AN ABSTRACT OF THE THESIS OF

Lucia Sy-Jing Lo Chao for the degree of Master of Science

in Food Science and Technology presented on July 16, 1979

Title: INFLUENCE OF FISH ON THE BIOAVAILABILITY OF PLANT IRON IN THE ANEMIC RAT.

Abstract approved: _____
Dr. Dennis T. Gordon

The composition of the diet in iron nutrition may be more important than the iron it contains. The relative biological value (RBV) of endogenous iron in wheat bran, soybean isolate and spinach were found to be 149, 71 and 57%, respectively. These values were obtained using the rat fed control diets which did not contain a fiber source and containing $FeSO_4$ (RBV=100%). Fish and fish oil substituted for casein and corn oil in diets containing wheat bran, soybean isolate and spinach, reduced, but not significantly, the RBV's to 137, 58 and 48%, respectively. A "meat factor" enhancing effect by fish on non-heme iron uptake could not be demonstrated although it was anticipated. One reason may be the rat's unacceptability as an animal model. A second explanation is the negative effect of polyunsaturated lipid on iron bioavailability. Uptake and retention of ^{59}Fe in animals, partially repleted on plant iron

with and without fish, were not influenced by dietary ingredients. Retention of radioactive iron was highly correlated with starting hemoglobin concentration at time of ^{59}Fe ingestion ($r = -0.9788$). Two important but contradictory observations from this experiment are: 1) food with low iron bioavailability may not interfere with the uptake of other dietary iron; and 2) use of an extrinsic label may not be applicable under all conditions in studying iron assimilation. Components of wheat bran, soybean isolate and spinach all appeared to enhance iron ($FeSO_4$) uptake. The RBV of iron in purified diets containing 1.74% cellulose-cellufil, 0.66% phytic acid and 2.10% oxalic acid was 138, 133 and 157%, respectively. Wheat bran and fiber components appear to be beneficial for growth and iron absorption in the rat. The exact nature of iron in foods and the interaction of dietary ingredients on iron absorption are still not clearly defined.

Influence of Fish on the Bioavailability of Plant Iron in the Anemic Rat

by

Lucia Sy-Jing Lo Chao

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1980

APPROVED:

Professor of Food Science and Technology in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented July 16, 1979

Typed by Charlene Mutschler for Lucia Sy-Jing Lo Chao.

ACKNOWLEDGEMENTS

I wish to thank:

Dr. Dennis T. Gordon for his valuable guidance during the study and in the preparation of this manuscript.

Mr. G. Louis Roberts and the staff of the Oregon State University Seafoods Laboratory in Astoria, Oregon, for their generous help and friendship.

Miss Rosemary Riggs for providing excellent facilities at the Small Animal Laboratory, Oregon State University, during the course of this investigation.

Dr. David L. Willis for the loan of Model 446 Armac Detector (in Room 332, Weniger Hall, Oregon State University) and his technical assistance in conducting the radioactive experiment.

Mr. John W. Amberg for allowing the use of the Small Animal Facility (in Room 605 and 605B, Weniger Hall, Oregon State University) during the radioactive experiment.

Oregon State University Computer Center for the grants of computing funds and for the excellent computing and support facilities they have provided.

Miss Suzi Maresh for her generous cooperation in conducting the computer programs.

National Oceanic and Atmospheric Administration (maintained by the U.S. Department of Commerce) Institutional Sea Grant 04-7-158-44085 for the partial funding of this project.

My parents, Mr. and Mrs. Char Song Lo, and my husband, Rong-Yue Chao, for their ever continuing love, encouragement and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIXES

Appendix

INFLUENCE OF FISH ON THE BIOAVAILABILITY OF PLANT IRON IN THE ANEMIC RAT

INTRODUCTION

Iron is an essential trace element in all living organisms.

There is a high incidence of iron deficiency anemia both in the United States (HEW, 1972 and 1974) and throughout the world (WHO, 1968 and 1972). It is specially high among infants (Dairy Council Digest, 1972), rapidly growing adolescents (HEW, 1976) and young women of child-bearing age (Anon., 1974). While iron deficiency may result from a number of diverse causes, the most important factor is inadequate intake or assimilation of dietary iron (Cook, 1978).

Changes in food habits and products alter dietary iron levels. Simple iron fortification of various foods has not been uniformly effective (Miller, 1977). The use of inappropriate iron salts and dietary factors such as phosphate (Peters et al., 1971), tannin (Disler et al., 1975a), phytate (Hussain et al., 1959; Turnbull et al., 1962; Hallberg and Solvell, 1967; Haghshenass et al., 1972) and fiber (Björn-Rasmussen, 1974; Reinhold et al. 1975; Ismail-Beigi et al., 1977) is believed to diminish iron absorption. Therefore, optimizing food iron intake and its bioavailability may offer the best long range approach.

Numerous investigators have demonstrated differences in availability of various food iron or supplements. Animal sources of iron (heme) are reported to have higher bioavailability than plant sources (non-heme) (Layrisse et al., 1969; Layrisse and Martinez-Torres, 1971). This is partially due to the high absorption of the porphyrin ring and its

association with animal protein (Layrisse et al., 1968; Martinez-Torres and Layrisse, 1971 and 1973; Layrisse and Martinez-Torres, 1972; Cook and Monsen, 1975 and 1976a). Among adjuvants which have been shown to increase non-heme iron absorption, ascorbic acid (Kuhn et al., 1968; Callender et al., 1970; Layrisse et al., 1974; Disler et al., 1975b) appears to be the best.

The principle of variety in nutrition appears to be sustained in iron nutriture. Meat in the diet not only provides highly available iron, but it, along with foods containing ascorbic acid, enhances the absorption of other dietary iron sources. Cereals and vegetables are examples of non-heme iron sources providing approximately 55% (9.7 mg) of a young adult daily requirement (RDA, 1974), but its availability is collectively estimated to be less than 5%.

Marine products do not occupy a large proportion of the American diet. However, recommendations have been made to increase the consumption of fish for a number of reasons (Dietary Goals for the United States, 1977). The substitution of fish in the diet will have a tendency to reduce heme iron intake. However, a protein enhancing effect may also exist when this food is consumed with diet containing a non-heme iron.

The objective of this study was to evaluate the effect of fish on the uptake of non-heme iron from three plant products: wheat bran, spinach and soybean isolate. The quantity of iron provided by these foods is high, but bioavailability is suspected to be low. Each of these plant sources contains ingredients believed to interfere with iron

utilization, i.e., fiber, oxalate and phytate. The relationship of these individual ingredients on iron uptake was also investigated. The rat was used as an experimental model.

REVIEW OF LITERATURE

Historical Perspective

Iron (Fe, its chemical symbol from the Latin ferrum) is one of the most abundant elements in the earth's crust after oxygen, silicon and aluminium.

The ancient Greeks were aware of the health-giving properties of iron. It has been a favorite health tonic and a major ingredient in many old time medicines. Iron was found to be effective for the treatment of anemia in man as early as the seventeenth century in England. Reasons for its beneficial properties became clear when it was discovered to be a component of the body and later, the blood (McCollum, 1957). Subsequently it was shown to be a part of the respiratory-pigment hemoglobin. Experimental evidence of the essential nature of iron in nutrition was first provided by the French chemist, Boussingault, in 1867 (McCollum, 1957).

Iron Deficiency

Nutritional anemia is recognized as the most widespread deficiency state in man. Whereas inadequate amounts of vitamin B_{12} or folate may also contribute to anemia, the major cause is a lack of iron (WHO, 1968 and 1972; HEW, 1972 and 1974). The shortage of this essential mineral might be the result of blood loss, parasitic infestation, or inadequate absorption due to functional disorders.

However, insufficient iron assimilation is the most prevalent cause.

Negative iron balance is manifested by a specific sequence (NRC, 1979). In the initial stage, iron stores become depleted and absorption increases. As long as there is enough storage iron to supply bone morrow requirements, the transferrin iron binding capacity and plasma iron levels remain normal. Although there is impairment of erythropoiess due to the lack of iron, it may not result in recognizable anemia in the second stage. An overt anemia occurrs only in the third stage of iron deficiency and is characterized by hemoglobin deficient erythrocytes, microcytic hypochromic anemia.

Iron nutriture may be evaluated by a variety of laboratory parameters. Newer measurements such as transferrin saturation, red blood cell protoporphyrin concentrations, bone marrow hemosiderin values and serum ferritin levels are being used as indicators of iron stores (Finch, et al., 1974). The initial measurement in indicating severity of the iron deficiency state is the circulating hemoglobin level. However, it falls short in identifying marginal or potential states of anemia in which the hemoglobin level may not have fallen. It does not differentiate between anemia caused by the lack of folate, vitamin B_{12}, or iron. Measurement of transferrin saturation or determination of red blood cell protoporphyrin identifies iron-deficient erythropoiesis at twice the frequency compared to hemoglobin levels alone (WHO, 1972). Their chief limitation is their inability to distinguish between iron deficiency and an internal block in iron transport due to infection. Chemical determination of non-heme iron, either from bone marrow or liver biopsies, provides good precise quantitative information, but such drastic methods have little practical application. A

new method, believed to overcome many previous problems, is measurement of serum ferritin levels which appear to best reflect differences in iron stores (Cook, et al., 1974).

Iron Chemistry

Some of the chemical properties of iron are presented to aid the interpretation of the behavior of iron in biological systems.

Iron is a transition metal which has several oxidation states ranging from -2 to 6. The ferrous (2+) and ferric (3+) forms are the only states which are stable in an aqueous environment. The rare exception is the possibility of having either Fe^{4+} or Fe^{5+} in peroxidase (Spiro and Saltman, 1974).

Ferrous iron may either be oxidized to its ferric form or reduced to metallic iron. In acid solutions, ferrous and ferric ions are hydrated as $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$, respectively. With increasing pH, the water molecules will release protons to form hydroxy-iron species. At neutral pH, ferrous and ferric ions will precipitate as $Fe(OH)_2$ and $Fe(OH)_3$ with corresponding solubilities of 10^{-1} and 10^{-18} M (Spiro and Saltman, 1974). This fact implies that ferric iron is much less soluble than ferrous. As with most hydroxide precipitates, the solubility equilibrium is not rapidly established and this may be of nutritional significance. In the stomach, the acid environment provides for a soluble and hydrated form of iron. This form may become an insoluble deposit upon passing into the alkaline medium of the small intestine. However, the slow development of precipitates and existence of some soluble hydroxides make absorption possible. The

different ferrous and ferric iron concentrations present in aqueous solutions at various pH's have been reported by Forth and Rummel (1973).

The precipitation of iron at an alkaline pH can be prevented by the reduction of iron to the more soluble ferrous state or by the formation of complexes. Iron forms complexes with a number of chelating agents. An octahedral configuration is preferred, but other geometries with 4, 5 and 7 metal-ligand bonds also exist (Spiro and Saltman, 1974). Common metabolic intermediates including amino acids, sugars, organic acids and nucleotides are effective ligands with ferric ions. More complex ligands, such as molecules containing porphin and sulphur, are capable of complexing both ferrous and ferric ions (Spiro and Saltman, 1974). Although these complexes may render iron available for absorption, some complexes, such as those formed by the chelating agents ethylenediaminetetraacetic acid (EDTA) and phosphates, may be so stable they exclude the iron from the acceptor sites in the intestinal mucosa. The stability of various iron complexes formed in a heterogeneous food system is in part responsible for conflicting data on the iron bioavailability from foods.

Iron Metabolism

An understanding of iron metabolism is essential to bioavailability studies. This subject has been reviewed by a number of investigators (Bothwell and Finch, 1962; Turnbull, 1974; Underwood, 1977; NRC, 1979).

Total Body Iron and Functional Distribution

The total iron content of the animal body varies between 25 and 75 mg/kg body weight. This level depends upon age, sex, nutrition, state of health and species. The normal adult man has approximately 49 mg Fe/kg body weight while the adult woman has 38 mg Fe/kg. Variations in iron content within and between species are due to circulating hemoglobin mass and differences in iron stores. Iron reserves of approximately 1000 mg in men are gained between the ages of 15 and 30. Women maintain lower stores of 300 mg through their adult life until menopause.

Several iron-containing compounds in the body perform important metabolic functions. Blood hemoglobin constitutes the largest fraction of body iron and functions as an oxygen carrier for all organs and tissues. The ability to transport gases is accounted for by the presence of iron in the heme structure. In the normal human, hemoglobin concentration depends on ambient oxygen tension, hemoglobin affinity for oxygen and circulating testosterone levels (NRC, 1979).

Some heme iron compounds other than hemoglobin are: myoglobin which functions as an oxygen carrier and reservoir for muscle; cytochromes that aid in electron transport; and the enzymes, catalase and peroxidase, which cleave peroxides.

Metalloflavo proteins, in which the iron is not in the form of heme, are involved in oxidative metabolism. These metalloflavo proteins include reduced nicotinamide adenine dinucleotide, succinate and α-glycerophosphate dehydrogenase. Some iron-containing compounds require iron

only as a co-factor. An example is aconitase, an enzyme of the tricarboxylic acid cycle. It catalyzes the interconversion of citric, isocitric and aconitic acid. Other research has shown that iron, in a loosely bound form, is required for the hydroxylation of proline and lysine in protocollagen, an essential step in synthesis of collagen (NRC, 1979). Transferrin, another non-heme iron compound, has a major function in iron transport and in the body's defense mechanisms against infection (Underwood, 1977).

Excess iron within the body is stored in the form of ferritin and hemosiderin. Ferritin, which is found mainly in liver, holds iron in a readily available form for use as needed. Hemosiderin is extremely insoluble and is considered to be masses of aggregated ferritin.

Iron Balance

It is estimated that the major daily loss of iron occurs via the gastrointestinal tract (blood, discarded epithelial mucosa cells and bile). This amounts to about 8.5 µg/kg body weight. Excretion of iron from the skin and urine is estimated to be 3.5 µg/kg. Menstral blood loss in adult females average 20 µg Fe/kg (NRC, 1979). To balance this physiological loss, a constant influx of iron from the diet is required. In man, only 5% of the average daily dietary iron intake of 18 mg must be absorbed to have iron balance. Woman, ingesting 10 mg of iron daily because of low caloric intake and other considerations, requires 15% absorption (Cook, 1978).

It is apparent that there is a limited capacity of dietary iron absorption to meet iron balance. Again, this fact is especially true in target groups such as women during the period of pregnancy, child delivery and lactation. A second target group is children during periods of active growth.

Other animals present few problems in maintaining iron balance because of their higher intake and more efficient absorption. However, swine appear to be an exception due to their very rapid growth and low iron stores at birth (Underwood, 1977).

Iron Absorption and Its Regulation

Dietary iron absorption is influenced by several parameters. These include iron stores, levels of ingested iron, and the action of the stomach and intestine thereon. Studies on iron absorption do not agree. This discordancy may be due to attempts to correlate results between animal models and man (Moore et al., 1944). Thus, results obtained from one species cannot be generalized to other species.

Body iron stores appear to be the most important influence on the absorptive capacity of the intestinal mucosa (Pirzio-Biroli and Finch, 1960; Cook et al., 1969). Low iron stores augment absorption while excess iron stores depress absorption (Bothwell and Finch, 1962; Kuhn et al., 1968; Cook et al., 1969).

The amount of iron absorbed increases as higher levels are presented to the intestinal mucosa. This relationship is log-linear at all dose levels in men (Bothwell and Finch, 1962), but it is linear

only above a threshold dose in rats (Wheby et al., 1964).

Various luminal factors have been demonstrated in humans and laboratory animals to affect iron absorption. Reduction or elimination of gastric juice decreases absorption (Cook et al., 1964). The administration of hydrochloric acid to human subjects with achlorhydria increases absorption (Cook et al., 1964). Similiar enhancement has been observed in rats given hydrochloric acid (Whitehead and Bannerman, 1965). It is probable that the primary role of gastric juice is to provide an acid environment which insures solubility. Under physiological conditions, it is likely that other substances in the gastric juice might facilitate or inhibit absorption. At the present time, these unidentified substances are thought to be mucoproteins secreted within the stomach (Morgan et al., 1969). Their role in iron binding is not yet clear. Hübers et al. (1971) found that iron in the brush border of rats was doubled in iron-deficient animals compared with iron-adequate. This observation suggests that the brush border might be one point of control. In addition, several iron-binding proteins have been isolated from intestinal mucosa (Pearson and Reich, 1969; Worwood et al., 1971; Pollack and Lasky, 1976). However, the nature of these proteins and their relationship to the transport mechanism for ironare not yet clear. From isolated intestinal loops, iron absorption is diminished by cycloheximide which depress protein synthesis (Greenberger, 1973), and increased by phenobarbitone which stimulates intestinal protein synthesis (Thomas et al., 1972). This evidence shows that protein synthesis may also influence intestinal absorption of iron. There is an inverse

relationship between amounts of iron absorbed and amounts of iron held as ferritin within the mucosal cell. The binding of iron by ferritin in intestinal mucosal cell is believed only to hold iron until absorbed and not to be involved in regulating absorption.

Other factors which have been documented to affect iron absorption include the rate of erythropoiesis, hypoxia, saturation of transferrin, hepatic diseases and the motility of the gastrointestinal tract (Turnbull, 1974; NRC, 1979).

Factors Affecting Iron Availability

The uptake of dietary iron is limited. Inorganic salts of iron are generally more assimilated than iron from non-heme foods (Fritz et al., 1970). Ferrous salts are believed to be better absorbed than ferric (Moore et al., 1944; Brise and Hallberg, 1962a; Waddell, 1974). However, there is no complete agreement on this issue(Fritz et al.,1970). The bioavailability of iron salts has been found to be highly correlated with their solubility (Pla et al., 1976) and inversely correlated with particle size or reduction state (Shah and Belonje, 1973a; Motzok et al., 1975).

Iron from animal sources (heme) is more efficiently absorbed than plant iron (non-heme) (Layrisse et al., 1969; Layrisse and Martinez-Torres, 1971). Absorption of non-heme iron is improved in diets containing animal foods (Layrisse et al., 1968; Martinez-Torres and Layrisse, 1971 and 1973; Layrisse and Martinez-Torres, 1972; Cook and Monsen, 1975 and 1976a). The addition of ascorbic acid either as

a chemically synthesized compound (Kuhn et al., 1968; Disler et al., 1975b) or as a substance naturally occurring in fruits and their juices (Callender et al., 1970; Layrisse et al., 1974) promotes non-heme iron absorption.

Ascorbic acid has been shown to improve the absorption of ferrous and ferric salts (Brise and Hallberg, 1962b), non-heme iron (Layrisse et al., 1974) and iron in eggs (Callender et al., 1970) containing natural inhibitors. This acid has also been shown to be beneficial for iron utilization in the presence of phosphates (Forth and Rummel, 1973). Other organic acids such as citric, tartaric, aspartic and glutamic increase iron absorption (Groen et al., 1947). Several amino acids are also effective in enhancing iron uptake, i.e., histidine, cysteine, lysine and methionine (Martinez-Torres and Layrisse, 1970; Van Campen, 1973; Miller, 1974). Amine and Hegsted (1975) found the retention of iron to be increased by various carbohydrates in the following order: lactose>sucrose>starch. They also indicated highly saturated fat (coconut oil) aided iron absorption. Other compounds reported to increase iron assimilation include: fructose (Pollycove et al., 1972), sorbitol (Loria et al., 1962) and alcohol (Millar, 1971).

Poor absorption of non-heme iron is believed to be due to the presence of natural inhibitors, i.e., phytate, phosphate, oxalate and carbonate, which yield insoluble precipitates with iron (HEW, 1974). Recent reports have stated that fiber has an adverse effect on iron utilization in man (Reinhold et al., 1975) and rat (Stiles et al., 1976). High intakes of calcium, zinc, cadmium, copper and manganese may obstruct iron absorption through competition for binding sites

(Underwood, 1977). Substances in tea (Disler et al., 1975a), corn (Layrisse et al., 1973; Martinez-Torres and Layrisse, 1973), eggs (Callender et al., 1970) and milk (Carmichael et al., 1975) also reduce absorption. Some chelating agents that may interfere with iron absorption include EDTA (Forth and Rummel, 1973; Cook and Monsen, 1976b), desferroxamine (Kuhn et al., 1968; Martinez-Torres and Layrisse, 1971; Martinez-Torres et al., 1974) and cholestyramine (Greenberger, 1973). Several varieties of antiacids and clay have also been documented to impair iron absorption (Turnbull, 1974; NRC, 1979).

A final theory concerning bioavailability of iron is the pool concept. Dietary iron can be classified as either heme or non-heme. All sources of iron other than those contained in a porphyrin structure are called non-heme (plants and salts). The absorption and initial intestinal metabolism of iron from these two pools are believed to be independent. Unlike non-heme iron, heme iron is absorbed intact, at a much higher efficiency into the mucosal cells, and is not changed by other dietary factors (Bjorn-Rasmussen et al., 1972; Layrisse and Martinez-Torres, 1972).

Iron Transport and Internal Exchange

All absorbed iron is converted into the ferric state in intestinal mucosal cells. Ferric iron is either attached to ferritin or transported into the circulating system and bound to transferrin

(Cook and Finch, 1975). This blood protein is a β-globulin with two binding sites,each capable of restraining one atom of iron. It shuttles iron back and forth between body tissue without being metabolized itself. A diversification of transport capabilities exists. Iron resulting from normal hemoglobin decay is chelated with transferrin. This carrier protein delivers iron to bone marrow for erythropoeisis or to the reticuloendothelial and hepatic system for storage. Other systems receiving iron via transferrin are the placenta during fetal development, myoglobin and cellular iron containing enzymes.

Transferrin concentration is described in terms of Total Iron Binding Capacity (TIBC). This value represents a full complement of adsorbed iron, or transferrin that is totally complexed with iron. Physiologically, only about one third of transferrin is saturated with iron. The amount of iron normally bound to transferrin is synonymous with serum iron values. The remainder represents a latent or unbound reservoir (Bothwell and Finch, 1962), commonly referred to as the Unsaturated Iron Binding Capacity (UIBC). Serum iron + UIBC = TIBC. The TIBC in normal adults ranges from 300-400 μg Fe/100 ml serum (Ramsay, 1957). Elevation in UIBC is found in iron deficiency and accelerated erythropoiesis. In these cases, the number of transferrin binding sites increases. Reductions occur in infection, protein malnutrition, iron overload and protein loss such as in nephrosis. Serum iron in the normal individual may change widely during the day, but it is not influenced by season, exercise or normal meals (Moore, 1973).

Although the amount of circulating plasma iron is only 3 to 4 mg, the Plasma Iron Turnover Rate (PITR) is rapid. Normal values for PITR range from 25 to 40 mg/day. About 70-90 percent of this iron is incorporated into red blood cells for hemoglobin synthesis (Moore, 1973). The senescent or defective erythrocyte is catabolized within the reticuloendothelial cell and its iron is either returned to plasma transferrin or deposited within the cell as ferritin. When iron uptake by the erythron decreases, the hepatocytes accept and store iron in the form of ferritin. Another storage form is hemosiderin which, unlike ferritin, can be identified by light microscopy. The ratio between ferritin and hemosiderin differs according to the total iron stores within the cell. At lower concentrations of tissue iron, the former compound predominates. At higher concentrations, most of the iron is found in later form (NRC, 1979). The only other known pathway of iron tranport in humans is the exchange of plasma iron with the extravascular transferrin pool (Moore, 1973).

An important characteristic of iron metabolism is its conservation and reutilization of more than 90 percent of total body iron. Therefore, daily requirements are dictated by replacing the small but constant amount lost. This physiological iron loss averages to about 0.9 mg of iron per day in man and 1.5 mg per day in woman (Cook, 1978). Iron is unique in that once absorbed, there is no normal route of excretion.

Food Sources of Iron

Iron is widely distributed in foods. Daily per capita food iron supply from the four food groups is listed in Table 1. Although

Table 1. Daily per capita food iron supply[1].

Food Group	mg iron/capita/day	Percent
Meat, poultry, fish	5.6	32
Flour, cereal products	5.3	30
Vegetables, fruits, legumes, nuts	4.4	25
Dairy products, eggs	2.3	13
TOTAL	17.6	100

[1]Value based on 1970 U.S. food consumption figures and 1980 food consumption estimates.

non-heme iron of plant origin provides 55% of daily food iron, its availability is low in comparison with heme iron of animal origin. Bioavailability of diverses food iron sources in man and rat is shown in Table 2.

The problem of unsatisfactory iron nutrition is the sum of many factors. Plant iron concentrations are altered greatly by changes in environmental conditions (Speirs et al., 1944). Different forms of iron may exist in the same plant. Morris and Ellis (1976) found that iron in wheat bran consists of monoferric phytate and an uncharacterized iron form extractable in a butanol-water-salt solvent system. The relative amounts of distinctive iron forms in the same plant may vary with changes in growing conditions (Price, 1968). The biological availability of iron from soybeans has been shown to vary with degree of plant maturity (Welch and Van Campen, 1975). Iron was less available in immature soybeans than in mature soybeans. The consumption of highly refined products and the technique of varied food preparation have also lead to unsatisfactory iron nutrition. Approximately 76 percent

Table 2. Bioavailability[1] of food iron in man and rat.

Source	Iron level mg/100 g	Man ----% Bioavailability----	Rat
Animal - heme			
Meat	3.0	22	30
Liver	6.5	11-22	70
Fish	0.3	10	7
Egg	2.3	1.5-3	30-40
Vegetable - non-heme			
Rice	2.0	1	25
Spinach	3.1	1	20-25
Maize	1.0	1-3	30-60
Soybean	2.7	7	30-60
Wheat	2.9	4-5	15-20
Other - non-heme			
Ferrous sulfate		2.5-8.5	50
Ferric orthophosphate		0.7-3.6	5-25
Sodium iron pyrophosphate		1.0	10-20
Reduced iron		4.2-9.1	15-30

[1]Values reported are mean levels obtained from numerous scientific reports.

of the iron in whole grain wheat is lost during the milling process (Czerniejewski et al ., 1964). Much of this loss is restored but with questionable bioavailability.

Cooking of vegetables in different quantities of water and for different lengths of time may result in iron losses in excess of 20 percent (Sheets et al., 1931). The amount of incidental food iron is also reduced because of modern food handling, processing, storage and cooking procedures. Changes in food habits, lower caloric intake as the consequence of decreased physical activity, and the use of food additives such as phosphate are factors contributing to lower net dietary iron available to the body. The solution to the problem of low dietary iron has often been stated in terms of increased fortification. The pros and cons of this practice continue to be debated (Nutr. Today, 1972).

Enrichment and Fortification Programs

During World War II, cereal enrichment was initiated to restore several B-vitamins and iron to original levels. Cereal-grain products were chosen because they provided a significant amount of the dietary caloric intake in the U.S. The Food and Nutrition Board of the National Academy of Sciences sets fortification levels of iron at 8.81 mg/100 g for cereal-grain products (NAS, 1974). The enrichment program helped to reduce vitamin-deficiency diseases, but had little effect on iron-deficiency anemia (Miller, 1977). One reason of this uneffectiveness was due to the fact that the original iron level in whole grains was low.

A typical American diet furnishes about 6 mg of iron per 1000 kcal (HEW, 1974). At this level, certain segments of the population, young women and children, are not receiving their recommended dietary allowance (RDA, 1974). A proposal to increase the iron level in enriched flour and bread was withdrawn by the Food and Drug Administration since such an increase was "not proven to be safe, needed or effective" (Anon., 1977). Data from the Economic Research Service of the U.S. Department of Agriculture have shown that flour consumption decreased about 20 percent from 1950 to 1972 (Burke, 1974). This trend has further reduced the average intake of dietary iron.

Another problem preventing the effectiveness of the fortification program was the poor bioavailability of some sources used as iron supplements. Among iron sources permitted by regulation, compounds most often used are reduced iron, ferrous sulfate, ferric orthophosphate and sodium iron pyrophosphate (Waddell, 1973).

As consumption of cereal products declined, efforts have been made in looking to other foods as vehicles for fortification. Such items as common salt, sugar, beverages, infant foods and even ethnic foods, i.e., fish sauce, which are consumed in sufficient quantitities by a significant portion of the population have been considered for fortification (INACG, 1977). No single iron source is suitable for all products. In selecting a food for fortification, the factors of bioavailability, technical feasibility, food quality, consumer's acceptability and cost must all be considered.

Bioavailability Studies

Bioavailability has been defined as the ratio between the quantity of iron in a sample versus the amount determined by biological assay to

be utilized in the body. Extensive studies have been conducted on the bioavailability of various iron sources in animals and humans. Hemoglobin repletion and uptake of radioactive iron are the two most widely used methods. The preferred method would be long term balance studies.

The hemoglobin repletion test proposed by Fritz (Pla and Fritz, 1970 and 1971; Fritz and Pla, 1972) has been accepted as a standard method of determining inorganic and non-heme iron bioavailability by the Association of Official Analytical Chemists (AOAC). The method has been continually re-evaluated and improved. This method is based on hemoglobin repletion by the anemic rat fed the test iron source diet. Reagent grade ferrous sulfate is used as the reference standard, and results are reported in terms of relative biological value (RBV). This value is defined as the ratio, $\frac{\text{equivalent ppm of iron } (FeSO_4)}{\text{ppm iron of test source}} \times 100$, which gives the same hematological response. Thus, any sample containing iron which is less available than ferrous sulfate will have an RBV less than 100. Conversely, a sample which is a better source of iron than ferrous sulfate will have an RBV greater than 100. Bioavailability of iron from ferrous sulfate in rats is approximately 50%. Thus, RBV is only intended for comparative purposes.

The use of radioisotopes of iron was first described by Hahn and co-workers (1943). It was developed as a highly accurate technique for measuring iron absorption from single or mixed foodstuffs. By this method, iron absorption is determined in man or animal after ingesting a trace amount of ^{59}Fe or ^{55}Fe, added intrinsically or extrinsically with dietary ingredients. The absorbed radioiron is measured using a whole-body counting apparatus. This technique offers the advantage of measuring iron uptake in non-anemic subjects.

Plant Components Suspected to Interfer with Iron Bioavailability

Phytate, fiber and oxalate have been postulated to affect mineral utilization. These substances are found in the three plant products, wheat bran, spinach and soybean isolate, selected for iron bioavailability studies.

Phytate

Phytic acid was first described by Pfeffer in 1872. However, the exact structure (Figure 1) was only established recently (Johnson and Tate, 1969). The proper chemical designation of phytic acid is

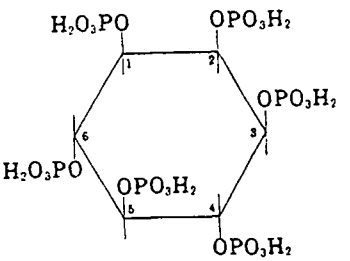

Figure 1. Structure and configuration of phytic acid.

myo-inositol 1, 2, 3, 4, 5, 6-hexakis (dihydrogen phosphate) (IUPAC-IUB, 1968). It occurs primarily in plant tissues such as seeds and whole grains (de Boland et al., 1975).

Since phytic acid has a large number of phosphoric radicals in its structure , it can form simple salts (with one metal) or mixed salts (with several metals in the same molecule), as well as metallic or protein complexes (Gontzea and Sutzescu, 1968a). While the compounds with alkali metals (sodium, potassium) are soluble in water, those

resulting from calcium, iron, magnesium, copper, zinc and lead are practically insoluble. The mixed salt with calcium and sodium is known as phytin. At pH 7.4,phytate forms complexes with metals in the following order: $Cu^{++} > Zn^{++} > Co^{++} > Mn^{++} > Fe^{++} > Ca^{++}$ (Vohra et al., 1965). The phytate-protein complexes can be formed on either side of the isoelectric point of the protein (Cosgrove, 1966; Saio et al., 1967). The size and tertiary structure of the protein affect the extent of phytic acid complexing. Hill and Tyler (1954) demonstrated reduction in the solubility of casein with the addition of phytate, but found that no complex was formed with hydrolyzed casein.

Phytic acid and phytates can be decomposed by prolonged heat treatment such as in bread making, acid hydrolysis or enzymatic action by a phosphatase called phytase (Gontzea and Stuzescu, 1968a). Phytase is found in foodstuffs containing phytic acid. Its activity varies widely from one plant species to another even though phytic acid contents are similar. Wheat and rye are rich in phytase, barley has an intermediate level, and oats and maize show a very low phytase activity.

Soybean, which generally contains the highest quantity of phytic acid, has the poorest amount of phytase. Within the same species, phytase activity can vary during maturity (Welch and Van Campen, 1975) and be unevenly distributed in the different parts of the grain (Gontzea and Stuzescu, 1968a).

The most important antinutritional properties of phytate in foods is its ability to combine with metal ions. Zinc deficiencies in chicks and rats (Lease, 1966; Oberleas et al., 1966), magnesium

deficiencies in man (Seelig, 1964) and calcium deprivation in rats, pigs and men (Nicolaysen and Njaa, 1951) have been induced or aggravated by high levels of dietary phytates. Decreased iron availability has also received much attention. Many investigations have shown that sodium phytate decreases iron absorption in man (Hussain et al., 1959; Turnbull et al., 1962; Hallberg and Solvell, 1967). In rats, Fuhr and Steenbock (1943) demonstrated slightly less hemoglobin was produced from ferric chloride when the optimal amount of phosphorus was supplied as phytic acid rather than a mixture of mono- and di-potassium phosphate. They also showed that ferric phytate regenerated 19% less hemoglobin than an equal amount of iron from ferric ammonium sulfate. Davies and Nightingale (1975) observed significantly reduced whole body retention of iron in rats fed sodium phytate.

Ruminants appear able to utilize phytic acid, due to the phytase activity of their microflora in the rumin (Cosgrove, 1966).

Fiber

The role of fiber in nutrition and health has received much attention in recent years. However, there is still no agreement concerning the definition of fiber. Crude fiber (CF), reported in many food tables, has been defined as the residue of plant food after sequential extraction with solvent, dilute acid and dilute alkaline. Van Soest (1973a) has drawn attention to this unappropriate term and its methods of analysis. Dietary fiber (DF) was defined as the remanents of the plant cell walls resistant to the alimentary enzymes

of man (Trowell, 1974). The polysaccharides in dietary fiber are celluloses, hemicelluloses, pectins, gums and mucilages. Some components are not polysaccharides. In this latter group are lignins, waxes, sterols, and products of non-enzymatic browning, plus proteins, fats, and carbohydrates not digested because of their intimate association with the plant cell walls.

The main components of the dietary fiber in which nutritional and health scientists are interested are cellulose, hemicellulose, lignin and pectin. Methods for the determination of different kinds of fiber in food have not been unified. The fiber analyses developed by Van Soest (Van Soest, 1965and 1973a; Van Soest and Wine, 1967 and 1968) were used in this study. These methods provide for the determination of neutral detergent fiber which includes cellulose, hemicellulose and lignin, and acid detergent fiber which gives cellulose and lignin.

Cellulose, the only true fibrous component of the plant cell walls, is a polymer of linear chain of 1→4' linked β-D-glucopyranose residues. It is not attacked by any enzyme of the digestive juices. Some 15% of dietary cellulose has been assessed to undergo bacterial degradation in the large bowel (Southgate and Durnin, 1970). Hemicellulose consists of a wide variety of polysaccharide polymers, i.e., a mixture of unbranched and branched pentose and hexose sugars. It is easily dissolved in hot diluted mineral acid. The molecules of hemicellulose are usually much smaller and more amorphous than those of cellulose. Hemicellulose is also not hydrolyzed by intestinal enzymes. Bacterial action in the large intestine has been estimated to break down approximately 85% of the total hemicellulose present (Southgate and Durnin, 1970).

Pectin is a partially methoxylated polygalacturonic acid. It forms a gel, and its cross-linked structure helps it withstand osmotic repulsions. The association between neighboring molecules is affected by the number of methyl esters and the presence of calcium which neutralizes negative charges and influences hydrogen bonds (Eastwood, 1973). Pectin is nearly completely digestible by man (Fetzer et al., 1977). Good plant sources of pectin are apples and the inner skin of oranges. Lignin is not a carbohydrate but an extremely complex aromatic polymer based on phenylpropane units. It is not hydrolyzed by strong acids or alkalis, nor by bacterial degradation in the colon (McCance and Lawrence, 1929).

The properties that contribute to the nutritional interest of fiber and its components are bulk effect, water holding capacity, organic absorption, fermentability and cation exchange capacity. As a bulking agent in the intestinal tract, fiber induces a faster transit time (Eastwood et al., 1973). This faster time might reduce the opportunity for production of microbal toxins or carcinogens, or further shorten the length of exposure to any toxin that may have been ingested. The antitoxic properties of fiber in rat and mouse rations have been reviewed by Ershoff in 1974. Hemicellulose and cellulose can adsorb water and thus increase stool weight, while lignin can absorb organic materials such as bile acids (Eastwood, 1973). Administration of pectin can effectively lower serum cholesterol concentration (Kay and Truswell, 1977). The fermentable portion of fiber residues primarily in the polysaccharide fractions (Van Soest, 1973b). It is through microbial action that energy incorporated in fiber,

resistant to digestive enzymes, becomes available. After fermentive digestion, the production of a microbial cell mass that has its own hydration and adsorption capacities will thus replace the fermented fiber (Fitt et al., 1972). Acidic polysaccharides with uronic acid moieties can act as cation exchangers, i.e., can bind metals (Eastwood, 1973). This property of fiber has been thought to raise an undesirable effect on mineral absorption.

There is evidence that fiber fractions can bind appreciable amounts of dietary calcium (Branch et al., 1975). Increased fecal zinc, iron and phosphorus, but not calcium occurred in subjects fed diet containing cellulose (Reinhold et el., 1975). Negative balances of calcium, magnesium, zinc and phosphorus were observed in subjects who consumed increased fiber and phosphorus in the form of whole meal bread (Reinhold et al., 1976). A decrease in iron absorption was also reported in subjects who consumed bread containing wheat bran (Björn-Rasmussen, 1974).

Oxalate

Oxalic acid, HOOC-COOH, has a greater dissociation constant ($K_1 = 6.5 \times 10^{-2}$ and $K_2 = 6.1 \times 10^{-5}$) than other organic acids due to the proximity of two carboxylic radicals. Its natural salts with sodium or potassium are water soluble, while those with alkaline earths or heavy metals are less soluble. The calcium salt is practically insoluble at neutral or alkaline pH but becomes soluble in acid (Gontzea and Sutzescu, 1968b).

Oxalic acid is found widely in the plant kingdom, both in the free state and more commonly in the form of salts (Oke, 1969). Its content depends on the species, climate and soil conditions. Kohman (1939) analyzed 53 samples of spinach, including practically all commercial and many experimental variations grown in various locations in the United States. The anhydrous oxalic acid content ranged from 4.5 to 12.6%. The distribution of oxalic acid inside a plant is also uneven, but generally the leaves are higher than the other parts.

Studies have been made of the effects of light, temperature and ionic balance on oxalate formation in spinach (Kitchen et al., 1964). These authors concluded that oxalate formation resulted from a photosynthetic process. It appeared that the oxalate formed was not utilized and tended to accumulate in the plant. Low temperature and high soil cation concentrations increased oxalate formation.

In biochemical studies, oxalate appears to be principally a metabolic end product in both plants and animals. Excreted oxalate is thought to be derived from ascorbic acid, glycine, and dietary oxalate or its precursors in vegetables, glycolic or glyoxylic acid. Absorption of oxalate is very poor, ranging from 1-6% under nonfasting conditions in man (Fassett, 1973).

The negative effect of oxalate on calcium utilization in many animals and humans has been reviewed by Gontzea and Suszescu (1968b). However, Fassett (1973) has concluded that this problem will not occur unless there is a very high intake of oxalate, associated with low calcium and vitamin D, over a prolonged period. Again, due to bacterial degradation, ruminants may ingest quantities of oxalate that would be

harmful to other species (Fassett, 1973).

The effect of dietary oxalate on the absorption of other essential metals (e.g. zinc and iron) has received relatively little attention. Two factors suggest that iron in plants containing high levels of oxalate (e.g. spinach) may not be readily available. These factors are: 1) the insolubility of the majority of the simple oxalates; and 2) the coprecipitation of transition metals with oxalates of calcium and other divalent metals (Krishnamurty and Harris, 1961).

MATERIALS AND METHODS

Experimental Design

The design of the experiment is summarized as follows:

I. Purpose: Evaluate the effects of fish on the bioavailability of plant iron in the anemic rat.

II. Experimental animal: Male rats (7 animals per group).

III. Experimental groups: Eighteen experimental groups, one negative and one positive control carried through depletion and repletion periods.

IV. Experimental periods:

Period 1. Initial iron depletion period of 33 days, followed by 14 days on respective test diets.

Period 2. Three days after termination of Period I, seven groups (6 animals each) were administered a dose of ^{59}Fe. A whole body counter was used to determine iron retention each day for 5 days.

V. Parameters measured:

Period 1. a) Food consumption.
b) Body weight.
c) Hemoglobin concentration.

Period 2. a) Food consumption.
b) Body weight.
c) Hemoglobin concentration.
d) ^{59}Fe intake and retention over 5 days.
e) Liver weight and ^{59}Fe accumulation.

Diets

Protein, lipid, oxalate, phytate, fiber and iron content of dietary ingredients are presented in Table 3. Additional information about type and sources of dietary ingredients are listed in Appendix I. Fish oil was obtained by cooking fillets of turbot, free of all skin and waste, in a steam jacketed kettle. The oil that rose to the top was removed, washed twice in distilled water and centrifuged. Butylated hydroxy toluene (BHT) was added to obtain 0.02%.

The diets were formulated to contain ca. 17.13% protein (Kjeldahl N x 6.25) and 5% lipid. The major component of each diet was D-glucose monohydrate. Other major protein and lipid components were reduced appropriately during substitution of the plant products into the diet. The composition of the 20 diets is shown in Table 4. All diets were supplemented with vitamin E (DL-α-tocopherol acetate), vitamin D (cholecalciferol) and vitamin K (menadione) above levels provided in the vitamin premix (see Table 4). This supplementation was in accordance with current recommendations of the American Institute of Nutrition (Bieri et al., 1977).

There were 5 control diets: negative control (No. 1), control (No. 2, 3 and 4) and positive control (No. 20) with respective iron contents of 0, 6.25, 12.5, 25.0 and 35.0 µg Fe/g added as ferrous sulfate. The 15 test diets contained either ferrous sulfate or endogenous iron provided by the plant material. These latter diets furnished 25.0 (No. 5 thru 16), 14.5 (No. 17) and 14.0 (No. 18) µg Fe/g. Diet No. 19 contained 5.2 µg Fe/g supplied by the fish. After being prepared, all diets

Table 3. Protein, lipid, oxalate, phytate, fiber and iron content of dietary ingredients.

	Protein[1]	Lipid	Oxalate	Phytate	Fiber				Iron
					NDF[2]	ADF[3]	HC[4]	C[5]	
	%--------								µg/g
Wheat bran[6]	15.1	4.5	-	3.41	41.30	10.87	30.43	8.25	119
Spinach	32.8	8.6	14.0	0.22	21.84	8.52	13.32	4.74	167
Soybean isolate[7]	90.1	0.3	-	1.28	0.95	-	0.95	-	170
Fish[8]	73.1	26.9	-	-	-	-	-	-	22
Casein	85.7	-	-	-	-	-	-	-	-

[1]Kjeldahl N x 6.25.
[2]Neutral detergent fiber.
[3]Acid detergent fiber.
[4]Hemicellulose; HC = NDF - ADF.
[5]Cellulose; C = ADF - L.
[6]Lignin 3.2%; pectin 3.0% (Mullen, 1978).
[7]Supro 610, Ralston Purina Co.
[8]Turbot - Arrowtooth flounder (Atheresthes stomias).

Table 4. Diet composition[1,2] (% by weight).

Diet No.	D-glucose monohydrate	Casein	Corn oil	Freeze dried fish	Fish oil	Wheat bran	Spinach	Soybean isolate Supro-610	Cellufil	Pectin	Lignin	Phytic acid	Oxalic acid
1 Control-negative	69.61	20.00	5.00	-	-	-	-	-	-	-	-	-	-
2 Control+6.25ppm Fe	68.50	20.00	5.00	-	-	-	-	-	-	-	-	-	-
3 Control+12.5ppm Fe	68.50	20.00	5.00	-	-	-	-	-	-	-	-	-	-
4 Control+25.0ppm Fe	68.50	20.00	5.00	-	-	-	-	-	-	-	-	-	-
5* Wheat bran	52.10	16.35	4.05	-	-	21.00	-	-	-	-	-	-	-
6* Wheat bran+fish	49.07	-	-	23.44	-	21.00	-	-	-	-	-	-	-
7 Control+cellufil	66.77	20.00	5.00	-	-	-	-	-	1.74	-	-	-	-
8 Control+cellufil+pectin	66.14	20.00	5.00	-	-	-	-	-	1.74	0.63	-	-	-
9 Control+cellufil+pectin +lignin	65.47	20.00	5.00	-	-	-	-	-	1.74	0.63	0.67	-	-
10 Control+cellufil+pectin +phytic acid	65.48	20.00	5.00	-	-	-	-	-	1.74	0.63	-	0.66	-
11 Control+phytic acid	67.84	20.00	5.00	-	-	-	-	-	-	-	-	0.66	-
12* Spinach	61.04	14.26	3.20	-	-	-	15.00	-	-	-	-	-	-
13* Spinach+fish	61.29	-	-	16.71	0.50	-	15.00	-	-	-	-	-	-
14 Control+oxalic acid	66.40	20.00	5.00	-	-	-	-	-	-	-	-	-	2.10
15 Control+cellufil +oxalic acid	62.65	20.00	5.00	-	-	-	-	-	3.75	-	-	-	2.10
16* Soybean isolate	69.26	4.55	5.00	-	-	-	-	14.70	-	-	-	-	-
17* Soybean isolate 50% +fish 50%	70.62	-	-	14.39	1.15	-	-	7.35	-	-	-	-	-
18* Soybean isolate 50% +casein 50%	68.88	12.28	5.00	-	-	-	-	7.35	-	-	-	-	-
19* Fish	70.07	-	-	23.44	-	-	-	-	-	-	-	-	-
20 Control+35.0ppm Fe	68.50	20.00	5.00	-	-	-	-	-	-	-	-	-	-

[1]Diets contained in addition: mineral mix 5.0% (obtained from J.T.Baker, containing in g/100g diet: $CaHPO_4$, 1.765; NaCl, 0.259; K citrate, 0.734; K_2SO_4, 0.174; $MgSO_4$, 0.248; glucose·H_2O, 1.787; in mg/100g diet: $ZnSO_4 \cdot 7H_2O$, 13.193; $MnSO_4 \cdot H_2O$, 16.616; $CuSO_4$, 2.358; KIO_3, 0.034; Na_2SeO_3, 0.022; $CrK(SO_4) \cdot 12H_2O$, 1.090), vitamin premix 0.70% (obtained from United State Biochemical Corp., containing in mg/100g diet: thiamin HCl, 0.7; riboflavin, 0.7; pyridoxine, 0.7; nicotinic acid, 3.15; D-calcium pantothenate, 2.1; folic acid, 0.063; D-biotin, 0.014; cyanocobalamin, 0.945 ug/100g; retinyl palmitate, 630 I.U./100g; calciferol, 70 I.U./100g; menaquinone, 1.575 ug/100g; diets further supplemented with: 30 I.U. cholecalciferol/100ug; 1.5 I.U. DL- -tocopherol acetate/100g; 3.425ug menadione (vitamin K oil snluble)/100g, all obtained from United State Biochemical Corp.), choline chloride 0.07% and DL-methionine 0.30%.

[2]Exogenous iron added as $FeSO_4 \cdot 7H_2O$ except those diets marked with an asterisk (*) which have iron supplied by test materials.

were placed in plastic bags and kept refrigerated.

Animals

Weanling male Long-Evans rats, weighing 39±6 g, were obtained from Charles River Breeding Laboratories (North Wilmington, MA). Upon receipt, animals were 23 days old. The rats were housed individually in stainless steel cages with wire mesh floors. Lighting was regulated automatically to provide 12 hours of light (7:00 a.m. to 7:00 p.m.) and 12 hours of darkness. Feed (in aluminum cups) and distilled water (in rubber stopper glass bottles) were supplied ad libitum.

Iron Bioavailability

Initially, the rats were fed the iron free diet for 33 days. At this time, the hemoglobin concentration was measured in blood obtained by amputating the tip of the tail. The depleted animals were then allotted to 19 groups of 7 animals each in such a manner that the mean and standard deviation of hemoglobin concentration were similar; 4.6±1.0 g/100 ml. One group of rats was maintained on the iron free diet and the remaining 18 groups were placed on the test diets. After a 14-day repletion period, hemoglobin concentration was again determined. This protocol is similar to the procedures outlined by Fritz and Pla (1972).

To further evaluate the effect of dietary components on iron absorption, it was decided to measure uptake from animals previously maintained on test diets and hopefully less anemic. This goal was accomplished by use of radioactive iron (^{59}Fe).

Uptake and Retention of Radioactive Iron (^{59}Fe)

Groups selected for conducting the radioactive portion of the experiment were those fed diets No. 5, 6, 12, 13, 17, 18 and 20. This experiment was to further evaluate the effect of fish on plant iron uptake. Labeled rations were prepared by adding 1 ml of the ^{59}Fe solution[1] to 100 g of each diet previously formulated. After being thoroughly blended for 2-1/2 minutes in a small mixing apparatus, 7 g of diet containing 1.4 μ Ci ^{59}Fe was given to each rat which had been withheld from food for 12 hr following a 3-day acclimation period. A similar quantity of ^{59}Fe solution was injected by micro syringe in a piece of styrofoam, 17 cm in length and 8 cm in diameter. This mock animal form was used as an external standard to verify the radioactivity of the original material and establish the operating parameter for the detector.

Whole-body radioactivity was counted initially within 4 hr of oral dose administration. Counting was repeated on approximately daily intervals until excretion of the unassimilated iron was essentially complete and retention had reached a "plateau value" - 110 hr. The experiment was terminated by sacrificing the animals, and determining blood hemoglobin, liver weight and liver ^{59}Fe levels.

Radioactivity was measured with a model 446 Armac Whole Body Liquid Scintillation Detector (Appendix 2) assembled by Packard Instrument Co. (Des Planes, IL). Since the location of the sample

---

[1] In the form of ferrous sulfate at the level of 200 μ Ci/10 ml in 0.1 M sulfuric acid solution and having a specific activity of 26.4 m Ci/mg (catalog/log No. NEZ-049-S1401K3, New England Nuclear, Boston, MA).

in the counting chamber affects the counting efficiency significantly, live rats were placed in disposable cups that limited their movement in an attempt to insure accurate repositioning with successive assays. Livers, in plastic tubes, were placed in a plastic sample holder. The detector was calibrated with ^{137}Cs having a gamma emission of 0.662 millelectron volts (MeV). Whole-body measurements obtained with the 1.10 and 1.29 MeV gamma emissions of ^{59}Fe were corrected for isotopic decay (half-life 45.6 days) and for variations in counting efficiency by reference to a ^{60}Co standard source.

Blood Drawing and Hemoglobin Determination

Animals were subjected to blood drawing at the end of depletion, regeneration and radioactive experimental periods. The rats were immobilized in a stainless steel animal holder. Prior to blood drawing, the tail was warmed in a beaker containing warm water to help blood circulation. Blood taken from the tail was analyzed for hemoglobin by the cyanmethemoglobin method (Crosby et al., 1954) using a Brusch and Lomb Spectronic 20 colorimeter. Drabkin reagent and hemoglobin standard were supplied by Sigma Chemical Co. (St. Louis, MO, Appendix III).

The cyanmethemoglobin standard solution yields a transmittance equivalent to 18 g Hb/100 ml whole blood. Zero, 2.0, 4.0 and 6.0 ml of cyanmethemoglobin standard were pipeted into 6.0, 4.0, 2.0 and 0.0 ml of Drabkin's solution, respectively, to construct a calibration curve. These mixtures have transmittance levels corresponding to blood hemoglobin of 0.0, 6.0, 12.0 and 18.0 g/100 ml. Samples

were assayed by adding 20 ul whole blood to tubes containing 5 ml Drabkin's solution. The pipets were rinsed several times. Contents of both sets were well mixed and allowed to stand 15 minutes at room temperature. Drabkin's solution served as the blank with transmittance measured at 545 nm. Sample hemoglobin levels were otained directly from the calibration curve of transmittance (% T) vs. hemoglobin concentration (g/100 ml).

Analytical Procedures

Iron

The wet-ashing procedure and analysis by atomic absorption spectrophotometer (AAS) were used respectively for preliminary sample digestion and iron content determinations (Gordon, 1978a).

Two gram samples were predigested in 100 ml Kjeldahl flasks containing 15 ml HNO_3 until completely charred. After the addition of 5 ml HNO_3 and 1.5 ml $HClO_4$, the digestion was allowed to proceed until clear, and continued for an additional 20 min. The colorless digest was cooled to room temperature, and diluted to 25 ml with distilled water.

A Perkin-Elmer Model 403 atomic absorption spectrophotometer (AAS), using a single element hallow cathode lamp, was employed. Absorption of diluted samples was measured at 252.7 nm (Perkin-Elmer Corp., 1973). Iron levels were determined from the calibration curve prepared from an iron standard[2] diluted in distilled water.

[2]1000 µg Fe/ml, Harleco, 60th and Woodland Ave., Philadelphia, PA 19143.

Protein

Protein content was measured according to the AOAC Kjeldahl organic nitrogen method (AOAC, 1975), using a micro-Kjeldahl apparatus. Protein levels are reported as Kjeldahl nitrogen x 6.25.

Lipid

The method of Folch *et al.* (1957) was adapted for lipid determination. Lipid was determined gravimetrically from an aliquot of the chloroform:methanol (2:1; v/v) sample extracts.

Oxalate

The method of Franco and Krinitz (1973) was used for the measurement of oxalate. The procedure consists of sample preparation, oxalic acid precipitation and calcium determination by AAS.

Approximately 10 g of sample was analytically weighed, and mixed in 300 ml distilled water for 15 minutes. After the addition of 55 ml 6N HCl and 2 drops of caprylic alcohol (antifoam agent), the mixture was boiled for 15 minutes. The cooled solution was transferred quantitatively to a 500 ml volumetric flask, diluted to volume with distilled water, mixed, and allowed to stand overnight. The contents of the flask were mixed again and filtered[3], discarding the first 10 ml.

---

[3]Whatman No. 30 fast quantitative paper.

Tungstophosphoric acid reagent[4] (5 ml) was added to 25 ml of filtrate. After standing $\geq$ 5 hr followed by filtration[3], 20 ml of filtrate was pipeted into a 50 ml conical centrifuge tube and ammonium hydroxide was added to achieve a pH 4-4.5. Buffer solution[4] (5 ml) was added and the mixture was allowed to stand overnight. Centrifugation for $\geq$ 15 min at 1700 rpm and washing of the precipitate[4] were repeated 3 times. After each centrifugation, the precipitate was drained completely each time.

The precipitate was quantitatively transferred to a 10 ml volumetric flask and completed to volume with 10% H_2SO_4. Appropriate dilutions were made to be within the range of standard calcium solutions[4]. Both sample and standards, containing 20% lanthamum solution[4] by volume, were read on a Perkin-Elmer Model 403 AAS. Operating parameters for calcium measurements were as described in the instructional manual (Perkin-Elmer Corp., 1973).

The calcium concentration was used to calculate percent oxalate on the assumption that calcium:oxalate complex exists in a 1:1 molar ratio.

Phytate

Phytate was determined indirectly after its precipitation with iron and analysis for phosphorus (de Boland *et al.*, 1975).

A finely ground sample (2 g) was extracted for 18 hr at room temperature with a solution (98 ml) containing 1.2% HCl and 10% Na_2SO_4. After a 10 min centrifugation at 2,500 rpm, 10 ml of the extract was

[4]See Appendix IV. Preparation of reagents used in oxalate determination.

diluted with 10 ml of water and treated with 5 ml of 0.4% $FeCl_3$ in 6% HCl containing 5% Na_2SO_4. The precipitate that formed, after standing 10 min, was centrifuged for 10 min at 2,500 rpm and washed with 15 ml of 0.6% HCl containing 5% Na_2SO_4. Digestion with 5 ml HNO_3 and 3 ml H_2SO_4 was completed after the water had evaporated and the solution turned clear. The digest was transferred quantitatively to a 50 ml volumetric flask and diluted to volume with water.

Phosphorus determination was by the Fiske and SubbaRow method (1925). To a 1 ml water blank, sample and standards[5], was added: 1 ml 1% ammonium molybdate in 1 N H_2SO_4[6], 0.5 ml freshly prepared and filtered Fiske and SubbaRow reducer[7] and 3 ml distilled water. The blue chromophor developed within 15 min. Absorbances of sample and standard solutions were read at 660 nm against the water blank on a Beckman DB Spectrophotometer. Sample phosphorus contents were determined from a standard curve. Phytate was calculated on the assumption it contains 28.2% phosphorus (Johnson and Tate, 1969).

Fiber

Fiber analysis was by the method of Van Soest (Van Soest, 1965 and 1973a; Van Soest and Wine, 1967 and 1968). These procedures provide for the following determinations: neutral detergent fiber (NDF) which includes cellulose, hemicellulose and lignin; and acid detergent fiber(ADF)

---

[5]Diluted 1, 2, 3 and 4 ml of stock solution (250 µg P/ml. 0.274445 g dried KH_2PO_4 plus 2 drops chloroform to 250 ml with water) in 100 ml water.

[6]One g ammonium molybdate plus 5.5 ml concentrated H_2SO_4 to 100 ml with water.

[7]Obtained from Sigma Chemical Co., St. Louis, MO.

which is the sum of lignin and cellulose. Cellulose is determined by subtracting lignin, independently assayed, from ADF. Hemicellulose is obtained indirectly by subtracting ADF from NDF.

Determination of ADF. A 1g of sample (S) was analytically weighed,and transfered into a 200 ml refluxing flask containing 100 ml acid detergent solution[8] at room temperature. It was then heated to boiling and refluxed for 60 min from onset of boiling. The contents of the flask were transferred quantitatively to a 50 ml weighted coarse porosity pyrex crucible (W_1). Filtration was completed using minimum suction. The filtered mat was broken up and soaked 30 sec with hot (90-100°C) water and dried under vacuum. This operation was repeated once with water and twice with acetone. The remaining mat was dried overnight at 100°C and weighted (W_2). Percent ADF was calculated by the formula: $100\ (W_1-W_2)/S$.

Determination of Lignin. One g acid washed asbestos[9] was added to the crucible containing ADF. The contents of the crucible were covered with cool (15°C) 72% H_2SO_4 and stirred with a glass rod to a smooth paste, breaking all lumps. The crucible was refilled with 72% H_2SO_4 and stirred hourly as the acid drained. After 3 hr, the acid was removed under vacuum and the residue was washed with hot water until acid-free to pH paper. Crucibles were dried at 100°C overnight and weighted (W_3). All organic materials were removed by ashing at 500 C,

---

[8]Dissolve 20 g technical grade cetyltrimethyl ammonium in 1.00 N previously standardized H_2SO_4 (1 liter).

[9]Asbestos, medium fiber; J. T. Baker.

and the crucibles were weighted (W_4). A 1 g asbestos blank was carried through the entire procedure to record any loss in weight upon ashing (W_5). Percent acid insoluble lignin was calculated by the formula: $\frac{(W_3-W_4-W_5)}{S} \times 100$, in which S = sample weight.

Determination of NDF. A 0.5 - 1.0 g of sample was analytically weighed int a 200 ml refluxing flask in the following order: 100 ml cool (room temperature) neutral detergent solution[10], 2 ml technical grade decahydronaphthalene and 0.5 g reagent grade sodium sulfite. The flask was heated to boiling, then refluxed 60 min from onset of boiling. The contents of the flask were transferred quantitatively to 50 ml tared crucible and filtered under low vacuum. The mat was broken up, washed twice with hot water and twice with acetone. Final residue was dried at 100°C overnight and weighed as NDF.

Statistical Analysis

One way analysis of variance (ANOVA) and Least Significant Difference (LSD) were done to compare the means of various determinations among the experimental groups. Student's t-test was carried out to compare any two test meals (Snedecor and Cochran, 1976). All significant differences were tested at the 5% level. Correlation coefficients were determined using a linear regression program. All statistical computation was conducted at the Computer Center, Oregon State University.

---

[10]To 1 liter distilled water add: 30 g sodium lauryl sulfate, USP; 18.61 g disodium dihydrogen ethylenediaminetetraacetic dihydrate, reagent grade; 6.81 g sodium borate decahydrate, reagent grade; 4.56 g disodium hydrogen phosphate, anhydrous, reagent grade; and 10 ml 2-ethoxyethanol (ethylene glycol, monoethyl ether), purified grade. Agitate to dissolve. Check pH to range 6.9-7.1.

RESULTS AND DISCUSSION

Animals assigned to 19 groups at the start of repletion trial had uniform body weights and hemoglobin levels (Table 5). Increases in body weight, hemoglobin concentration and feed consumption during the 14-day repletion period are reported in Table 5.

Growth

Among test diets containing 25 µg Fe/ g, the highest growth rate was observed in animals consuming wheat bran (diets No. 5 and No. 6). Higher growth was also observed in groups (diets No. 7, No. 8, No. 9 and No. 10) receiving fiber components, i.e., cellufil, pectin and lignin. Spinach in the diet (No. 12 and No. 13) had a negative effect on growth. Growth of animals on other diets (No. 11, No. 14 and No. 15) was intermediate. Animals receiving soybean isolate (diets No. 17 and No. 18), containing 14 µg Fe/g, had equivalent growth to control diet No. 3 with 12.5 µg Fe/g. The poor growth rate of animals on the diet (No. 19) containing fish was attributed to its very low iron level. Fish and fish oil in diets with plant materials (No. 6, No. 13 and No. 17) did not significantly alter growth when compared with diets containing casein and corn oil (No. 5, No. 12 and No. 18).

Hemoglobin Levels

A normal hemoglobin concentration of rat is approximately 14 g/100 ml. This value was observed with the positive control diet (No. 20).

Table 5. Initial and final body weight and hemoglobin, and total feed consumption during 14-day iron repletion period[1].

Diet No.	Body weight (g) Initial	Body weight (g) Final	Hemoglobin (g/100ml) Initial	Hemoglobin (g/100ml) Final	Total feed consumption (g)
1	118±13	138±14^{a}	4.6±1.1	2.6±1.1^{i}	130±12^{r}
2	125±25	176±24^{bcdef}	4.6±1.1	4.2±1.0^{j}	175±25^{t}
3	109±16	164±21^{abcd}	4.6±1.1	6.4±1.0^{k}	172±19^{t}
4	101±22	176±30^{bcdef}	4.6±1.1	9.2±1.6^{lm}	207±26^{uvw}
5	131±26	213±30^{h}	4.6±1.0	10.5±0.8^{nop}	252±22^{y}
6	121±22	211±22^{gh}	4.6±1.0	9.9±0.2^{lmno}	238±12^{xy}
7	115±17	191±19^{dfgh}	4.6±1.0	11.0±1.2^{op}	218±28^{vwx}
8	114±32	189±49^{defgh}	4.6±1.0	9.2±1.8^{lm}	234±38^{xy}
9	110±16	202±27^{fgh}	4.7±1.0	10.2±1.0^{mno}	227±16^{wxy}
10	108±17	196±15^{fgh}	4.7±1.0	9.5±1.3^{lmn}	221±18^{wx}
11	117± 8	189±28^{dfgh}	4.7±1.0	10.9±1.3^{op}	235±22^{xy}
12	111±27	160±30^{abc}	4.7±1.0	6.8±1.3^{k}	171±35st
13	105±14	169±16^{bcde}	4.6±1.0	5.9±0.8^{k}	182±22^{tu}
14	126±22	184±38^{cdefg}	4.6±1.0	13.4±1.6^{q}	184±46^{tu}
15	102±14	170±26^{bcde}	4.6±1.0	11.7±1.7^{p}	191±21^{tu}
16	107±20	177±35^{bcdef}	4.6±1.0	8.8±2.0^{l}	193±31^{tuv}
17	102±15	168±12^{bcde}	4.7±1.0	4.9±0.6^{jk}	186±13^{tu}
18	108±29	163±28^{abcd}	4.7±1.0	5.5±0.9^{k}	179±28^{tu}
19	111±15	155±18^{ab}	4.7±1.0	2.1±0.2^{i}	144±18^{rs}
20	202± 6*	249±19*	13.3±0.4*	14.1±1.4^{q}	246±35^{xy}

[1]Values reported as mean ± SD for 7 animals; values not followed by the same superscript letter are significantly different at $P \leq 0.05$.

*Significantly different from all other groups at $P \leq 0.05$.

During the repletion period, no test group attained this level. The 25 ppm iron concentration of test diets was selected so animals would not reach normal hemoglobin status by the 14th day.

Among diets No. 5 through No. 16, the lowest hemoglobin level was observed in diets No. 12 and No. 13 containing 15% spinach. The highest hemoglobin level was reached on diet No. 14 containing 2.1% oxalic acid, an equivalent amount provided by the spinach diets. These extremes were significantly different from all other diets regenerating intermediate hemoglobin levels. It appears that the chemical form of the iron complex in spinach is different from the suspected iron-oxalate chelate resulting when the two latter ingredients are mixed in a diet. Oxalic acid by itself showed a marked improvement on iron uptake.

Fish in diets with wheat bran and soybean isolate, versus those containing casein and corn oil, did not significantly alter hemoglobin levels. However, in the diet containing spinach, fish and fish oil did retard hemoglobin regeneration significantly. An enhancement of plant iron uptake by adding fish and fish oil to all diets was anticipated.

Feed Consumption

Among diets No. 5 through No. 16, total feed consumption was high in those containing fiber or its components. The consumption of diets containing spinach and soybean isolate was low. For the spinach diet, palatability could have been one reason. Other diets were consumed

at intermediate level.

Animals receiving their total protein and lipid complement from fish with no added iron consumed the lowest quantity of food. There was no significant difference in feed intake upon adding fish and fish oil to diets having wheat bran, spinach or soybean isolate. Food consumption between repleted animals and those receiving wheat bran during the two-week test period was approximately the same. However, the later diets resulted in significantly higher growth rates. It is believed that wheat bran or its components have a positive effect on iron utilization and subsequently growth. Although very speculative, the prospect of a growth factor associated with fiber in rats, as observed for fibrous fraction of alfalfa by Knehans et al. (1979) in guinea pigs, is an exciting possibility. Spiller and Amen (1976) have also considered an uncharacterized growth stimulant in plants to be related to a fiber component.

Relative Biological Value (RBV)

Three methods were used in determining the bioavailability of iron. Relative biological value (RBV), as described by Pla and Fritz (1970), evaluates test iron by comparing its ability to replenish hemoglobin against the ability of iron from ferrous sulfate in anemic animals. The ratio of test iron to iron from ferrous sulfate is computed to produce an equivalent hematological response. A more realistic value was gained through construction of a standard dose-response curve. This curve was obtained by plotting graded iron levels ($FeSO_4$) against final hemoglobin concentrations (Pla and Fritz, 1971). A straight line

was obtained through the use of 4 control diets (No. 1, No. 2, No. 3 and No. 4) with different iron levels. The linear regression equation of this standard curve was: [Fe] = -9.8687 + 3.7154 x Final Hb, (r = 0.9959). Through the substitution of final hemoglobin from each of the test diets into the equation, the amount of equivalent ferrous sulfate was determined. Since true hemoglobin concentration is directly related to body weight, the product of these two parameters is believed to be a better response indicator (Shah and Belonje, 1973). By this criteria, the linear regression equation in determining equivalent ppm of $FeSO_4$ was: [Fe] = -8.2242 + 0.0204 X (Final Hb x Final body weight), (r = 0.9966). Both methods were used to calculate the RBV for iron in each test diet. These values are reported as RBV-1 and RBV-2 in Table 6. The term RBV-1 refers only to final hemoglobin and RBV-2 reflects changes in both final hemoglobin and final body weight. Statistical analysis indicated that these two RBV's are highly correlated (r = 0.9435). However, RBV-2 values are slightly higher than RBV-1. A third method to evaluate iron bioavailability will be discussed later.

Iron Bioavailability from Wheat Bran

The RBV-2 of wheat bran iron is 1-1/2 times that from ferrous sulfate. This higher RBV is contradictory to the widely supported hypothesis that phytate and/or fiber are responsible for the poor absorption of iron in diets containing wheat bran (Haghshenass et al., 1972; Björn-Rasmussen, 1974; Reinhold et al., 1975). Cook et al. (1973) showed that iron in whole wheat was absorbed by man to a greater extent

Table 6. Relative biological value (RBV) of plant iron with and without fish and ferrous iron ($FeSO_4$) as influenced by plant components.

Diet No.	RBV-1[1,3]	RBV-2[2,3,4]
5	117	149*ab
6	108	137*abc
7	124*	138*abc
8	97	113 cd
9	112	135*abc
10	102	119 cd
11	123*	133*abc
12	62*	57*e
13	48*	48*e
14	160*	157*a
15	134*	127*bc
16	91	91 d
17	58*	58*ef
18	76*	71*f
19	-40*	-28*g

[1]Equivalent ppm of $FeSO_4$ based on final Hb determined from the equation: [Fe] = -9.8687 + 3.7154[4] x Final Hb ,(r = 0.9959).

[2]Equivalent ppm of $FeSO_4$ based on final Hb x final body weight determined from the equation: [Fe][4] = -8.2242 + 0.0204 x (Final Hb x final body weight), (r = 0.9966).

[3] $RBV = \frac{\text{Equivalent ppm of iron}(FeSO_4)}{\text{ppm iron of test source}} \times 100$; mean values of 7 animals.

[4]Values not followed by the same superscript letter are significantly different at $p \leq 0.05$.

*Significnatly different from 100 at $p \leq 0.05$.

than added inorganic iron. Elvehjem et al. (1933) considered whole wheat a moderately available source of iron in the rat. Morris and Ellis (1976) suggested the iron in wheat bran would be highly available because of its high content of monoferric phytate, a source they found to have a high level of assimilation. In the present study, iron from wheat bran was demonstrated to be highly available. To the author's knowledge, this study is the first one actually determining the availability of endogenous wheat bran iron by using the rat as the experimental animal.

It is known that rats possess an intestinal phytase (Pileggi, 1959) which may have hydrolyzed some of the phytate present. Also, wheat bran contains its own phytase (Ranhotra and Loewe, 1975). The net contribution of these two phytates in aiding the absorption of iron through hydrolysis is unknown. The good solubility and stability of phytate over the entire pH range in the intestine of the rats may be responsible for good absorption. O'Dell et al. (1972) has suggested that phytate complexes in wheat bran might be in a more soluble form. These soluble phytates, including monoferric phytate, may possibly be bound to the cationic sites of proteins or other cellular compounds. The release of their iron is enhanced through solubilization by an ion exchange type mechanism rather than by hydrolysis of the phytate. Furthermore, phytate may only interfer with mineral nutrition when it is above one percent in the diet (Erdman and Forbes, 1977). The percent of phytate in the wheat bran diet was 0.66. From this study, it is concluded that the phytate present in wheat bran has no adverse effect on iron absorption.

Persson et al. (1976) have demonstrated decreased serum iron levels after the consumption of diets containing bran or whole wheat products.

Björn-Rasmussen (1974) found an occurrance of reduced iron absorption when wheat bran was added into the diet. Results from Reinhold et al. (1975) questioned the inhibitory effect of phytate and suggested that fiber was the main factor for the diminished dietary iron absorption. Recently, Sandstead et al. (1978) found that iron balance was not affected by wheat bran intake. The enhanced iron uptake from the wheat bran diet in this study cannot be fully explained by the soluble iron complex, monoferric phytate, and/or other factors discussed previously. Fiber, as a component of wheat bran, appears to play a very positive role in mineral nutriton.

From a physiological point of view, the incorporation of fiber in food requires more chewing action which stimulates the secretion of saliva and gastric juice. The increased volume of ingested food, due to fiber mass and its water binding capacity will cause the stomach to become more distended. This enlargement provokes the release of the hormone gastrin. Among many actions of gastrin, it stimulates gastric motility and acid-pepsin secretion. Moreover, bulkier stomach contents may, on entry into the duodenum, be more powerful stimulators of the digestive hormones, secretin and cholecystokinin. These hormones promote the secretion of bile and pancreatic juices (Davenport, 1966).

The increased stimulation of gastric juice provides more hydrochloric acid which in turn insures a better acid environment. Hydrychloric acid influences iron absorption by facilitating the release of food iron into solution (Kirch et al., 1947) and/or the formation of soluble iron chelates (Conrad and Schade, 1968). In addition,

gastric motility may increase the accessibility of iron complexes to gastric juice through more thorough mixing. Acid pepsin, the chief enzyme of gastric juice, converts protein into proteoses and peptones. It may produce free amino acids which have been shown to have an enhancing effect on iron absorption (Martinez-Torres and Layrisse, 1970; Van Campen, 1973; Miller, 1974). Finally, as the chyme enters the duodenum, fiber as a bulk agent will indirectly stimulate bile and pancreatic juices. Bile has been demonstrated to increase ferrous iron absorption in the dog (Wheby et al., 1962) and rat (Webling and Holdsworth, 1966). It was shown that inorganic iron forms complexes with bile which are soluble at neutral pH and thus help to keep iron in solution (Jacobs and Miles, 1970). Pancreatic secretions have also been thought to modify iron absorption, but sufficient evidence is lacking to support this idea.

There is another possible explanation of how fiber may promote iron uptake. Blocking agents to iron absorption may be adsorbed by fiber either chemically or physically and be excluded from the digestion and absorption process in the small intestine.

Fiber is a heterogeneous complex of polysaccharides and lignin. Its exact role or action on mineral nutriture is difficult to explain. The effect of fiber may vary accordingly to its own chemical nature and the presence or absence of other dietary components.

Iron Bioavailability from Spinach[11]

Among groups receiving 25 μg Fe/g diet, animals that had spinach as their sole iron source had the lowest RBV's. The RBV-2 of this group was only about half of the control group. The low iron absorption may be the result of low iron intake as the consequence of low food consumption.

It has been determined that oxalic acid exists in quantities 2-7 times higher than calcium in spinach on a milliequivalent basis (Gontzea and Sutzescu, 1968). In addition to binding with calcium, this excess oxalic acid could complex with other minerals. The endogenous iron in spinach may also be available to chelate with this excess oxalic acid. Spinach is high in soluble oxalate (Oke, 1969). Thus, there may be two forms of iron chelators in spinach: one form which is totally unavailable; and a second form which is apparently soluble and leads to partial hemoglobin regeneration as observed. Some iron in spinach may also form insoluble compounds with other ligands such as phosphate.

The quantity of dietary fiber in the spinach diets was only about 1/3 of the wheat bran diets. Probably due to this lower fiber content and/or different physico-chemical nature of its "fibers," the iron enhancing effect of wheat bran fiber was not apparent in spinach.

---

[11] The ancient Chinese people have recognized that spinach is rich in iron - an essential ingredient for regeneration of blood. Soup made of spinach and pork liver is still used today for anemic subjects such as women after child birth. This latter therapy appears to be a very early application of the "meat factor" effect.

Iron Bioavailability from Soybean Isolate

The RBV-2 of the endogenous iron from soybean isolate was found to be 91 (diet No. 16) and 71% (diet No. 18) when fed at two levels. When the soybean isolate provided almost the full protein complement of the diet at an iron level of 25 ppm, the higher value was observed. The lower value resulted when 50% of the dietary protein was supplied by casein. It was anticipated that equal RBV's would be obtained. Reasons for a different relative hemoglobin regeneration in similar test materials at different iron levels are not readily apparent.

Results of this study are in general agreement with other investigations. These findings indicated RBV of iron from soybean isolate to be 70-125% (Fritz et al., 1970), 86% (Theuer et al., 1971) and 82-100% (Rotruck and Luhrsen, 1979). Evaluating the same soybean material, Supro 610, Steinke and Hopkins (1978) found a RBV of 66.5 $\pm$ 6.4%. Difference in experimental condition and type of product evaluated can easily account for variations in results. Rackis et al. (1977) have concluded that processing conditions of soybean protein products alter iron bioavailability. Lower RBV may be due to the formation of protein-phytate-mineral complexes during manufacture (Smith and Rackis, 1975). Some trypsin inhibitor (TI) activity remains if heat treatment is not sufficient. This TI activity may affect fat absorption, metabolism of sugar and amino acids, and digestibility of proteins (Rutkowski, 1977) The activity which ultimately alters iron utilization. Species differences also complicate results. Davis et al. (1962) found good utilization of iron from soybean isolates by the chick, while Fitch et al. (1964) observed

poor utilization by the monkey. Man has been reported to utilize iron from soybean well (Layrisse et al., 1969). Welch and Van Campen (1975) reported no direct correlation between phytate content in soybean seeds and iron bioavailability. Their observations also suggest that naturally occurring phytate will not interfere with the availability of iron.

Iron Bioavailability from Fish

Iron absorption from fish produced negative RVB's. This negative effect is primarily due to the very low iron content of fish and subsequently the low iron content of the diets. Chemical analyses has shown that about 14% of the total fish muscle iron was in the insoluble portion (hemosiderin) and the remaining fraction corresponds to ferritin (Martinez-Torres et al., 1975). Ferritin iron acts as non-heme iron and is affected by many dietary factors (Martinez-Torres et al., 1974 and 1975; Layrisse et al., 1975). Iron atoms of ferritin are both structurally and functionally heterogeneous. This heterogeneity is due to the fact that they can occupy either surface or interior sites in the micelle structure of the molecule. Those atoms that happen to be at the surface at any given time are immediately available for release (Hoy et al., 1974). All these facts lead to the conclusion that the contribution of fish iron to the diet is meager.

Effect of Fish and Fish Oil on Plant Iron Uptake

Fish has been reported to enhance absorption of non-heme iron

in human subjects (Layrisse et al., 1968 and 1974; Cook and Monsen, 1976a). In the present study, however, this enhancing effect was not demonstrated. This lack of expected enhancing effect may be due to the rat's unacceptability as a model to measure a "meat factor" effect. During the comparison of diets No. 5 to No. 6, No. 12 to No. 13, and No. 17 to No. 18, it was noticed that the RBV-2's in diets containing fish and fish oil were lower, but not significantly. Evidence from two separate studies by Gordon (1978b and 1979) has shown that increasing levels of unsaturation, especially as found in marine oil, have a negative effect on iron bioavailability. At 5% dietary lipid, hemoglobin regeneration was significantly lower in anemic animals fed salmon oil versus corn oil or beef fat. The difference increased with increasing salmon levels ranging from 5 to 20 percent. The difference in iron absorption between beef fat and corn oil at increasing lipid levels was not significantly different.

Lipid oxidation is believed to be the primary cause of the blocking effect of marine oil on iron uptake. The exact relationship between dietary lipid oxidation and iron uptake is unknown. However, this problem was anticipated. Diets were supplemented with additional vitamin E and antioxidants (Table 4) and kept refrigerated prior to use.

Chemical analysis has shown that the amino acid content of fish protein is comparable to casein (Gordon, unpublished data), and the protein efficiency ratio (PER) is higher in fish protein (3.55) than casein (2.50)(FAO, 1970). Thus, lower iron uptake due to possible amino acid imbalance in diets containing fish is discounted. However, some fish protein products have been reported to be inferior to milk

protein when fed to undernourished children (Graham et al., 1966) and baby pigs (Barnes et al., 1966). It was suggested that this difference was due to better proteolytic digestion of milk protein under conditions in which digestive enzymes are deficient (Barnes et al., 1966). Therefore, this poor digestion may be one cause in reduced iron absorption by anemic rats fed fish protein.

Effect of Fiber, Phytate and Oxalate on Iron Bioavailability

In addition to the examination for a possible "meat factor" effect in fish, an investigation of the components of plant products suspected to inhibit iron absorption was also performed. These plant components include fiber (cellufil, pectin and lignin), phytate and oxalate which were fed at levels comparable to amounts found in the corresponding wheat bran, soybean and spinach diets. Some components were fed singularly and some were fed in combination.

All control diets (No. 2, No. 3 and No.4) were made without dietary fiber, cellufil. This absence of cellufil is believed to be responsible for the high RBV's of test diets in general containing any type of fiber. When RBV-2's of diets No. 4 and No. 7 are compared, the latter is significantly higher. The positive effect of fiber alone on enhancing iron uptake is demonstrated by the fact that feed consumption between the two groups was approximately the same. All subsequent comparisons are made against diet 4. The addition of pectin (from orange), the RBV-2 of diet No. 8 decreased but not significantly. In the presence of all three fiber components, cellufil, pectin and lignin (diet No. 9), the RBV-2

increased again to the value of the diet containing only cellufil (diet No. 7). This increase may be due to the added iron contamination from lignin. A diet containing only lignin was not evaluated. Phytic acid by itself (diet No. 11) had a significantly higher RBV-2. However, a lower RBV-2 was obtained when phytic acid was added, together with cellufil and pectin (diet No. 10). Oxalic acid in the diet (No. 14) had the highest RBV-2. This high RBV-2 was reduced significantly when cellufil was present (diet No. 15). The RBV in latter diet was approximately the same as in the diet containing cellufil alone (diet No. 7).

From these results, cellulose appears to have a beneficial effect on iron uptake. The functions of acting as a bulk agent and aiding in the absorption of iron would appear to warrent in recommending its use in the diet. Lignin has no apparent effect, while pectin seems to have a negative effect in iron absorption. This negative effect may be due to the latter forming substances which complex with protein (Bing, 1976) and indirectly affecting the absorption of metals. Another hypothesis is that highly esterified pectin may undergo enzymatic, acid or alkali hydrolysis to produce pectic acids. These acids are able to complex with divalent cations (Southgate, 1973). Thus, iron may complex with these pectic acids and become unavailable. The impairment of growth without necessary a concomitant decrease in food intake by pectin supplements mentioned by Spiller and Amen (1976) was not apparent (see Table 5) in this study.

Purified sodium phytate has been reported to decrease whole body retention of iron in rats (Davies and Nightingale, 1975). Cowan et al. (1966) and Ranhotra et al. (1974) observed no effect on iron availability in rats fed sodium phytate. The increased iron absorption

observed in the present study with sodium phytate cannot be readily explained. One reason may be that the added sodium phytate preferentially binds other minerals and thereby facilitates the enhanced uptake of iron. Also, in comparing studies, the source of the sodium phytate may have been different. Thus, the exact identity of a phytate salt may not be known and is difficult to define.

Oxalic acid did not exhibit the inhibition expected. The great promoting effect in iron absorption by oxalic acid is really amazing. It is not obvious why oxalic acid failed to supress iron absorption. An explanation is offered below.

Simple iron oxalate is formed during diet preparation. When this diet reaches the acid environment of the stomach, the oxalate is dissociated. As the chyme moves from the acid environment of the gastric region into the intestine, it is neutralized. However, excess oxalic acid in the medium probably may serve as a powerful ligand to protect against iron oxidation. A second reason may be that oxalic acid reduces precipitation by replacing the hydroxy groups and thus forming very stable and soluble complexes. In addition, the phenomenon of coprecipitation of iron with oxalates of calcium or other metals may be reduced. The iron-oxalate complexes now are available to the intestinal mucosa for transport into the blood stream.

The appropriate molar ratio between iron and oxalate may be important in the formation of large quantities of soluble complexes. For example, EDTA in the proportion of 2 moles of EDTA per mole of iron reduced iron uptake (Cook and Monsen, 1976b), whereas EDTA, when in a mole to mole ratio, increased iron uptake by two-fold. Evidently, for optimum iron uptake,

the molar ratio of iron to oxalate may differ significantly from iron to EDTA.

A similar enhancing effect by oxalate on zinc availability was reported by Welch et al. (1977) in rats. Oxalate has also been demonstrated to significantly enhance Cr^{3+} transport in all sections of the rat intestine i.e., duodenum, jejunum and ileum (Chen et al., 1973).

Finally, the presence of both oxalic acid and cellufil in the diet did not show a synergetic effect in iron absorption. These two compounds together had the same enhancing effect as cellufil alone in the diet.

Special Remarks

Theoretical and actual dietary iron and actual protein levels are listed in Appendix V. There were no significant differences in protein contents among all test diets. Variations between theoretical and actual iron levels determined after diet preparation were approximately equal and highly correlated ($r = 0.9587$). This statistical analysis insured that all previous discussions were not altered by variable actual iron or protein contents.

True Iron Bioavailability

Iron bioavailability studies based on RBV's is only a relative measurement. A more realistic value is true iron bioavailability (Table 7), i.e., the efficiency of converting dietary iron into

Table 7. True iron bioavailability (% efficiency).

Diet No.	Fe intake[1] (mg)	Hb gain[1] (g)	Hb-Fe gain[1] (mg)	Efficiency[1] (%)
1	-	-	-	-
2	1.094	0.115	0.385	36
3	2.148	0.364	1.219	57
4	5.164	0.762	2.551	50
5	6.254	1.084	3.631	58
6	5.957	1.022	3.424	57
7	5.461	1.044	3.551	65
8	5.861	0.848	2.840	48
9	5.675	1.038	3.479	61
10	5.532	0.914	3.060	55
11	5.864	1.002	3.357	58
12	4.282	0.393	1.316	30
13	4.561	0.342	1.145	25
14	4.593	1.194	3.999	83
15	4.768	1.003	3.359	71
16	4.836	0.689	2.307	49
17	2.703	0.227	0.761	28
18	2.510	0.244	0.817	35
19	0.748	-0.121	-0.369	-57
20	8.595	0.559	1.873	21

[1]Mean values of 7 animals.

hemoglobin iron (Mahoney et al., 1974). This procedure accounts for differences in body weight gain and iron consumed due to unequal food intakes. In Table 7, iron intake (mg) was calculated as the product of iron level (μg/g) multiplied by total food consumption (g). Hemoglobin gain (g) was determined as the difference between initial and final body hemoglobin content. Both initial and final total hemoglobin (g) were calculated as the product of body weight (g) multiplyied by 0.065 g blood per g body weight multiplied by hemoglobin concentration (g/ml). Hemoglobin iron gain (mg) is the product of hemoglobin gain and 3.35 mg iron per gram hemoglobin. The efficiency % (true iron bioavailability) is the ratio of hemoglobin iron gain versus iron intake x 100.

In this experiment, 57 and 50% of the iron in diets containing 12.5 and 25 ppm $FeSO_4$, respectively, were converted to hemoglobin. This conversion efficiency is in agreement with Mahoney et al. (1974) and Fritz et al. (1970). The poor conversion in diet No. 2 containing 6.25 ppm Fe cannot be explained.

True bioavailability is thus generally only one-half of reported RBV's. Statistically, there was no significant difference between 2 x % efficiency and RBV's among groups 5 through 18. The value of 2 x % efficiency was more correlated with RBV-1 ($r = 0.9836$) than with RBV-2 ($r = 0.9328$).

Whole Body Iron Retention

The technique of extrinsic labeling, i.e., adding radioactive

iron to test food, is used to estimate the bioavailability of food iron (Björn-Rasmussen et al.. 1972 and 1974; Layrisse et al., 1974; Monsen, 1974). By this procedure, radioactive iron enters a common non-heme iron pool with resulting isotopic exchange between the tracer and the food iron. Although the food iron exceeds the radioactive iron by many hundred times, newly incorporated radioactive iron can be readily distinguished from that already existing in the body. This technique is particularly useful in studying factors that enhance or inhibit absorption of non-heme food iron in animals of varying iron status (Monsen, 1974).

Measurements of radioactivity were made on each animal at approximately daily intervals. Sufficient individual counts were obtained to reduce the net counting rate error to less than ± 1% (Wang et al., 1975). The data were first corrected for radioactive decay and then converted to percent retention based on 100% ^{59}Fe administered 4 hr after ingestion. Mean and standard deviation of counting rate and percent retention in each test group were calculated (Appendix VI).

Whole body retention of ^{59}Fe added in diets containing three plant products was in the following order: soybean isolate = spinach > wheat bran. Rentention in the latter group was equivalent to that observed in the positive control group maintained on adequate iron levels throughout the experiment. Fish and fish oil in the diets did not significantly alter the retention (Figure 2 and Table 8). These results indicate that all three plant materials did not interfer with the availability of exogeneous ^{59}Fe as would be expected from preliminary repletion studies.

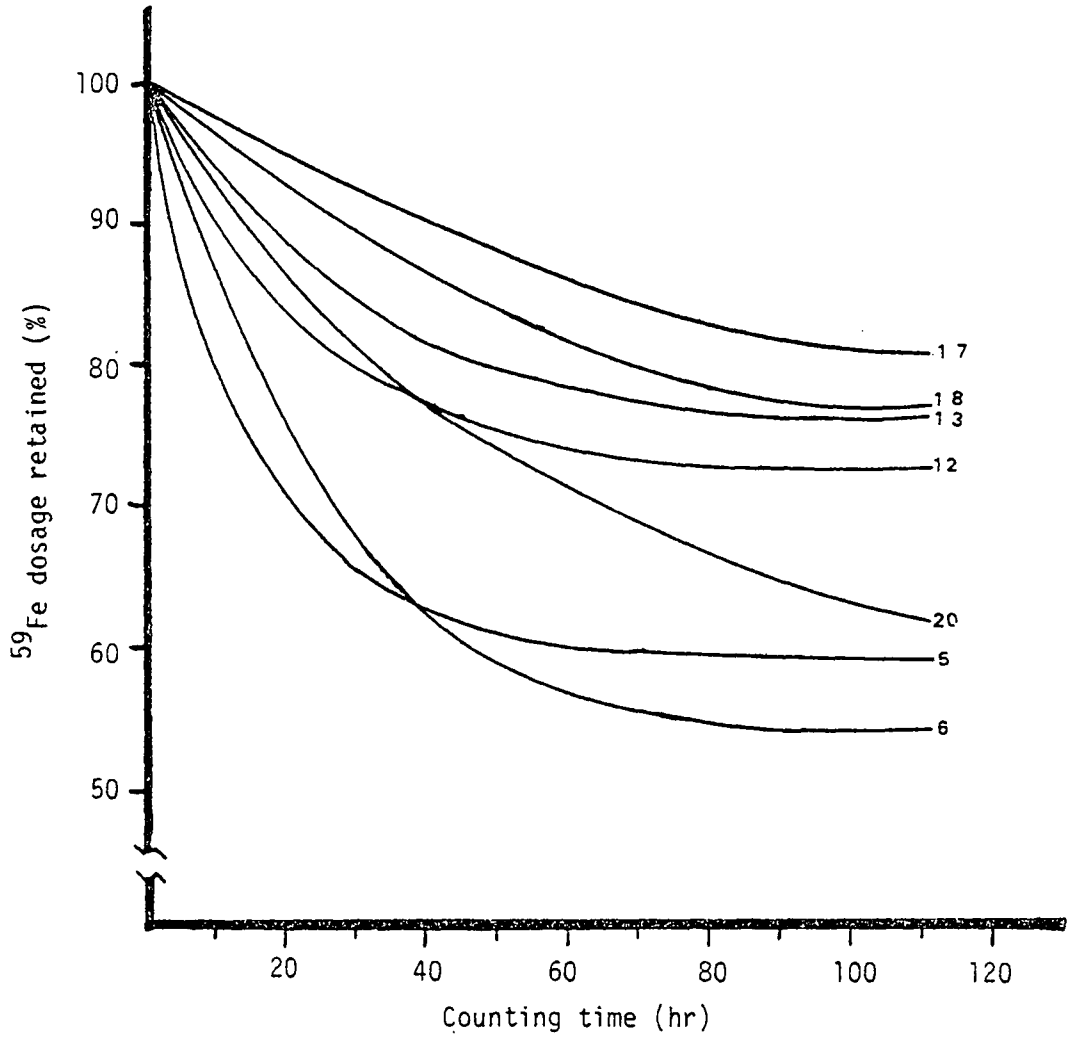

Figure 2. Radioactive iron (^{59}Fe) absorption decay profile.

Table 8. Percent whole body retention of ^{59}Fe in rats fed plant foods with and without fish.

Diet no.	Major composition	^{59}Fe retained after 110 hrs (%)[1]
17	Soybean + fish + fish oil	80[a]
18	Soybean + casein + corn oil	77[a]
13	Spinach + fish + fish oil	76[a]
12	Spinach + fish + corn oil	73[a]
5	Wheat bran + casein + corn oil	58[b]
6	Wheat bran + fish + fish oil	54[b]
20	$FeSO_4$ + casein + corn oil[2]	62[b]

[1]Mean value of 6 animals; values not followed by the same superscript letter are significantly different at $p \leq 0.05$.

[2]Positive control, non-anemic animal.

It was hoped that starting hemoglobin levels in all groups would have been higher when the animals were given the radioactive iron. However, it is believed that the varying starting hemoglobin levels did not distract from the experimental objective and actually contributed valuable information. Monsen (1974) in her work showing the uniform uptake of extrinsic and intrinsic food iron by the rat, did have equal starting hemoglobin levels. The data herein suggests that although iron availability is low from a specific food (e.g. spinach), this food may not interfere with other dietary sources of iron. A second area of concern is the use and significance of extrinsically labelled iron. Results of this experiment indicate that the ^{59}Fe did not mix with a common iron pool. Work of two other research groups (Amine and Hegsted,

1974; Wien et al., 1975) has also indicated possible reservations with use of the extrinsic label in the rat.

A linear relationship was obtained between starting hemoglobin concentration and percent iron retention (Figure 3; r = -0.9788). Only the positive control animals deviated from this regression line. The highest absorption occurred in the most anemic rats.

During the period of ^{59}Fe measurement, all groups had the same growth rate and with no significant change in hemoglobin level. A slight decrease in hemoglobin concentration was observed in groups fed soybean isolate diets and spinach with casein plus corn oil. Feed consumption again was higher in animals consuming wheat bran either with or without fish. Other groups, including the positive control, had no significant difference in food intake (Table 9).

Liver Iron Accumulation

Since liver is the main body iron storage organ, it serves also as an indication in estimating iron absorption.

Total liver weight and percent ^{59}Fe accumulated per g of liver is shown in Table 10. As previously observed with whole body counts, two groups resulted in which these parameters are compared. Animals on the soybean isolate and spinach diets had lower liver weights but higher iron accumulation. The opposit was observed in the second group of animals fed wheat bran or maintained on the positive control diet. Again, fish and fish oil had no effect on iron

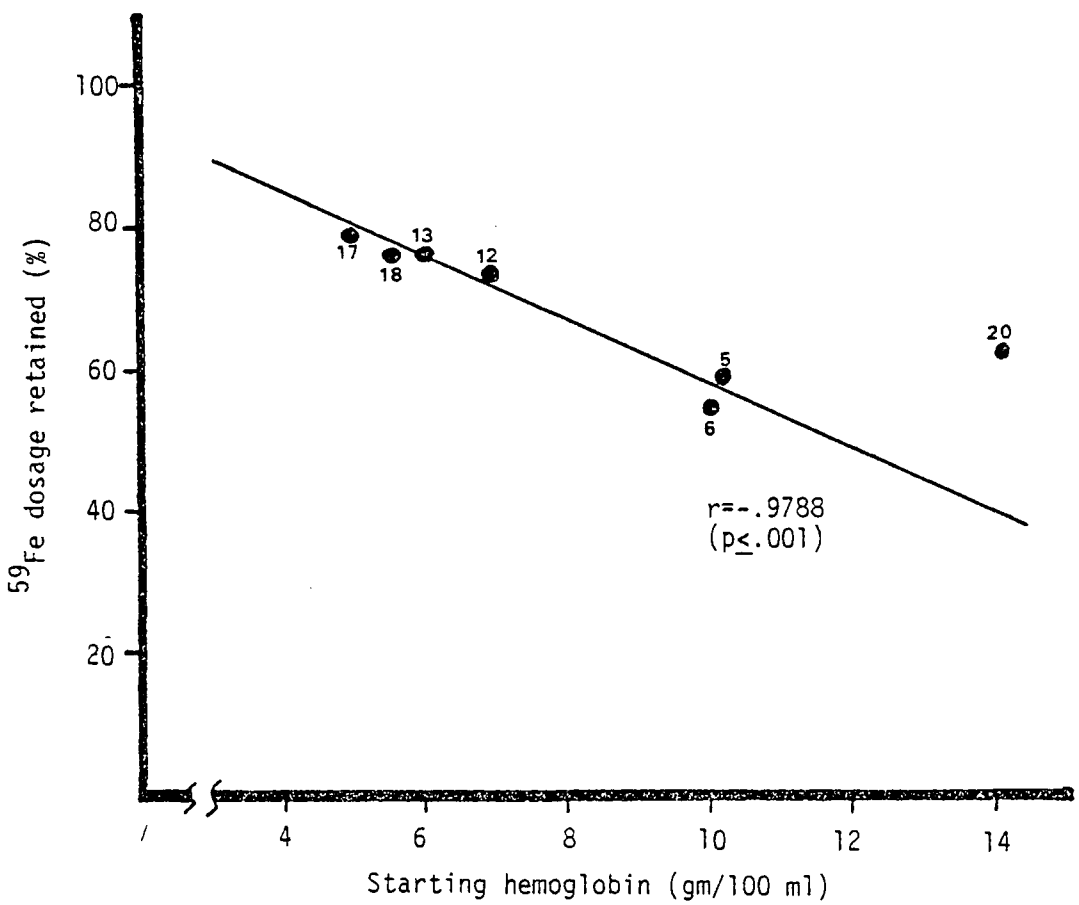

Figure 3. Whole body retention of ^{59}Fe after five days versus starting hemoglobin level.

Table 9. Body weight, hemoglobin and feed consumption prior to and after ^{59}Fe administration[1].

Diet No.	Body weight (g) Start ^{59}Fe (Day 70)	Body weight (g) End ^{59}Fe (Day 78)	Hemoglobin (g /100 ml) Start ^{59}Fe (Day 70)	Hemoglobin (g /100 ml) End ^{59}Fe (Day 78)	Total feed consumption[2] (8 days)(g)
17	168±12^{a}	196±13^{d}	4.9±0.6^{g}	4.4±0.8^{g}	126±20^{lm}
18	163±28^{a}	197±26^{d}	5.5±0.9^{gh}	4.9±1.0^{g}	116±18^{l}
13	169±16^{a}	187±22^{d}	5.9±0.8^{h}	6.0±0.8^{h}	121±11^{lm}
12	160±30^{a}	199±26^{d}	6.8±1.3^{i}	6.4±0.8^{hi}	129±23^{lm}
5	213±30^{b}	246±24^{e}	10.5±0.8^{j}	10.5±0.9^{j}	168±16^{n}
6	211±22^{b}	252±25^{e}	9.9±0.2^{j}	9.8±1.3^{j}	167±26^{n}
20	249±19^{c}	279±15^{f}	14.1±1.4^{k}	14.1±0.6^{k}	141±14^{m}

[1]Values reported as mean ± SD for 6 animals; values not followed by the same superscript lettering are significantly different at $p \leq 0.05$.

[2]The administration of ^{59}Fe prepared in 7 g of diet on Day 73 was not included.

accumulation in any diet containing different irons. The relationship between starting hemoglobin level and ^{59}Fe accumulation was linear (Figure 4; $r = -0.9798$). A high correlation existed between iron retention (^{59}Fe) and liver accumulation ($r = 0.9797$).

Table 10. Liver weight and ^{59}Fe accumulation[1].

Diet No.	Liver weight (g)	^{59}Fe accumulation per g of liver (%)
17	7.5[a]	0.57[c]
18	6.8[a]	0.61[c]
13	6.7[a]	0.55[c]
12	7.4[a]	0.51[c]
5	9.8[b]	0.29[d]
6	10.5[b]	0.28[d]
20	10.4[b]	0.31[d]

[1]Mean values of 6 animals; values not followed by the same superscript letter are significantly different at $p \leq 0.05$.

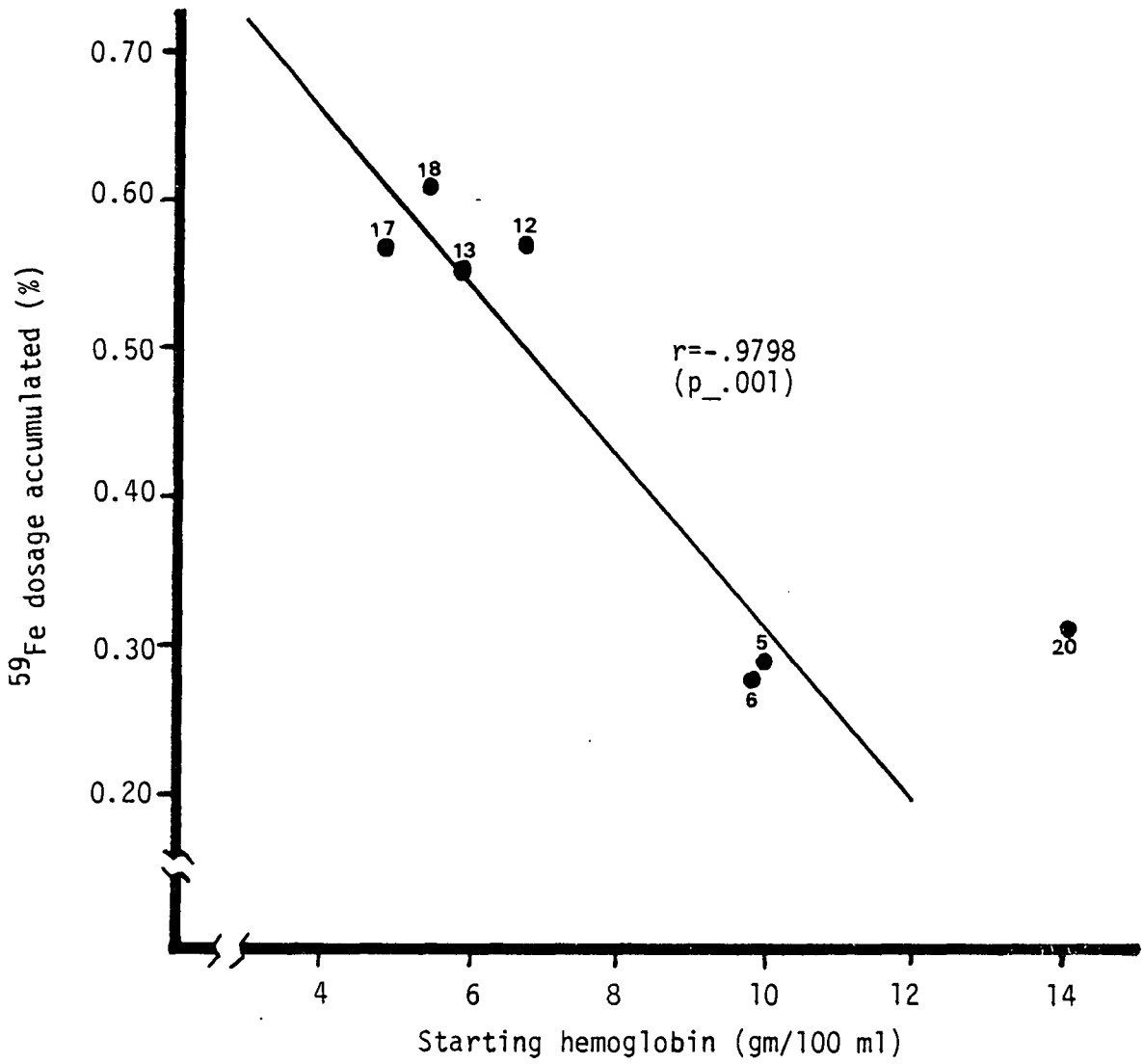

Figure 4. ^{59}Fe accumulation per gram of liver after five days versus starting hemoglobin level.

SUMMARY AND CONCLUSION

The effect of fish on the bioavailability of iron from three plant sources, wheat bran, spinach and soybean isolate was determined. In these plant materials, dietary components, fiber, oxalate and phytate suspected to reduce iron absorption were also evaluated. The rat was used as an experimental model. Availability of endogenous plant iron was measured by the hemoglobin repletion technique. Radioactive iron (^{59}Fe) was also used to evaluate the effect of diet on iron uptake.

Animals which developed anemia over a thirty day period were fed plant foods and components individually and in combination for 14 days. In the comparison of hemoglobin regeneration and hemoglobin times body weight in test animals versus controls fed on iron standard ($FeSO_4$), two relative biological values (RBV) were obtained (RBV, $FeSO_4$ = 100%). The firsts value, based on hemoglobin level alone, was designated RBV-1. The second value, RBV-2, referred to hemoglobin times body weight. These two values were highly correlated ($r = 0.9435$). The efficiency - true bioavailability - of utilizing dietary iron is approximately one-half of the RBV.

Iron bioavailability from wheat bran was high (RBV-2 = 149%), intermediate in soybean isolate (RBV-2 = 71%) and low in spinach (RBV-2 = 57%). Substituting fish and fish oil for casein and corn oil lowered but not significantly, the RBV-2 for wheat bran (137%), soybean isolate (58%) and spinach (48%). While an enhancement of iron uptake due to a "meat factor" effect was anticipated, one reason for this reversal might be, the use of an unacceptable model (the rat) for such investigations.

A second explanation could be the negative effect of polyunsaturated lipid, as present in marine products, on dietary iron uptake.

Wheat bran increased both body growth and iron absorption. This observation was generally true of all fiber components tested. Iron uptake was enhanced by cellulose-cellufil (138%) but decreased not significantly by cellulose and pectin (113%). In the rat, wheat bran and its components were found to be beneficial to iron absorption.

Phytic (133%) and oxalic acid (157%) demonstrated enhanced iron absorption, while naturally occurring phytate in wheat bran or soybean isolate apparently had no adverse effect on iron utilization. The reason for low iron assimilation from spinach (57%) may not be all due to oxalate. The importance of the chemical nature of iron in foods and its interaction with other dietary ingredients is again exemplified.

Measuring the uptake and retention of radioactive iron (^{59}Fe) by animals previously maintained on plant diets with and without fish led to some unexpected observations. Instead of extrinsic iron uptake being influenced by dietary ingredients, retention had the highest correlation with starting hemoglobin concentration ($r = -0.9788$). Thus while endogenous iron uptake is variable in foods, i.e., wheat bran, soybean isolate and spinach, and is influenced by other dietary factors, i.e. fish and fish oil, the uptake of an extrinsic iron source is not. Two important findings from this experiment are: 1) food with low iron bioavailability may not interfere with the uptake of other dietary iron; and 2) use of an extrinsic label to estimate effects of food and other dietary components on iron assimilation may not be applicable under all conditions. It is realized that this conclutions oppose each other.

Extrapolation of these results to human nutrition is inappropriate in view of documented specie differences. These experiments have indicated the importance of dietary interaction on nutrient uptake. Additional research should be directed at determining the relationship between the rat and higher animals, i.e., pig and baboon, with the ultimate goal of aiding the nutritional status of man.

BIBLIOGRAPHY

Amine, E.K. and Hegsted, D.M. 1974. Biological assessment of available iron in food products. J. Agr. Food Chem. 22:470.

Amine, E.K. and Hegsted, D.M. 1975. Effect of dietary carbohydrates and fats on inorganic iron absorption. J. Agr. Food Chem. 23:204.

Anon. 1974. Diet study points to iron deficiency among women. Food Prod. Develop. 8(5):79.

Anon. 1977. Iron fortification increases withdrawn by FDA. Food Prod. Develop. 11(10):8.

AOAC. 1975. Official Method of Analysis. 12th ed. Association of Official Analytical Chemists, Washington, DC, Sec. 2.049.

Barnes, R.H., Pond, W.G., Kwong, E. and Reid, I. 1966. Effect of severe protein-calorie malnutrition in the baby pig upon relative utilization of different dietary proteins. J. Nutr. 89:355.

Bieri, J.G., Stoewsand, G.S., Briggs, G.M., Phillips, R.W., Woodard, J.C. and Knapka, J.J. 1977. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr. 107:1340.

Bing, F.G. 1976. Dietary fiber - in historical perspective. J. Am. Diet. Assoc. 69:498.

Björn-Rasmussen, E. 1974. Iron absorption from wheat bread. Influence of various amounts of bran. Nutr. Metabol. 16:101.

Björn-Rasmussen, E., Hallberg, L. and Walker, R.B. 1972. Food iron absorption in man. I. Isotopic exchange between food iron and inorganic iron salt added to food: studies on maize, wheat and eggs. Am. J. Clin. Nutr. 25:317.

Björn-Rasmussen, E., Hallberg, L., Isaksson, B. and Arvidsson, B. 1974. Food iron absorption in man: applications of the two-pool extrinsic tag method to measure heme and non-heme iron absorption from the whole diet. J. Clin. Invest. 53:247.

Bothwell, T.H. and Finch, C.A. 1962. Iron Metabolism. Boston, MA: Little, Brown and Company, 440 p.

Branch, W.J., Southgate, D.A.T. and James, W.P.T. 1975. Binding of calcium by dietary fibre: its relationship to unsubstituted uronic acids. Proc. Nutr. Soc. 34:120A.

Brise, H. and Hallberg, L. 1962a. Absorbability of different iron compounds. Acta Med. Scand. 171 (suppl. 376):23.

_____. 1962b. Effect of ascorbic acid on iron absorption. Acta Med. Scand. 171 (Suppl. 376):51.

Burke, M.C. 1974. Changes in U. S. Food Consumption, 1909-1962. Conf. May 22.

Callender, S.T., Marney, S.R. and Warner, G.T. 1970. Egg and iron absorption. Brit. J. Hematol. 19:657.

Carmichael, D., Christopher, J., Hegenauer, J. and Saltman, P. 1975. Effect of milk and casein on the absorption of supplemental iron in the mouse and chick. Am. J. Clin. Nutr. 28:487.

Chen, N.S.C., Tsai, A. and Dyer, I.A. 1973. Effect of chelating agents on chromium absorption in rats. J. Nutr. 103:1182.

Conrad, M.E. and Shade, S.G. 1968. Ascorbic acid chelates in iron absorption: a role for hydrochloric acid and bile. Gastroenterology 55:35.

Cook, J.D. 1978. Food iron availability. National Livestock and Meat Board, Food Nutr. News. 49(3).

Cook, J.D. and Finch, C.A. 1975. Iron nutrition: Medical Progress. West J. Med. 122:474.

Cook, J.D. and Monsen, E.R. 1975. Food iron absorption. I. Use of a semi-synthetic diet to study absorption of nonheme iron. Am. J. Clin. Nutr. 28:1289.

_____. 1976a. Food iron absorption in human subjects. III. Comparison of the effect of animal proteins on nonheme iron absorption. Am. J. Clin. Nutr. 29:859.

_____. 1976b. Food iron absorption in man. II. The effect of EDTA on absorption of dietary nonheme iron. Am. J. Clin. Nutr. 29:614.

Cook, J.D., Brown, G.M. and Valberg, L.S. 1964. The effect of achylia gastrica on iron absorption. J. Clin. Invest. 43:1185.

Cook, J.D., Layrisse, M. and Finch, C.A. 1969. The measurement of iron absorption. Blood 33:421.

Cook, J. D., Minnich, V., Moore, C.V., Rasmussen, A., Bradley, W.B. and Finch, C.A. 1973. Absorption of fortification iron in bread. Am. J. Clin. Nutr. 26:861.

Cook, J.D., Lipschitz, D.A., Miles, L.E.M. and Finch, C.A. 1974. Serum ferritin as a measure of iron stores in normal subjects. Am. J. Clin. Nutr. 27:681.

Cosgrove, D.J. 1966. Chemistry and biochemistry of inositol polyphosphates. Rev. of Pure and Appl. Chem. 16:209.

Cowan, J.W., Esfahani, M., Salji, J.P. and Azzam, S.A. 1966. Effect of phytate on iron absorption in the rat. J. Nutr. 90:423.

Crosby, W.H., Munn, J.I. and Furth, F.W. 1954. Standardizing a method for clinical hemoglobinometry. U.S. Armed Forces Med. J. 5:693.

Czerniejewski, C.P., Shank, C.W., Bechtel, W.G. and Bradley, W.B. 1964. The minerals of wheat, flour and bread. Cereal Chem. 41:65.

Dairy Council Digest. 1972. Iron-deficiency anemia in infants and preschool children. An interpretive review of recent nutrition research. Published by Nat. Dairy Council 43(1).

Davenport, H.W. 1966. Physiology of the Digestive Tract. 2nd ed. Year Book Medical Publishers, Chicago, 230 p.

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

Davis, P.N., Norris, L.C. and Kratzer, F.H. 1962. Interference of soybean proteins with the utilization of trace minerals. J. Nutr. 77:217.

de Boland, A.R., Garner, G.B. and O'Dell, B.L. 1975. Identification and properties of " phytate " in cereal grains and oilseed products. J. Agr. Food Chem. 23:1186.

Dietary Goals for the United States. 1977. Select Committee on Nutrition and Human Needs. United States Senate, 95th Congress, 1st Session, George McGavern, Chairman.

Disler, P.B., Lynch, S.R., Charlton, R.W., Bothwell, T.H., Walker, R.B. and Mayet, F. 1975a. Studies on the fortification of cane sugar with iron and ascorbic acid. Brit. J. Nutr. 34:141.

Disler, P.B., Lynch, S.R., Charlton, R.W., Torrance, J.D., Bothwell, T.H., Walker, R.B. and Mayet, F. 1975b. The effect of tea on iron absorption. Gut 16:193.

Eastwood, M.A. 1973. Vegetable fibre : its physical properties. Proc. Nutr. Soc. 32:137.

Eastwood, M.A., kirkpatrick, J.R., Mitchell, W.D., Bone, A. and Hamilton, T. 1973. Effects of dietary supplements of wheat bran and cellulose on faeces and bowel function. Brit. Med. J. 4:392.

Elvehjem, C.A., Hart, E.B. and Sherman, W.C. 1933. The availability of iron from different sources for hemoglobin formation. J. Biol. Chem. 103:61.

Erdman, J.W. and Forbes, R.M. 1977. Mineral bioavailability from phytate containing foods. Food Prod. Develop. 11(10):46.

Ershoff, B.H. 1974. Antitoxic effects of plant fiber. Am. J. Clin. Nutr. 27:1395.

FAO. 1970. Amino acids. In : Amino Acid Content of Foods. Food and Agriculture Organization of the United Nations ed.

Fassett, D.W. 1973. Oxalate. In : Toxicants Ocurring Naturally in Foods. 2nd ed. pp. 346-362. National Academy of Sciences, Washington, DC.

Fetzer, S.G., Kies, C. and Fox, H.M. 1977. Gastric disappearance of dietary fiber by adolescent boys. Annual Meeting of American Association of Cereal Chemists, San Francisco, CA Octuber 23-26.

Finch, C.A., Cook, J.D., Monson, E.R. and Sood, S.K. 1974. The problem of nutritional iron deficiency. Ind. J. Nutr. Diet. 11:296.

Fiske, C.H. and SubbaRow, Y. 1925. The calorimetric determination of phosphorus. J. Biol. Chem. 66:375.

Fitch, C.D., Harville, W.E., Dinning, J.S. and Porter, F.S. 1964. Iron deficiency in monkeys fed diets containing soybean protein. Proc. Soc. Exp. Biol. and Med. 116:130.

Fitt, T.J., Hutton, K., Thompson, A. and Armstrong, D.G. 1972. Binding of magnesium ions by isolated cell walls of rumen bacteria and the possible relation to hypomagnesemia. Proc. Nutr. Soc. 31:100A.

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497.

Forth, W. and Rummel, W. 1973. Iron absorption. Physiol. Rev. 53:724.

Franco, V. and Krinitz, B. 1973. Determination of oxalic acid in foods. J. A.O.A.C. 56:164.

Fritz, J.C. and Pla, G.W. 1972. Application of the animal hemoglobin repletion test to measurement of iron availability in foods. J. A.O.A.C. 55:1129.

Fritz, J.C., Pla, G.W., Roberts, T., Boehne, J.W. and Hove, E.L. 1970. Biological availability in animals of iron from common dietary sources. J. Agr. Food Chem. 18:647.

Fuhr, I. and Steenbock, H. 1943. The effect of dietary calcium, phosphorus and vitamin D on the utilization of iron. 1. The effect of phytic acid on the availability of iron. J. Biol. Chem. 147:59.

Gontzea, I. and Sutzescu, P. 1968a. Substances depressing utilization of minerals. I. Phytic acid. In : Natural Antinutritive Substances in foodstuffs and Forages. pp. 55-84. S.Karger-Basel (Switzerland)-New York.

Gordon, D.T. 1978a. Atomic absorption spectrometric and colorimetric determination of iron in seafoods. J. A.O.A.C. 61:715.

_____. 1978b. Bioavailability of iron from seafoods. J. Agr.Food Chem. (Submitted for publication).

_____. 1979. Unpublished results.

Graham, G.G., Baertl, J.M. and Cordane, A. 1966. Studies in infantile malnutrition. V. The effect of dietary protein source on serum proteins. Am. J. Clin. Nutr. 18:16.

Greenberger, N.J. 1973. Effects of antibiotics and other agents on the intestinal transport of iron. Am. J. Clin. Nutr. 26:104.

Groen, J., VanDerBreek, W.A. and Veldman, H. 1947. Absorption of iron compounds from the small intestine in the rat. Biochimica et Biophysica Acta 1:315.

Haghshenass, M., Mahloudji, M., Reinhold, J.G. and Mohammadi, N. 1972. Iron-deficiency anemia in an Iranian population associated with high intake of iron. Am. J. Clin. Nutr. 25:1143.

Hahn, P.F., Bale, W.F., Ross, J.F., Balfour, W.M. and Whipple, G.H. 1943. Radioactive iron absorption by gastrointestinal tract. Influence of anemia, anoxia and antecedent feeding distribution in growing dogs. J. Exp. Med. 78:169.

Hallberg, L. and Solvell, L. 1967. Absorption of hemoglobin iron in man. Acta Med. Scand. 181:335.

HEW. 1972. Ten State Nutrition Survey, 1968-1970. U.S. Dept. of Health, Education, and Welfare, Washington, DC, Publication No. (HSM) 72-8132.

___. 1974. Preliminary Findings of the First Health and Nutrition Examination Survey, United States, 1971-1972. U.S. Dept. of Health, Education, and Welfare, Washington, DC, Publication No. (HRA) 74-1219-1.

___. 1976. Iron Nutriture in Adolescence. U.S. Dept. of Health, Education, and Welfare, Washington, DC, Publication No. (HSA) 77-5100.

Hill, R. and Tyler, C. 1954. The reaction between phytate and protein. J. Agr. Sci. 44:324.

Hoy, T.G., Harrison, P.M., Shabbir, M. and Jmacara, I.G. 1974. The release of iron from horse spleen ferritin to 1,10-phenanthroline. Biochem. J. 137:67.

HÜbers, H., HÜbers, E., Forth, W., Leopold, G. and Rummel, W. 1971. Binding of iron and other metals in brush borders of jejunum and ileum of the rat in vitro. Acta Pharmacol. Toxicol. 29 (Suppl. 4):22.

Hussain, R. and Patwardhan, V.N. 1959. The influence of phytate on the absorption of iron. Ind. J. Med. Res. 47:676.

INACG. 1977. Guidelines for the iron deficiency anemia. A report of the International Nutritional Anemia Consultative Group.

Ismail-Beigi, F., Reinhold, J.G., Faraji, B. and Abadi, P. 1977. Effects of cellulose added to diets of low and high fiber content upon the metabolism of calcium, magnesium, zinc and phosphorus by man. J. Nutr. 107:510.

IUPAC-IUB. 1968. The nomenclature of cyclitols. Eur. J. Biochem. 5:1.

Jacobs, A. and Miles, P.M. 1970. The formation of iron complexes with bile and bile constituents. Gut 11:732.

Johnson, L.F. and Tate, M.E. 1969. Structure of " phytic acids ". Can. J. Chem. 47:63.

Kay, R.M. and Truswell, A. 1977. Effect of citrus pectin on blood lipids and fecal steroid excretion in man. Am. J. Clin. Nutr. 30:171.

Kirch, E.R., Bergeim, O., Kleinberg, J. and James, S. 1947. Reduction of iron by foods in artificial gastric digestion. J. Biol. Chem. 171: 687.

Kitchen, J.W., Burns, E.E. and Langnston, R. 1964. The effects of light, temperature and ionic balance on oxalate formation in spinach. Proc. Am. Soc. Hortic. Sci. 85:465.

Knehans, A.W., Kincaid, R.L., Regan, W.O. and O'Dell, B.L. 1979. AN unrecognized dietary factor for guinea pigs associated with the fibrous fractions of plant products. J. Nutr. 109:418.

Kohman, E.F. 1939. Oxalic acid in foods and its behavior and fate in the diet. J. Nutr. 18:233.

Krishnamurty, K.U. and Harris, G.M. 1961. The Chemistry of the metal oxalato complexes. Chemical Rev. 61:213.

Kuhn, I.N., Layrisse, M., Roche, M. Martinez-Torres, C. and Walker, R.B. 1968. Observations on the mechanism of iron absorption. Am. J. Clin. Nutr. 21:1184.

Layrisse, M. and Martinez-Torres, C. 1971. Food iron absorption. Iron supplementation of food. Prog. Hematol. 7:137.

_____. 1972. Model for measuring dietary absorption of heme iron : test with a complete meal. Am. J. Clin. Nutr. 25:401.

Layrisse, M., Martinez-Torres, C. and Roche, M. 1968. Effect of interaction of various foods on iron absorption. Am. J. Clin. Nutr. 21:1175.

Layrisse, M., Cook, J.D., Martinez-Torres, C., Roche, M., Kuhn, I.N., Walker, R.B. and Finch, C.A. 1969. Food iron absorption. A composition of vegetable and animal foods. Blood 33:430.

Layrisse, M., Martinez-Torres, C., Cook, J.D., Walker, R.B. and Finch, C.A. 1973. Iron fortification of food. Its measurement by the extrinsic tag method. Blood 41:333.

Layrisse, M., Martinez-Torres, C. and González, M. 1974. Measurement of the total daily dietary iron absorption by the extrinsic tag model. Am. J. Clin. Nutr. 27:152.

Layrisse, M., Martinez-Torres, C., Renzy, M. and Leets, I. 1975. Ferritin iron absorption in man. Blood 45:689.

Lease, J.G, 1966. Effect of autoclaving sesame meal on its phytic acid content and on the availability of its zinc to the chick. Poultry Sci. 45:237.

Loria, A., Medel, L.S. and Elizondo, J. 1962. Effect of sorbitol on iron absorption in man. Am. J. Clin. Nutr. 10:124.

Mahoney, A.W., VanOrden, C.C. and Hendrick, D.G. 1974. Efficiency of converting food iron into hemoglobin by the anemic rat. Nutr. Metab. 17:223.

Martinez-Torres, C. and Layrisse, M. 1970. Effect of amino acids on iron absorption from a staple vegetable food. Blood 35:669.

_____. 1971. Iron absorption from veal muscle. Am. J. Clin. Nutr. 24:531.

_____. 1973. Nutritional factors in iron deficiency - food iron absorption. Clin. Haematol. 2:339.

Martinez-Torres, C., Leets, I., Renzi, M. and Layrisse, M. 1974. Iron absorption by humans from veal liver. J. Nutr. 104:983.

Martinez-Torres, C., Leets, I. and Layrisse, M. 1975. Iron absorption by humans from fish. Archivos Latin-americano de Nutricion 25:199.

Miller, D.F. 1977. Cereal enrichment / Pellagra - USA ... in perspective. Abst. 62nd. An Meeting Am. Assoc. Cereal Chemists, Oct. 23-27, San Francisco.

Miller, J. 1974. Metabolism of iron by rats fed wheat diets supplemented with lysine and methione. Nutr. Rep. Int. 10:1.

Monson, E.R. 1974. Validation of an extrinsic iron label in monitoring absorption of non-heme food iron in normal and iron-deficient rats. J. Nutr. 104:1490.

Moore, C.V. 1973. Iron. In : Modern Nutrition in Health and Disease. pp. 297-323. R.S. Goodhart and M.E. Shils eds. 5th ed. Lea and Febiger, Philadelphia.

Moore, C.V., Dubach, R., Minnich, V. and Roberts, H.K. 1944. Absorption of ferrous and ferric radioactive iron by human subjects and by dogs. J. Clin. Invest. 23:755.

Morgan, O.S., Gatenby, P.B.B., Weir, D.G. and Scott, J.M. 1969. Studies on an iron-binding component in human gastric juice. Lancet 1:861.

Morris, E.R. and Ellis, R. 1976. Isolation of monoferric phytate from wheat bran and its biological value as an iron source to the rat. J. Nutr. 106:753.

Motzok, J., Pannell, M.D., Davies, M.J. and Ross, H.V. 1975. Effect of particle size on the biological availability of reduced iron. J. A.O.A.C. 58:99.

Mullen, J.D. 1978. Dietary fiber sources for human studies. Am. J. Clin. Nutr. 31 (Suppl.) : S103.

McCance, R.R. and Lawrence, R.D. 1929. The carbohydrate content of foods. Special Report Series, Medical Research Council, London.

McCollum, E.V. 1957. A History of Nutrition. Boston, Houghton Mifflin Co. 451p.

NAS. 1974. Proposed fortification policy for cereal-grain products. ISBN 0-309-02232-0. National Academy of Sciences, Washington, DC.

Nicolaysen, R. and Njaa, L.R. 1951. Investigations on the effect of phytic acid on the absorption of calcium in rats, pigs and men. Acta Physiol. Scand. 22:246.

NRC. 1979. Iron. Committee on Medical and Biological Effects of Environmental Pollutants, Division of Medical Sciences Assembly of Life Sciences, National Research Council. Univ. Parc Press, Baltimore. 248p.

Nutr. Today. 1972. March/April 7(2):2-3,4-9,9,13-16,19-24 and 28-32.

Oberleas, D., Muhrer, M.E. and O'Dell, B.L. 1966. Dietary Metal-complexing agents and zinc availability in the rat. J. Nutr. 90:56.

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

Oke, O.L. 1969. Oxalic acid in plants and in Nutrition. World Rev. Nutr. Diet. 10:262.

Pearson, W.N. and Reich, M.B. 1969. Studies of ferritin and a new iron-binding protein found in the intestinal mucosa of the rat. J. Nutr. 99:137.

Perkin-Elmer Corp. 1973. Analytical Methods for Atomic Absorption Spectrophotometry. Perkin-Elmer Corp., Norwalk, CT.

Persson, I., Raby, K., Fønt-bech, P. and Jensen, E. 1976. Effect of prolonged bran administration on serum levels of cholesterol, ionized calcium and iron in the elderly. J. Am. Gerontol. Soc. 24:334.

Peters, T., Jr., Apt, L. and Ross, J.F. 1971. Effect of phosphates upon iron absorption studied in normal human subjects and in an experimental model using dialysis. Gastroenterology 61:315.

Pileggi, V.J. 1959. Distribution of phytase in the rat. Arch. Biochem. Biophys. 80:1.

Pirzio-Biroli, G. and Finch, C.A. 1960. Iron absorption III. The influence of iron stores on iron absorption in the normal subject. J. Lab. Clin. Med. 55:216.

Pla, G.W. and Fritz, J.C. 1970. Availability of iron. J. A.O.A.C. 53:791.

_____. 1971. Collaborative study of the hemoglobin repletion test in chicks and rats for measuring availability of iron. J. A.O.A.C. 54:13.

Pla, G.W., Fritz, J.C. and Rollinson, C.L. 1976. Relationship between the biological availability and solubility rate of reduced iron. J.A.O.A.C. 59:58.

Pollack, S. and Lasky, F.D. 1976. A new iron-binding protein isolated from intestinal mucosa. J. Lab. Clin. Med. 87:670.

Pollycove, M., Saltman, P., Fish, M., Newman, R. and Tono, M. 1972. Ferric fructose absorption in man. Clin. Res. 20:183.

Price, C.A. 1968. Iron compounds and plant nutrition. Ann. Res. Plant Physiol. 19:239.

Rackis, J.J. and Anderson, R.L. 1977. Mineral availability in soy protein products. Food Prod. Develop. 11(10):38.

Ramsay, W.N.M. 1957. The determination of the total iron-binding capacity of serum. Clin. Chimica Acta 2:221.

Ranhotra, G.S. and Loewe, R.J. 1975. Effect of wheat phytase on dietary phytic acid. J. Food Sci. 40:940.

Ranhotra, G.S., Loewe, R.J. and Pergot, L.V. 1974. Effect of dietary phytic acid on the availability of iron and phosphorus. Cereal Chem. 51:323.

RDA. 1974. Recommended Dietary Allowances. 8th ed. Food and Nutrition Board National Research Council, National Academy of Sciences, Washington, DC.

Reinhold, J.G., Ismail-Beigi, F. and Faradji, B. 1975. Fibre Vs. phytate as determinant of the availability of calcium, zinc and iron of bread stuffs. Nutr. Rep. Int. 12:75.

Reinhold, J.G., Faradji, B., Abadi, P. and Ismail-Beigi, F. 1976. Decreased absorption of calcium, magnesium, zinc and phosphorus by humans due to increased fiber and phosphorus consumption as wheat bread. J. Nutr. 106:493.

Rotruck, J.T. and Luhrsen, K.R. 1979. A comparative study in rats of iron bioavailability from cooked beef and soybean protein. J. Agr. Food Chem. 27:27.

Rutkowski, A. 1977. Evaluation of soybean protein isolates and concentrates as meat additives to provide a basis for increasing utilization of soybeans. Project No. PL-ARS13. Inst. of Food Eng. and Biotechnology, Agriculture-Technical Univ. of Olsztyn, Olsztyn, Poland.

Saio, K., Emiko, K. and Watanabe, T. 1967. Protein-calcium-phytic acid relationship in soybean. Part I. Effects of calcium and phosphrus on solubility characteristics of soybean meal protein. Agr. Biol. Chem. 31:1195.

Sandstead, H.H., Muñoz, J.M., Jacob, R.A., Klevay, L.M., Reck, S.J., Logan, G.M., Jr., Dintzis, F.R., Inglett, G.E. and Shvey, W.C. 1978. Influence of dietary fiber on trace element balance. Am. J. Clin. Nutr. 31(Suppl.):S180.

Seelig, M.S. 1964. The requirement of magnesium by the normal adult. Am. J. Clin. Nutr. 14:342.

Shah, B.G. and Belonje, B. 1973a. Bioavailability of reduced iron. Nutr. Rep. Int. 7:151.

_____. 1973b. Bioassay for iron source additives. J. Inst. Can. Sci. Tech. Aliment 6(1):37.

Sheets, O., Frazier, E. and Dickins, D. 1931. Conservation of iron in vegetables by methods of preparation and cooking. Miss. Agr. Exp. Stn. Bull. 291.

Smith, A.K. and Rackis, J.J. 1975. Phytin elimination in soybean protein isolation. J. Am. Chem. Soc. 79:633.

Snedecor, G.W. and Cochran, W.G. 1976. Statistical methods. 6th ed. The Iowa State Univ. Press, Ames, Iowa. 593p.

Southgate, D.A.T. 1973. Fibre and the other unavailable carbohydrates and their effects on the energy value of the diet. Proc. Nutr, Soc. 32:131.

Southgate, D.A.T. and Durnin, J.V.G.A. 1970. Calorie conversion factors. An experimental re-assessment of the factors used in the calculation of the energy value of human diets. Brit. J. Nutr. 24:517.

Speirs, M. et al. 1944. Effect of fertilizer and environment on the iron content of turnip greens. South Coop. Ser. Bull. 2.

Spiller, G.A. and Amen, R.J. 1976. Fiber in Human Nutrition. Plenum Press, New york and London. 278p.

Spiro, T.G. and Saltman, P. 1974. Inorganic Chemistry. In : Iron in Biochemistry and Medicine. pp. 1-28. A. Jacobs and M. Worwood eds. Academic Press, London and New york.

Steinke F.H. and Hopkins, D.T. 1978. Biological availability to the rat of intrinsic and extrinsic iron with soybean protein isolates. J. Nutr. 108:481.

Stiles, L.W., Rivers, J.M., Hackler, L.R. and Van Campen, D.R. 1976. Altered mineral absorption in the rat due to dietary fiber. Fed. Proc. 35:744.

Theuer, R.C., Kemmerer, K.S., Martin, W.H., Zoumas, B.L. and Sarett, H.P. 1971. Effect of processing on availability of iron salts in liquid infant formula products- Experimental soy isolate formula. J. Agr. Food Chem. 19:555.

Thomas,F.B., McCullough, F.S. and Greenberger, N.J. 1972. Effect of phenobarbital on the absorption of inorganic and hemoglobin iron in the rat. Gastroenterology 63:590.

Trowell, H. 1974. Definitions of fibre. Lancet 1:503.

Turnbull, A. 1974. Iron absorption. In : Iron in Biochemistry and Medicine. pp.370-404. A. Jacobs and M. Worwood eds. Academic Press, London and New York.

Turnbull, A., Cleton, F., Finch, C.A., Thompson, L. and Martin, J. 1962. Iron absorption IV. The absorption of hemoglobin iron. J. Clin. Invest. 41:1897.

Underwood, E.J. 1977. Iron. In : Trace Elements in Human and Animal Nutrition. pp.13-55. 4th ed. Academic Press, New York, San Francisco and London.

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

Van Soest, P.J. 1965. Use of detergents in analysis of fibrous feeds III. Study of effects of heating and drying on yield of fiber and lignin in forages. J. A.O.A.C. 48:785.

_____. 1973a. Collaborate study of acid-detergent fiber and lignin. J. A.O.A.C. 56:781.

_____. 1973b. The uniformity and nutritive availability of cellulose. Fed. Proc. 32:1804.

Van Soest, P.J. and Wine, R.H. 1967. Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell-wall constituents. J. A.O.A.C. 50:50.

_____. 1968. Determination of lignin and cellulose in acid - detergent fiber with permanganate. J. A.O.A.C. 51:780.

Vohra, P., Gray, G.A. and Kratzer, F.H. 1965. Phytic acid - metal complexes. J. Soc. Exp. Biol. Med. 120:447.

Waddell, J. 1973. The bioavailability of iron sources and their utilization in food enrichment. LSRO/FASEB Pub. PB-224122. Nat. Tech. Info. Service, U.S. Dept. of Commerce.

_____. 1974. Bioavailability of iron sources. Food Prod. Develop. 8(2):80.

Wang, C.H., Willis, D.L. and Loveland, W.D. 1975. Nuclear statistics. In : Radiotracer Methodology in the Biological, Environmental and Physical Sciences. pp.294-309. Prentice-Hall, Inc. Englewood Cliffs, New Jersey.

Webling, D.D A and Holdsworth, E.S. 1966. Bile and the absorption of strontium and iron. Biochem. J. 100:661.

Wein, E.M., Van Campen, D.R. and Rivers, J.M. 1975. Factors affecting the concentration and bioavailability of iron in turnip greens to rats. J. Nutr. 105:459.

Welch,R.M.and Van Campen, D.R. 1975. Iron bioavailability to rats from soybeans. J. Nutr. 105:253.

Welch, R.M., House, W.A. and Van Campen, D. 1977. Effects of oxalic acid on availability of zinc from spinach lesves and zinc sulfate to rats. J. Nutr. 107:929.

Wheby, M.S., Conrad, M.E., Hedberg, S.E. and Crosby, W.H. 1962. The role of bile in the control of iron absorption. Gastroenterology 42:319.

Wheby, M.S., Jones, L.G. and Crosby, W.H. 1964. Studies on iron absorption. Intestinal regulatory mechanisms. J. Clin. Invest. 43:1433.

Whitehead, J.S.W. and Bannerman, R.M. 1965. Absorption of iron by gastrectomized rats. Gut 5:38.

WHO. 1968. WHO Group of Experts on Nutritional Anaemias. World Health Organization. Geneva, Switzerland. Tech. Rep. Ser. 405.

____. 1972. WHO Group of Experts on Nutritional Anaemias. World Health Organization. Geneva, Switzerland. Tech. Rep. Ser. 503.

Worwood, M., Edward, A. and Jacobs, A. 1971. Non-ferritin iron compound in rat small intestinal mucosa during iron absorption. Nature 229:409.

APPENDIX

Appendix I. Source of dietary ingredients.

Dietary Ingredients	Description	Source
D-glucose monohydrate		J. T. Baker Chemical Co., Philipsburg, NJ.
Ferrous sulfate	$FeSO_4.7\ H_2O$	Ibid
Casein		U. S. Biochemicals Co., Cleveland, OH
Corn oil		Ibid
Cellufil	Hydrolyzed	Ibid
Pectin	Citrus, polygalacturonic acid methylester	Ibid
Fish	Freeze dried turbot-Arrowtooth flounder (Atheresthes stomias) fillets without skin, bone or offal.	Obtained fresh Astoria, OR
Fish oil	Rendered from turbot fillets.	Ibid
Wheat bran	Food grade, ground	American Association of Cereal Chemists, Certified RO 7-3691
Spinach	Commercial freeze-dried	Oregon Freeze Dried Foods, Inc., Albany, OR
Soybean isolate	Supro-610	Ralston Purina Co., St. Louis, MO
Lignin	Orzan G, lignin sulfonate	Crown Zellerbach Corp., Camas, WA
Phytic acid	Inositol hexaphosphoric acid from corn type V sodium salt	Sigma Chemical Co., St. Louis, MO
Oxalic acid	Sodium salt crystalline	Ibid

Appendix II. Counter settings for measuring ^{59}Fe radioactivity in Model 446 Armac whole body liquid scintillation detector.

Item	Settings
1. Main power	H.V.
2. High voltage switch	On
3. High voltage adjust coarse	3
4. High voltage adjust fine	0
5. High voltage indicating meter	1425 volts
6. Timer switch	1 min
7. Mode selector switch	Auto
8. Spectrophotometer display switch	Red/Green
9. Sample changer switch	Repeat
10. Lower discriminator control	100
11. Upper discriminator control	700
12. Gain control	25 percent
13. Approx. background	2600 cpm
14. Counter efficiency	38 percent

Appendix III. Preparation of Drabkin's and cyanmethemoglobin standard solutions.

A. Drabkin's reagent, Stock No. 525-2

Contains a dry mixture, 1.25 g, consisting of sodium bicarbonate, 100 parts, potassium ferricyanide, 20 parts, and potassium cyanide, 5 parts. Store in dark at room temperature.

B. Brij-35 solution, Stock No. 430 AG-6

Contains Brij-35, 30 g/100 ml. Store at room temperature.

C. Hemoglobin standard, Stock No. 525-18

Contains lyophilized human methemoglobin, 36 mg. Store in refrigerator at 0-5^{o}C.

D. Drabkin's solution

Reconstitute Drabkin's reagent, stock No. 525-2, with 1000 ml water. Add 0.5 ml 30% Brij-35 solution, stock No. 430AG-6, and mix well. Store in amber bottle at room temperature. (Stable for at least 6 months.)

E. Cyanmethemoglobin standard solution

Reconstitute vial of hemoglobin standard, stock No. 525-18, with 50.0 ml Drabkin's solution. Mix well and allow to stand for at least 30 minutes. Store in refrigerator at 0-5^{o}C (stable for at least 6 months).

Appendix IV. Preparation of reagents used in oxalate determination.

Tungstophosphoric acid reagent

Dissolve 2.5 g sodium tungstate in mixture of 4 ml H_3PO_4 and 50 ml water and dilute to 100 ml with water.

Buffer solution

pH 4.5. Dissolve 2.5 g anhydrous calcium chloride in 50 ml glacial acetic acid (1 + 1). Dissolve 33 g sodium acetate in water and dilute to 50 ml with water. Mix solution (1 + 1).

Washing liquid

Dilute 12.5 ml glacial acetic acid to 250 ml with water. Add pulverized calcium oxalate, shake, and let stand. Repeat addition and shaking until solution is saturated. Cool to $+4^{o}C$ to avoid supersaturation and store in refrigerator. Just before use, filter portion to be used. Keep cold during filtration and use.

Calcium solution

0.05 mg/ml. Slurry 2.497 g calcium carbonate (primary standard grade) into 1 L volumetric flask with 300 ml water. Carefully add 10 ml concentrated HCl. After CO_2 is completely released, dilute to volume with water. Dilute 5 ml solution above to 100 ml with water.

Lanthanum solution

5%. Wet 5.865 g La_2O_3 with water, very slowly add 25 ml concentrated HCl until material is dissolved, and dilute to 100 ml with water.

Standard calcium solutions

Pipet 0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 ml of calcium solution into individual 50 ml volumetric flasks. Pipet 10 ml lanthanum solution into each flask and dilute to volume with water.

APPendix V. Theoretical versus actual dietary iron and Kjeldahl nitrogen protein in test diets.

Diet No.	Iron Theoretical (µg/g)	Iron Actual[1] (µg/g)	Kjeldahl N Protein[1,2] Actual (%)
1	-	7.0	18.00
2	6.25	12.7	17.92
3	12.5	18.0	18.10
4	25.0	30.3	18.20
5	25.0	32.0	18.23
6	25.0	33.9	18.56
7	25.0	28.1	18.06
8	25.0	28.7	17.98
9	25.0	28.8	17.95
10	25.0	32.4	18.17
11	25.0	30.3	18.02
12	25.0	25.0	17.96
13	25.0	28.1	17.95
14	25.0	28.8	18.06
15	25.0	29.4	18.50
16	25.0	26.2	17.53
17	14.5	14.8	16.76
18	14.0	16.3	17.94
19	5.2	9.4	19.5
20	35.0	45.0	18.00

[1]Mean value of duplicate determinations.

[2]Diets formulated to contain 17.13% Kjeldahl N protein (theoretical).

Appendix VI. Whole body counts per minute of ^{59}Fe retention in rats over 5 days.

Diet No.	0 Hrs.	15 Hrs.	39 Hrs.	62 Hrs.	86 Hrs.	110 Hrs.
5	469250±168454[1] (100)[2]	348832±155956 (73±11)	286558±105993 (62±8)	276341±100315 (60±8)	269785±97900 (58±7)	269128±98392 (58±8)
6	623217±125588 (100)	500852±112209 (80±9)	376229±100643 (61±8)	349924±86916 (56±8)	338018±84161 (54±8)	337031±82135 (54±8)
12	508067±227269 (100)	435787±203859 (85±5)	393449±173708 (77±3)	376792±165416 (74±2)	367199±158293 (73±3)	366086±155217 (73±3)
13	517450±100862 (100)	466258±107204 (90±3)	420048±85819 (81±4)	400852±80009 (78±4)	395499±83606 (76±4)	391317±78590 (76±5)
17	360150±203885 (100)	343717±190002 (96±4)	315207±164313 (90±6)	299499±153234 (85±7)	288845±147680 (82±7)	280993±144496 (80±7)
18	467400±144969 (100)	456853±150495 (97±5)	394987±110313 (86±7)	372441±98658 (81±9)	352309±86614 (77±11)	349557±86150 (77±10)
20	478783±123380 (100)	446152±124009 (93±4)	369020±110251 (77±9)	334774±92235 (70±8)	299088±61563 (64±8)	292375±60937 (62±8)

[1]Mean ± SD in counts per minute.

[2]Mean ± SD in percent counts per minute.