-based diet compared to those fed a starch-based diet. No differences due to dietary carbohydrates were found in copper supplemented rats. Another study conducted by Johnson and Gratzek (1986) showed that male rats fed copper deficient diets with 20% sucrose or fructose rather than starch or glucose had lower growth rates, higher mortality rates, higher heart and liver weights and were more anemic after 3-4 weeks feeding. The mortality rate of copper deficient rats fed fructose or sucrose was greater than 60% after 7-9 weeks. In contrast, the mortality rate of copper deficient rats fed starch or glucose generally ranged from 0-30% (Fields et al., 1984). Results from these studies indicate that fructose may be the dietary component which has an adverse effect on copper status. However, in rats fed a marginal copper diet (2.5 μg/g), Johnson et al. (1988) found no significant differences in copper absorption due to different carbohydrates in a single meal. These results showed that carbohydrate component of the diets can affect copper bioavailability on a long term feeding.

Zinc-copper interactions. Zinc is an essential nutrient required for numerous metalloenzymes, cell replication and differentiation, reproduction, growth and many other essential functions (Hambidge et al., 1986). Zinc and copper are two elements with very similar molecular weight, and their electronic structures are quite close; therefore, they may compete with each other for absorption. Several investigators have reported inhibition of copper absorption and deposition of copper in the tissues when the dietary or intraluminal Zn : Cu ratio was from 500 : 1 to 1000 : 1 (Van Campen and Scaife, 1967; Evans et al., 1974). O'Dell (1985) reported that high zinc diets (120 or 240 μg/g daily) significantly lowered the activities of liver Cu, Zn-superoxide dismutase and heart cytochrome c oxidase, even when the diet contained an adequate level of copper (6 μg/g).

Oestreicher and Cousins (1985) conducted a series of experiments to test the influence that copper and zinc exert on each other's absorption by using an isolated, vascularly perfused rat intestine technique. The dietary copper and zinc concentrations were held at 6 mg/kg and 30 mg/kg, respectively, while the metal concentrations in the luminal perfusate were changed (from 1 to 36 mg/L and from 5 to 180 mg/L for copper and zinc, respectively). Results indicated that medium and high luminal zinc concentrations (30 and 180 mg/L, respectively) significantly decreased copper absorption when the luminal copper concentration was low (1 mg Cu/L). The results of luminal copper concentration on zinc absorption showed that medium and high copper concentrations (6 and 36 mg/L, respectively) in the lumen perfusate significantly decreased zinc absorption when the luminal zinc concentration was also high (180 mg

Zn/L). In contrast, there was no significant difference on zinc or copper absorption when the luminal zinc and copper concentrations were 36 and 6 mg/L, respectively. Results of studies in humans were similar to those obtained in animal studies. Fischer et al. (1984) reported that consumption of 50 mg Zn/day for 6 weeks by young men significantly decreased Cu, Zn-superoxide dismutase activity in red blood cells. However, Turnland et al. (1988) observed that relatively small changes in zinc intake did not appear to affect copper absorption. For example, an increase in dietary zinc from 5.5 to 16.5 mg Zn/day, which is approximately the RDA for zinc (15 mg/day), did not increase fecal losses of copper or decrease the absorption of copper in young men fed 1.3 mg Cu/day. The results obtained from these studies indicate that a competition and/or inhibition of copper or zinc uptake into intestinal cells occurs when the luminal concentration of either metal is very high, whereas no copper-zinc antagonism occurs at the absorptive level when the copper and zinc intakes are near requirement level.

Bioavailability of trace elements

Bioavailability can be defined as the proportion of the total mineral in a food, meal or diet that is utilized for normal body functions. It involves various stages, each of which is affected by different dietary and physiological factors (Fairweather-Tait, 1992). The amount of a mineral that is available for absorption is dependent upon dietary composition, gastrointestinal secretions, and luminal interactions. Several studies indicated that the competitive interaction between minerals has an effect on bioavailability of minerals and it can occur in the food during

harvesting, processing or storage or in the lumen of the intestine during the gastrointestinal phase of digestion. It also can occur as minerals bind to the brush border of the intestinal cell or are incorporated into the intestinal cell (Mertz, 1980; Sandstead, 1981). Clydesdale (1989) reported that the interaction between minerals in the gastrointestinal tract was directly related to their electronic structure, that is, those elements whose physical and chemical properties are similar will act antagonistically to each other biologically.

In foods, the mechanisms involved in mineral-mineral interactions which have the potential to affect bioavailability have been broadly defined into four categories (Clydesdale, 1988):

1. Mineral displacement: Displacement of a mineral from a complex with another mineral to form a soluble (available) or insoluble (unavailable) complex. This category would include mineral reactions with fiber or phytate. For instance, lignin, an inhibitor, provides a good example of this since it readily binds most minerals, causing them to precipitate and then become unavailable. However, the inhibition of lignin on mineral was variable in several studies. This variability may have occurred because in some studies only one mineral was present and thus it would be inhibited, whereas in other studies other minerals were also present which may compete for binding.
2. Polymineral-ligand complexes: The addition of a second or third mineral to a soluble mineral-ligand complex causing precipitation by forming a polymineral-ligand complex. This category includes the phenomena which occur when calcium and zinc are present together with phytate. Platt et al. (1987) analyzed the solubility of calcium in the

presence of phytate added with or without zinc. When the phytate/calcium/zinc ratio was 3 : 6 : 1, 19% of the calcium and 50.2% of the zinc remained soluble. Another study conducted by Graf and Eaton (1984) indicated that calcium exhibited a bimodel effect on the solubility of zinc. For example, low concentrations of calcium increase the solubility of zinc whereas high concentrations of calcium potentiate the precipitation of zinc by phytate.

3. Polymineral-polyligand: The addition of a mineral causing a mineral-ligand complex to form and/or more than one mineral binding to more than one substrate (ligand) and forming a polymineral-polyligand complex. This third category includes complexes such as an iron-zinc-phytate-protein complex, the protein-phytate-zinc complex and the zinc-phytate complex. When only one mineral and one ligand are present in the system, precipitation may occur. This complex is usually mineral-specific and ligand-specific so that this ligand will not form a complex with all minerals. In addition, this complex may remain soluble unless another mineral and/or another ligand combines with this complex to form insoluble polymineral-polyligand complexes. Platt et al. (1987) analyzed the interactions of iron, alone and in combination with zinc, copper, with a phytate-rich, fiber-rich (PRFR) fraction of wheat bran and a sodium phytate sample under gastrointestinal pH conditions. Results showed that 31.2% of iron remained soluble when iron was added alone to the PRFR fraction; the solubility of protein and phytate was also decreased. This result indicated that an insoluble iron-phytate-protein may have been formed. In contrast, iron in the sodium phytate sample was completely

soluble. It is probable that the iron-phytate complex is soluble. When both iron and zinc were added to PRFR samples, there was a large decrease in iron solubility (8.2%) and the solubility of protein and phytate. Zinc solubility was also very low (10.6%). This result indicated that an insoluble zinc-iron-phytate-protein complex was formed whereas zinc and iron remained soluble in the sodium phytate sample. This demonstrated that phytate complexes existed when protein is not present. In addition, when copper was added with iron there was little change in iron solubility in either the PRFR or phytate samples. However, the solubility of copper was lower in the PRFR (65.9%) and higher in the sodium phytate (93.6%) sample. In this situation, there are several factors that would affect solubility of minerals such as proteins, fiber or phytate and the presence of other minerals which may either increase or decrease solubility.

4. Enzyme susceptibility of complexes: The formation of a polymineral-ligand complex which changes the susceptibility of the mineral-ligand bonds to cleavage by digestive enzymes. Digestive enzymes in the gastrointestinal tract may increase the solubility of minerals by cleaving mineral-ligand bonds. Although some mineral-ligand complexes are susceptible to degradation by digestive enzymes, thus releasing the mineral for absorption, it is possible that some complexes form which are not susceptible to digestive enzymes. The total bioavailability of a mineral depends on the proportion of mineral in complexes that can be degraded by digestive enzymes.

Legumes

Legume production and consumption are increasing as more people become aware of legumes' nutritional benefits. Morrow (1991) reported that the 1990 per capita consumption levels of dry beans was 7.2 lb, an increase of 30.9 % over the previous year. Legumes are high in complex carbohydrates, protein, certain vitamins (thiamin and niacin), minerals (phosphorus, potassium, calcium, zinc, copper and magnesium), and dietary fiber, especially water-soluble fiber. The high content of water soluble fiber is particularly effective in lowering cholesterol in the blood, while water insoluble fiber provides bulk, promoting the movement of food through the intestines at a faster rate. In addition, beans are a good alternative to red meat. Since beans offer more protein per dollar than other foods, they become an important economical source of protein in the diet of many developed and developing countries (Morrow, 1991). However, legumes also contain antinutritional factors such as trypsin inhibitors, lectins, protease inhibitors, polyphenols or phytates. Since legumes contain a considerable amount of phytate, concern has been expressed that their content of phytate may have an adverse effect on mineral metabolism, particularly on trace elements (Reddy et al., 1982).

Phytate in legumes

Chemistry of phytic acid. Phytic acid was discovered by Hartig in 1855 and through extensive studies by many researchers, it is accepted that phytic acid is myoinositol 1, 2, 3, 4, 5, 6-hexaphosphate with an empirical formula of $C_6H_{18}O_{24}P_6$ (Reddy et al., 1982; Gibson and Ullah, 1990). The

name of phytic acid has been used interchangeably in the literature with the term phytin which more correctly refers to the mixed Ca-Mg-K salts of the acid in plants. A partially dissociated Anderson-based structure for phytic acid might occur at neutral pH. At neutral pH, the phosphate groups have negatively charged oxygen atoms so that cations can chelate between two phosphate group or within a phosphate group (Reddy et al., 1982). Figure 2 is the structure of phytic acid and phytic acid chelate at neutral pH.

Occurrence. Phytic acid widely occurs in plant seeds and/or grains (O'Dell, 1979), root and tubers (McCance and Widdowson, 1935), and fruits and vegetables (Larbi and M'Barek, 1985). Phytic acid occurs primarily as a salt of mono- and divalent cations in discrete regions of cereal grains and legumes. It rapidly accumulates in seeds and/or grains during ripening period, accompanied by other storage substance such as starch and lipids (Nahapetian and Bassiri, 1975). The accumulation site of phytate in cereals such as wheat, rice, barley and rye is near the outside of the seed coat, whereas in legumes, phytate is distributed throughout the entire protein complex of the seed (O'Dell et al., 1972; Reddy et al., 1982). Phytate represents most of the phosphorus in cereals and legumes. Values for the concentration of phytate in various foods have been summarized by Reddy et al. (1982). In general, the amount of phytate varies from 0.14% (rice, polished short grain) to 1.16% (barley) in cereals and from 0.28% (garbanzo bean) to 2.06% (red kidney bean) in legumes.

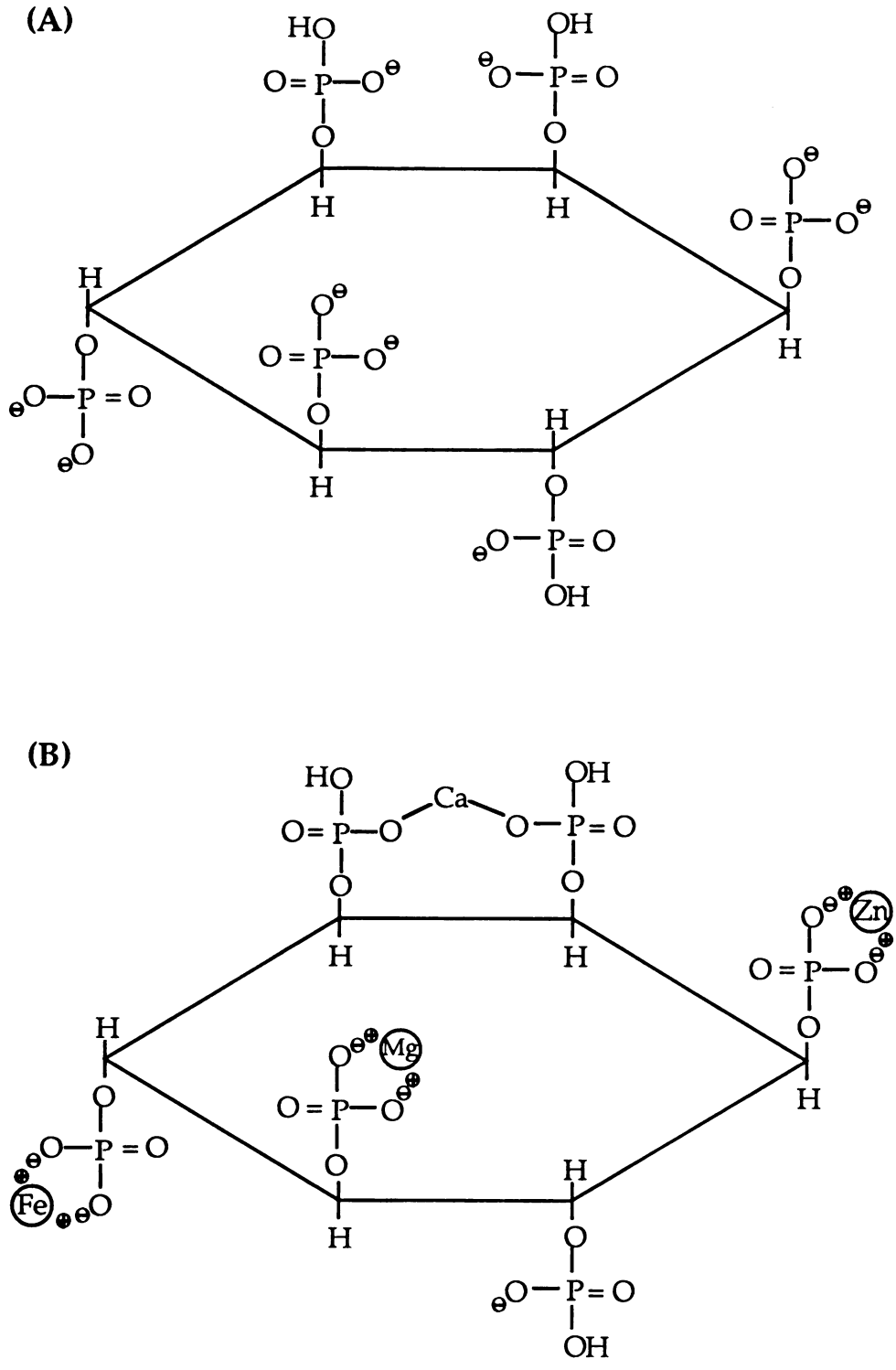

Figure 2. (A) Structure of Phytic acid,
(B) Phytic acid chelate at neutral pH

Functions and uses of phytate. Several physiological roles have been suggested for phytic acid in plants. These include its role as a storage of phosphate, an energy source, a source of myoinositol and an initiator of dormancy (Gibson and Ullah, 1990). Besides having a role in the physiological function, phytate may have an effect on the production of aflatoxin. Gupta and Venkatasubramanian (1975) reported that phytate in soybean seeds prevented aflatoxin production by making zinc, which is necessary for aflatoxin production, unavailable to the mold. In addition, Graf et al. (1987) suggested that phytate may serve as a natural antioxidant in seeds during dormancy based on its ability to bind iron to form an iron chelate thus inhibiting iron-catalyzed hydroxyl radical formation and suppressing lipid peroxidation.

Sands et al. (1986) summarized the food and medical applications of phytic acid and suggested several additional uses for phytic acid. Because of the chelating and antioxidant properties of phytic acid, it has been proposed for use as an additive to preserve a variety of foods. A number of medical applications have been suggested for phytic acid based on its interaction properties, and its antineoplastic action has become the topic of intense investigation at present. Phytic acid has been shown to have antineoplastic action in colon carcinogenesis. Graf and Eaton (1985) reported that inositol hexaphosphate consumption is considered to be a major etiologic factor for the approximate 50% reduction in the incidence of large intestinal cancer in Finland compared to Denmark. Since Finnish people consume 20-40% more phytate than do Danes, it was suggested that diets high in phytate suppress colonic carcinogenesis and other inflammatory bowel diseases by inhibiting intracolonic hydroxyl radical

generation via the chelation of iron. Moreover, Shamsuddin and his co-workers have done a series of experiments to investigate the antineoplastic action of phytic acid (Ullah and Shamsuddin, 1990; Sakamoto et al., 1993; Vucenik and Shamsuddin, 1994; Shamsuddin et al., 1988 and 1992). They demonstrated that phytate exerted chemopreventive and chemotherapeutic effects in rodent colon and mammary carcinogenesis models. However, Thompson (1988) indicated that the use of antinutrients such as phytic acid as therapeutic agents needs to be carefully considered because of the adverse effects associated with their high intakes.

Digestion and bioavailability of phytate in humans and animals. In mature cereal grains, legumes, and oil seeds, the major portion of the total phosphorus is present in the form of phytic acid, and the availability of phosphorus when present in the form of phytate depends on the species, the age of animal, and the level of phytase activity in the intestinal tracts of the species. Due to the rumen microorganisms, ruminants were able to utilize most of the dietary phytate. Nelson et al. (1976) studied the hydrolysis of natural phytate phosphorus from soybean meal, sorghum grains, and corn meal in the intestinal tract of calves and steers. They found that the initial phytate hydrolysis occurred in the rumen and was complete before the feed reached the other parts of the digestive system. In general, biological availability of dietary phytate phosphorus to ruminants is 80% or greater (Reddy et al., 1982). However, the biological availability of phytate phosphorus to nonruminants is poor compared to ruminants. Reddy et al. (1982) reported that the biological values of

phytate phosphorus for pigs were from 25% to 40%. Lei (1992) reported that phytate phosphorus from barley and corn was about 17% and 8% digestible, respectively, for growing pigs when phytate was the major source of phosphorus in the diets.

It has been reported that rats can utilize phytate phosphorus. The average of phytate phosphorus that can be utilized by rats is 27% to 41% (Reddy et al., 1982). A study found that rats adapt to low phosphorus diets by an increase in phytate digestion (Moore and Veum, 1983). The adaptation may result from increased synthesis of phytase or alkaline phosphatase by intestinal microorganisms when there is a low level of phosphorus in the diet. Although the number of experiments to determine the bioavailability of phosphorus from legumes phytate in humans have been limited, Sandberg et al. (1982, 1986, and 1987) reported that 40% to 60% of the phytate phosphorus from wheat bran was available to man. They also indicated that the hydrolysis of phytate by human phytase or alkaline phosphatase does occur in the stomach and small intestine, and is not due to microbial phytase activity.

Phytase. Phytase, myoinositol hexaphosphate phosphohydrolase, is an enzyme which is widely distributed in plants, animals, and fungi. Phytase is capable of hydrolyzing myoinositol hexaphosphate to inorganic orthophosphate and a series of lower phosphoric esters of myoinositol or to free myoinositol (Reddy et al., 1982). Maiti et al. (1979) reported that degradation of phytate by phytase occurs in a stepwise manner starting with dephosphorylation from position 6 followed by removal of phosphorus from positions 5 and 4, 1 and 3, or 1 and 4, with the phosphate

at position 2 being stable. Recently, Gibson and Ullah (1990) suggested that there are two major types of phytase. One is 3-phytase (E. C. 3. 1. 3. 8) which initiates the removal of phosphate groups attached to position 1 or 3 of myoinositol. The other one is 6-phytase (E. C. 3. 1. 3. 26) which first frees the phosphate at the 6 position. Phytase characterized from microorganisms and the fungi belong to 3-phytase, while the 6-phytase is found in seeds of higher plants. In general, both types of activity are referred to as phytase.

The pH and temperature optima for phytase from certain cereals and legumes are different. According to Reddy et al. (1982), the optimal pH for phytase activity appears to be in the range 4-7.5. The optimum temperature for phytase activity varies from source to source but appears to be in a high temperature range (45-60 °C). The unit of phytase activity described in this thesis is defined as the amount of enzyme that liberates 1 µmol of inorganic phosphorus from sodium phytate per minute at pH 5.15 and 55 °C.

It has been shown that the bioavailability of trace elements can be improved by the presence of supplemental phytase in diets which contain a high amount of phytate; this will be discussed in the following section on phytate-mineral interactions.

Phytate-mineral interactions

Under certain condition, phytate may form insoluble complexes with di- and trivalent cations at neutral pH potentially rendering these minerals unavailable for intestinal absorption (Graf and Eaton, 1984). The insolubility of metal-phytate complexes may have adverse nutritional

implications since it has been claimed to interfere with the bioavailability of calcium, zinc, and iron in humans (Morris, 1986). The formation of these complexes is pH dependent. Oberleas (1973) reported that although the decreasing order of metal complexion is $Cu^{++} > Zn^{++} > Co^{++} > Mn^{++} > Fe^{+++} > Ca^{++}$ at pH 7.4, maximum precipitation of zinc-phytate or phytate-zinc-calcium occurs at pH 6.0 which is the approximate pH of the duodenum. Nutritionally, mineral-phytate binding at pH 6.0 is more important since the duodenum is the place where maximum absorption of divalent metal ions takes place.

Interactions with zinc. Phytic acid is thought to be the primary inhibitory factor in soybean products that results in reduced zinc bioavailability. A reduction in phytate is reported to improved the bioavailability of soybean zinc (Ellis and Morris, 1981; Lonnerdal et al., 1988; Zhou et al., 1992). A negative effect of phytate on zinc bioavailability was first shown by O'Dell and Savage (1960) who reported that the phytic acid associated with plant proteins was responsible for decreased availability of zinc in diets prepared from natural plant proteins. They found that animals fed animal protein-based diets containing the same level of zinc grew normally without any deficiency symptoms compared to those fed on plant protein-based diets. To verify this, they conducted a second experiment in which phytic acid from soybean was added to a casein-based diet and the growth response of chicks fed this diet was compared to those fed soybean proteins as the only source of protein. Results showed that phytic acid decreased the bioavailability of zinc with the casein-based diet and produced symptoms similar to those observed in

animals fed soybean protein diets containing a comparable level of phytate. Therefore, they concluded that the zinc requirement was increased in the presence of soybeans in animal diets.

Momcilovic et al. (1976) evaluated the biological availability of zinc in bovine milk and soy protein-based infant formulas using total femur zinc of growing rats. A zinc deficient diet containing egg white protein was supplemented with graded levels of zinc (0, 3, 6, 9, 12 μg/g) from zinc sulfate, bovine milk, or soy protein-based infant formulas. By using a slope-ratio bioassay model, the relative biological availability of zinc in bovine milk-based formula and soy-based formula was 0.86 and 0.67, respectively compared to zinc sulfate (zinc sulfate = 1). It was evident from these results that the relative biological availability of zinc in soy-based formula was about 20% less than that in the milk-based formula. In a similar study, Forbes and Parker (1977) measured zinc bioavailability from full fat soy flour using a slope-ratio assay procedure in male weanling rats. These researchers reported that when the log of total femur zinc content was used as a criterion of response, zinc from the flour was utilized 34% as efficiently as was zinc from zinc carbonate. In a second experiment, however, when zinc carbonate was added to a whole fat soy flour diet, the zinc utilization was 94% as efficient as when zinc carbonate was added to an egg white diet. Results indicated that zinc bioavailability in full fat soy flour was lower than zinc carbonate and it was increased by adding zinc carbonate.

A study conducted by Welch and House (1982) demonstrated that both endogenous phytate in plant proteins and added phytate from sodium phytate impaired zinc absorption. In the first experiment, rats

were given either immature soybean seeds (0.6% phytic acid) or mature soybean seeds (1.7% phytic acid) which were intrinsically labeled with ^{65}Zn. A single meal of the intrinsically labeled soybean supplement was supplied after a 16-hour fast. The results showed that approximately 89% of the ^{65}Zn was absorbed from the immature and 60% from mature seeds. In the second experiment, sodium phytate (1.7% phytic acid) was added to a basal egg white diet to simulate the phytate level in the diet containing mature soybean seeds. The rats were fed a zinc deficient diet for five days before the 16 hours fast and administration of a single meal. ^{65}Zn as zinc sulfate was added extrinsically in the case of the basal diet and as intrinsic label in the case of the soybean seeds. Results showed that the phytate supplement decreased the absorption of ^{65}Zn from both sources. From these experiments, it is clear that both endogenous and added phytate impair zinc absorption.

From these studies, it is clear that phytate has a negative effect on zinc bioavailability. A question arises with regard to whether the factor or factors responsible for the low zinc bioavailability in cereals and legumes can be either removed or overcome. Lonnerdal et al. (1988) evaluated the effect of phytate removal from soybean formula on zinc absorption using suckling rat pups and infant rhesus monkeys as animal models. The absorption of zinc from human milk, whey-predominant formula (60 : 40 whey : casein), casein-predominant formula (18 : 82 whey : casein), soy formula and dephytinized soy formula was determined by whole body counting. In infant rhesus monkeys, zinc absorption was 65% from human milk, 60% from whey-predominant formula, 46% from casein-predominant formula and only 27% from conventional soy formula.

However, the zinc absorption from dephytinized soy formula was increased to 45%. Similar results were obtained in suckling rat pups. Zinc absorption was 60% from human milk, 52% from whey-predominant formula, 53% from casein-predominant formula compared to 16% from conventional soy formula and 47% from dephytinized soy formula. Results suggested that the low bioavailability of zinc from soy formula was attributed to its phytate content and it could be overcome by the removal of phytate. These results were confirmed by Zhou et al. (1992) who investigated zinc bioavailability in rats fed either egg white-based or soybean isolate-based diets. Two commercial soybean isolates containing either normal (2.20%) or reduced levels (0.47%) of phytic acid were used. Results showed that zinc bioavailability from soybean isolate-based diets containing normal levels of phytic acid was significantly reduced compared to the egg white-based diets; furthermore, the reduced phytic acid soybean isolate-based diets resulted in a significant increase in zinc bioavailability compared to the soybean isolate-based diets containing normal phytic acid level. These results coupled with other reports indicate that phytic acid is the primary inhibitory factor in soybean products that results in reduced zinc bioavailability, and that phytate reduction in soybean protein increases zinc bioavailability.

In human studies, Prasad (1976) reported that patients who were characterized by dwarfism and hypogonadism showed an improvement in growth and sexual maturity in response to zinc supplementation of a hospital diet which consisted mainly of phytate-rich cereals and beans. Analysis of foods consumed by humans suffering from zinc deficiency symptoms revealed that the amounts of zinc from foods were adequate

according to published nutrient requirement literature. Later it was realized that the presence of phytate in plant products was an important factor in the reduction of zinc absorption. Several studies on the effect of phytate on zinc deficiency in humans were reported from developing countries since people in these countries consume diets primary consisting of mixtures of cereals and legumes (Reinhold, 1971 and 1973; Reinhold et al., 1981). The average daily phytate intake may be higher when compared to developed countries, where the diets contain primary animal proteins. Thus, zinc deficiency in humans in Middle Eastern countries was in part caused by the high phytate contents of breads that are the staple food of the poor.

Several researchers evaluated zinc absorption in humans from composite meals containing varied levels of phytate (Turnlund, et al., 1984; Sandstrom, et al., 1980; Morris and Ellis, 1985). Most of these studies concluded that high levels of phytate in the meals decrease zinc absorption. Turnlund et al. (1984) studied the effects of α-cellulose and phytate on zinc absorption in young men. A semipurified liquid formula diet supplying 15 mg of zinc daily was given, and zinc absorption was measured by monitoring fecal excretion of a stable isotope of zinc. Results showed that average zinc absorption was 34% for the basal diet, but dropped to 17.5% when 2.34 g of phytate as sodium phytate was added to basal semipurified liquid formula. They concluded that phytate inhibits zinc absorption and high levels of dietary phytate could result in zinc deficiency in humans. However, from this study, it is not known whether or not the effect would be the same if the same level of phytate were consumed in a natural food diet. Morris et al. (1988) investigated mineral

absorption of adult men consuming whole or dephytinized wheat bran which contained 2 or 0.2 g phytic acid, respectively. Results showed that apparent absorption tended to be lower when whole bran was consumed. Sandstrom et al. (1985) observed that retention of a radioactive tracer of zinc in humans was inversely related to the amount of phytate in meals that were based on cereal grains.

The molar ratio of phytate to zinc in phytate-rich food has been suggested as a predicator of the availability of zinc. This concept was first proposed by Oberleas and Prasad (1976). Later, this hypothesis was supported and demonstrated by Davies and Olpin (1979) and Morris and Ellis (1980). The latter investigators used semipurified diets to test the bioavailability of dietary zinc at different phytate : zinc molar ratios in rats. Growth and femur zinc were used as parameters to evaluate zinc bioavailability. They observed that growth was not adversely affected when the dietary phytate : zinc molar ratio was about 12 or less and the dietary zinc was near the minimum requirement (12 ppm). When the phytate : zinc molar ratio was greater than 12, growth was not depressed if the dietary zinc concentration was 2.5 or 5 times the minimal requirement for growth. However, the accumulation of zinc in femurs was depressed. In agreement with the findings of Morris and Ellis, Lo et al. (1981) found that, in general, ratios of approximately 12 and above reduced zinc bioavailability. Lo and co-workers determined the zinc bioavailability in isolated soy protein by giving an oral dose of ^{65}Zn and measuring the liver uptake and disappearance from the gastrointestinal tract of rats. Results showed that when rats were fed a low zinc diet (5 μg Zn/mg) for 2 weeks before the absorption trial , the isolated soy protein source had an negative

effect on zinc absorption. In a four hour period, ^{65}Zn absorption was 12.7% when administered with soybean protein compared to 30.4% when given with egg white. The liver uptake was 4.2% for soybean protein diet compared to 10.1% for egg white. The phytate:zinc ratio in soybean protein was 12.5 and zinc absorption was 40% of that in the egg white protein which contained no phytate. In contrast, when rats were fed adequate dietary zinc prior to ^{65}Zn administration, the percentage absorption and liver uptake were the same for both proteins. From these experiments, it was demonstrated that phytate had a marked effect on zinc absorption when the zinc status of the animal was low and the ratio of phytate : zinc was high but had little effect when the phytate : zinc ratio was 9 or less and the zinc status was adequate.

In addition to phytate : zinc molar ratios, Morris and Ellis (1980) also showed the importance of the calcium content of the diet to the phytate : zinc molar ratio on zinc bioavailability. Growth of rats was not affected by phytate : zinc molar ratios of 12 or less if the level of dietary calcium was 0.75% but was depressed if the level of calcium was 1.75%. In contrast, when the dietary zinc concentration was 2.5 or 5 times higher than the minimal requirement, growth was not depressed at the dietary calcium levels of 0.75% and 1.75%. These findings were confirmed by Graf and Eaton (1984), who suggested that calcium ions potentiated zinc ion precipitation only at high phytate : zinc ratios.

Interactions with copper. Although the effect of phytate on the absorption and availability of copper has been studied, compared to zinc the effect of phytate on copper bioavailability was less pronounced and the

results were often conflicting. Johnson, P. E. et al. (1988) evaluated copper bioavailability in sunflower seeds, peanuts, cooked garbanzo beans, cooked shrimp and cooked beef compared to copper sulfate using an extrinsic ^{67}Cu label. Rats were fed a diet containing 2.5 ppm copper for 2 weeks before administration of a test meal. The amount of copper absorbed from sunflower seeds, peanuts, cooked garbanzo beans, cooked shrimp, and cooked beef was 46%, 41%, 30%, 50%, and 46%, respectively. Copper absorption from the control diet which contained copper sulfate was 46%. These results suggested that copper bioavailability in cooked garbanzo beans tended to be lower compared to other foods in a single meal. In an earlier study, Davies and Nightingale (1975) found that the addition of 1% phytate as sodium phytate to an egg white diet significantly decreased copper absorption. The whole body retention of copper was also significantly reduced by 57% in rats when they received a diet containing a phytate to copper molar ratio of 40.

In contrast, the results of Lee et al. (1988) suggested that phytic acid increased copper bioavailability in rats by its ability to bind other dietary components, such as zinc, that compete with copper at the site of intestinal absorption. In their study, depletion and repletion feeding techniques were used to evaluate the biologically available copper. Liver copper, liver Cu, Zn-superoxide dismutase activity, serum copper and serum ceruloplasmin were used as physiological indicators to determine the bioavailability of copper in the diet. In the repletion period, rats were fed diets containing either 1.4, 3.0, 5.2, or 10.5 μg Cu/g as copper carbonate and 0, 0.4, or 0.8% phytic acid as sodium phytate at each copper level. The results of this study indicated that phytic acid added to the diets containing

5.2 µg/g copper significantly enhanced copper utilization during the 3d repletion period, whereas there were no differences among the other levels of copper. This enhanced utilization was evident for both amounts of dietary phytate tested and for all copper indices examined.

In an experiment conducted by Lo et al. (1984), the bioavailability of copper in isolated soybean protein was evaluated with growing rats using a depletion and repletion feeding approach. After a 21 day copper depletion period, rats were fed diets containing different levels of copper either from copper carbonate or isolated soybean protein for one week. Serum and liver copper contents were used as indicators to determine the bioavailability of copper. Results suggested that copper was available equally from isolated soybean protein and copper carbonate.

Turnlund et al. (1985) studied the effects of phytate on copper absorption in young men. The young men were given a liquid formula diet with and without 2.35g of phytic acid as sodium phytate. The average copper absorption was 35% (25.3%-45.3%) from the basal diet and 31.4% (22%-44.6%) from the diet containing added phytic acid. They concluded that high levels of phytate do not affect copper absorption. Morris and Ellis (1985) similarly concluded that intake of 0.5, 1.7 or 2.9 g of phytic acid daily had no effect on apparent absorption of copper in adult men.

Summary

There is considerable potential for increased utilization of cereals and legume-based foods in developed countries, and a high consumption of these foods already exists in developing countries. These plant products, however, contain considerable quantities of phytic acid which may affect mineral bioavailability.

Phytic acid, myoinositol hexaphosphate, is a storage form of phosphorus in plants and is common constituent in cereals and legumes. Its molecule is highly charged with six phosphate groups extending from the central inositol ring structure and therefore is an excellent chelator of cations. At neutral pH, phosphate groups of phytate have either one or two negatively charged oxygen atoms, and it is apparent that various cations could strongly chelate between two phosphate groups or weakly within a phosphate group.

Numerous studies have suggested that phytate in plant foods reduces bioavailability of essential trace elements. The negative effect of phytate on zinc bioavailability is clear and occurs in both human and animal studies. The degree of its effect on zinc bioavailability depends on the phytate : zinc molar ratio and zinc nutritional status of the animal. Whether the phytate is added to the diet as sodium phytate or as endogenous phytate seems to make little difference in its effect on zinc absorption.

Compared to zinc, the effect of phytate on copper utilization has not been studied extensively and results often are conflicting. Results from experiments reported in the literature indicate that further studies are need to clarify the effect of phytic acid on copper bioavailability. In

addition, most of the available information related to the effect of food phytate on mineral utilization were from studies on soybean or soybean products. The data on soybean may not be completely applicable to other types of beans. The effect of phytate in other legumes on mineral utilization should be also investigated.

The objectives of this study were:

1. to evaluate the bioavailability of copper in kidney beans and garbanzo beans by comparing the utilization of copper from copper carbonate, kidney bean or garbanzo bean.
2. to determine the effect of phytate in beans on copper utilization.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

General procedures. Weanling male Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed individually in stainless steel wire cages in a temperature (21-23 °C) and humidity (68-70%) controlled room with 12-hour cycles of light and dark (light cycle, 7 am-7 pm). Deionized water was available ad libitum using water bottles with plastic caps and stainless steel drinking tubes. Food intakes and body weights were recorded twice a week. Experimental protocol and procedures were approved by the All University Committee on Animal Use and Care at Michigan State University.

Experiment 1

Animals and treatments. The purpose of this experiment was to evaluate the utilization of copper in kidney beans and garbanzo beans. After an adjustment period of 2 days, 24 weanling male Sprague-Dawley rats were randomly allocated to the following three diet treatment groups: 1) control, 2) kidney bean and 3) garbanzo bean. Beans were selected for the experiment based on literature values for their phytate content:

garbanzo bean, 0.65%; kidney bean, 2.06% (Reddy et al., 1982). Rats were fed the respective diets for 3 weeks. After an overnight fast at the end of the feeding period, rats were anesthetized with methoxyflurane and blood collected by open cardiac puncture using 5 ml syringes rinsed with 1000 units/ml sodium heparin solution. Blood samples were centrifuged (Centrific Centrifuge, Fisher Scientific, Itasca, IL) at 200 x g for 20 min. Serum was removed immediately and stored at -20 °C for later determination of ceruloplasmin activity and serum copper and zinc. Liver and kidneys were removed, washed with cold saline, blotted, weighed and frozen on dry ice. Tissues were stored at -20 °C and freeze-dried (Unitrap II, Virtis Co., Gardiner, NY) for later analysis. On the first day of the experiment, four additional rats were killed and blood and tissues obtained for baseline analyses.

Diets. Table 1 shows the compositions of diets used in this experiment. The diets were formulated according to the general recommendations of the American Institute of Nutrition (Reeve et al., 1993) with exception of the vitamin mix and the mineral mix. Diets provided adequate and approximately equal amounts of total energy, protein and fat. Protein was supplied by egg white or a combination of egg white and beans. The amount of beans used in garbanzo bean and kidney bean diets was seleted to provide approximately 2 mg Cu/kg which is lower than the AIN-93G recommendation (6 mg/kg). The same amount of copper was provided by copper carbonate in the control diet. The purpose of using a lower copper level was to maximize the chance of showing differences in bioavailability. A copper-, zinc- and iron- free

TABLE 1

Compositions of diets, Experiment 1

Ingredient	Control	Kidney bean	Garbanzo bean
	(g/100g)		
Kidney bean[1]	—	30.7	—
Garbanzo bean[2]	—	—	32.7
Egg white[3]	20.0	11.5	12.7
Cornstarch	39.8	19.6	15.8
Dyetrose[4]	13.2	13.2	13.2
Sucrose	10.0	10.0	10.0
Solka-Floc (BW 200 cellulose)	5.0	3.1	3.7
Choline bitartrate	0.14	0.14	0.14
L-cystine	0.3	0.3	0.3
AIN-76 vitamin mix[4]	1.0	1.0	1.0
Mineral mix[5] (Fe, Zn, Cu free)	3.5	3.5	3.5
Soy oil	7.0	7.0	7.0
	(mg/100g)		
Zinc carbonate	2.30	0.62	0.28
Ferric citrate	21.10	11.20	10.30
Cupric carbonate	0.38	—	—

[1] Pigeon Co-op Co., Pigeon, MI

[2] Manchester Co., Shawnee Mission, KS

[3] Teklad Test Diet Co., Madison, WI

[4] Dyets Inc., Bethlehem, PA

[5] Supplies (g/kg mineral mix): $CaHPO_4$, 500; NaCl, 74; $K_3C_6H_5O_7.H_2O$, 220; K_2SO_4, 52; MgO, 24; $MnCO_3$, 3.5; KIO_3, 0.01; $Na_2SeO_3.5H_2O$, 0.01; $CrK(SO_4)_2.12H_2O$, 0.5; sucrose, 125.93.

mineral mix was prepared to provide the recommended amount of minerals for rats. The amount of zinc carbonate, ferric citrate and cellulose added to the diets was adjusted for amounts of zinc, iron and fiber provided by the beans so that the total amount of these minerals and insoluble fiber in the diets would be equal. The copper, zinc and iron content of experimental diets was analyzed by atomic absorption spectrophotometry before feeding.

Bean preparation. Kidney beans (Pigeon Co-op Co., Pigeon, MI) and garbanzo beans (Manchester Co., Shawnee Mission, KS) were cooked in deionized water for 4 hours at a ratio of 1 : 2 and 1 : 3 (beans : water), respectively. The cooked beans were dried in a forced air convectional drying oven (Proctor & Schwartz Inc., Philadelphia, PA) at 60°C for 48 hours. The dried beans were ground to fine powder using a micro-mill (Chemical Rubber Co., Cleveland, OH) and analyzed for protein, copper, zinc, iron before incorporation into the diets.

Experiment 2

Animals and treatments. The purpose of the second experiment was to clarify results obtained in experiment 1 using a different experimental approach. After a 2 day adjustment period, four rats were killed on day 1 of the feeding study to obtain baseline values. The remaining 32 rats were fed a copper deficient basal diet (0.12 mg Cu/ kg) for 4 weeks. Rats were trained to meal feed by restricting food intake (9:00-5:00 pm). The purpose of the meal feeding was to avoid differences in

food intake that had occurred in experiment 1. During the fourth week, food intakes were recorded daily to obtain the average daily food intake. At the end of 4 weeks, blood samples were taken from tail arteries to determine serum ceruloplasmin. Rats were divided into 4 groups (n=8) of equal mean weight and fed the respective experimental diets for 7 days. The amount of diet fed (15 g) was based on the average daily food intake during the fourth week of the basal copper deficient diet. The dietary treatments were: 1) control (basal copper deficient diet with the addition of copper at 2 mg /kg diet as copper carbonate), 2) control diet with the addition of sodium phytate (Sigma Chemical, St. Louis, MO) at 1.53 g/kg diet, 3) kidney bean diet and 4) dephytinized kidney bean diet. The copper content of the bean diets was 2 mg/kg provided by the beans. At the end of the four weeks of copper depletion, 1 ml blood was taken from tail artery into a 1 ml syringe rinsed with sodium heparin for ceruloplasmin activity determination. At the third day of copper repletion period, blood samples were taken from the tail artery again. After seven days of copper repletion, rats were anesthetized and killed by open cardiac puncture. Blood and tissues were collected as previously described in experiment 1.

Diets. Composition of the basal copper deficient diet and four test diets is listed in Table 2. Diets were prepared in the same manner as in experiment 1 and formulated to provide adequate and equal amounts of total energy, protein and fat. Protein sources in the experimental diets were supplied by egg white or a combination of egg white and kidney beans. The protein content of the diets was analyzed using standard Kjeldahl nitrogen analysis (AOAC, 1984). In this experiment, a AIN-93G

TABLE 2

Compositions of diets, Experiment 2[1]

Ingredient	Basal Dephytinized	Control	Control +phytate	Kidney bean	Kidney bean
		(g/100g)			
Kidney bean	—	—	—	30.0	30.0
Egg white	20.0	20.0	20.0	11.6	11.6
Cornstarch	39.8	39.8	39.7	20.2	20.2
Dyetrose	13.2	13.2	13.2	13.2	13.2
Sucrose	10.0	10.0	10.0	10.0	10.0
Solka-Floc (BW 200 cellulose)	5.0	5.0	5.0	3.0	3.0
Choline bitartrate	0.14	0.14	0.14	0.14	0.14
L-cystine	0.3	0.3	0.3	0.3	0.3
AIN-76 vitamin mix	1.0	1.0	1.0	1.0	1.0
AIN-93 mineral mix[2] (Fe, Zn, Cu free)	3.5	3.5	3.5	3.5	3.5
Soy oil	7.0	7.0	7.0	7.0	7.0
Sodium phytate[3]	—	—	0.1	—	—
		(mg/100g)			
Zinc carbonate	2.30	2.30	2.30	0.60	0.60
Ferric citrate	21.00	21.00	21.00	11.00	11.00
Cupric carbonate	—	0.32	0.32	—	—

[1]The sources of ingredients in this experiment were the same as in experiment 1 with the exception of mineral mixture, sodium phytate and phytase.

[2]Dyets Inc., Bethlehem, PA

[3]Sigma Chem. Co., St. Louis, MO

mineral mix without copper, zinc and iron was purchased from Dyets (Bethlehem, PA). Zinc carbonate, ferric citrate and copper carbonate were premixed with sucrose and then incorporated into the test diets and basal copper deficient diet as indicated in Table 2. Concentrations of copper, zinc and iron in experimental diets were analyzed by atomic absorption spectrophotometry.

Bean and Dephytinized bean preparation. Kidney beans were obtained from the same source as in experiment 1. Beans were mixed with deionized water in a ratio of 1 : 1.2 (beans : deionized water) and soaked for 2 hours. Beans were then cooked by a conventional home cooking procedure for 2 hours and dried in an air oven at 100°C for 48 hours. The dried beans were ground to a fine powder using a coffee mill (Robert Krups, Denver, CO).

Dephytinzed beans were prepared according to procedures described by Sandberg and Svanberg (1991) modified as follows: 200 g of ground kidney bean and 2 g wheat phytase (Sigma Chem. Co., St. Louis, MO) were suspended in 300 ml of deionized water. The pH was adjusted to 4.5 using 1N hydrochloric acid and then gradually increased to 5.15, the optimal pH for phytase in wheat. The suspension was incubated at 55°C in a water bath with mild mechanical agitation. The suspension was removed after 17 hours, dried in an oven for 24 hours and ground using a coffee mill (Robert Krups, Denver, CO).

Beans were analyzed for protein, copper, zinc, iron before they were mixed with other ingredients in the preparation of the complete diet.

Inositol phosphates (Ip3 to Ip6) were also determined using ion exchange chromatography and high pressure liquid chromatography techniques.

Analytical methods

Protein content in diets. Protein content of the diets was determined by the standard Kjeldahl nitrogen analysis using a modification of the AOAC (1984) method. Concentrated sulfuric acid (5 ml) and one catalyst table (potassium sulfate and selenium; Tecator Co., England) were added to each of the digestion tubes containing preweighed samples. The samples were digested using gradually increased temperatures until digestion was complete. The protein content was calculated on a dry weight basis using a nitrogen conversion factor of 6.25.

Tissue and diet copper and zinc determination. Diets and tissues were digested using a modified wet-ash procedure (Clegg et al., 1981). Duplicate samples of diets, freeze-dried livers, kidneys and tibias were weighed and put into 50 ml Erlenmeyer flasks. Following the addition of 10 ml of concentrated nitric acid, flasks were heated at low temperature for 30 min to oxidize the more reactive organic matrix without an overly vigorous reaction. The temperature was raised after the initial chemical reaction subsided. For some samples additional nitric acid was needed. When the nitric acid digest had been reduced to near dryness, hydrogen peroxide (30%) was then added to oxidize the remaining particles. After evaporation of hydrogen peroxide, a white ash was obtained. The flask contents were cooled and diluted with 0.1N HCl (Cu: 10 ml, Zn: 20 ml).

Samples were analyzed for copper, zinc and iron by atomic absorption spectrophotometry (Perkin-Elmer model 2380, Norwalk, CT). Mineral standards in appropriate concentrations were prepared from stock standard solutions (J. T. Baker Chem. Co., Phillipsburg, NJ) and analyzed by atomic absorption spectrophotometry.

The accuracy of the ashing procedure and the atomic absorption spectrophotometry was checked by analyzing NIST bovine liver-1577b (National Institute of Standards and Technology, Gaithersburg, MD).

Serum copper and zinc determination. Serum samples were diluted and analyzed for copper and zinc by atomic absorption spectrophotometry. Serum was diluted with an equal volume of deionized water for copper analysis and diluted 1 : 5 with deionized water for zinc analysis. Copper and zinc standards were prepared by diluting the stock standard solution with 10% (v/v) or 5% (v/v) glycerol, respectively. A 10% or 5% glycerol solution was used in order to maintain similar viscosity in samples and standards. Absorbance of samples was recorded and compared to standard solutions treated in a similar manner.

Determination of serum ceruloplasmin activity. Ceruloplasmin activity of serum samples was determined by the colorimetric procedure of Schosinsky et al. (1974). This method is based on the oxidase activity of ceruloplasmin on diamine groups using o-dianisidine dihydrochloride as substrate (Sigma Chem. Co., St. Louis, MO). This reagent is converted to a yellowish-brown product in the presence of ceruloplasmin and oxygen at

pH 5. Acidification stops the enzymatic reaction, and a stable purplish-red solution is formed that absorbs maximally at 540 nm.

Determination of phytate phosphorus in beans. A modified method of ion-exchange procedure (Graf and Dintzis, 1982) and colorimetric measurement (Fiske and Subbarow, 1925) was used for determination of phytic acid. Dried ground bean samples (0.5g) were extracted in 20 ml 0.5M hydrochloric acid using vigorous mechanical agitation for 2 hours at 20 °C. The mixture was centrifuged at 1800 rpm for 30 min (International Centrifuge, Model UV) and supernatant decanted, frozen overnight and filtered through a 0.45 micrometer membrane filter (Gelman Sciences, Ann Arbor, MI) under pressure. The filtrate was diluted with 10 ml distilled deionized water and passed through an ion exchange column containing 0.65 ml resin (AG 1-X8, 200-400 mesh) at 0.4 ml/min follow by 10 ml of 0.025 M HCl. Inositol phytates were removed and collected from the column with ten 1 ml portion of 2M HCl. The final eluent was then digested with 5 ml nitric acid and 5 ml hydrogen peroxide until white ash was obtained. The ash was dissolved in 10 ml deionized water, and a 0.5 ml aliquot was then taken for phosphorus determination (Fiske-Subbarow, 1925). The final sample was allowed to stand for 15 min for color formation; absorbance was read at 640 nm (Spectronic 21, Bausch & Lomb). A standard curve was prepared by diluting a stock standard solution (100 µg P/ml) made from potassium acid phosphate. A factor of 3.55 was used to convert phytate phosphorus into total phytate content.

HPLC analysis of inositol phosphates in beans. The purpose of determining the inositol phosphates in kidney beans and dephytinized kidney beans was to quantify the inositol hexaphosphates in the kidney beans and dephytinized kidney beans and evaluate the extent of degradation of phytic acid in dephytinized kidney beans used in experiment 2. Phytate content in kidney bean and dephytinized kidney bean was determined by ion exchange chromatography and high pressure liquid chromatography (HPLC) techniques using modifications of methods described by Graf and Dintzis (1982) and Sandberg and Ahderinne (1986). Samples were prepared using the same ion exchange procedure as described previously for phytate phosphorus determination. The final eluent was evaporated to dryness and diluted with 1 ml of HPLC water. A 100 µmole/ml stock sodium phytate solution was made by dissolving sodium phytate (Sigma, St. Louis, MO) in HPLC water. Standard solutions contained 1, 2, 5, 10 and 20 µmol/ml of phytate. The mobile phase consisted of 0.05M formic acid : methanol (46 : 54) to which was added 1.5 ml/100 ml of tetrabutyl ammonium hydroxide. The pH was adjusted to 4.3 by addition of 9M sulfuric acid. The mobile phase was filtered through a millipore filter (0.45 µm) under vacuum and degassed by sonication for 20 min. A reverse phase Supelcosil LC-18 column (25 cm x 4.6 mm) (Supelco, A ROHM and HAAS Co., Bellofonte, PA) 5 micron particle size was equilibrated with the mobile phase for one hour each day prior to beginning the sample analysis. Samples were analyzed using a Model M45 Waters HPLC pump (Waters Associates, MA), Rheodyne injector (Model 7010, Rheodyne Inc. CA) with a 20 µl loop at a flow rate of 2 ml/min. Inositol phosphates were detected using a differential refractometer

(Model R401, Waters Associates, MA). Retention times and peak areas were measured with an analog to digital board and Peak Simple II integration software (SRI Instruments, CA). The linearity of phytate concentration versus peak area was determined using 20 µl injections of standard solutions (1-20 µmol/ml) and a standard curve was obtained by calculating the regression equation for peak area vs standard solutions. Phytate content of the samples were computed from sample peak areas and the regression equation.

Statistical methods

All data were analyzed by one-way analysis of variance. If the difference was significant at $P< 0.05$, the Bonferroni/Dunn test was used for comparisons among groups. Tests were conducted with statistical software (Statview version 4.01, Abacus Concepts, Inc., 1992-1993).

RESULTS

Experiment 1

Copper, zinc, iron, phytic acid and protein content of diets. The analyzed amounts of copper, zinc, iron were similar in all diets (Table 3). The accuracy of analytical procedures was verified by analysis of NIST bovine liver. The analyzed copper, zinc and iron concentrations in bovine liver were within the range of certified values. The two kind of beans were selected to represent beans with low and high contents of phytate. Literature values for the phytate content of garbanzo and kidney beans are 0.65% and 2.06%, respectively (Reddy et al., 1982). However, the analyzed values for the beans used in this experiment were low (0.4% and 0.6% for garbanzo bean and kidney bean, respectively) and also similar to each other. Concentrations of phytate in the diets containing garbanzo bean and kidney bean (Table 1) were 0.13% and 0.19%, respectively (Table 5). The analyzed protein concentrations of kidney beans and garbanzo beans were 22.1% and 17.8%, respectively. The amount of protein in the two bean diets was then adjusted with egg white to be the same as in the control diet .

Body weight, weight gain, food intake, efficiency of food utilization and total mineral intake. The mean final body weight of rats in the

TABLE 3

Analyzed mineral and phytic acid content of diets, Experiment 1

Group	Phytic acid	Copper	Zinc	Iron
	(mg/g)		(μg/g)	
Control	—	2.53	12.64	37.40
Garbanzo bean	1.3	2.37	11.93	36.25
Kidney bean	1.9	2.15	12.00	39.00

control group (208±2.0 g) was significantly lower than that of the garbanzo bean (233±5.6 g) and kidney bean (236±4.8 g) groups (Table 4). There was no significant difference in the final body weight of the garbanzo bean and kidney bean groups. Rats fed the control diet gained significantly less weight than rats fed the garbanzo bean and kidney bean diets. The lower total weight gain of rats in the control group, which was 80 % of that in the garbanzo bean group and 78% of that in the kidney bean group, may be related in part to differences in food intake, since their food intake was significantly less than that in the bean groups. However, decreased efficiency of food utilization was also observed in rats fed the control diet compared to the garbanzo bean or kidney bean diets.

The total dietary intakes of copper, zinc and iron are shown in Table 5. Because of differences in food intake, the total copper and zinc intakes were significantly ($P< 0.05$) lower in rats fed the control diet than in those fed the garbanzo bean or kidney bean diets.

Organ wet weights, dry weights and % body weight of organs. Rats fed the garbanzo bean and kidney bean diets had significantly higher liver and kidney weights than rats fed the control diet (Table 6). However, there were no significant differences in the mean wet weights of the livers and kidneys between rats fed garbanzo bean and kidney bean diets. The same trend was observed in the mean liver and kidney dry weight values for rats fed the three diets. These results could be related to differences in body weights. Body weights of rats in garbanzo bean and kidney bean diets were also significantly higher than those fed the control diet. Therefore, when the liver and kidney weights were expressed per 100 g body weight,

TABLE 4

Body weights and food intake of rats, Experiment 1[1]

Group	Initial body weight (g)	Final body weight[2] (g)	Weight gain[2] (g)	Food intake[2] (g/d)	Food efficiency ratio[2,3] (%)
Control	94±2	208±2.0[a]	114±1.3[a]	13.2±0.2[a]	36.6±0.5[a]
Kidney bean	92±3	233±5.6[b]	141±4.3[b]	14.7±0.3[b]	41.6±1.0[b]
Garbanzo bean	91±3	236±4.8[b]	145±4.8[b]	14.6±0.3[b]	41.6±0.9[b]

[1]Values are means ± SEM (n=7 in control group and n=8 in kidney bean and garbanzo groups).

[2]Values in the same column with different superscript letters are significantly different ($p<0.05$) by Bonferroni/Dunn test.

[3]Food efficiency ratio (%) = weight gain/total food intake x 100.

TABLE 5

Total mineral intake from diets, Experiment 1[1]

Group	Total food intake (g)	Total copper intake[2,3] (µg)	Total zinc intake[2,4] (µg)
Control	312±6[a]	786±15[a]	3940± 71[a]
Garbanzo bean	339±8[b]	882±19[b]	4406± 93[b]
Kidney bean	349±7[b]	858±21[b]	4286±103[b]

[1]Values are means ± SEM (n=7 in control group and n=8 in kidney bean and garbanzo groups).

[2]Values in the same column with different superscript letters are significantly different ($p < 0.05$) by Bonferroni/Dunn test.

[3]Total copper intake = copper content in diet (µg/g as fed) x total food intake (g).

[4]Total zinc intake = zinc content in diet (µg/g as fed) x total food intake (g).

TABLE 6

Organ wet weights and dry weights, Experiment 1[1]

Group	Wet weight[2]		Dry weight[2]	
	Liver	Kidney	Liver	Kidney
	(g)		(g)	
Control	9.1±0.3[a]	1.6±0.0[a]	2.8±0.1[a]	0.4±0.0[a]
Garbanzo bean	10.1±0.3[b]	1.8±0.0[b]	3.1±0.1[b]	0.4±0.0[b]
Kidney bean	10.1±0.3[b]	1.8±0.1[b]	3.1±0.1[b]	0.4±0.0[b]

[1]Values are means ± SEM (n=7 in control group and n=8 in kidney bean and garbanzo groups).

[2]Values in the same column with different superscript letters are significantly different ($p<0.05$) by Bonferroni/Dunn test.

there were no significant differences between groups (Table 7).

Ceruloplasmin activity, serum concentrations of copper and zinc. Serum ceruloplasmin activity was significantly different among groups : control > garbanzo bean > kidney bean (Table 8). A similar pattern in serum copper was observed although the difference between the garbanzo bean and kidney bean groups were not statistically significant. Serum zinc concentrations followed the same trend as ceruloplasmin activity with statistically significant differences between groups and rats fed the control diet having the highest concentrations (Table 8).

Tissue concentrations of copper and zinc. The concentration of copper in livers and kidneys of rats were unaffected by dietary treatments (Table 9). The total kidney copper in rats fed garbanzo bean or kidney bean diets was significantly higher than that in rats fed the control diet. Total liver copper also was higher in the bean-fed rats although the differences were not statistically significant. The dietary treatments did not significantly affect zinc concentration and total zinc content of kidney. The total liver zinc content is significantly higher in rats fed bean diets although liver zinc concentration were not significant in all groups (Table 10).

Experiment 2

Copper, zinc, iron, phytic acid and protein content of diets. Table 11 lists the mineral and phytate content of the depletion and repletion diets.

TABLE 7

Organ wet weight to body weight ratio, Experiment 1[1]

	Ratio (%)	
Group	Liver	Kidney
Control	4.4±0.1	0.8±0.0
Garbanzo bean	4.3±0.0	0.8±0.0
Kidney bean	4.3±0.1	0.8±0.0

[1]Organ ratio (%) = (organ wet weight / body weight) x 100.

TABLE 8

Ceruloplasmin activity, serum copper and zinc concentration, Experiment 1[1]

Group	Ceruloplasmin activity[2] (U/L)	Serum copper concentration[2] (µg/dl)	Serum zinc concentration[2] (µg/dl)
Control	107.7±2.7[a]	88.3±4.9[a]	188.0±7.2[a]
Garbanzo bean	94.4±2.9[b]	76.3±1.7[b]	155.7±4.4[b]
Kidney bean	84.6±2.0[c]	72.4±2.6[b]	140.1±3.1[c]

[1]Values are means ± SEM (n=7 in control group and n=8 in kidney bean and garbanzo bean groups).

[2]Values in the same column with different superscript letters are significantly different ($p < 0.05$) by Bonferroni/Dunn test.

TABLE 9

Concentration and total content of copper in tissues, Experiment 1[1]

Group	Liver	Kidney	Tissue total copper[2] Liver	Kidney
	(μg/g dry weight)		(μg)	
Control	11.1±0.3	15.8±0.5	31.1±1.4	6.0±0.1[a]
Garbanzo bean	10.8±0.5	15.8±0.3	33.9±1.9	6.8±0.2[b]
Kidney bean	10.8±0.6	16.2±0.4	33.8±2.1	6.9±0.2[b]

[1]Values are means ± SEM (n=7 in control group and n=8 in kidney bean and garbanzo groups).

[2]Values in the same column with different superscript letters are significantly different ($p < 0.05$) by Bonferroni/Dunn test.

TABLE 10

Concentration and total content of zinc in tissues, Experiment 1[1]

Group	Liver (μg/g dry weight)	Kidney (μg/g dry weight)	Tissue total zinc: Liver (μg)	Tissue total zinc: Kidney (μg)
Control	37.0±1.6	24.2±1.6	103.0±4.9[a]	9.3±0.7
Garbanzo bean	38.4±1.4	23.3±0.8	120.5±4.9[b]	9.0±0.4
Kidney bean	40.9±2.1	24.3±0.9	127.2±6.8[b]	10.3±0.4

[1]Values are means ± SEM (n=7 in control group and n=8 in kidney bean and garbanzo groups).

TABLE 11

Analyzed mineral and phytate content of diets, Experiment 2

Diet	Phytic acid	Copper	Zinc	Iron
	(mg/g)		(μg/g)	
Depletion diet	—	0.12	11.42	43.44
Repletion diets				
Control	—	1.94	12.86	35.61
Control+ phytate[1]	1.4	2.36	13.02	33.04
Kidney bean[2]	1.4	2.20	12.41	33.89
Dephytinized[3] kidney bean	ND	2.10	13.01	35.43

[1]1.53g of sodium phytate (Sigma, St. Louis, MO) was added to the control diet to make the phytate content equal to the amount of phytate in the kidney bean diet.

[2]The phytate content in this diet was calculated as total phosphorus and then converted it to total phytate.

[3]The phytate content in dephytinized kidney beans was not detectable (ND) using HPLC analysis.

The amounts of copper, zinc and iron were similar in all repletion diets although the copper content was somewhat lower in the control diet. The analyzed concentration of phytate in kidney beans was 0.5%. HPLC analysis showed no phytic acid and little evidence of lower inositol phosphates in the dephytinized kidney beans. The amount of sodium phytate added to the control+phytate diet was based on the amount of phytate in the kidney bean diet (0.14%). The kidney beans used in this experiment were from the same source as in the first experiment; therefore, the protein content of the beans was assumed to be the same.

Body weight, weight gain, food intake and efficiency of food utilization. There were no significant differences in the mean initial and final body weights among groups in the repletion period (Table 12), although the final body weights in the control and control+phytate groups tended to lower than those in the kidney bean and dephytinized kidney bean groups. The mean body weights at the beginning of the depletion period also were not significantly different. The total weight gain and efficiency of food utilization in rats fed both the kidney bean and dephytinized kidney bean diets were significantly higher than those fed the control and control+phytate diets. These results were not due to differences in food intake since food intake in the four groups was controlled. The food intake of rats in each group was limited to the average food intake of all rats during the last week of the depletion period (15 g/day).

TABLE 12

Body weights and food intake of rats during the repletion period, Experiment 2[1]

Group	Initial body weight[2] (g)	Final body weight[2] (g)	Weight gain[3] (g)	Food intake (g/d)	Food efficiency ratio[3,4] (%)
Control	237±10	262±9	25±3[a]	15	27.8±3.0[a]
Control+ phytate	237± 7	264±6	27±1[a]	15	30.3±1.6[a]
Kidney bean	237± 8	276±6	39±2[b]	15	43.1±2.8[b]
Dephytinized kidney bean	237± 9	272±9	36±2[b]	15	40.6±2.4[b]

[1]Values are expressed as means±SEM (n=7 in dephytinized kidney bean group and n=8 in control, control+phytate and kidney bean groups).

[2]Initial body weights represent weight at the end of the depletion period, and the final body weights were measured at the end of the repletion period.

[3]Values in the same column with different superscript letters are significantly different ($p<0.05$) by Bonferroni/Dunn test.

[4]Food efficiency ratio (%) = weight gain/total food intake x 100.

Organ wet weights, dry weights and % body weight of organs. The mean wet weights and dry weights of the livers, kidneys and tibias in rats fed the control, control+phytate, kidney bean and dephytinized kidney bean diets were not significantly different (Table 13 and 14). Similarly, when the liver, kidney and tibia weights were expressed per 100 g body weight, the differences also were not statistically significant.

Ceruloplasmin activity, serum copper and zinc concentration. During wk 3 of the copper depletion period, rats were randomly chosen to test serum ceruloplasmin activity. The range of ceruloplasmin activity was from 3.1 U/L to 51.3 U/L (n=9). The ceruloplasmin activity values obtained in baseline rats ranged from 62.2 U/L to 103.4 U/L. Although the values of ceruloplasmin activity in the copper depleted rats were low compared to the values of baseline rats, individual variation was high. Consequently, the depletion period was extended one more week before the repletion period was started. After four weeks of copper depletion, the serum ceruloplasmin activity was determined in all rats, and rats were divided into four groups having similar mean ceruloplasmin values ranging from 3.3 to 6.4 U/L (Table 15). After consuming the repletion diets providing approximately 2 µg Cu/g for 3 days, rats showed an increase in ceruloplasmin activity irrespective of copper source in the diet. Rats fed either the control or dephytinized kidney bean diets had significantly higher ceruloplasmin activity than rats fed the control+phytate or kidney bean diets. The serum ceruloplasmin activity was not increased further by an additional four days of feeding the marginal copper diets; thus, at 7 days differences between groups were unchanged. Results in this study

TABLE 13

Organ wet weights and dry weights, Experiment 2[1]

Group	Wet weight			Dry weight		
	Liver	Kidney (g)	Tibia	Liver	Kidney (g)	Tibia
Control	11.1±0.6	2.2±0.1	0.8±0.0	3.4±0.2	0.5±0.0	0.4±0.0
Control+ phytate	10.9±0.4	2.1±0.1	0.7±0.1	3.3±0.2	0.5±0.0	0.4±0.0
Kidney bean	11.1±0.5	2.2±0.1	0.8±0.1	3.4±0.2	0.5±0.0	0.4±0.0
Dephytinized kidney bean	11.0±0.5	2.3±0.1	0.8±0.0	3.3±0.3	0.5±0.0	0.4±0.0

[1]Values are expressed as means±SEM (n=7 in dephytinized kidney bean group and n=8 in control, control+phytate and kidney bean groups).

TABLE 14

Organ wet weight to body weight ratio of rats, Experiment 2[1]

Group	Ratio (%)		
	Liver	Kidney	Tibia
Control	4.2±0.1	0.8±0.0	0.3±0.0
Control+ phytate	4.1±0.2	0.8±0.0	0.3±0.0
Kidney bean	4.0±0.2	0.8±0.0	0.3±0.0
Dephytinized kidney bean	4.0±0.2	0.8±0.0	0.3±0.0

[1]Organ ratio (%) = (organ wet weight / body weight) x 100.

TABLE 15

Ceruloplasmin activity at initial, 3d and 7d of repletion period, Experiment 2[1]

Group	Initial[2]	Repletion[3]	
		3d	7d
	U/L		
Control	6.4±2.6	113.4±2.1[a]	113.3±2.6
Control+ phytate	3.4±2.6	91.3±3.3[b]	95.2±4.0[b]
Kidney bean	3.3±1.9	82.4±3.9[b]	83.2±5.0[b]
Dephytinized kidney bean	6.4±1.3	114.0±2.3[a]	115.9±1.9[a]

[1]Values are expressed as means±SEM (n=7 in dephytinized kidney bean group and n=8 in control, control+phytate and kidney bean groups).

[2]Values represent the ceruloplasmin activity at the end of the copper depletion period.

[3]Values in the same column with different superscript letters are significantly different ($p<0.05$) by Bonferroni/Dunn test.

demonstrated that 3 days of copper repletion at 2 μg Cu/g of intake is sufficient to replete serum ceruloplasmin. Changes in serum copper values were consistent with those in serum ceruloplasmin activity (Table 16). When rats were fed the control or dephytinized kidney bean diets, serum copper concentrations were significantly higher than in rats fed the control+phytate or kidney bean diets. There were no significant differences in serum zinc among the four groups.

Tissue concentrations of copper and zinc. The concentrations and total contents of copper and zinc in livers, kidneys and tibias are given in Tables 17 and 18. The concentrations and total contents of copper and zinc in livers, kidneys, tibias were not significantly different among groups. However, the copper concentrations and total liver copper in rats fed control and dephytinized kidney bean diets tended to be higher as compared to rats fed the control+phytate and the kidney bean diets. The same pattern was observed in zinc concentration and total zinc content of livers and tibias.

Inositol phosphate analyses using high pressure liquid chromatography. There was a strong linear relationship between integrated values of refractive index detector response and amount of inositol hexaphosphates injected ($Y=231.50X-182.22$, $r=0.99$). Peak areas of kidney bean and dephytinized kidney beans extracts were measured and the concentrations of inositol hexaphosphates in the kidney beans and dephytinized kidney beans were calculated by the equation obtained from the standard calibration curve. Results showed that there was 0.5% of

TABLE 16

Serum copper and zinc concentrations after 7 days of repletion, Experiment 2[1]

Group	Serum copper[2]	Serum zinc
	µg/dl	
Control	106.6±3.3[a]	154.0±5.6
Control+ phytate	87.0±4.5[b]	158.1±5.3
Kidney bean	84.3±4.6[b]	152.2±8.7
Dephytinized kidney bean	110.5±2.5[a]	150.9±3.5

[1]Values are expressed as means±SEM (n=7 in dephytinized kidney bean group and n=8 in control, control+phytate and kidney bean groups).

[2]Values in the same column with different superscript letters are significantly different ($p<0.05$) by Bonferroni/Dunn test.

TABLE 17

Concentration and total content of copper in tissues, Experiment 2[1]

Group	Liver (µg/g dry weight)	Kidney (µg/g dry weight)	Tibia (µg/g dry weight)	Tissue total copper: Liver (µg)	Tissue total copper: Kidney (µg)	Tissue total copper: Tibia (µg)
Control	8.5±0.3	9.7±0.4	1.3±0.2	29.1±1.2	5.1±0.3	0.6±0.1
Control+ phytate	8.3±0.2	8.8±0.3	1.7±0.5	27.2±1.3	4.6±0.2	0.6±0.2
Kidney bean	8.3±0.3	9.4±0.5	1.2±0.5	27.7±1.6	5.1±0.3	0.5±0.3
Dephytinized kidney bean	9.1±0.6	10.3±0.3	1.4±0.3	29.4±2.1	5.6±0.2	0.7±0.2

[1]Values are expressed as means±SEM (n=7 in dephytinized kidney bean group and n=8 in control, control+phytate and kidney bean groups).

TABLE 18

Concentration and total content of zinc in tissues, Experiment 2[1]

Group	Liver (µg/g dry weight)	Kidney (µg/g dry weight)	Tibia (µg/g dry weight)	Tissue total zinc: Liver (µg)	Tissue total zinc: Kidney (µg)	Tissue total zinc: Tibia (µg)
Control	30.4±0.9	26.8±0.8	180.3±7.2	100.1±7.4	14.1±0.5	78.5±2.3
Control+ phytate	27.2±2.5	26.2±1.7	178.2±9.9	89.9±6.3	13.7±0.8	69.5±2.0
Kidney bean	25.8±1.2	26.3±0.8	175.8±8.6	86.4±6.7	14.2±0.3	70.3±6.5
Dephytinized kidney bean	28.6±2.0	27.6±1.5	183.0±3.0	94.2±8.5	14.8±0.5	78.4±4.2

[1]Values are expressed as means±SEM (n=7 in dephytinized kidney bean group and n=8 in control, control+phytate and kidney bean groups).

phytic acid in kidney beans and phytic acid in dephytinized kidney beans was undetectable.

DISCUSSION

In experiment 1, when food intakes were not controlled, rats fed the kidney bean and garbanzo bean diets tended to ingest more food than the control group. The factor(s) responsible for this apparent preference for the bean diets are not clear but may have included differences in palatability or digestibility of the diets. This phenomena was also observed by Zhou et al. (1992) who reported that soybean protein-fed rats consumed greater amounts of diet than egg white fed rats. In experiment 1, higher total weight gains in the bean groups were also observed and it was thought that this difference was related to higher food intakes. In experiment 2, however, total weight gains were also significantly higher in the kidney bean and dephytinized kidney bean groups compared to the control and control+phytate groups. In this experiment food intakes were controlled, and theoretically, the total weight gains should be the same in all groups. Although diets were planned to be as similar as possible, small compositional differences in the diets may have contributed in part to the differences in weight gain.

Several parameters were used to evaluate copper and zinc status in this study. Results in experiment 1 showed that ceruloplasmin activity was significantly different among groups (control > garbanzo bean > kidney bean), and serum copper was significantly lower in the garbanzo

bean and kidney bean groups compared to the control group. The concentrations of copper in liver, kidney and tibia were not significantly different, although the liver copper concentrations were somewhat lower in the garbanzo and kidney bean groups. These results suggest that the availability of copper in garbanzo and kidney beans is lower than that of copper carbonate. A feeding trial of more than 3 weeks may be needed to show significant differences in tissue content in response to small differences in bioavailability.

The results in experiment 2 using a different experimental approach generally support findings in the first experiment. Serum copper and serum ceruloplasmin activity in the kidney bean and control+phytate groups were significantly lower than in the control and dephytinized kidney bean groups. The concentrations of liver, kidney and tibia copper were not significantly different among groups, although the kidney concentrations tended to be lower in the kidney bean and control+phytate groups compared to control and dephytinized kidney bean groups. Results indicated that whether the phytate is added to the diet as sodium phytate or as endogenous phytate seems to make little difference in its effect on copper bioavailability and the low copper bioavailability in diets containing phytate can be overcome by the removal of phytate. It is speculated that the one week of repletion is not enough to show the difference in tissue copper. The overall results from experiments 1 and 2 indicated that copper was less bioavailable in the diets that contained phytate than in those that did not contain phytate.

These data are in agreement with the observations of Johnson et al., (1988) and Davies and Nightingale (1975). Davies et al. (1975) found that

dietary phytate significantly reduced the average daily accumulation and whole body retention of copper. In their study, the amount of sodium phytate fed (1%) was higher than the level of phytate in the present experiment (0.13-0.19%). Although the phytate content of their diets was higher, the copper level also was higher (25 µg Cu/g diet), approximatly 12 times higher than the copper level used in this study (2 µg Cu/g diet). Johnson et al. (1988) used a single test meal which contained 20 µg copper extrinsically labeled with ^{67}Cu to evaluate the bioavailability of copper in various foods. Weanling rats were fed diets containing 2.5 ppm copper and 20 ppm zinc for 16 days before the test meals were given. Results showed that only 65% of copper in garbanzo beans was absorbed compared to copper sulfate.

Other research in rats and humans has shown no effecf of phytate on copper bioavailability or, in one study (Lee et al., 1988), an enhancing effect. These conflicting results may be related to differences in experimental conditions. Lo et al (1984) did not observe any difference in copper bioavailability between copper carbonate and isolated soy protein using a slope-ratio assay. In their study, rats were fed a copper deficient diet (0.53 µg Cu/g diet) for 3 weeks followed by 7 days of repletion diets containing 0.5, 1.0, 1.5 or 2.0 µg Cu/g diet from copper carbonate or various types of soy protein isolate. The phytate content in the diets ranged from 0.05% to 0.22% and was similar to that used in the present experiment. Serum and liver copper also were used as indicators to evaluate copper bioavailability in their study. They did not report the serum concentration of copper at the end of the depletion period, however, and it may be that rats were less responsive to the experimental diets because they were not

as depleted as those in the present study which were depleted for a total of 4 weeks. At the end of 3 weeks of copper depletion, ceruloplasmin activity was still high in some rats in experiment 2 although the average activity was decreased. At the end of 4 weeks depletion ceruloplasmin activity in all rats was markedly decreased. Other characteristics of the diets also may have affected the results, such as the amount of zinc used in the two studies. Diets contained 50 μg Zn/g diet in their study which was higher than the amount used in the present study (12 μg/g). It is possible that in the presence of a high level of zinc in the diet, phytate is more likely to form a phytate-zinc complex leaving less available to complex with copper.

Conflicting results were reported by Lee and co-workers (1988) who concluded that copper bioavailability was enhanced by the presence of phytate. In their study, rats were fed copper deficient diets (<1 μg Cu/g diet) for 4 weeks and then provided required amounts of copper (5 μg Cu/g diet) for 3 days. The level of copper in their study was higher than the level used in this study (2 μg Cu/g diet). The phytate content of diets (0.4 and 0.8%) were also higher than the content of phyate (0.12-0.18%) in the present study. Differences in total copper intake may influence the outcome. In their study, diets contained 20 μg Zn/g which was also higher than the amount used in present study (12 μg/g). It is possible that phytate is more likely to form a complex with zinc allowing more available copper to be absorbed; thus an enhancing effect occurred because copper level was higher.

Two groups of investigators did not observe any negative effect of phytate on copper absorption in humans (Morris and Ellis, 1985; Turnlund

et al., 1985).Relative to body weight the amount of phytate fed in these experiments was less than that used in animal experiments. In addition, the effect of phytate may be different in humans than in rats. Differences between species have been demonstrated for dietary factors affecting non-heme iron absorption. Reddy and Cook (1991) reported that whereas meat enhanced and tea inhibited iron absorption in humans, minimal effects of these substanecs were observed in rats.

In experiment 2, ceruloplasmin activity increased rapidly within 3 days and no further increases were observed at day 7. This result indicates that ceruloplasmin activity is very sensitive to copper status. Although the lower ceruloplasmin activity and serum copper in rats fed diets containing phytate were statistically significant, they may have limited biological significance. In experiment 1, the values of ceruloplasmin activity in baseline rats ranged from 80 to 126.3 U/L and were 82.2 to 103.4 U/L in experiment 2. The values in the baseline rats were considered as normal values because rats had been fed copper adequate diets. In both experiments, final ceruloplasmin values for all groups were within the range of baseline values although ceruloplasmin activity in the rats fed diets containing phytate were lower.

Although an inhibitory effect of phytate on zinc bioavailability is well established (O'Dell and Savage, 1960; Lonnerdal et al., 1988; Zhou et al., 1992), there was limited evidence of an effect on zinc in the experimental conditions used in this study. In experiment 1, serum zinc concentrations were significantly higher in the control group than the garbanzo bean and kidney bean groups. Although there were no significant differences in liver and kidney zinc concentrations between

groups, the liver zinc concentrations tended to be lower in the garbanzo bean and kidney bean groups. A longer period of feeding may have been needed to show significant differences in tissues. The higher total liver and kidney zinc contents in garbanzo bean and kidney bean groups might be explained by the higher total zinc intake in these groups.

In experiment 2, there were no significant differences in serum, liver, kidney, and tibia zinc concentrations. Since diets containing phytate were only fed for one week there may not have been sufficient time for the phytate to show an inhibitory effect. Another explanation could be a decreased zinc requirement in older rats. It is known that zinc is required for growth; therefore, it is more likely to see the effect of dietary treatment in growing rats than in older rats. In experiment 2, rats were approximately 7 weeks old before the experimental diets were initiated, and rats of that age are considered as adult. It is assumed that zinc requirement is lower for adult rats, therefore, the effect of dietary treatment would be less and a longer period of feeding is necessary in order to show differences.

Perhaps the most likely explanation for the weak evidence of an effect of phytate on zinc bioavailability is the low phytate : zinc ratio in the diets. It has been reported that the phytic acid : zinc molar ratio in the diet can be used as a predictor for the effect of phytic acid on zinc bioavailability (Oberleas and Prased, 1976; Morris and Ellis, 1980). In experiment 1, the phytic acid : zinc molar ratios were 10.7 and 15.5 for the garbanzo bean and kidney bean diets, respectively. In experiment 2, the phytic acid : zinc molar ratios were 10.5 and 11.0 in the control+phytate and kidney bean diets, respectively. Values greater than 12 have been reported to reduce

accumulation of zinc in femurs but not depress growth (Davies and Olpin, 1979; Morris and Ellis, 1980). Moreover, Zhou et al. (1992) reported that the total tibia zinc gain tended to be lower when the phytate : zinc molar ratio was between about 10 to 16 but the differences were not statistically significant. They concluded that zinc utilization is not inhibited when the ratio ranges from 10 to 16. Results in the present study confirm such a conclusion. Lo et al. (1981) reported that there was no effect on zinc bioavailability when the ratio was less than 9 and zinc status was adequate.

Work performed by Graf and Eaton (1984) demonstrated the importance of dietary calcium on the effect of the phytate : zinc molar ratio on zinc bioavailability. They indicated that a low calcium content in the diet (0.75%) had no effect on zinc bioavailability when the phytate : zinc molar ratio was 12 or less, whereas when the calcium content of the diet was increased to 1.75%, growth of rats was significantly depressed. In the present study, calcium concentrations were low and similar in all diets: 0.50, 0.51 and 0.52% in control, kidney bean and garbanzo bean diets, respectively in experiment 1 and 0.50, 0.50, 0.51 and 0.51% in control, control+phytate, kidney bean and dephytinized kidney bean diets in experiment 2, respectively.

The lower phytate contents of beans used in these experiments compared to literature values might be explained by food processing. It has been reported that phytate can be hydrolyzed during various types of food processing including cooking, soaking and fermentation (Chang, 1977; Reddy et al., 1982; Sandberg, 1991). During such processes hydrolysis occurs and various inositol phosphates are formed. Sandberg (1991) reported that soaking of wheat bran resulted in hydrolysis of 95% of the

phytate within one hour and complete degradation within two hours and concluded that the naturally occurring phytase in cereals are activated by soaking under optimal conditions (pH 4.5-5.5, 60°C). Chang (1977) also reported that incubation of presoaked beans in water at 60 °C for 10 hours lowers their phytate content by 90%. Therefore, it is speculated that part of the phytate in the beans used for the diets in this experiment was hydrolyzed to lower inositol phosphates during cooking and were not detected in the analysis for phytic acid.

CONCLUSION

The overall results of this study indicate that copper bioavailability is lower in diets containing phytate than those without phytate. In experiment 2, when phytate was added to the control diet, bioavailability of copper was lower compared to the copper carbonate in the control diet. In addition, results also indicate that when phytate was removed from kidney beans, copper bioavailability increased and was similar to that of copper carbonate. Therefore, it is concluded that whether the source of phytate is sodium phytate or garbanzo or kidney beans seems to make little difference, and the adverse effect of phytate on copper bioavailability can be overcome by removal of phytate. Although serum copper and ceruloplasmin activity were depressed, it is unclear if these changes have any biological significance. The values of ceruloplasmin activity in the present study were within the normal range. In other words, rats were not copper deficient; however, the diet was fed for a relatively short period of time. Therefore, a longer period of feeding would be needed to evaluate long term effects of phytate on tissues.

Bioavailability of zinc in this study was not inhibited by phytate. Although the serum zinc was significantly lower in the two bean groups in experiment 1, no differences were found in liver, kidney and tibia zinc concentrations. The phytate : zinc molar ratio used in present study

ranged from 10 to 16 and the ratio may have been too low to show difference in tissues. It is concluded that zinc bioavailability is not inhibited when the phytate : zinc ratio is less than 16.

FUTURE RESEARCH

It is well documented that expression of the phytate : zinc content of diets on a molar basis is a satisfactory means of predicting dietary zinc bioavailability. Results of numerous studies have demonstrated that an increase in the phytate : zinc molar ratio will decrease zinc bioavailability. In addition, some investigators have reported that the calcium content of diet to phytate : zinc molar ratio is also a good indicator to predict the zinc bioavailability since zinc bioavailability can be further decreased by high dietary calcium to form a calcium, zinc and phytate complex. However, few studies have been done to evaluate the phytate molar relationship with respect to copper. Using phytate : copper molar ratio to evaluate copper bioavailability can be further studied. In addition, according to the literature the copper is likely to interact with other minerals such as zinc and iron; therefore, the interactions among phytate, calcium, zinc, iron and copper expressed as molar ratio to evaluate the bioavailability of copper should be further investigated.

LIST OF REFERENCES

LIST OF REFERENCES

Association of Official Analytical Chemists. (1990) In: Official Methods of Analysis, 14th ed. (Williams, S. ed.), pp. 153-156. Association of Official Analytical Chemists Inc., Arlington, VA.

Bronner, F. & Yost, J. H. (1985) Saturable and nonsaturable copper and calcium transport in mouse duodenum. Am. J. Physiol. 249: G108-112.

Chang, R. (1977) Phytate removal from whole dry beans by enzymatic hydrolysis and diffusion. J. Food Sci. 42: 1098-1101.

Clegg, M. S., Keen, C. L., Lonnerdal, B. & Hurley, L. S. (1981) Influence of ashing techniques on the analysis of trace elements in animal tissue. I. Wet ashing Biol. Trace Element Res. 3: 107-115.

Clydesdale, F.M. (1989) The relevance of mineral chemistry to bioavailability. Nutr. Today 24: 23-30.

Clydesdale, F.M. (1988) Minerals: their chemistry and fate in food. In: Trace Minerals in Foods (Smith, K.T. ed.), pp. 57-94. Marcel Dekker Inc., New York, NY.

Davies, N.T. & Nightingale, R. (1975) The effects of phytate on intestinal absorption and secretion of zinc and whole body retention of zinc, copper, iron and manganese in rats. Br. J. Nutr. 34: 243-258.

Davies, N.T. & Olpin, S.E. (1979) Studies on the phytate:zinc molar contents in diets as a determinant of zinc availability to young rats. Br. J. Nutr. 41: 591-603.

DiSilvestro, R.A. & Cousins, R.J. (1983) Physiological ligands for copper and zinc. Ann. Rev. Nutr. 3: 261-288.

Ellis, R. & Morris, E.R. (1981) Relation between phytic acid and trace metals in wheat bran and soybean. Cereal Chem. 58: 367-370.
Evans, G.W., Grace, C.I. & Hahn, C. (1974) The effect of copper and cadmium on ^{65}Zn absorption in zinc deficient and zinc supplemented rats. Bioinorg. Chem. 3: 115-123.

Fairweather-Tait, S.J. (1992) Bioavailability of trace elements. Food Chem. 43: 213-217.

Fields, M., Ferretti, R.J., Reiser, S. & Smith, J.C. (1984) The severity of copper deficiency in rats is determined by the type of dietary carbohydrate. Proc. Soc. Exp. Biol. Med. 175: 530-537.

Fields, M., Holbrook, J., Scholfield, D., Rose, A., Smith, J.C & Reiser, S. (1986) Development of copper deficiency in rats fed fructose or starch: weekly measurements of copper indices in blood. Proc. Soc. Exp. Biol. Med. 181: 120-124.

Fields, M., Holbrook, J., Scholfield, D., Smith, J.C., Reiser, S. & Los Alamos Medical Research Group. (1986) Effect of fructose or starch on copper-67 absorption and excretion by the rat. J. Nutr. 116: 625-632.

Finley, E.B. & Cerklewski, F.L. (1983) Influence of ascorbic acid supplementation on copper status in young adult men. Am. J. Clin. Nutr. 37: 553-556.

Fischer, P.W.F., Giroux, A. & L'Abbe, M.R. (1984) Effect of zinc supplementation on copper status in adult man. Am. J. Clin. Nutr. 40: 743-746.

Fiske, C.H. & Subbarow, Y.S. (1925) Quantitative determination of phytate in the presence of inorganic phosphate. J. Biol. Chem. 66: 375-400.

Forbes, R. & Parker, H.M. (1977) Biological availability of zinc in and as influenced by whole fat soy flour in rat diets. Nutr. Rep. Intl. 15: 681-688.

Gibson, D.M. & Ullah, A.B.J. (1990) Phytases and their actions on phytic acid. In: Inositol Metabolism in Plants, pp. 77-83. Wiley-Liss Inc., NY.

Graf, E. & Dintzis, F.R. (1982) Determination of phytic acid in foods by high-performance liquid chromatography. J. Agric. Food Chem. 30: 1094-1097.

Graf, E. & Eaton, J.W. (1985) Dietary suppression of colonic cancer: fiber or phytate. Cancer 56: 717-718.
Graf, E. & Eaton, J.W. (1984) Effects of phytate on mineral bioavailability in mice. J. Nutr. 114: 1192-1198.

Graf, E., Empson, K.L. & Eaton, J.W. (1987) Phytic acid: a natural antioxidant. J. Biol. Chem. 262: 11647-11650.

Gupta, S.K. & Venkatasubramanian, T.A. (1975) Production of aflatoxin on soybeans. Appl. Microbiol. 29: 834-836.

Hambidge, K.M., Casey, C.E. & Krebs, N.F. (1986) Zinc. In: Trace Elements in Human and Animal Nutrition, 5th Ed. (Mertz, W., ed.), vol. 2, pp. 1-138. Academic Press, Orlando, FL.

Johnson, M.A. (1986) Interaction of dietary carbohydrate, ascorbic acid and copper with the development of copper deficiency in rats. J. Nutr. 116: 802-815.

Johnson, M.A. & Gratzek, J.M. (1986) Influence of sucrose and starch on the development of anemia in copper and iron deficient rats. J. Nutr. 16: 2443-2452.

Johnson, M.A. & Murphy, C.L. (1988) Adverse effects of high dietary iron and ascorbic acid on copper status in copper deficient and copper adequate rats. Am. J. Clin. Nutr. 47: 96-101.

Johnson, P.E., Stuart, S.M.A. & Bowman, T.D. (1988) Bioavailability of copper to rats from various foodstuffs and in the presence of different carbohydrates. Proc. Soc. Exp. Biol. Med. 187: 44-50.

Larbi, A. & M'Barek, E. (1985) Dietary fiber and phytic acid levels in the major food items consumed in Morocco. Nutr. Rep. Intl. 31: 469-476.

Lee, D.Y., Schroeder, J. & Gordon, D.T. (1988) Enhancement of copper bioavailability in the rat by phytic acid. J. Nutr. 118: 712-717.

Lei, X. (1992) Supplemental microbial phytase improves utilization of phytate phosphorus and other minerals by young pigs. Doctoral thesis, Michigan State Universuty, East Lansing, Michigan.

Lo, G.S., Settle, S.L., Steinke, F.H. & Hopkins, D.T. (1981) Effect of phytate:zinc molar ratio and isolated soybean protein on zinc bioavailability. J. Nutr. 111: 2223-2235.

Lo, G.S., Settle, S.L. & Steinke, F.H. (1984) Bioavailability of copper in isolated soybean protein using the rat as an experimental model. J. Nutr., 114: 332-340.

Lonnerdal, B., Bell, J.G., Hendrickx, A.G. Burns, R.A. & Keen, C.L. (1988) Effect of phytate removal on zinc absorption from soy formula. Am. J. Clin. Nutr. 48: 1301-1306.

Maiti, L.B., Majumdar, A.L. & Biswas, B.B. (1979) Further characterization of phytase from *phaseolus aureus*. Phytochemistry 18: 316-321.

McCance, R.A. & Widdowson, E.M. (1935) Phytin in human nutrition. Biochem. J. 29: 2694-2698.

Mertz, W. (1980) Mineral elements: New perspectives. J. Am. Diet. Assoc. 77: 258-263.

Momcilovic, B., Belonji, B., Giroux, A. & Shah, B.G. (1976) Bioavailability of zinc in milk and soy protein based infant formulas. J. Nutr. 106: 913-917.

Moore, R.J. & Veum, T.L. (1983) Adaptive increase in phytate digestibility by phosphorus-deprived rats and the relationship of intestinal phytase (EC 3. 1. 3. 8) and alkaline phosphatase (EC 3. 1. 3. 1) to phytate utilization. Br. J. Nutr. 49: 145-152.

Morris, E.R. (1986) Phytate and dietary mineral bioavailability. In: Phytic Acid, Chemistry and Applications (Graf, E. ed.), pp. 57-76. Pilatus Press, Minneapolis, MN.

Morris, E.R. & Ellis, R. (1985) Trace element nutriture of adult men consuming three levels of phytate. In: Trace Elements in Man and Animals (TEMA-5, Mills, C.F., Bremner, S. & Chester, J.K., eds.), pp. 443-446. Commonwealth Agricultural Bureaux, Aberdeen, Scotland.

Morris, E.R. & Ellis, R. (1980) Effect of dietary phytate:zinc molar ratio on growth and bone zinc response of rats fed semipurified diets. J. Nutr. 110: 1037-1045.

Morris, E.R., Ellis, R., Steele, P. & Moser, P.B. (1988) Mineral balance of adult men consuming whole or dephytinized wheat bran. Nutr. Res., 8: 445-458.

Morrow, B. (1991) The rebirth of legumes. Food Technol. 45 (9): 120-121.

Nahapetian, A. & Bassiri, A. (1975) Changes in concentrations and interrelationships of phytate, phosphorus, magnesium, calcium, zinc and iron in wheat during maturation. J. Agric. Food Chem. 23: 1179-1184.

Nelson, T.S., Daniels, J.B. & Shielda, L.G. (1976) Hydrolysis of natural phytate phosphorus in the digestive tract of calves. J. Animal Sci. 42: 1509-1521.

Oberleas, D. (1973) Phytates. In: Toxicants Occurring Naturally in Foods. National Academy of Sciences, pp. 363-372. Washington, D.C.

Oberleas, D. & Prasad, A.S. (1976) Factors affecting zinc homeostasis. In: Trace Elements in Human Health and Disease, Vol I, Zinc and Copper (Prasad, A.S., ed.), pp 155-162, Academic Press, New York.

O'Dell, B.L. (1985) Bioavailability of copper and interactions among trace elements. In: Trace Elements in Nutrition of Children (Chandra R. K., ed.), pp 41-62. Nestle Nutrition, Vevey/Raven Press, NY.

O'Dell, B.L. (1990) Copper. In: Present Knowledge in Nutrition (Brown M.L., ed.), pp. 261-267. International Life Sciences Institute, Nutrition Foundation, Inc., Washington, DC.

O'Dell, B.L. (1979) Effect of soy protein on trace mineral bioavailability. In: Soy Protein and Human Nutrition (Wilcke, H.L., Hopkins, D.T. & Waggle, D.H., ed.), pp. 187-207. Academic Press, New York.

O'Dell, B.L., de Boland, A. & Koirtyohann, R. (1972) Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J. Agric. Food Chem. 20: 718-721.

O'Dell, B.L. & Savage, J.E. (1960) Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med. 103: 304-305.

Oestreicher, P. & Cousins, R.J. (1985) Copper and zinc absorption in the rats: mechanism of mutual antagonism. J. Nutr. 115: 159-166.

Prasad, A.S. (1976) Deficiency of zinc in man and its toxicity. In: Trace Elements in Human Health and Disease. I. Zinc and Copper (Prasad, A.D., ed.), pp. 1-23. Academic Press, New York, NY.

Platt, S.R., Nadeau, D.B., Gifford, S.R. & Clydesdale, F.M. (1987) Prospective effect of milk on mineral precipitation by sodium phytate. J. Food Sci. 52: 240-241.

Reddy, N.R., Sathe, S.K. & Salunkhe, D.K. (1982) Phytates in legumes and cereals. In: Advances in Food Research (Chichester, C.O., ed.), 28: 1-91. Academic Press, New York.

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.

Reeves, P.G., Nielsen, F.H. & Fahey, G.C. Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition. Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123: 1939-1951.

Reinhold, J.G. (1971) High phytate content of Iranian bread: a possible cause of zinc deficiency. Am. J. Clin. Nutr. 24: 1204-1206.

Reinhold, J.G. (1973) Phytate concentrations of leavened and unleavened breads. Ecol. Food Nutr. 1: 187-192.

Reinhold, J.G., Faradji, B., Abadi, P. & Ismail-Beigi, F. (1981) An extended study of the effect of Iranian village and urban flat breads on the mineral balances of two men before and after supplementation with vitamin D. Ecol. Food Nutr. 10: 169-177.

Sakamoto, K., Venkatraman, G. & Shamsuddin, A.M. (1993) Growth inhibition and differentiation of HT-29 cells in vitro by inositol hexaphosphate (phytic acid). Carcinogenesis 14: 1815-1819.

Sandberg, A. S. (1991) The effect of food processing on phytate hydrolysis and availability of iron and zinc. In: Nutritional and Toxicological Consequences of Food Processing (Friedman, M., ed.), pp. 499-508. Plenum Press, New York.

Sandberg, A.S. & Ahderinne R. (1986) HPLC method for determination of inositol tri-, tetra-, penta- & hexaphosphates in foods and intestinal contents. J. Food Sci. 51: 547-549.

Sandberg, A.S., Andersson, H., Carlson, N.G. & Sandstrom, B. (1987) Degradation products of bran phytate formed during digestion in the human small intestine: effect of extrusion cooking on digestibility. J. Nutr. 117: 2061-2065.

Sandberg, A.S., Andersson, H., Kivisto, B. & Sandstrom, B. (1986) Extrusion cooking of a high fiber cereal product. 1. Effects on digestibility and absorption of protein, fat, starch, dietary fiber and phytate in the small intestine. Br. J. Nutr. 55: 245-254.

Sandberg, A.S., Hasselblad, C. & Hasselblad, K. (1982) The effect of wheat bran on the minerals in the small intestine. Br. J. Nutr. 48: 185-191.

Sandberg, A.S. & Svanberg, U. (1991) Phytate hydrolysis by phytase in cereals: effects on in vitro estimation of iron availability. J. Food Sci. 56: 1330-1333.

Sands, S.H., Biskobing, S.J. & Olson, R.M. (1986) Commercial aspects of phytic acid: an overview. In: Phytic acid: Chemistry and Applications (Graf, E., ed.), pp. 119-125. Pilatus Press. Minneapolis, MN.

Sandstrom, B. & Cederblad, A. (1980) Zinc absorption from composite meals. II. Influence of the main protein source. Am. J. Clin. Nutr. 33: 1778-1783.

Sandstrom, B., Arvidsson, B., Cederblad, A. & Bjorn-Rasmussen, E. (1980) Zinc absorption from composite meals. I. The significance of wheat extraction rate, zinc, calcium and protein content in meals based on bread. Am. J. Clin. Nutr. 33: 739-745.

Sandstrom, B., Navert, B. & Cederblad, A. (1985) Effect of soy protein on zinc absorption from composite meals. In: Trace Elements in Man and Animals (TEAM-5, Mills, C.F., Bremner, S. & Chesters, J.K. eds.), pp. 440-442. Commonwealth Agricultural Bureaux, Aberdeen, Scotland.

Shamsuddin, A.M., Baten, A. & Lalwani, N.D. (1992) Effects of inositol hexaphosphate on growth and differentiation in K-562 erythroleukemia cell line. Cancer Lett. 64: 195-202.

Shamsuddin, A.M., Elsayed, A.M. & Ullah, A. (1988) Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate. Carcinogenesis 9: 577-580.

Sandstead, H.H. (1981) Trace element interactions. J. Lab. Clin. Med. 98: 457-462.

Schosinsky, K.H., Lehmann, H.P. & Beeler, M.F. (1974) Measurement of ceruloplasmin from its oxidase activity in serum by use of *o*-dianisidine dihydrochloride. Clin. Chem. 20: 1556-1563.

Thompson, L.U. (1988) Antinutients and blood glucose. Food Technol. 42 (9): 123-126.

Turnland, J.R., Wada, L., King, J.C., Keyes, W.R. & Acord, L.L. (1988) Copper absorption in young men fed adequate and low zinc diets. Biol. Trace Elem. Res. 17: 31-41.

Turnland, J.R., Wada, L., King, J.C., Keyes, W.R., Gong, B. & Michel, M.C. (1984) A stable isotope study of zinc absorption in young men : effects of phytate and α-cellulose. Am. J. Clin. Nutr. 40: 1071-1077.

Ullah, A. & Shamsuddin, A.M. (1990) Dose-dependent inhibition of large intestinal cancer by inositol hexaphosphate in F344 rats. Carcinogenesis 11: 2219-2222.

Van Campen, D. & Gross, E. (1968) Influence of ascorbic acid on the absorption of copper by rats. J. Nutr. 95: 617-622.

Van Campen, D.R. & Scaife, P.O. (1967) Zinc interference with copper absorption in rats. J. Nutr. 91: 473-476.

Vucenik, I. & Shamsuddin, A.M. (1994) [^{3}H] Inositol hexaphosphate (phytic acid) is rapidly absorbed and metabolized by murine and human malignant cells in vitro. J. Nutr. 124: 861-868.

Welch, R.M. and House, W.A. (1982) Availability to rats of zinc from soybean seeds as affected by maturity of seed, source of dietary protein and soluble phytate. J. Nutr. 112: 879-885.

Zhou, J.R., Fordyce, E.J., Raboy, V., Dickinson, D.B., Wong, M.S., Burns, R.A. & Erdman, J.W. (1992) Reduction of phytic acid in soybean products improves zinc bioavailability in rats. J. Nutr. 122: 2466-2473.