

From the Departments of Nutritional Research and Prosthetic Dentistry
and the Biophysics Laboratory, Umeå University, Umeå, Sweden

Iron Absorption and Iron Status Related to Diet - An Experimental and Epidemiological Study.

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av rektorsämbetet
vid Umeå Universitet för avläggande av doktorsexamen i odontologisk vetenskap
kommer att offentligen försvaras i sal B, Tandläkarhögskolan
9 tr, fredagen den 29 september 1995, klockan 09.00

av

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Umeå 1995

Abstract

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The absorption of iron from the diet is a major determinant of the iron status of an individual. Accurate measures of iron absorption are thus essential in the determination of the effects of diet composition on iron absorption and status.

The aim of these studies have firstly been to investigate different methods to measure iron absorption and effects of diet in both rats and humans. Secondly to describe variations in iron status in a general population and to relate the variations to diet.

Experimental studies in rats: The most important factors determining the availability of iron from the diet to growing rats appears to be the concentration of iron in the diet and the iron status of the rats. The type of cereal grain in the diet and the diet phytate and fiber concentrations were of far less importance. The absorption of ^{59}Fe from test meals, the iron balance over a period of a few days and the blood hemoglobin and serum and liver iron concentrations were in good general agreement on the effects of diet and iron status on the bioavailability of iron from grain diets. The extent of coprophagy in rats is not affected by diet or iron status and did not affect iron absorption appreciably.

Effect of dairy products on iron absorption in man: The effect of milk and fermented milk on iron absorption was studied in nine ileostomy subjects. We found no decrease in iron absorption during two three-week periods on a high calcium (milk) diet. The results of the present study differ from those studies in which the absorption of radioiron from test meals was measured, which have shown a pronounced effect of high calcium levels. We believe that these differences in results were caused by differences in experimental design and choice of measurement method. Our results support earlier findings that the iron status of the subject rather than the bioavailability of dietary iron is the major factor determining absorption of dietary non-heme iron.

Measurement of iron absorption from single meals and daily diets in humans using radioiron: Ten ileostomy subjects were given the same composite diet for all three meals each day for five consecutive days (meal proportion ratio 1:2:4 for breakfast, lunch and dinner respectively). The iron absorption from a low-fiber diet measured from the morning meals (^{59}Fe) was almost 80% higher than the average iron absorption measured from all meals (^{59}Fe) during the last two days on this diet. The iron absorption from a high fiber diet eaten at breakfast was almost 50% higher than the daily average absorption. This suggests that all meals of the day should be labeled with radioiron and not just the morning meal.

Population studies of diet and iron status: In a randomly selected population in Northern Sweden lower iron status was associated with a high intake of dairy products in women (25-44 yr.). The striking result was however that iron status was not related to other major variations in dietary intake, particularly intake of energy and iron. No correlation could be demonstrated between iron status measured as ferritin, serum iron or TIBC and the estimated intakes of calcium, fiber, ascorbic acid, meat, tea or coffee, all of which are known to be factors which influence the bioavailability of iron. On the population level, factors other than diet, e.g. menstruation and metabolic factors, were more important than diet as determinants of iron status.

Key words: iron status, iron absorption, bioavailability, dietary fiber, phytate, calcium, radioiron, diet.

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by

Per Tidehag



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ISBN 91-7197-079-4

Printed in Sweden by
Nyheterernas Tryckeri KB
Umeå 1995

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To myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble, or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

SIR ISAAC NEWTON

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Key words: iron status, iron absorption, bioavailability, dietary fiber, phytate, calcium, radioiron, diet.

Preface

This thesis is based on the following original articles. In this summary, they will be referred to by their Roman numerals.

- I Moberg Wing, A., Wing, K., Tidehag, P., Hallmans, G. and Sjöström, R.
The effect of the iron status in rats on the accumulation of cadmium from diets with and without wheat bran. *Nutrition Research* 12: 1205-1215, 1992.
- II. Wing, K., Wing, A., Tidehag, P., Hallmans, G., Sunzel, B. and Sjöström, R.
The availability of iron, zinc and cadmium to rats from composite diets with different cereal grains. *Accepted for publication in Nutrition Research* 1995.
- III. Tidehag, P., Hallmans, G., Sjöström, R., Sunzel, B., Wetter, L. and Wing, K.
The extent of coprophagy in rats with differing iron status and its effect on iron absorption. *Laboratory Animals* 22: 313-319, 1988.
- IV. Tidehag, P., Sandberg, A-S., Hallmans, G., Wing, K., Türk, M., Holm, S. and Grahn, E. The effect of milk and fermented milk on iron absorption and iron status in ileostomy subjects. *Accepted for publication in American Journal of Clinical Nutrition* 1995.
- V. Tidehag, P., Hallmans, G., Wing, K., Sjöström, R., Ågren, G., Lundin, E. and Zhang, J- X. A comparison of iron absorption from single meals and daily diets using radioiron (^{55}Fe / ^{59}Fe). *Accepted for publication in British Journal of Nutrition* 1995.
- VI. Tidehag, P., Johansson, I., Lindahl, B., Asplund, K. and Hallmans, G.
Iron status related to age, sex, diet and metabolic factors. A population-based study. *Submitted for publication in International Journal of Epidemiology*.

Introduction

In the evolution of human society, the use of metals in making both objects of art and objects of practical importance passed through several ages: gold and copper were succeeded by bronze which was succeeded by iron. The significance of iron is perhaps best summarized in the words of Rudyard Kipling as cited by Philip Aisen (1977):

"Gold is for the mistress - silver for the maid -
Copper for the craftsman cunning at this trade!
Good! Said the Baron, sitting in his hall,
But **iron** - Cold **iron** - is master of them all".

However, this book is not about the iron age or the importance of the Celts in its development but it is concerned with the importance of iron in human nutrition. Iron is the fourth most common element and the second most common metal in the earth's crust. It is believed that large amounts of reduced iron (Fe^{2+}) were available in the atmosphere during the earlier chemical evolution of the last 500 million years. Iron then probably functioned mostly as a catalyst in protein or metal-protein complexes. Presently iron is closely involved in the transport of oxygen and carbon dioxide, and also in various tissue enzymes such as cytochromes and enzymes necessary for function of the immune system in the body. The importance of iron may lie in its ability to interchange between two different stable forms, ferrous (Fe^{2+}) and ferric (Fe^{3+}), and its unique capacity to form complexes having redox potentials that are highly coordination-dependent (Neilands 1991). This ability might however also be rather harmful since the single-electron transfer reactions of iron can lead to the production of harmful free radicals and their derivatives such as peroxide, superoxide and the hydroxyl radical (Weinberg 1989, Ramdath & Golden 1989). To protect against this, a very intricate system of iron-binding proteins including transferrin, ferritin and hemosiderin is active during transport and storage of iron (Crichton & Charleoteaux-Wauters 1987).

The iron in our present day environment is almost exclusively found in the oxidized state (Fe^{3+}) which is not readily accessible to man while other forms of life, such as plants, have developed mechanisms to reduce this highly insoluble and unavailable form of iron into the more usable Fe^{2+} . While man has a very limited capacity to absorb iron from the diet he also has a unique ability to conserve body iron. Thus only a small fraction of the iron normally present in the diet must be absorbed each day. Despite this low requirement, iron overload is rare and iron deficiency is very common, especially in certain parts of the world and in particular age groups (Expert Scientific Working Group 1985). Heme iron, which is found mostly in meat, is absorbed well while nonheme iron, which is found mainly in plant foods, is absorbed differently. Meat has, however, almost disappeared from the diet of many people, especially in developing countries but also in the Western parts of the world. It is the iron in most vegetable staples which is particularly inaccessible and it may be that current nutritional

problems are due to the agricultural revolution over the last ten thousand years and man's genetic constitution which is still unchanged from the time when he was a hunter.

Iron metabolism

The new-born infant has a total of about 250 mg iron in the body. The average adult has about 3-5 grams, depending on body size and gender. Females have on the average less iron, about 35 mg/kg b.w., as compared to males who have about 50 mg/kg b.w. Of this, approximately two-thirds is utilized as functional iron such as that in hemoglobin (60%), myoglobin (5%) and various heme and nonheme enzymes (5%). The remainder is found in storage as ferritin (20%) and hemosiderin (10%).

The major pathway of iron metabolism is a nearly closed cycle in which iron passes from plasma transferrin to the red blood cell (RBC) precursors in the bone marrow where it is incorporated into hemoglobin. Mature RBCs then enter the circulation where they remain nearly 4 months before they age metabolically and are engulfed by phagocytes. Iron which is then released from hemoglobin returns to plasma transferrin, thus completing one cycle and beginning another. From this iron cycle, small quantities of iron are diverted for use in other iron compartments. A slow, continuous exchange takes place between the storage and transport compartments. Normally, very small quantities of iron are present in most cells of the body, in plasma and in other extracellular fluids, and the body rigorously conserves its iron.

Iron Compartments

Hemoglobin

Approximately 2.5 g of iron is in hemoglobin, an iron protein that contains 0.34% iron by weight. Normally, virtually all hemoglobin iron is contained within RBCs or their precursors in the bone marrow.

Transport

Iron is distributed in the body by the plasma. Normally, there is a total of approximately 2.5 mg of iron in plasma. The majority of the plasma iron is bound to a specific transport protein called transferrin and this fraction is usually called "plasma iron" or "serum iron". Hemoglobin is also present in plasma as a result of intravascular hemolysis or the lysis of red cell precursors in the marrow. This is usually about 2% of the total plasma iron. A small amount (<1%) plasma iron is also present in ferritin, the concentration of which correlates well with the total body iron stores.

Transferrin

Transferrin is synthesized mostly in the liver (Lane 1967). The main role of transferrin in serum is to transport iron to the erythroid marrow for hemoglobin synthesis and to other body tissues as well. Transferrin also minimizes the loss of iron from the body by conserving any surplus by deposition in tissues specially adapted for iron storage. The transferrin molecule has two iron binding sites. Each of these sites may bind one Fe^{3+} . Normally over 90% of the transferrin bound iron is supplied to the erythron (prestages of erythrocytes) for incorporation into hemoglobin (Fairbanks & Beutler 1990). Transferrin has a half-life of about 8 days in man (Awai & Brown 1963).

The iron-transferrin complex binds to specific transferrin receptors on the cell surface. It is then internalized and the iron is released from transferrin in the cytosol. The number of transferrin receptors increases in the presence of iron deficiency and decreases in iron excess. The most important clinical application for the transferrin-receptor assay is in distinguishing iron deficiency anemia from chronic inflammatory disease.

The transferrin concentration is usually estimated by determining the maximum iron-binding capacity of the plasma. This is called the total iron binding capacity, TIBC. The serum TIBC varies in disorders of iron metabolism. It is often increased in iron deficiency and decreased in chronic inflammatory disorders, malignancies and also often in hemochromatosis. Because normally only one-third of the iron-binding sites of transferrin are occupied by Fe^{3+} , serum transferrin has considerable reserve iron binding capacity. This is called the serum unsaturated iron binding capacity (UIBC). The serum iron concentration shows the Fe^{3+} bound to serum transferrin and does not include the iron contained in serum as free hemoglobin. Thus, the serum iron concentration as measured by standard laboratory techniques can, for all practical purposes be taken as representing the transferrin iron concentration. In normal individuals there is considerable day-to-day variation in the serum iron concentration. It is decreased in many but not all patients with iron deficiency anemia and in chronic inflammatory disorders such as acute infection. Acute or recent hemorrhage, including that due to blood donation also results in low serum iron concentration.

Hemoglobin iron

A small amount of hemoglobin (not counting the hemoglobin incorporated into the RBCs) is normally present in plasma as a result of erythrocyte degradation. It is bound to haptoglobin, transported to the liver and taken up by the hepatocytes (Hershko *et al.* 1972), where it is broken down and stored or returned to the plasma for reutilisation (Garby & Noyes 1959).

Plasma ferritin

With the development of sensitive immunoradiometric assays, a small amount of ferritin was shown to be present in the plasma of normal individuals and a close relationship was demonstrated to exist between the size of the body iron stores and the plasma ferritin

concentration. There are two conditions in which the plasma ferritin concentration is increased out of proportion to the size of the tissue iron stores. One of these is inflammation, whether related to infection or neoplasia (Jacobs & Worwood 1975). The other is associated with liver disease, in which an inflammatory component may be added to hepatocyte damage

Storage

Ferritin

Ferritin is the major iron storage protein. It consists of an outer apoferritin shell with a central ferric oxyhydroxide (FeOOH) crystalline core. The core crystal may maintain as many as 4500 iron atoms, but usually contains less than 3000 atoms. The oxidation (uptake) or reduction (release) of iron takes place very rapidly, which means that ferritin is both a very efficient iron trap and a good source of iron for metabolic requirements. It is found in nearly all cells of the body, although predominantly in the hepatocytes of the liver and in the macrophage system of the bone marrow.

Hemosiderin

The other form of storage iron is partially deproteinized ferritin called hemosiderin. As opposite to ferritin it is insoluble in aqueous solution. Iron is only slowly released from hemosiderin, probably because it occurs in relatively large aggregates and therefore has a much smaller surface/volume ratio. Like ferritin, hemosiderin is primarily located in the cells of the reticulo-endothelial system, especially in the liver, spleen and bone marrow as well as skeletal muscle.

Tissue iron

Myoglobin very closely resembles a subunit of hemoglobin. It is mainly found in muscle tissue and is involved both in oxygen transport and storage. Total myoglobin iron content is normally approximately 130 mg.

Iron status, iron deficiency and iron overload

Iron status is best assessed using a combination of measures. Iron deficiency is usually associated with one of the following: decreased serum ferritin concentrations, serum iron concentration or serum transferrin saturation ($= 100 \times \text{serum iron} \div \text{TIBC}$), or blood hemoglobin concentration and/or increase erythrocyte protoporphyrin concentration or serum total iron binding capacity. Each can be classified either as an indicator of iron in the storage compartment (serum ferritin concentrations and total iron binding capacity) or as an indicator of iron in the functional compartment (erythrocyte protoporphyrin concentration, serum transferrin saturation, and blood hemoglobin concentration). In Table 1 are reference intervals for some common iron parameters given:

Table 1. Reference intervals for some common iron status parameters.

	men	women
serum ferritin	20-300 $\mu\text{g/L}$	15-200 $\mu\text{g/L}$
serum iron	13-36 $\mu\text{mol/L}$	10-29 $\mu\text{mol/L}$
blood hemoglobin	130-170 g/L	115-150 g/L
serum TIBC	46-70 $\mu\text{mol/L}$	46-70 $\mu\text{mol/L}$

Iron deficiency is more common among infants, children, teenagers and women of childbearing age, while healthy adult males are rarely iron deficient. Deficiency is caused by several factors, usually by a combination of increased need (rapid growth in the young population, menstruation and pregnancy in fertile women) and insufficient uptake, which in turn may depend on other factors such as a decreased caloric intake and/or a larger fraction of calories deriving from food ingredients which contain less easily absorbed iron (nonheme iron).

The earliest stage of iron deficiency is characterized by loss of storage iron (as indicated by ferritin) and is called iron depletion or prelatent iron deficiency. The concentrations of serum iron and the iron-carrying serum protein transferrin are normal at this stage. When iron stores are exhausted (serum ferritin < 12 $\mu\text{g/L}$), serum iron decreases and serum transferrin, which is usually measured as total iron-binding capacity (TIBC), increases. If the hemoglobin concentration is still normal, this stage is called iron deficiency without anemia or latent iron deficiency. When the iron stores are completely exhausted, there is not sufficient iron for hemoglobin synthesis. This final stage is called iron deficiency anemia or manifest iron deficiency.

Symptoms frequently associated with iron deficiency anemia include palor, weakness, fatigue, dyspnea, palpitations, sensitivity to cold, abnormalities in the oral cavity and gastrointestinal tract, and reduced capacity for work (Conrad 1987). It further appears that even mild/prelatent iron deficiency may have significant health consequences which can be attributed to decreases in essential body iron and limitations in tissue oxidative capacity (Baynes & Bothwell 1990, Cook 1990, Cook & Lynch 1986).

Iron overload is associated with increases in non-protein bound iron resulting from the physiologic iron-binding capacity being overwhelmed (Weinberg 1989). Disadvantages with overload are for example increased risk for bacterial infection and cardiomyopathy. Overload can result from inborn errors in metabolism leading to hyperabsorption of iron or inadequate synthesis of the iron-binding proteins. Hereditary hemochromatosis is preeminent as a cause of morbidity and mortality among genetic diseases (Weinberg 1989). Surveys in several industrialized countries have indicated that 0.3 to 0.88% are homozygous for this disease while the frequency of heterozygotes is 10 to 16% (Carpenter & Mahoney 1992). Overload can also result from excessive absorption of dietary iron due to various causes including chronic ingestion of greater than adequate amounts of dietary iron, especially heme iron (Weinberg 1989). These observations concerning the prevalence of iron overload have also

raised the question as to whether or not general fortification of food with inorganic iron is beneficial (Weinberg 1989).

Iron requirements

Up to 40 mg of iron are required each day by the body, mainly to replace hemoglobin that has been destroyed. Obligatory losses also include iron lost by sloughing off of cells from different epithelial surfaces in the body, such as skin, gastrointestinal tract and urinary tract. Fortunately recycling of iron is so efficient that only 1 to 1.5 mg is lost from the body of the average adult each day and needs to be provided by intestinal absorption.

During infancy the requirements are about 0.5 mg/day, increasing to 0.8 mg/day in childhood and about 1.6 mg/day for boys and 1.4 mg/day for girls during adolescence (Bothwell *et al.* 1979). Studies in adult males have shown iron losses to be about 12-14 μ g/kg b.w./day in subjects with adequate iron status (Green *et al.* 1968). One blood donation of 400-500 ml blood per year represent an increased iron requirement of about 0.5 mg/day.

In fertile women, losses due to menstruation must be added to the obligatory losses. These losses are rather constant in the individual woman but show large variations among women (Hallberg & Nilsson 1964). The amount of iron lost is between 0.45 mg and 1.0 mg/day (Bothwell *et al.* 1979) and this difference may be the major factor determining the variation in iron requirements and iron stores in the female population. While there are no menstrual losses during pregnancy, the total increased need for iron during pregnancy is about 1000 mg. Most of this iron is needed during the latter half of pregnancy (Hallberg *et al.* 1966). This increased need results in a physiological increase in iron absorption which, under normal circumstances, is large enough to meet the increased demands, provided that the dietary intake is sufficient (Barrett *et al.* 1994).

The diet is the sole source of iron to the body and it must replace all losses as well as provide iron for growth and the establishment of a reserve store. In order to absorb this amount of iron (1-1.5 mg) from the diet, a daily dietary intake of about 10-20 mg/day is needed. The absorbed amount depends to a large extent on the availability of the iron in the diet, often called bioavailability. Lately the expression nutrient density has been used to express the adequacy of a diet with respect to specific nutrients (Windham *et al.* 1983). With iron, the most useful estimator of nutritional adequacy is, however, not total iron density but rather the density of bioavailable iron in the diet (Hallberg 1981a-b). In Table 2, the density of bioavailable iron (mg/1000 kcal) necessary to provide the required iron is presented based on data for energy intake in different sex and age segments of the population (Carpenter & Mahoney 1992). It is however important to keep in mind that many factors exert influence on the absorptive process and that iron bioavailability will be the result of the summation of these effects.

Table 2. Human iron requirements. From Carpenter and Mahoney (1992)

Population segment (sex and age)	Daily requirement		
	Iron (mg)	Energy (kcal)	Bioavailable iron (mg/1000 kcal)
Males			
1-10 years	1,0	1800	0,56
11-18 years	1,2	2750	0,44
19 + years	1,0	2800	0,36
Females			
1-10 years	1,0	1800	0,56
11-18 years	1,5	2200	0,68
19-50 years	1,5-2,4	2200	0,68-1,09
50 + years	1,0	1900	0,53
Pregnant	4,0-6,0	2500	1,6-2,4

Iron absorption

Early observations by McCance and Widdowson (1937) led to the conclusion that the mechanisms controlling iron balance are active mainly via absorption as the capacity of the body to excrete iron is very limited. Iron absorption is often increased in iron-deficient individuals. Several other mechanisms are also active in regulating iron status including the reutilization of iron from hemoglobin and the use of stored excess iron in ferritin.

In order to discuss the absorption of dietary iron, it is helpful to classify the many different chemical forms of iron found in the diet into two groups, nonheme and heme iron, because of the differences in their mechanisms of absorption.

Nonheme iron

Most of the nonheme iron in our diet originates from plant foods, but also approximately 40-50% of the iron in meat is nonheme iron. The common oxidation states of iron are either ferrous (Fe^{2+}) or ferric (Fe^{3+}). It is generally agreed that the ferrous iron is readily absorbed while the ferric iron is poorly absorbed as it slowly forms large polynuclear complexes with hydroxide ions, water and other anions present in the diet. At acid pH, the redox potential for iron in aqueous solutions favors the ferrous state and at higher pH the equilibrium favors the ferric state. The low pH in the stomach reduces a proportion of the nonheme iron in ferric state to the ferrous state. A major role is played by the hydrochloric acid in the gastric juice (Schade *et al.* 1968). The importance of this gastric phase has long been recognized, and maximal nonheme iron absorption has been demonstrated to occur when iron is injected directly into the stomach as compared to the duodenum or jejunum (Rhodes *et al.* 1968).

It has been demonstrated that all nonheme iron in the different ingredients in a meal forms a common pool of iron in the stomach (Hallberg & Björn-Rasmussen 1972). This common-pool-concept is based on a series of studies (Björn-Rasmussen *et al.* 1974, Hallberg &

Björn-Rasmussen 1972) where it was found that the absorption of radioiron from an intrinsically labeled single food item in a meal was almost identical to the iron absorption from the same food item labeled extrinsically. This indicates that all available nonheme iron in the diet forms a common pool in which a free exchange of all iron occurs. This complete exchange takes place at low pH in the stomach where nonheme iron is soluble. Nonheme iron entering this common pool will form complexes with other food constituents (phytate, fiber etc.), form chelates with various ligands (ascorbic acid, citrate etc.) and possibly bind to digestive components such as gastrin or mucin. Some of these components will enhance while others will inhibit iron absorption (Baynes & Bothwell 1990, Conrad *et al.* 1991, Hallberg 1981b, Torre *et al.* 1991). The more important of these food components will be discussed in greater detail below.

Mechanisms of nonheme iron uptake in the intestine

Iron balance is regulated primarily by keeping the absorptive process in harmony with the body requirements. This is a very complex process and the mechanisms involved in mucosal uptake of dietary iron and the transfer through the mucosal cell into plasma are only partly understood. A transferrin-receptor mechanism is believed to mediate the transport of iron into non-intestinal cells. A number of different mechanisms for the transfer of iron from the intestinal lumen to plasma via the mucosal cells have been proposed on the basis of the results of different studies.

Hahn *et al.* (1943) demonstrated using radioiron that the absorption of iron was regulated by some type of mucosal receptor which prevented absorption when it became saturated with iron. The receptor was believed to be ferritin and this hypothesis was supported by the fact that the protein was demonstrated electron microscopically to be present in the mucosal cells of animals with large iron stores. However, immunological studies of the quantity of apoferritin within intestinal mucosa showed that this was incorrect (Johnson *et al.* 1983).

Transferrin has been suggested to act as a shuttle protein which is secreted into the intestinal lumen, binds iron and then reenters the cell (Huebers *et al.* 1983). This hypothesis was questioned, however, when it was demonstrated that the gene for transferrin not was expressed in duodenal mucosal cells (Pietrangelo *et al.* 1992) and neither transferrin nor transferrin receptors could be found in the apical portion of the intestinal cells where iron-binding substances have been demonstrated (Diponkar *et al.* 1986).

Recently, another model for protein regulation of iron absorption was proposed by Conrad *et al.* (1994), who demonstrated that intestinal mucins can bind multiple iron molecules at acid pH (Conrad *et al.* 1991). In the iron-mucin complex that is formed, iron remains soluble even when the pH is raised, thus making the iron more available for absorption by the absorptive intestinal cells. Different proteins have been suggested to bind to the iron from the intestinal lumen when it has been transported into the cell and then serve as a carrier protein within the cell. Two examples are mobilferrin (Conrad *et al.* 1991) which has been shown to be biochemically different from both ferritin and transferrin, and aconitase

(Beinert & Kennedy 1993) a two-faced protein functioning as both an enzyme and an iron regulatory factor.

Transferrin receptors located on the basolateral membranes of the absorptive cell have been suggested to permit iron to enter the cell from plasma similar to cells in other organs, thereby regulating iron uptake (Diponkar *et al.* 1986). When iron is absorbed in excess, ferritin synthesis is stimulated and the iron is deposited in ferritin to prevent damage to the cell from ionic iron (Kuhn 1991). At very high concentrations in the intestinal lumen, the cells ability to regulate iron absorption is overwhelmed and the amount of iron absorbed continues to increase with the concentration of the administered iron. This can be seen in acute iron poisoning following an overdose of oral iron.

In rats the transfer of iron through the mucosal cell is measurable within 15 seconds after the introduction of iron into the intestinal lumen (Wheby *et al.* 1964). In humans the maximum rate of transfer occurs during the first 30 minutes and continues at a much slower rate for 12-24 hours (Hallberg & Sölvell 1960).

The major site of absorption of iron is in the small intestine, where the most active site of absorption is the duodenum and the upper jejunum (Weby 1970). In this part of the intestine, the pH rises toward neutral, and ferric iron will undergo hydrolysis and precipitate irreversibly (Spiro & Saltman 1974). The absorption rate is lower in the more distal part of the intestine. This is probably caused both by an increase in pH and by different properties of the intestinal mucosa (Dowley *et al.* 1960). A slower transit time in the distal parts of the intestine might to some extent compensate this. It has also been shown that drugs which prolong the transit time also cause increased iron absorption in rats, probably by slowing intestinal motility and by increasing the duration of exposure of luminal iron to absorptive cells (Schade *et al.* 1969).

Heme iron

The main sources of heme iron in the diet are hemoglobin and myoglobin in blood products or meat. Heme iron is absorbed by the intestinal mucosa as the intact heme complex after it has been released from the globin. In vivo studies have shown that the release from globin occurs in the duodenum due to the action of duodenal enzymes, especially trypsin (Wheby *et al.* 1970). Heme iron is not freed from the porphyrin ring within the intestinal lumen but it is released within the mucosal cell where the porphyrin ring is broken down by heme oxygenase (Raffin *et al.* 1974).

Heme iron is absorbed very effectively as it is not subject to influence from other nutrients to the same extent as is nonheme iron. The splitting of heme is the limiting step in the absorption of heme iron and the absorption is fairly constant, about 20-25% in both iron-deficient and iron-replete subjects (Hallberg & Rossander 1982a, Hallberg *et al.* 1979a).

Dietary factors enhancing nonheme iron absorption

Below a number of substances which have been found to either enhance or inhibit iron absorption will be presented. The results of different studies are in many cases contradictory most likely due to differences in models, methods and/or study design. These differences will be further discussed in the Results and Discussion section.

Ascorbic acid

Ascorbic acid appears to be the factor which is most potent in enhancing the absorption of nonheme iron in single-meal studies. This was first reported by Moore and Dubach (1951) and later by several other authors (Hallberg *et al.* 1986, Hashimoto *et al.* 1992, Hunt *et al.* 1990). There are two mechanisms for this effect of nonheme iron on absorption. Firstly it prevents the formation of insoluble and unabsorbable iron compounds and secondly reduces ferric iron (Fe^{3+}) to ferrous (Fe^{2+}) iron (Hallberg *et al.* 1989).

The long-term-effect of ascorbic acid on iron status in the body is however less clear. Adding high amounts of ascorbic acid to daily diets for a longer period have in several studies failed to increase ferritin levels (Cook *et al.* 1984, Hunt *et al.* 1994, Malone *et al.* 1986) although single meal absorption measures using radioiron have in the same subjects indicated that ascorbic acid enhances the iron absorption.

Meat

Half of the iron in meat is in the form of heme iron. The absorption of heme iron is, as stated above, influenced very little by other food components in the diet with the exception of meat which enhances absorption (Hallberg 1983) and calcium which inhibits iron absorption (Hallberg *et al.* 1993). Meat also increases the absorption of nonheme iron (Hallberg *et al.* 1979b, Johnson & Walker 1992). The mechanism behind this enhancing effect of meat on both heme and nonheme iron absorption is as yet unknown. It has however been hypothesized by Zhang *et al.* (1990) that meat enhancement of iron absorption is by multiple mechanisms involving both gastric acidity effects and chelation. Initially, meat may enhance nonheme iron absorption by stimulating production of gastric acid, and thereby promoting iron solubilization within the stomach. Thereafter, a meat factor(s) may chelate the solubilized iron in the acidic environment of the stomach and thereby maintain iron solubility during intestinal digestion and absorption. Fish and poultry also have an enhancing effect on nonheme iron absorption (Morris 1983).

Alcohol

Alcohol increases the absorption of nonheme iron slightly in man (Hallberg & Rossander 1982b). It has been shown that chronic alcohol abusers have increased serum ferritin concentrations and calculated total body iron compared with nondrinkers (Ballot *et al.* 1989) although it is controversial whether the alcohol affects the actual absorption process of iron.

Dietary factors inhibiting nonheme iron absorption

Calcium

Calcium has been shown to inhibit iron absorption in both rats (Barton *et al.* 1983) and man (Cook *et al.* 1991b, Dawson Hughes *et al.* 1986, Deehr *et al.* 1990, Glerup *et al.* 1995). Giving 165 mg Ca as milk, cheese or calcium chloride reduced absorption by 50-60% in single-meal measurements with maximum inhibition of iron with approximately 300 mg calcium in the meal (Hallberg *et al.* 1991). Further increasing the amount of dairy products above a basal level of 300 mg appears to have no further inhibiting effect on iron absorption (Galan *et al.* 1991). The duration of the inhibitory effect of calcium on iron absorption has been shown to be less than two hours (Glerup *et al.* 1993). Calcium inhibits the absorption of both heme and nonheme iron in a comparable way and it is thus likely that this inhibition by calcium occurs after the heme iron is freed from the porphyrin ring (Hallberg *et al.* 1993).

This negative effect of calcium on iron absorption was however not seen in a study of changes in iron status measured as plasma ferritin and other parameters in 109 free-living healthy, premenopausal women eating self-chosen diets. Ingestion of 1000 mg Ca as the carbonate daily with meals over a twelve-week period did not appear to be harmful to their iron status (Sokoll & Dawson Hughes 1992).

Phytate

Phytate occurs naturally primarily as a salt in many plants. Its role is probably as a store for phosphorus and other minerals. The estimated daily intake from a western diet is in the range of 110-230 mg phytate (Harland & Oberleas 1987). At pH values occurring in foods and during digestion, the phytate molecule will be negatively charged, indicating a potential for binding positively charged metal ions such as iron, zinc and calcium.

The negative effect of bran on iron absorption was first demonstrated by McCance and Widdowson (1942) using white bread and brown bran bread. This effect was earlier believed to be due to its high content of phytate which has been demonstrated in a number of more recent studies (Brune *et al.* 1992, Hallberg 1987, Hallberg *et al.* 1987, Rossander-Hulthén *et al.* 1990).

Brune *et al.* (1989a) investigated the possibility that long-term consumption of a high bran and phytate diet would induce changes in the intestine which would bring about an adaptation to the inhibitory effects of phytate on iron absorption. They examined vegetarians and non-vegetarians and found no intestinal adaptation to a high phytate intake and concluded that the satisfactory iron stores in the vegetarian group were due to a high consumption of ascorbic acid. Craig (1994) also concluded that a well-balanced vegetarian diet allows for an adequate iron status, although an increased prevalence of iron-deficiency anemia might be found in children/teenagers on vegetarian diets (Sanders & Reddy 1994). Several methods of preparations of cereal grains including soaking, germination and fermentation, have been shown to completely reduce the phytate content of cereals and vegetables under

optimal conditions (Larsson & Sandberg 1992) and could thereby eliminate their effects on iron absorption.

Despite the dominating evidence that phytate inhibits iron absorption there are still several major difficulties in drawing conclusions from different studies due to different experimental conditions and methods used. Most studies which have demonstrated negative effects of phytate on iron absorption have measured iron absorption using radioiron methods while a balance study in ileostomy subjects found no inhibitory effect of phytate on iron absorption (Sandberg *et al.* 1982). Similarly a long-term study over a 12-week period in geriatric patients (Sandström *et al.* 1983) found no differences in serum mineral levels when ordinary wheat bran was replaced by a low-phytate bran.

Phenolic compounds

The majority of phenolic compounds are of plant origin, and they are mainly found in the leaves, stems and flowers. They are reactive substances and form iron-phenolic complexes in the intestine thereby making the iron less available for absorption. Tannins are large polyphenols which can precipitate proteins. A number of foods such as coffee, tea, beans, spinach and aubergine contain such phenolic compounds (Brune *et al.* 1989b).

Measurement of iron absorption

The absorption in the intestine is as has been discussed above dependent on several factors. Both the iron requirement of the individual, which differs with age, gender and several other factors, and the amount and bioavailability of dietary iron, which is affected by various dietary factors, are important in determining the amount of iron absorbed. In order to issue accurate recommendations to optimize dietary uptake of iron, it is therefore important to have accurate and reliable methods for measuring iron absorption from both individual foodstuffs and whole meals.

Animal studies

Data are very inconclusive as to whether animals and in particular rats are a suitable model for the study of the effects of various factors in humans diets on iron absorption in man. The results from similar studies in rats and humans have often given inconclusive results, indicating differences in response to dietary factors between the two species. The rat's ability to produce vitamin C and their habit of coprophagy have been evoked as possible explanations for these differences.

Human studies

The use of humans in iron absorption studies is definitely the model of choice as no species-translation has to be made when interpreting the results. Prior to the use of radioisotopes, the methods used in humans were time-consuming and sometimes not as accurate, which made the choice of species more difficult. The development of different

radioiron techniques have altered this situation. A variety of techniques used to measure iron absorption will be discussed below. Most of these methods can also be applied to studies in animals.

Classic chemical balance technique

This classical method measures the difference between the iron ingested and the iron remaining in the fecal output. It is only used to measure iron absorption from diets and cannot be used in studies of iron absorption from single meals. The method is very demanding as long balance periods are needed to reduce errors due to inaccurate fecal demarcation. Iron absorption is represented by the small difference between ingested iron and excreted iron which makes the method very sensitive to measurement errors and even minor contamination dramatically may affect the results. For these reasons, the method is used only rarely. For a review see Moore (1968).

Metabolic balance technique using ileostomy subjects

The use of well-established, conventional ileostomy subjects in balance measures makes it possible to use shorter balance periods due to the fact the transit time of the small intestine is short. Undigested products of bran have been shown to be excreted on the day of consumption (Sandberg *et al.* 1981). This makes it possible to avoid the difficulties in feces demarcation in subjects with intact colon. The collection of the ileostomy contents in external appliances further reduces the risk for errors due to incomplete collection and contamination of feces which plague the classic chemical balance technique. The method has been used in a few mineral and metabolic studies (Sandberg *et al.* 1982, Zhang *et al.* 1992b), but is not very commonly used due to the limited availability of suitable test subjects.

Radioiron measurements

Most of the present knowledge concerning iron metabolism has been obtained with radioactive isotopes of iron. The radioisotope which is used most often is ^{59}Fe which has many of the characteristics of an ideal radioactive tracer. It has an half-life of 45.6 days, which makes it possible to monitor changes in iron metabolism for a period of several weeks. It can be measured using not only whole-body but also liquid scintillation counting. A second form of radioiron is ^{55}Fe , which has a half-life of 2,6 years. It is more difficult to measure than ^{59}Fe due to very weak emissions which require elaborate and relatively tedious methods in the preparation samples for counting, but it is useful in that it makes it possible to employ two radioiron tags in tandem. It is measured most often using liquid scintillation counting according to a method described by Eakins and Brown (1966).

Extrinsic tag method

The radioiron method used today to measure nonheme iron absorption from a composite meal is based on the results of several studies (Björn-Rasmussen *et al.* 1974, Hallberg & Björn-

Rasmussen 1972) in which the absorption of radioiron from an extrinsically labeled single food item was shown to be almost identical to the absorption from the same food item labeled intrinsically with radioiron. This result formed the basis for the common-pool concept. The absorbed fraction of a radioiron tracer added extrinsically to any food item (with a few exceptions) in a meal will thus give a measure of the amount of nonheme iron absorbed from the entire meal.

The tracer is added to a food or composite meal. There are two conditions which must be fulfilled in order to ensure complete isotope exchange with the nonheme iron in the food. The tracer and the food must be thoroughly mixed before absorption occurs and the tracer and the nonheme iron compounds in the meal must be in a suitable form, that is, they must be sufficiently dissociable and soluble. By using two radioiron tracers (^{59}Fe and ^{55}Fe) in the same study, comparisons between meals with and without dietary factors which affect iron absorption can be made with the same individuals acting as their own controls. The method was initially used to measure absorption from whole meals, but has later often been used to measure absorption from single food stuffs (Björn-Rasmussen 1974, Brune *et al.* 1992a). The method is often used with an enhancing or inhibitory substance added to a meal compared to a meal without this substance. In many cases an iron salt is added to the one of the diets in order to obtain equal iron concentrations in the diets. However, the validity of the results of such experiments has been questioned due to the fact that the added iron salt is highly soluble and should therefore be far more available for absorption than the iron which is endogenous to the control diet.

As heme iron forms a pool in the gastrointestinal tract independent of nonheme iron it cannot be labeled with an extrinsic tag of inorganic iron, but must be labeled with biosynthetically radioiron-labeled hemoglobin (Layrisse & Martínez-Torres 1972). This means that heme iron and nonheme iron can be independently and simultaneously labeled with extrinsic tags of radioheme and radioiron (Hallberg & Björn-Rasmussen 1972).

The state of the art

Summarizing the results of iron absorption studies reported in the literature, it appears evident that dietary factors exert an influence on the amount of iron absorbed by the intestine. It has been shown that diets containing calcium, phytate or phenolic compounds exert a negative influence while those containing either ascorbic acid or meat exert a positive influence on iron absorption. The strength and thus the practical importance of these influences are open to discussion. However, results from studies using different experimental models, species and methods for the measurements of iron absorption have in many cases produced disparate results. Measurements in animals tend to show less influence of dietary factors on iron absorption than similar measurements in humans and the influences of dietary factors are less pronounced in long-term studies and in studies with whole diets, than they are in measurements made with single meals or single food stuffs.

Aims of the Thesis

The general aim of this thesis was to further investigate the effects of different dietary factors on iron absorption and iron status in man. A number of different questions had to be answered in order to sort out the complexity of this general aim. Thus the particular aims of the separate studies in this thesis were:

1. to study the effect of the addition of wheat bran to a low-fiber diet on the availability of iron for absorption in rats with different iron status;
2. to compare the availability of iron from different whole grain flours with that from endosperm wheat flour for absorption in rats;
3. to determine the extent to which coprophagy may influence the results of iron absorption measurements in rats;
4. to study the effect of dietary calcium on the absorption of iron from a complete diet in ileostomy subjects;
5. to compare iron absorption measured by administration of radioiron in one meal or in several meals of both low- and high-fiber diets; and
6. to describe variations in iron status by age and gender and to relate these variations to variations in diet in a randomly selected middle-aged population.

Materials and Methods

Experimental studies in animals

Animals

Three-weeks old, male, albino rats of the Sprague-Dawley strain (Anticimex, Sollentuna, Sweden), were used in **studies I-III**.

Experimental design

The purpose of **study I** was to investigate the effect of the addition of wheat bran to a low-fiber diet on the availability of iron for absorption in rats with different iron status. Three groups of twelve rats each were fed one of three different diets with endosperm wheat flour with different iron concentrations for 19 days in order to establish groups with low, marginal and high iron status (Fig. 1). On day 20, the rats were deprived of food but not water for twelve hours and each iron status group was divided into two subgroups of six rats

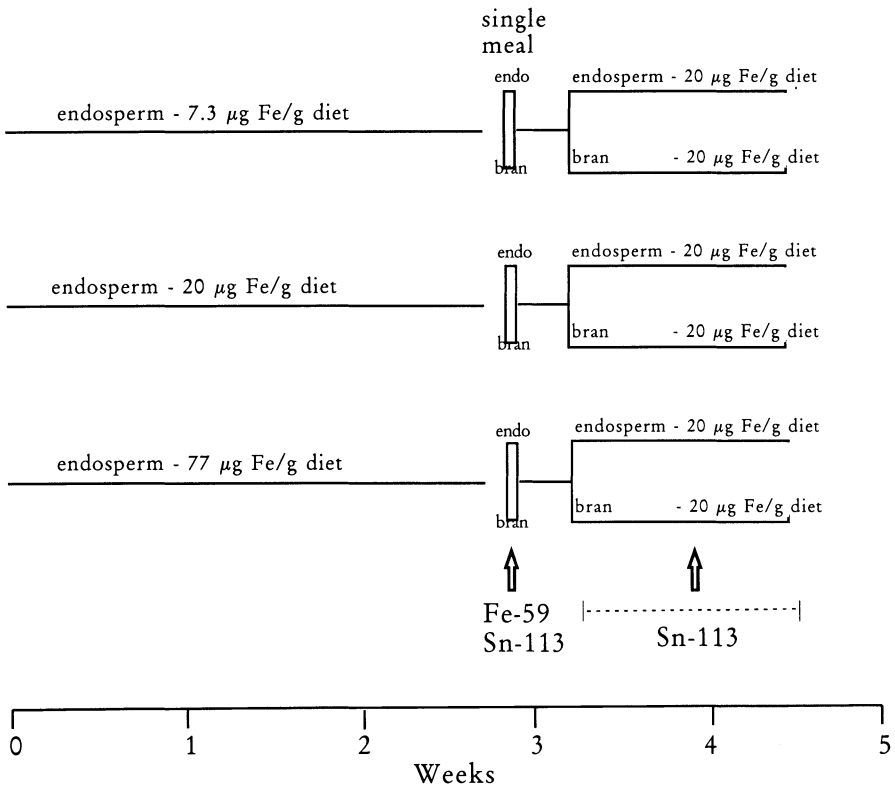


Figure 1. The experimental design of study I.

each. One subgroups in each iron status group was then given a single meal (5 g) of the endosperm diet while the other subgroup was given a diet in which half of the wheat endosperm flour was replaced by wheat bran. The iron concentrations in both diets were equal, 20 $\mu\text{g Fe/g}$ diet. The iron in the diet was labeled with ^{59}Fe for iron absorption measurements and ^{113}Sn -labeled microspheres was added as a non-absorbable marker for the diet (Wing *et al.* 1992). The rats had free access to this meal and water for two hours. During the next three days the groups were again given their primary diets and for the last twelve days of the experiment the rats received diets corresponding to the single meals. These diets were labeled with ^{113}Sn microspheres only. During three days of this period all feces were collected and balance measures were performed. During the entire study, blood (20 μl) was taken from a tail vein once a week for the determination of hemoglobin.

The purpose of **study II** was to compare availability of iron from different whole grains flours with that from endosperm wheat flour for absorption in rats. Forty-five rats were divided into five groups and fed one of five diets composed of one part endosperm wheat or whole grain wheat, oats, barley or rye flour and one part non-fat dried milk with vitamins and maize oil. The endosperm wheat flour used had been supplemented with 65 mg elemental iron/kg flour. The rats were fed these diets and deionized water *ad libitum* for 35 days. On day 36, they were placed in metabolic cages and deprived of the diets but not water for fifteen hours. Each of the rats was then given a 5 g test meal of their own grain diet labeled with ^{59}Fe . They had free access to this test meal and deionized water for six hours.

The purpose of **study III** was to determine the extent to which coprophagy may influence the results of iron absorption measurements in rats. In the **first experiment** in study III the number of rats, the experimental design and the diets were identical with those used in study I (Fig. 1). Following the single meal on day 20, the retention of the ^{113}Sn -labeled microspheres was determined daily for three days in a whole-body scintillation detector as a relative measure of coprophagy in rats with different iron status given endosperm or bran meals.

In the **second experiment** in study III the experimental design was similar to that in the experiment in study I with some few differences (Fig. 2). The initial diet period used to establish different iron status in the three groups was only 14 days. After ten days ^{57}Co -labeled microspheres were added to the diets as a non-absorbable marker for the recycled feces. On day 15 the three groups were each divided into two sub-groups and deprived of diets but not water for a 15 hour period. One subgroup in each iron status group (six rats) was then given a diet in which half of the wheat endosperm flour was replaced by wheat bran. The other subgroup was given the original low-fiber diet to which ferrous sulphate was added to approximate the iron level in the bran diet. The test meals were labeled with ^{59}Fe for measurements of iron absorption and ^{113}Sn -labeled microspheres as a marker for the diet as described above. The rats had free access to this test meal and deionized water for two hours.

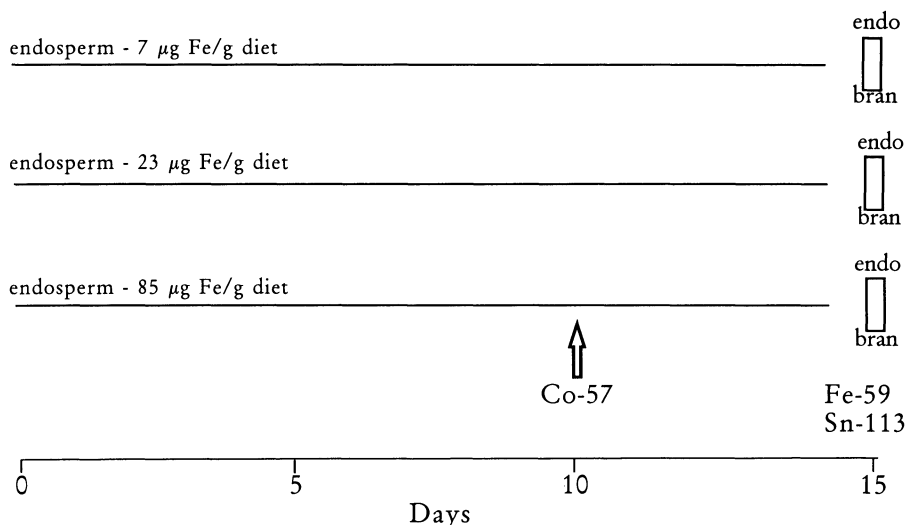


Figure 2. The experimental design of the second experiment in study III.

Sample collection

At sacrifice, the animals were killed by exsanguination under ether anesthesia. The blood was collected and a serum sample was prepared. The liver and the gastro-intestinal tract were quickly removed. The GI tract was divided into the stomach, the duodenum (the first five cm of the small intestine) and the remainder of the intestine, which was further divided in **study III**. In **studies I and III**, the contents of the duodenum were flushed with four ml deionized water using a syringe and then the duodenum was opened lengthwise and the mucosa with its mucus was gently scraped free from the wall using the backside of a scalpel blade.

Hemoglobin measurements

The blood hemoglobin concentrations were determined spectrophotometrically at 543 nm using the cyanide method (International Committee for Standardization in Human Blood 1965). A standard solution of cyanmethemoglobin was used as a reference.

Whole body measurements

In **study II**, the carcass and all of the samples from each rat were divided into three groups of samples for the measurement of the ^{59}Fe activities. The stomach and its contents were measured as one group. The intestine, including the duodenal mucosa and wall, with the intestinal contents formed the second group of samples. The third group was comprised of the carcass and all of the remaining samples. The ^{59}Fe activity in the remaining test meal were also measured. The fractional absorption of ^{59}Fe six hours after the introduction of the test meal was calculated as follows:

$$\text{Fraction } ^{59}\text{Fe absorbed} = \frac{^{59}\text{Fe activity in carcass} + \text{samples}}{^{59}\text{Fe activity in carcass} + \text{samples} + \text{intestine} + \text{intestinal contents}}$$

The amounts of iron absorbed from each of the test meals (mg Fe/kg diet) were calculated by multiplying the fractional absorption by the diet iron concentration. The details of this procedure has been described previously (Wing *et al.* 1992).

The ^{113}Sn activity in the rats in the **first experiment in study III** was used as a relative measure of coprophagy in the six groups of rats. The ^{113}Sn activity was measured in a whole body scintillation counter as described previously (Wing *et al.* 1992). The percentage of ^{113}Sn retained was calculated as follows:

$$\% \text{ } ^{113}\text{Sn retained} = \frac{(^{113}\text{Sn activity in the rat}) \times 100}{\text{Initial } ^{113}\text{Sn after test meal}}$$

Scintillation measurements

The ^{57}Co , ^{113}Sn and ^{59}Fe activities in the samples of the intestine as well as samples of the test meals in **study III** were determined using a scintillation counter (Wing *et al.* 1992).

Iron determinations

The concentrations of iron in the diets and selected tissue samples were determined using dry ashing and atomic absorption spectrophotometry (Hallmans & Wing 1978). Known amounts of iron dissolved in 0.6 M HCl were used as standards.

Calculation of balance measures

The amount of diet represented by the feces samples were calculated by dividing the ^{113}Sn activity in the feces by the ^{113}Sn activity/g diet. The iron balance could thus be calculated as:

$$\text{Fe balance} = \text{Fe concentration in diet} \times \frac{^{113}\text{Sn activity in feces}}{^{113}\text{Sn activity / g}} - \text{Fe in feces}$$

Calculation of retention of ^{57}Co -labeled feces or ^{113}Sn -labeled diet and the rate of coprophagy

The ^{57}Co in the stomach and the small intestine represent coprophagy of the earlier diet ingested together with the ^{113}Sn -labeled test meal. The amount of feces (represented by ^{57}Co)

or diet (represented by ^{113}Sn) in the intestine from the stomach or pylorus through two-thirds of the ileum in the rats in the **second experiment in study III** was thus calculated as:

amount of diet

$$(\text{or test meal}) \text{ retained (g)} = \frac{{}^{57}\text{Co (or } {}^{113}\text{Sn) activity in the intestine samples}}{{}^{57}\text{Co (or } {}^{113}\text{Sn) activity / g original diet}}$$

The coprophagy in the second experiment is defined as:

$$\% \text{ coprophagy} = \frac{(\text{g } {}^{57}\text{Co - labeled diet retained}) \times 100}{\text{g } {}^{57}\text{Co - labeled diet} + \text{g } {}^{113}\text{Sn - labeled diet retained}}$$

Calculation of iron (^{59}Fe) absorption

The whole body retention of ^{59}Fe in **study I** was calculated by dividing the retention measured four days after introduction of the single meal with the retention three hours after the single meal.

The relative absorption of ^{59}Fe into the body two hours after introduction of the test meal in the **second experiment in study III** was calculated as follows:

$$\text{Fraction } {}^{59}\text{Fe absorbed} = 1 - \frac{{}^{59}\text{Fe intestine}}{({}^{113}\text{Sn intestine}) \times ({}^{59}\text{Fe} / {}^{113}\text{Sn})_{\text{test meal}}}$$

Determinations of fiber and phytic acid concentrations in the diet.

In the determination of dietary fiber content in the diets and test meals an enzymatic method as described by Asp *et al.* (1983) was used. In the determination of phytic acid concentrations a method by Sandberg *et al.* (1986) was used.

Experimental studies in humans

Subjects

In study IV nine ileostomy subjects and in study V ten ileostomy subjects were used.

Diets and experimental design

The purpose of study IV was to investigate the effect of dietary calcium from milk on the absorption of iron from a complete diet in ileostomy subjects. The ileostomy subjects were recruited from the patients with ileostomies at the University hospital. Criteria for selection were a well-established ileostomy with stable function and volumes of excreta within normal range as well as good general health as indicated by a case history and routine medical examination including blood and urine tests. The experiment lasted for eight weeks, divided into four periods of 1, 3, 3 and 1 week respectively (Fig. 3).

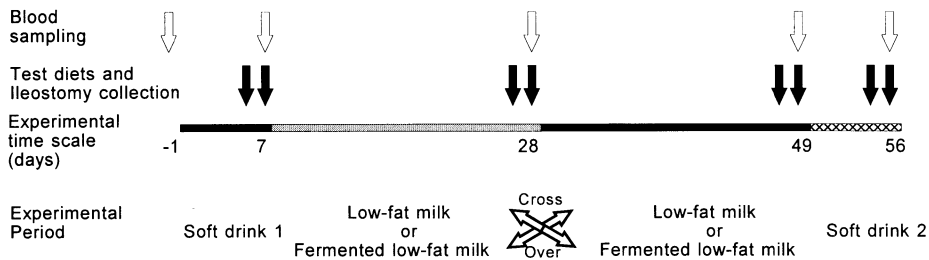


Figure 3. The experimental design in study IV.

The subjects lived at home except for the last two days in each of the four diet periods when they were admitted to the Research Ward. During the entire eight weeks, the subjects consumed 1000 ml beverage daily in addition to coffee. The beverage was distributed evenly among the three main meals and an evening snack. During the first and last weeks of the eight-week experiment, the beverage was a colorless soft drink. During the two intervening three-week periods the beverages were low-fat milk and fermented low-fat milk (Verum® Hälsofil, Norrmejerier, Umeå, Sweden) given in a formally randomized, cross-over design. When the subjects lived at home, they were instructed orally and in writing to eat a diet similar to their usual diet with some restrictions in order to limit the intake of dietary fiber and phytate as well as that of calcium in excess of the calcium in the beverage.

During the test diet and collection days, the subjects were admitted to the Research Ward and stayed overnight at a nearby patient hotel. The daily menu was identical for each of the test days and each of the four diet periods. The subjects were given either 0.75, 1.0, 1.25 or 1.5

portions of the diet according to their estimated individual energy consumption based on dietary interviews made prior to the study and FAO/WHO/UNU recommendations (1985).

The purpose of **study V** was to compare iron absorption measured by administration of radioiron in one meal or in several meals of both low- and high-fiber diets. Two study periods of five successive days each were separated by 16 days. During the first experimental period, the ileostomy subjects were given a low fiber diet (LFD) and during the second a high fiber diet (HFD). The LFD and HFD diets were identical except for the bread. The wheat-flour based bread was supplemented with 2.37 mg Fe/piece of soft bread as FeSO_4 in 100 ml 0.01 M HCl to the dough adding a total of 5.5 mg Fe / portion of bread (1 portion = 2.33 pieces of bread). The subjects were given individually calculated portions of the diets as in **study IV**. The same diet was consumed at each of three meals each day for all five experimental days in both periods. The breakfast consisted of one-seventh, lunch of two-sevenths and dinner of four-sevenths of the total intake during the day. On days 1 and 2 of each five-day experimental diet period, the subjects ate the experimental diet in their own homes according to explicit instructions. On days 3, 4 and 5, the subjects were admitted to the Research Ward and stayed overnight in a nearby hotel. During both day 4 and day 5 of each of the two experimental periods the subjects were given the breakfast meals labeled with ^{55}Fe and all three meals each day labeled with ^{59}Fe .

^{59}Fe and ^{55}Fe activity measurements

Before the first diet period in **study V**, possible background activity at the gamma energies of ^{59}Fe in each subject was measured in the whole body counter at the University Hospital in Umeå, Sweden. At the time of the whole body measurement, a 20 ml blood sample was drawn to determine the activities of ^{55}Fe and ^{59}Fe . Analyses of ^{55}Fe and ^{59}Fe in blood were made using the method of Eakins and Brown (1966) as described by Bothwell *et al.* (1979) with the minor modifications described in **study V**.

Nineteen days after the administration of each radioiron-labeled test diet, the whole body and blood activity measurements were repeated and the activities from the immediately previous measurements were subtracted as background. The day after the third whole body measurement, the subjects received reference doses of both ^{59}Fe and ^{55}Fe . Three mg ferrous iron as FeSO_4 labeled with both radioisotopes was administered together with 30 mg ascorbic acid in 10 ml 0.01 mol/L HCl. They received the reference doses on two consecutive mornings after a 12 h overnight fast. Nineteen days after the administration of the reference doses, the whole body and blood activity measurements were again repeated and the activities from the immediately previous measurements were subtracted as background. The ratio of ^{55}Fe to ^{59}Fe activity in the blood sample was multiplied by the whole body retention of ^{59}Fe to calculate the whole body retention of ^{55}Fe .

Collection and preparation of ileostomy effluents and diet samples

In **study IV** ileostomy effluents were collected from 9 a.m. on each test day to 7 p.m. the following day on both test days in all four diet periods. Ileostomy bags were changed every two hours between 7 a.m. and 9 p.m. as well as during the night when necessary. The bags were immediately frozen on dry ice and stored at -20° C. The ileostomy effluents were then freeze-dried to constant weight and the effluents from each 24-h period were weighed, pooled, homogenized and placed cold until analyzed for their contents of phytate, iron and calcium. Duplicate meals from each of the test days in each diet period were freeze-dried, pooled and processed in the same way as the ileostomy effluents.

Determination of iron status variables

In **study IV and V** venous blood samples were drawn in the morning after an overnight fast to determine the blood hemoglobin and plasma iron and ferritin concentrations. In **study IV** TIBC was also determined. In **study V** samples were also taken every hour of day during both diet periods for metabolic measures (not presented in study V).

During **study IV** a total of 220 ml blood was taken and in **study V** a total of 525 ml was taken from each subject for different analyses.

The hemoglobin concentration was determined spectrophotometrically at 543 nm using the cyanide method (International Committee for Standardization in Human Blood 1965). The plasma iron and ferritin concentrations and the TIBC in all the thawed plasma samples were determined on the same occasion by the University Hospital. Ferritin was determined by an enzyme-linked immunosorbent assay using immunological reagents (DAKO, Copenhagen) and calibrated against WHO standard 80/578. A subject was classified as being iron deficient when the serum ferritin concentration was less than 12 µg/L (Cook 1990).

Determination of iron, calcium, inositol phosphates and dietary fiber concentrations in diets and ileostomy contents

The concentrations of iron in the diets and ileostomy contents were determined by atomic absorption spectrophotometry (Hallmans & Wing 1978). Inositol tri-, tetra-, penta- and hexaphosphates were determined *ad modum* Sandberg and Adherinne (1986) and Sandberg *et al.* (1989). Dietary fiber was analyzed using the enzymatic method of Asp *et al.* (1983).

Calculations and statistical analyses

Apparent absorption (intestinal balance) of iron and calcium in **study IV** was calculated by subtracting the iron and calcium contents of the ileostomy effluents from the iron and calcium intake respectively for each 24 h period on the test diet. The results of apparent iron and calcium absorption and for phytate excretion were tested using a two-way ANOVA for repeated measurements in the same individuals. If the analysis of variance was found to be significant for a particular variable ($p \leq 0.05$), the data for pairs of test day/diet period

combinations were further tested using a Scheffé's test for paired observations. Pearson product-moment correlation coefficients were calculated for iron absorption and plasma ferritin and tested using a t-test. In each of the statistical tests, we chose to reject the null hypothesis at the 5% level ($p \leq 0.05$).

In **study V** the data describing the plasma ferritin are presented as geometric means and their standard errors since the data describing plasma ferritin is skewed. The statistical tests on mean ferritin concentrations were also performed using the logarithms of the ferritin values. The differences among time points for ferritin and blood hemoglobin concentrations were tested using a one-way analyses of variance for repeated measurements within subjects (Snedecor & Cochran 1980). If the one-way analysis was significant differences between pairs of time points for plasma ferritin and hemoglobin were tested using a paired t-test. Differences between measures of iron absorption (^{55}Fe and ^{59}Fe) were also tested with a paired t-test. Pearson product-moment correlation coefficients were calculated for retention of ^{55}Fe and ^{59}Fe . In each of the statistical tests, we chose to reject the null hypothesis at the 5% level ($p \leq 0.05$).

Epidemiological study in humans

Subjects

In **study VI** the participants in the northern Sweden MONICA study in 1990 were included. In the MONICA study continuously updated population registers were used to randomly select 1000 men and 1000 women (25-64 years old) living in the provinces of Västerbotten and Norrbotten. The overall response rate was 79.2%.

Study design

The purpose of **study VI** was to describe variations in iron status by age and gender and to relate these variations to variations in diet in a randomly selected middle-aged population. All participants received by mail a questionnaire including a medical history. The questionnaire was returned at the medical examination during which blood pressure and anthropometric measurements were registered according to MONICA standards (World Health Organization 1985). All subjects were requested to fast for at least four hours in advance of the medical examination.

A food frequency questionnaire was included in the general questionnaire sent to all invited participants ($n=2000$) in the counties of Västerbotten and Norrbotten in northern Sweden. In addition the participants in the county of Västerbotten ($n=645$) were requested to complete a seven-day food record. The daily intake of meat, fruits, vegetables and ascorbic acid were estimated as factors stimulating iron absorption and the intake of dairy products, tea, coffee, dietary fiber and calcium as factors inhibiting iron absorption.

The food frequency questionnaire contained questions on 49 food items; seven on the consumption frequency of various types of fats, seven on milk and dairy products, four on

bread and cereals, nine on fruit, vegetables and root fruits and four on snacks, soft drinks and coffee. Three questions on spirit, wine and beer consumption were included in the list of beverages. The remaining 15 questions assessed potato, rice, pasta, meat and fish intake. Average consumption frequencies during the last year were reported on a nine level scale ranging from never eaten to eaten four or more times per day. The reported consumption frequencies were converted to number of intakes per day. The average number of intakes per day of dairy products, fruits and vegetables were calculated and evaluated in relation to iron status.

The participants were individually instructed for 15 minutes by one of four dietitians in how to complete the seven-day food record. The dietitians had been trained to give identical information. The participants were asked to complete the diary carefully and maintain their ordinary dietary habits during the period of registration. Preprinted alternatives of food items with a 4-scale selection of portion sizes were given for breakfast, lunch and dinner. Portion sizes for lunch and dinner meals were defined according to a 4-level photo-scale. In addition to the precoded alternatives, space was available for open registration of additional items at the main meals and for snacks and small meals. For a few items, such as biscuits, standard sizes were used. Standard portions were calculated according to tables from the National Food Administration in Sweden (1988). The average number of intakes per day of meat, tea and coffee and the average intake of energy, fiber, alcohol, calcium, ascorbic acid and iron were calculated using software (Mats Rudans Lättdata, Västerås, Sweden) adapted to Food Composition Tables from the National Food Administration (1988).

Statistical analyses were carried out using the Statistical Analyses System software package, SAS Inst, Carry, MO USA (1988). Means and their 95 percent confidence intervals variables were calculated for continuous variables for groups stratified by gender and age according to definitions given in the results section. Age-adjustment was accomplished by analysis of covariance. The individuals within each group were ranked into quartiles based on the distribution for each variable, such as daily intake of energy, iron, alcohol, fiber etc. Differences among quartile groups with respect to average values for serum iron, serum ferritin and TIBC were tested using a one-way ANOVA. When the ANOVA rejected the null-hypothesis that all four quartile groups were samples from the same population the difference between means for the highest and lowest quartile groups were tested with a Student's t-test. All tests were two-tailed and p-values below 0.05 were considered statistically significant.

Results and Discussion

Studies in animals

Negative effects of phytate and fiber on iron absorption have been demonstrated in the rat. Ranhotra *et al.* (1979) found a reduction in iron absorption when high-fiber breads were fed to rats. However, the magnitude of this inhibition was unrelated to the amount of phytate phosphorus or dietary fiber present in the diet. Results from experiments by Fairweather Tait (1982) indicate higher absorption from FeSO_4 than from the endogenous Fe present in bread, both expressed as mg Fe absorbed and fractional Fe absorption. Using balance and single meal radioisotope measures, she found no differences in Fe absorption among three different breads with fiber contents of 16.1, 38.1 and 72.4 g/kg but with the same phytate concentration (6.3-6.4 g/kg). Andersson *et al.* (1983) used the exact same bread in balance measures in humans during 3 x 24 days in 6 subjects and did not find any influence of fiber concentration on iron balances. This indicates indirectly that the inhibitory effect of fiber is most likely due to the phytate in the fiber fraction and further supports the results of Morris and Ellis (1980) who found that iron absorption in rats was higher from dephytinized bran.

Some studies in rats have failed to demonstrate a negative effect of phytate on iron absorption. Hunter (1981) found that iron-deficient rats could absorb sufficient iron from the diet for hemoglobin synthesis when sodium phytate was added to their diets at levels up to 4% by weight. The phytate content in the bran-enriched diet used in **study I** was almost 7 times higher than that the low-fiber diet. Nevertheless, there were no differences in iron absorption from the two diets measured using the radioisotope technique in any of the three iron status groups. In the same experiment a balance period (twelve days) was also carried out in the same rats beginning on day 26 (Fig. 1). Iron absorption measured using the radioisotope technique showed good agreement with iron balance measurements (unpublished results, $r=0.780$, $p<0.001$).

It is well known that fractional absorption of iron is higher in iron-deficient animals than in iron-adequate animals (Underwood 1977) and this was also seen in this study (Table 3). The total mean iron concentrations in the serum, liver and duodenal mucosa in the high iron

Table 3. The means for the six subgroups and their standard errors for iron absorption measured as isotope retention and balance measures. In all subgroups $n=6$. The results of two-way analyses (F,P) are presented.

Iron status	Low		Marginal		High		ANOVA	
	Endo	Bran	Endo	Bran	Endo	Bran	Iron status	Diet
Isotope retention	15.3±0.6	15.4±0.3	13.9±0.5	13.8±0.6	7.0±0.7	9.5±0.7	$F_{\text{iron}}=32.3$	$F_{\text{diet}}=0.7$
μg Fe/ g diet							$P_{\text{iron}}<0.001$	$P_{\text{diet}}>0.05$
Balance measure	14.4±0.3	13.3±0.3	13.1±0.2	11.8±0.5	10.1±0.3	11.6±0.5	$F_{\text{iron}}=82.8$	$F_{\text{diet}}=3.2$
μg Fe/g diet							$P_{\text{iron}}<0.001$	$P_{\text{diet}}>0.05$

status group at the end of the experiment were significantly higher than those in both the marginal and low iron status rats (study I, Table 2). Thus the regulation of iron absorption did not fully compensate for the higher iron concentration in the diet fed to the high iron status group. Based on earlier unpublished results we had expected to find little or no inhibition of iron absorption by bran in the low and possibly also the marginal iron status groups and a marked inhibition by bran in the high iron status group. However, we found that the rat's iron status did not affect the relative availability of iron between wheat bran and endosperm diets. Our only findings in this direction were that groups given bran showed a tendency toward a lower mean hemoglobin concentration in blood and lower iron concentrations in serum, liver and duodenum, but none of these differences were significant.

One explanation for the small differences or absence of differences between the low-fiber diet and the wheat bran diet in **study I** could be the form in which iron and phytate is found in wheat. Ellis and Morris (1979) have reported that monoferric phytate is the most common form of iron in wheat, a form that is water soluble, and readily available to rats compared to ferric phytate with 2 to 4 mole of iron/mole of phytate. A second explanation is that there is a high need of iron in growing rats which may substantially reduce the inhibitory effect of phytate on iron absorption.

It might be that different grains and fractions of grains show differences in iron absorption related to the concentrations of iron and phytate, but that it is the combined effect of many factors in the diet which determines the total amount of iron available to the organism. Studies in humans have demonstrated that absorption of iron from rolls made with whole grain oats (Rossander-Hulthen *et al.* 1990) or rye (Brune *et al.* 1992) also have lower availability of iron than that from rolls made with iron-supplemented endosperm wheat and they have shown the fractional availability of iron to be negatively correlated to the phytate concentration.

In order to investigate if the effects of the fiber source show similar effects on the availability of iron we performed a study in which we fed diets with different cereal grains to rats for five weeks and studied the effects on iron absorption and iron status (**study II**). In order to make it possible to compare the results with results made in humans we chose to measure ^{59}Fe from a single meal, as well as blood hemoglobin concentration and the iron concentrations in the serum, liver and duodenal mucosa after five weeks on the diets. The hemoglobin concentration and the accumulation of iron in the liver replaced the balance measurements used in **study I**. The diets used in **study II** were supplemented with a vitamin mixture only and the mineral contents were left unaltered. However, the endosperm wheat flour used was that which was commercially available in Sweden at the time and it was sold supplemented with 65 mg elemental Fe/kg flour.

The study design should make it possible to decide whether or not variations in iron availability among the diets, if in fact they are appreciable, are related to the naturally occurring variations in the concentrations of known factors in the diets. As iron, fiber and

phytate concentrations varied by a factor of 3-3.5 among these diets there should be sufficient variation to make possible a comparison of their effects on the availability of iron for absorption. The fractional absorption of iron (^{59}Fe) varied significantly among the grain diets and it was lower in the whole wheat, oats and rye diets, which contained higher phytate and fiber concentrations than the endosperm wheat and barley diets. Moreover, the rye diet, which had higher phytate and fiber concentrations than the barley diet and almost the same iron concentration, showed a significantly lower availability of iron than the barley diet.

The absorption of iron (mg/kg diet) and the concentrations of iron in serum, blood (Hb), liver and duodenal mucosa varied considerably among the diet groups and in general followed the diet iron concentration (Table 4).

Table 4. The values represent the means and their standard errors ($n = 9$). F and p represent the results of a one-way analysis of variance among the group means for each variable. $\text{LSD}_{0,01}$ is the least significant difference at $p \leq 0.01$. Group means for the same variable with different letter superscripts differ by more than $\text{LSD}_{0,01}$. An LSD value in parentheses indicates that Bartlett's test of variances is significant for that particular variable ($p \leq 0.01$). For each of these variables, group means with different letter superscripts are significantly different ($p \leq 0.01$) in a t-test for independent samples with different variances. Modified from Wing *et al.* (1995).

Diet group	Body wt increase g/5 wks.	Phytic acid mmol	Iron mg	Fractional iron (^{59}Fe) absorption %	Iron absorption mg/kg dw diet	Serum iron mg/L	Hb g/L	Liver iron mg/kg ww	Duodenum iron mg/kg dw
Endosp. wheat	230,8 ^{a,b} 5,6	2,2	44,0	50,8 ^b 2,8	22,5 ^d 1,2	2,51 ^c 0,28	125,4 ^b 4,9	110,0 ^b 19,7	86,2 ^b 12,3
Whole wheat	237,3 ^{a,b} 2,4	7,3	20,9	45,4 ^{a,b} 0,5	9,5 ^c 0,15	1,97 ^{b,c} 2,1	131,9 ^b 4,4	38,8 ^a 5,6	55,1 ^{a,b} 4,6
Oats	255,6 ^b 6,1	6,9	36,9	43,9 ^{a,b} 2,5	11,8 ^c 0,7	2,33 ^c 0,15	136,5 ^b 2,1	67,7 ^{a,b} 13,3	66,9 ^b 6,3
Barley	220,4 ^a 6,1	3,2	14,4	49,8 ^b 2,0	7,2 ^b 0,3	1,36 ^{a,b} 0,13	99,4 ^a 2,8	31,0 ^a 1,6	45,1 ^a 1,3
Rye	227,2 ^a 6,8	6,4	13,8	36,7 ^a 1,6	5,1 ^a 0,2	0,96 ^a 0,13	86,7 ^a 4,8	28,9 ^a 1,0	41,9 ^a 1,5
F	4,1			5,9	97,5	13,8	30,4	9,8	7,5
p	0,008			<0,001	<0,001	<0,001	<0,001	<0,001	<0,001
$\text{LSD}_{0,01}$	25,5			8,9	(2,6)	0,67	15,1	(41,9)	(25,2)

The iron concentrations in the barley and rye diets were below the requirement of the rats (Warner & Breuer, 1972) and the groups fed these diets showed below normal iron status (Hb). While the differences in fractional iron absorption were significant among the grain diet groups, the differences were relatively small. Therefore the concentration of the iron in the

diet was by far the most important factor in determining the amounts of iron available to the rats for absorption.

There are several possible explanations for the lack of dramatic effect of phytate or fiber on iron absorption in this study. In most studies in which comparisons are made among diets, the iron concentrations in the diets are made equal by adding exogenous mineral to one or more diets in order to study the effects of other components such as phytate. The addition of a readily available iron salt to the diet distorts comparisons of the availability of the iron among the diets. It has moreover been indicated that the commonly accepted nonheme Fe pool may be separated into two: a simple inorganic nonheme Fe salt pool and an organically complex nonheme Fe pool (Zhang *et al.* 1989, Fig. 4). Two different mechanisms of Fe absorption have been suggested to operate simultaneously in the intestinal lumen (Gitlin & Cruchaud 1962). In the first, absorption is limited by the amount of carrier while, in the second, the process is simple diffusion. This proposal has been supported by Geisser and Muller (1987) who found that ferrous sulphate and ferric-hydroxide polymaltose (ferripolymaltose) appear to have totally different Fe absorption or distribution mechanisms. Ferrous sulphate appear to be absorbed by passive diffusion, which is limited only by the membrane surface area and the Fe concentration gradient, while the absorption of ferripolymaltose appear to have an active transport mechanism, which is energy dependent. Once in the mucosal cells, all three Fe pools, including the iron from hemoglobin appear to enter the bloodstream via the same pathway, which is regulated by Fe requirements.

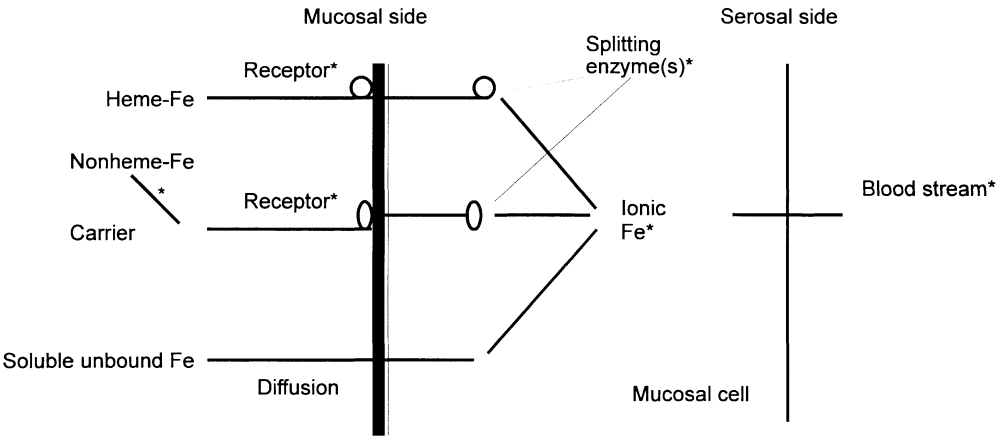


Figure 4. Suggested mechanism of iron absorption (three Fe pool hypothesis). * Control points for Fe absorption. After Zhang *et al.* (1989).

Even though a slight impact of phytate on iron absorption was seen in this study it is not of the magnitude seen in humans. This lack of inhibition might to some extent be caused by coprophagy, a habit that has been observed to occur to some degree in most animals. This led

to the suggestion that the rat might not to be a suitable model for iron availability studies since they have been reported to recycle between 45 and 65% of their fecal material (Barnes *et al.* 1957), which may cause overestimation of iron absorption. Preventing coprophagy could therefore make rodent data more generalizable to higher animals. Many of the early studies exploring the effect of coprophagy have used different kinds of anal cups. While these cups may prevent coprophagy, their presence can also cause stress to the animals which in turn may affect food intake, absorption, metabolism and weight gain. Zhang *et al.* (1992a) have in a recent study demonstrated a valuable way to bypass this by using sham-ring collars (controls for the stress effect) as an extra control group to compare with a treatment group having collars. They concluded that the effect of coprophagy on iron bioavailability varies with the diets, and attributed between 8 and 25% of the iron absorbed to coprophagy.

In study III we did not attempt to hinder coprophagy but rather to measure it and to relate the rate of coprophagy to iron status and diet. In order to evaluate the possible influence of coprophagy on iron absorption studies in the rat and its implications on their interpretation, we assessed the extent of coprophagy and its association with iron status and iron absorption in two experiments. In the first experiment we used ^{113}Sn -labeled microspheres as a non-absorbable marker for the test meal and could then measure the fraction of the test meal remaining in the intestine at different times after ingestion. These results indicated that the low iron status group had a higher retention of the test meal two and three days after ingestion (34% compared to 7-15% for the marginal and high iron status group on day 2 and 24% compared to 2-5% on day 3). The prolonged retention in the low iron status group could have been caused by an increased rate of coprophagy, but it could also be the result of prolonged intestinal emptying time. No differences were seen between the endosperm and bran subgroups within each iron status group.

As we found it difficult to draw any conclusions from these data alone, we designed a second experiment with one non-absorbable radioactive marker for the initial diet that the rat might recycle and a second marker for the test meal. In order to determine whether or not coprophagy affected iron absorption ^{59}Fe was also added to the test meal. We found coprophagy to occur in all groups at a mean rate of between 5 and 22% (Fig. 5), which is a considerably lower rate than those previously reported in the literature (Barnes *et al.* 1957).

We did not find that coprophagy correlated to the iron status of the rat (study III, Fig. 4). The bran added to one of the two test meals did not affect neither the extent of coprophagy or the absorption of ^{59}Fe . No conclusions about the long-term effect of bran on coprophagy can be drawn from this study as this was not studied. If coprophagy is a response to a physical need in the rodent in order to obtain sufficient nourishment, it would be likely to affect absorption of complex iron more than highly soluble or ionic iron, since the latter would have been absorbed to a higher percentage during the first passage throughout the gastrointestinal tract. The long-term effect of bran diets on coprophagy should therefore be further investigated.

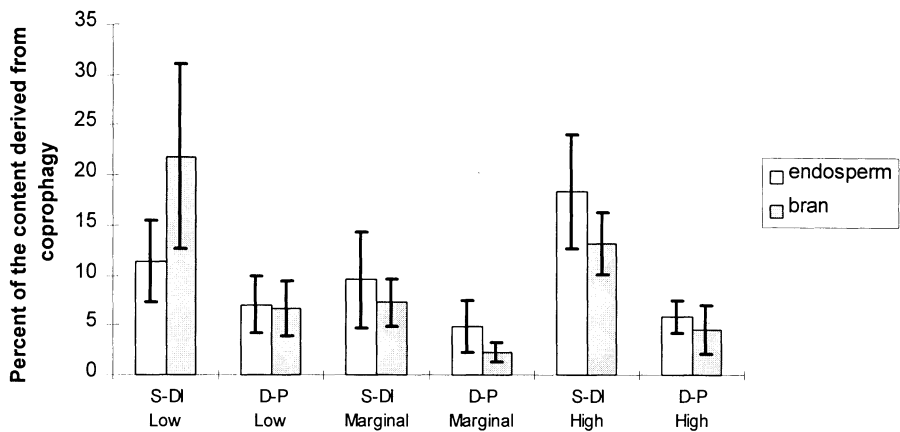


Figure 5. The mean degree of coprophagy in the 3 groups expressed as percentage of the intestinal content from the stomach to the distal ileum (S-DI) and from the pylorus to the distal ileum (D-P; without the stomach) which derived from feces. The brackets represents 1 standard error of the mean

The validity of the rat model in the study of the availability of minerals in human diets, particularly that of iron, has been widely debated and intensively studied without reaching a consensus. Reddy and Cook (1991) used identical methods and diets in absorption studies to compare the significance of known dietary enhancers (ascorbic acid and meat) and inhibitors (tea, bran and soy protein) on iron absorption in rats and humans. They found that rats should not be used to estimate the quantitative importance of dietary factors in human iron nutrition since their absorptive response was far lower than it was in humans. Quite contrary to this, a comparison of data obtained in depletion-repletion studies in rats showed good agreement with iron absorption from similar diets in humans measured using the radioiron-labeled single meal method (Forbes *et al.* 1989).

Some of the differences seen can possibly be explained by the fact that the animal used in these studies were growing whereas the human subjects were adults. The additional iron requirements may have enhanced iron absorption in the rats. Still, the obvious distinction found in many iron absorption studies between rats and humans suggests a more fundamental difference in the mechanism of iron absorption. One possible difference between rats and human that has been discussed is the rats ability to synthesize its own ascorbic acid, a substance shown to be a forceful enhancer of iron absorption. This was recently investigated by Reddy and Cook (1994) in iron absorption studies with normal rats and with a genetic strain which lacks the ability to synthesize the vitamin. They did not find any dramatic changes in these vitamin C-deficient rats and normal rats in sensitivity to dietary enhancers or inhibitors of nonheme iron. Therefore, the absorptive response in rats to dietary factors that affect iron absorption could not be attributed to the rats ability to produce their own ascorbic acid.

Experimental studies in humans

If the results of single meal studies are valid and accurate with respect to the strength of the effects of enhancers and inhibitors then it should be possible to verify their effects with measurements of iron balance or changes in the iron status in subjects on diets similar to the single meals. Iron balance is the sum of mineral absorption, endogenous excretion and reabsorption. In its ordinary form, the chemical balance technique is subject to large systematic and random errors due mainly to the problems of incomplete collection of feces and inaccurate demarcation of feces corresponding to the period of intake. These problems have been brought under control by the use of fecal markers in a study by Andersson *et al.* (1983) but this method still requires very long balance periods, which makes compliance difficult and the studies very costly.

These difficulties can be reduced if balance studies are performed on ileostomy subjects for whom the collection of ileostomy contents is routine and essentially complete. The use of ileostomy subjects for studies of mineral absorption was introduced by Sandberg *et al.* (1982). The demarcation of ileostomy contents is facilitated by the shortness of the intestinal transit time which has been demonstrated by the fact that undigested components are excreted within the day of consumption (Sandberg *et al.* 1981). Thus the measurement of iron balance in ileostomy subjects should be sufficiently accurate to be used as a corroborative measure of iron absorption. This supposition is supported by the results of **study IV** which showed a negative correlation between iron balance and plasma ferritin similar to the correlation between iron absorption and plasma ferritin seen in earlier studies and the nearly 100% recovery of dietary phytic acid in the ileostomy content.

The aim of **study IV** was to investigate the effect of a relatively high dietary calcium intake on the absorption of iron from a complete, low-phytate diet using the chemical balance technique in ileostomy subjects at the end of each of four periods with either a soft drink, low-fat milk or fermented low-fat milk as the beverage with all meals. No differences in iron absorption were seen among these four periods. There was a tendency for the values on the second day to be higher than on the first day during the soft drink periods and vice versa during the milk (high calcium) periods. This interaction was not significant. The apparent iron absorption (mg or %) did not differ significantly between the two test days in each diet period, among the four diet periods or between the low and high calcium diets. This is in direct contrast to the results of single meal isotope studies which have demonstrated a 50 - 60 % reduction in iron absorption from a composite low-phytate meal to which 300 mg calcium was added either as CaCl_2 , milk or cheese (Hallberg *et al.* 1991, 1992).

As the calculated daily iron requirements for men and menstruating women are approximately 1 and 2 mg iron respectively (Bothwell *et al.* 1979), Hallbergs *et al.* results predict a decrease in apparent iron absorption (balance) by as much as 0.5 mg /day in men and 1 mg Fe/day in menstruating women. Provided the amount of hemoglobin remains unchanged, such losses should result in reductions in body iron stores of the same order of magnitude. These iron losses should be reflected in decreases in the plasma ferritin

concentration, which has been demonstrated to vary directly with body iron stores at the rate of about 1 μg for each 0.12 mg Fe/ kg body weight (Cook 1990). In a 80 kg man the plasma ferritin concentration would decrease by about 0.05 $\mu\text{g/L}$ for each day on such a diet $([0.5 \text{ mg Fe/day}] / 80 \text{ kg} / [0.12 \text{ mg Fe/kg}])$ while in a 60 kg woman the decrease would be as much as 0.14 μg ferritin/L $([1.0 \text{ mg Fe/day}] / 60 \text{ kg}/[0.12 \text{ mg Fe} / \text{kg}])$. Thus, over a period of six weeks, the ferritin should decrease by between 2.2 and 5.8 $\mu\text{g/L}$.

The results of **study IV** appear to be both self-contradictory and in disagreement with the above predictions from earlier studies. Iron balance showed little or no effect of calcium on iron absorption while the decrease in plasma ferritin by 8.9 $\mu\text{g/L}$ during the six weeks on the milk diets appears to demonstrate an overwhelming effect, corresponding to a loss of about 80 mg Fe or 2 mg Fe/day which is twice the measured iron balance (Table 5).

Table 5. Means and their standard errors for ferritin on the day before the study began and the last day of each of the four diet periods, *i.e.* in chronological order, are presented together with the change in ferritin during each diet period in the study. The results of one-way analyses of variance for repeated measurements in the same individuals are also presented. Means with unlike letter superscripts are significantly different from one another ($p\leq0.05$).

	Day before study began	Soft drink period 1	Milk period 1	Milk period 2	Soft drink period 2	ANOVA
Plasma ferritin, $\mu\text{g/L}$	54.0 \pm 20.2 ^a	52.1 \pm 19.4 ^a	47.8 \pm 18.0 ^{a,b}	43.2 \pm 17.6 ^b	45.9 \pm 17.0 ^{a,b}	$F_{\text{diet}} = 5.16; \text{df} = 4; 32$ $p_{\text{diet}} < 0.01$
Plasma ferritin, $\mu\text{g/L}$ (geometric mean)	36.4 $\times \div$ 1.33	34.4 $\times \div$ 1.35	31.8 $\times \div$ 1.34	25.5 $\times \div$ 1.40	29.7 $\times \div$ 1.36	$F_{\text{diet}} = 6.25; \text{df} = 4; 32$ $p_{\text{diet}} = 0.001$
Change in Ferritin/diet period, $\mu\text{g/L}$		-1.9 \pm 1.9	-3.9 \pm 2.0	-5.0 \pm 2.8	2.7 \pm 1.5	$F_{\text{diet}} = 2.43; \text{df} = 3, 24,$ $p_{\text{diet}} > 0.05$

One explanation for this discrepancy may be the loss of iron due to blood sampling. Forty ml of blood were taken at the start of the study and 45 ml at the end of each diet period. By the beginning of the second high calcium diet period, a total of 130 ml had been taken, causing an average loss of 66.5 mg of iron $(3.35 \text{ mg Fe} / \text{ g} / \text{L Hb} \times \text{blood volume (L)} \times \text{mean Hb in blood (g / L Hb)})$. If a change in plasma ferritin can be assumed to follow blood loss within a period of one to two weeks, the plasma ferritin should have dropped by 7.7 $\mu\text{g/L}$ by the end of this period. The actual mean decrease in plasma ferritin by the end of the third period was 10.8 $\mu\text{g/L}$. However, the plasma ferritin in one male subject had decreased 33 $\mu\text{g/L}$ over the three periods, a decrease far too large to be caused by the blood loss and a small change in iron balance. If this subject is eliminated from the calculation, the average decrease in plasma ferritin is 8.3 $\mu\text{g/L}$ which

corresponds to 69.4 mg storage iron. The difference between this and the iron lost with blood samples is only 3 mg, or less than 0.1 mg Fe/day over the two three-week high calcium diet periods. Thus both the apparent iron absorption and the plasma ferritin concentration appear to show a very weak effect of high diet calcium (1.4 g Ca/day) on iron balance and iron status.

Other studies have shown little or no effect on iron absorption of increasing the calcium concentration in the diet. Turnlund *et al.* (1990) demonstrated that the addition of milk to a cereal-based diet did not affect apparent iron absorption (balance) in young women and Galan *et al.* (1991) showed that increasing the proportion of dairy products in a representative French meal did not cause any measurable effect on iron absorption measured using radioisotopes. However, the diet used in the former study contained other inhibitors of iron absorption such as phytate and the French diet initially contained more than 300 mg calcium per meal. As no additional effect on iron absorption has been demonstrated using single-meal measurements when calcium has been added to diets with more than 300 mg calcium (Hallberg *et al.* 1991, 1992), it was perhaps not to be expected that the addition of calcium to the diets in the studies of Turnlund *et al.* and Galan *et al.* would cause appreciable decreases in iron absorption and balance.

In contrast to the diets in the studies cited above, the subjects' control diets in the present study were intentionally low in both phytate and calcium. During periods in the study when they lived at home, the subjects ate self-selected diets. They were instructed both orally and in writing to restrict their consumption of dairy products, bread, fruits, vegetables and sweets in order to prevent the ingestion of factors known to appreciably enhance or inhibit iron absorption. These restrictions were made to prevent the subjects from eating food enhancing or inhibiting iron absorption, but did in fact also prevent them from eating between meals. A small fraction of the reduced ferritin level could have been caused by these restrictions, mainly the lack of vitamin C intake from fruit and vegetables. A dietary history was taken for the days preceding each of the four test diet periods in order to determine compliance with these restrictions. There was no evidence of the ingestion of iron in quantities which would have affected the results of the balance studies or the ingestion of amounts of known inhibitors and enhancers of iron absorption which would have appreciably confounded the effect of the calcium in the diet on the absorption of iron. The phytate (IP3-IP6) content of the test diet was only 132 $\mu\text{mol/day}$, most of which derived from bread, coffee and potatoes. The meals and evening snack initially contained only about 40 mg calcium each. When the soft drink was exchanged for low-fat milk or low-fat fermented milk, about 300 mg calcium was added to each of the three main meals and the evening snack. Thus the composition of the diet in **study IV** was similar to that of the single meals in studies which have shown a marked effect of calcium on iron absorption.

In the studies in which the most dramatic and significant effects of calcium on iron absorption have been demonstrated, the initial calcium content has been considerably lower than 40 mg/meal (Hallberg *et al.* 1991, 1992). Rather than demonstrating an inhibition of iron

absorption by high calcium concentrations in the diet, those studies may have demonstrated increased iron absorption in the control groups due to extremely low diet calcium concentrations. It may be that when the diet calcium concentration is reduced to a level where most of the calcium is absorbed and the body's need for calcium is still not met, iron may be absorbed instead of calcium. The low calcium intake during the soft drink periods (0.16 g Ca/day) resulted in much lower apparent absorption of calcium (balance) than did the more normal calcium intake during the two milk periods (1.4 g Ca/day). However, the fractional apparent absorption of calcium was increased only slightly and not significantly during the soft drink periods in response to the low calcium intake. The low calcium content of the soft drink diet also caused the iron:calcium molar ratio to increase approximately ten-fold over that in the two milk diets, but the ratio was less than 0.06 in the soft drink diet. While it appears unlikely that these changes in fractional absorption and molar ratio would be sufficient to affect iron absorption, a further reduction of the calcium content by a factor of four as in the studies of Hallberg *et al.* (1991, 1992), may very well affect iron absorption.

One additional principal difference between studies in which calcium has been shown to markedly impair iron absorption and **study IV** is the different methods used to measure iron absorption. In this study, iron absorption was measured as intestinal iron balance from a test diet over a two-day period after at least one week of adaptation to the imposed calcium intake level while earlier studies used radioiron-labeled single meals. Thus there is a possibility that differences in the methods for measuring iron absorption may explain the differences in the results. It is well known that there is a marked variation in absorption of iron not only between different, apparently healthy, individuals but also in the same individual on different days, and that absorption measurements made over a period of time show compensation for low iron absorption from one meal by higher absorption from others (Sandberg *et al.* 1982). In a recent study by Gleerup *et al.* (1995), nonheme iron in all meals over a period of ten days were labeled with radioisotopes of iron. The inhibitory effect of calcium was compared when the same amount of calcium was distributed in two ways, with either mainly breakfast and evening meals or more evenly with all meals. About 30-50% more iron was absorbed when less calcium-rich products were served with the main meals. This was also less than the reduction of 50-60% demonstrated in single meal studies. Thus studies of iron absorption from diets rather than single meals, regardless of the method of measurement used, appear to be in agreement on a more limited effect of calcium on iron absorption.

Cook *et al.* (1991a) labeled the iron in 28 meals over a period of 14 days and compared the measurement of iron absorption with single-meal measurements in the same subjects. They found that the difference between an inhibiting and enhancing diet was much smaller when measured over a 14 day period. The single-meal method thus appears to overestimate the net effects of enhancers and inhibitors of iron absorption compared to their effects in an ordinary diet. Cook *et al.* concluded that, the iron status of the subjects rather than the bioavailability of dietary iron was the major factor determining the fractional absorption of dietary non-heme iron. This conclusion is supported by the results of the present study.

One disadvantage with the ileostomy model is the difficulty in recruiting a sufficient number of subjects who are willing to participate and also are in good health. It is thus very difficult to perform this kind of study in a strictly controlled manner using a large number of subjects. However, there is reason to believe that using the subjects as their own controls at least partially compensates for the low number of subjects. While there is a large interindividual variation in both iron status and iron absorption the two are well correlated (study IV, Fig. 1) and the four repeated measures of balance are also well correlated (Table 6). Thus the intraindividual variation is low and the interindividual variation in iron status should not confound the effects of diet (calcium) on iron absorption.

Table 6. Pearson's product-moment correlations in study IV between iron balance during different diet periods tested using t-test (p).

	Soft drink period 1	Low-fat milk	Fermented low-fat milk	Soft drink period 2
Soft drink period 1				
Low-fat milk	0.904 (p=0.001)			
Fermented low-fat milk	0.926 (p<0.001)	0.818 (p=0.007)		
Soft-drink period 2	0.899 (p=0.001)	0.774 (p=0.014)	0.894 (p=0.001)	

As the subjects acted as their own controls a paired test was chosen. We did not find any significant differences in iron absorption between diet periods. With the variations found in the material, a 29% decrease in absorption from the combined calcium groups compared to the combined control-groups was calculated to be required to reach the 0.05 significance level

$$\text{significant decrease} = t\text{-value (5\% - level)} \times \frac{\text{S.D.}}{\sqrt{n}} = 2.306 \times \frac{0.400}{\sqrt{9}} = 0.306698$$

As this decrease was calculated using absolute numbers, the value of 0.307 must be divided by the mean balance value for the soft drink period which is 1.07 in order to obtain the decrease in percent ($\approx 29\%$). If the four periods are treated separately the differences (percent decrease) necessary to obtain a significant t-test result ($p\leq 0.05$) vary from 33 to 57%.

Thus the sample size should have been sufficient to detect a biologically relevant difference and certainly adequate to detect decreases had they been as large as those reported earlier (Gleerup *et al.* 1995, Hallberg *et al.* 1991).

It appears that absorption methods using radioiron, especially the single-meal method, overestimate the net effect of calcium on iron absorption compared with both balance and radioiron measurements from a whole diet. The aim of **study V** was thus to compare

radioiron measurements from one meal with radioiron measurements from all meals during the day using the same diets in all meals. The experiment was repeated with both a low-fiber and a high-fiber diet. We found that the absorption values obtained for a low-fiber diet (LFD) for the single meal were on average 78% higher than those obtained for the entire

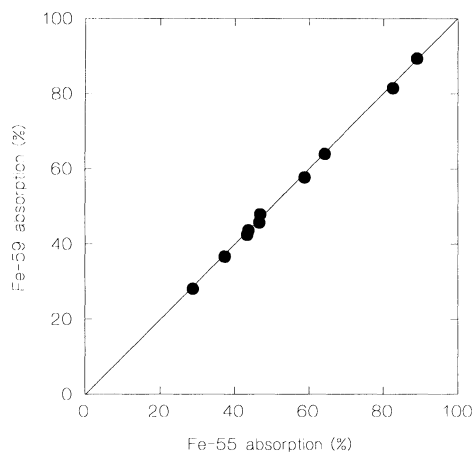


Figure 6. Percent absorption of ⁵⁵Fe and ⁵⁹Fe added in the same reference dose of three mg ferrous iron as FeSO₄. The solid line is the line of identity.

day’s diet. The iron absorption values for the high-fiber diet (HFD) were 48% higher for the single meal than for the entire day’s diet, but this difference was not significant. As the absorption of the reference doses of ⁵⁵Fe and ⁵⁹Fe was almost identical (Fig. 6), there is no reason to believe that a method error was responsible for these systematic differences in iron absorption from single meals labeled with ⁵⁵Fe and daily diets labeled with ⁵⁹Fe. This is also supported by the good agreement between single meal and daily diet in the two diet periods (Table 7).

Table 7. Pearson’s product-moment correlations in study V between single-meal and daily diet during low-fiber diet (LFD) and high-fiber diet (HFD) periods tested using t-test (p).

	LFD daily diet	HFD daily diet	Reference dose ⁵⁹ Fe
LFD single meal	0.774 (p=0.009)		0.176 (p=0.627)
HFD single meal		0.890 (p≤0.001)	0.407 (p=0.244)
Reference dose ⁵⁵ Fe	0.152 (p=0.675)	0.336 (p=0.343)	0.999 (p≤0.001)

The ratio of the two absorption measurements from the LFD and the HFD (1.78 vs. 1.48) were not significantly different from each other. As the study was made within the framework of a larger study which was not designed primarily to compare the LFD and HFD, no cross-over was used. The iron status of the subjects, as reflected in the plasma ferritin concentrations, decreased between the LFD and HFD periods due to blood sampling. This as well as unnoticed changes in other parameters may have occurred between the test periods, but it is unlikely that the change in iron status is the main cause of the comparatively low absorption ratio during the high fiber period.

There may be several explanations which separately or combined may explain the differences observed between the results of the single meal design and the daily diet design. One of them may be that the single meal was given after a 12-hour period of fasting (overnight) compared to 4 hours without food before the lunch and dinner meals. This difference in fasting period length may have caused greater absorption from the breakfast meal than the average for the same diet throughout the whole day. This would appear to contradict the results of Björn-Rasmussen *et al.* (1974) who found a higher absorption of iron from lunch and dinner compared to that of a breakfast meal. The meals in that study were however not identical. The lunch and dinner contained fish and meat, which are supposed to stimulate iron absorption, while the breakfast contained more phytic acid, which is supposed to inhibit iron absorption. Identical meals were used in a recent study from Taylor *et al.* (1995) and they also found, in agreement with Björn-Rasmussen *et al.*, that there was no significant difference in the percentage iron absorption from the same meals eaten in the morning (with a prior twelve-hour fasting period) and when eaten at in the middle of the day (with a prior four-hour fasting period).

A second factor which may explain the differences in results is that the single meal given in the morning represented only one-seventh of the total daily intake and therefore only one-seventh of the total iron intake. In the present study, the ratios of iron, calcium, phytic acid and fiber to one another were equal in all meals in order to avoid the possibility that differences in absorption between the single meal and daily diet design could be caused by differences in relative proportions of enhancers and inhibitors among meals as was the case in the studies of Gleerup *et al.* (1995) and Cook *et al.* (1991a), for example. As the intake of iron in the morning was comparatively low, this may have given rise to a higher fractional iron absorption from the morning meal.

A third explanation of the results might be related to the sequencing of the differences in accumulation and transfer of iron within/through the intestinal mucosa. It is known that the uptake of iron by the mucous membrane of the small intestine is a function of the concentration and availability of iron within the lumen as well as the iron requirements of the body. It is further known that the need of the body influences the proportion of iron which is later transferred into plasma. Not all the iron taken from the lumen into the intestinal cells is necessarily transferred into plasma. A variable proportion may be stored within the mucosal cell, and later discarded when the cell exfoliates (Charlton *et al.* 1965,

Conrad & Crosby 1963). Radioisotope studies have demonstrated that a prolonged radio-iron excretion occurs in normal subjects after oral intake. This indicates that a part of the iron is stored in the mucus and the mucosal cells of the intestinal mucosa and excreted when cells are desquamated (Björn-Rasmussen *et al.* 1980). In iron-depleted humans the iron transfer across the mucosal cell has been shown to be at a maximum during the first 30 minutes after the introduction of iron into the intestinal lumen (Hallberg & Sölvell 1960). After this rapid absorption phase, transfer continues at a much slower rate for 12 to 24 hours, during which time some or all of the iron transferred has been temporarily stored within the intestinal cell as ferritin (Charlton *et al.* 1965).

How can the differences in results between the single meal method and the reference method be explained in relation to isotope transfer and/or exchange in the intestinal mucosa? One hypothesis may be that the isotopes do not always measure and/or represent what we believe them to measure and/or represent. The present results might be an illustrative example of this. The nonheme iron from the breakfast meal in the present study is labeled with ^{55}Fe and ^{59}Fe . The radioiron in the meal will be transferred to the intestinal mucosa together with and in proportion to the diet iron, and then establish a new equilibrium between radioiron and iron present in the meal and iron already situated in the mucosa. During the next meal, lunch, iron will also be transported from the lumen into the intestinal cells. The iron in this meal is labeled with ^{59}Fe isotopes but not with ^{55}Fe . A portion of the ^{55}Fe from the breakfast meal present in the intestinal mucosa or within the intestinal cells may however be transferred into the plasma during the rapid phase of absorption following this lunch meal. This iron should represent iron absorbed from the lunch meal, although all ^{55}Fe absorption will represent a false increase in absorption from the breakfast since all ^{55}Fe retained represents iron absorbed from the breakfast meal.

In addition, about 30% of the cells in the intestine will be renewed during the night's fasting, and consequently some of the iron within the cells will be lost. This means that more ^{59}Fe from the dinner will be lost than ^{55}Fe from breakfast before they get a chance to be absorbed. In summary, the seven times larger dose of ^{55}Fe given in the morning will have a higher possibility to be absorbed during the day than the ^{59}Fe where the main amount is given during dinner. A simplified model of this is illustrated in Figure 7, where the iron absorption into plasma is assumed to be 10% of the amount available in the cell. The fractional iron transport into the cell is also assumed to be constant at all meals and 10% of the cells are assumed to be desquamated every 5 hours. This would give a ratio for ^{55}Fe : ^{59}Fe of 1.38. This is less than what we have seen in **study V** but this could be one contributing factor among others explaining the differences in results. It should however be pointed out that this is a very simplified model, where a number of factors are held constant. It should also be noted that we have assumed that 90% of the iron on or in the mucosal cells is not absorbed but is available for exchange with the iron from the next meal. Should a lesser fraction be left behind or should some of this iron not be exchangeable with the iron in the next meal, the calculated ratio should be lower than 1.38.

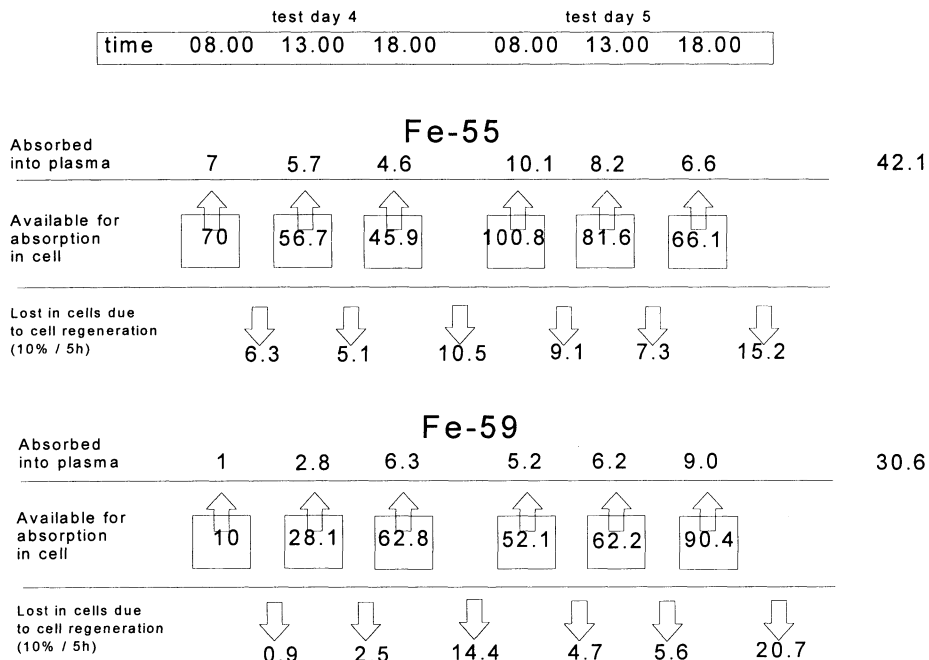


Figure 7. Simplified model for absorption of ^{55}Fe and ^{59}Fe during the two test days.

The differences (although non-significant) in iron absorption ratios (single meal:daily diet) between the LFD and HFD may also be explained by the fact that ferrous sulphate was added to the LFD in order to even out differences in iron content between the LFD and HFD. In the present study, 5.5 mg iron as FeSO_4 was added to 1.0 daily portion of the LFD and it thus constituted almost half of the LFD iron content. This added iron may be more easily and quickly absorbed than iron endogenous to the diet as suggested by Zhang *et al.* (1989). It may further be that, in addition to the transfer of iron from the meal to the intestinal mucosa, there may also be an exchange between iron present in the meal with no net transfer. This means that "excess" amounts of radioiron may accumulate in the mucosa and at least partly later be absorbed. This iron does not represent true absorption but will not interfere with absorption studies if all types of iron from different diets behave in the same way. If, however, iron sulphate has a greater tendency to exchange with iron in the intestinal mucosa as compared to iron bound to inhibiting factors such as phytate, this may further exaggerate the inhibitory effect of the phytate. If so, an overestimation of iron absorption from the LFD would result and would most likely be interpreted as an inhibitory effect of the HFD. This may also partly explain the large differences between the iron absorption from LFD and HFD seen in other single meal studies.

It appears that the single-meal method in some situations is associated with a tendency to exaggerate the effect of inhibitors and enhancers. The modified method in which all meals of the day are labeled just may prove to reduce this effect somewhat. It would appear that much of the differences in the effect of calcium seen in different studies may be the result of differences in study design and the use of different methods. It is important to use methods that measure the absorption from a composite diet over a sufficient period of time and not only from one meal in order to adequately evaluate the long-term consequences of different dietary factors on iron absorption and the iron status of the individual. A longer period with many meals will allow for the different dietary factors found in normal meals to fully interact.

Epidemiological studies in humans

To further attempt to estimate the combined effect of different dietary factors on iron absorption in an varied Scandinavian diet we therefore decided to study a randomly selected middle-aged population in northern Sweden (study VI) within the framework of the WHO MONICA Project (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease). The purpose of this study was to describe variations in iron status associated with age and gender and to relate variations in iron status (measured as serum ferritin, serum iron and TIBC) to dietary (fiber, meat, tea, coffee, alcohol, fruit, vegetables and dairy products) and metabolic factors. The dietary intake was recorded in 1583 randomly selected men and women, 25-64 years of age. A self-administered food frequency questionnaire and a 7-day food record method were used to estimate dietary intake.

As has been discussed previously, it is of importance to obtain information of the dietary intake of iron and dietary components that influence the iron absorption over a considerable length of time so that random day-to-day and seasonal variation can be minimized. This estimation can only be done by dietary history methods combined with adequate parameters that measure iron status. The dietary history method used in this study is subject to problems with compliance, memory and false judgment, but is widely used in epidemiological studies and has proved to be reliable (Johansson *et al.* 1994). The estimated daily intake of food items possibly affecting iron status were based on the food frequency questionnaire (n=1575) for fruit, vegetables and various dairy products, whereas intake of other items was assessed by 7-day food records in a subsample (n=646).

We found, in agreement with Takkunen *et al.* (1975) that a high intake of dairy products in women (25-44 yr.) was associated with lower iron status. The most striking result, however, was that iron status was not related to differences in dietary intake, especially the intake of energy and iron. No correlation could be demonstrated between iron status, measured as serum iron, ferritin or TIBC, and the estimated intakes of calcium, fiber, ascorbic acid, meat, tea or coffee, all known to be factors which influence the bioavailability of iron. On the population level, we found that factors other than diet, e.g. menstruation and metabolic factors, were more important as determinants of iron status/stores. A plausible

explanation for our findings is that almost all subjects consumed a diet containing a mixture of inhibitory and stimulatory factors. Individuals that were high-consumers of inhibitory factors like fiber, calcium and dairy products, also had a higher intake of iron as compared to low-consumers of these inhibitors.

Summary and conclusions

It appears evident that the main factor determining the absorption of iron from cereal grain diets in growing **rats** is the iron concentration of the diet. The great inhibitory effect of phytate seen in some animal and many human studies could not be confirmed as only a slight and non-significant effect of phytate was seen. This effect was not dependent on the iron status of the rat as we had anticipated. We found that coprophagy occurred in all animals without any obvious correlation to their iron status. We found good agreement between absorption measurements using a radioiron-labeled single-meal technique and classical iron balance measures. It would appear that the rat is a less suitable model for accurately measuring iron absorption due to these factors.

Based on our results in **humans**, we find it likely that the amount of iron available for absorption to the body and the resulting iron status will be affected only marginally if variations in the calcium content of the diet or meals within a diet are within normal physiological limits. We believe that the differences between the results of the present study and those of radioisotope studies which have shown pronounced effects of high calcium levels are the result of differences in experimental design and measurement method including the fact that the most dramatic differences in iron absorption have been seen in studies using extremely low diet calcium levels as controls.

On the basis of the results of a method study in humans we recommend that radioiron-labeled test diets should be given at all meals during test days and not as single meals in the morning after an overnight fast as the values obtained with the single meal technique may be inflated. In some situations the single meal technique may exaggerate the effects of dietary factors on iron absorption as suggested by Cook *et al.* (1991a). We therefore recommend that the results of single meal studies as a measure of iron absorption should be interpreted with caution.

The findings in the epidemiological study suggest that dietary iron intake may be a less important factor than menstrual blood loss and possible metabolic factors in relation to iron status in a randomly selected population consuming a normal western diet. The iron content of the diet appears to be a limiting factor on iron status only at very low diet iron levels or in individuals with a high iron loss.

Acknowledgments

The present thesis is based on research conducted at the Department of Nutritional Research and Prosthetic Dentistry and the Biophysics Laboratory at Umeå University and has involved much of their staff. Many colleagues and co-workers have contributed in different ways and the work has extensively depended on the positive attitudes from all personnel of these Departments. In particular, I would like to thank the following persons.

Göran Hallmans, my tutor and friend for his invaluable support and involvement. It has been a time filled with hard work and laughter and I will always remember our fruitful discussions in your kitchen, drinking pots of coffee.

Kenneth Wing, my second tutor and friend, for your support and guidance, constructive suggestions, and your never-ending patience with my way of treating your native language.

Ingegerd Johansson, my co-author and friend, for introducing me to the field of epidemiology and always finding time for discussions and writing.

Rolf Sjöström, for your invaluable help in designing studies and then making them work, and your excellent work in always refining our analyzes. Also for your effort in trying to design homogenous diets in the blender to the subjects in study IV and V, and in helping me understand what I don't understand, *i.e.* the slide-rule and complex statistics.

Eva Lundin and Jie-Xian Zhang for excellent co-operation in carrying out the human studies. I think we make quite a team together!

Bo Bergman, Solveig Gustafsson and all the personnel at the Department of Prosthetic Dentistry for all their help and support, both moral and financial, during this period.

All the study subjects that participated, with honor, in study IV and V even though they had to eat the same diet week after week. Without you, this would never have been possible.

My co-authors for much help and for many fruitful discussions.

Margareta Tagewall for all your help with just about everything.

Margareta Holmgren, Inger Sjöström, Åsa Ågren and Ann-Marie Åhrén for their invaluable help with analyzing samples and handling data. And for always being supportive.

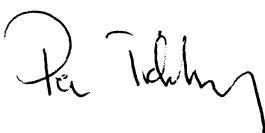
Bo Sunzel, my brother of arms and true friend. Without your encouragement and positive spirit this would not have been finished during this century.

Johan Gunne and Donald Sellman, both for giving guidance and sharing many happy moments.

Britt-Mari my wife for her outstanding patience and love during all these years. Also to my boys Viktor and Ludvig for remembering dad although he never seemed to be at home.

My parents, Karin and Rolf for always supporting me.

Umeå in August 1995



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