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MEAT EFFECTS ON NONHEME IRON ABSORPTION

by

Yunji Kim

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of the requirements for the degree

of

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ABSTRACT

Meat Effects on Nonheme Iron Absorption

by

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Utah State University, 1991

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Studies were undertaken to investigate if gastric acidity and iron chelation to a meat component enhance nonheme iron absorption. Cereal meals, with and without added proteins, were gavaged into iron-deficient rats. The role of iron chelation was investigated by adding sodium phytate, an iron chelator implicated with decreased iron absorption, to the meals. The role of gastric acidity was investigated by treating the rats with cimetidine, which inhibits gastric acid production. In rats with normal acid production, beef, pork and chicken enhanced iron absorption when phytate had been added to the meals, suggesting a role for chelation in meat enhancement of iron absorption. However, the enhancement by beef and pork was insignificant in cimetidine-treated rats given the cereal + phytate meals, indicating that gastric acid production also plays a role in meat enhancement of iron absorption. Fish and egg white were sometimes inhibitory to iron absorption and, therefore, did not fit the pattern of enhancement demonstrated by beef, pork, and chicken. In a separate experiment, gastric acidity was not directly altered by the protein source included with cereal meals. No significant effects of the various proteins on iron absorption from cereal + phytate meals were observed in a final experiment involving iron-replete rats. *In vitro* iron

solubilizing capacity of beef, pork, chicken, and egg white was positively correlated with enhanced iron absorption by iron-deficient rats.

Studies were performed to 1) investigate if ferric iron bound in complex with iron-solubilizing meat components is absorbable, 2) compare the relative iron-solubilizing capacity of meats, and 3) investigate the physicochemical and compositional characteristics of the meat components responsible for the iron solubilizing capability of meat. Iron-solubilizing components of beef were isolated from pH 2 HCl homogenates into dialysis bags (MWCO, 6-8 K). Radiolabelled iron complexes were then generated using ferric iron and either the ILC (isolated low-molecular-weight components) from undigested beef or ascorbate. The bioavailabilities of radioiron in these complexes or as ferric iron were measured as radioiron absorption into the blood one hour after injection into ligated duodenal loops of rats. Iron absorption values were ascorbate-ferrous complexes > beef ILC-ferric complexes > ferric iron (p <.05). In separate experiments, ILC from 0.1 g of various dietary protein sources (beef, pork, chicken, fish, or egg white) were added to 400 μg ferric iron in pH 2 HCl, the pH raised to 7.2, and soluble iron determined in the supernatant after centrifugation at 2,500 g for 10 min. Iron solubilizing capabilities of ILC were pork > beef > chicken > fish > egg white (p <.05). In a final series of experiments, the compositional and physicochemical characteristics of the ILC from the various dietary proteins were investigated.

(145 pages)

INTRODUCTION

Iron deficiency is the most common nutritional problem in both developing and developed countries. Although most iron present in the diet is in inorganic forms, it is not readily absorbed. Many studies have demonstrated dramatic changes in bioavailability of inorganic iron as a result of interactions among foods or food processing. Consumption of foods in certain combinations can either enhance or inhibit absorption. Thus, the absolute iron content of a diet can be less important in terms of nonheme iron bioavailability than is the composition of the diet.

Meat, fish, or poultry increases nonheme iron absorption two- to four-fold when eaten in the same meal. One g meat is equivalent to 1 mg ascorbic acid in its ability to promote nonheme iron absorption. This enhancing effect has been shown only in the presence of cellular animal proteins such as beef, veal, pork, lamb, liver, fish, and chicken. Non-cellular animal proteins do not exhibit this enhancing effect and some protein sources such as soy protein have been found to depress iron absorption. Even though extensive research has focused on identifying the nature of meat enhancer, it has not been identified.

A hypothesis concerning the mechanisms of meat enhancement on iron absorption has been proposed which involves: 1) chelating nonheme iron with amino acid(s), polypeptide(s), protein(s), or other meat component(s), 2) stimulating secretion of gastrin or other gastric factor which maintains ingested iron soluble in the intestine, and 3) stimulating secretion of gastric acid.

The objective of the research presented in this dissertation was to expand knowledge about meat enhancement of inorganic iron absorption by testing the previous hypotheses. This information may contribute to improvement of iron nutrition. The major objective was accomplished by addressing the following specific objectives:

  1. Determine the relationship between iron solubilization *in vitro* and iron

absorption *in vivo*.

2. Determine the relationship between enhancement of gastric acidity by meat and iron absorption *in vivo*.

3. Determine the bioavailability of iron complexes formed with isolated low-molecular-weight components from undigested beef.

4. Determine the composition of isolated low-molecular-weight components and their chemical characteristics.

PART 1: REVIEW OF LITERATURE

FUNCTIONS OF IRON IN MAN

Iron is an essential metal to virtually all forms of living organisms. Iron in the human body is classified as functional and storage iron. About two-thirds of the iron in the body is functional iron. It is found in hemoglobin, transferrin, myoglobin, and enzymes (Bothwell et al., 1979). About one-third of the body iron is found in storage forms such as tissue ferritin and hemosiderin (mainly in the spleen, liver, and bone marrow), and a small amount is associated with the blood transport protein transferrin. Almost all the iron in the body is bound to protein (Worwood, 1977). It is present in enzymes responsible for the transport of oxygen (hemoglobin and myoglobin), electron transport (cytochromes), and the activation of oxygen (oxidases and oxygenases) (Hallberg, 1984).

Oxygen and carbon dioxide transport

The role of hemoglobin in the blood is to transport oxygen from the lungs to the tissues and to transport carbon dioxide from the actively metabolizing cells to the lungs for excretion. A decrease in the hemoglobin content and, therefore, the iron content of red blood cells reduces their oxygen-carrying capacity (Cook et al., 1990). Myoglobin binds oxygen in the muscle cells and stores it until it is needed by the mitochondria (Pike and Brown, 1984). Myoglobin has a higher affinity for oxygen than does hemoglobin and is found in muscle cells where it functions as an oxygen store, releasing oxygen to cytochrome oxidase when the supply of oxygen is insufficient for the needs of the tissues (Perutz, 1970; 1972). Iron carries out its role of oxygen and carbon dioxide transport primarily as a part of both hemoglobin in the blood and myoglobin in the muscle but also as part of several tissue enzymes essential in cell respiration. The exchanges are involved primarily in the release of energy within the cell (Guthrie, 1989).

Electron transport

Mitochondria contain an electron transport system which transfers electrons from substrate to molecular oxygen with the simultaneous generation of adenosine triphosphate. The cytochromes (cytochrome a, a_3, b, c_1, c, b_5, and p-450) are components of this pathway, and in the process of electron transfer their iron atoms are alternately oxidized and reduced (Zubay, 1983). Cytochromes are not the only iron-containing proteins in oxidative phosphorylation. A number of iron-sulfur proteins (complex III Fe-S protein, succinate dehydrogenase Fe-S protein, and succinate dehydrogenase flavoprotein) also take part (Worwood, 1977). Due to iron's function in electron and oxygen transport, if iron levels fall below normal, energy production is reduced contributing to the lethargy, fatigue and reduced performance associated with iron deficiency (Edgerton et al., 1972; Viteri, 1989).

Immune system

The role of iron in the immune system has been confusing and contradictory. Iron is essential for both the growth of infectious microorganisms as well as the enzymes of the immune system that destroy harmful organisms (Guthrie, 1989; Skikne, 1988). Iron therapy has been associated with a significant increase in incidence of infection. Fletcher (1971) observed that iron in excess of the transferrin-binding capacity enhanced *E. coli* growth in fresh serum. On the other hand, if iron is not available, there is a decrease in the production of iron-containing enzymes and other immune substances needed to destroy infectious organisms. In iron deficiency, impairment of immune function has been found to be related to cell-mediated immunity. The two main factors important in bactericidal activity of polymorphonuclear leukocytes are myeloperoxidase activity and the oxidative burst (Prasad, 1979). Myeloperoxidase is a heme-containing enzyme whose concentration in the polymorphonuclear leukocytes is decreased in iron deficiency (Murakawa et al., 1987). Lactoferrin in human milk is an

iron-binding substance that is especially effective against *E. coli* organisms in the gastrointestinal tract of the infant.

Other biological functions

Iron plays a role in the following biochemical processes: conversion of ß-carotene to vitamin A, synthesis of collagen, formation of purines as part of nucleic acid, removal of lipids from the blood, detoxification of drugs in the liver, production of antibodies, synthesis of carnitine which is an amino acid required for mitochondrial oxidation of fatty acids, and synthesis of neurotransmitters (Guthrie, 1989; Hallberg, 1984).

CHEMICAL PROPERTIES OF IRON

Alkali metals such as sodium, potassium, and chloride are present completely ionized as ions in solution, whereas iron, copper, and zinc exist almost completely in some form of complex (nonionized) state in biological systems. Iron exists in two distinct oxidation states-ferrous and ferric. In a medium with a pH value slightly above 3, solubility of ferric iron decreases because the sparingly soluble ferric hydroxides are formed. The formation of sparingly soluble hydroxides and phosphates of iron in the intestinal lumen can be prevented by compounds that form complexes and or chelates with iron (Forth and Rummel, 1973; Kaltwasser et al., 1987). Thermodynamically, chelates have increased solubility constants as formed with multidentate as compared with several monodentate ligands. This can be explained by the greater positive entropy change during the formation of the multidentate chelate ring. The effective stability of iron complexes can be influenced by interfering reactions in various segments of the gastrointestinal tract (Forth and Rummel, 1973; Valberg and Flanagan, 1983). The availability of complex-bound iron depends on the effective stability of the complexes, which is related to the thermodynamic stability constant and intraluminal factors such as pH or the presence of competing metals and ligands in food. Soluble salts, which remain stable through the varying conditions imposed during passage through the gastrointestinal tract, will be the most available. Iron is released from the complexes if the effective stability is low. If the effective stability is high, absorption of iron depends on the extent to which iron is released from the intact complexes for absorption, i.e. on the physicochemical properties of the complexes. The nature of ligands that form chelate compounds with iron is an important determinant of iron absorption (Valberg and Flanagan, 1983). If the iron complex is small enough and tightly bound, it may be absorbed as an intact complex (Forth and Rummel, 1973). As long as ferric salts are maintained in solution, their

oxidation state is not a barrier to absorption (Johnson and Jacobs, 1990; Marx and Aisen, 1981).

Solubility of iron in the intraluminal medium of the gastrointestinal tract is a prerequsite for its absorption. Several factors have been demonstrated to influence the solubility and, therefore, bioavailability of iron. These factors include pH, chemical form of iron, oxidoreductive acitivity of iron, capacity of iron to form complexes, food digestability, food processing conditions, and nutrient interactions. The amount of iron available for absorption from the diet is related to the relative concentrations of soluble iron present as ionic iron and low-molecular-weight iron complexes to insoluble iron present as insoluble precipitates, macromolecules, or high-molecular-weight complexes (Clydesdale, 1983; Smith, 1983). Examples of low-molecular-weight complexes include soluble ferrous sulfate, ferrous fumarate, ferric citrate, sodium iron-EDTA, and iron ascorbate complexes. Examples of insoluble and poorly available forms of iron precipitates include iron oxide, iron hydroxide, and certain iron-fiber and iron-phosphoprotein complexes. When iron ions are in solution at neutral pH, the formation of insoluble hydroxides is favored with a resulting difference in the solubility of the two hydroxides formed: the solubility for ferric hydroxide at pH 7.0 is 10^{-37} M, while that for ferrous hydroxide is 10^{-15} M or approximately 10^{20} greater than for the ferric form (Spiro and Saltman, 1974). Derman et al. (1977) showed that ferric hydroxide does not readily exchange with extrinsically added iron.

IRON BALANCE

The total body iron stores are approximately 4-5 g, with an average daily requirement of 1.0 mg/day (Hallberg, 1984). Daily requirements are based on the estimated obligatory loss of iron from desquamated epithelial cells of the gut and skin and from excretion via the gut, kidney, and skin (Finch and Hueber, 1986). There is little iron excreted in the urine (0.1 mg/day) unless a patient develops a sustained intravascular hemolysis in which the free hemoglobin in the blood exceeds the binding capacity of heptoglobin so that iron is lost in the urine.

Previously, it was believed that the quantity of iron in the body was controlled solely by regulation of absorption and that excretion played a passive role. McCance and Widdowson (1937; 1938) suggested and confirmed that the intestine has no power of regulating by excretion the amount of iron in the body. The daily obligatory loss of cells from the skin and gut in secretions such as sweat and bile provides a limited but selective loss of body iron (Dubach et al., 1959; Green et al., 1968; Weintraub et al., 1965).

Variations in physiologic iron requirements occur in several metabolic conditions, including bleeding, pregnancy, and iron overload. Iron losses during menstruation may average 1.0 mg/day or more (Crichton and Charloteaux-Wauters, 1987). During pregnancy, the daily requirement in the second and third trimester is approximately 4-6 mg (Hallberg, 1981). Variable absorptive capabilities of the gastrointestinal tract are exhibited during different states of iron homeostasis. Absorption rises to a maximum of 3-4 mg/day when the body is depleted of its iron stores, and declines to less than 0.5 mg daily when iron overload exists (Rossander et al., 1979). Weintraub et al. (1964) suggested the iron content of the epithelial cell is important in the regulation of its ability to absorb iron.

The body has three mechanisms for maintaining iron balance and preventing the development of iron deficiency: 1) the continuous reutilization of iron from cells catabolized in the body; 2) the presence of a specific storage protein, ferritin, which makes it possible to store iron in the body to meet excessive iron demands as in late pregnancy; and 3) the regulation of the absorption of iron affected by actual requirements with an increased iron absorption in the presence of iron deficiency and a decreased iron absorption in states of iron overload.

IRON ABSORPTION

Iron absorption occurs primarily in the duodenum and the most proximal part of the jejunum (Conrad and Crosby, 1963; Conrad et al., 1964; Duthie, 1964). The exact mechanism which regulates the absorptive process has not been fully established. Absorption may be thought of as the sum of intestinal lumen-related effects and intestinal mucosal cell-related effects. In other words, iron absorption is the sum of uptake and transfer (Manis and Schachter, 1962). The behavior of iron during digestion and absorption differs for heme and nonheme iron. Heme iron-containing proteins are digested to release the iron-porphyrin heme moiety. It has been suggested that the brush border may contain receptors for heme (Tenhunen et al., 1980). Heme is thought to be absorbed intact, after which the porphyrin ring is degraded and the iron presumably becomes indistinguishable from absorbed nonheme iron (Weintraub et al., 1968; Wheby et al., 1970). It is not clear whether uptake of inorganic iron as ferric or ferrous is via the same mechanism or by discrete mechanisms. Proposed mechanisms suggest that uptake is via the same ferrous-specific mechanism with prerequisite reduction of ferric (Barrand et al., 1990; Wollenberg and Rummel, 1987), or uptake of ferrous and ferric iron is by discrete mechanisms (Johnson and Jacobs, 1990; Simpson et al., 1986).

To be absorbed, iron must be taken up by the intestinal mucosal cell and subsequently be moved across the cell, across the serosal membrane, and to the blood where it is picked up by transferrin. Iron-binding protein, transferrin, in the intestinal mucosa was proposed as an important mediator of intestinal iron absorption (Huebers et al., 1983). Evidence contrary to that hypothesis was reviewed by Peters et al. (1988). Osterloh et al. (1987) and Schumann et al. (1986) have shown by immunoassays only trace levels of transferrin exist in the gut lumen with no significant increase in situations of enhanced iron absorption. Osterloh et al. (1985) reported that no transferrin

synthesis by the enterocytes occurs. Generally, the role of transferrin appears in picking up a portion of the iron that has been internalized into the mucosal cell, and transferrin then transports the iron through the portal venous system and the liver, and deposits it in the bone marrow. Iron absorption is affected by several factors including intraluminal, mucosal, and corporeal factors.

Intraluminal factors

The physicochemical form of inorganic iron affects iron absorption (Martinez-Torres and Layrisse, 1973; Moore, 1961). Dietary constituents which solubilize iron may enhance absorption whereas compounds that cause precipitation or molecular aggregation of iron decrease absorption (Benjamin et al., 1967; Charley et al., 1963; Conrad et al., 1966; Conrad and Schade, 1968). Some dietary constituents and intestinal secretions tend to bind iron to maintain it in a soluble monomeric form at the alkaline pH of the small intestine (Conrad and Schade, 1968). Certain sugars, amino acids, and amines decrease both the precipitation and polymerization of iron in aqueous solutions by interfering with the formation of water bridges between iron molecules. Carbonates and macromolecules which are commonly found in the diet decrease iron absorption (Moore, 1961; Spiro and Saltman, 1974).

Gastrointestinal secretions play a role in altering iron absorption. Gastric hydrochloric acid solubilizes ferric iron to make it available for chelation with substances that enhance absorption in the less acid environment of the small intestine (Conrad and Schade, 1968). Bile enhances iron absorption because it contains significant quantities of ascorbic acid and other substances that reduce and chelate iron (Conrad and Schade, 1967; Jacobs and Miles, 1970). Pancreatic bicarbonate diminishes iron absorption, and its relative absence in cystic fibrosis and chronic pancreatitis may explain the increased iron absorption observed in these conditions (Benjamin et al., 1967; Davis and Badenoch, 1962; Tonz et al., 1965). Intestinal enzymes which release sugars and amino acids from

food are believed to enhance iron absorption indirectly by forming tridentate iron chelates which remain soluble in the small intestine with a low-molecular-weight complex (Van Campen, 1972; 1973).

Mucosal factors

Nonheme iron is absorbed in experimental animals by a mechanism in which two processes operate simultaneously: the first is limited by the impermeability of the intestinal mucosa and the concentration of available iron in the lumen; whereas the second process has a rate limited capacity and displays saturation kinetics and competitive inhibition (Thomson et al., 1971; Thomson and Valberg, 1971; Wheby et al., 1964). Valberg and Flanagan (1983) showed that iron absorption takes place mainly by simple diffusion when iron stores are replete; whereas, binding sites are involved in the transport of iron when iron reserves are low. Hahn et al. (1943) postulated that iron absorption was regulated by a mucosal receptor that blocked absorption when it became satiated with iron.

The movement of iron across the intestinal mucosa involves two major steps: 1) iron uptake from the lumen, and 2) subsequent transfer of all or a portion of this iron to the body (Manis and Schachter, 1962; Wheby et al., 1964). When iron solutions containing iron-ascorbate complexes were placed in or perfused through duodenal loops of iron-loaded rats *in vivo*, iron uptake in the first 30 min was directly proportional to the concentration of available iron in the lumen (Thomson and Valberg, 1971; Wheby et al., 1964). Radioiron placed in the duodenum of the rat was found in the carcass within 15 sec and as much as 40% of the 30 min iron uptake occurred within the first minute (Thomson et al., 1971; Weintraub et al., 1968). Starvation and the administration of endotoxin decrease the uptake of mucosal iron from the gut lumen without concomitant changes in the intestinal iron content (Conrad and Crosby, 1963; Cortell and Conrad, 1967). Parmley et al. (1978) showed that specific staining of ferric and ferrous iron in the intestinal mucosa of normal, iron-loaded, and iron-deficient animals at the ultrastructural

level has shown marked differences in the nonferritin mucosal iron content. Although it appears that the total quantity of iron in the mucosa may play a role in the absorption of iron, the amount of this iron that is bound to mucosal receptors that facilitate absorption may play a more important role (Parmley et al., 1985). Mucosal uptake may be a passive process in which the state of iron repletion fails to affect iron uptake at the cell surface (Howard and Jacobs, 1972; Savin and Cook, 1978). In contrast, Cox and Peters (1978), using human duodenal biopsy specimens, demonstrated that uptake into the cell may be an active process dependent on metabolic energy, with saturation kinetics in the range of iron concentrations found in the intestinal lumen under physiologic circumstances. With larger amounts of iron, a process suggestive of passive nonspecific diffusion of iron occurs (Conrad and Crosby, 1963; Conrad et al., 1964). Transfer of iron from intestinal cells into the plasma can be diminished without changes in the mucosal iron content.

Corporeal factors

Accelerated red blood cell production is related to enhanced iron absorption whether the cause be bleeding, hemolysis, or hypoxia. It is tempting to postulate that an erythrocyte-stimulating factor acts as messenger to the gut to increase iron absorption. Beutler and Bullenweiser (1960) have searched for a blood factor that signals the gut to enhance or diminish iron absorption. Clinical observations indicate that the hemoglobin concentration and plasma levels of iron and transferrin do not perform these functions (Beutler and Bullenweiser, 1960; Conrad and Crosby, 1963). Most corporeal factors that alter iron absorption do not exert an effect for several days (Smith and Pannacciulli, 1958; Weintraub et al., 1968).

While corporeal factors must be the most important regulators of iron absorption, the way they inform the duodenum to transfer appropriate amounts of iron into the plasma is unknown. Certain hormones such as erythropoietin, thyroid, and

pituitary extracts enhance iron absorption (Beutler and Bullenweiser, 1960; Mendel, 1961).

IRON UTILIZATION AND STORAGE

Once iron is incorporated into hemoglobin, it remains in this state until the hemoglobin is degraded in the reticuloendothelial cells. Thus, iron can return from hemoglobin to the plasma by one of several routes. The majority of hemoglobin remains intact within the erythrocyte for the 120-day life-span of the cell. A short-term reflux of iron is derived from either ineffective erythropoiesis in the bone marrow or when reticulocytes are decreased from a volume of 130 μ^3 to 90 μ^3 as they mature into an erythrocyte or both (Cook et al., 1970). Iron in the reticuloendothelial cells is derived predominantly from the catabolism of heme. The major flow of iron in the body is unidirectional, passing in order from transferrin to the erythroid marrow to red blood cells to reticuloendothelial cells. When iron is released from the reticuloendothelial cells into the plasma, the iron is again bound to transferrin and the majority of it is returned to the erythroid marrow for heme synthesis. There is only a limited exchange with the storage iron in the reticuloendothelial cell under physiological circumstances (Finch et al., 1970). The largest nonheme iron stores in the body are in the reticuloendothelial organs (liver, spleen, bone marrow) where iron is stored either as ferritin or hemosiderin.

IRON DEFICIENCY

Iron deficiency is one of the most prevalent nutritional disorders in the world. This can be caused by a variety of factors. Inadequate dietary intake of iron often results from low-calorie diets, food restrictions, or single food diets. In industrialized countries, the reduction of energy expenditure in recent decades has led to a reduction in dietary intake of both energy and iron. In developing countries, the bioavailability of iron from the diet is often very low, due mainly to a low content of factors facilitating non-heme iron absorption (meat, fish, and ascorbic acid) (Hallberg, 1984). Depending on the underlying cause, iron deficiency may develop very rapidly, within a matter of weeks, or over the course of months to years. Factors associated with blood loss contribute to iron deficiency very quickly, whereas dietary deficiencies and inadequate absorption of iron affect iron status very gradually (Cook et al., 1990).

Certain symptoms that occur in other chronic anemias are often observed in iron-deficient subjects in the absence of anemia. These include headache, fatigue, heartburn, changes in appetite, vasomotor disturbances, muscular cramping, dyspnea, palpitation, menorrhagia, reduced work performance, and hypothermia (Beard and Borel, 1988; Conrad and Barton, 1981; Viteri, 1989).

FACTORS AFFECTING IRON BIOAVAILABILITY

Absorption enhancing factors

Ascorbic acid (Carlson and Miller, 1983; Gillooly et al., 1984; Hallberg and Rossander, 1982[a]; Morck et al., 1982; Rossander et al., 1979) and meat (Björn-Rassmussen and Hallberg, 1979; Cook and Monsen, 1976; Gordon and Godber, 1989; Hurrel et al., 1988; Layrisse et al., 1969) have consistently been shown to enhance iron bioavailability. An acid environment promotes iron absorption by maintaining nonheme iron in the more soluble, ferrous, form which aids in the formation of chelates. Ascorbic acid, citric acid, hydrochloric acid, and certain amino acids aid in the absorption of nonheme iron by preventing the formation of insoluble ferric hydroxide and by forming soluble complexes with ferric iron (Hallberg and Rossander, 1982[a]). Hallberg and Rossander (1982[b]) demonstrated that a meal consumed with orange juice enhanced nonheme iron absorption by 85 %. The effectiveness of ascorbic acid in enhancing iron absorption may be due to its involvement in modifying all three factors which would enhance iron absorption. Ascorbic acid lowers the food pH, can reduce Fe^{3+} to Fe^{2+}, and forms a chelate with Fe^{3+} at acid pH which remains soluble in the alkaline environment of the small intestine (Conrad and Schade, 1968). The promoting influence on iron absorption of ascorbic acid is dose dependent (Björn-Rasmussen and Hallberg, 1974; Sayers et al., 1973).

The increased bioavailability of nonheme iron by animal protein has been called the meat factor effect. The enhancing effect of meat was first reported by Layrisse et al. (1968). Layrisse et al. (1973) showed that in healthy human subjects, the absorption of fortification iron added to a vegetable food was limited, reaching 0.30 mg with an intake of 60 mg of fortification iron. However, a supplement of 5 mg iron eaten with veal muscle resulted in absorption of 0.85 mg iron. Veal muscle also increased the

absorption of intrinsically labelled rabbit hemoglobin from 12% to 22% (Martinez-Torres and Layrisse, 1971). Martinez-Torres and Layrisse (1970) found that fish or a mixture of amino acids similar to the amino acid composition of fish increased the iron absorption from black bean from 1.4% to 2.7-3.1% in healthy human subjects. They also found that only cysteine enhances iron absorption from black beans. Cook and Monsen (1976) have shown that all sources of animal protein are not equivalent for increasing nonheme iron absorption. Substitution of beef, lamb, pork, liver, fish, and chicken for the egg ovalbumin in a semisynthetic meal resulted in a significant 2-to 4-fold increase in iron absorption whereas no increased iron absorption was observed with the substitution of milk, cheese, or egg. Björn-Rasmussen and Hallberg (1979) used the extrinsic tag technique in which the iron retention was measured by a whole body counter. Only nonheme iron retained in the body was measured in this case. They reported that beef, fish, chicken, and calf thymus increased the ^{59}Fe absorption to about the same extent (2.97-4.37 times). However, egg albumin, which has approximately the same amino acid composition as meat and fish, did not enhance the absorption of food iron. They concluded that the absorption promoting effect of meat on nonheme iron can be neither a general protein effect nor an effect of the presence of nucleoproteins in meat products. Gordon and Godber (1989) observed that absorption of iron by rats fed diets containing beef tended to be higher, compared with animals fed diets containing lactalbumin. Worthington-Roberts et al. (1988) compared the iron status of women on diets that differed by the protein source, but were not significantly different by iron content. They found that women regularly consuming red meat had significantly better iron status as compared to women who consumed chicken and fish or milk and vegetables as the main source of dietary protein. The importance of animal protein in iron nutrition is recognized in World Health Organization recommendations for daily iron intake. In adult women, an intake of 28 mg per day is recommended when less

than 10 % of the dietary energy is derived from animal protein, as compared with 14 mg

per day when more than 25 % of the energy is from animal protein (World Health

organization, 1970). However, some studies could not demonstrate meat enhancement

in iron absorption. Chao and Gordon (1983) studied the effects of fish protein and fish

oil on the utilization of endogenous iron in plant food fed to anemic rats. They found no

meat enhancement in iron absorption. Fritz et al. (1970) also could not see meat

enhancement in iron absorption.

Although meat enhancement of nonheme iron absorption is well documented, the

mechanism(s) by which meat enhances nonheme iron absorption is controversial. To

determine the mechanism of enhanced iron absorption by animal tissue protein, several

investigators studied the effects of single amino acids on absorption and found that

histidine, lysine, and cysteine increased the absorption of ferric iron in isolated intestinal

segments of rats (Van Campen, 1972; Van Campen and Gross, 1969). Removal or

modification of their ionizing groups (decarboxylation of histidine, removal of the

epsilon-amine group of lysine, and substitution of H or OH for the sulfurdryl group of

cysteine) resulted in a loss of the ability of these amino acids to enhance $FeCl_3$ uptake in

rats (Van Campen, 1973). Van Campen (1973) suggested that the enhancing effect of

the unaltered amino acids is due to their ability to form tridentate ligands and satisfy the

octahedral configuration of Fe^{3+}. The enhancing effect of meat on iron absorption may

be due to a component not related to the protein content (Hallberg and Björn-

Rasmussen, 1972). A mechanism to explain meat enhancement has been proposed as

protein that enhances iron bioavailability by releasing peptides during digestion which

form soluble, low-molecular-weight complexes with dietary iron that readily release iron

to mucosal receptors (Berner et al., 1985). The enhancing effect of meat is most often

recognized and is most pronounced in diets which are otherwise inhibitory to iron

absorption (Björn-Rasmussen and Hallberg, 1979; Hurrel et al., 1988).

Absorption inhibiting factors

Iron bioavailability from many plant sources is generally low, the factor or factors responsible have not been clearly identified (Layrisse et al., 1969; Lynch et al., 1984). Polyphenolic compounds, including tannins, are widely distributed in plants and are thought to contribute to the decrease in iron absorption observed when tea and coffee are consumed with various diets (Bindra and Gibson, 1986; Disler et al., 1975). Morck et al. (1983) demonstrated that tea taken with a meal reduced the absorption of iron by 64% and that coffee reduced absorption by 39%. Although coffee stimulates the secretion of gastric juices which enhance iron absorption, polyphenols in coffee bind iron and offset this positive influence. Factors decreasing iron absorption may reduce the pool of exchangeable iron by forming more insoluble or undissociated iron compounds. Gillooly et al. (1983) observed marked inhibition of the geometric mean absorption when tannic acid was added to a broccoli meal.

Björn-Rasmussen and Hallberg (1974) and Simpson et al. (1981) demonstrated an inhibiting effect of bran on iron absorption likely due to content of phytate. Sodium phytate can be used experimentally as a physiological inhibitior of iron absorption. Phytates are commonly found in whole grains, bran, and soy products and decrease the bioavailability of dietary iron (Macfarlane et al., 1988; Siegenberg et al., 1991). Hallberg (1981) and Turnbull et al.(1962) have reported sodium phytate inhibits iron absorption. Phytates occur in a number of plants as a phosphorus storage compound. Most of the phytates are bound to cations such as potassium and magnesium. For many years, it has been assumed that phytates present in bran are the inhibiting factor since ferric phytate complex is poorly soluble, especially at low pH, and addition of sodium phytate markedly inhibits iron absorption both from food iron and iron salts. However, recent findings suggest that the phytates in bran may not be the cause of the inhibitory effect (Morris and Ellis, 1976; Simpson et al., 1981).

The role of calcium and phosphate in iron absorption is controversial. Monsen and Cook (1976), using an extrinsic iron-tracer technique, observed that retention of nonheme iron was impaired by 53-73% when moderate amounts of both calcium (178 mg) and phosphorus (138 mg) were added to the test meal, whereas iron retention was not affected by addition of an equivalent amount of either calcium or phosphate alone. On the other hand, Snedeker et al. (1982) found no significant difference in iron retention from meals to which calcium, with or without phosphorus, had been added. Dawson-Hughes et al. (1986) found that the addition of either calcium carbonate or hydroxyapatite significantly reduced iron retention.

Oxalates (Christian and Greger, 1985) and the food additive, ethylenediamine-tetracetic acid (EDTA), bind iron tightly and inhibit absorption (Monsen, 1988). Dietary fibers have been associated with decreased nonheme iron absorption. A slight reduction in absorption was observed with hemicellulose preparations, but pectins, guar gum, and cellulose had no effect (Monsen, 1988).

POSSIBLE MECHANISMS OF MEAT ENHANCEMENT IN IRON ABSORPTION

Meat enhancement of nonheme iron absorption by chelation

One possible mechanism of meat enhancement of iron absorption is based on meat increasing iron solubility by chelation. Iron solubility is of critical importance to iron bioavailability since it is generally agreed that only soluble iron can be absorbed. Recent reports show that ferric iron is also well absorbed if presented as soluble low-molecular-weight ferric iron ($Fe(NO_3)_3$) or soluble high-molecular-weight ferric hydroxide polymers and chelates (Berner et al., 1985; 1986; Geisser and Muller, 1987). Soluble iron polymaltose, in which a ferric oxide polymer core is bonded to disaccharide groups has similar bioavailability to ferrous salts (Jacobs et al., 1984; Johnson and Jacobs, 1990). Iron presented as inorganic ferric or ferrous salts may also be absorbed, although the more soluble ferrous salts are absorbed much more rapidly (Bezkorovainy, 1989). Iron exists in two distinct oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}). In aqueous solution of low pH each oxidation states exists as hydrate, $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$. As the pH is raised, protons are lost until at neutral and alkaline pH each oxidation state exists as hydroxides, $Fe(OH)_2$ and $Fe(OH)_3$. The hydroxides are much less soluble than the hydrates, $Fe(OH)_3$ being 10^{15} less soluble than $Fe(OH)_2$ ($10^{-16}M$ vs. $10^{-1}M$) (Clydesdale, 1983).

In vitro digestion of meals and subsequent analysis of soluble iron has shown good correlation with *in vivo* determination of iron bioavailability (Hurrel et al., 1988; Schricker et al., 1981). Soluble iron after *in vitro* digestion is measured as iron which is dialyzable across a membrane with a molecular weight cut-off of 6,000-8,000. The dialyzable iron from various meals increases upon addition of beef (Kane and Miller, 1984). Meat components that are smaller than M.W. 6,000-8,000 may contribute to

iron absorption by forming soluble meat-iron complexes. Carpenter and Mahoney (1989) reported that proteolytic digestion of meat is not necessary for iron solubilization. They suggested meat enhancer may be a tissue component other than proteins since meat enhancer is not dependent on, nor inhibited by proteolytic digestion.

Meat enhancement of nonheme iron absorption
by stimulation of gastric acid secretions

Gastric acid is considered to be one of the most important luminal factors necessary for optimal nonheme iron absorption. The hydrochloric acid in gastric juice increases nonheme iron absorption (Jacobs et al., 1964; Jacobs and Owen, 1968; Mignon et al., 1965). Other components of gastric juice may also facilitate iron absorption. An endogenous component of gastric juice with the ability to bind iron and possibly affect the intestinal absorption of the metal has been studied by a number of workers (Davis et al., 1966; Jacobs and Miles, 1969; Koepke and Stewart, 1964; Smith et al., 1969; Wynter and Williams, 1968). Beutler et al. (1963) noted that gastric juice prevented ferrous iron from precipitating at an alkaline pH. Koepke and Stewart (1964) reported that gastric juice from anemic dogs enhanced the absorption of iron by healthy dogs. Although the presence of such a gastric iron-binding substance was confirmed by other workers, they were unable to verify any consistent alteration in iron-binding levels in disease states (Jacobs and Miles, 1969; Smith, 1968; Smith et al., 1969; Wynter and Williams, 1968). Rudzki and Deller (1973) reported that the iron binding substance was a glycoprotein containing approximately 90% sugar residues and 10% amino acid residues. The binding of inorganic iron by macromolecular constituents such as glycoproteins has been demonstrated *in vitro*. Although the binding is weak and probably ionic in nature (Swan and Glass, 1973; Wynter and Williams, 1968), it is agreed that the complexes are not precipitated when the pH is raised (Beutler et al., 1963; Davis et al., 1966; Jacobs and Miles, 1969; Wynter and Williams, 1968). The

evidence from absorption studies is conflicting. Jacobs and Owen (1968) found no difference between the effects of gastric juice and HCl of similar molarity on iron absorption in achlorhydric subjects, but Jacobs and Owen (1969) reported that iron absorption was potentiated significantly by gastric juice even when the effect of the acid was eliminated.

When the iron was injected directly into the duodenum and jejunum, less iron was absorbed than when injected into the stomach. Bezwoda et al. (1978) reported that gastric juices with pH values above 2 had a very limited capacity to solubilize the iron in bread. However with gastric juice pH below 2, iron solubilization increased linearly with decreasing pH. They suggested that pH is the only factor in gastric juice that is of importance in modifying the absorption of nonheme iron. Barry et al. (1981) reported iron absorption falls only when acid secretion is markedly reduced. Goldberg et al. (1963) demonstrated that iron-deficient patients with histamine-fast achlorhydria absorbed less supplemental and dietary iron than did those with a normal acid output.

McArthur et al. (1988) observed that average gastrin rise is 65%-75% less and acid secretion is 30%-40% less with soy than beef. This result indicate, that the source of dietary protein in a meal may be an important determinant of gastric acid secretion and gastrointestinal hormone release. Potent intragastric stimulants of acid secretion act largely by stimulating release of gastrin. In dogs, cysteine, phenylalanine, and tryptophan were the most potent individual amino acids for stimulation of gastrin release (Strunz et al., 1978). In man, tryptophan and phenylalanine were the best stimulants of gastrin release and acid secretion, but cysteine was ineffective (Taylor et al., 1982). Ippoliti et al. (1976) observed milk is a good stimulant of gastrin and acid secretion in man. Saint-Hilaire et al. (1960) concluded, from their survey, that the component of food producing the greatest effect on the secretion of acid was protein. Since protein generally is a better buffer than is carbohydrate or fat, a correlation was sought between

the acid response to the foods and their buffering capacity.

Meat enhancement of nonheme iron absorption by a dual mechanism

A single mechanism does not explain how meat enhances nonheme iron absorption in all studies. It is most likely that increased secretion of gastric acid and other gastric factors, and iron solubilization by a meat enhancer work synergistically to maximize nonheme iron absorption (Zhang et al., 1990). Food acts as an antacid or neutralizing substance, and especially protein is considered as a main factor giving buffering capacity of food (Osmon et al., 1957). Gastric acid production stimulated by food causes iron solublization in the stomach. Solubilized iron binds with meat enhancer and meat enhancer-iron complexes maintain solubility in the small intestine. Meat may provide a carrier which transports iron to the mucosal membrane by forming a meat-iron complex (Björn-Rasmussen and Hallberg, 1979). Kroe et al. (1966) demonstrated the additive effect of low pH and chelating amino acids on enhanced ^{59}Fe absorption. Björn-Rasmussen and Hallberg (1979) reported that although beef enhanced iron absorption in achlorhydric patients less than normal subjects, the relative enhancement was about the same. These indicate both gastric secretion and meat enhancer play roles in nonheme iron absorption.

POTENTIAL OF THE RAT MODEL FOR PREDICTING IRON BIOAVAILABILITY FOR HUMAN

There is controversy concerning the use of rats in determining iron bioavailability. The responses of this species to ascorbic acid and heme iron are of interest. The absorptive response of rats to ascorbic acid is less than in humans. This may be due to the high content of ascorbic acid in the luminal secretion of rats. Shah et al. (1983) reported that the anemic rat is not a suitable model for normal man since the absorption of heme iron by man is much higher than that of inorganic or non-heme iron. This difference has been demonstrated with enhancement of iron bioavailability in rats from meat after heat processing (Jansuittivechakul et al., 1985; 1986). Iron absorption in humans from over-cooked meat was less than iron absorption from meat heated to a lesser extent (Martinez-Torres et al., 1986). Turnbull et al. (1962) and Hussain et al. (1965) have shown that iron from heme is absorbed by iron-adequate people as well as from an inorganic source. Iron-deficient people absorbed heme iron about one-third as efficiently as they absorbed ferrous-sulfate or ferrous-ascorbate. Insensitivity of rats to factors that influence the absorption of nonheme iron in humans has been reported (Reddy and Cook, 1991; Schricker et al., 1981; 1983).

In contrast, Mahoney and Hendricks (1984) reported that rats and humans respond similarly to many dietary and physiological factors known to affect iron utilization. Responses of two species were highly correlated ($r = .94$), and the average human response was 68% that of the rats. Gordon and Godber (1989) mentioned that there are similarities between the two species with regard to the effect of animal protein on iron absorption. They suggested that valuable information can be obtained from rat studies with properly designed experiments and can be carefully extrapolated to results observed in the human.

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PART 2.   ALTERATION OF IRON ABSORPTION BY VARIOUS SOURCES OF DIETARY PROTEIN: INFLUENCES OF GASTRIC ACID PRODUCTION, IRON STATUS AND DIETARY PHYTATE

ABSTRACT

Studies were undertaken to investigate if gastric acidity and iron chelation to a meat component are involved with meat enhancement of iron absorption. Cereal meals, with and without added proteins, were gavaged into iron-deficient rats. The role of iron chelation was investigated by adding phytate, an iron chelator implicated with decreased iron absorption, to the meals. The role of gastric acidity was investigated by treating the rats with cimetidine which inhibits gastric acid production. In rats with normal acid production, beef, pork, and chicken enhanced iron absorption only when phytate had been added to the meals, suggesting a role for chelation in meat enhancement of iron absorption. However, the enhancement by beef and pork were insignificant in cimetidine-treated rats given the cereal + phytate meals, indicating that gastric acid production also plays a role in meat enhancement of iron absorption. Fish and egg white were sometimes inhibitory to iron absorption and, therefore, did not fit the pattern of enhancement demonstrated by beef, pork, and chicken. In a separate experiment, measurable gastric acidity was not directly altered by the protein source included with cereal meals. No significant effects of the various proteins on iron absorption from cereal + phytate meals were observed in a final experiment involving iron-replete rats. *In vitro* iron solubilizing capacity of beef, pork, chicken, and egg white was correlated with iron absorption only in iron-deficient rats *in vivo* . Fish demonstrated an exaggerated solubilization value in the *in vitro* system.

INTRODUCTION

Among the dietary factors, only ascorbic acid (Carlson and Miller, 1983; Gillooly et al., 1984; Hallberg and Rossander, 1982; Morck et al., 1982; Rossander et al., 1979) and meat (Björn-Rasmussen and Hallberg, 1979; Cook and Monsen, 1976; Gordon and Godber, 1989; Hurrel et al., 1988; Layrisse et al., 1969) have consistently been shown to enhance iron bioavailability. Meat serves a twofold purpose in iron absorption: it is a source of highly bioavailable iron as heme, and it has the ability to enhance both heme and nonheme iron absorption (Kane and Miller, 1984). However, the mechanism by which meat enhances nonheme iron absorption is still unknown. One possible mechanism by which meat enhances nonheme iron absorption is based on meat increasing iron solubility by chelation. Iron solubility is critical to iron bioavailability since it is generally agreed that only soluble iron can be absorbed. Meat component(s) may chelate iron solubilized from food by acid conditions within the stomach and thereby maintain iron solubility once neutral pH conditions are encountered within the intestinal lumen. Some studies suggest that the meat factors which chelate iron are proteolytic digestion products. Other studies have identified a factor in meat that is able to chelate iron but is released independent of digestion (Carpenter and Mahoney, 1989). Although the meat factor responsible for enhancing iron absorption has not been positively identified, there is general agreement that different protein sources affect iron availability differently (Björn-Rasmussen and Hallberg, 1979; Cook and Monsen, 1976).

Meat may also enhance nonheme iron absorption by stimulating gastric acid production and thereby promoting the solubilization of iron from the food matrix. Acid secretion depends both on the amounts of food protein or amino acids (Taylor et al., 1982) and the specific protein source (Ippoliti et al., 1976). McArthur et al. (1988) reported that in humans, meat stimulated 30-40% more gastric acid production and 65-

75% more gastrin, the primary physiological stimulant of gastric acid secretion, than soy protein. Saint-Hilaire et al. (1960) concluded, from their survey, that the component of food producing the greatest effect on the secretion of acid was protein. Zhang et al. (1990) have previously reviewed the research in this area and reported a high correlation (r =.94 and r =.91) for increases in nonheme iron absorption in two independent human studies as compared with gastric acid secretion in a third study for five dietary protein sources. They hypothesized that meat enhancement results from alteration of iron solubility through a dual mechanism involving both gastric acidity and iron chelation effects.

This study was intended to test the hypothesis that meat enhancement is via a dual mechanism involving both iron chelation and gastric acidity effects. The approach was to investigate the specific alterations in absorption of iron caused by various dietary proteins influenced by three factors known to modify iron absorption, i.e., gastric acidity, iron status, and dietary phytate.

MATERIALS AND METHODS

Experimental design

The effects of various dietary proteins (beef, pork, chicken, fish, and egg white) on iron absorption from cereal meals were observed in rats. To explore the possible role of chelation in meat enhancement of iron absorption, test meals were prepared with or without added phytate. To explore the role of gastric acid production in meat enhancement of iron absorption, rats were treated with cimetidine. In experiment 1, all possible combinations of treatments (6 meals x 2 phytate levels x 2 cimetidine treatments) were used. Each treatment was given to 5 iron-deficient rats. In experiment 2, only the phytate-containing meals were used. Each treatment was given to 5 iron-replete rats. In experiment 3, gastric acidity was measured in sixty iron-deficient rats after gavaging the test meals. In experiment 4, four retail packages of animal protein were selected and used to prepared ILC. Iron solubilizing capacity of animal proteins were measured with the *in vitro* system. Data obtained from experiment 1-4 were statistically analyzed.

Animals

Sprague-Dawley, 21-day-old male rats (Simonson Laboratories, Gilroy, CA), were divided into two groups and were fed diets of low or adequate iron to establish iron-deficient and replete status animals (APPENDIX C). Iron status of the rats was manipulated by varying the iron concentration in the diet. The iron-deficient diet was prepared which contained about 15 mg iron/kg of diet preparation. The iron-adequate diet was prepared by adding 35 mg iron/kg to the basal iron-deficient diet. Rats were individually housed in stainless steel cages with wire-mesh bottoms and fronts. Housing was in a temperature-controlled room (72°F) with a 12-h light:dark cycle. The rats were fed the assigned diets and deionized water *ad libitum* for 4 wk. The rats were fasted one-

day before gavaging the test meals. Plastic collars (i.d. 2.5 cm, o.d. 10 cm) were placed on the rats to prevent coprophagy (Zhang et al., 1991).

Cimetidine treatment

Cimetidine (Sigma Chemical Co., St. Louis, MO), 250 mg, was dissolved into 1.1 ml of 1.0 mol HCl/L at room temperature and then 2.0 ml 0.1 mol NaOH/L and 10 ml demineralized water were added. This cimetidine solution, 16 ml/kg of body weight, was gavaged 1 h before gavaging the test meals to 24 h fasted rats (Brimblecombe et al., 1975; Galbraith and Jellinck, 1989). A control solution without cimetidine was prepared and gavaged to the normal rat group.

Preparation of test meals

An extrinsically-labelled meal (1.5 ml) consisting of 1) cereal meal: 0.12 g ground whole wheat cereal (Shredded Wheat, Nabisco, East Hanover, NJ), 15 μg Fe^{3+} from $FeCl_3$, 1.5 μCi $^{59}Fe^{3+}$ from $^{59}FeCl_3$ (Du Pont, NEN Products, Boston, MA) and demineralized water to a volume of 1.5 ml, or 2) cereal + phytate meal: the cereal meal plus phytate (150 μg sodium phytate, Sigma), or 3) test meal: either cereal or cereal + phytate plus 0.07 g of protein as muscle meats from one of four species or as egg white. Each protein was added as a demineralized water slurry prepared using a polytron tissue homogenizer (Kinematica® GMBH, Switzerland). Meats used were beef (round eye steak), pork (rib loin), chicken (breast), and fish (trout). The ratio of iron:sodium phytate:animal protein (1:10:~4700) used was similar to that used previously in the meals in humans (Björn-Rasmussen and Hallberg, 1979).

Protein determination

The crude protein contents of meats were determined by Kjeldahl (APPENDIX D). Meat, 0.2 g, was measured and digested with addition of Tecalor Special Kjeltabs S3.5, Hoganas, Sweden. The digested sample was then analyzed for nitrogen using a

Tecator Kjeltec Auto 1030 Analyzer.

Iron absorption experiment

Hemoglobin levels of rats were measured using the cyanmethemoglobin method (Crosby et al., 1954; APPENDIX E) one day before the iron absorption experiment. Rats were then assigned to various treatment groups so that there was no significant difference in hemoglobin level among groups receiving the various meals. Rats were fasted 24-h before gavaging test meals, but demineralized water was provided. During fasting, a plastic collars (i.d.; 2.2 cm, o.d.; 10 cm) were placed on the rats to prevent coprophagy. Half of the animals used for experiments 1 and 2 were treated with cimetidine 1 h before gavaging test meals to inhibit gastric acid production. Test meals, 1.5 ml, were gavaged to rats and the plastic collar was placed until collecting blood samples. Duplicate 20 μl blood samples were collected from the retro-ocular capillary bed 1 d after gavaging using a heparinized capillary tube. Radioactivity of the blood samples was measured for determination of iron absorption.

Iron solubilizing capacity

Duplicate 20 μl blood samples were placed in test tubes with 5 ml of demineralized water. ^{59}Fe activity of the blood samples was counted in a gamma counter (Packard Auto-Gamma Model 2000 Series, Meriden, CT). Standard curves .002, .004, .008, .016, .032, .050, and .100 μCi of ^{59}Fe per tube were prepared from the original stock solution and used with all ^{59}Fe counting data to correct for decay and counting efficiency. The percentage of the ^{59}Fe administered that was absorbed into the blood was calculated as follows:

^{59}Fe absorbed in blood (%) = [(μCi ^{59}Fe/0.02 ml blood x g body weight x 0.067 ml/g)/μCi ^{59}Fe administered] x 100.

Measurement of gastric acidity

In experiment 3, cereal meals containing the various proteins were gavaged to iron-deficient rats. Gastric acidity was directly measured using a semi-micro pH probe (Corning, Medfield, MA) subsequent to sacrificing the rats at 30, 45, 60, or 90 min after gavaging the test meals.

Isolation of ILC

Beef (round eye steak), pork (rib loin), chicken (breast), fish (trout), and egg, purchased at a local market were trimmed of visible fat. Meat, 12.5 g, was homogenized with 70 ml 0.01 mol HCl/L using a polytron homogenizer (Kinematica® GMBH, Switzerland). The pH was adjusted to 2.0 with 0.1 N/L HCl and the total weight raised to 100 g with 0.01 mol HCl/L. A dialysis bag with a MWCO 6-8 K (Spectrum, Medical Industries, Inc., Los Angeles, CA) containing 25 ml 0.01 mol HCl/L was put into the homogenized meat slurry. The mixture was incubated at 37°C with constant shaking for 3 h. The dialysis bag was taken from the meat slurry and rinsed with demineralized water. The contents (ILC) in the dialysis bag were assayed for dialyzable iron.

Determination of soluble iron

In experiment 4, 0.2 ml ILC was mixed with 1ml $FeCl_3$ (from 80 mg Fe/L stock solution) and then kept at room temperature for 20 min. Demineralized water (2.4 ml) and 0.2 ml 0.25 mol/L pH 8.5 Tris buffer were added to the mixture and shaken. The mixture was incubated at 37°C for 1 h. The supernatant after centrifugation at 2,500 x g for 10 min was assayed for soluble iron. Soluble iron was determined spectrophotometrically by ferrozine assay (APPENDIX F).

Statistical analysis

Data were analyzed statistically using ANOVA in a randomized block design.

Whenever F was significant, $p < .05$, means were compared by Fischer's least significant difference (LSD) test.

RESULTS

Effect of iron status and gastric acidity in iron absorption

Iron status and gastric acidity were confirmed as significant factors in iron absorption from experiments 1 and 2. Iron absorption was greater ($p < .001$) for iron-deficient rats (Hb 8.4 ± 0.9 g/dl; mean $\pm$ SD) than iron-replete rats (Hb 12.4 ± 1.6 g/dl) in both control and cimetidine treated groups. Iron absorption was less ($p < .001$) for cimetidine treated rats in both the iron-replete and iron-deficient groups. Modification of iron status, gastric acidity, and dietary phytate variously influenced the significant ($p < .05$) effects of the dietary proteins on iron absorption as compared to cereal meals (Fig. 1-6, Table 1, Appendix L).

Iron absorption in iron-deficient rats (experiment 1)

Enhancement of iron absorption by meat added to the cereal + phytate meal was observed for iron-deficient rats (Fig. 1); beef ($62.8 \pm 2.7\%$; mean $\pm$ SEM), pork ($65.0 \pm 1.5\%$), and chicken ($59.7 \pm 4.9\%$) significantly ($p < .05$) increased iron absorption as compared to the cereal + phytate meal ($49.0 \pm 2.4\%$), but no significant difference was observed among meats. Fish ($55.0 \pm 1.1\%$) and egg white ($52.5 \pm 3.9\%$) did not increase or inhibit iron absorption significantly compared to the cereal + phytate meal. Iron absorption by iron-deficient rats from all meat-containing cereal meals was not different from the cereal meal ($60.1 \pm 2.5\%$) although absorption from the egg white-containing cereal meal ($42.8 \pm 9.9\%$) was significantly inhibited (Fig. 2). Iron absorption values from beef, pork, chicken, and fish were $59.3 \pm 2.0\%$, $61.9 \pm 7.4\%$, $62.4 \pm 4.0\%$, and $62.1 \pm 2.5\%$ respectively. Chicken ($35.1 \pm 2.8\%$) and fish ($32.6 \pm 2.8\%$) significantly enhanced iron absorption by cimetidine-treated iron-deficient rats from cereal + phytate meal as compared to the cereal + phytate meal ($20.4 \pm 5.1\%$) (Fig.
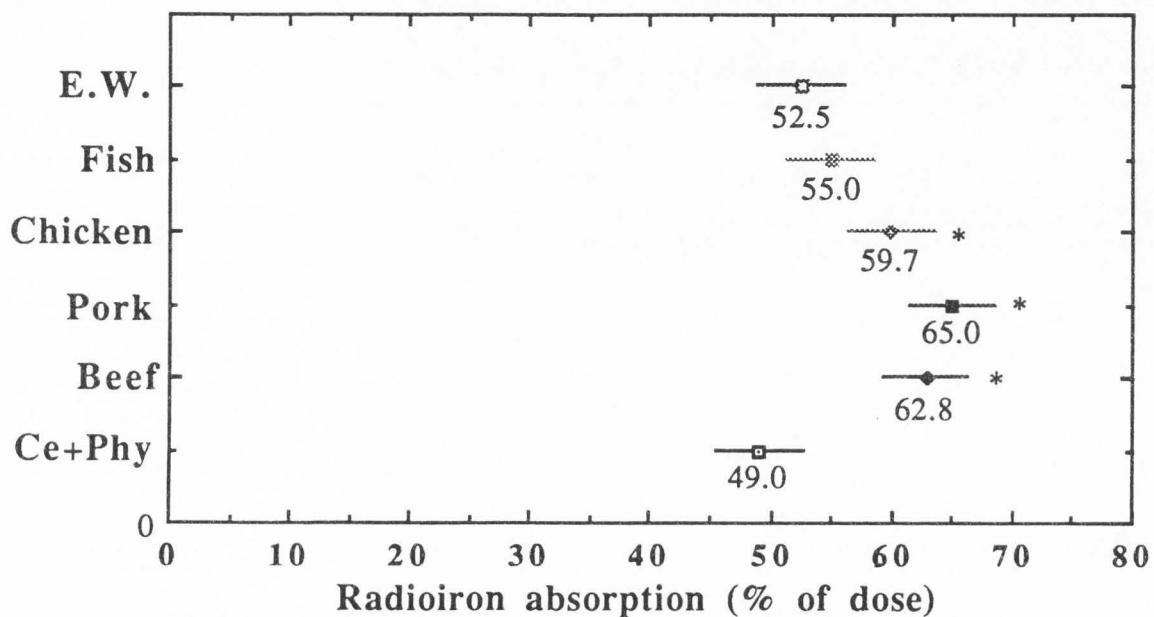
Figure 1. Absorption of radioiron from cereal + phytate meals, with or without added proteins, by iron-deficient rats. Bars represent mean ± 1/2 Fischer's LSD value. Treatments with * are significantly different from Ce + Phy (cereal + phytate) meal (p <.05). Values under each bar represent mean value (%) of radioiron absorption from each treatment. E.W. =egg white.
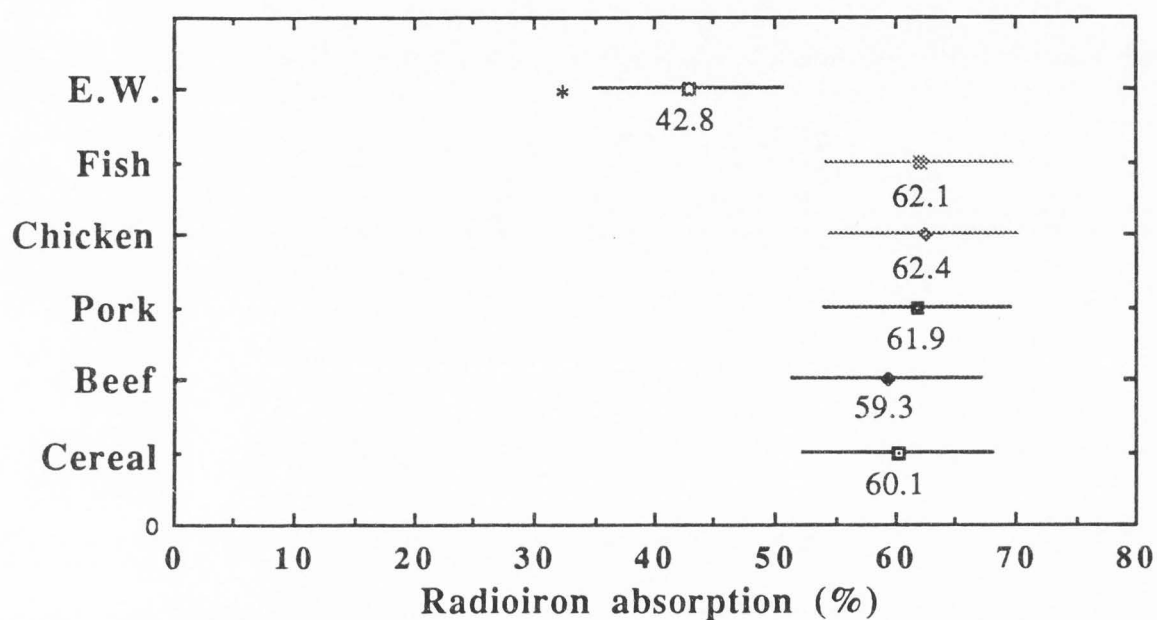
Figure 2. Absorption of radioiron from cereal meals, with or without added proteins, by iron-deficient rats. Bars represent mean ± 1/2 Fischer's LSD value. Treatment with * is significantly different from cereal meal (p <.05). Values under each bar represent mean value (%) of radioiron absorption from each treatment. E.W. =egg white.
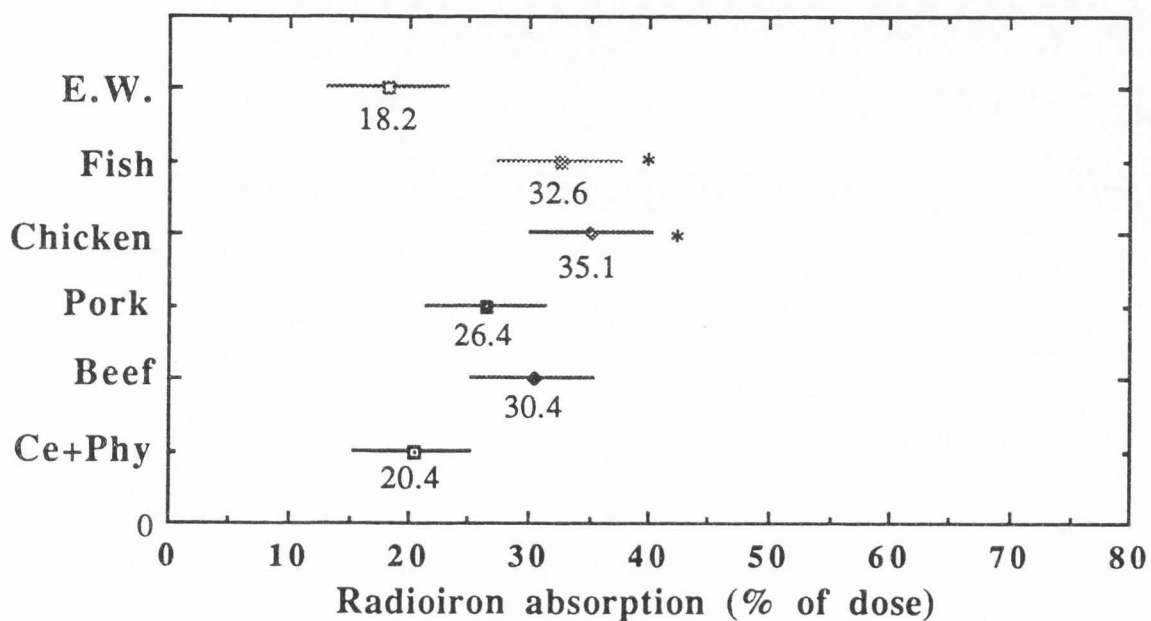
Figure 3. Absorption of radioiron from cereal + phytate meals, with or without added proteins, by cimetidine-treated, iron-deficient rats. Bars represent mean ± 1/2 Fischer's LSD value. Treatments with * are significantly different from Ce + Phy (cereal + phytate) meal ($p < .05$). Values under each bar represent mean value (%) of radioiron absorption from each treatment. E.W. =egg white.
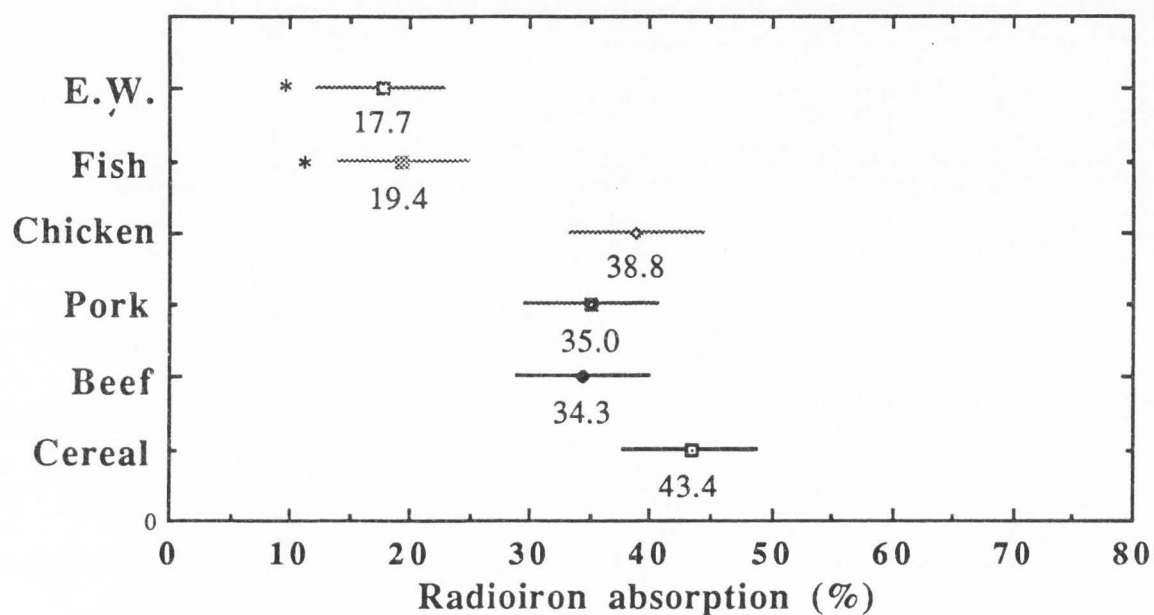
Figure 4. Absorption of radioiron from cereal meals, with or without added proteins, by cimetidine-treated, iron-deficient rats. Bars represent mean ± 1/2 Fischer's LSD value. Treatments with * are significantly different from cereal meal ($p < .05$). Values under each bar represent mean value (%) of radioiron absorption from each treatment. E.W.=egg white.
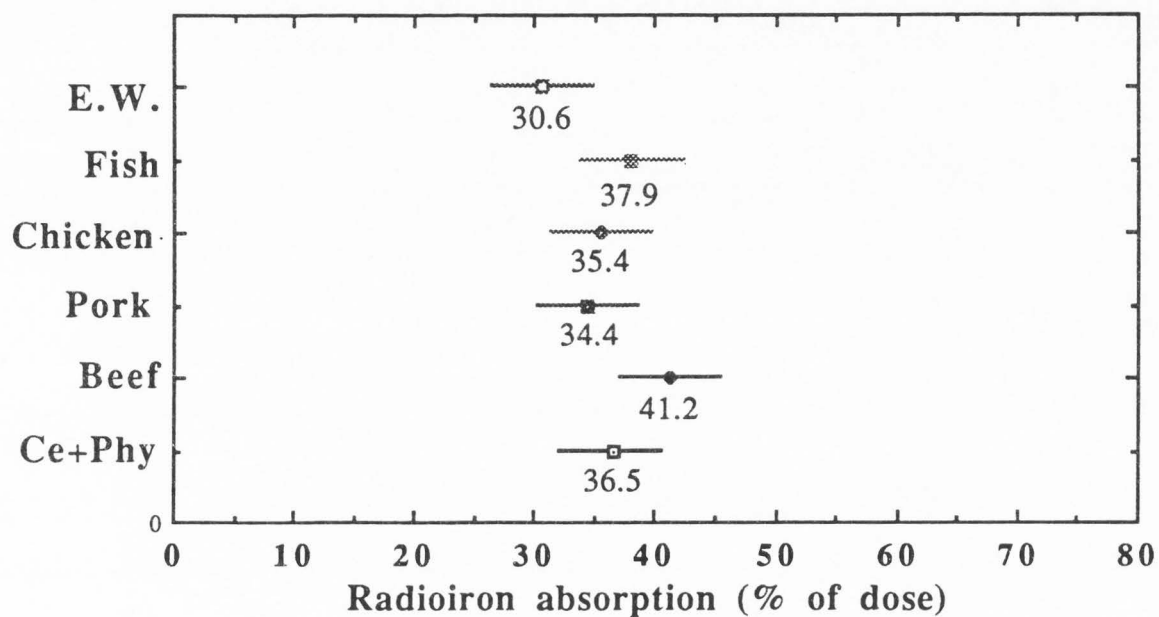
Figure 5. Absorption of radioiron from cereal + phytate meals, with or without added proteins, by iron-replete rats. Bars represent mean ± 1/2 Fischer's LSD value. Values under each bar represent mean value (%) of radioiron absorption from each treatment. E.W. =egg white.
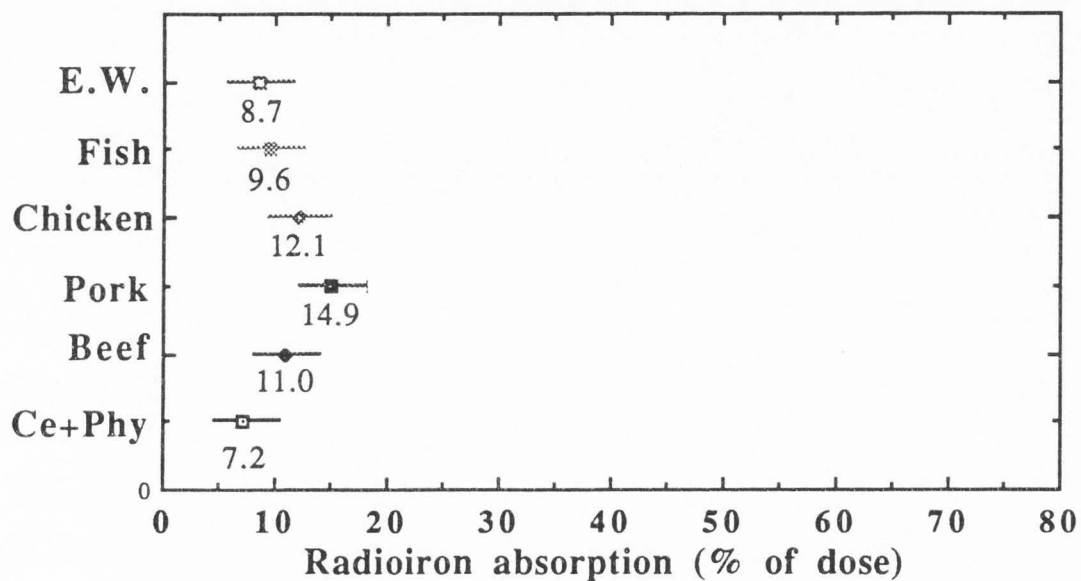
Figure 6. Absorption of radioiron from cereal + phytate meals, with or without added proteins, by cimetidine-treated, iron-replete rats. Bars represent mean ± 1/2 Fischer's LSD value. Values under each bar represent mean value (%) of radioiron absorption from each treatment. E.W. =egg white.

Table 1. Meat effects on iron absorption in different conditions of iron status, acidity and dietary phytate.

| | Iron-deficient | | | | Iron-replete | |
| | Cimet-[1] | | Cimet+[2] | | Cimet- | Cimet+ |
	Phy+[3]	Phy-[4]	Phy+	Phy-	Phy+	Phy+
Beef	E[5]	NS[6]	NS	NS	NS	NS
Pork	E	NS	NS	NS	NS	NS
Chicken	E	NS	E	NS	NS	NS
Fish	NS	NS	E	I	NS	NS
Egg white	NS	I[7]	NS	I	NS	NS

~No - see ANOVA table p = .356

[1] No cimetidine treatment.
[2] Cimetidine treated to inhibit gastric acid production.
[3] Phytate added meals.
[4] Meals without addition of phytate.
[5] Enhanced radioiron absorption ($p < .05$) as compared to respective control meal.
[6] Not significant.
[7] Inhibited radioiron absorption ($p < .05$) as compared to respective control meal.

3). Iron absorptions from the meat-containing cereal + phytate meals were not significantly different. Egg white (17.7 ± 1.7%) and fish (19.4 ± 2.6%) significantly inhibited iron absorption by cimetidine-treated iron-deficient rats from the cereal meal (Fig. 4).

Iron absorption in iron-replete rats (experiment 2)

For iron-replete rats receiving cereal + phytate meals, no significant alteration in iron absorption from cereal + phytate meals containing various animal protein was observed as compared to the cereal + phytate meal in both normal-treated (Fig. 5) and cimetidine-treated (Fig. 6) groups. Although mean differences were observed among the meats, they were not significant statistically (p >.05). Iron absorption from beef (41.2 ± 4.5%) or fish-containing (37.9 ± 3.5%) cereal + phytate meals was higher than from cereal meal (36.5 ± 3.6%). Egg white (30.6 ± 1.3%), chicken (35.4 ± 3.3%), and pork (34.4 ± 2.0%) inhibited iron absorption as compared to cereal + phytate meal.

All the animal tissue protein sources increased iron absorption by cimetidine-treated iron-replete rats as compared to cereal + phytate meal (7.2 ± 1.7%), although differences were not significant statistically. Iron absorptions from beef, pork, chicken, fish, and egg white were 11.0 ± 2.5%, 14.9 ± 2.9%, 12.1 ± 1.2%, 9.6 ± 2.0%, and 8.7 ± 1.7% respectively.

Gastric acidity measurement (experiment 3)

No significant (p >.05) differences in gastric acidity were observed in iron-deficient rats among cereal meals containing each of the various dietary protein sources (Fig. 7).

Iron solubilizing capacity of meats *in vitro* (experiment 4)

ILC from various meats based on the same protein content, 2.8 g, were demonstrated differences in iron solubilizing capacity (Fig. 8). Iron solubilizing capacity

was pork > beef = fish > chicken > egg white ($p < .05$).
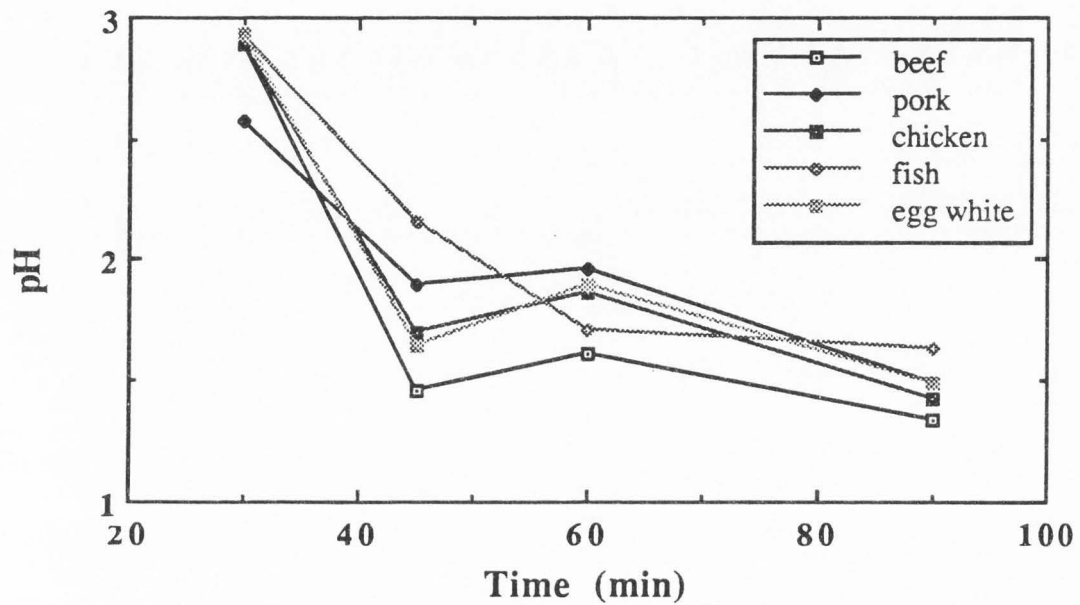
Figure 7. Gastric acidity change with time in iron-deficient rats from gavage of meals containing various animal proteins. No significant difference was observed among meals containing different animal proteins ($p > .05$).
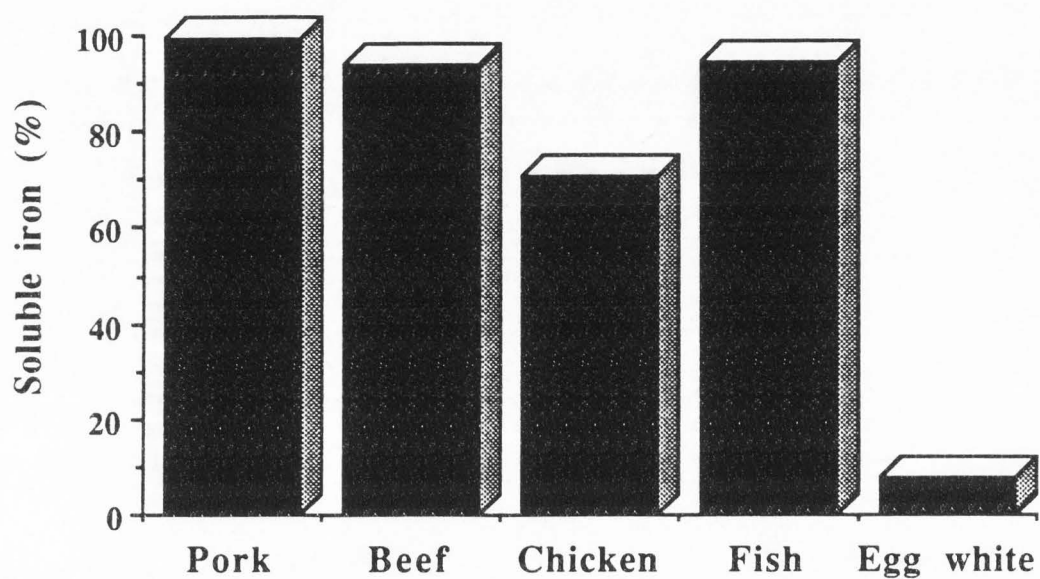
Figure 8. *In vitro* iron solubilizing capacity of ILC from undigested animal proteins. Iron solubilizing capacities were pork (99.7 ± 0.20%; mean ± SEM) > beef (93.9 ± 0.64%) = fish (94.4 ± 0.70%) > chicken (70.6 ± 1.70%) > egg white (7.80 ± 0.70%) (p <.05).

DISCUSSION

Iron-deficient rats absorbed iron from meals about 1.3 to 2.0 times more than iron-replete rats depending on the conditions involving various animal protein included with the meals, gastric acid production, and presence of dietary phytate (Fig. 1-6, Table 1). Iron status is a significant factor in iron absorption (Conrad and Crosby, 1962). There is an inverse relationship between iron stores and iron absorption (Thomson et al., 1971; Wheby et al., 1964). Several factors are related in regulating iron absorption in different iron status of animals. For example, stomach emptying rate is one of those regulating factors in iron absorption; iron-deficient rats have been observed to release stomach contents more slowly than normal rats (Magnusson et al., 1979).

In iron-deficient rats under normal gastric acid production beef, pork, and chicken enhanced iron absorption with cereal + phytate meals. However, inclusion of the above meat to the meal did not increase iron absorption significantly by iron-deficient rats when either of the following conditions was changed: 1) iron-deficient rats were treated by cimetidine which means that gastric acid secretion of the rats was blocked or, 2) phytate was not added to the meals. Gastric acid is considered to be one of the most important luminal factors necessary for optimal nonheme iron absorption. Patients with iron deficiency anemia and histamine-fast achlorhydria have a diminished ability to absorb nonheme dietary iron (Goldberg et al., 1963; Skikne et al., 1981). Inorganic iron from food will become soluble under an acidic condition in the stomach, and soluble iron is available to bind chelators. Politz and Clydesdale (1988) have reported that the chelate must have a stability constant for iron greater than the iron hydroxides, but the constant must be such that it allows the release of the iron to mucosal acceptors for absorption. Meat enhancement in iron absorption from food may not be observed without other competitive chelator because soluble iron in the stomach can bind any available meat
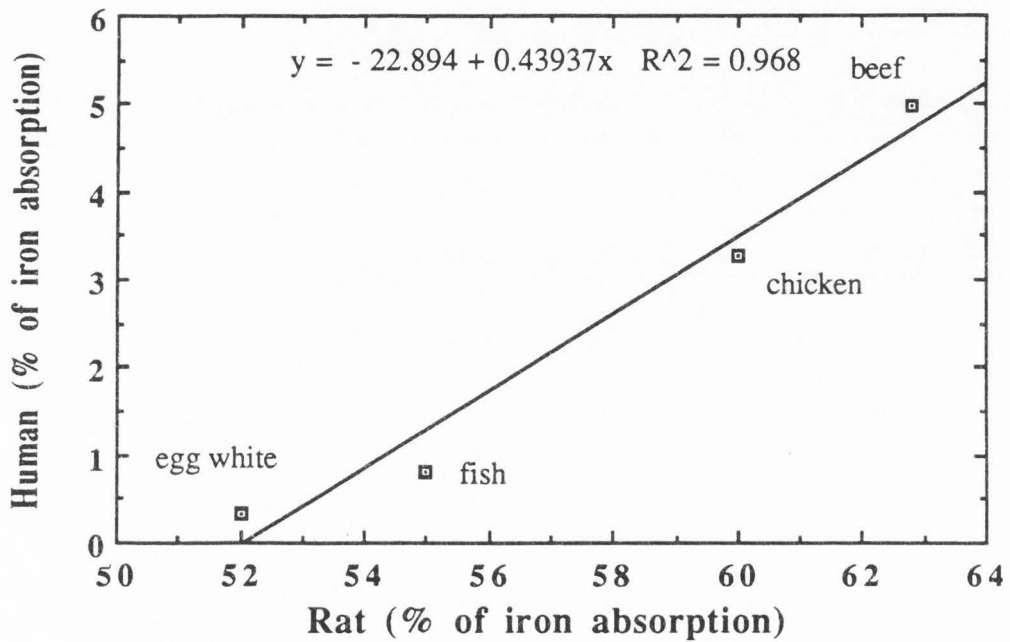
Figure 9. Correlation of meat effect on nonheme iron absorption in rats (data from Fig. 1) and human (Björn-Rasmussen and Hallberg, Nutr. Metab., 23, 192, 1979).

components which may not that tightly bind with iron compared to binding of iron with phytate. Phytate is believed to inhibit iron absorption by binding iron to form insoluble complexes. Although some studies found no effect (inhibition) on iron absorption of dietary fiber using meals containing meats and vegetables (Kelsay et al., 1979; Sandstead et al., 1978), Björn-Rasmussen et al. (1974) and Mameesh et al. (1970) reported that dietary fiber may depress iron absorption when meals are low in meat and vegetables and high in cereal food. Factors which chelate iron in soluble form, thereby precluding iron binding tightly to insoluble fiber, would be expected to enhance absorption. Binding to dietary fiber is always relative to attraction of the iron to other complexing agents in the diets (Leigh and Miller, 1983). Some of the ingredients in meat appear to be required to produce low-molecular-weight compounds which bind iron from food in acidic conditions such as in the stomach and, therefore, maintain the iron in soluble form when passing through the small intestine (Hazell et al., 1978). The extent of meat degradation to low-molecular-weight compounds depends on the types of meat as well as the time of degradation (Latunde-Dada and Neale, 1986).

High correlation was observed between iron absorption by normal iron-deficient rats and that of humans (Björn-Rasmussen and Hallberg, 1979) (Fig. 9). This suggests that there are similarities between the human and the rat with regard to the effect of animal protein on iron absorption in certain conditions. Valuable information can be obtained from rat studies with proper experimental design and careful extrapolation (Gordon and Godber, 1988; Buchowski et al., 1991).

That food acts as an antacid or neutralizing substance as it is first consumed and then stimulates gastric acid secretion is common knowledge. Food protein and amino acids and the specific protein source are main factors stimulating acid secretion (Korman et al., 1971; Osmon et al., 1957; Richardson et al., 1976). Osmon et al. (1957) and Saint-Hilaire et al. (1960) have observed that the time needed to reduce

stomach pH to 3.0 was different when different protein sources (beef, chicken, fish, egg, milk, or soy bean) were consumed by humans. However, we could not repeat their results using the rats (Fig. 7). The disadvantage of using rats for the study of gastric acid production is their continuous interdigestive secretion and sensitivity to stress which inhibits gastric acid secretion (Lane et al., 1957). Possibly, no differences in gastric acid secretion with various animal protein-containing meals might be observed in rats due to this characteristic of the rat. From our result of gastric acidity measurement, meat enhancement was apparently not proportional to direct stimulation of acid production since there were no significant differences in gastric acidity. The results from this study suggest that meat enhancement of iron absorption may be due to chelation of iron with a meat enhancer(s) under acid condition in the stomach and the iron-meat complex will maintain soluble in alkaline environment of intesitine for absorption. Meat enhancement on iron absorption observed in this study can be explained by synergistical work of increased secretion of gastric acid and iron chelation by a released meat factor(s) which confirms the suggestion by Zhang et al. (1990).

Egg white and fish did not fit the pattern for enhancement of absorption demonstrated by beef, pork, and chicken. Fish was observed to demonstrate anomalous behavior when given to cimetidine-treated iron-deficient rats, acting as an enhancer for the phytate added meal and as an inhibitor for the without addition of phytate. However, in the presence of a strong inhibitor of iron absorption such as phytate, inhibition by components of meat may be insignificant. In this case, proteins from fish may even enhance iron absorption by stimulating gastric substances production. Significant effects of egg white were always inhibitory. This result was consistent with other reports. When added to a protein free meal, egg white reduced iron absorption by 72% in humans (Hurrell et al., 1988).

The data suggest that the *in vitro* method used in this study is capable of distinguishing among meats of iron enhancement since each animal protein sources showed different iron solubilizing capacity (Fig. 8). From comparison of rat *in vivo* and *in vitro* method, the trend was consistent between iron absorption *in vivo* and iron solubility *in vitro* from beef, pork, and chicken (Fig. 10). Fish demonstrated exaggerated value in the *in vitro* system compare to the *in vivo* system. To observe factors in iron absorption from food, several *in vitro* methods have been proposed (Jacobs and Greenman, 1969; Miller et al., 1981; Ranhotra et al., 1971; Rao and Prabhavthi, 1978) since *in vitro* methods have advantages for rapid screening and testing of availability of iron from diets. It is hard to say that any one of the *in vitro* methods presented can estimate iron availability. Schricker et al. (1981) suggested that incorrect estimate of iron availability in *in vitro* methods can be decreased by using two *in vitro* methods. Variability of iron availability from food among *in vitro* methods is mainly due to selection of conditions for *in vitro* assays of mineral. Correlation of *in vivo* and *in vitro* assays may depend on choice of conditions for *in vivo* studies (Johnson, 1989). Similarly, data from the *in vitro* method used in this study was correlated highly only in normal iron-deficient rats with the cereal + phytate meal containing animal proten.
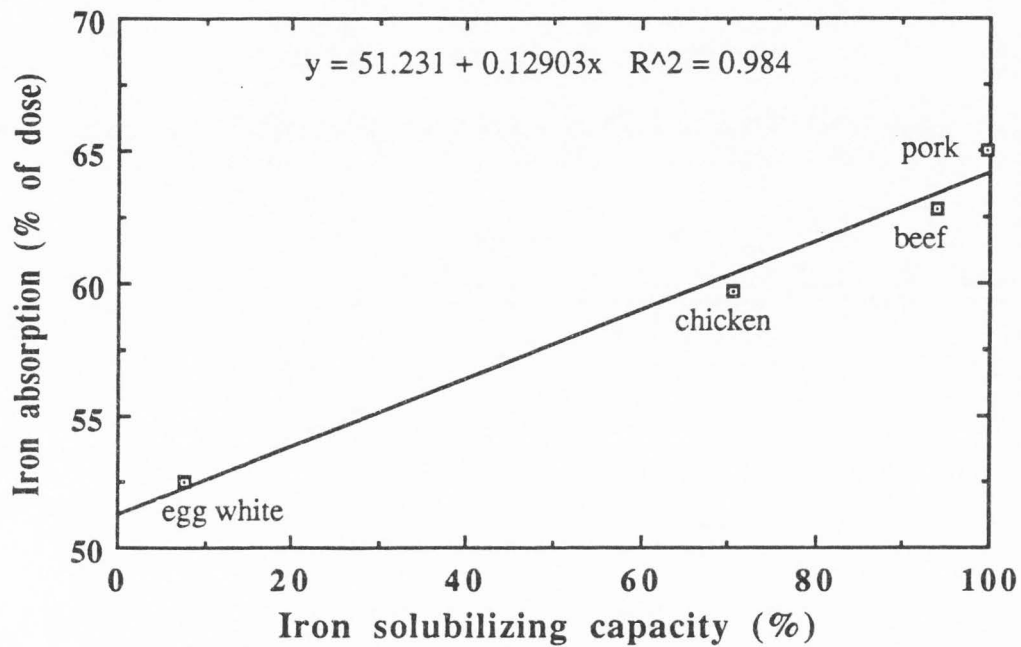
Figure 10. Correlation of iron absorption by iron-deficient rats (Fig. 1) and iron solubilizing capacity of animal protein *in vitro* (Fig. 8).

CONCLUSIONS

We conclude that results of these experiments were consistent with the hypothesis that meat enhancement of iron absorption is by a dual mechanism involving both gastric acidity effects and iron chelation effects by one or more meat factor.

Normal acid production was observed to be critical for enhancement of iron absorption by beef and pork. However, enhancement was apparently not related to direct stimulation of acid production since no significant differences in gastric acidity were caused by the various meals.

Egg white and fish did not fit the pattern for enhancement of absorption demonstrated by beef, pork, and chicken since they can be inhibitory to iron absorption.

Data of iron availability from the *in vitro* method used in this study were correlated in iron-deficient rats *in vivo*, although fish demonstrated exaggerated value in the *in vitro* system.

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PART 3.   IRON-SOLUBILIZING ISOLATE OF MEAT:
PHYSIOLOGICAL, COMPOSITIONAL AND
PHYSICOCHEMICAL CHARACTERIZATIONS

ABSTRACT

Studies were performed to 1) investigate if ferric iron bound in complex with iron-solubilizing meat components are absorbable, 2) compare the relative iron-solubilizing capacity of meats, and 3) investigate the physicochemical and compositional characteristics of the meat solubilizing components. Iron-solubilizing components of beef were isolated from pH 2 HCl homogenates into dialysis bags (MWCO of 6-8 K). Radiolabelled iron complexes were then generated using ferric iron and the isolated low-molecular-weight components (ILC) from undigested beef or ascorbate. The bioavailabilities of radioiron in these complexes or as ferric iron were measured as radioiron absorption into the blood one hour after injection into ligated duodenal loops of rats. Iron absorptions were ferrous-ascorbate complexes > ferric-ILC complexes > ferric iron ($p < .05$). In separate experiments, ILC from 0.1 g of beef, pork, chicken, fish, or egg white were added to 400 μg ferric iron in pH 2 HCl, the pH raised to 7.2, and soluble iron determined in the supernatant after centrifugation at 2,500 g for 10 min. Iron solubilizing capabilities of ILC were pork > beef > chicken > fish > egg white ($p < .05$). In a final series of experiments the compositional and physicochemical characteristics of the ILC from the above dietary protein sources were investigated.

INTRODUCTION

Availability of dietary iron is affected by several enhancing and inhibiting factors present in foods. Meats, especially the red meats of beef and pork, are dietary components that enhance iron absorption via a presently obscure mechanism termed "the meat effect" (Björn-Rasmussen and Hallberg, 1979; Cook and Monsen, 1976[b]; Gordon and Godber, 1989; Hurrel et al., 1988; Layrisse et al., 1969; Worthington-Roberts et al., 1988). Studies employing single food items tagged biosynthetically with radioiron have shown that inorganic iron absorption from vegetable or cereal foods is usually less than 5% as compared with 15-20% absorption from animal sources such as beef, liver, and fish.

The factor(s) responsible for this meat enhancement has not been identified, but has been hypothesized to be a combination of meat influences on gastric functioning and chelation of dietary iron by a meat component (Zhang et al., 1990). The amount of iron solubilized during *in vitro* digestion of meat-containing meals correlates with *in vivo* determinations of iron bioavailability and led to the suggestion that amino acids or peptides released from meat during digestion may be the meat factor (Kane and Miller, 1984; Martinez-Torres et al., 1981; Schricker et al., 1981). Soluble iron was defined for these *in vitro* digestions as iron that is dialyzable across a membrane with a molecular weight cut off (MWCO) of 6-8 K. Other evidence has demonstrated the presence of an iron-solubilizing factor(s) in meat independent of digestion (Carpenter and Mahoney, 1989). These components are also dialyzable across a membrane with MWCO of 6-8 K and referred to in the remainder of this paper as isolated low-molecular-weight components (ILC). Our most recent studies suggest a role for these iron-chelating components in meat enhancement of iron absorption. However, 1) the bioavailability of iron bound in such complexes has not been established, nor 2) has the digestion-

independent iron-solubilizing capacity of various meats been examined, nor 3) has the compositional and chemical characteristics of the meat components responsible for iron solubilization been investigated. These studies were intended to address these deficiencies.

MATERIALS AND METHODS

Experimental design

Bioavailability of iron from a beef ILC-iron complex. Thirty-six, six-week-old iron-deficient (7.9 ± 0.7 g Hb/ dl; mean $\pm$ SD) rats were used. Iron status of the rats was observed by measuring hemoglobin concentration in duplicate samples of fresh blood using the cyanmethemoglobin method (Crosby et al., 1954) (APPENDIX E). One hour after injection of radiolabelled test solutions (iron complexes) into the small intestinal ligated loops, blood samples were taken and radioiron absorption measured. Three rats for each treatment (ferrous-ascorbate, ferric-ILC complex and ferric chloride) were used in the experiment with four replicate trials on different days. Rats were randomly assigned for each treatment.

Iron solubilizing capacity of meat. Beef (round eye steak), pork (rib loin), chicken (breast), fish (trout), and egg were purchased at a local market. Four separate retail packages of each protein source were selected. One retail sample for each protein source was used for measurement of iron solubilizing capacity on each of four separate days. Iron solubilizing capacity was measured using ILC prepared from the undigested meats.

Physicochemical and compositional characteristics of ILC. ILC from beef, pork, chicken, fish, or egg white were prepared from undigested meats and analyzed for protein, amino acid, phosphorus, iron, carnosine, and inosine monophosphate.

Isolation of ILC

Beef (round eye steak), pork (rib loin), chicken (breast), fish (trout), and egg, purchased at a local market, were trimmed of visible fat. Weighed meat, 12.5 g, was homogenized with 70 ml 0.01 mol HCl/L using a polytron homogenizer (Kinematica®

GMBH, Switzerland). The pH was adjusted to 2.0 with 0.1 mol HCl/L, and the total weight raised to 100 g with 0.01 mol HCl/L. A dialysis bag with a MWCO of 6-8 K (Spectrum, Medical Industries, Inc., Los Angeles, CA) containing 25 ml 0.01 mol HCl/L was put into the homogenized meat slurry. The mixture was incubated at 37°C with shaking for 3 h. The dialysis bag from the meat slurry was rinsed with demineralized water, and the contents (ILC) were assayed for dialyzable iron. Amounts of ILC are expressed either 1) in volume units, which is how the liquid ILC was actually measured for the experiments, or 2) in weight units corresponding to the wet-weight of the protein source from which a specific amounts of ILC was produced (PS; gram wet-weight of protein source).

Preparation of iron complexes

The formulations used to prepare the iron solutions to be injected into rat intestinal loops are given in Table 2. Iron standard $FeCl_3 \cdot 6H_2O$ in dilute HCl (Ricca Chemical Co., Arlington, TX) and ILC (contents in the dialysis bag), 0.01 mol HCl/L, or 100 mmol/L ascorbic acid (in 0.01 mol HCl/L) were mixed and set at 37°C incubator for 30 min. Demineralized water and pH 8.5, 0.25 mol/L Tris buffer were added and mixed. The final pH of test solutions was 7.2. After preparation, all iron complexes were incubated at 37°C for 1 h.

Animals

Sprague-Dawley weanling male rats, 21-day-old (Simonson Laboratories, Gilroy, CA) were fed low-iron diet (15 mg Fe/kg diet) (APPENDIX C). The rats were individually housed in stainless steel cages with wire-mesh bottoms and fronts. Housing was in a temperature-controlled room (72°F) with a 12 h light:dark cycle. Rats were fed the diet and deionized water *ad libitum* for 3 wk.

Table 2. Formulation of iron complexes injected into *in situ* ligated loop.

Treatment	Ferrous-ascorbate (ml)	Ferric-ILC[1] (ml)	Ferric (ml)
$FeCl_3$ [2]	0.25	0.25	0.25
ILC	-	0.05	-
0.01 mol HCl/L	0.05	0.05	0.10
Ascorbic acid (100 mmol/L in 0.01 mol HCl/L)	0.05	-	-
Demineralized water	0.605	0.615	0.615
0.25 mol/L Tris buffer (pH 8.5)	0.045	0.035	0.035
Total volume (ml)	1.00	1.00	1.00

Final pH: 7.2.
[1] Isolated low-molecular-weight components.
[2] 80 mg Fe/L in 0.01 mol HCl/L and $^{59}FeCl_3$ (0.5 μCi/ml).

Iron absorption from ligated intestinal segments

Animals were starved one-day before onset of the *in situ* iron absorption measurements. Animals were anesthetized by intraperitoneal injection of 4 mg sodium pentabarbital (Anthony Products Co., Arcadia, CA) per 100 g of body weight. The animals were laparatomized, and 20 cm of the duodenum, beginning 1 cm below the pyloric valve, was ligated using cotton string. Test solutions (ferrous-ascorbate, beef ILC-ferric complex, and ferric chloride), 0.5 ml previously prepared in test tubes using radiolabelled iron (Table 2), were injected into the ligated loops and the abdomen closed using Michel clips. The animals were then maintained in a quiet room at 32°C for 1 h and the animals exanguinated by decapitation. Duplicate 20 μl blood samples were obtained and used for determination of radioiron absorption.

Determination of radioiron absorption

Duplicate 20 μl blood samples were placed in test tubes with 5 ml of demineralized water. The ^{59}Fe activity of blood samples was counted in a gamma counter (Packard Auto-Gamma Model 2000 Series, Meriden, CT). All ^{59}Fe counting data were corrected for decay and counting efficiency. The percentage of the ^{59}Fe administered that was absorbed into blood was calculated as follows:

^{59}Fe absorbed in blood (%)= [(μCi ^{59}Fe/0.02 ml blood x g body weight x 0.067 ml blood/g body weight)/μCi ^{59}Fe administered] x 100.

Determination of iron solubilizing capacity

ILC, 0.2 ml, was mixed with 1 ml $FeCl_3$ (from 80 or 160 mg Fe/L stock solution) and then kept at room temperature for 20 min. Demineralized water (2.4 ml) and 0.25 mol/L pH 8.5 Tris buffer (0.2 ml) were added to the mixture and shaken. Final iron concentration was 20 or 40 mg Fe/L and final pH was about 7.2. The mixture was incubated at 37°C for 1 h. Supernatant after centrifugation at 2,500 x g for

10 min was assayed for soluble iron. Soluble iron was determined spectrophotometrically by ferrozine assay (APPENDIX F).

Effect of pH on iron solubility

Various ratios of ILC and $FeCl_3$ in 0.01 mol HCl/L were mixed and held at room temperature for 20 min. The pH was adjusted by adding 0.25 mol/L pH 10 Tris buffer and raised to 10 ml volume with demineralized water. Soluble iron was determined using the procedure for determination for iron solubilizing capacity.

Effect of molar ratio of ILC:iron on iron solubility

Two different iron concentrations (40, 20 mg Fe/L) were used, and the ILC:iron ratio was varied by changing the amount of ILC. Tris buffer, 0.25 mol/L pH 8.5, was used to adjust the pH to 7.2, and demineralized water was used to adjust the final volume to 10 ml.

Iron Determination

Iron was quantified using ferrozine color reagent (3-2(pyridyl)-5,6-diphenyl-1,2,4-triazone p,p'disulfonic acid, Aldrich Chemical Co., Milwaukee, WI). To 0.5 ml samples 1.25 ml of 0.02% ascorbic acid in 0.1 mol HCl/L was added, mixed, and let set in the dark for 10 min. Next, 1 ml ammonium acetate (10% solution) and 1.25 ml of 1 mM/L color reagent in demineralized water were added to the samples with mixing, and the samples were placed in the dark for 20 min. Finally, 1 ml of demineralized water was added with mixing, and absorbance was read at 562 nm in a Shimadzu UV 2100U Spectrophotometerm (Shimadzu Corporation, Kyoto, Japan) (Carter, 1971).

Protein determination

Protein was determined spectrophotometrically (Lowry et al., 1951). Absorbance was measured at 540 nm (APPENDIX G).

Amino acid analysis

ILC was concentrated for amino acid analysis by lyophilizing 25 ml of the ILC and then adding 1 ml of 6 mol HCl/L. The mixture was flame-sealed in a 5 ml ampule after flushing with nitrogen while in an ultrasonic water bath. Ampules were held in a heat block at 110°C for 20 h. After hydrolysis, the samples were filtered through a 0.2 μm syringe filter into a test tube from which 10 μl were placed into another test tube. Nitrogen gas was gently blown across the 10 μl sample until it was dry. Dried samples were rehydrated with 250 μl of Beckman Na-S dilution buffer, filtered through a 0.2 μm syringe filter, loaded into a sample cartridge, and placed in a Beckman System 6300 Amino Acid Analyzer. Standards were included which allowed amino acid concentrations to be calculated by a Beckman 7000 data system (APPENDIX H).

Phosphorus determination

ILC (25 ml) was lyophilized and then wet-ashed by adding H_2SO_4 and HNO_3. Phosphorus was detected by the AOAC (1984) methodology using molybdate and aminonaphtol sulfonic acid to form a colored complex (APPENDIX I).

Nonheme iron content

ILC was concentrated ten-fold by lyophilization and examined by ferrozine for presence of nonheme iron. Duplicate 0.5 ml samples were used for ferrozine assay.

Heme iron content

ILC was concentrated ten-fold by lyophilization and then used for absorption spectra from 400 to 700 nm to determine presence of heme iron. The Hornsey method was used to quantify heme iron (Hornsey, 1956).

Total iron determination

ILC (25 ml) was wet-ashed by adding H_2SO_4 and H_2NO_3. Wet-ashed ILC was

added to 2.5 ml demineralized water, and then 0.5 ml samples, in duplicate, were applied to ferrozine assay.

Carnosine determination

ILC, 0.1 ml, and 0.9 ml OPA (o-phthalaldehyde) reagent (Sigma, St. Louis, MO) were mixed and filtered through 0.2 μm syringe and then applied to HPLC (Beckman, Somerset, NJ). Test parameters were as follows: column, 5 μm ultrasphere ODS high resolution end-capped column (250 mm x 4.6 mm); column temperature, ambient; detection, fluorescence 310 nm for excitation and 375 nm for emission; flow rate, 0.7 ml/min; mobile phase, 50% B (methanol-acetonitrile (60:40, v/v)) in A (0.4% triethylamine in 0.06 mol KH_2PO_4/L) (Carnegie et al., 1983; Kasziba et al., 1988; APPENDIX J). Reference compound, L-carnosine (N-ß-alanyl-L-histidine), was purchased from Sigma.

Inosine monophosphate determination

ILC was filtered through 0.2 μm syringe filter and then applied to HPLC (Beckman Instruments). Test parameters were as follows: column, 5 μm ultrasphere ODS high-resolution end-capped column (250 mm x 4.6 mm); column temperature, ambient; detection, UV 254 nm; flow rate, 1.5 ml/min; mobile phase, linear gradient from 0% to 25% of high-concentration eluent (methanol-water (60:40, v/v)) by volume in 30 min and low-concentration eluent (20 mmol KH_2PO_4/L) (Krstulovic et al., 1979; APPENDIX K). Reference compound, inosine 3',5'-cyclic mono phosphate (sodium salt), was purchased from Sigma.

Statistical analysis

Data were analyzed statistically using ANOVA in a randomized block design. When F was significant (p <.05), means were compared by Fischer's least significant difference (LSD) test.

To obtain a prediction equation for iron solubilizing capacity from analysis of ILC or from amino acid composition of ILC, multiple regression was done using Statview, software on Macintosh, at confidence interval 95%. To observe association of iron solubilizing capacity and each components of ILC, simple regression was also done.

RESULTS

Iron absorption from ligated loop of small intestine of rat

Iron bound in complexes with beef ILC was available for absorption from ligated segments of small intestine (Fig. 11). Ferrous-ascorbate was absorbed significantly (p <.001) more than any other iron complex (APPENDIX L). Absorption was $18.8 \pm 2.2\%$ (mean ± SEM) of dose. Although not as available as ferrous-ascorbate complexes, ILC-iron ($4.9 \pm 0.6\%$) was more available (p <.05) for absorption than uncomplexed ferric iron ($2.2 \pm 0.33\%$).

Iron solubilizing capacity of meats

There were significant differences in iron solubilizing capacity among meats (p <.001) (APPENDIX L). Iron solubility from pork, beef, chicken, fish, and egg white was $99.9 \pm 0.1\%$ (mean ± SEM), $93.6 \pm 3.5\%$, $75.8 \pm 1.8\%$, $64.6 \pm 3.6\%$, and $50.9 \pm 0.9\%$ respectively (Table 3). Iron solubilizing capacity of ILC from protein sources showed consistent (overall CV =5.42%) value over different animal samples and different times.

pH effect on iron solubility

Iron solubility of ILC-iron complexes was observed at different pH condition (Fig. 12). Minimum solubility was observed approximately from pH 4 to pH 6.2 for all iron concentrations used. By increasing pH above 6.2, iron solubility was increased for 20 and 15 mg Fe/L with ILC treatment. In contrast, the 32 mg Fe/L with ILC treatment showed nearly zero solubility above pH 6.2. Solubility of ferric iron, as a reference, was zero at above pH 4.5.
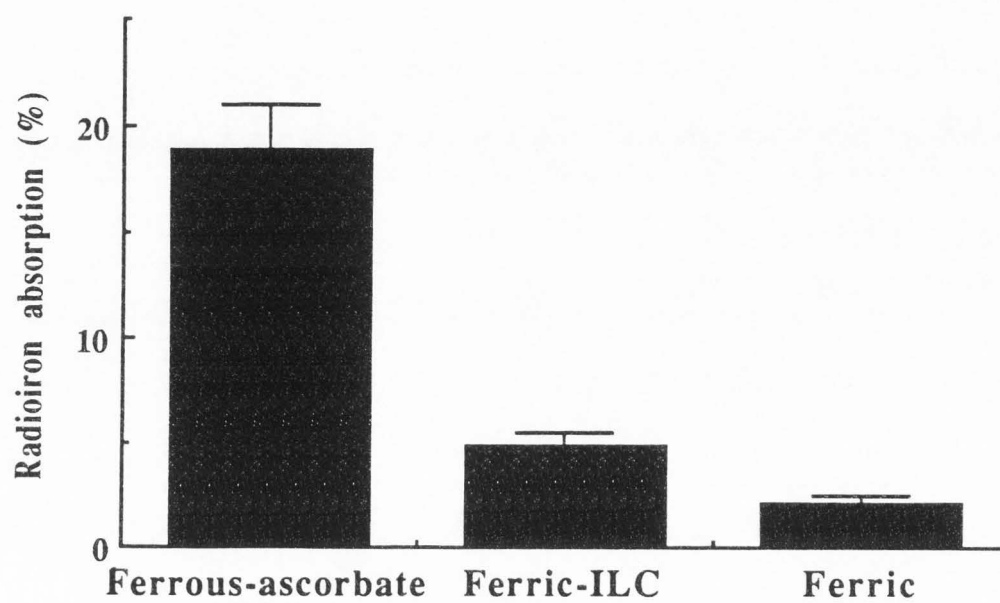
Figure 11. Radioiron absorption from iron solutions injected into ligated segments of rat small intestine. Error bars represent one standard error mean. Iron absorptions were ferrous-ascorbate > ferric-ILC (isolated low-molecular-weight components) > ferric (p <.05).

Table 3. Precision of *in vitro* method to evaluate iron solubilizing capacity of meat.

Protein source	n	ISC[1] (mean ± SD)	CV(%)
Beef	8	93.7 ± 3.45	3.68
Pork	8	99.9 ± 0.16	0.16
Chicken	8	75.8 ± 3.66	4.83
Fish	8	64.6 ± 6.10	9.40
Egg white	8	50.0 ± 1.89	3.78
Overall			5.42[2]

[1] Iron solubilizing capacity.

[2] CV for pork was excluded due to indications that the capacity of assay was exceeded with pork.
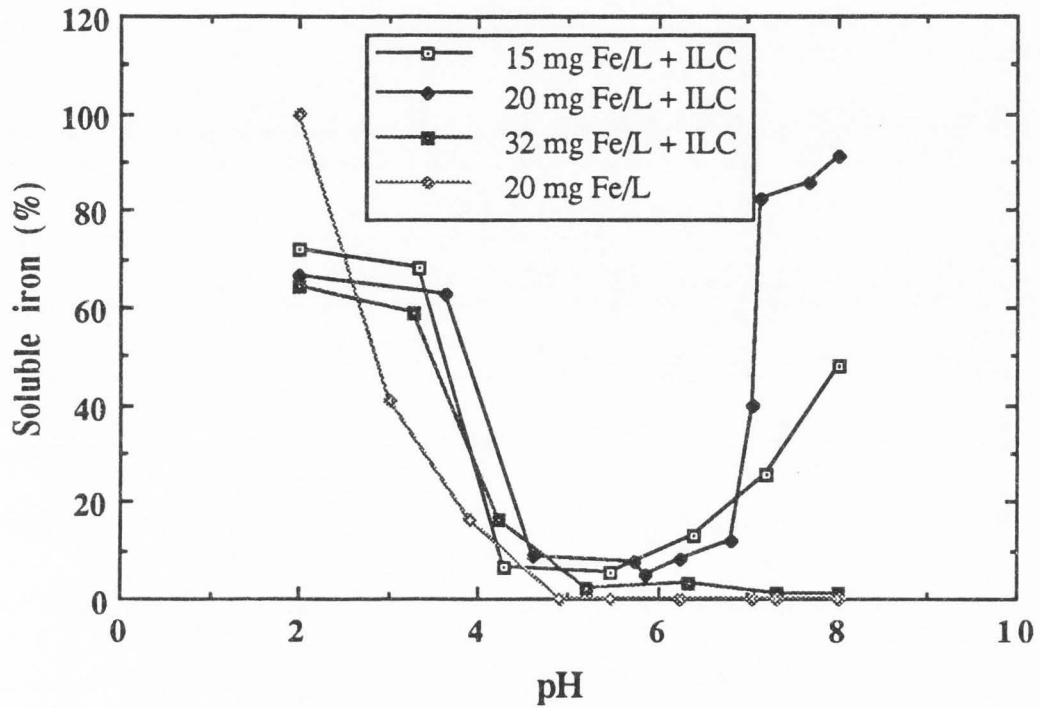
Figure 12. Iron solubility of ILC from undigested beef and iron complex at various pH conditions. Amounts of ILC was fixed to 0.5 ml (equivalent to 0.25 g wet-weight protein source), and final volume fixed to 10 ml. Iron concentration was varied by adding 2.5 ml of $FeCl_3$ from stock solutions of the appropriate concentration.

Effect of ratio of ILC and iron on solubility

For both iron concentrations used, 20 and 40 mg Fe/L, maximum solubilities of 90 and 9% ,respectively, were observed when the ratio of ferric (mg):ILC (PS) source was 0.8 (Fig. 13). With 20 mg Fe/L concentration, solubility was increased by increasing the ratio of ferric:ILC from 0.2 to 0.8. Iron solubility decreased when the ratio increased above 0.8 by decreasing content of ILC in test solutions. When iron concentration was 40 mg Fe/L, percent iron solubility was much less than in 20 mg Fe/L.

Composition of ILC

ILC included different amounts of protein (Table 4). Beef ILC (512 μg protein/PS) showed more protein content than other ILCs. ILC from pork, chicken, fish, and egg white contained 414, 440, 452, and 92 μg/PS, respectively.

The phosphorus content of chicken ILC was 17.8 μg/PS which was 1.6 times more than other muscle meats. Beef (11 μg/PS), pork (11.2 μg/PS), and fish (11.8 μg/PS) showed similar amounts of phosphorus in ILC. Egg white, 12.5 g, generated 3.2 μg phosphorus/PS.

Heme iron was not detected in ILC since no peak was observed from the absorption spectra from 400 to 700 nm. No nonheme iron was detected in any ILC before ashing.

Total iron contents in ILCs were measured after wet-ashing ILCs. The total iron content of beef, pork, chicken, and fish ILC were 0.44, 0.64, 0.38, and 0.38 μg/PS, respectively. No iron was detected in egg white ILC.

Carnosine (N-ß-alanyl-L-histidine) was detected in ILC from muscle protein. It was not detectable in ILC from egg white. Pork ILC contained 12.2 μg carnosine/PS which was higher than ILC from other proteins used in this study. Carnosine contents of ILC from beef, chicken, and fish were 6.6, 3.2, and 4.4 μg/PS, respectively.
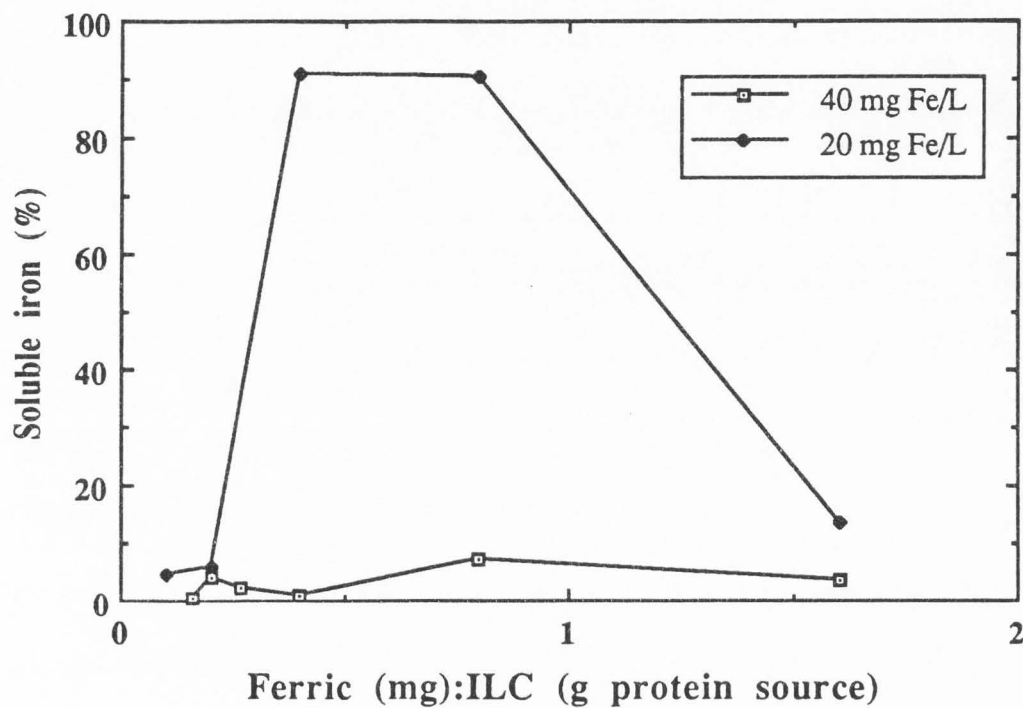
Figure 13. Effect of iron:ILC ratio on solubility at pH 7.2. Volume of test solution (10 ml) and amount of iron were fixed. Amount of ILC (isolated low-molecular-weight components) was varied to give different ratio of iron and ILC.

Table 4. Identified components of isolated low-molecular-weight components (ILC) from various undigested animal proteins.

ILC	Protein (μg/PS[1])	Phosphorus (μg/PS)	Carnosine (μg/PS)	IMP[2] (μg/PS)	Heme iron (μg/PS)	Total iron (μg/PS)
Beef	512	11	6.6	26.0	ND[3]	0.44
Pork	414	11.2	12.2	84.0	ND	0.64
Chicken	440	17.8	3.2	ND	ND	0.38
Fish	452	11.8	4.4	ND	ND	0.38
Egg white	92	3.2	ND	ND	ND	ND

[1] g wet-weight protein source
[2] Inosine monophosphate
[3] Not detectable

Inosine monophosphate (IMP), one of the nucleotides in muscle, was detected in beef ILC (26 μg/PS) and pork (84 μg/PS). It was not detectable in ILC from chicken, fish, or egg white.

The four major amino acids (glutamic acid, glycine, alanine, and histidine) had similar molar percentages among ILC from beef, pork, and chicken (Table 5). Overall, amino acid composition of ILC from beef, pork, and chicken was similar. Fish and egg white showed a different pattern from other muscle meats.

Regression study

Carnosine (r =.904) and total iron content (r =.908) were determined as highly correlated factors to iron solubilizing capacity by simple regression (Table 6). Among amino acids, glutamic acid (r =.919), alanine (r =.923), and histidine (r =.936) were highly correlated to iron solybilizing capacity (Table 7).

To predict iron solubilizing capacity from these various components of ILC, which were individually correlated with iron solubilizing capacity, a predictive equation was obtained by multiple regression. The best fitting model was; iron solubilizing capacity (%) =49.306 +2.833[ala] +0.142[his] +3.926[glu] +1.75[carnosine] +0.077[IMP]; R^2 =.967.

Table 5. Amino acid composition of isolated low-molecular-weight components (ILC) from various undigested animal proteins.

Amino Acid	Beef	pork	chicken	fish	egg white
			(Molar % of the total)		
Asp	3.0	4.1	5.2	2.7	6.2
Thr	2.5	2.8	3.9	3.8	5.3
Ser	3.6	3.6	5.5	3.9	7.9
Glu	21.8	16.7	13.7	6.0	10.6
Pro	2.4	3.0	2.9	2.1	12.7
Gly	17.5	21.7	18.2	34.8	6.9
Ala	17.2	11.9	9.2	12.7	6.2
Cys	0.3	0.8	0.4	0.3	-
Val	3.2	3.3	3.4	3.9	7.0
Met	1.0	1.1	1.6	1.2	3.6
Ile	1.7	1.9	2.2	1.4	6.6
Leu	3.4	3.7	4.2	3.3	10.6
Tyr	1.2	1.2	1.6	2.1	5.0
Phe	1.5	1.7	1.7	2.1	5.0
His	14.7	16.8	18.1	13.8	1.9
Lys	2.9	3.6	5.1	5.6	1.1
Arg	2.1	2.1	3.1	1.6	2.9
Total	100	100	100	100	100

Table 6. Associations between iron solubility and identified components of isolated low-molecular-weight components (ILC) from beef, pork, chicken, fish and egg white [x =concentration of components (μg/g wet-weight protein source), y =iron solubility (%)].

Component	Correlation (r)	Intercept (β_0)	Slope (β_1)
Protein	0.717	42.8	0.087
Phosphorus	0.497	69.8	0.301
Carnosine	0.904	54.4	4.06
IMP[1]	0.811	65.8	0.454
Total iron	0.908	46.5	81.5

[1]Inosine monophosphate

Table 7. Associations between iron solubility and molar percentage of amino acid of isolated low-molecular-weight components (ILC) from beef, pork, chicken, fish and egg white (x =amino acid, y =iron solubility).

Amino acid	Correlation (r)	Intercept (β_0)	Slope (β_1)
Asp	0.602	58.5	2.94
Thr	0.521	58.1	3.46
Ser	0.587	57.1	2.70
Glu	0.919	51.9	0.91
Pro	0.805	44.9	7.00
Gly	0.638	53.4	0.60
Ala	0.923	45.7	1.29
Cys	0.851	57.5	28.0
Val	0.774	46.5	5.38
Met	0.698	51.9	11.4
Ile	0.780	50.7	8.14
Leu	0.743	49.8	4.31
Tyr	0.594	54.4	9.66
Phe	0.731	46.4	10.3
His	0.936	45.0	1.34
Lys	0.451	61.6	2.17
Arg	0.655	57.2	5.30

DISCUSSION

Iron solubility is a critical physicochemical factor controlling iron absorption since it is generally agreed that only soluble iron can be absorbed (Clydesdale, 1983; Forth and Rummel, 1973; Johnson, 1989). Unfortunately, the neutral pH found within the intestine will cause the precipitation of ferric iron, the predominant form of inorganic dietary iron, thereby precluding iron absorption. Dietary factors that can form complexes with ferric iron during the gastric phase of digestion, and subsequently maintain ferric iron soluble at the neutral pH found within the intestine, have the potential to enhance iron absorption, provided the iron is not bound so tightly so as to exclude uptake. Therefore, absorption of complexed iron is expected to be dependent not only on solubility, but also on the stability of the iron chelate. First, the chelate must have a stability constant for iron greater than the iron hydroxides in order to prevent hydroxide formation and maintain solubility. Second, the constant must be low enough that it allows the release of the iron to mucosal acceptors for absorption (Politz and Clydesdale, 1988). Previous observations have documented that components from beef fulfill the first requirement in that they are capable of maintaining ferric iron in solution at neutral pH. However, it had not been determined whether ferric complexes formed with the meat components would be released to the intestine for uptake. *In situ* absorption by rats (Fig. 11) showed that iron bound in complexes with beef ILC was available for absorption. Thus, beef contains components that have the characteristics necessary to enhance iron absorption.

Estimation of iron solubilization is a major criterion because solubility is a first premise in iron absorption. Thus, solubility is a clue which suggests the possibility that meat components-iron complexes may be available on absorption. Ability to solubilize iron *in vitro* has been suggested to be predictive of enhancement of iron absorption

(Jacobs and Greenman, 1969; Miller et al., 1981; Ranhotra et al., 1971; Rao and Prabhavthi, 1978). *In vitro* solubility measurements are reasonably good predictors of the enhancing effect of ascorbic acid (Miller and Berner, 1989). Kane and Miller (1984) measured dialyzable iron (MW <6,000-8,000) after pepsin digestion. Slatkavitz and Clydesdale (1988) reported that pepsin digestion products with MW< 10,000 solubilized significantly more iron than those with MW >10,000. In contrast, pancreatin digestion products of MW <10,000 were not effective iron-solubilizing agents. Politz and Clydesdale (1988) reported that molecular range of 6,200-2,500 solubilized significantly more iron than all the other peptide fractions after pepsin digestion. Carpenter and Mahoney (1989) have observed that iron chelation by meat components does not depend on nor is inhibited by proteolytic digestion. They concluded that meat contains a factor(s) that solubilizes iron independent of proteolytic digestion. In most cases, solubility is measured under intestinal conditions, usually after exposure to a lower pH environment similar to that of the stomach. *In vitro* approaches to the estimation of iron availability rely on the assumption that iron must be in solution to be absorbed. This is a weakness of *in vitro* estimation because soluble iron is not always absorbed depending on characteristics of binding with a chelator. Iron-solubilizing capacities of animal proteins *in vitro* were significantly different among protein sources (Table 3). The *in vitro* method used in this study was able to distinguish differences in iron-solubilizing capacities among animal protein sources. Measurements of *in vitro* iron-solubilizing capacity of ILC from undigested animal proteins were repeatable since the overall CV was less than 6%.

To predict iron bioavailability with *in vitro* techniques, it is important to understand the physicochemical characteristics of ILC-iron complexes under conditions which appear during the digestive process. A number of observations have been made about iron binding capacity of gastric juice (Beutler et al., 1963; Davis et al., 1967;

Smith et al., 1969). Swan and Glass (1973) mentioned that the iron bond which may form in alkaline pH is weak, and is probably of ionic nature. Rudzki and Deller (1973) extracted a specific iron binder from gastric juice and characterized it as a glycoprotein. The iron binding characteristics of ILC over pH 2 to pH 8 (Fig. 12) were similar to observations on gastric juice by Wynter and Williams (1968). They also observed similar binding in various protein solutions such as trypsin, egg albumin, alkaline phosphatase from intestinal mucosa and acid phosphatase from wheat germ and urine, and concluded that the iron binding phenomenon was a nonspecific ionic effect. Parallel with our observations of ILC-iron complexes, solubility of gastric juice-iron complexes was decreased by increasing pH from 2 to 4, but it was increased again around neutral pH.

The effect of food additives such as EDTA on iron absorption depends on its molar ratio to iron (Cook and Monsen, 1976[a]). When present in small amounts, it actually enhances iron absorption, but as the molar ratio exceeds 2:1, absorption of iron progressively declines. Similarly, Spiro et al. (1967) demonstrated that citrate ion, when present in equimolar concentration, chelates ferric iron at low pH with loss of its hydroxylic proton. As the solution is neutralized, the characteristic brown color of polymerized iron appears. This brown color was observed in some solutions of iron-ILC complexes. Iron concentration was observed as a critical factor determining solubility of iron in the presence of ILC (Fig. 12, 13). On the other hand, low ratio (<.4) ferric iron (mg):ILC (PS), which means excess ILC in solution did not increase iron solubility either. Both the ratio of ferric:ILC and total amount of iron were important. This is similar to observations with other iron chelators such as EDTA and citrate.

Diets low in protein are associated with a low iron uptake, and the amount of iron absorbed is roughly dependent on the amount of protein in the diet (Klavins et al.,

1959; 1962). Kroe et al. (1963) suggested that one of the iron enhancing factors of protein could be the availability of increased amounts of amino acids derived from high dietary protein. It has since been shown that amino acids have a chelating effect upon iron (Albert, 1950; 1952). Van Campen (1972) have observed that histidine increased ^{59}Fe retention when added to the dosing solution, but did not when added to the diet. Kroe et al. (1966) have found that amino acids have an influence on iron absorption independent of pH, and that, for any given pH, greater iron absorption occurs if the iron is administered with an amino acid such as glutamine or histidine. It has been proposed that certain amino acids and intermediary products of cellular meat digestion chelate with soluble iron and deliver the iron to the gut mucosa. Non-cellular animal proteins do not appear to form chelates that enhance nonheme iron absorption. To identify the components of ILC that may be contributing to iron solubilization, ILC was examined for the presence of iron-binding components. Subsequently, the amount of these components in the ILC of various dietary proteins was correlated with their *in vitro* iron solubilizing capacity. Amino acid composition of ILC from beef, pork, and chicken was similar, although fish and egg white showed differences from other muscle meats (Table 5). Among the amino acids found in ILC, alanine (r =.923), histidine (r =.936), and glutamic acid (r =.919) were highly correlated with iron solubility (Table 7).

From analysis of amino acid composition of ILC, histidine was suspected to play a role in iron solubilizing as a component of a certain polypeptide or as a free amino acid because of high molar percentage in ILC from cellular animal protein. There is a very small portion in ILC from egg white. Histidine and alanine, which are components of carnosine, were highly correlated with iron solubilizing capacity of ILC. The ratio of the histidine dipeptides which are present in skeletal muscle has been proposed as a useful aid to the identification of the species or origin of meat used in processed meats (Carnegie et al., 1982; Margolis, 1974). Carnosine (ß-alanylhistidine) is a histidine-

containing dipeptide and should chelate copper in tissues where this dipeptide is present in high concentration (Brown, 1981). Although the biological role of carnosine has not been established, a proposed function is action as a defense mechanism against oxidative stress through a variety of mechanisms, including chelation of metal ions (Brown, 1981; Kohen et al., 1988).

Carnosine and total iron content were highly correlated with iron solubility (Table 6). The iron in ILC was not in porphyrin ring or free non-heme iron. The iron possibly comes from heme; however, there was no detectable porphyrin ring or other heme protein in ILC by spectral analysis. Total iron which was highly correlated with iron solubilizing capacity may indicate the extent of degradation of heme.

CONCLUSIONS

Iron complexes formed with ILC from beef were available for absorption in the *in situ* ligated small intestine of rats. Iron absorption was ferrous-ascorbate complexes > ferric-beef ILC complexes > ferric iron (p <.05).

Iron solubilizing capacities of animal proteins in the *in vitro* system were pork > beef > chicken > fish > egg white (p <.05). These results support the hypothesis that meat enhancement of iron absorption may be due to its iron solubilizing ability.

Prediction equation for iron solubility of protein sources obtained was: iron solubility (%) =49.306 +2.833[ala] +0.142[his] +3.926[glu] +1.75[carnosine] +0.077[IMP]; R^2 =.967.

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PART 4: APPENDICES

APPENDIX A

Vitamin mixture

Ingredients	Amounts (g/kg)
Vitamin A concentrate (500,000 I.U./g)	1.8
Vitamin D concentrate (850,000 I.U. I.U/g)	0.125
Niacin	4.25
Riboflavin	1.0
Pyridoxine hydrochloride	1.0
Thiamin hydrochloride	1.0
Vitamin B_{12} (mg/kg)	1.35
Ascorbic acid	45.0
Calcium pantothenate	3.0
Biotin (mg/kg)	20.0
Folic acid (mg/kg)	90.0
Alpha tocopherol (250 I.U./g)	22.0
Ascorbic acid	45.0
Inositol	5.0
Choline chloride	75.0
Menadione	2.25
p-Aminobenzoic acid	5.0

Nutrition Biochemicals Corp., Cleveland, OH.

APPENDIX B

Mineral mixture

Ingredients	Amounts (g/kg)
Potassium chloride	296.7
Magnesium carbonate	121.0
Manganese sulfate	12.7
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.7
Copper sulfate ($CuSO4 \cdot 7H2O$)	1.6
Potassium iodide	0.8
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.1
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	28.0
Glucose	538.4

APPENDIX C

Diet composition (g/kg)

Ingredient	Basal diet (iron-deficient)	Iron-supplement diet
Casein	198	198
Corn oil	100	100
Cellulose	50	50
NaH_2PO_4	26.9	26.9
$CaCO_3$	17	17
Vitamin mixture[a]	20	20
Mineral mixture[b]	11.6	11.6
Iron added with ferrous sulfate (mg/kg)		35
Dextrose	576.5	576.5

[a]: APPENDIX A

[b]: APPENDIX B

APPENDIX D

Protein determination by Micro Kjeldahl Method

Digestion

1) Samples (0.1-0.2 g) were accurately weighed into digestion tube.

2) One Tecalor Special Kjeltabs S3.5 (3.5 g K_2SO_4, 0.0035 g Se), Hoganas,

Sweden, was added to each tube.

3) Five ml concentrated H_2SO_4 was added.

4) Samples were oxidized by heating until a clear solution was obtained.

5) Let cool down and demineralized water, 50 ml, was added.

Determination

The digested samples were titrated for nitrogen using a Tecator Kjeltec Auto

1030 Analyzer. The procedure was as outlined in the instruction manual for the

Auto 1030 Analyzer.

Calculation

Protein content was calculated as follows:

Protein (%) = 14.01 x Normality of HCl x 6.25 x (ml titrant-ml blank) x 100/mg

APPENDIX E

Cyanmethemoglobin method for determination of hemoglobin concentration

Preparation of Drabkin's reagent

Sodium bicarbonate, 1 g, Potassium cyanide, 52 mg, and Potassium ferricyanide, 198 mg, were weighed, then dissolved and diluted to 1 L in a volumetric flask with demineralized water.

Determination

1) Drabkin's solution, 5 ml, was transferred to test tube.

2) Whole blood, 20 μl was added to the test tube and mixed.

3) The assay mixture was kept for 10 min.

4) Absorbance was measured at 540 nm and concentration calculated from the standard curve.

Preparation of standard curve

Standards (Fisher Scientific Company, Orangeburg, NY) containing 5.5, 13.1 and 17.1 g hemoglobin/dl were used. Standard, 20 μl, was added to the test tube which contained 5 ml of Drabkin's solution and followed the same procedure as the determination.

APPENDIX F

Nonheme iron determination by Ferrozine assay

Reagents

Ferrozine color reagent

Ferrozine color reagent (3-2(pyridyl)-5,6-diphenyl-1,2,4-triazone-p, p'-disulfonic acid, Aldrich Chem. Co., Milwaukee, WI) was dissolved in demineralized water to make 1mM solution.

Ammonium acetate (10% solution).

Ascorbic acid (dissolved into 0.1 N HCl to make 0.02% solution).

Determination

1) Ascorbic acid, 0.02% solution, 1.25 ml, was added to sample, 0.5 ml appropriately diluted. Mixture was mixed and set for 10 min at room temperature.

2) One mililiter ammonium acetate solution was added and mixed.

3) Ferrozine color reagent,1.25 ml, was added and mixed and placed in the dark for 20 min.

4) Demineralized water, 1 ml, was added.

5) Absorbance was measured at 562 nm.

6) Concentration of sample was calculated from standard curve.

Preparation of standard curve

Standards were prepared by dilution of 1000 mg/L $FeCl_3$ (Ricca Chemical Co., Arlington, Texas). Absorbance was measured from 0, 2, 4, 6, 8, and 10 mg/L. Same procedure as determination was followed.

APPENDIX G

Protein determination by Lowry method

Reagents

Solution A

Anhydrous sodium carbonate, 20 g, sodium hydroxide, 4.0 g, and

sodium potassium tartarate tetrahydrate, 0.2 g were dissolved in the

demineralized water, 1L.

Solution B

Cupric sulfate pentahydrate, 6 g, was dissolved in the demineralized

water, 1L.

Solution C

Solution A and Solution B were mixed in a ratio 50:1.

Folin & Ciocalteu phenol reagent (Sigma Chemical Co., Saint Louis,

MO) was diluted with 5% sulfuric acid in a 1:1 ratio.

Preparation of standard stock solution

Bovine albumin stock solution (650 μg/ml) was prepared by dissolving bovine

albumin crystal (Fisher Scientific Company,, Orangeburg, NY). Standard protein

solutions were prepared by diluting 1, 2, 3, and 4 ml of the stock solution each to 10

ml.

Preparation of standard curve

1) Solution C, 1.0 ml, was added to 0.2 ml sample and then 0.1 ml folin &

Ciocalteu reagent was added and immediately mixed.

2) The assay mixture was left to stand 30 min for color development. Blank

consisted of solution C, diluted Folins & ciocalteus phenol reagent, and 0.2
ml of deionized water.

3) Absorbance was determined at 660 nm against the blank.

Determination

1) Same procedure as in preparation of standard curve.

2) Concentration of sample was determined from the standard curve.

APPENDIX H

Amino acid analysis

Procedure

1) Duplicate samples of ILC from beef, pork, chicken, fish, and egg white were
prepared.

2) Each sample was prepared for amino acid analysis by lyophilizing 25 ml of
the ILC and then adding 1 ml of 6 N HCl.

3) The mixture was flame-sealed in a 5 ml ampule (Wheaton #176779) after
flushing with nitrogen while in an ultrasonic water bath.

4) Ampules were held in a heat block at 110°C for 20 h (Moore, 1963; Moore
and Stein).

5) After hydrolysis, samples were filtered through a 0.2 μm syringe filter
(Gelman Acrodisc LC 13 PVDF) into a test tube from which 10 μl were
placed into another test tube.

6) Nitrogen gas was gently blown across the 10 μl sample until it was dry,
approximately 3 min.

7) Dried samples were rehydrated with 250 μl of Beckman Na-S dilution buffer
filtered through a 0.2 μm syringe filter (Gelman Acrodisc LC 13 PVDF),
loaded into a Beckman sample cartridge and placed in a Beckman System
6300 Amino Acid Analyzer.

8) Standards were included which allowed amino acid concentrations to be
calculated by a Beckman 7000 data system.

APPENDIX I

Phosphorus determination by the Vanado-Molybdate Colorimetric Method

Reagents

Molybdovanadate reagent

1) Ammonium molybdate, 40 g, was dissolved in 400 ml hot demineralized water and cooled.

2) Ammonium metavanadate, 2 g, was dissolved in 250 ml hot demineralized water and 450 ml, 70% $HClO_4$ was added.

3) Molybdate solution was gradually added to vanadate solution with stirring, and diluted to 2 L.

Standard phosphate solution

A stock solution containing 3.834 g potassium dihydrogen phosphate per liter was prepared. Twenty five milliliters of the stock solution were diluted to 250 ml (0.2 mg P_2O_5/ml).

Determination

1) Sample, 25 ml of ILC, were wet-ashed with adding 10 ml H_2SO_4 3-4 times.

2) Demineralized water, 2 ml, was added to ashed sample.

3) A few drops of ammonia solution were added and then adjusted to pH 5 with nitric acid diluted to 1:2 with demineralized water. Then the flasks were diluted to volume and mixed.

4) Twenty ml of the vanadate-molybdate reagent were added. Then the flasks were diluted to volume and mixed.

5) The samples were allowed to stand for 10 min and the absorbance was measured at 400 nm.

APPENDIX J

Carnosine determination by HPLC

Reagents

> 1) OPA (o-phthalaldehyde) reagent from Sigma Chemical Co., St. Louis, MO.
>
> 2) Solvent A: 0.06 M potassium phosphate mono basic dissolved in HPLC-grade
> water. this was used to prepare a solution of 0.4% triethylamine.
>
> 3) Solvent B: methanol-acetonitrile (60:40, v/v).
>
> 4) Reference compound: L-carnosine (ß-ala-His) from Sigma Chemical Co., St.
> Louis, MO.

Instrumentation

> Beckman Model 110A HPLC system (Somerset, NJ), equipped with the
> following components: 2070 Spectrofluorometeric detector (Varian, Walnut
> Creek, CA), Beckman 427 Integrator and 5-μm Ultrasphere ODS high-resolution
> end-capped column (250 mm x 4.6 mm) from Beckman Instruments.

Chromatography

> The mobile phase composition, 50% B in A, was adjusted pH to 6.4, filtered
> (membrane filters, pore size 0.45 μm) and degassed. The flow rate was 0.7
> ml/min. The column temperature was ambient. Detection wavelengths were set at
> 310 nm for excitation and 375 nm for emission.

Sample treatment

> ILC in duplicate was mixed with OPA reagent (1:1, v/v). Mixture was filtered
> through 0.2 μm syringe filter (Gelman Acrodisc LC 13 PVDF). Filtered mixture,
> 20 μl was injected to HPLC system.

APPENDIX K

Inosine mono phosphate determination by HPLC

Reagents

1) Reference compound: Inosine 3':5'-cyclic mono phosphate sodium salt from Sigma Chemical Co.

2) Low concentration eluent: 20 mM/L KH_2PO_4 in HPLC-grade water and adjusted pH to 3.7 with a dilute phosphoric acid solution.

3) High concentration eluent: 60/40 (v/) mixture of anhydrous methanol and water.

Instrumentation

Beckman Model 110A HPLC system (Somerset, NJ), equipped with the following components: Variable-wavelength detector, Beckman 164 Variable Wavelength Detector, Beckman 427 Integrator and 5-μm Ultrasphere ODS high-resolution end-capped column (250 mm x 4.6 mm) from Beckman Instruments.

Chromatography

A linear gradient from 0% to 25% of the high-concentration eluent by volume in 30 min was used. Detection wavelength was 254 nm. The column temperature was ambient. All eluent was filtered through membrane filters, pore size 0.45 μm (FP Verice Membrane Filter, Gelman Sciences, Ann Arbor, MI) and degassed before use.

Sample treatment

ILC in duplicate was filtered with a 0.2 μm syringe filter (Gelman Acrodisc LC 13

PVDF) and 20 μl filtered sample was loaded to HPLC system.

APPENDIX L

Analysis of Variance Tables

Table 8. Analysis of variance for ^{59}Fe absorption of iron-deficient and iron-replete rats by gavage phytate added meals in different gastric acid production conditions.

Source of Variance	df	M.S.	F	Sig. level
Iron status (I)	1	11,303.996	238.988	< 0.001
Gastric acidity (A)	1	23,312.141	492.826	< 0.001
Protein sources (F)	5	299.690	6.336	< 0.001
I x A	1	160.036	3.383	> .05
I x F	5	87.137	1.842	> .05
A x F	5	41.941	0.887	> .05
I x A x F	5	105.988	2.241	< .05
Error	94	47.303		
Total	117*			

* 2 missing observations

Table 9. Analysis of variance for ^{59}Fe absorption of iron-deficient rats by gavage with or without phytate added meals in different gastric acid production conditions.

Source of Variance	df	M.S.	F ratio	Sig. level
Phytate (P)	1	241.9	3.57	> .05
Gastric acidity (A)	1	20,428.5	301.60	> 0.001
Protein sources (F)	5	421.2	6.22	> 0.001
P x A	1	81.3	1.20	> 0.1
P x F	5	214.7	3.17	< .05
A x F	5	81.3	1.20	> 0.3
P x A x F	5	130.2	1.92	> .05
Error	81	67.7		
Total	104*			

* 15 missing observations

Table 10. Analysis of variance for 59 Fe absorption from meals containing various animal proteins with addition of dietary phytate by iron-deficient rats.

Source of variance	df	Adj. M.S.	F ratio	p value
Protein sources	5	191.95	4.23	0.007
Error	24	45.40		
Total	29			

Table 11. Analysis of variance for ^{59}Fe absorption from meals containing various animal proteins without addition of dietary phytate by iron-deficient rats.

Source of variance	df	Adj. M.S.	F ratio	p value
Protein sources	5	97.35	1.20	0.356
Error	15	81.14		
Total	20			

Table 12. Analysis of variance for ^{59}Fe absorption from meals containing various animal proteins with addition of dietary phytate by cimetidine treated iron-deficient rats.

Source of variance	df	Adj. M.S.	F ratio	p value
Protein sources	5	277.08	3.62	0.013
Error	24	76.56		
Total	29			

Table 13. Analysis of variance for ^{59}Fe absorption from meals containing various animal proteins without addition of dietary phytate by cimetidine treated iron-deficient rats.

Source of variance	df	Adj. M.S.	F ratio	p value
Protein sources	5	350.50	4.44	0.008
Error	18	78.97		
Total	23			

Table 14. Analysis of variance for ^{59}Fe absorption from meals containing various animal proteins with addition of dietary phytate by iron-replete rats.

Source of variance	df	Adj. M.S.	F ratio	p value
Protein sources	5	63.95	1.26	0.313
Error	23	50.56		
Total	28			

Table 15. Analysis of variance for ^{59}Fe absorption from meals containing various animal proteins with addition of dietary phytate by cimetidine treated iron-replete rats.

Source of variance	df	Adj. M.S.	F ratio	p value
Protein sources	5	33.16	1.38	0.268
Error	23	24.02		
Total	28			

Table 16. Analysis of variance for ^{59}Fe absorption from ferrous-ascorbate, ferric-ILC (isolated low-molecular-weight components) and ferric by *in situ* ligated loop segments of iron-deficient rats.

Source of variance	df	Adj. M.S.	F ratio	*p* value
Replication (R)	3	11.22	0.51	0.678
Iron (I)	2	852.62	38.95	0.000
R x I	6	12.37	0.57	0.754
Error	23	21.89		
Total	34			

Table 17. Analysis of variance for iron solubilizing capacity of isolated low-molecular-weight components (ILC) from animal protein sources based on wet basis weight.

| Source of variance	df	Adj. M.S.	F ratio	*p* value
Protein sources	4	6682.1	308.39	0.000
Error	74	21.7		
Total	78			

APPENDIX M

Chromatogram
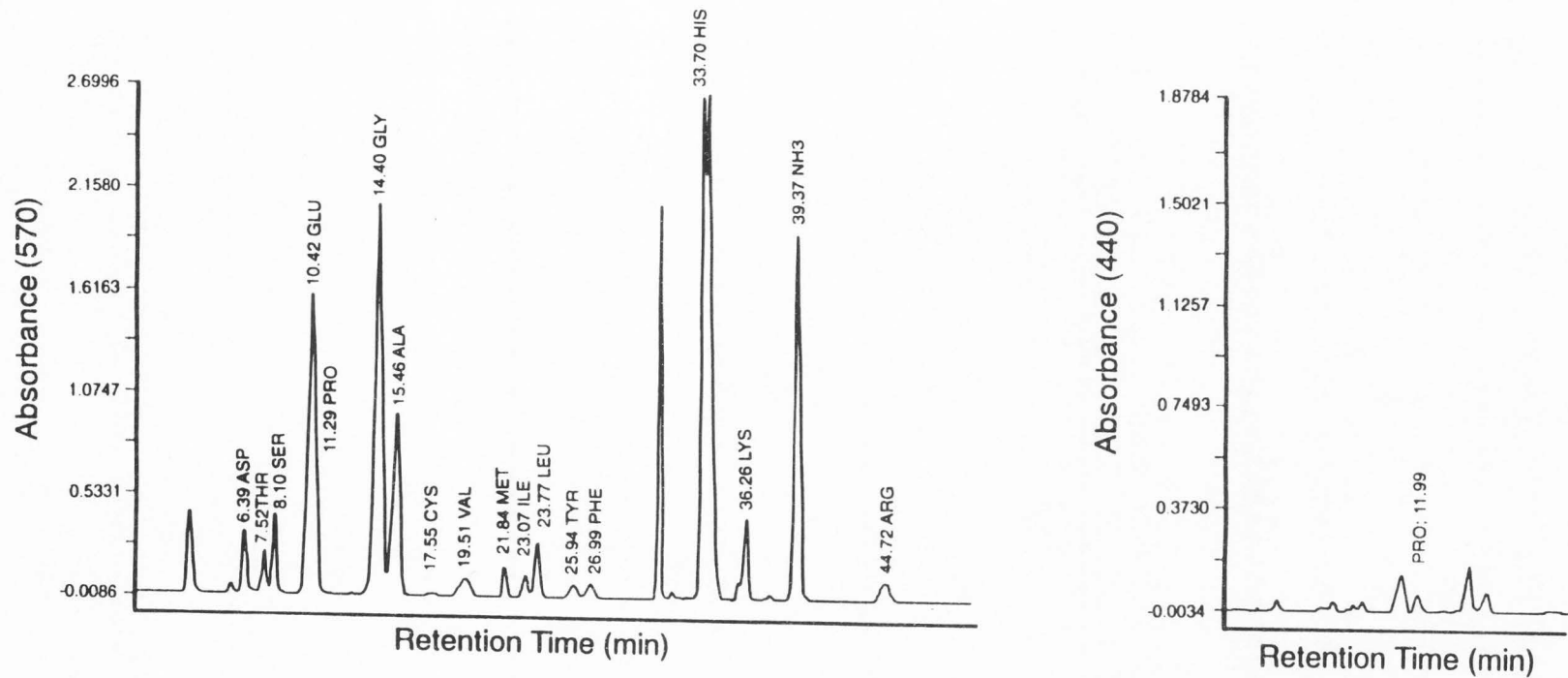
Figure 14. Chromatograms at 570 nm and 440 nm of amino acid analysis of isolated low-molecular-weight components (ILC) from undigested beef. Values on tops of peaks are retention times in minutes.
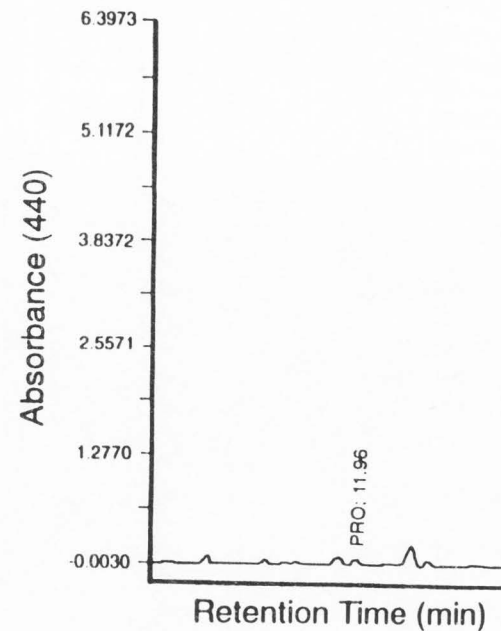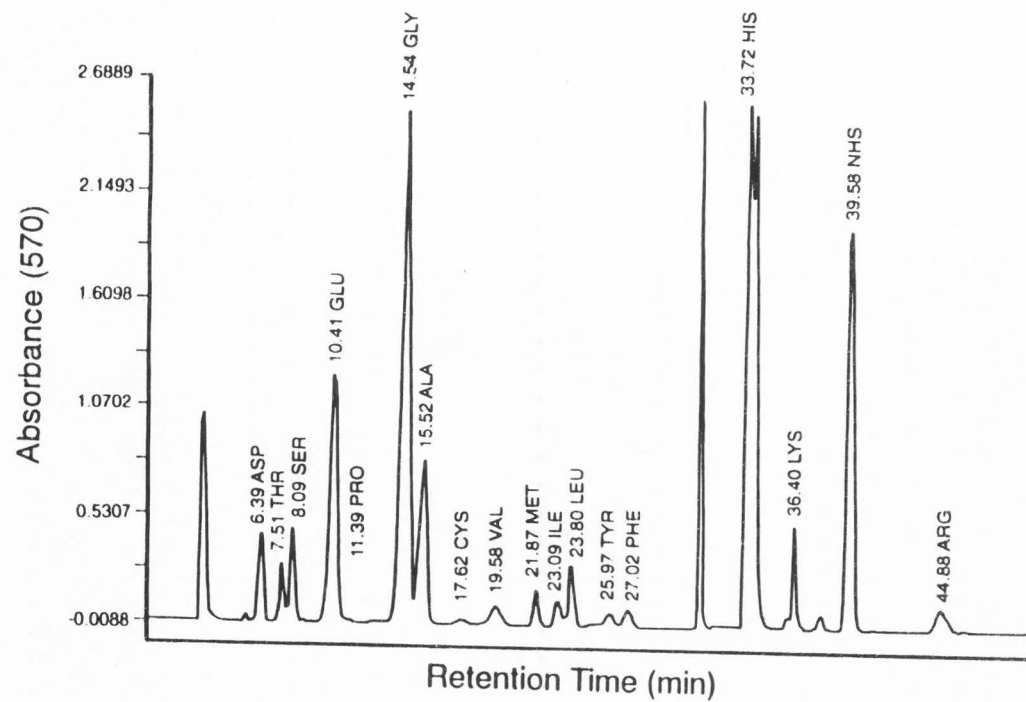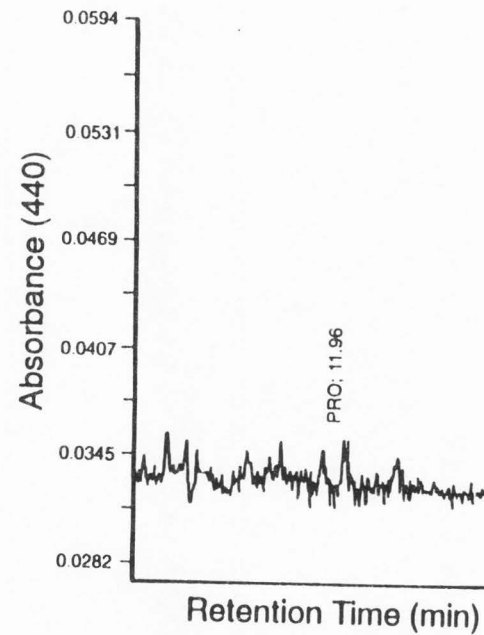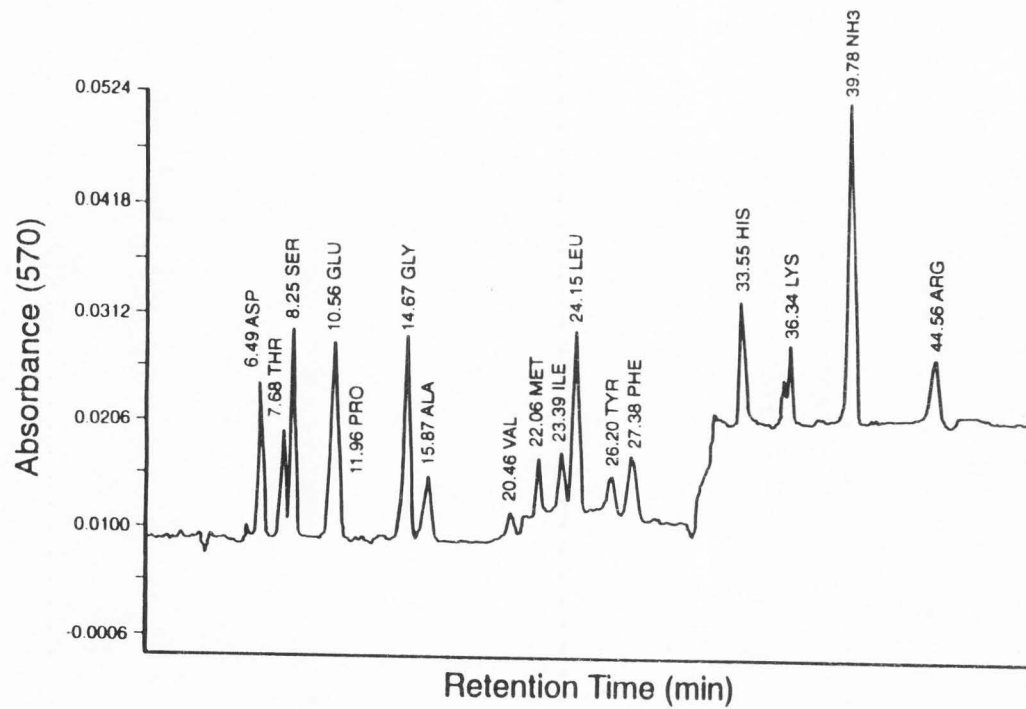
Figure 15. Chromatograms at 570 nm and 440 nm of amino acid analysis of isolated low-molecular-weight components (ILC) from undigested pork. Values on tops of peaks are retention times in minutes.
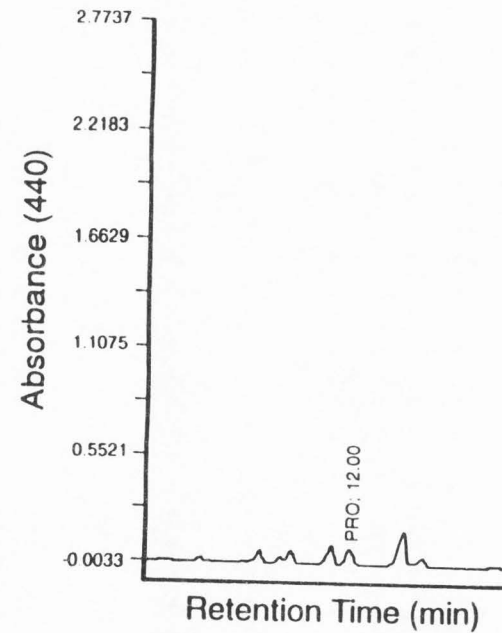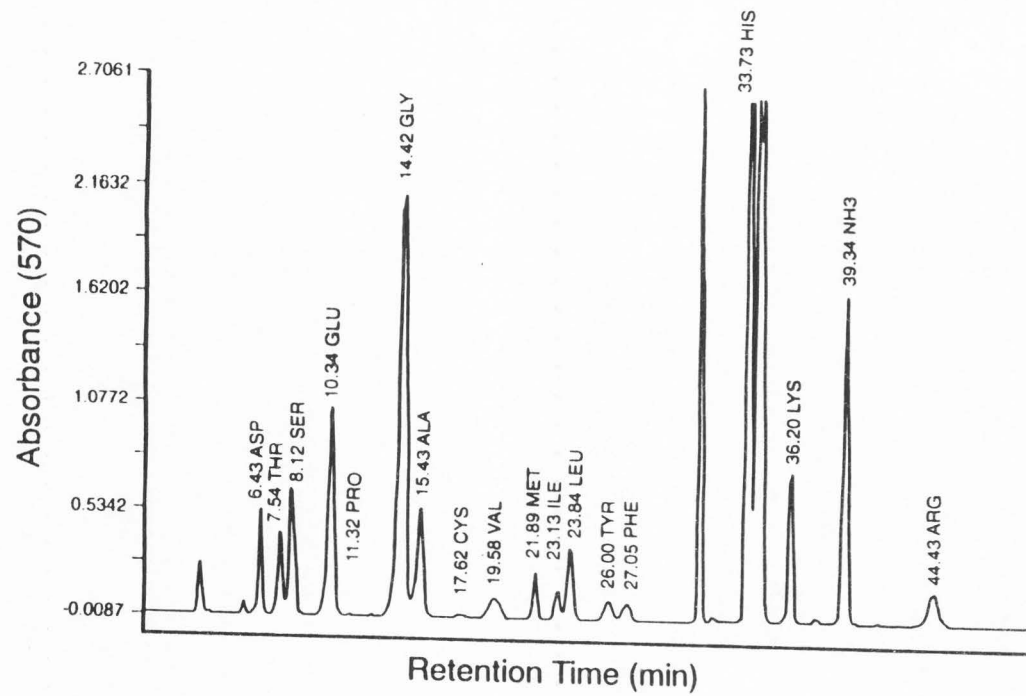
Figure 16. Chromatograms at 570 nm and 440 nm of amino acid analysis of isolated low-molecular-weight components (ILC) from undigested chicken. Values on tops of peaks are retention times in minutes.
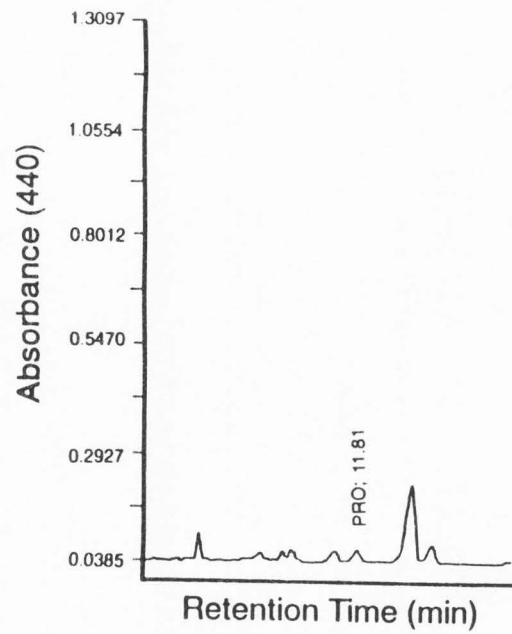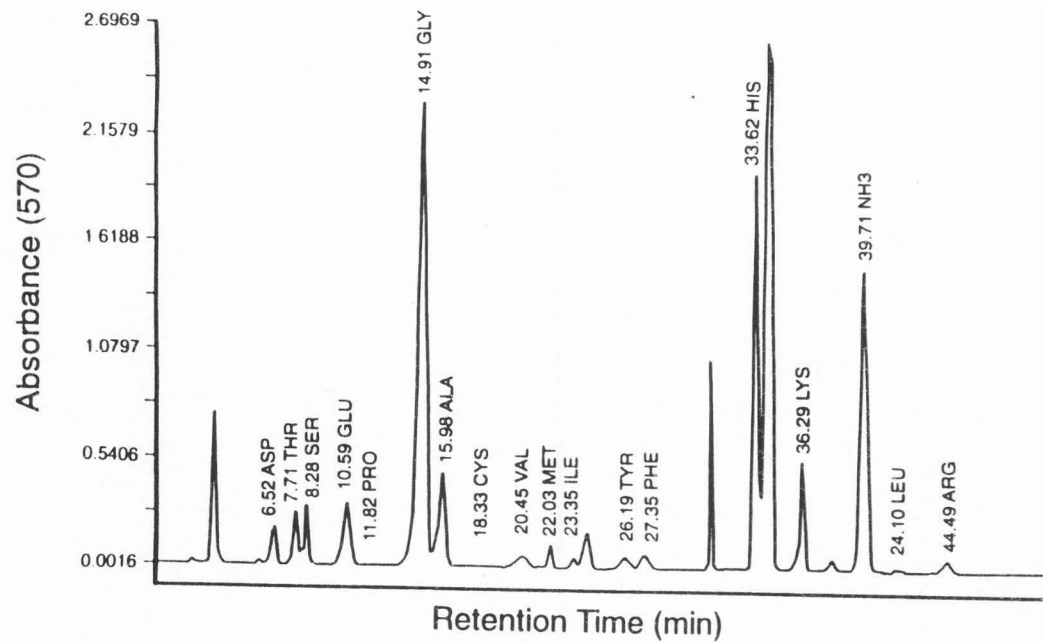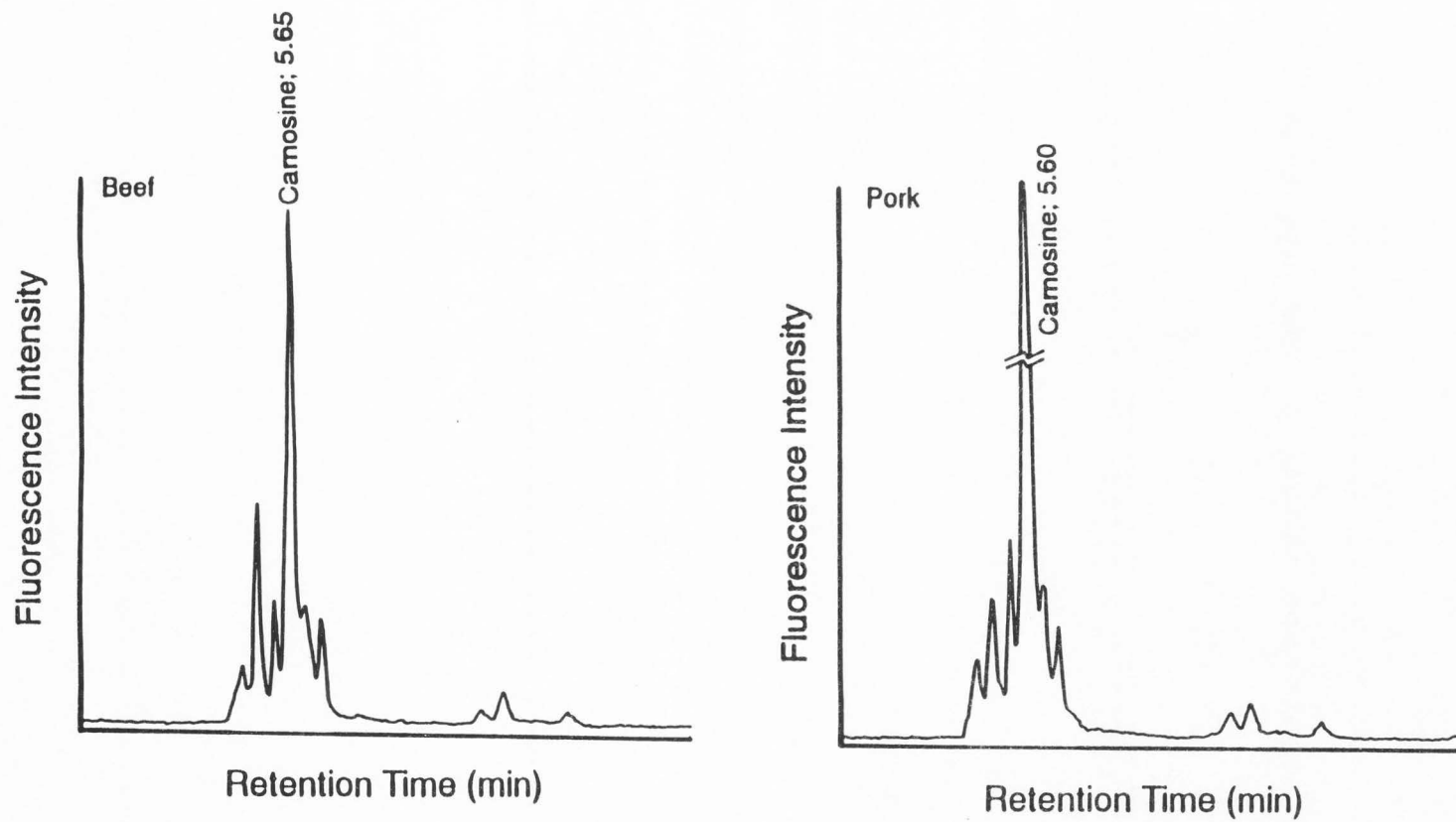
Figure 17. Chromatograms at 570 nm and 440 nm of amino acid analysis of isolated low-molecular-weight components (ILC) from undigested fish. Values on tops of peaks are retention times in minutes.

Figure 18. Chromatograms at 570 nm and 440 nm of amino acid analysis of isolated low-molecular-weight components (ILC) from undigested egg white. Values on tops of peaks are retention times in minutes.

Figure 19. HPLC chromatograms of carnosine determination on isolated low-molecular-weight components (ILC) from undigested beef and pork. Values on tops of peaks are retention times in minutes.
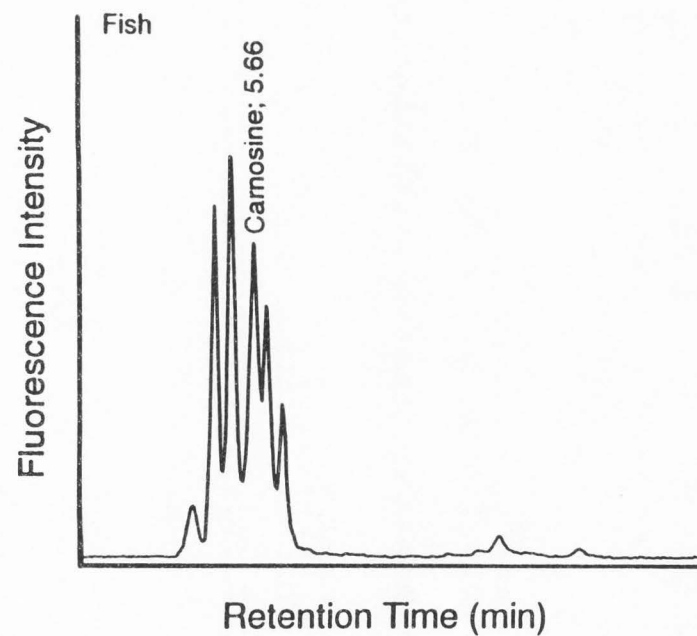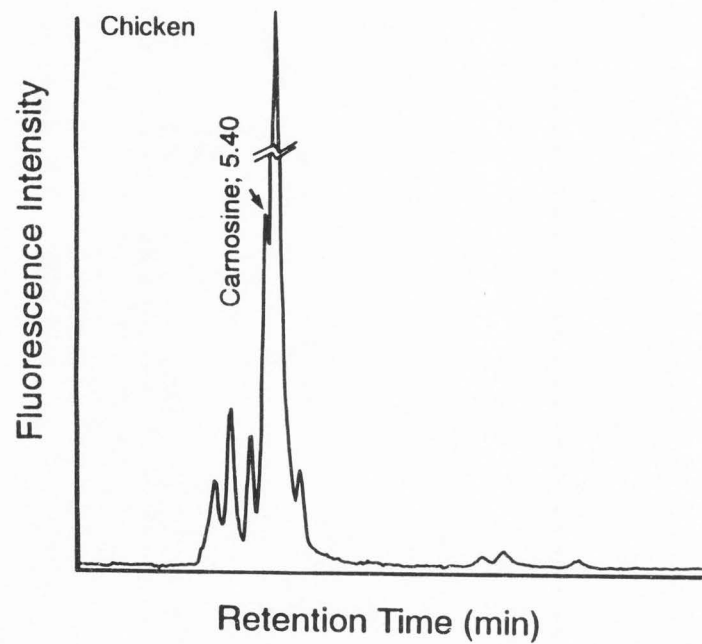
Figure 20. HPLC chromatograms of carnosine determination of isolated low-molecular-weight components (ILC) from undigested chicken and fish. Values on tops of peaks are retention times in minutes.
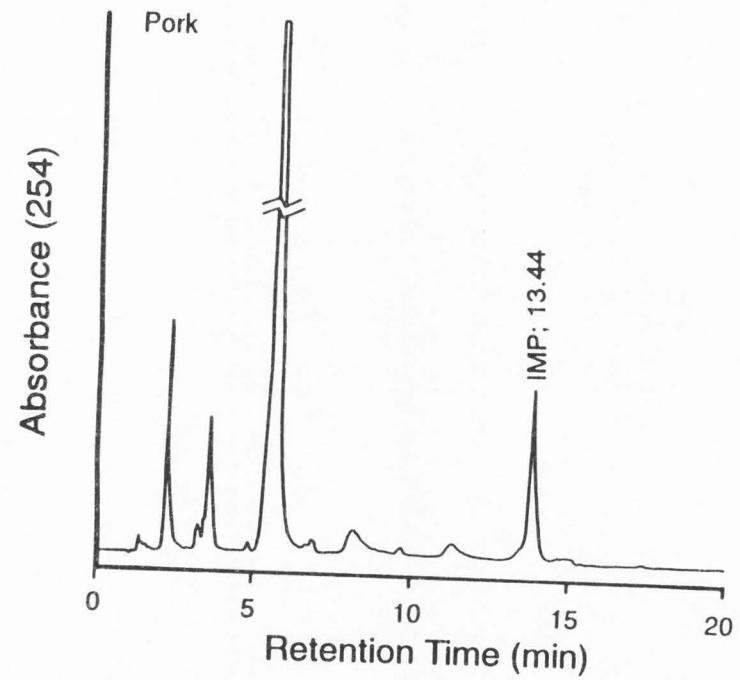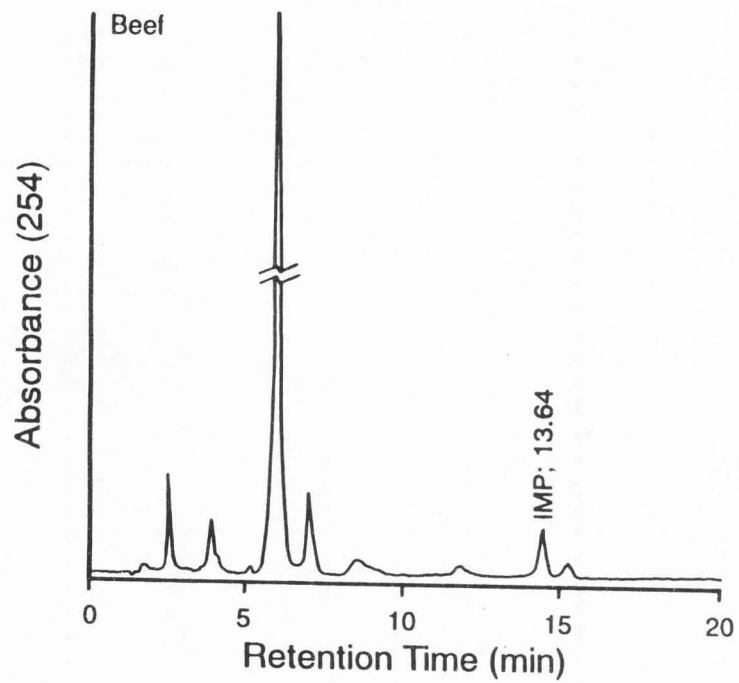
Figure 21. HPLC chromatograms of inosine monophosphate determination on isolated low-molecular-weight components (ILC) from undigested beef and pork. Values on tops of peaks are retention times in minutes.

VITA

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