DIETARY FACTORS
INFLUENCING NON-HEME IRON ABSORPTION

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presented by

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***

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***

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***

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***

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***

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***

Always last, never least: my family and friends whose continuous love and support made me move mountains – and Switzerland has many!
“Indeed the management of iron in our body has been compared to the handling of fire in our homes. Confinement of fire to the furnace, the fireplace, and the stove provides essential services. Allowing fire to invade the walls of our homes ensures destruction.”

Eugene D. Weinberg
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## 1 Iron

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<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AD</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C(4)S</td>
<td>Chondroitin (4-)sulfate</td>
</tr>
<tr>
<td>CPP(s)</td>
<td>Caseinophosphopeptide(s)</td>
</tr>
<tr>
<td>CTM</td>
<td>Consensus transport motif</td>
</tr>
<tr>
<td>DCT1</td>
<td>Divalent cation transporter 1</td>
</tr>
<tr>
<td>Dcytb</td>
<td>Duodenal cytochrome b</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent metal transporter 1</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary Reference Intakes</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin gene</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agricultural Organization</td>
</tr>
<tr>
<td>Fe$_2$-Tf</td>
<td>Diferric transferrin</td>
</tr>
<tr>
<td>FEP</td>
<td>Free erythrocyte protoporphyrin</td>
</tr>
<tr>
<td>GAG(s)</td>
<td>Glycosaminoglycan(s)</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>Hamp</td>
<td>Hepcidin antimicrobial peptide</td>
</tr>
<tr>
<td>HAMP</td>
<td>Hepcidin antimicrobial peptide gene</td>
</tr>
<tr>
<td>HCP1</td>
<td>Heme carrier protein 1</td>
</tr>
<tr>
<td>Heph</td>
<td>Hephaestin gene</td>
</tr>
<tr>
<td>HFE</td>
<td>Gene mutated in hereditary hemochromatosis</td>
</tr>
<tr>
<td>HFE gene product</td>
<td></td>
</tr>
<tr>
<td>HFE2</td>
<td>Alternative name for the $HJV$ gene</td>
</tr>
<tr>
<td>HH</td>
<td>Hereditary hemochromatosis</td>
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<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor-1</td>
</tr>
<tr>
<td>HJV</td>
<td>Hemojuvelin gene</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HT</td>
<td>Heart transplantation</td>
</tr>
<tr>
<td>ID</td>
<td>Iron deficiency</td>
</tr>
<tr>
<td>IDA</td>
<td>Iron deficiency anemia</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IMP</td>
<td>Integrin-mobilferrin-paraferritin pathway</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IP</td>
<td>Inositol phosphate</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron responsive element</td>
</tr>
<tr>
<td>IRE-BP</td>
<td>Iron responsive element-binding protein</td>
</tr>
<tr>
<td>IREG1</td>
<td>Iron regulated mRNA 1</td>
</tr>
<tr>
<td>IRP1/2</td>
<td>Iron regulatory protein 1/2</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>LIP</td>
<td>Labile iron pool</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>LPI</td>
<td>Labile plasma iron</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MTP-1</td>
<td>Metal transfer protein-1</td>
</tr>
<tr>
<td>NHE</td>
<td>Na(^+)/H(^+) exchanger</td>
</tr>
<tr>
<td>Nramp2</td>
<td>Natural resistance associated macrophage protein 2</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non-transferrin-bound iron</td>
</tr>
<tr>
<td>OA</td>
<td>Oxalic acid</td>
</tr>
<tr>
<td>PA</td>
<td>Phytic acid</td>
</tr>
<tr>
<td>PP</td>
<td>Polyphenols</td>
</tr>
<tr>
<td>RBV</td>
<td>Relative biological value</td>
</tr>
<tr>
<td>RE</td>
<td>Reticuloendothelial</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SF</td>
<td>Serum ferritin</td>
</tr>
<tr>
<td>SFT</td>
<td>Stimulator of Fe Transport</td>
</tr>
<tr>
<td>SFT</td>
<td>Stimulator of Fe Transport gene</td>
</tr>
<tr>
<td>SSM</td>
<td>Semisynthetic meal</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR1/2</td>
<td>Transferrin receptor 1/2</td>
</tr>
<tr>
<td>TfR1/2</td>
<td>Transferrin receptor 1/2 gene</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>Tumor necrosis factor-(\alpha)</td>
</tr>
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## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TS</td>
<td>Transferrin saturation</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WBC</td>
<td>Whole body counting</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</tbody>
</table>

Summary

Background
Non-heme iron bioavailability in humans is influenced by a variety of dietary factors. Ascorbic acid and meat have been repeatedly shown to enhance iron absorption, whereas polyphenols, phytic acid, calcium as well as milk and soy proteins depress it. Organic acids such as citric, malic, and oxalic acid have produced equivocal results. How meat exerts its beneficial effect on iron absorption has so far eluded researchers. Most studies provide evidence in favor of meat proteins as the source of enhancer(s), but more recent in vitro data also suggest glycosaminoglycans, a minor muscle tissue component, as a potential “meat factor”. Oxalic acid is assumed to largely contribute to the poor bioavailability of spinach iron; yet experimental evidence to justify this claim is scarce.

Aim
The aim of this PhD project was to evaluate selected dietary components with regard to their influence on non-heme iron bioavailability. Firstly, experiments were undertaken to isolate and characterize iron-binding peptides from meat that may serve as enhancers of iron absorption. Secondly, purified glycosaminoglycans were assessed as to their potential “meat factor” properties. Lastly, the role of oxalic acid in human iron absorption was addressed.

Design
In study series 1, an extensive set of in vitro experiments was performed to obtain information on low-molecular-weight iron-binding meat peptides that could act as promoters of iron absorption. Simulated gastrointestinal digestion with pepsin and pepsin/pancreatin, ultrafiltration, gel filtration with an added radioiron tracer, amino acid analysis, Caco-2 cell culture, and liquid chromatography-mass spectrometry (LC-MS) were employed to characterize iron-binding peptides from water-soluble (sarcoplasmic) and salt-soluble (myofibrillar) meat protein extracts.

Study 2 comprised two human stable isotope studies comparing iron absorption from a semisynthetic meal (maltodextrin, corn oil, egg albumin, water) with or without purified glycosaminoglycans of either the unsulfated (hyaluronic acid) or sulfated
Summary

(chondroitin sulfate) type. Absorption was quantified by measuring erythrocyte incorporation of $^{57}$Fe and $^{58}$Fe 14 d after test meal administration.

Iron absorption from kale (low in native oxalic acid) with or without added potassium oxalate was determined in study 3 and compared to iron absorption from spinach (high in native oxalic acid).

Results

Study series 1: Low-molecular-weight peptides (< 10 kDa) set free upon digestion of myofibrillar proteins from beef, chicken, cod, lamb, and pork muscle tissue showed extensive iron-solubilizing capacity. Between 84-98% of the total iron in the assay appeared in the ultrafiltrable fraction of these proteins. Based on results from beef, chicken, and cod, pepsin digestion alone was sufficient to produce such peptides. Gel filtration of the myofibrillar peptides tagged with $^{59}$Fe revealed a fraction of 2 kDa molecular weight as the major iron-binding fraction. As indicated by amino acid analysis, glutamic and aspartic acid residues were enriched in the iron-binding peptides. LC-MS of peptides separated by gel filtration and coupled with a SwissProt database search matched some of the peptide masses to fragments of the major myofibrillar protein myosin.

Study series 2: Addition of the unsulfated glycosaminoglycan sodium hyaluronate (300 mg) to a semisynthetic meal consisting of maltodextrin, corn oil, egg albumin, and water did not significantly affect its iron bioavailability (21.2% vs 19.5%, $P = 0.42$). Replacing sodium hyaluronate with equimolar amounts of the sulfated glycosaminoglycan chondroitin sulfate (360 mg) yielded very similar results (19.4% vs 20.3%, $P = 0.64$). Percentage iron absorption was not significantly different between the semisynthetic meals containing either sodium hyaluronate or chondroitin sulfate ($P = 0.50$). The study design would have allowed detecting a change in iron absorption of about 30% at 80% power.

Study series 3: Iron absorption from meals containing 100 g wheat bread rolls and 150 g kale, a vegetable low in native oxalic acid (< 0.01 g/100 g fresh weight), was 11.0% and did not differ from kale meals with 1.26 g added potassium oxalate (11.2%). Normalization for reference spinach meal iron absorption yielded absorption percentages of 10.7% and 11.5%, respectively, the difference remaining non-
significant \((P = 0.86)\). Iron absorption from the spinach meals was 8.4\% in the first study and 8.0\% in the second study. The 24-28\% lower absorption from the spinach compared to the kale meals did not reach statistical significance \((P > 0.16)\), which could be expected as the study was designed to pick up a 30\% difference at 80\% power.

**Conclusions**

1. The present findings provide further evidence that low-molecular-weight peptides set free upon digestion of myofibrillar meat proteins bind and solubilize iron. These results are in line with literature reports and extend the published data mostly on chicken muscle, to include beef, cod, lamb, and pork. Importantly, pepsin digestion was sufficient to obtain such peptides. The gel filtration experiments identified a new group of iron-binding peptides of 2 kDa molecular mass enriched in aspartic and glutamic acid which could explain the iron-binding properties. The results would support a theory of early release, in the stomach, of peptides by pepsin which bind with iron and prevent interaction with inhibitors of iron absorption such as phytic acid and polyphenols. The peptides remain soluble at the higher pH of the duodenum and transport iron to the absorptive sites. Certain iron-binding peptides within the 2 kDa group of peptides identified in this study would be expected to be part of the “meat factor”. Nevertheless, other peptides not identified in this study, and perhaps enriched in cysteine and/or histidine, could also be part of the “meat factor”.

2. Meat carbohydrates, namely glycosaminoglycans derived from the intercellular matrix, have been proposed as an alternative “meat factor” based on Caco-2 iron uptake experiments. The two human absorption studies evaluating hyaluronic acid and chondroitin sulfate – which may be regarded representative of unsulfated and sulfated glycosaminoglycans, respectively – did not support this suggestion. No differences in iron absorption from semisynthetic meals with or without added glycosaminoglycans were observed. Unsulfated and sulfated glycosaminoglycans as represented by hyaluronic acid and chondroitin sulfate do not enhance iron absorption and it would seem unlikely that minor glycosaminoglycans would influence iron absorption. However, we only tested two glycosaminoglycan structures out of a large number of possible compounds. Furthermore, glycosaminoglycan
carbohydrates are known to be susceptible to oxidation, and the effect of isolation of the purified compound on its chemical structure remains unknown.

3. Large amounts of oxalic acid, given as soluble potassium oxalate, did not influence iron absorption from a vegetable meal low in native oxalic acid. It is therefore unlikely that oxalic acid from plant foods would interfere with iron absorption. The lower iron absorption from spinach reported in the literature presumably relates to its high polyphenol and calcium content rather than the high levels of native oxalic acid. However, the different solubility properties of ferrous and ferric oxalate warrant further investigation.
Zusammenfassung

Hintergrund

Zielsetzung

Design
Zusammenfassung

Abschnitt 2: Zwei Humanstudien mit stabilen Isotopen dienten dem Vergleich der Glykosaminoglykane Hyaluronsäure (unsulfatiert) und Chondroitinsulfat (sulfatiert) bezüglich ihres Einflusses auf die Eisenabsorption aus einem semisynthetischen Shake (Maltodextrin, Maiskeimöl, Eialbumin, Wasser). Die Anreicherung der stabilen Eisenisotope $^{57}$Fe und $^{58}$Fe in den Erythrozyten 14 Tage nach Gabe der Testmahlzeiten diente als Mass für die Absorption.


Ergebnisse


Abschnitt 2: Zusatz des unsulfatierten Glykosaminoglykans Na-Hyaluronat (300 mg) zu einem semisynthetischen Shake aus Maltodextrin, Maiskeimöl, Eialbumin und Wasser hatte keinen signifikanten Einfluss auf die Eisenabsorption (21.2% vs 19.5%, $P = 0.42$). Austausch des Na-Hyaluronats durch eine äquimolare Menge des sulfatierten Glykosaminoglykans Chondroitinsulfat (360 mg) erbrachte sehr ähnliche Resultate (19.4% vs 20.3%, $P = 0.64$). Die prozentuale Eisenabsorption unterschied sich ebenfalls nicht zwischen den zwei Glykosaminoglykanen ($P = 0.50$). Das
Zusammenfassung

Studiendesign hätte den Nachweis einer signifikanten Absorptionsänderung von 30% mit achtzigprozentiger Macht gestattet.

Abschnitt 3: Die Eisenabsorption aus einer Mahlzeit mit 100 g Weizenbrötchen und 150 g Grünkohl, der arm an natürlicher Oxalsäure ist, betrug 11.0% (Studie I) und änderte sich nicht nach Zugabe von 1.26 g Kaliumoxalat (11.2%, Studie II). Bezogen auf eine über die zwei Studien gemittelte Absorption von 8.2% aus der Kontrollmahlzeit, die statt Grünkohl Spinat enthielt, ergaben sich Absorptionsraten von 10.7% beziehungsweise 11.5%. Der Unterschied blieb statistisch nicht signifikant ($P = 0.86$). Aus der Spinatmahlzeit wurde das Eisen zu 8.4% (Studie I) und 8.0% (Studie II) absorbiert. Die um 24-28% niedrigere Absorption aus Spinat gegenüber Grünkohl erreichte keine statistische Signifikanz ($P > 0.16$), da die Studie auf die Erfassung eines mindestens dreissigprozentigen Unterschieds mit einer Macht von 80% ausgelegt war.

Schlussfolgerungen

Zusammenfassung

vermutlich einen erhöhten Gehalt an Cystein und/oder Histidin aufweisen, am „meat factor“ beteiligt sind.


Introduction

It is highly reactive, toxic when free (Hentze et al. 2004) and possibly involved in a variety of pathologies like cancer (Toyokuni 1996; Huang 2003), diabetes (Salonen et al. 1998), and coronary heart disease (Salonen et al. 1992; Tuomainen et al. 1998; Gaenzer et al. 2002), yet virtually no living thing on earth can do without it – iron. Except for a few lactic acid bacteria (Bruyneel et al. 1989; Weinberg 1997), all organisms have made iron an essential nutrient by incorporating it into central proteins of the intermediary metabolism (Lauble et al. 1992; Lodish et al. 2004), the respiratory chain (Lodish et al. 2004), and the DNA synthesis/repair machinery (Kolberg et al. 2004). Its redox chemistry favors the involvement of iron in a number of hydroxylation and electron transfer reactions. At the same time, this requires intricate mechanisms to avoid oxidative damage, because once out of control, iron catalyzes the formation of the very short-lived, i.e., extremely reactive hydroxyl radical (Nappi and Vass 2002). Ensuing pro-oxidative modifications of proteins, fatty acids, and DNA wreak havoc on a subcellular and cellular level, provoking the above-mentioned pathological conditions.

Nutritionally, iron supply is critical for several population groups, deficiency levels being highest in countries of the developing world (WHO 2002). Diet diversification/modification (Gibson and Hotz 2001), fortification (Hurrell 1997b), and supplementation (Baltussen et al. 2004) are means to improve iron status, but usefulness and feasibility of these strategies vary with the extent of deficiency and the level of intervention (individual, subgroup, or population). Conversely, people suffering from iron overload disorders like hereditary hemochromatosis are requested to remove excess iron from the body to avoid organ damage and associated pathologies (Beutler 2006). Dietary strategies to minimize iron intake and bioavailability are insufficient and need to be complemented with iron chelator therapy and regular phlebotomies to achieve healthy body iron levels (Hash 2001).

The present thesis is based on a literature review and three manuscripts. The first part of the review illustrates the absorption, transport, and physiological role of iron in the human body and describes pathophysiological consequences of iron deficiency and overload. Subsequent chapters are dedicated to in vitro and in vivo measures of iron bioavailability. Nutritional issues related to iron are discussed thereafter, focusing
on dietary enhancers and inhibitors of iron absorption. Special emphasis is placed on muscle tissue components that might contribute to the as yet elusive “meat factor”, an iron absorption-enhancing entity in meat. The first manuscript deals with the characterization of iron-binding meat peptides in vitro, employing simulated gastrointestinal digestion, ultrafiltration, chromatographic techniques, mass spectrometry, and the Caco-2 cell culture model of intestinal absorption. Manuscript 2 reports on a human study assessing the potential of purified sulfated and unsulfated glycosaminoglycans (GAGs), a minor component of muscle tissue, to constitute a part of the meat factor. Iron absorption was studied from a semisynthetic meal containing egg albumin, corn oil, and maltodextrin served with or without added GAGs. Finally, results of the influence of oxalic acid (OA) on human iron absorption are presented in manuscript 3. Wheat bread meals were fed with spinach (high native OA), kale (low native OA), and kale with added OA from potassium oxalate in amounts equal to spinach OA content.
Literature Review

1 Iron

1.1 METABOLIC ASPECTS
The adult human body contains 3-5 g of iron, approximately two thirds of which are found in the oxygen carrier molecules hemoglobin and myoglobin as well as in several enzymes (NARINS 1980; AISEN et al. 2001). The remainder is sequestered in the storage proteins ferritin and hemosiderin in the liver, spleen, and bone marrow. Daily plasma iron turnover ranges between 20-25 mg although only about 3 mg iron circulate in the steady state as transferrin-bound iron (Fig. 1).

![Systemic iron homeostasis](image)

**Figure 1** Systemic iron homeostasis. The major functional iron pool is as heme in the erythrocytes. At the end of their lifespan (~120 d), the red cells are digested by reticuloendothelial macrophages and their iron released as inorganic iron onto transferrin (Fe$_2$-Tf). From there, it may be distributed into peripheral tissues, recycled via the bone marrow for newly forming erythrocytes, or stored in the liver as ferritin (adapted from (HENTZE et al. 2004)).

The high metabolic iron demand is largely balanced by efficient regeneration (~90%) of iron released from senescent erythrocytes (BONNET et al. 1960). Actual body iron losses amount to approximately 1 mg/d in healthy, adult men (GREEN et al. 1968; HALLBERG 1981a; HALLBERG et al. 1998) and are normally adequately covered by
dietary iron absorption (Cook 1990; Hulten et al. 1995). However, regular blood losses cause menstruating women to face an additional loss of 0.5-1.5 mg iron per day (Hallberg and Rossander-Hulten 1991), which is more difficult to balance by dietary means. Likewise, children and adolescents require 0.5-1.5 mg in excess of their daily iron losses to satisfy the extra needs associated with growth. Finally, the total iron cost of pregnancy has been estimated at 1040 mg (Picciano 2003), with average daily requirements reaching as high as 4-6 mg during the second and third trimester (Carpenter and Mahoney 1992).

It is well known that host-related factors such as body iron stores or pregnancy influence non-heme iron absorption in humans (Table 1). Today, many of the underlying mechanisms are beginning to be elucidated and hypothetical signaling networks developed. How regulation of iron homeostasis is brought about is best understood by looking at the proteins of iron metabolism and the consequences of their genetic defects. Beforehand, a summary of the physiological roles of iron clarifies why the body undertakes the risk to acquire this potentially toxic metal.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect on absorption</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of body iron stores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- low</td>
<td>marked inverse effect</td>
<td>(Baynes et al. 1987)</td>
</tr>
<tr>
<td>- normal, high</td>
<td>minor inverse effect</td>
<td></td>
</tr>
<tr>
<td>Rate of erythropoiesis</td>
<td>positive correlation</td>
<td>(Skikne and Cook 1992)</td>
</tr>
<tr>
<td>Physiological state</td>
<td>increased absorption in pregnancy</td>
<td>(Whittaker et al. 2001)</td>
</tr>
<tr>
<td>Iron content of mucosal cells</td>
<td>exposure to iron reduces subsequent absorption</td>
<td>(Fairweather-Tait and Minski 1986)</td>
</tr>
<tr>
<td>High altitude, hypoxia</td>
<td>increased absorption</td>
<td>(Cook et al. 2005)</td>
</tr>
<tr>
<td>Secretion of gastric juice</td>
<td>positive correlation</td>
<td>(Bezwoda et al. 1978)</td>
</tr>
<tr>
<td>GI secretions (bile, pancreatic secretions, mucus)</td>
<td>increased absorption in presence of amino acids, peptides, ascorbic acid, and mucoproteins</td>
<td>(Bothwell et al. 1979)</td>
</tr>
</tbody>
</table>
1.1.1 Physiological roles of iron

1.1.1.1 Oxygen carrier

The most prominent role of iron in the human body is that of an oxygen carrier and reservoir in the hemoglobin and myoglobin molecules. Hemoglobin (Fig. 2) is a tetrameric protein complex consisting of two α-globin and two β-globin chains with each chain containing a hydrophobic pocket into which a heme ring is inserted. Embedded as the central atom in the heme moiety (protoporphyrin IX, Fig. 3a), ferrous iron coordinates with the four nitrogen atoms of the porphyrin ring and a histidine residue in the respective globin chain. The residual coordination site is left for reversible oxygen binding, but may also interact with other ligands like carbon monoxide (CO) or nitric oxide (NO). Oxidation of the heme iron to the ferric form yields so-called methemoglobin (also known as hemiglobin), which is not capable of binding oxygen. Since formation of methemoglobin is a spontaneous but unwanted process, an enzyme called methemoglobin reductase is in place to restore functional hemoglobin (KUMA and INOMATA 1972; TOMODA et al. 1980) and with it oxygen transport.

![Figure 2](image)

**Figure 2** Human hemoglobin – tetrameric complex consisting of two α-globin (red) and two β-globin (golden) chains and four heme molecules (green) inserted in their respective pockets; central dots in the heme moieties represent coordinately bound iron (from wikipedia).

The affinity of hemoglobin to oxygen depends on several factors: pH, partial oxygen pressure (pO₂), oxygen saturation, and the concentration of 2,3-bisphosphoglycerate
(JENSEN 2004). These factors concordantly facilitate oxygen release and carbon dioxide uptake at the site of oxygen consumption, i.e., peripheral tissues and organs, whereas in the lungs the reverse process is promoted. In addition, deoxygenated hemoglobin is involved in NO production from nitrite (NO$_2^-$), thus contributing to blood flow regulation (CRAWFORD et al. 2006). Controversy exists about hemoglobin as a reservoir for NO (in the form of S-nitroso-hemoglobin or iron-nitrosyl-hemoglobin) or as being allosterically regulated by NO in terms of oxygen affinity (GOW et al. 1999; HUANG et al. 2001; GLADWIN et al. 2002; STEPURO and ZINCHUK 2006).

In contrast to hemoglobin, myoglobin is a monomeric protein, i.e., consisting of a single globin chain, and – as the name suggests – acts as a muscle cell-specific oxygen carrier (WITTEMBERG and WITTEMBERG 2003). Its oxygen affinity is slightly higher than that of hemoglobin thus facilitating oxygen inflow into the myocyte. Further members of the globin family are the neuron-specific neuroglobin and the ubiquitous cytoglobin, both capable of binding oxygen (FAGO et al. 2004) and possibly i) regulating oxygen transport, ii) detoxifying reactive oxygen species (ROS), and/or iii) acting as hypoxia sensors (BURMESTER et al. 2002; SUN et al. 2003; XU et al. 2006).

1.1.1.2 Redox chemistry

Its redox properties predestine iron to participate in a variety of electron transfer processes and enzymatic hydroxylation reactions. Chemically, a reduction can be described as the uptake of one or more electrons, the opposite being true for an oxidation. Consequently, redox reactions are characterized by the coupling of an oxidation and a reduction leading to the transfer of electrons from an electron donor to an electron acceptor. In the human body, such redox reactants may be complex organic molecules such as flavin adenine dinucleotide (FAD/FADH$_2$) or simple atoms like the transition metals nickel (Ni$^{+}$/Ni$^{2+}$), copper (Cu$^+$/Cu$^{2+}$), or iron (Fe$^{2+}$/Fe$^{3+}$). At several stages of the respiratory chain, iron functions as an intermediate, i.e., first as an electron acceptor and then as a donor, for the transfer of electrons from one molecule to another (BEARD 2001). As a result, electrons are passed down along a cascade of redox-active proteins ultimately leading to the reduction of molecular oxygen and the formation of ATP, the universal cellular energy currency (LODISH et al. 2004).
1.1.1.3 Enzyme cofactor
Iron is an essential cofactor to a number of enzymes, e.g. aconitase (BRAZZOLLOTTO et al. 1999), ribonucleotide reductase (KOLBERG et al. 2004), or the cytochrome P450 family of oxygenases (POULOS 2005). Within these enzymes, iron is normally present either as heme (Fig. 3a) or in the form of iron-sulfur (Fe-S) clusters (Fig. 3b). Its major function lies in the activation of molecular oxygen for hydroxylation reactions (BOLLINGER and KREBS 2006), but electron transfer reactions as occur in the respiratory chain are similarly important.

![Heme and iron-sulfur prosthetic groups](lodish2004)

**Figure 3**  Heme (a) and iron-sulfur (b) prosthetic groups (from LODISH et al. 2004).

It is obvious from Figure 3b that the Fe-S clusters contribute substantially to protein conformation (ROUAULT and TONG 2005; IMLAY 2006). Since conformation is closely linked to enzyme functionality, the availability of iron dictates enzymatic activity. This is illustrated by the cytosolic isofrom of the enzyme aconitase, which contains a cubane 4Fe-4S cluster (Fig. 4, left) in its active state (DUPUY et al. 2006). Upon iron deprivation or oxidative attack, the 4Fe-4S cluster turns into a 3Fe-4S structure (Fig. 4, right), thus changing protein conformation and causing loss of aconitase activity (BRAZZOLLOTTO et al. 1999). Interestingly, when cluster disassembly proceeds, the enzyme assumes a new role as the so-called iron-regulatory protein 1 (HAILE et al. 1992) discussed in section 1.2.2.3.
1.1.2 Molecular biology of iron metabolism

Since the body lacks mechanisms to actively excrete iron (McCance and Widdowson 1937; Hallberg 1981a; Ganz and Nemeth 2006), iron status is largely regulated at the level of absorption. Some may consider the transfer of surplus body iron into mucosal cells followed by cell sloughing a directed means of iron removal. Yet fecal ferritin excretion – although responsive to body iron stores (Skikne et al. 1995) and dietary iron bioavailability (Hunt and Roughead 2000) – does not seem to substantially contribute to body iron balance (Hunt and Roughead 2000). Proteins involved in enterocyte iron uptake show high abundance in the duodenum (Frazer and Anderson 2005; Mackenzie and Garrick 2005; Petrak and Vyoral 2005; Wessling-Resnick 2006), the major site of iron absorption (Whelby 1970). Corresponding gene expression levels are modulated by an intricate network of factors signaling body iron needs. The cast of proteins involved in iron absorption in the small intestine is dealt with in order of appearance during the absorptive process from the gut lumen to the bloodstream. Subsequently, major options of iron distribution and pathways of storage and release are reviewed. A summary of the mechanisms regulating iron absorption rounds off the molecular aspects of normal iron metabolism.
1.1.2.1 Iron absorption
Assuming dietary non-heme iron to reach the duodenum – the major site of iron absorption (Wheelby 1970) – in the ferric state, it first needs to be reduced to the ferrous form through the action of a plasma membrane ferric reductase. The existence of such a reductase had first been demonstrated in animals (Raja et al. 1992; Pountney et al. 1994) and cell culture studies (Ekmeckioğlu et al. 1996). However, it was not until 2001 that McKie and co-workers (2001) finally identified the gene (Dcytb) and protein (Dcytb) sequence of a molecular entity capable of reducing iron in mouse duodenum. The name Dcytb (duodenal cytochrome b) was chosen because of its similarity to other proteins of the cytochrome b\textsubscript{561} family of plasma membrane reductases. Dcytb is a putative diheme protein that is highly expressed in the apical membrane of duodenal enterocytes and shows adaptive response to iron status variations (McKie et al. 2001; Latunde-Dada et al. 2002). Ascorbic acid (AA) seems to be the primary electron donor for iron reduction in this system (Raja et al. 1992; McKie et al. 2001; Latunde-Dada et al. 2002), but NADH and FMNH\textsubscript{2} might also play a role (Pountney et al. 1994). Whether AA exerts its enhancing effect on iron absorption at this stage remains to be demonstrated.

Recently, Dcytb knockout mice (Dcytb\textsuperscript{-/-}) were shown to have normal iron absorption and to exhibit a phenotype indistinguishable from wild-type mice (Gunshin et al. 2005), thus questioning the importance of Dcytb in the absorption of dietary iron. However, inherent limitations of the model system put the results into perspective. First, the mouse strain employed in the studies reportedly does not become anemic on an iron-deficient diet known to induce anemia in other strains. Second, mice endogenously synthesize ascorbic acid, a well known enhancer of iron absorption (Hallberg et al. 1986; 1989a), and feed antioxidants may have further contributed to iron reduction. Third, even if Dcytb is dispensable in mice, this might not be the case for other species including humans. Finally, further ferric reductases (Van Duijn et al. 1998; Lee et al. 2000) may be active to maintain sufficient iron bioavailability in the absence of Dcytb. In addition to enzymatic means, ferric reduction can also be accomplished by luminal AA or other reducing agents, e.g. glutathione or cysteine (Barrand et al. 1990), but their relative contribution is unknown.

Reduced iron is internalized by the divalent metal transporter 1 (DMT1, also known as DCT1 or Nramp2) that displays electrogenic Fe\textsuperscript{2+}/H\textsuperscript{+} symport (Fleming et al. 1997;
Such a proton-driven uptake mechanism is well known in the intestinal absorption of di- and tripeptides (Daniel 1996) and may also be active in niacin uptake (Nabokina et al. 2005). The protons required to drive these processes are derived from the activity of the Na\(^+\)/H\(^+\) exchanger (NHE) present in the enterocyte apical membrane (Hoogerwerf et al. 1996). NHE creates an acidic microclimate on the outer cell surface (Lucas and Blair 1978), the proton gradient then being exploited to translocate nutrients into the enterocyte. DMT1 (Fig. 5) is an integral membrane protein of the intestinal brush border with twelve putative transmembrane domains, two predicted extracellular \(N\)-linked glycosylation sites in the fourth extracellular loop and a ‘consensus transport motif’ (CTM) in the fourth intracellular loop (Gruenheid et al. 1995; Gunshin et al. 1997). The CTM is characteristic for several transport proteins of bacterial and mammalian origin (Vidal et al. 1993; Cellier et al. 1994; Cellier et al. 1995). The importance of functional DMT1 in mammalian iron absorption is evidenced by \(mk\) mice and Belgrade rats, which both develop anemia due to mutations in their gene coding for the DMT1 protein (Canonne-Hergaux et al. 2000).

![Figure 5](image)

**Figure 5** Putative membrane topology of the divalent metal transporter 1 (DMT1) with 12 transmembrane domains (1-12), the intracellular amino and carboxy termini, two putative \(N\)-linked glycosylation sites in the fourth extracellular loop, and an intracellular ‘consensus transport motif’ (CTM) in the fourth intracellular loop (from (Gunshin et al. 1997)).

As the name suggests, DMT1 may not be specific for iron. Gunshin et al. (1997), using heterologous expression in *Xenopus* oocytes, demonstrated comparable induction of an electrical current in the presence of Zn\(^{2+}\), Cd\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\), and Co\(^{2+}\).
Ni\textsuperscript{2+} and Pb\textsuperscript{2+} yielded somewhat lower responses. However, electrical current measurements do not necessarily reflect absorption, and later uptake studies in erythroleukemia-like K562 (CONRAD et al. 2000) and intestinal Caco-2 cells (TANDY et al. 2000) failed to confirm transport of Zn\textsuperscript{2+} by DMT1. In contrast, Mn\textsuperscript{2+}, Co\textsuperscript{2+}, and Cd\textsuperscript{2+} were internalized by DMT1 in Caco-2 cells (FORBES and GROS 2003) and a 100-fold excess of Co\textsuperscript{2+} or Cd\textsuperscript{2+} significantly inhibited DMT1-mediated Fe\textsuperscript{2+} uptake by > 50\% (TANDY et al. 2000). Additionally, Mn\textsuperscript{2+} uptake increased upon transient overexpression of DMT1 in HEK263T kidney cells and was diminished when DMT1 was blocked with a selective antibody (CONRAD et al. 2000).

In addition to the non-heme ferrous iron absorption pathway, evidence for a specific system for ferric iron absorption exists (CONRAD et al. 2000). Dietary ferric iron would bind to mucin in the stomach (CONRAD et al. 1991) to be delivered to β\textsubscript{3}-integrin residing in the duodenal mucosal membrane (CONRAD et al. 1993) and transferred to mobilferrin and ferritin or the paraferritin protein complex in the enterocyte cytoplasm (CONRAD et al. 1992). Hence, this route has been termed integrin-mobilferrin-paraferritin (IMP) pathway (CONRAD and UMBREIT 2000). Gastrointestinal mucins have long been implicated in iron absorption. Mucus secretion is stimulated by acid (RAMIREZ et al. 2004) and dietary peptides (CLAUSTRE et al. 2002), possibly hinting at a synergistic effect of gastric acid (BEZWODA et al. 1978) and mucins on iron solubilization. POWELL et al. (1999) proposed exchange kinetics between mucins and metals of varying valency as an important factor in mineral bioavailability. Notably, the iron-binding capacity of mucin from dog stomach – albeit generally moderate – was highest in a uronic acid-rich fraction (BELLA and KIM 1973).

Although pulse-chase experiments demonstrated sequential binding to molecules of the IMP pathway in K562 cells (CONRAD et al. 1994), it remains unclear how iron is internalized by β\textsubscript{3}-integrin. If ferric iron from β\textsubscript{3}-integrin is reduced by Dcytb followed by DMT1-mediated import and subsequent transfer to mobilferrin, this would obviate the need for postulating a separate absorption pathway for ferric iron. However, blocking β\textsubscript{3}-integrin with a specific antibody or chelating ferric iron with an impermeable chelator abolished ferric iron uptake into K562 cells (CONRAD et al. 2000), whereas ferrous iron uptake was unaffected by this treatment. In turn, blocking DMT1 with a selective antibody diminished ferrous but not ferric iron uptake,
and only ferrous iron uptake increased upon transient overexpression of DMT1 in HEK293T kidney cells. Since the molecules supposed to mediate ferric iron uptake in K562 cells are present in the human duodenal mucosa, the IMP pathway may indeed present an alternative route of intestinal non-heme iron absorption. The relative contribution of the IMP and DMT1 pathways seems to depend on the luminal redox state (CONRAD et al. 2000). Recent reports of severe microcytic anemia in subjects with partially defective DMT1 (BEAUMONT et al. 2006; IOLASCON et al. 2006; LAM-YUK-TSEUNG et al. 2006), however, support an essential role of the latter. Additionally, the poor solubility of ferric iron at pH > 3 makes itself a rare substrate at the relatively high intestinal pH.

Once inside the cell, non-heme iron is shuttled into a common pool together with iron derived from heme. Intact heme enters the intestinal mucosal cell by a separate, endocytotic process via the heme receptor HCP1 recently identified in the apical membrane of mouse duodenal enterocytes (SHAYEGHI et al. 2005). Subsequently, microsomal heme oxygenase 1 (HO-1) splits the porphyrin ring to release inorganic iron into the cytosol (RAFFIN et al. 1974), presumably in a DMT1-mediated fashion. This common pool of intracellular non-heme iron is not to be confused with that forming from extrinsic and intrinsic non-heme iron in the stomach and duodenum during meal digestion (COOK et al. 1972). The precise mechanisms of intracellular iron transport remain poorly understood, but mobilferrin has been suggested to be involved in the trafficking machinery (CONRAD et al. 1996).

Non-heme iron within the enterocyte may be processed in three ways. The fraction not immediately needed by the body is stored in ferritin, the major iron storage protein throughout the human body (HARRISON and AROSIO 1996), and lost upon epithelial sloughing. Metabolic demands of the enterocyte are covered by another fraction. For the remaining iron to reach its functional compartments of hemoglobin, myoglobin, and iron-dependent enzymes in non-intestinal cells, it first needs to cross the enterocyte basolateral membrane. Transfer (Fig. 6) is accomplished by the interplay of the export protein ferroportin1 (DONOVAN et al. 2000), also known as IREG1 (McKie et al. 2000) or MTP1 (ABBoud and Haile 2000), and the membrane-bound multi-copper ferroxidase hephaestin (VULPE et al. 1999; FRAZER et al. 2001). Ferroportin1 shows sequence homology to the DMT1 protein family (ABBoud and
HAILE 2000), which lends support to its function as a metal transporter. Most probably, ferrous iron serves as a substrate for ferroportin1 spanning the basolateral membrane and is oxidized by hephaestin before it is loaded onto transferrin (Tf) for distribution. A mouse mutant in hephaestin develops anemia with concomitant enterocyte iron loading (VULPE et al. 1999), demonstrating the vital role of this protein in the export process. The highly similar expression patterns of ferroportin1 and hephaestin in the rodent gut (DONOVAN et al. 2000; FRAZER et al. 2001) emphasize the close functional link between these molecules.

Figure 6 Proteins involved in enterocyte apical iron absorption and basolateral transfer. Dietary non-heme iron, if not already in the ferrous form, is reduced by the membrane ferric reductase Dcytb prior to proton-coupled internalization via DMT1. Heme iron, upon binding to the meanwhile identified heme receptor HCP1 (SHAYEGHI et al. 2005), takes a separate, endosomal pathway into the enterocyte. Heme oxygenase 1 splits the porphyrin ring within the endosome to release ferrous iron, which is most likely moved across the endosomal membrane by DMT1. Cytosolic iron is either stored in ferritin or exported basolaterally through the interplay of IREG1 and the ferroxidase hephaestin to be loaded onto transferrin (Tf) for distribution. Dcytb, duodenal cytochrome b; DMT1, divalent metal transporter 1; IREG1, ferroportin1; Fe$^{2+}$Tf, diferric transferrin; HO, heme oxygenase 1 (from (MACKENZIE and GARRICK 2005)).

1.1.2.2 Iron distribution

In the blood, extracellular iron mainly travels bound to Tf (> 97%) (DAS et al. 1996), a very minor fraction occurring as non-Tf-bound iron (NTBI) complexed to small organic molecules like citrate (GROOTVELD et al. 1989). The monomeric Tf protein has a molecular weight of ~80 kDa, contains two glycosylation sites (SPIK 1982), and may be divided into two homologous N- and C-lobes, which are stabilized by intrachain
disulfides (HIROSE 2000). Each lobe binds one atom of Fe\(^{3+}\) (HIROSE 2000), and the bicarbonate anion has been found to influence binding kinetics (LIN et al. 1993). Intriguingly, replacement of bicarbonate by oxalate locks iron in Tf (HALBROOKS et al. 2004), potentially compromising iron metabolism. It is not known, though, whether this effect would have any consequences on intestinal iron absorption.

Cellular uptake of Tf-bound iron (holo-Tf) is accomplished by interaction with a complementary transferrin receptor (TfR) residing in the surface membrane. Two isoforms of TfR, namely TfR1 and TfR2, have been described in humans and seem to play different roles in the regulation of body iron distribution and homeostasis (HENTZE et al. 2004). Since TfR2 is assumed to have a rather regulatory function, it will be discussed in more detail in section 1.1.2.3. Generally, TfR is a protein homodimer capable of binding two holo-Tf molecules. Upon binding, the holo-Tf-TfR complex is internalized by a highly regulated process called clathrin-mediated endocytosis (SORKIN 2004) to form an endosomal compartment (Fig. 7). Clathrin-coated pits initiate endocytosis by gathering holo-Tf-TfR complexes and budding towards the cell interior. Subsequent acidification of the endosome to a pH of ~5.5 by a H\(^+\)-ATPase causes proton-driven decarbonation and conformational changes in the Tf-TfR complex that favor iron dissociation (HEMADI et al. 2006). The suggestion that H\(^+\)-ATPase plays a direct role in iron export into the cytosol (LI et al. 1995) is not supported by more recent data (SAVIGNI and MORGAN 1996). Iron exits from the endosome in the ferrous form via DMT1 (FLEMING et al. 1998b), which had been co-internalized with the holo-Tf-TfR complex. The necessary prior reduction of ferric to ferrous iron is likely mediated by Steap-3, a recently identified flavin-NADPH-dependent, membrane-bound oxidoreductase (OHGAMI et al. 2005). Endosomal apo-Tf-TfR is recycled to the surface membrane, where the blood pH of 7.4 stimulates the release of apo-Tf into the circulation (DAUTRY-VARSAT et al. 1983).
Figure 7  Schematic representation of the Tf-TfR cycle for iron delivery into cells (from (ANDREWS 1999)).

Depending on the cell type, cytosolic iron may be stored as ferritin or routed to the mitochondria for incorporation into Fe-S proteins or heme synthesis (HENTZE et al. 2004). The latter process is of prime importance in erythrocyte precursor cells, so-called reticulocytes, which take up iron by the endocytotic Tf-TfR cycle (LEVY et al. 1999) or by a transferrin-independent mechanism similar to that in the gut (HODGSON et al. 1994; INMAN et al. 1994; CANONNE-HERGAUX et al. 2001). Again, the IMP pathway may have an additional role in non-Tf iron uptake (CONRAD et al. 1994; CONRAD et al. 1996). How iron crosses the mitochondrial membrane is still a conundrum, but frataxin – a protein associated with the degenerative disease Friedreich’s ataxia – and members of the ABC (ATP-Binding Cassette) transporter family may be partially involved (CHUNG and WESSLING-RESNICK 2003). Frataxin regulates the biogenesis of Fe-S-containing proteins and, when defective, causes mitochondrial iron overload and neurodegeneration (BECKER and RICHARDSON 2001). Iron not used for Fe-S cluster synthesis is inserted into protoporphyrin IX by action of the enzyme ferrochelatase (CROUSE et al. 1996) to form heme. The ferrochelatase seems to widen the inner space of the porphyrin ring by bending the planar molecule, before ferrous iron is fitted into position from one side with concomitant release of
two hydrogen ions on the other (DAILEY et al. 2000). Variable frataxin levels have been speculated to provide a switch between the two pathways of mitochondrial iron use (HENTZE et al. 2004).

Surplus iron in the body – be it derived from the diet as a consequence of the hepatic first-pass effect or from elimination of senescent red blood cells – is shuffled into the storage compartments of liver, spleen, and macrophages (ANDREWS 1999). Since free iron is toxic, the iron storage protein ferritin sequesters iron to avoid cell damage. Ferritin has a molecular weight of approximately 450 kDa and consists of 24 subunits made up by heavy (H) and light (L) chains of 21 and 19.5 kDa molecular weight, respectively (AISEN et al. 2001). The H-chain has ferroxidase activity important for iron deposition (TREFFRY et al. 1992), whereas the L-chain controls the structure of the crystalline iron core and reduces non-specific iron hydrolysis (LEVI et al. 1994). The protein subunits assemble into a rhomboid shell, which can be filled with up to 4500 iron atoms stored as micellar ferric oxy-hydroxide (CHIANCONE et al. 2004). Depending on the tissue, H- and L-chains contribute in varying proportion to the ferritin heteropolymer (HARRISON and AROSIO 1996) and may thus govern different uses of the stored iron. Notably, crystal structures of the ferritin iron core differ between animals, bacteria, and plants, a major criterion being the P:Fe ratio. Pea (Pisum sativum) ferritin showed a ratio of 2.83 (WADE et al. 1993), whereas horse spleen ferritin and human ferritin were cited to display ratios of 1:8 and 1:20, respectively. Such differences impact on crystal continuity and may affect iron mobilization from the crystal core (HARRISON and AROSIO 1996). With heavy iron loading, an increasing part of the soluble ferritin is degraded into water-insoluble hemosiderin, presumably as a result of incomplete proteasomal ferritin breakdown (RICHTER 1986). This may be viewed as a biological protective mechanism to minimize oxidative damage, because hemosiderin iron contributes much less to free radical formation than ferritin (O'CONNELL et al. 1986). However, these authors point out that hemosiderin may likewise simply result from oxidative attack on ferritin.

In times of need, iron is mobilized from ferritin in a redox process involving dihydroflavins (JONES et al. 1978), NO and glutathione (WATTS and RICHARDSON 2002), AA (BOYER and McCLEARY 1987), or the enzyme xanthine oxidase (BIEMOND et al. 1986; BOLANN and ULVIK 1987). In the presence of oxygen, xanthine oxidase...
oxidizes hypoxanthine and xanthine to uric acid with concomitant production of hydrogen peroxide and superoxide anions ($O_2^{-}$) (Porrás et al. 1981). The $O_2^{-}$ radical, in turn, transfers its excess electron onto ferric iron to yield soluble Fe$^{2+}$ available for incorporation into functional proteins. AA also seems to act indirectly by generating $O_2^{-}$ (Boyer and McCleary 1987). Dihydroflavins like FADH$_2$, in contrast, may access the ferritin iron core through pores in the protein shell to cause iron reduction and release (Jones et al. 1978). Of note, the xanthine oxidase pathway is not entirely dependent on $O_2^{-}$ as demonstrated by residual ferric reduction in the presence of superoxide dismutase (Bolann and Ulvik 1987). Cytosolic transport of ferritin-released ferrous iron remains poorly characterized, but export is presumably effected by ferroportin1 (Nemeth et al. 2004b). In analogy to hephaestin-mediated iron oxidation in enterocytes, liver cells and reticuloendothelial macrophages use the ferroxidase ceruloplasmin, a homolog of hephaestin, to generate ferric iron for transport by Tf (Harris et al. 1999; Richardson 1999). Ceruloplasmin is produced in the liver and flows freely in the circulation to regulate tissue iron efflux. The copper dependence of hephaestin and ceruloplasmin provides a direct link between the metabolism of these two trace elements. Experimental evidence suggesting a role for ceruloplasmin in iron uptake (Mukhopadhyay et al. 1998) was refuted in a subsequent study (Richardson 1999). In mice challenged with phlebotomy, rather than mobilizing storage iron ceruloplasmin up-regulated iron absorption by stimulating enterocyte basolateral iron transfer (Cherukuri et al. 2005). No similar effects have yet been described in humans.

### 1.1.2.3 Regulatory elements of iron metabolism

The striking differences in iron absorption between iron-replete and iron-deficient subjects as reported by Martinez-Torres et al. (1971; 1974) indicate the body’s ability to adapt to the metabolic demand. Iron homeostasis is regulated on the level of absorption, which inevitably draws the attention to the duodenal enterocyte. How does the body communicate its actual iron needs to the absorptive epithelium and what are the molecular rearrangements within the enterocyte that gauge the amount of iron reaching the circulation? Whereas heme iron absorption is affected very little by dietary or host physiological factors, non-heme iron absorption may vary as much as 15-fold (Lynch et al. 1989). Thus, a look at the proteins involved in non-heme iron absorption, storage, and transport should reveal the underlying molecular strategies.
Increased erythropoiesis and low iron stores are classical stimulators of iron absorption and decades of research have been directed at identifying the “erythroid regulator” and “stores regulator” (FINCH 1994). Since hypoxia and inflammation also affect iron absorption and metabolism, a “hypoxia regulator” and an “inflammatory regulator” may likewise be postulated (HENTZE et al. 2004). Although the signaling pathways are not yet fully understood, they seem to converge on the level of the liver-expressed peptide hepcidin (Fig. 8). Hepcidin, first isolated from human urine as an antimicrobial peptide (PARK et al. 2001), was later shown to respond to body iron stores (PIGEON et al. 2001; MAZUR et al. 2003). Additionally, anemia and hypoxia cause hepcidin levels to fall, whereas inflammation and infection stimulate its release (NICOLAS et al. 2002). Erythropoietic and hypoxic signals involve the hormone erythropoietin, which is produced in the kidney and liver and induces red cell maturation. Hypoxia is sensed in that an iron- and oxygen-dependent prolyl hydroxylase continually tags hypoxia-inducible factor-1α (HIF-1α) for proteasomal degradation by attaching a hydroxyl group to a critical proline residue (ZHOU et al. 2002). Under low pO₂, this hydroxylation is abrogated and HIF-1α becomes available for interaction with a β-subunit, thus forming the active HIF-1 heterodimer. This complex translocates into the cell nucleus, where it binds two other proteins, namely hepatocyte nuclear factor-4 and p300, to stimulate EPO\(^1\) gene expression through a hypoxia response element (HRE). Other genes such as Tfr have also been shown to contain HREs recognized by functional HIF-1 (BIANCHI et al. 1999). Considering the iron dependence of the prolyl hydroxylase reaction, it is easily conceivable how changing iron levels affect gene expression.

\(^1\) EPO denotes the gene coding for erythropoietin
Hepcidin-mediated regulation of iron homeostasis. When iron absorption is high, plasma holo-Transferrin (holo-Tf) levels increase. Subsequent saturation of Transferrin receptor 1 (TfR1) on hepatocytes causes competitive dissociation of HFE from TfR1. Kupffer cells may serve as an additional source of HFE. HFE then signals increased hepcidin production by an as yet unknown mechanism. Hepcidin halts cellular iron egress from duodenal enterocytes, hepatocytes, and reticuloendothelial macrophages (dashed lines) by internalizing Ferroportin1 for degradation. Excess holo-Tf is also sensed by TfR2 on hepatocytes, which provides another signal for hepcidin stimulation. Finally, hemojuvelin is a third, seemingly essential modulator of the hepcidin response (from (FLEMING and BACON 2005)).

In the intestine, hepcidin facilitates internalization and degradation of basolateral ferroportin1 (NEMETH et al. 2004b), thus cutting iron export into the circulation. A similar effect is observed in hepatocytes and reticuloendothelial macrophages. In the case of bacterial infection, limiting the availability of plasma iron impedes survival of the invading microorganism(s). Inflammation/infection triggers the hepcidin response in an HFE-dependent fashion (ROY et al. 2004) and is mediated by the cytokine interleukin-6 (IL-6) (NEMETH et al. 2003; NEMETH et al. 2004a). HFE was first
identified as the protein product of the gene mutated in hereditary hemochromatosis (HH) (FEDER et al. 1996). In the duodenal crypt, HFE forms a ternary complex with Tfr1 and β2-microglobulin located in the basolateral membrane (WAHEED et al. 1999) and is competitively displaced by circulating holo-Tf (LEBRON et al. 1999; GIANNETTI and BJORKMAN 2004). Consequently, large amounts of body iron cause increased levels of holo-Tf, in turn leading to higher basolateral iron uptake. The initial hypothesis derived from these observations was that loading of immature enterocytes at the crypt basis with iron in times of plenty would down-regulate the expression of genes involved in iron absorption during differentiation and vice versa (WAHEED et al. 2002; GIANNETTI and BJORKMAN 2004). The response lag of 2-3 days coincides with the maturation period of newly formed enterocytes (Fig. 9) and was thus considered indicative of such a programming mechanism. However, FRAZER and colleagues (2004) observed a similar lag phase in hepcidin synthesis and CHEN et al. (2003) showed expression of Heph$^2$ and SLC40A1$^3$ genes responsible for iron export to depend on systemic signals but not enterocyte iron status. Instead, experimental data from Hfe knockout mice (MUCKENTHALER et al. 2003) and HH patients (BRIDLE et al. 2003) suggest that HFE is more important as a modulator of hepcidin output in the liver, and Kupffer cells may serve as the primary HFE source (MAKUI et al. 2005).

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$^2$ Heph denotes the gene coding for the ferroxidase protein hephaestin
$^3$ SLC40A1 encodes ferroportin1
Figure 9  Maturation of small intestinal epithelial cells during migration from the crypt to the villus tip. a) Schematic representation of the small intestinal anatomy with stem cells at the bottom of the crypt, a proliferative region in the crypt wall, and a region of differentiation running along the villus wall. Around the crypt-villus junction, the cells switch from proliferative to differentiation mode, causing cell polarization as demonstrated by the formation of microvilli on the luminal side and the insertion of proteins involved in nutrient breakdown and uptake into the apical membrane (CHANTRET et al. 1988). b) Histological sections of murine small intestine indicating the migration of newly developing enterocytes (stained in black) along the crypt-villus axis to reach the villus tip after about 3 days. The life cycle ends with exfoliation of dead cells from the tip (adapted from (LODISH et al. 2004)).

Two other proteins called TfR2 and hemojuvelin have also been implicated in the regulation of liver hepcidin. TfR2, a homolog of TfR1, is mainly expressed in the liver (KAWABATA et al. 1999) and responds to circulating levels of holo-Tf but not apo-Tf or NTBI (ROBB and WESSLING-RESNICK 2004). Since Tfr1−/− mice die in utero, TfR2 does not compensate for TfR1. Much rather, it has a distinct role as a monitor of body iron status by sensing changes in the concentration of holo-Tf. Apparently, TfR2 levels are modulated posttranslationally by increasing protein stability (JOHNSON and ENNS 2004). How this effect is mediated and what the exact events downstream of TfR2 stabilization are remains to be elucidated. Mutations in the HJV gene (also known as HFE2) coding for hemojuvelin result in severe, early-onset hemochromatosis (PAPANIKOLAOU et al. 2004) similar to that observed in subjects with defective hepcidin (ROETTO et al. 2003). Again, little is known about the underlying signaling pathway(s), but the HJV expression pattern resembles that of HAMP4 (PAPANIKOLAOU et al. 2004), suggesting a close functional link. Notably, inflammatory stimuli elicit down-regulation of hepcidin irrespective of functional hemojuvelin, whereas iron-

4 HAMP (hepcidin antimicrobial peptide) is the name of the gene coding for hepcidin
sensing in terms of hepcidin response is defective in HJV-mutant mice (NIEDERKOFLER et al. 2005). Hemojuvelin may thus communicate body iron levels to hepcidin but is not required in the cytokine-hepcidin pathway of inflammation.

Probably independent of liver signals, enterocyte iron exerts an immediate effect termed “mucosal block” on further iron absorption by creating a negative feedback loop via the ubiquitous IRE-IRP system (EISENSTEIN and BLEMINGS 1998; PANTOPOULOS 2004). Iron-responsive elements (IREs) are conserved stem-loop structures (Fig. 10) found in a number of mRNAs for proteins involved in iron metabolism, e.g. ferritin (HENTZE et al. 1987) and TfR (KOELLER et al. 1989).

![Figure 10](image)

**Figure 10** Iron-responsive element (IRE) consensus structures (adapted from (PANTOPOULOS 2004)).

If the IREs are located in the 5'-untranslated region (5'-UTR) of the mRNA, binding of complementary iron-regulatory proteins (IRPs) blocks translation of the message (Fig. 11). On the other hand, mRNAs with one or more IREs in the 3'-UTR are stabilized when IRPs are bound, resulting in higher levels of the respective protein product. Two IRP isoforms, IRP1 and IRP2, have been described, which react differently to changes in intracellular iron levels. Under iron-replete conditions, IRP1 assumes the function of cytosolic aconitase, an enzyme involved in glutamate synthesis (MCGAHAN et al. 2005) and cellular redox regulation (NARAHARI et al. 2000).
IRP2, in turn, is constantly targeted to proteasomal breakdown when iron abounds (SAMANIEGO et al. 1994). When the intracellular iron levels decrease, the IRPs gain IRE-binding activity. With regard to iron absorption, the major outcome of IRPs binding to their cognate IREs is stabilization of the TfR and DMT1 mRNAs, resulting in higher TfR and DMT1 protein levels and increased iron uptake from apical and basolateral membranes. At the same time, ferritin mRNA translation is blocked. IRP detachment, on the other hand, allows translation of the ferritin message with resultant sequestration of excess iron in ferritin, whereas the TfR and DMT1 mRNAs are degraded. The activity of the IRPs is not only regulated by iron, but also responds to H₂O₂ (SUREDA et al. 2005) and NO (Kim and Ponka 2003) in that they cause Fe-S-cluster degradation (IRP1) and increased proteasomal breakdown (IRP2). Contradictory findings have been reported for NO in the regulation of IRP activity and cellular iron metabolism, but they are likely to have arisen from the different chemical forms of NO, namely the nitroxyl anion (NO⁻), the nitrosonium cation (NO⁺), and the redox species NO⁺ (PANTOPOULOS 2004).

![Figure 11](image)

**Figure 11** The IRE-IRP system as exemplified by ferritin and TfR mRNAs. When iron levels are low (Fe↓), IRPs bind to and stabilize mRNAs carrying one or more IREs in their 3'-UTR, resulting in increased translation of the message and subsequent higher iron absorption. At the same time, IRE-IRP complexes in the 5'-UTR block translation. With rising intracellular iron (Fe↑), the blockade of mRNAs with an IRE-containing 5'-UTR is removed and translation initiated. On the other hand, mRNAs with 3'-IREs are degraded by RNase. TfR, transferrin receptor; IRE, iron-responsive element; IRP, iron-regulatory protein; UTR, untranslated region (adapted from (PANTOPOULOS 2004)).

Caco-2 data provide evidence that it is not total enterocyte iron but a small fraction called labile iron pool (LIP) that carries out regulatory function. Overexpression of the
ferritin IRE caused ferritin levels to rise and TfR levels to fall (GARATE and NUNEZ 2000). The resulting decrease in the LIP induced DMT1-mediated iron uptake irrespective of total intracellular iron, i.e., the sum of ferritin iron and iron in the LIP. Similar regulatory effects of the LIP were reported in HepG2 hepatocytes, which showed accelerated ferritin synthesis with rising intracellular labile iron (STURM et al. 2005). Of note, 5'-IREs at a distance greater than 67 nucleotides from the cap side may not be repressible by IRP binding (ROUAULT et al. 1987). This is the case in intestinal ferroportin1 mRNA, which has been shown not to respond to variations in intracellular iron (CHEN et al. 2003).

The putative Stimulator of Fe Transport (SFT), first described in K562 cells, human peripheral blood lymphocytes, spleen, thymus, and small intestine, has been proposed to play a role in Tf-bound and non-Tf-bound iron transport (GUTIERREZ et al. 1997). Studies in Caco-2 cells did not show a correlation between iron uptake and SFT expression, irrespective of iron pretreatment (TALLKVIST et al. 2000), and Hfe knockout mice with iron overload had SFT mRNA levels similar to their wild-type counterparts (KNUTSON et al. 2001). In contrast, the significant up-regulation of SFT mRNA reported in HH patients (YU et al. 1998) has been implicated to contribute to the etiology of iron overload. Species differences such as the rate of disease progression, the degree of liver iron-loading, or the propensity to develop fibrosis were suggested to explain the divergent observations (KNUTSON et al. 2001). A potential influence of phlebotomy may have further skewed the results. However, the perception of SFT changed completely, when GEHRKE et al. (2003b) revealed a close nucleotide sequence homology to intron 6/exon 7 of UbcH5A, which belongs to a family of ubiquitin5-conjugating enzymes and is involved in the proteasomal degradation of HIF-1. Of note, IRP2 is also degraded in the proteasome under iron-replete conditions (IWAI et al. 1998) and may thus provide a link between UbcH5A and iron metabolism.

5 Ubiquitin residues are attached to proteins destined for breakdown in the proteasome complex. The process involves the sequential action of three enzyme systems (E1, E2, E3) that activate and conjugate multiple ubiquitins to the target protein prior to its degradation CIECHANOVER, A. (1994). "The ubiquitin-proteasome proteolytic pathway." Cell 79(1): 13-21.
1.1.3 Pathophysiological conditions

Factors such as inadequate dietary supply, mucosal atrophy, inflammation, repeated blood transfusions, or defects in the homeostatic control of body iron may lead to the pathophysiological states of iron deficiency (ID) or iron overload. If ID is sustained, iron deficiency anemia (IDA) and its associated pathologies of reduced workload capacity (HAAS and BROWNLE 2001), increased morbidity and mortality around childbirth (BRABIN et al. 2001a; BRABIN et al. 2001b) as well as impaired cognitive and psychomotor development (GRANTHAM-MCGREGOR and ANI 2001) may ensue. Furthermore, immune function (BEARD 2001; OPPENHEIMER 2001) and thermoregulation (ROSENZWEIG and VOLPE 1999) may be compromised. On the other hand, leaving iron overload uncorrected may cause tissue and organ damage and increase the risk for cardiovascular events, diabetes mellitus, or cancer (FRAGA and OTEIZA 2002). The following two sections provide an overview of pathological derangements of iron homeostasis. Dietary factors associated with ID and iron overload are dealt with in the chapter on nutritional aspects.

1.1.3.1 Iron deficiency states

Iron deficiency is commonly divided into three overlapping stages (Table 2). In stage I, body iron stores are almost depleted (< 100 mg) as indicated by the absence of stainable bone marrow, an increase in total iron binding capacity, and a serum ferritin (SF) value below 12 µg/L (COOK and FINCH 1979; INSTITUTE OF MEDICINE 2001b). No adverse physiological consequences are observed at this point. With continued negative iron balance, stores become fully depleted and iron-deficient erythropoiesis occurs. Biochemical indicators of this second stage are decreased transferrin saturation (TS < 16%), a rise in free erythrocyte protoporphyrin (FEP) and/or increased levels of serum transferrin receptor. In the final stage, overt IDA develops as defined by hemoglobin levels below 130 g/L in men and 120 g/L in women and the presence of hypochromic, microcytic red cells. Since hemoglobin concentrations may drop in response to a variety of conditions – e.g. inflammation or other micronutrient deficiencies –, the diagnosis of IDA requires concomitant abnormalities in TS, FEP, and SF (COOK and FINCH 1979). In the case of infection, an increase in SF as part of the acute phase response may help distinguish this type of anemia from classical IDA.
High iron requirements due to growth, menstrual blood losses, or pregnancy put the respective population groups at an increased risk of ID (HERCBERG et al. 2001). Likewise, repeated blood donations are likely to result in ID in individuals who fail to maintain sufficient iron stores (SKIKNE et al. 1984). The reduced iron status primarily observed in runners may be explained by exercise-induced hemolysis and higher gastrointestinal blood losses (WEAVER and RAJARAM 1992), but might also be related to a physiological adaptation to physical training (NEWHOUSE and CLEMENT 1988).

Increased blood losses due to parasitic infestations such as *Schistosoma spp.* and hookworms are a common cause of ID and IDA in developing countries (STEPHENSON 1993; STOLTZFUS et al. 1997). In contrast, populations of industrialized countries, rather experience peptic ulcers, gastritis, hemorrhoids, or cancerous lesions as major pathological reasons for gastrointestinal blood losses (SKIKNE 1988). Chronic aspirin use may further complicate some of these conditions (PEURA 2004).

Long-standing infection with *Helicobacter pylori* causes lower gastric ascorbic acid levels and mucosal atrophy with subsequent achlorhydria (ANNIBALE et al. 2003), together potentially impairing iron absorption to the degree of ID/IDA (CARDENAS et al. 2006). Achlorhydria may likewise be cause or consequence of ID/IDA. A lack in iron has been reported to induce atrophic gastritis (DELAMORE and SHEARMAN 1965), and achlorhydric patients displayed profoundly lower iron absorption than healthy control subjects (CELADA et al. 1978). Patients with combined IDA and achlorhydria absorbed significantly less iron than normochlorhydric IDA patients, (GOLDBERG et al. 1963).
Moreover, IDA is a common condition after partial gastrectomy (Tovey and Clark 1980). Together with the observation that acidic but not neutralized gastric juice fully corrected the defective iron absorption in achlorhydric subjects (Cook et al. 1964), these findings strongly demonstrate the supportive role of gastric acid. However, blocking acid secretion by 60-80% with cimetidine reduced iron absorption by a modest 28% (Skikne et al. 1981), which indicates that gastric acid production is more than sufficient in healthy individuals. Only severe hypochlorhydria or achlorhydria would thus be expected to substantially impair iron absorption.

Chronic inflammatory bowel diseases (Crohn’s disease and ulcerative colitis) and the autoimmune disorder celiac disease cause pathological blood loss and a reduction in intestinal absorptive surface due to epithelial necrosis or villous atrophy. As a consequence, nutrient absorption is compromised and may lead to multiple deficiencies including iron (Gasche et al. 2004). On the other hand, inflammatory diseases are known to induce anemia independent of iron status. A likely explanation is the cytokine-stimulated increase in hepcidin activity (Nemeth et al. 2004a), which results in the sequestration of body iron in storage compartments and down-regulation of iron absorption. Under such conditions, mucosal atrophy and inflammation would synergistically exacerbate ID. However, adaptive up-regulation of proteins involved in iron absorption has been reported in patients affected by celiac disease (Barisani et al. 2004).

Genetic defects in iron absorption proteins like DMT1 and Hephaestin have been demonstrated to cause IDA in rats (Fleming et al. 1998b) and mice (Vulpe et al. 1999; Canonne-Herdeaux et al. 2000). Similar observations, although very rare, have been made in humans carrying heterozygous mutations in the DMT1 gene (Beaumont et al. 2006; Iolascon et al. 2006; Lam-Yuk-Tseung et al. 2006). These subjects display microcytic, hypochromic anemia in the face of liver iron overload, thus indicating defective iron regeneration for erythropoiesis. Oral iron does not correct the condition, so it either might be poorly absorbed in the intestine or wrongly shuttled into the liver. No reports exist on homozygous DMT1 mutations, possibly because such a genotype would be incompatible with life. Other rare defects causing anemia due to a lack in functional iron or increased hemolysis comprise atransferrinemia, aceruloplasminemia, and heme oxygenase-1 deficiency (Bosio et
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al. 2002; KAWASHIMA et al. 2002; KNISELY et al. 2004). Again, tissue iron overload may be present, highlighting perturbed homeostasis of theoretically sufficient iron.

Patients with hepcidin-overexpressing hepatic adenomas were reported to show iron-refractory anemia which resolved spontaneously after resection of the adenoma or liver transplantation (WEINSTEIN et al. 2002). This type of anemia is also observed in the case of inflammation/infection. LPS, a bacterial endotoxin and potent stimulator of inflammation, induces the cytokines IL-6 and TNF-α, resulting in hepcidin overexpression (NEMETH et al. 2004a; NIEDERKOFLER et al. 2005). Hepcidin, in turn, constantly signals lower iron absorption from the gut. In the liver, HJV mRNA is specifically down-regulated during inflammation, presumably to transiently offset sensing of the low serum iron necessary to limit pathogen growth (NIEDERKOFLER et al. 2005).

1.1.3.2 Iron overload conditions

Iron overload becomes clinically relevant if it exceeds normal body iron 5-10 times (LYNCH 1995). This may occur due to genetic defects, parenteral iron treatment, or secondary to repeated blood transfusions necessary in chronic kidney disease and thalassemia (GORDEUK et al. 1987). Excessive dietary intake is considered unlikely to cause iron loading in healthy individuals (COOK et al. 1984), although higher stores have been reported in elderly subjects consuming diets with very high iron bioavailability (FLEMING et al. 2002). Four types of genetically determined hemochromatoses have been described so far (Table 3), of which types 2 and 3 are rarely encountered (FRANCHINI 2006). Concurrently high levels of serum iron, serum ferritin and Tf saturation are characteristic for all but type 4.
Table 3  Genetic classification of hereditary hemochromatosis (adapted from (FRANCHINI 2006))

<table>
<thead>
<tr>
<th>Disease</th>
<th>Inheritance</th>
<th>Gene</th>
<th>Protein</th>
<th>Diagnosis</th>
<th>Clinic</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>AR</td>
<td>HFE</td>
<td>HFE</td>
<td>↑SI, ↑SF, ↑TS</td>
<td>Late onset (4&lt;sup&gt;th&lt;/sup&gt;-5&lt;sup&gt;th&lt;/sup&gt; decade), parenchymal iron overload</td>
<td>Phlebotomy, iron chelating therapy</td>
</tr>
<tr>
<td>Type 2</td>
<td>AR</td>
<td>HJV</td>
<td>Hemojuvelin</td>
<td>↑SI, ↑SF, ↑TS</td>
<td>Early onset (2&lt;sup&gt;nd&lt;/sup&gt; decade) with hypogonadism, cardiopathy</td>
<td>Phlebotomy, iron chelating therapy, HT</td>
</tr>
<tr>
<td>Type 2</td>
<td>AR</td>
<td>HAMP</td>
<td>Hepcidin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 3</td>
<td>AR</td>
<td>TIR2</td>
<td>Transferrin receptor 2</td>
<td>↑SI, ↑SF, ↑TS</td>
<td>Late onset (4&lt;sup&gt;th&lt;/sup&gt;-5&lt;sup&gt;th&lt;/sup&gt; decade), parenchymal iron overload</td>
<td>Phlebotomy, iron chelating therapy</td>
</tr>
<tr>
<td>Type 4</td>
<td>AD</td>
<td>SLC40A1</td>
<td>Ferroportin1</td>
<td>↑SF</td>
<td>RE iron overload, symptoms milder than HFE HH</td>
<td>Phlebotomy, erythropoietin</td>
</tr>
</tbody>
</table>

AR, autosomal recessive; AD, autosomal dominant; SI, serum iron; SF, serum ferritin; TS, transferrin saturation; HT, heart transplantation; RE, reticulendothelial; HH, hereditary hemochromatosis

The most common form of HH is a mutation in the HFE gene, which results in the exchange of a tyrosine (Y) for a cysteine (C) in position 282 (C282Y) of the HFE protein (FEDER et al. 1996). Substitution of aspartate (D) for histidine (H) in position 63 (H63D) is a second genotype frequently observed. More than 80% of all hemochromatotics are homozygous for the C282Y mutation, the remainder mainly showing compound heterozygosity in the C282Y and H63D polymorphisms (FEDER et al. 1996). Mutant HFE fails to properly interact with β<sub>2</sub>-microglobulin and TfR1 (FLEMING and BRITTON 2006) and possibly other targets involved in this signaling pathway, leading to the phenomenon of “anemic” enterocytes in the face of systemic iron overload (WOOD 2002). As a consequence, up-regulated DcytB activity (RAJA et al. 1996), DMT1 and ferroportin1 expression (ZOLLER et al. 1999; ZOLLER et al. 2001) as well as decreased intracellular ferritin stores (WHITTAKER et al. 1989) concordedly favor excessive iron absorption. In addition, low mucosal iron levels minimize the body’s capacity to remove excess iron by sloughing of iron-laden enterocytes. These observations lend support to the crypt cell hypothesis involving HFE as a communicator of body iron stores in the intestine (WAHEED et al. 1999). However, the inappropriately low hepcidin levels reported in HFE hemochromatotics (BRIDLE et al. 2003; GEHRKE et al. 2003a) implicate the liver as an equally important regulator in this condition.
HFE-associated HH (C282Y homozygosity) affects approximately 0.5% of individuals of Caucasian descent, another 10% being heterozygous for the mutation (WAALEN et al. 2005). Despite this high prevalence and the presence of biochemical abnormalities, relatively few individuals carrying the trait actually develop the full range of characteristic clinical symptoms: diabetes, hypogonadism, liver cirrhosis, and skin pigmentation (BEUTLER 2006). The reason for this is not entirely clear, but it indicates that functional HFE is not essential in iron metabolism. Modifiers of disease penetrance comprise host factors such as growth demands, menses, pregnancy, blood loss, alcohol intake, and certain diseases (PIETRANGELO 2004). Defects in other genes of iron metabolism, antioxidant defense, fibrogenesis, or tissue repair may further affect phenotype expression. Based on a daily positive iron balance of 3 mg due to excessive iron absorption (CROSBY 1963), it takes about 20-40 years to accumulate the 20-40 g of body iron associated with clinical symptoms (LYNCH 1995). Furthermore, it is possible that TfR2 partly compensates for defective HFE and vice versa (PIETRANGELO 2004). This may explain the late onset of disease, typically the 4th or 5th decade of life, in HFE HH. In the rare juvenile hemochromatoses, no compensatory mechanisms exist to balance defective hepcidin regulation, which is why disease progression is enhanced and the phenotype more severe.

Mutations in the SLC40A1 gene coding for ferroportin1 deserve special mention as they result in high serum ferritin levels in the face of low-normal Tf saturation (PIETRANGELO et al. 1999; MONTOSI et al. 2001). The failure to provide sufficient iron for erythropoiesis leads to mild anemia, while storage iron remains locked predominantly in the reticuloendothelial macrophages. Of note, Bantu siderosis, an iron overload condition frequently observed in South-African Blacks and possibly related to the consumption of iron-rich beer (BOTHWELL et al. 1964), is phenotypically similar to type 4 hemochromatosis. Indeed, the Q248H polymorphism in the ferroportin1 protein is highly prevalent in southern African subjects with iron overload (GORDEUK et al. 2003).

A feature often observed in iron overload, be it due to HH, thalassemia, or sickle cell disease, is the presence of elevated plasma NTBI. This low-molecular-weight form of iron has been implicated in the production of ROS (GROOTVELD et al. 1989) and may thus cause oxidative tissue damage and organ destruction as represented by the
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cirrhotic liver of late-stage hemochromatotics. Similarly, it may be involved in inflammatory processes mediated by cytokines such as IL-6, which are produced in response to oxidative stress. For example, chelation of iron by desferrioxamine blunted the cytokine release of cultured endothelial cells (Visseren et al. 2002) that would otherwise result in endothelial damage and possibly atherosclerosis. Especially problematic is the apparently unregulated accelerated uptake of NTBI into liver and heart as demonstrated in cell culture studies of hepatocytes and cardiac myocytes (Parkes et al. 1993; Parkes et al. 1995) as well as in rat liver (BrissoT et al. 1985). Ahmed et al. (1986) reported NTBI levels of 15-18% in patients with sickle cell disease or β-thalassemia compared to <6% in healthy control subjects. The increase in NTBI only occurred when Tf saturation exceeded 80%, hinting at a close link to severe iron overload. More recent data, however, showed the presence of NTBI at Tf saturation levels in the range of 30-60% (Breuer et al. 2000b). This indicates that conditions other than chronic iron overload may contribute to plasma NTBI. Nevertheless, it should be pointed out that NTBI is of heterogeneous nature and a highly redox-active fraction termed labile plasma iron (LPI) becomes particularly prominent at a Tf saturation > 70% (Breuer et al. 2000a; Poottrakul et al. 2004).

1.2 Nutritional Aspects

According to the 2nd WHO World Health Report (WHO 2002), ID ranks among the 10 leading risks of disease burden, regionally and globally. Of no surprise, developing countries with an overall high mortality are struck hardest. It has been estimated that ID affects one third of the world’s population (Stephenson et al. 2000), making it the most widespread micronutrient deficiency. Major causes of ID comprise low iron intake, high demands, and low bioavailability. Low intake may result from overall food scarcity, unavailability of iron-rich foods, poverty, or poor dietary habits. The latter two hold for population groups in developing and industrialized countries alike. High demands arise from growth spurts, pregnancy, and increased blood loss, which occurs with pronounced menstruation, parasitic infestation, bleeding gastrointestinal ulcers, etc. (Hallberg 1981a; Monsen 1988). Finally, low iron bioavailability is typically observed in monotonous plant-based diets as consumed by low socioeconomic strata of third world populations (Skikne 1988). In contrast, the high prevalence of hereditary hemochromatosis in Europe (Beutler 2006) may require
individuals carrying mutations in their *HFE* gene to minimize iron intake and bioavailability. This seems to hold mainly for C282Y/H63D compound heterozygotes (Hunt and Zeng 2004). Unless such genetic defects are present, diets with highly bioavailable iron are considered unlikely to cause iron overload as suggested by several authors (Cook et al. 1984; Hulten et al. 1995). In elderly subjects, however, increased iron stores were related with the consumption of ≥ 7 servings/wk of red meat, > 21 servings/wk of fruit, or > 30 mg/d of supplemental Fe (Fleming et al. 2002). Whether this translates into a higher disease risk awaits clarification. In the following sections, dietary recommendations and iron intake data, important food sources, and the role of bioavailability in adequate iron nutrition are discussed.

### 1.2.1 Food sources, intake data, DRI

Dietary iron comes in two forms, heme and non-heme iron, which are absorbed by distinct pathways and in different proportions. Heme iron is essentially of animal origin and constitutes 30-60% of meat and fish iron (Cook and Monsen 1976; Schrick et al. 1982a; Kongkachuichai et al. 2002). Its high bioavailability of 15-35% makes it an important contributor to total absorbed iron despite the relatively low dietary abundance of 10-15% (Carpenter and Mahoney 1992). The remaining 85-90% of iron in the diet are derived from non-heme sources of plant or animal origin and largely affected by enhancers and inhibitors (see chapters 1.2.2.2 and 1.2.2.3, respectively). In developing countries, contamination iron from the soil may add substantially to total iron intake but little to iron absorption due to its poor bioavailability (Hallberg et al. 1983).

Daily iron requirements have been estimated to amount to ~1 mg in healthy, adult men (Green et al. 1968), and this figure forms the basis for the Dietary Reference Intakes (DRI) issued by the Institute of Medicine for the USA and Canada (Table 4). Based on an average iron absorption of 18%, the calculated DRI assumes to cover the iron needs of 97.5% of the target population. Since the diet of infants aged 7 through 12 months often is poor in meat but rich in cereals and vegetables (Davidsson et al. 1997), a bioavailability estimate of 10% is used for this subpopulation (Institute of Medicine 2001b). The low DRI value for infants below 7 months of age is explained by the considerable iron endowment and high mobilization from endogenous stores in healthy, term infants.
Table 4  DRI for iron (mg/d) by age and gender as recommended by the Institute of Medicine (2001b)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age</th>
<th>DRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td>0-6 months</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>7-12 months</td>
<td>11</td>
</tr>
<tr>
<td>Children</td>
<td>1-3 years</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>4-8 years</td>
<td>10</td>
</tr>
<tr>
<td>Males</td>
<td>9-13</td>
<td>8</td>
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<tr>
<td></td>
<td>14-18</td>
<td>11</td>
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<tr>
<td></td>
<td>19-30</td>
<td>8</td>
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<tr>
<td></td>
<td>31-50</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>51-70</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>&gt;70</td>
<td>8</td>
</tr>
<tr>
<td>Females</td>
<td>9-13</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>14-18</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>19-30</td>
<td>18</td>
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<tr>
<td></td>
<td>31-50</td>
<td>18</td>
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<tr>
<td></td>
<td>51-70</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>&gt;70</td>
<td>8</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>≤18</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>19-30</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>31-50</td>
<td>27</td>
</tr>
<tr>
<td>Lactating</td>
<td>≤18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>19-30</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>31-50</td>
<td>9</td>
</tr>
</tbody>
</table>

The Food and Agricultural Organization (FAO) of the United Nations together with the WHO has also published intake recommendations for iron (Table 5), which are again based on an estimated requirement coverage of 97.5% of the target population (FAO/WHO 2001). In this case, figures are broken down by the degree of dietary iron bioavailability. High bioavailability (≥ 15%) would be observed from diets containing ample amounts of meat, fish, and ascorbic acid. With decreasing meat and AA intake and rising consumption of plant foods rich in polyphenols and phytic acid, percentage bioavailable iron continuously drops to ≤ 5%. Diets with intermediate to high iron bioavailability may be considered representative of industrialized countries, whereas diets of poor bioavailability are typically consumed by populations from developing countries. The following chapters deal with the dietary enhancers and inhibitors of iron absorption and how their influence is assessed.
Literature review

Table 5  FAO/WHO iron intake recommendations (mg/d) by age and gender group according to dietary iron bioavailability (from FAO/WHO 2001)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Age (years)</th>
<th>Dietary iron bioavailability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High (15%)</td>
</tr>
<tr>
<td>Children</td>
<td>0.5-1</td>
<td>6.2a</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>4-6</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>7-10</td>
<td>5.9</td>
</tr>
<tr>
<td>Males</td>
<td>11-14</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>18+</td>
<td>9.1</td>
</tr>
<tr>
<td>Females</td>
<td>11-14b</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>11-14</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>15-17</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td>18+</td>
<td>19.6</td>
</tr>
<tr>
<td>Post menopause</td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>Lactating</td>
<td></td>
<td>10.0</td>
</tr>
</tbody>
</table>

a highly variable bioavailability during this period
b non-menstruating

1.2.2 Bioavailability

The term bioavailability has been defined in many ways across different disciplines, but even within the nutritional sciences several definitions exist (Wienk et al. 1999). The most commonly used defines bioavailability as “the fraction of the ingested nutrient that is absorbed and subsequently utilized for normal physiological functions” (Fairweather-Tait and Hurrell 1996). Approximately 90% of dietary iron is consumed in the non-heme form, however, due to its low bioavailability it constitutes only ~50% of the iron actually absorbed (Carpenter and Mahoney 1992; Hunt 2002). In subjects with low iron stores, the contribution of non-heme to total iron absorption gradually increases with the level of depletion (Cook 1990). As non-heme iron is influenced substantially by diet-derived absorption enhancers and inhibitors, meal composition plays a vital role in adequate iron nutrition (Hallberg 1981a; Hurrell 1997a). The nutritive value of a specific food or diet is usually based on its nutrient density per unit energy. In the case of iron, Hallberg (1981b) introduced the concept of bioavailable nutrient density as a more meaningful figure, taking into
account the amount of heme and non-heme iron in the diet, their relative bioavailability, and the individual’s iron status. These three factors, in conjunction with host physiological characteristics other than iron status (Table 1), determine overall iron absorption (HALLBERG 1981a; COOK 1990; HUNT 2002).

1.2.2.1 Iron bioavailability assessment methods

Human absorption studies with radioactive or stable iron isotopes are the gold standard for measuring iron bioavailability in man. However, such studies are costly, labor-intensive, and may require sophisticated instrumentation. To circumvent these issues, a number of in vitro techniques and animal models have been developed for rapid, inexpensive screening of the potential bioavailability of dietary iron from various meals or the usefulness of different iron fortification compounds (NARASINGA RAO and PRABHAVATHI 1978; MILLER et al. 1981; AOAC 1990; GARCIA et al. 1996a; GLAHN et al. 1996). The different in vitro and in vivo approaches with their merits and drawbacks are summarized below.

1.2.2.1.1 Iron solubility

Iron solubility is considered a prerequisite for iron to be bioavailable (DISLER et al. 1975a; MILLER and BERNER 1989) and its determination is simple, rapid, and inexpensive. Basically, iron solutions or complex meal digests are centrifuged and the supernatant iron quantified relative to the total iron content of the mixture. Since supernatant iron may exist as high-molecular-weight (HMW) complexes which are deemed unavailable for absorption (TERATO et al. 1973), ionizable iron is sometimes used as a more specific indicator of absorbable iron (NARASINGA RAO and PRABHAVATHI 1978). Unfortunately, experimental parameters vary between labs and limit comparability of results. Measurement of iron solubility can be considered useful for studying the bioavailability of iron fortification compounds or the enhancing effect of AA, but is clearly insufficient to assess the influence of dietary proteins (MILLER and BERNER 1989).

1.2.2.1.2 Iron dialyzability

Assuming that only low-molecular-weight complexes of iron are available for absorption (TERATO et al. 1973), an in vitro steady-state iron dialyzability method was developed to quantify this fraction from different meals (MILLER et al. 1981). A continuous-flow setup (MINIHANE et al. 1993) was later proposed to better mimic in
vivo conditions but has not received much attention. In general, meals are subjected to simulated gastrointestinal digestion with pepsin at pH 2 and pancreatin/bile salts at pH 6-7.5. A dialysis bag containing bicarbonate in sufficient amounts to raise meal pH to ~5 is placed into the meal slurry after pepsin digestion thus mimicking intestinal alkalization. This is necessary because pancreatic enzymes would be inactivated at a pH < 5 (MURTHY et al. 1980). The choice of pore size of the dialysis membrane is mostly guided by the findings of TERATO et al. (1973) that iron chelates and polymers < 10 kDa are well absorbed in vitro and in vivo. Accordingly, molecular weight cut-offs commonly range between 6 and 15 kDa (SCHRICKER et al. 1982b; VAN DYCK et al. 1996; ENGLE-STONE et al. 2005). Enzyme levels are based on systematic analyses and selected to achieve significant protein digestion within the time of incubation (NARASINGA RAO and PRABHAVATHI 1978; MILLER and SCHRICKER 1982). Iron recovered from the dialysis bag at the end of the pancreatin digestion step is related to total meal iron content and the percentage used as an estimate of iron available for absorption.

Results from iron dialyzability studies correspond reasonably well with human absorption data (SCHRICKER et al. 1981; LUTEN et al. 1996; SANDBERG 2005), although bioavailability tends to be overestimated in some cases (HURRELL et al. 1988). Prominent exceptions are milk (TURNLUND et al. 1990) and tea (VALDEZ et al. 1992), which inhibit iron absorption in humans (see chapters 1.2.2.3.4. and 1.2.2.3.1., respectively) but display relatively high iron availability in vitro. A major advantage of the dialyzability approach is the controllability of experimental parameters such as digestion time or enzyme concentration (WIEJNK et al. 1999). Final pH adjustment and exact timing of the digestion phases were identified as the most critical parameters for obtaining reproducible results within and between laboratories (LUTEN et al. 1996). For example, WOLFGOR et al. (2002) reported improved target pH values and reduced variability in dialyzable iron with the use of PIPES buffer in place of sodium bicarbonate. Nevertheless, several physiological factors are not considered in the dialysis system (Table 6) that may substantially affect iron bioavailability in humans.
Table 6  Factors potentially influencing iron bioavailability that are not considered in the dialyzability model (adapted from (SANDBERG 2005))

| Gastrointestinal transit time (depends on meal composition) |
| Different absorptive capacities of duodenum, jejunum, ileum, and colon |
| Composition of digestive juices (depends on meal composition) |
| Potential role of intestinal microflora |
| Active iron absorption by the epithelium |
| Body iron status |
| Availability of heme iron |

It is clear that no in vitro method will ever be able to precisely imitate in vivo conditions. Furthermore, one should bear in mind that iron dialyzability simulates only the phase prior to absorption in the gut (SANDBERG 2005). Thus, the direction of response observed in this model rather than the magnitude of effect should be considered indicative of meal iron bioavailability (FAIRWEATHER-TAIT et al. 2005).

1.2.2.1.3 Caco-2 uptake
Caco-2 cells are derived from a human colonic adenocarcinoma and upon differentiation express a phenotype resembling that of small intestinal enterocytes (PINTO et al. 1983). When grown into a confluent, polarized monolayer comparable to the epithelial lining of the small intestine, they can be used for intestinal bioavailability studies of nutrients, pharmaceuticals, or toxic substances (AUDUS et al. 1990; DELIE and RUBAS 1997; INGELS and AUGUSTIJNS 2003). Brush border alkaline phosphatase and sucrase-isomaltase are frequently used in cell culture studies as markers of intestinal epithelial cell differentiation (PINTO et al. 1983; ZWEIBAUM et al. 1983; ROUSSET 1986; JUMARIE and MALO 1991; BRISKE-ANDERSON et al. 1997; SIAVOSHIAN et al. 1997; TURCK et al. 2004). With regard to iron absorption, the gene expression of divalent metal transporter 1 and hephaestin increased during Caco-2 cell maturation (BEDRINE-FERRAN et al. 2004). Analogous effects were reported for DMT1 on the mRNA and protein level (HAN et al. 1999; SHARP et al. 2002).

First reports on the usefulness of the Caco-2 cell line for the study of iron absorption came up in the early 1990s, when ALVAREZ-HERNANDEZ and colleagues (1991; 1994) and HALLEUX and SCHNEIDER (1994) demonstrated regulated iron uptake under a variety of conditions. Studies of food iron bioavailability followed soon thereafter and
correlated reasonably well with results from human absorption studies (GARCIA et al. 1996a; GLAHN et al. 1996). Numerous methodological differences exist between laboratories, but two basic setups may be distinguished. In one (GARCIA et al. 1996a), cells are grown on permeable membranes so that iron uptake from the apical or basolateral side and transport through the cells can be studied. The other (GLAHN et al. 1996) uses the insert as a dialysis membrane and measures only soluble low-molecular-weight iron traversing the membrane and taken up into cells grown on the plastic well bottom. To address the issue of surface-bound iron being interpreted as iron taken up by the cells, GLAHN et al. (1995) developed a procedure to remove surface iron without affecting cell viability or intracellular iron. Later on, ferritin formation was established as a surrogate marker of cell iron uptake, circumventing the issues of surface-bound iron and radioiron handling (GLAHN et al. 1998b).

A valid cellular model of the iron-absorbing epithelium would require the presence of the respective proteins of iron absorption and metabolism. Indeed, Dcytb (HAN and WESSLING-RESNICK 2002), DMT1 (SHARP et al. 2002), ferroportin1 (MARTINI et al. 2002), HFE (TALLKVIST et al. 2000), and hephaestin (HAN and WESSLING-RESNICK 2002) as well as the IRE-IRP system (ARREDONDO et al. 1997) and components of the Tf-TfR cycle (NUNEZ et al. 1997) have all been detected in Caco-2 cells. Furthermore, reduction of ferric to ferrous iron was reported a necessary condition for non-heme iron uptake (HAN et al. 1995b), and hepcidin, the liver-derived negative regulator of iron absorption, decreased uptake activity (YAMAJI et al. 2004). However, iron uptake does not necessarily reflect bioavailable iron, since iron absorbed by the enterocyte may be stored in ferritin and lost upon cell desquamation. Growing cells on permeable inserts and measuring basolateral iron excretion partly addresses this issue, but this approach will remain incomplete unless we know and include all systemic factors regulating iron handling in the enterocyte. Moreover, it should be borne in mind that Caco-2 cells originate from a tumor and thus carry metabolic traits quite distinct from non-transformed enterocytes (SHARP 2005).

Compared to iron dialyzability, the Caco-2 model adds a physiological component to the study of iron bioavailability. Nevertheless, the direction of response rather than the magnitude of effect should be considered indicative of meal iron bioavailability (FAIRWEATHER-TAIT et al. 2005; LYNCH 2005). Careful meal preparation that closely
mimics human study procedures seems critical to obtain qualitatively, if not even semi-quantitatively correct data (AU and REDDY 2000; YUN et al. 2004).

1.2.2.1.4 Rat hemoglobin repletion test
Hemoglobin is the major protein of the functional body iron compartment, and blood levels successively decrease on a low iron diet after depletion of storage iron. Thus, the capacity of an iron fortification compound to restore hemoglobin levels in anemic rats is considered indicative of its bioavailability. The Association of Official Analytic Chemists (AOAC) has developed a standardized protocol (AOAC 1990) to measure hemoglobin repletion efficiency and express it as relative biological value (RBV). Young, male rats are maintained on an iron-deficient diet until anemia develops (at least 4 weeks). Subsequently, the rats are randomized into like groups and receive diets enriched with three different dosage levels of either ferrous sulfate (usual reference diet) or the studied compound. After a 2-week repletion phase, hemoglobin levels are determined and for each compound plotted against the iron dose administered. The RBV of a fortification compound is given by the ratio of the slopes expressed relative to ferrous sulfate, which is set at 100%.

The hemoglobin repletion test gives reasonable agreement of relative bioavailability with human studies for fortification iron. However, it is not sensitive enough to pick up differences in the bioavailability of dietary iron. Total iron consumed and overall absorption in humans is low compared to fortification or supplementation iron. Consequently, the high iron absorption in anemic rats leaves a much smaller window for detecting such comparatively subtle changes (REDDY and COOK 1991). These authors also demonstrated that the rat is not a good model for the study of dietary factors influencing iron absorption, which is why little attention is paid to rat studies within this review.

1.2.2.1.5 Chemical balance
The chemical balance technique is a direct measure of iron absorption and has been used extensively in the first half of the 20th century (MCCANCE and WIDDOWSON 1937; WIDDOWSON and MCCANCE 1937; 1942; MCCANCE et al. 1943). Generally, subjects are kept on a defined diet for a period of days to weeks so that intake of the mineral under study is known, and urine and stools are collected to assess excretion of that mineral. The difference between mineral intake and excretion represents apparent
absorption, which differs from true absorption in that it does not take into account endogenous losses, e.g. from sloughed intestinal epithelial cells. For iron, the major excretory pathway is via feces, obviating the need for urine collection. However, percentage iron absorption is usually low (< 10%) and thus severely limits the sensitivity of this method to detect differences in iron bioavailability. This problem is exacerbated by contamination from exogenous iron sources, and incomplete fecal collection may cause overestimation of actual iron bioavailability. Finally, adaptation to the diet may change the percentage of absorbed iron (this limitation applies to all methods).

1.2.2.1.6 Radioiron isotope studies
Generally, three approaches exist to study iron absorption with radioactive isotopes: whole body counting, fecal excretion, and erythrocyte incorporation. Whole body counting (WBC), as the name says, measures radiation emitted from the whole body of a test person given on oral radioiron dose. The first measurement is usually taken 1 h after dose administration and set as the 100% value, followed by a second count 10-14 d later when the steady state is reached (Wienk et al. 1999). After correction for radioactive decay, the percentage radiation relative to the initial count gives the amount of retained iron. Counting is normally done using moveable detectors that scan over the body or by larger detectors covering almost all of the body (Hansen et al. 2001). Successful applications include the quantification of iron losses and requirements (Bonnet et al. 1960; Saito et al. 1964), the biological half-life of iron (Bonnet et al. 1960), and iron absorption (Bæch 2002; Bæch et al. 2003a). In WBC, special care must be taken to minimize background radiation, especially when low levels of radiolabel are to be quantified. Appropriate shielding of the detector or the whole measurement system can be achieved with thick steel walls that may be additionally lined with lead. While the major advantages of WBC are its non-invasiveness (Hansen et al. 2001) and the possibility to record body radiolabel distribution maps (Davidsson et al. 1989), the equipment is quite expensive and its handling requires a considerable level of expertise.

Fecal monitoring, like the chemical balance approach, only measures apparent absorption unless parenteral administration of a second iron isotope is used to correct for endogenous losses (Walczyk 2001). Rare earth elements may be used as non-absorbable markers to assess completeness of stool collection
Additionally, unabsorbed food-grade dyes like Brilliant Blue or Carmine serve as visual indicators of the collection period. Again, the generally low absorption of dietary iron makes it difficult to pick up differences in bioavailability.

The most common approach to measure iron absorption is by quantifying incorporation of an iron label into erythrocytes 14 d after administration. Measuring the amount of absorbed iron transferred into its main functional compartment, this parameter can be considered a true indicator of iron bioavailability (BNF IRON TASK FORCE 1995). Meals may be labeled with radioisotopes in two different ways, i.e., intrinsically or extrinsically. The method of intrinsic tagging involves biosynthetic labeling of the food of choice. To this end, plants are i) grown in a nutrient solution enriched with $^{55}$Fe or $^{59}$Fe, ii) the radioiron solution injected directly into the plant, or iii) the isotopes given as foliar feed (FAIRWEATHER-TAIT and FOX 1996). The label is then taken up and assumed to be incorporated into functional and storage compartments as would soil iron. When assessing iron absorption from animal foods, a dose of radioiron is either administered orally or injected into the blood stream, peritoneum, or muscle of the animal and the animal kept for several weeks to permit tissue distribution of the label. The major advantage of the intrinsic tag approach is that it provides foods containing labeled iron in its biological form. However, it is very time-consuming and the rate of incorporation is low (MARTINEZ-TORRES and LAYRISSE 1971; MARTINEZ-TORRES et al. 1975; FAIRWEATHER-TAIT and Fox 1996). Furthermore, its use is restricted to studying iron absorption from single foods, because absorption from a complete diet may be inadequately reflected by intrinsic labeling of only one food component (COOK et al. 1972). Thus, human studies were greatly facilitated when it was shown that a small dose of inorganic radioiron added to a meal is a valid marker of non-heme iron absorption (COOK et al. 1972), as it forms a common pool with native food non-heme iron. An absorption ratio of 1.1 was observed between extrinsic and intrinsic radioiron over a dose range of 0.001-0.5 mg. This extrinsic tag concept was subsequently extended to the parallel study of heme and non-heme iron absorption from a complete diet by using differently radiolabeled hemoglobin and inorganic iron salts (BJORN-RASMUSSEN et al. 1974). Since heme iron follows a separate absorption pathway, a specific heme tag is necessary to adequately assess the bioavailability of this fraction.
Assuming that adult subjects with serum ferritin levels $> 15 \mu g/L$ incorporate 80% of an absorbed dose into erythrocytes 14 d after isotope administration (HOSAIN et al. 1967), the fractional absorption can be calculated based on blood volume determination from body weight and height (BROWN et al. 1962; EAKINS and BROWN 1966). For individuals with a serum ferritin $\leq 15 \mu g/L$, erythrocyte incorporation increases to 100% (HOSAIN et al. 1967). Intraindividual variation is usually minimized by comparing the iron bioavailability from different meals within the same subject using two different radioisotopes ($^{55}$Fe and $^{59}$Fe) and feeding the differently labeled meals on consecutive days. Interindividual absorption values – which inversely correlate with iron stores measured as serum ferritin (COOK et al. 1974; DISLER et al. 1975a) – are usually normalized correcting for Fe status or Fe absorption from ferrous sulfate (LAYRISSE et al. 1969). Adult subjects with depleted iron stores, yet non-anemic, on average absorb 40% iron from a solution containing 3 mg ferrous iron and 30 mg AA (HALLBERG 1981a; MAGNUSSON et al. 1981), yielding the following equation for correcting observed absorption values:

$$\text{corrected food Fe absorption} = \frac{\text{calculated food Fe absorption} \times 0.40}{\text{calculated reference Fe absorption}}$$

A second approach, which has been found more effective in reducing interindividual variation in adults, is to use a serum (or plasma) ferritin value of 40 $\mu g/L$ (COOK et al. 1991a), and more recently 30 $\mu g/L$ (REDDY et al. 2000), as a basis for correction. The equation reads as follows:

$$\log A_c = \log A_0 + \log F_0 - \log 30$$

where $A_c$ is corrected iron absorption, $A_0$ is observed absorption, and $F_0$ is measured serum ferritin. Correction for reference dose absorption or serum ferritin works well in adults but seems of little use in infants (DAVIDSSON et al. 2000b). Systematic analyses have demonstrated higher iron absorption percentages from single meals as compared to complete diets (COOK et al. 1991a; TIDEHAG et al. 1996). This has to be taken into account when assessing the nutritional adequacy of a specific diet on the basis of contained inhibitors and enhancers of iron absorption.
In brief, radioisotopes are advantageous in that they are i) real tracers due to the low amounts needed, ii) easy to measure, and iii) hardly affected by background radiation. Nevertheless, the associated ionizing radiation increasingly sparks health concerns and makes it more and more difficult to obtain ethical approval (Hansen et al. 2001; Fairweather-Tait and Dainty 2002). Consequently, stable isotope methods are gaining importance and protocols are developed to overcome inherent limitations (Woodhouse and Abrams 2001; Chen et al. 2005).

1.2.2.1.7 Stable iron isotope studies
Most of what has been said about radioiron absorption studies also holds for the stable isotope approach, which has been demonstrated to yield highly comparable data (Barrett et al. 1994a). The major difference, however, is the amount of label used. Minute amounts of the radioactive tracer are sufficient to detect even low levels of absorption. In contrast, stable iron isotopes have to be added in quantities that may change the total iron content of a meal considerably (Fairweather-Tait and Dainty 2002). Using large doses of iron isotope label is not an issue in the study of fortification compounds, which are extrinsic by definition and commonly added to foods poor in or devoid of intrinsic iron. However, when assessing the bioavailability of dietary iron – as opposed to fortification iron –, the contribution of the extrinsic tag to total meal iron should not exceed the natural variation of the food iron content, which is estimated at 20%. The amounts actually needed depend on the natural abundance of the respective isotope, its expected absorption percentage, and the sensitivity of the measurement technique (Woodhouse and Abrams 2001). Unless measurement sensitivity and analytical precision is improved, complicated meal and feeding designs as well as thorough validation studies prior to broad-scale application are necessary. Furthermore, stable isotopes may be considered prohibitively expensive in the absence of substantial funding. These aspects may explain the scarcity of stable isotope studies evaluating the bioavailability of dietary non-fortification iron in adults. However, stable isotopes do not bear any radiation hazard, thus allowing studies in children and pregnant women not feasible with radioisotopes due to safety and ethical considerations. Another advantage is the stability of the label, which permits long-term studies of iron kinetics and individual iron requirements as well as “infinite” sample storage time without loss of signal (Woodhouse and Abrams 2001).
1.2.2.2 Enhancers of iron bioavailability

It has been known for decades that non-heme iron absorption is affected by meal composition. Two dietary components – ascorbic acid and meat/fish/poultry – have consistently increased iron bioavailability at normal intake levels from a wide range of meals (Layrisse et al. 1968; Martinez-Torres and Layrisse 1971; Martinez-Torres et al. 1974; Martinez-Torres et al. 1975; Cook and Monsen 1976; 1977; Bjorn-Rasmussen and Hallberg 1979; Hallberg et al. 1986; Siegenberg et al. 1991). Due to its elusive nature, the enhancing principle in meat has come to be termed the “meat factor”. Furthermore, some reports have focused on organic acids as potential absorption enhancers (Derman et al. 1980b; Gillooly et al. 1983; Hallberg and Rossander 1984; Ballot et al. 1987) but their role seems to be less clear (see review by Teucher et al. (2004)).

1.2.2.2.1 Ascorbic acid (AA)

Ascorbic acid has numerous physiological roles in the human body. It acts as an enzyme cofactor in collagen (Wu et al. 2000), catecholamine (Levin et al. 1960), and carnitine (Hulsee et al. 1978) biosynthesis as well as the α-amidation of various hormones (May and Eipper 1985) and seems to partly regulate cholesterol metabolism (Bjorkhem and Kallner 1976). In some cases, it operates indirectly by keeping critical protein sulfhydryl residues or metal cofactors like iron or copper in the reduced state. Owing to its strong reducing properties, AA may also directly inactivate reactive oxygen species (Drake et al. 1996; Carr and Frei 1999) and thus minimize oxidative damage to essential biomolecules like DNA, fatty acids, or proteins. The potential pro-oxidant effects of AA observed in vitro have not yet been unequivocally demonstrated to be of major concern in humans (Carr and Frei 1999; Reddy and Clark 2004).

Recommended intakes for AA in the healthy adult population range from 60-75 mg/d in the USA (Institute of Medicine 2001c) to 100 mg/d in Germany, Austria, and Switzerland (DGE et al. 2000), the upper safe intake level being estimated at 2 g/d (Institute of Medicine 2001c). Relevant food sources for AA comprise fresh citrus fruits, most green leafy vegetables, and potatoes (Table 7).
Table 7 AA content of commonly consumed fruits and vegetables (adapted from (JOHNSTON et al. 2001))

<table>
<thead>
<tr>
<th>Fruits</th>
<th>mg/100 g edible portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cherry</td>
<td>15-30</td>
</tr>
<tr>
<td>Black currant</td>
<td>150-200</td>
</tr>
<tr>
<td>Red currant</td>
<td>20-50</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>30-70</td>
</tr>
<tr>
<td>Kiwi fruit</td>
<td>80-90</td>
</tr>
<tr>
<td>Lemon</td>
<td>40-50</td>
</tr>
<tr>
<td>Orange</td>
<td>30-50</td>
</tr>
<tr>
<td>Pineapple</td>
<td>15-25</td>
</tr>
<tr>
<td>Strawberry</td>
<td>40-70</td>
</tr>
<tr>
<td>Tomato</td>
<td>10-20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vegetables</th>
<th>mg/100 g edible portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagus</td>
<td>15-30</td>
</tr>
<tr>
<td>Broccoli</td>
<td>80-90</td>
</tr>
<tr>
<td>Cabbage</td>
<td>30-70</td>
</tr>
<tr>
<td>Cauliflower</td>
<td>50-70</td>
</tr>
<tr>
<td>Kale</td>
<td>70-100</td>
</tr>
<tr>
<td>Potato</td>
<td>4-30</td>
</tr>
</tbody>
</table>

In terms of iron bioavailability, AA has repeatedly demonstrated a strong enhancing effect from various foods and meals. A positive linear relationship (Fig. 12) was reported between the iron absorption ratio and the amount of AA added using a semisynthetic meal made from maltodextrin, egg albumin, and corn oil (COOK and MONSEN 1977). Iron absorption significantly increased from 0.77% to 1.27% (1.7-fold) by adding 25 mg AA to the SSM, which corresponds to a molar AA:Fe ratio of 1.9:1. By increasing this ratio to 77:1 (1000 mg AA), a 9.6-fold increase in iron absorption was obtained. As pointed out by LYNCH and STOLTZFUS (2003) however, the highest relative increase was observed at molar AA:Fe ratios < 10:1. In a parallel study with a standard meal consisting of beef, potatoes, corn meal, peaches, ice milk, bread, and margarine, addition of 100 mg AA led to a 1.7-fold rise in iron absorption (from 4.05% to 6.78%). At the same dose, AA enhanced iron absorption 4.2-fold from the SSM (from 0.77% to 3.19%). Similarly, no further enhancement of iron absorption was induced by fish from a maize and papaya meal already containing 70 mg AA (LAYRISSE et al. 1974) or by adding 500 mg AA to a veal muscle meal (MARTINEZ-TORRES and LAYRISSE 1971) or 50 mg AA to a veal liver meal (MARTINEZ-TORRES et al. 1974). As COOK and MONSEN (1977) concluded, this difference indicates the effects of meat and AA are not additive and suggests a common mechanism.
However, this conclusion is not supported by studies consistently showing a further increase in iron absorption when combining meat/fish and AA compared to either enhancer alone (HALLBERG and ROSSANDER 1982b; a; HALLBERG et al. 1986; HALLBERG et al. 2003).

**Figure 12** Dose-dependent enhancing effect of ascorbic acid (AA) on iron absorption in humans (from data of (COOK and MONSEN 1977) as presented in (TEUCHER et al. 2004)).

**Mechanism of AA as an Fe absorption enhancer**

AA presumably complexes and/or reduces iron to the ferrous form (HURRELL 1997a), thereby rendering it more soluble and preventing interaction with absorption inhibitors. In addition, ferrous iron is the transport substrate for DMT1 in the duodenum. It has been postulated that an acidic pH is necessary to form soluble chelates from ferric iron and AA (CONRAD and SCHADE 1968). This is supported by Caco-2 iron uptake data indicating a higher response at pH 5.5 compared to pH 7 (GARCIA et al. 1996a). Furthermore, iron tended to polymerize above pH 5.5 despite the presence of a 5:1 molar excess of AA (LEIGH and MILLER 1983); increasing this ratio to 25:1 kept a major part of the iron in a LMW form up to pH 7. Considering that food first passes the low pH environment of the stomach and that iron absorption largely takes place in the upper duodenum, where slightly acidic chyme (FALLINGBORG 1999) first reaches the absorptive epithelium of the small intestine, this makes sense physiologically.
Food matrix effects
Iron absorption from wheat rolls containing graded doses of the absorption inhibitor phytic acid (7-878 mg phytate) improved 2-3-fold upon the addition of 50 mg AA, the effect being even more pronounced with 100 mg AA (HALLBERG et al. 1989a). Whereas the absolute increase in absorption was highest from the phytate-free rolls, relative increases rose with phytic acid content. Similar results were obtained for wheat rolls enriched with another class of absorption inhibitors, namely tannins (HALLBERG et al. 1989b). In another study, a dose of 25-30 mg AA given in a bread meal was shown to effectively overcome high levels of phytate (~200 mg) and tannic acid (420 mg) (SIEGENBERG et al. 1991). Addition of 100 mg AA to a SSM made with egg albumin or isolated soy protein increased iron absorption by 6-fold and 2-fold, respectively (MORCK et al. 1982), the molar AA:Fe ratio being ~25:1.

Further evidence for the importance of meal composition was provided by HALLBERG et al. (1986) who compared iron absorption from breakfast, hamburger, bran bun, Latin American, pizza, rice, and vegetarian meals with varying levels of native or synthetic AA. The diets varied considerably in total iron content (0.4-4.2 mg) as well as the presence of phytic acid and polyphenols. Nevertheless, 50 mg of AA consistently increased iron absorption from the different meals by 60-160%. Again, a positive linear relationship was observed between absorption ratio (w/wo AA) and meal AA content (25-500 mg AA), with no significant differences between crystalline and native AA (e.g. from orange juice or cauliflower). The latter aspect demonstrates that AA from solid foods is readily liberated in the gastrointestinal tract to improve iron absorption. Importantly, AA is effective only when consumed with the iron-containing meals (COOK and MONSEN 1977).

Among a plethora of fruits tested, only citrus fruits, guava, and papaya were able to potently enhance iron absorption (BALLOT et al. 1987). This effect was largely attributed to the fruits’ AA content, but high levels of citric acid could have had an added benefit. In parallel, vegetables high in AA, like cauliflower, broccoli and cabbage, demonstrated favorable iron bioavailability compared to those with a low AA content and/or rich in polyphenols and phytate (GILLOOLY et al. 1983).
Literature review

AA is the sole enhancer in vegetarians
Vegetarians, especially vegans, run a greater risk of becoming iron-deficient than non-vegetarians (Dwyer 1991; Waldmann et al. 2004), largely due to the copious intake of plant foods high in polyphenols and phytate and the concomitant absence of meat as a rich source and enhancer of bioavailable iron. Consequently, dietary strategies to improve iron status should focus on increasing AA intake to balance low iron bioavailability (Hallberg et al. 1989b). In this regard, the finding that adding 60-65 mg AA as cauliflower to vegetarian meals of low iron bioavailability increased iron absorption to a greater extent than did the addition of 75-90 g of meat (Hallberg and Rossander 1982a; 1984) underlines the usefulness of this approach. The largest effects are obtained at a dose of about 50-100 mg AA (Hallberg et al. 1989b).

AA and fortification iron
Similar to native food iron, absorption of fortification iron may be improved by concomitant AA intake. Again, effect size depends on the AA:Fe molar ratio (Derman et al. 1980a; Davidsson et al. 1998; Lynch and Stoltzfus 2003) and the presence of inhibitory substances (Cook et al. 1997), but is additionally governed by the physicochemical properties of the various fortification compounds (Forbes et al. 1989; Hurrell et al. 1991; Davidsson et al. 2000a; Teucher et al. 2004).

AA and in vitro iron bioavailability
The enhancing effect of AA has been reproduced in different in vitro assays of iron bioavailability. Iron uptake in Caco-2 cells from various meals increased markedly in the presence of AA at molar AA:Fe ratios of 0.8:1 and above (Garcia et al. 1996a; Glahn et al. 1999; Yun et al. 2004; Engle-Stone et al. 2005; Etcheverry et al. 2005), and AA is frequently employed as a positive control in such studies (Glahn et al. 1998a; Swain et al. 2002; Huh et al. 2004). Iron solubilization from pinto beans improved significantly from 10% to 51% when excess AA (10 mM) was added to the mixture, with a maximal effect in the pH range of 1.5-5 (Kojima et al. 1981). Notably, almost all supernatant iron was in the ferrous form, indicating the strong reducing effect of AA. However, such a high AA dose would only be achievable with AA supplements. Ionizable (Narasinga Rao and Prabhavathi 1978) and dialyzable (Miller et al. 1981) iron increased upon addition of AA to cereal, semisynthetic, or
beef meals, respectively. In contrast, JOVANI et al. (2000) did not detect any significant correlation between AA content and percentage dialyzable iron from soy-based infant formulas. The authors suggested that this might have been due to the high molar ratios tested, which were in the range of 5.3:1-13.0:1. However, in light of the linear relationship between absorption ratio and AA content up to a molar ratio of 77.3:1 reported in humans (COOK and MONSEN 1977) and the recent conclusion that the highly inhibitory nature of soy products might require higher AA:Fe ratios than other foods known to decrease iron absorption (TEUCHER et al. 2004), methodological inadequacies seem to provide a more likely explanation.

**Long-term effects of AA on iron absorption**

When assessing iron absorption from complete diets as opposed to single meals, the enhancing effect of AA is far less pronounced (HUNT et al. 1994; COOK and REDDY 2001). A possible explanation is that absorption is only increased if the body needs it. After a few days of higher absorption, hepcidin production increases, the ferroportin pathway closes, and the mucosal block becomes active. In addition, a highly diverse diet with plenty of inhibitory substances like polyphenols, phytic acid, and calcium may partly account for this observation. Consuming AA-rich foods with the main non-heme iron containing meals was shown in a 2 wk field study to be an effective means of improving iron absorption from a habitual diet (DIAZ et al. 2003), but no significant effect on iron status parameters was observed in a subsequent 8 mo efficacy trial (GARCIA et al. 2003). In contrast, the reported positive correlation between fruit consumption and iron status in the elderly (FLEMING et al. 2002) indicates that high intakes of AA may result in the expansion of iron stores over the long term.

**Effects of the AA stereoisomer erythorbic acid**

FIDLER and co-workers (2004) recently demonstrated that erythorbic acid, a stereoisomer of AA, possesses even stronger absorption-enhancing properties on fortification iron than AA. Technologically, erythorbic acid is used in similar ways as AA, i.e., as a water-soluble antioxidant (WALKER 1991), but the virtual lack of antiscorbutic activity (FABIANEK and HERP 1967) limits its applicability in food-fortification programs.
1.2.2.2 Organic acids

The role of organic acids other than AA, namely citric, lactic, malic, and tartaric acid, as potential enhancers of iron absorption has recently been reviewed by Teucher et al. (2004). All four acids have been shown to complex ferrous and/or ferric iron (Galdi and Valencia 1988; Suzuki et al. 1992). Common fruit and vegetable sources are listed in Table 8. Among the foods listed, fresh and dried grapes are the only relevant sources of tartaric acid, whereas sauerkraut is very high in lactic acid.

<table>
<thead>
<tr>
<th>Food</th>
<th>Organic acid (mg/100 g edible portion)</th>
<th>Citric</th>
<th>Malic</th>
<th>Tartaric</th>
<th>Lactic</th>
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</thead>
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Table 8 continued

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<th>Organic acid (mg/100 g edible portion)</th>
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<th>Malic</th>
<th>Tartaric</th>
<th>Lactic</th>
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<td>4680</td>
<td>200</td>
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</table>

*** = no values reported

**Citric acid**

Citric acid is a common constituent of fruits like oranges, peaches, strawberry, rhubarb, or pineapple, with concentrations ranging from 370 mg/100 g in peaches to 1080 mg/100 g in strawberries (BAILOTT ET AL. 1987). Somewhat lower values are reported by SOUCI ET AL. (1994) (see Table 8). Given as a laboratory ‘orange juice’ (100 ml water, 33 mg AA, 750 mg citric acid), citric acid increased iron absorption from a 200 g rice meal by 2-fold compared to a solution containing only AA (BAILOTT ET AL. 1987). Significantly higher absorption was also observed when 4 g citric acid was added to 100 ml commercial orange juice containing 30 mg native AA and 700 mg native citric acid compared to orange juice alone. Among the fruit juices tested in that study, those with a substantial AA content caused the strongest enhancement. However, the promoting effect of pineapple juice with an AA content of only 5.2 mg/100 ml was attributed to its high citric acid content of 770 mg/100 ml. Fruit citric acid content correlated significantly with iron absorption, although the link was less pronounced than for AA. Unexpectedly, the significant enhancing effect of strawberry, which was reported to contain on average 60 mg AA and 1080 mg citric acid per 100 g edible portion, was lost upon correction for reference dose absorption. It could be, though, that the polyphenol content of strawberry (Table 11, chapter 1.2.2.3.1) may have partially offset the absorption promoters.

Further evidence for citric acid as an absorption enhancer was provided by GILLOOLY and colleagues (1983) who reported a 3-fold higher absorption (8.5% vs. 2.8%) from a rice meal, to which 1 g citric acid had been added, than from rice alone. Dose-dependent increases in iron absorption upon the addition of citric acid to a soy-based
infant formula were observed in borderline iron-deficient adult females (DERMAN et al. 1987). Absorption percentages were 1.1, 2.6, 4.8, and 6.8 for formulas containing 0, 1, 2, and 4 g citric acid, respectively. In line with earlier findings (GILLOOLY et al. 1983), addition of very high doses of citric acid was necessary for a relevant enhancement to occur. This may cause undesirable sensory changes and the effect may only be moderate from inhibitory meals like those based on soy products.

An inhibitory effect of citric acid was found by HALLBERG and ROSSANDER (1984), who reported a reduction in iron absorption from 0.9% to 0.3%, when 1 g of citric acid was added to a basal meal containing black beans, maize, and rice. Likewise, GLAHN et al. (1998a) observed higher iron uptake (+46%) in Caco-2 cells when the citrate content of the tested infant formula was decreased by 67%. Differences in the food matrix may partly account for the divergent results.

Malic, lactic, and tartaric acids
In the study by GILLOOLY et al. (1983), 1 g of L-malic acid and tartaric acid caused a 2-fold increase of iron absorption from a simple rice meal. When vegetables containing one or more of these organic acids were assessed, no clear correlation could be made between organic acid content and iron absorption, although good bioavailability often concurred with higher AA levels. The enhancing effect of pumpkin was ascribed to its malic acid content. The dose of malic acid administered with this vegetable, however, was 7.5 mg at best, assuming cooking losses to be minimal and the actual content to correspond to the food database value reported. Lactic acid was proposed to have contributed substantially to the excellent iron absorption from sauerkraut. A positive effect on iron bioavailability of lactic acid had been reported earlier (DERMAN et al. 1980b), although the dose administered was much higher. In a very recent study (BERING et al. 2006), the improved iron absorption from fermented oat gruel was partly attributed to its lactic acid content, but the bacterial species used for fermentation seemed to have had an additional effect. These findings are at variance with those of BAYNES et al. (1990) who failed to observe an effect of lactic acid on iron absorption from a rice meal. A major drawback of the studies by BALLOT et al. (1987) and GILLOOLY et al. (1983) is that the organic acid contents of the fruits and vegetables were mostly taken from food composition tables, which are notoriously imprecise and may differ substantially from actual food...
content. Thus, only qualitative comparisons between organic acids regarding iron absorption should be made.

1.2.2.2.3 The “meat factor”

The first observations of a positive effect of meat on iron absorption date back to the late 1940s, when JOHNSTON and co-workers (1948) reported a doubling in absorption of dietary iron upon incorporation of additional beef into the test meals. Although beef failed to improve iron absorption from a spinach diet, it was demonstrated to be a superior source of bioavailable iron (MC MILLAN and JOHNSTON 1951). The insignificant results regarding absorption enhancement may be explained by the insensitivity of the chemical balance method employed in that study and the small sample size.

In a series of radioisotope absorption studies using an intrinsic tag, LAYRISSE and co-workers (1968) examined the effect of interaction of plant and animal tissues on human iron absorption from various foods. Portion sizes were based on food iron content. Black bean iron was 2.7-fold better absorbed when veal muscle (3 mg iron) was given with black beans (1 mg iron) compared to black beans (4 mg iron) alone. Replacing black beans with corn, enhancement with veal was still apparent, yet less pronounced (1.5-fold). Conversely, veal iron absorption decreased significantly by 10-30% in the presence of black beans or corn. However, the effect of corn could not be reproduced in a later study (MARTINEZ-TORRES and LAYRISSE 1971). Combining fish (1 mg iron) with corn (2 mg iron) increased corn iron absorption 3.1-fold compared to corn (3 mg iron) alone. Fish failed to significantly enhance the absorption of black bean iron, probably owing to the small sample size and the good iron status of the subjects. When the results were pooled with data from a subsequent study (MARTINEZ-TORRES and LAYRISSE 1970), fish was shown to significantly improve iron absorption from black beans by a factor of 2. Absorption of fish iron was unaffected by the addition of corn in one study (LAYRISSE et al. 1968), but reduced by 54% in another (MARTINEZ-TORRES et al. 1975), probably owing to differences in food quantities administered. No data were presented for the effect of black beans on fish iron absorption. It should be borne in mind that replacing part of the plant foods with meat or fish not only increased the amount of absorption enhancers, *i.e.*, the “meat factor”, but also lowered the levels of the absorption inhibitors phytic acid and polyphenols, the reverse being true for the meat/fish meals.
The reported changes in plant and animal tissue iron absorption were thus presumably due to a change in the ratio of enhancers to inhibitors rather than a single factor alone.

**The role of food matrix**
Protein in general inhibits iron absorption compared to a protein-free meal (Cook and Monsen 1976; Monsen and Cook 1979; Hurrell et al. 1988; 1989). Furthermore, the “meat factor” seems to require an inhibitory food matrix to become apparent. This is indicated by a study showing only slightly improved iron absorption when 100 g beef was added to a semisynthetic meal (SSM) containing maltodextrin, corn oil, and egg albumin (Morck et al. 1982), a protein with little influence on iron absorption. In contrast, absorption rose nearly 4-fold from the same beef meal, when egg albumin was replaced by isolated soy protein, which is highly inhibitory (Cook et al. 1981; Hallberg and Rossander 1982c). Likewise, Layrisse et al. (1974) failed to observe a further enhancement with 100 g fish added to a diet already containing 70 mg AA, which may indicate the importance of basal Fe absorption.

**Protein origin of the “meat factor”**
Interestingly, feeding black beans with an amino acid mixture as present in 100 g fish caused a 2.7-fold higher absorption than from black beans alone (Layrisse et al. 1968). This was the first evidence for meat protein as the likely source of the “meat factor”. A subsequent systematic analysis of aromatic, aliphatic, basic, and sulfur-containing amino acids revealed that cysteine was the sole amino acid capable of enhancing iron absorption to the extent observed with fish or an amino acid mixture (Martinez-Torres and Layrisse 1970). However, the amino acids administered were not heated as would happen during the cooking of food, and cysteine was given in gelatin capsules thus minimizing oxidation of critical sulfhydryl groups. Adding a mixture of amino acids as present in 100 g beef directly into the warm food enhanced iron absorption from a maize meal by 60%, which was significantly less than the effect of whole beef (Bjorn-Rasmussen and Hallberg 1979). Free cysteine had no significant effect in that study.

Focusing on animal proteins, meat (beef, lamb, pork), fish, and poultry were all shown to display equally high bioavailability in a standardized American hamburger diet and to enhance iron absorption from a SSM when substituted for egg albumin.
(Cook and Monse 1976). In contrast, the non-cellular animal proteins casein and egg albumin exhibited low iron bioavailability irrespective of whether they were administered as purified compounds or in the form of milk, cheese, or powdered egg. With regard to the enhancing properties of the various muscle tissues, red meats (2.96-3.77-fold increase) were more effective than chicken (2.43-fold) or fish (2.11-fold). The lesser potency of fish was also reported by others (LaRissee et al. 1968; Martinez-Torres et al. 1975). White fish contains largely non-heme iron (> 70%), with minor quantities of heme iron (Kongkachuchai et al. 2002; Huh et al. 2004), which may partly explain the relatively low iron absorption of 8-9% from a fish-only meal in iron-replete to borderline iron-deficient subjects (Martinez-Torres et al. 1975). The doubling in absorption when 500 mg AA was added to this meal underlines this contention, as only non-heme iron is affected by AA. In addition, cooking destroys some of the food heme iron (Martinez-Torres et al. 1986; Garcia et al. 1996b) and thus decreases overall iron bioavailability. Importantly, the “meat factor” effect has also been observed in infants (Engelmann et al. 1998) who are critically dependent on adequate iron nutrition for proper brain development (Roncagliolo et al. 1998; Grantham-McGregor and Ani 2001; Algarin et al. 2003). Moreover, a meat-containing diet was shown to better maintain iron status in exercising women than were iron supplements (Lyke et al. 1992).

Hurrell et al. (submitted for publication) examined the effect of chicken and beef and corresponding heme-free and heme-containing protein extracts on iron absorption from a SSM and a farina meal. When 30 g of protein were provided in the SSM, the chicken and beef extract still containing the heme fraction showed a significant enhancement over the protein-free control. After heme removal, however, the chicken extract further rose in its effect, but the beef extract went down to control values. Using only 15 g of protein, all beef preparations increased iron absorption by 1.5-2.5-fold compared to the protein-free control and by 3-4-fold compared to the egg albumin SSM. In a subsequent titration study with a farina meal, the heme-containing chicken extract showed a slight dose-response effect at levels of 10, 20, and 30 g of protein, whereas the corresponding beef extract gave inconsistent results. The observed increase in iron absorption with graded doses of pork meat added to a phytate-rich meal (Baeck et al. 2003a) underlines a potential dose dependency. Such effects, however, may only be important in single meal studies. Recently, Reddy
et al. (2006) reported only marginal changes in percentage iron absorption from the whole diet despite large variations in meat intake. Comparisons were made between a self-selected, a vegetarian, and a high-meat diet, each consumed over the course of 5 d. It is likely, though, that the large quantities of heme iron associated with the latter diet contribute substantially to replete iron stores (Fleming et al. 1998a). In contrast to the study by Reddy et al., a significant 1.5-fold increase in non-heme iron absorption was observed in a controlled 5-d feeding study when 180 g/d of Danish pork meat were added to a vegetarian diet low in AA and high in phytic acid (Kristensen et al. 2005). Strict surveillance of meal consumption may have accounted for the statistical significance.

Cysteine and low-molecular-weight peptides
The stomach contributes only ~10-20% to total protein digestion, brought about by the proteolytic enzyme pepsin (Guyton 1991). Consequently, a large part of the dietary protein reaches the duodenum in the form of peptides with varying chain lengths. On the basis that cysteine potently enhanced human iron absorption (Martinez-Torres and Layrisse 1970; Martinez-Torres et al. 1981), it has been hypothesized that low-molecular-weight (LMW) peptides, especially those containing cysteine, would form a vital component of the “meat factor” (Kane and Miller 1984; Taylor et al. 1986; Slatkavitz and Clydesdale 1988; Mulvihill and Morrissey 1998). Indeed, doses of cysteine as found in 100 g, 300 g and 500 g beef and equimolar amounts of reduced glutathione, a cysteine-containing tripeptide, stimulated iron absorption from corn meals to a similar extent (Layrisse et al. 1984). Feeding human subjects a maize/meat broth meal with a native cysteine content of 210 mg resulted in a 2.7-fold higher iron absorption than when the cysteine content was taken down to < 10 mg by oxidation (Taylor et al. 1986). However, the meat was digested with pepsin prior to feeding, which does not normally occur in human nutrition. Moreover, the oxidation procedure may have equally affected other molecules that would otherwise enhance or not inhibit iron absorption. Enhancement of iron uptake in Caco-2 cells by cysteine and reduced cysteinyl-glycine (Glahn and Van Campen 1997) supports a critical role for cysteine as part of the “meat factor”. The lack of effect of reduced glutathione in this model was suggested to relate to the amino group and the free sulfhydryl of cysteine as critical parameters for enhanced
uptake. It is likely that glutathione was at least partially hydrolyzed in the study by 
Layrisse et al. (1984), which may explain the observed enhancement.

A further approach in the search for a protein-derived “meat factor” involved muscle protein fractionation prior to the quantification of iron solubility or dialyzability. To this end, cytoplasmic and myofibrillar proteins were separated on the basis of their solubility in aqueous buffers of varying ionic strength. The major myofibrillar proteins actin and myosin are soluble in dilute salt solutions like KCl- or NaCl-buffers ≥0.3 M and relatively high in cysteine, with myosin containing 40-45 cysteine residues (Mannherz and Goody 1976) and actin 5 Cys residues (Elzinga et al. 1973). In contrast to bovine serum albumin (BSA), in which 34 of the 35 cysteines form disulfide bridges (Nakamura et al. 1997), myosin and actin carry only reduced cysteines (Hofmann and Hamm 1978). Actin, myosin, and all myosin fractions except light meromyosin exerted a positive effect on iron dialyzability from semisynthetic meals, which was related to the fractions’ cysteine content (Mulvihill et al. 1998; Mulvihill and Morrissey 1998). Addition of graded cysteine doses to actin proportionately increased dialyzable iron. In turn, blocking the myosin cysteines with N-ethylmaleimide caused a dose-dependent reduction. That dialyzability did not decrease to the level of the egg albumin control may be explained by the variable accessibility of the different cysteines for chemical modification (Mannherz and Goody 1976). Although actin and light meromyosin have about the same cysteine content, the former increased iron dialyzability whereas the latter slightly decreased it (Mulvihill et al. 1998). The authors suggested a role of protein structure in the formation of potential iron absorption enhancers, since light meromyosin and actin differ substantially in their content of α-helical structures (Mannherz and Goody 1976).

In a series of papers (Seth et al. 1999; Seth and Mahoney 2000b; Seth and Mahoney 2000a; Vattem et al. 2001; Diaz et al. 2002), the influence of dilute saline-soluble and saline-insoluble peptides from chicken muscle on iron solubility and binding capacity was assessed. Results hinted at cysteine and histidine residues as binding sites, because specific blocking of these residues diminished iron-binding by 67-79% and 30%, respectively (Seth and Mahoney 2000b; Vattem et al. 2001). Furthermore, the sulfhydryl content of the peptides was closely correlated with iron
dialyzability, and reduced cysteines presumably mediated the production of ferrous iron (Diaz et al. 2002). However, iron was largely associated with a high-molecular-weight fraction > 10 kDa (Seth and Mahoney 2000a). The positive influence of histidine on *in vitro* iron bioavailability is supported by studies in Caco-2 cells showing improved iron uptake in the presence of histidine-rich LMW beef peptides (Swain et al. 2002). In contrast, no enhancing effect of free histidine has been observed in humans (Martinez-Torres and Layrisse 1970; Layrisse et al. 1984).

Slatkavitz and Clydesdale (1988) reported significantly better iron solubilization by peptides of less than 10 kDa molecular weight compared to those > 10 kDa after pepsin digestion of acid-insoluble chicken muscle protein. In addition, prolonged exposure to pepsin and low pH followed by limited intestinal proteolysis at pH 5 – the iron added with the pancreatin – yielded maximal iron solubilization and reduction (Politiz and Clydesdale 1988). Peptides with a molecular weight in the range of 2500-6200 Da had the highest solubilization capacity. Consequently, digestion intermediates rather than end products might determine iron bioavailability, hinting at a potential role of protein digestion rate. The suggested mechanism is early release of absorption-enhancing peptides from meat that would prevent the binding of iron to inhibitors like phytate, polyphenols, or soy protein. Additional pancreatin digestion abolished the solubilization properties of the LMW peptides, but did not change the characteristics of the unfractionated digest (Slatkavitz and Clydesdale 1988). Notably, the iron-solubilizing fraction was not extractable with water, which is in line with human absorption data (Bjorn-Rasmussen and Hallberg 1979).

**Gastric acid secretion**

Another proposed mechanism by which meat enhances iron absorption concerns the stimulation of gastric acid secretion, which may result in increased solubilization of dietary non-heme iron. Hydrochloric acid is known to aid in iron absorption in that it contributes to ferric solubilization (Rhodes et al. 1968; Bezwoda et al. 1978) and reduction (Bergeim and Kirch 1949) and promotes iron chelation by AA (Conrad and Schade 1968). The general importance of the gastric phase is further evidenced by the high prevalence of IDA observed in partially gastrectomized patients (Tovey and Clark 1980). Notably, gastric acid output and gastrin secretion were significantly higher when meat was compared to soy protein (McArthur et al. 1988). Gastrin is
the primary endogenous stimulus for gastric acid secretion. Studies using cimetidine, a suppressant of gastric acid secretion, have likewise shown decreased iron absorption in man (SKIKNE et al. 1981). However, only prolonged severe hypochlorhydria or achlorhydria would be expected to substantially impact on iron status. Pharmacological stimulation of gastric acid secretion in healthy subjects resulted in a statistically insignificant doubling in iron absorption, whereas beef (20 g protein) significantly improved iron absorption 3.7-fold in achlorhydric subjects (BJORN-RASMUSSEN and HALLBERG 1979). In light of these findings, the meat factor seems to be independent of gastric acid secretion.

The role of iron valence
Ferric iron is insoluble above pH 3. Unless complexed in a soluble form or reduced to the more soluble ferrous iron, it precipitates at the high intestinal pH and is excreted unabsorbed (FORTH and RUMMEL 1973). Basal gastric secretions as well as AA-rich foods and beef had a superior effect on total soluble and percentage ferrous iron in the human stomach compared to milk or eggs (BERGEIM and KIRCH 1949). Furthermore, formation of ferrous iron was demonstrated to be a prerequisite for absorption in mice and rats (WOLLENBERG and RUMMEL 1987; BARRAND et al. 1990; RAJA et al. 1992). This is most likely due to ferrous iron being the substrate for the duodenal iron transporter DMT1. On these grounds, the role of iron valence on its bioavailability has been studied extensively (POLITZ and CLYDESDALE 1988; KAPSOKEFALOU and MILLER 1991; MULVIHILL et al. 1998; MULVIHILL and MORRISSEY 1998; VATTEM et al. 2001). The reducing agents AA, glutathione, and cysteine all caused substantial reduction of ferric to ferrous iron during in vitro digestion (KAPSOKEFALOU and MILLER 1991), reflecting their enhancing potential in humans (COOK and MONSEN 1977; LAYRISSE et al. 1984). Raw and cooked beef was less effective, but reduced significantly more iron than casein or egg albumin, both of which did not differ from the control. Similar results have been reported for different meat sources (MULVIHILL and MORRISSEY 1998). For BSA, dialyzable ferrous iron was found to be a better indicator than total dialyzable iron (KAPSOKEFALOU and MILLER 1991). BSA displayed high iron dialyzability but little ferric reduction, which is in line with a modest iron absorption enhancement in humans (HURRELL et al. 1988).
Table 9 Summary of human single meal absorption studies assessing the influence of meat on iron absorption

<table>
<thead>
<tr>
<th>Authors</th>
<th>Method</th>
<th>Compound</th>
<th>Meal</th>
<th>Subjects</th>
<th>Absorption¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>(BAECH et al. 2003a)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeSO₄ ⁵⁷FeCl₃ total Fe = 2.3-2.5 mg</td>
<td>60 g boiled, polished rice 100 g tomato sauce 50 g pea purée 50 g wheat roll 25-75 g pork patties 220 mg PA 64.3 mg Ca 7.4 mg AA</td>
<td>45 women 24±3 y All women received meat-free basal meal</td>
<td>25 g meat: 1.15-fold increase 50 g meat: 1.44-fold increase 75 g meat: 1.57-fold increase</td>
</tr>
<tr>
<td>(BAECH et al. 2003b)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeSO₄ ⁵⁷FeCl₃ total Fe = 2.8 mg</td>
<td>60 g boiled, polished rice 100 g tomato sauce 50 g pea purée 50 g wheat roll 75 g pork patties, heated at 70, 95, and 120°C 220 mg PA Ca not stated 33 mg AA</td>
<td>21 women 25±3 y</td>
<td>pork, 70°C: 1.4-fold increase² pork, 95°C: 1.3-fold increase² pork, 120°C: 2-fold increase²</td>
</tr>
<tr>
<td>(HALLBERG et al. 2003)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeSO₄ ⁵⁷FeCl₃ total Fe = 2.0 mg</td>
<td>Whole-wheat infant gruel (whole-wheat flour, milk powder) 48 g gruel powder 175 ml boiling water +/- powdered meat corresponding to 20 g lean meat</td>
<td>3 women 4 men 22-55 y 3 men were blood donors</td>
<td>1.85-fold increase²</td>
</tr>
<tr>
<td>(ENGELMANN et al. 1998)</td>
<td>Extrinsic tag, stable isotopes</td>
<td>FeSO₄ total Fe = 896.3 µg</td>
<td>Vegetable puree with added meat (60 g potato 25 g beef 10 g carrot 10 g broccoli 1 g corn starch 2 g corn oil 17 g water)</td>
<td>5 boys 3 girls 7-8 mo</td>
<td>~1.5-fold increase</td>
</tr>
<tr>
<td>(REDDY et al. 1996)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl₃ total Fe = 6.2 mg</td>
<td>SSM 67 g hydrolyzed corn starch 36 g corn oil 12 ml vanilla extract 30 g added protein (EA or beef)</td>
<td>21 women 18 men 19-37 y 8-12 per meal beef compared to protein-free SSM: 2.5-fold increase beef compared to SSM with EA: 5.6-fold increase</td>
<td></td>
</tr>
</tbody>
</table>
Table 9 continued

<table>
<thead>
<tr>
<th>Authors</th>
<th>Method</th>
<th>Compound</th>
<th>Meal</th>
<th>Subjects</th>
<th>Absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>(REDDY and COOK 1991)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl$_3$</td>
<td>75 g bun 68 g French fries 50 g milkshake +/- 80 g ground beef</td>
<td>7 subjects 18-40 y</td>
<td>~2-fold increase</td>
</tr>
<tr>
<td>(HURRELL et al. 1988)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl$_3$</td>
<td>60 g corn meal, 4.1 g butter, 92 g cooked ground beef</td>
<td>18 men 3 women 19-39 y</td>
<td>~3-fold increase</td>
</tr>
<tr>
<td>(HURRELL et al. 1988)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl$_3$</td>
<td>bread meal (60 g wheat flour, 82% extraction), 4 g butter, 92 g cooked ground beef</td>
<td>18 men 3 women 19-39 y</td>
<td>no change</td>
</tr>
<tr>
<td>(HALLBERG and ROSSANDER 1984)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl$_3$</td>
<td>Maize (80 g), rice (50 g), black beans (31 g), (+16 g total protein), 75 g cooked ground beef (+15 g protein) 250 mg PA</td>
<td>4 men 5 women 25-34 y</td>
<td>~2.6-fold increase</td>
</tr>
<tr>
<td>(BJORN-RASMUSSEN and HALLBERG 1979)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>~0.5 mg native iron from maize flour + FeSO$_4$ to make total Fe = 5.0 mg</td>
<td>Maize porridge (50 g maize flour +/- 20 g protein from beef, fish, or chicken</td>
<td>108 subjects 29-42 y</td>
<td>beef: 3.80-fold increase chicken: 3.24-fold increase fish (20 g protein): 4.37-fold increase fish (10 g protein): 1.52-fold increase</td>
</tr>
<tr>
<td>(AUNG THAN et al. 1976)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl$_3$</td>
<td>200 g boiled polished rice 40 g fish (catfish) 8 g fish paste 25 g beans 4 g onions garlic, chili, ginger, salt, 13 g groundnut oil</td>
<td>9 men 11 women 23-31 y</td>
<td>males: ~4.6-fold increase females: ~5.9-fold increase $^2$ 8.5-fold increase $^2$ 5.5-fold increase $^2$</td>
</tr>
<tr>
<td>(COOK and MONSEN 1976)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl$_3$</td>
<td>SSM (dextrimaltose, corn oil, EA) 20 g meat protein + 9.4 g egg albumin protein compared to 29.4 g EA protein</td>
<td>70 women 18-37 y</td>
<td>pork: 3.25-fold increase lamb: 3.25-fold increase beef liver: 3.86-fold increase chicken: 2.43-fold increase fish (cod): 2.05-fold increase</td>
</tr>
</tbody>
</table>
Table 9 continued

<table>
<thead>
<tr>
<th>Authors</th>
<th>Method</th>
<th>Compound</th>
<th>Meal</th>
<th>Subjects</th>
<th>Absorption1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(COOK and MONSEN 1976)</td>
<td>Extrinsic tag, radioisotopes</td>
<td>FeCl₃ total Fe = 4.1 mg</td>
<td>Standard meal (corn, potato, bread, margarine, ice milk, peaches) 20.2 g beef protein compared to 20.2 g EA protein</td>
<td>70 women 18-37 y 8-10 per meal</td>
<td>Beef vs EA 2.5-fold increase</td>
</tr>
<tr>
<td>(MARTINEZ-TORRES et al. 1974)</td>
<td>Intrinsic tag, radioisotopes</td>
<td>“Ferrous citrate” total Fe = 4.0 mg</td>
<td>Veal liver (3 mg Fe) + maize (1 mg Fe)</td>
<td>6 women 6 men 19-60 y</td>
<td>2.22-fold increase</td>
</tr>
<tr>
<td>(MARTINEZ-TORRES and LAYRISSE 1971)</td>
<td>Intrinsic tag, radioisotopes</td>
<td>“Ferrous citrate” total Fe = 4.0 mg</td>
<td>Veal muscle (3 mg Fe) + maize (1 mg Fe)</td>
<td>4 women 11 men 16-58 y</td>
<td>1.87-fold increase</td>
</tr>
<tr>
<td>(MARTINEZ-TORRES and LAYRISSE 1971)</td>
<td>Intrinsic tag, radioisotopes</td>
<td>“Ferrous citrate” total Fe = 4.0 mg</td>
<td>Veal muscle (3 mg Fe) + black beans (1 mg Fe)</td>
<td>2 women 9 men 12-58 y</td>
<td>2.21-fold increase</td>
</tr>
<tr>
<td>(MARTINEZ-TORRES and LAYRISSE 1970)</td>
<td>Intrinsic tag, radioisotopes</td>
<td>$^{59}$FeCl₃ total Fe = 2.5-3.5 mg</td>
<td>Black beans (2-3 mg Fe) +/- fish (0.5 mg Fe)</td>
<td>107 subjects 50 male, 57 female</td>
<td>2.08-fold increase</td>
</tr>
<tr>
<td>(LAYRISSE et al. 1968)</td>
<td>Intrinsic tag, radioisotopes</td>
<td>“Ferrous citrate” total Fe = 3.0 mg</td>
<td>Fish (1 mg Fe) + black beans (2 mg Fe)</td>
<td>8 men 23-49 y</td>
<td>1.7-fold increase</td>
</tr>
</tbody>
</table>

1 values are given as fold increase in iron absorption compared to meat-free meal
2 adjusted to a reference dose absorption of 40%
EA, egg albumin; SSM, semisynthetic meal

Heme iron absorption and the “meat factor”

The meat factor promotes iron absorption from both heme and non-heme iron sources (MARTINEZ-TORRES and LAYRISSE 1971; MARTINEZ-TORRES et al. 1974). Heme bioavailability is most likely improved by protein breakdown products that prevent polymerization of the heme molecules, which would render them unavailable (UZEL and CONRAD 1998). This mechanism may also have contributed to the slight enhancing effect of glutathione on hemoglobin iron absorption (LAYRISSE et al. 1984). In vitro data suggested the balance between the strength of the peptide-heme interaction and the heme polymerization rate to be even more important than peptide-heme interaction alone (VAGHEFI et al. 2002).
HAZELL et al. (1978) reported superior absorption in rats of LMW iron dialyzed from rat muscle peptic digests compared to an aqueous muscle extract. Their results also indicated some degree of heme iron breakdown from this meat source, whereas purified hemoglobin largely formed soluble hematin complexes, which were less well absorbed. However, the fact that heme iron absorption in humans is hardly affected by enhancers or inhibitors of non-heme iron absorption does not support the suggested formation of non-heme iron intermediates from heme prior to absorption. The comparative study by REddy and COOK (1991) on rat and human iron absorption from beef meals questions the general validity of rat studies in assessing the meat factor. Iron absorption in humans doubled with the addition of 80 g beef to a hamburger meal, whereas only minimal increases were observed in rats. Dialyzability and Caco-2 iron uptake experiments have so far more reliably paralleled the enhancing effect of meat on iron absorption in humans (MILLER et al. 1981; SCHRICKER et al. 1981; GARCIA et al. 1996a; GLAHN et al. 1996; MULVIHILL et al. 1998; AU and REddy 2000; SWAIN et al. 2002; ENGLE-STONE et al. 2005). Nevertheless, all in vitro and animal models of human iron bioavailability have also produced results deviating from human absorption data and thus in any case require cautious interpretation and extrapolation.

Glycosaminoglycans and the “meat factor”

Although the results of BJÖRN-RASMUSSEN and HALLBERG (1979) do not support a potency ranking of red meat, chicken, and fish (COOK and MONSEN 1976), they likewise demonstrate a consistent increase in iron absorption from maize porridge with all meat sources. It is interesting to note that if the amount of food component used to obtain 20 g of meat protein is normalized to 100 g in this study, the ranking of beef, chicken, and fish is recovered. At the same time, this might imply that there is some factor other than protein involved in the absorption enhancement, because fish yielded the highest mean absorption ratio and was used in the largest amounts gramwise. A study by HUH et al. (2004) lends support to this hypothesis by showing that a highly acidic carbohydrate fraction extracted from fish increased iron uptake in Caco-2 cells about 3-5-fold. The authors suggested that oversulfated glycosaminoglycans (GAGs) presumably derived from heparan sulfate proteoglycans interact with released food iron to prevent it from precipitation. Such metal-carbohydrate interactions are described for the GAG monomers D-glucosamine...
(Bhatia and Ravi 2003), D-glucosamine 6-sulfate, D-glucuronic acid, and L-iduronic acid (Whitfield and Sarkar 1991) as well as for the intact macromolecules chondroitin sulfate (Ishida et al. 1992) and hyaluronic acid (Mercé et al. 2002). Intriguingly, only cellular animal protein, i.e., muscle tissue, enhances iron absorption, and GAGs form an essential part of the intercellular connective tissue.

However, the vast body of literature on dietary factors influencing iron bioavailability provides little evidence to support the hypothesis that carbohydrates from muscle tissue play a vital role as “meat factors”. This may be partly due to the fact that meat is generally not considered a relevant carbohydrate source (1-2% of wet weight, (Pearson and Young 1989)). Furthermore, chemical analyses revealed that the free carbohydrates in meat comprise glycogen, glucose-6-phosphate, glucose, fructose, and ribose (Belitz and Grosch 1992). While fructose was unable to influence iron absorption in humans (Heinrich et al. 1974), the other compounds remain to be tested.

Proteoglycans are extracellular matrix structures consisting of a peptide backbone to which one or more sulfated GAG side chains are attached (O-linked via serine or threonine). These GAGs are linear polymers of repeating disaccharides, in most cases containing an O-sulfated N-acetylhexosamine and uronic acid (Pearson and Young 1989). Seven types of GAGs have been identified: hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparan sulfate, heparin, and keratan sulfate. They differ in disaccharide composition, chain length, and degree of sulfation (Table 10). Despite large variations in the number of repeats – 50-10000 for hyaluronic acid compared to 5-40 for keratan sulfate – a representative figure is 50. Proteoglycans may contain one or two different types of glycosaminoglycans and the number of GAG side chains varies between one and over 100, resulting in up to 10000 charged (anionic) groups per proteoglycan molecule. Accordingly, relative molecular weights range from 50000 to several millions, of which 50-95% is made up by carbohydrates. Besides the O-linked GAGs, N-linked oligosaccharides composed of D-xylose, D-mannose, L-fucose, and sialic acid may be found in fully processed proteoglycans (Pearson and Young 1989).
### Table 10 Composition of repeating disaccharide units of glycosaminoglycans (GAGs) (modified from (PEARSON and YOUNG 1989))

<table>
<thead>
<tr>
<th>GAG</th>
<th>Disaccharide units (n)</th>
<th>Repeating disaccharide (A-B)</th>
<th>Sulfates per disaccharide unit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>monosaccharide A</td>
<td>monosaccharide B</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>50-10000</td>
<td>β-D-GlcUA</td>
<td>β-D-GlcNAc</td>
</tr>
<tr>
<td>Chondroitin 4-sulfate</td>
<td>20-60</td>
<td>β-D-GlcUA</td>
<td>β-D-GalNAc</td>
</tr>
<tr>
<td>Chondroitin 6-sulfate</td>
<td>20-60</td>
<td>β-D-GlcUA</td>
<td>β-D-GalNAc</td>
</tr>
<tr>
<td>Dermatan Sulfate¹</td>
<td>30-80</td>
<td>β-D-GlcUA</td>
<td>β-D-GalNAc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-L-IdUA</td>
<td>β-D-GalNAc</td>
</tr>
<tr>
<td>Heparan sulfate (HS)¹</td>
<td>10-60</td>
<td>β-D-GlcUA</td>
<td>α-D-GlcNAc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-L-IdUA</td>
<td>α-D-GlcNAc</td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>5-40</td>
<td>β-D-GlcNAc</td>
<td>β-D-Gal</td>
</tr>
</tbody>
</table>

¹ these GAGs may contain two different types of disaccharide units
GlcNac, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; IdUA, iduronic acid; GlcUA, glucuronic acid; Gal, galactose

In addition to proteoglycans, extracellular matrix glycoproteins (e.g. fibronectin and laminin) constitute another oligosaccharide source in meat as they contain 1-60% carbohydrates in the form of numerous relatively short, branched oligosaccharide chains (usually < 15 monomers). These chains are of variable composition, have a mannose core and frequently terminate with sialic acid (PEARSON and YOUNG 1989). Furthermore, they are always N-linked to asparagine residues of the protein portion. While the family of fibronectins is quite heterogeneous in its carbohydrate structure, laminin is especially rich in galactosamine. As mentioned before, proteoglycans also contain such N-linked oligosaccharides (CARRINO et al. 1999).

Collagen, another extracellular matrix protein, contains carbohydrate units that are linked to the collagen molecule via hydroxylysine residues. The degree of glycosylation is dependent on the type of collagen. Type IV collagen, localized mainly in basement membranes, displays the largest amount of hydroxylysines (i.e., carbohydrate binding sites) among the 11 collagen subtypes, with about 80% being glycosylated (PEARSON and YOUNG 1989).

The total content of glycosaminoglycans in bovine muscle tissue was found to approximate 0.1% on a wet weight basis with dermatan sulfate and heparan sulfate being the most abundant subtypes (PEDERSEN et al. 1999).
consumption from the European Prospective Investigation into Cancer and Nutrition (EPIC) study show that total meat intakes range between 46-127 g/d in women and 79-242 g/d in men (LINSEISEN et al. 2002). Assuming that the glycosaminoglycan content in meat of different species does not vary greatly, the estimated GAG intake for non-vegetarians would thus amount to 46-127 mg/d in women and 79-242 mg/d in men. In mouse muscle tissue, the unsulfated GAG hyaluronic acid (HA) constitutes about 70% of total GAGs (WATANABE et al. 1986), the remainder being sulfated variants like dermatan sulfate or heparan sulfate. Assuming a similar distribution in muscle tissue from other mammalian species, daily HA and sulfated GAG intakes probably range between 32-169 mg/d and 14-73 mg/d, respectively. HA consists of repeating disaccharide units of N-acetyl-glucosamine and D-glucuronic acid. Chondroitin 4-sulfate (C4S) differs from HA in that N-acetyl-glucosamine is replaced by N-acetyl-galactosamine and the C4 of the galactosamine is sulfated. Whereas HA is the only unsulfated GAG, C4S can be viewed as representative of all sulfated GAGs. Interestingly, iron-chondroitin sulfate complexes (Condrofer®, Blutal®) have been used in the treatment of iron deficiency anemia (BARONE et al. 1988; ALLEGRA et al. 1991) and hyaluronic acid has been shown in vitro to bind iron (MERCE et al. 2002). Soluble HA-iron complexes were obtained at a molar ratio of 3:1 (HA disaccharide:Fe).

1.2.2.3 Inhibitors of iron bioavailability
The list of dietary factors inhibitory to iron absorption comprises mainly plant constituents, thus explaining the generally low iron bioavailability from plant-based diets (HALLBERG 1981a; HURRELL 1997a). Milk protein is the only true animal food with the potential to decrease iron absorption (HURRELL et al. 1989), whereas the detrimental role of calcium is unspecific to its source but may depend on the chemical form (MONSEN and COOK 1976; COOK et al. 1991b). Interestingly, soy protein seems to be a double-edged sword when comparing its effects on heme and non-heme iron (LYNCH et al. 1985).

1.2.2.3.1 Polyphenols (PP)
Cancer, cardiovascular disease, and osteoporosis are among the diseases polyphenols have been implicated to prevent or at least ameliorate (SCALBERT et al. 2005). One supposed mechanism is the scavenging of reactive oxygen species, since PP are known to have strong antioxidant potential. Although a large part of the
evidence comes from \textit{in vitro} and animal studies, epidemiological data on the beneficial health effects of vegetables, fruits, and legumes support an important role for these minor plant components. Rich food sources (Table 11) are, for example, dark berries, aubergines, legumes, cocoa, tea, and red wine (\textit{MANACH et al. 2004}). Fig. 13 depicts basic PP structures.

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
Polyphenol class & Source & Polyphenol content by wt or vol (mg/kg fresh wt or mg/L) \\
\hline
Hydroxybenzoic acids & Blackberry & 80-270 \\
& Strawberry & 20-90 \\
Hydroxycinnamic acids & Blueberry & 2000-2200 \\
& Kiwi & 600-1000 \\
& Cherry & 180-1150 \\
& Plum & 140-1150 \\
& Aubergine & 600-660 \\
& Apple & 50-600 \\
& Coffee & 350-1750 \\
Anthocyanins & Aubergine & 7500 \\
& Blackberry & 1000-4000 \\
& Black currant & 1300-4000 \\
& Black grape & 300-7500 \\
& Cherry & 350-4500 \\
& Rhubarb & 2000 \\
& Strawberry & 150-750 \\
\hline
\end{tabular}
\caption{Polyphenol content of commonly consumed foods (adapted from (\textit{MANACH et al. 2004})). The trimeric procyanidin represents condensed tannins.}
\end{table}

\textbf{Figure 13} Basic structures of selected polyphenol subclasses (adapted from (\textit{MANACH et al. 2004})). The trimeric procyanidin represents condensed tannins.
In contrast to their health benefits, PP have been demonstrated to have a detrimental influence on dietary non-heme iron absorption in man. Iron from black beans – a PP-containing food – has been reported to be poorly available (LAYRISSE et al. 1968; LAYRISSE et al. 1969), but the first observations directly linking PP with low iron bioavailability were made with tea, which was found to markedly decrease absorption from iron solutions as well as from bread and rice meals or uncooked hemoglobin, but not from cooked hemoglobin or crystallized heme (DISLER et al. 1975a; DISLER et al. 1975b). The effect was ascribed to the formation of insoluble iron-tannate complexes as indicated by the blackish discoloration of a ferric chloride solution mixed with tea. Similar color changes have been reported in PP-rich vegetables upon iron addition (GILLOOLY et al. 1983). Since tea only affected heme iron absorption from uncooked hemoglobin, DISLER et al. (1975a) suggested that tea polyphenols had a tanning effect on the native globin molecule and thus interfered with hemoglobin digestion and subsequent heme release in the human gastrointestinal tract. Whatever the mechanism, the relevance to human nutrition is probably
marginal as meat is virtually always cooked before consumption. Nevertheless, tea decreased iron absorption from a hamburger meal containing 82 g minced meat by 62% \cite{hallback1982b}, emphasizing its strong inhibitory potential even in meals with initially high iron bioavailability. Absorption from a SSM was reduced by 83% with added tea \cite{reddy1991}, and increasing tea strength threefold caused a further 50% inhibition. This is paralleled by findings on coffee \cite{morck1983}. The high absolute iron absorption from the hamburger meal given with red wine \cite{hallback1982b} may be explained by the large contribution of the wine to total meal iron.

In most studies, coffee was less inhibitory than tea, probably owing to differences in PP composition. This aspect has been addressed by Brune \textit{et al.} \cite{brune1989} who systematically evaluated the effects of tannic, gallic, and chlorogenic acids as well as catechin on iron absorption from bread meals. Equimolar amounts of gallic acid (14.7 mg) and chlorogenic acid (30.5 mg) caused a 52% and 33% reduction, respectively, with gallic acid being equally inhibitory as tannic acid per mol galloyl groups. The content of galloyl groups was also found indicative of the extent of inhibition by oregano (69%) and tea (61%). Spinach (38%) had a less pronounced effect, which may have been due to its AA content. Furthermore, tannic acid reduced iron absorption in a dose-dependent manner. A significant 20% decrease was already visible at 5 mg, and the effect leveled off above 50 mg tannic acid. Similar results have been reported by Siegenberg \textit{et al.} \cite{siegenberg1991}. Catechin, on the other hand, did not affect iron bioavailability at a dose of 25 mg \cite{brune1989}, which is the molar equivalent to the amounts of gallic acid and chlorogenic acid stated above. Since chlorogenic acid and catechin share structural similarities, the authors suggested that the poor solubility of catechin prevented interaction with the iron.

Additional evidence for diminished iron availability with different PP-containing beverages was provided by Hurrell \textit{et al.} \cite{hurrell1999} and Cook \textit{et al.} \cite{cook1995}, who observed a reduction in iron absorption of ≥ 50% with coffee, black and herb teas, cocoa, and red wine added to bread meals. The effect of the different drinks was dependent on their PP content (Fig. 14), which is in line with findings from vegetable meals \cite{gillooly1983}. However, no special importance could be attributed to PP structure as had been reported earlier \cite{brune1989}. The authors
concluded that any beverage containing 20-50 mg PP would reduce iron absorption from a bread meal by 50-70%. With a total PP content of 100-400 mg, the reduction would be 60-90%. Diets with a high iron bioavailability may be affected to a lesser degree as indicated by a study, in which rosemary and green tea extracts (~35 mg PP) lowered iron absorption by 20-30% from a pasta/meat sauce meal (SAMMAN et al. 2001).

Figure 14  Dose-dependent inhibition of non-heme iron absorption from bread meals given with different polyphenol-containing beverages relative to a bread meal with water; Δ = white wine, • = black tea, ▲ = red wine, ▲ = herb teas, ■ = cocoa (from (HURRELL et al. 1999)).

Inhibitory effects have also been reported for meals containing solid sources of PP like nuts (MACFARLANE et al. 1988) or certain vegetables (GILLOOLY et al. 1983). GILLOOLY et al. (1983) observed a significant inverse relationship between vegetable PP content and iron absorption from rice meals. This correlation became even closer when only condensed, i.e., non-extractable PP, were considered. Wheat germ, aubergines, and certain legumes – all high in PP – exerted the strongest negative effects. Additionally, 500 mg tannic acid mixed into a broccoli meal with excellent iron bioavailability (29.7%) took the absorption down to 1.5%.

As mentioned above, the mechanism of inhibition by PP is most likely due to formation of insoluble PP-iron complexes. Iron uptake studies using Caco-2 cells
support the unavailability of such complexes at molar ratios occurring in the diet (AU and REDDY 2000; GLAHN et al. 2002; YUN et al. 2004). Likewise, coffee and tea solubilized significantly less iron from a bean iron suspension compared to orange juice (KOJIMA et al. 1981), which is known to facilitate iron absorption (BALLOT et al. 1987). Although this is not a standard assay of iron bioavailability, it hints at the poor iron solubilization properties of PP in a food matrix.

However, when the effect on dialyzable iron of tea and coffee was compared to water, tea alone caused a significant decrease from a standard hamburger meal (MILLER et al. 1981). Coffee, instead, improved iron dialyzability. The superior dialyzability of iron from aqueous coffee and tea solutions compared to cereal-based milk meals mixed with these beverages (BROWN et al. 1990) indicates the important role of meal composition in iron bioavailability. Polyphenols are known to precipitate protein (CHARLTON et al. 2002). Presumably, protein-iron-PP complexes too large to pass the dialysis membrane were formed in the presence of the cereal meals, whereas the simple aqueous suspensions contained low-molecular-weight PP-iron complexes that were dialyzable. Importantly, this only means that the complexes had a molecular weight below the membrane cut-off of 6-8 kDa. In humans, they may still be unavailable for absorption due to the strength of complexation. Of note, only the black and green teas caused a true reduction in iron dialyzability (~50%) from the cereal meal; coffee had no effect (BROWN et al. 1990). The lack of inhibition by the herb teas tested may be explained by their very low condensed PP content. Cocoa, like green and black tea rich in PP, acted differently in that it decreased iron dialyzability by 77% compared to water, but only by 28% compared to the cereal meal with water. Compositional differences in PP structures may account for a major part of the reported divergence between beverages.

1.2.2.3.2 Phytic acid (PA)
Phytic acid is an abundant component of plant-based diets (Table 12) and occurs mainly in legumes, whole grains, seeds, and nuts (REDDY and SATHE 2002), where it acts as a phosphate store or phosphate sink (CHERYAN 1980). In humans, PA may have a role as a lipid- and cholesterol-lowering or cancer-preventive agent (ZHOU and ERDMAN 1995; MIDORIKAWA et al. 2001; VUCENIK and SHAMSUDDIN 2003). The antioxidant effect of PA observed in foods (EMPSON et al. 1991) may be important for its anti-cancer properties by minimizing metal-catalyzed ROS formation. When it
comes to mineral metabolism, however, PA assumes the role of an anti-nutritive factor because it powerfully decreases the bioavailability of minerals and trace elements like calcium, zinc, and iron (CHERYAN 1980; ZHOU and ERDMAN 1995; SANDBERG 2002; HUNT 2003; HURRELL 2004).

### Table 12 Relevant food sources of phytic acid (adapted from (REDDY and SATHE 2002))

<table>
<thead>
<tr>
<th>Food source</th>
<th>Phytate [% dry basis]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cereals and cereal-based foods</strong></td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>0.39-1.35</td>
</tr>
<tr>
<td>Corn</td>
<td>0.75-2.22</td>
</tr>
<tr>
<td>Oat</td>
<td>0.42-1.16</td>
</tr>
<tr>
<td>Barley</td>
<td>0.38-1.16</td>
</tr>
<tr>
<td>Rye</td>
<td>0.54-1.46</td>
</tr>
<tr>
<td>Wild rice</td>
<td>2.20</td>
</tr>
<tr>
<td>Polished rice</td>
<td>0.14-0.60</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>0.25-1.37</td>
</tr>
<tr>
<td>Oat meal</td>
<td>0.89-2.40</td>
</tr>
<tr>
<td>Oat bran</td>
<td>0.60-1.42</td>
</tr>
<tr>
<td>Whole wheat breads</td>
<td>0.43-1.05</td>
</tr>
<tr>
<td>Corn tortillas</td>
<td>0.11-0.95</td>
</tr>
<tr>
<td><strong>Beans and bean-based foods</strong></td>
<td></td>
</tr>
<tr>
<td>Soybeans</td>
<td>1.00-2.22</td>
</tr>
<tr>
<td>Peas</td>
<td>0.22-1.22</td>
</tr>
<tr>
<td>Lentil</td>
<td>0.27-1.05</td>
</tr>
<tr>
<td>Linseed</td>
<td>2.15-2.78</td>
</tr>
<tr>
<td>Peanuts</td>
<td>1.05-1.76</td>
</tr>
<tr>
<td>Chickpeas</td>
<td>0.28-1.26</td>
</tr>
<tr>
<td>Kidney beans</td>
<td>0.89-1.57</td>
</tr>
<tr>
<td>Soy flour</td>
<td>1.24-2.25</td>
</tr>
<tr>
<td>Tofu</td>
<td>1.46-2.90</td>
</tr>
<tr>
<td>Peanuts, toasted and salted</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>Tubers, fruits, leafy vegetable, nuts</strong></td>
<td></td>
</tr>
<tr>
<td>Potato</td>
<td>0.01-0.18</td>
</tr>
<tr>
<td>Yam</td>
<td>0.04-0.29</td>
</tr>
<tr>
<td>Cassava</td>
<td>0.10-0.19</td>
</tr>
<tr>
<td>Avocado</td>
<td>0.51</td>
</tr>
<tr>
<td>Mango</td>
<td>0.14</td>
</tr>
<tr>
<td>Spinach</td>
<td>0.01-0.07</td>
</tr>
<tr>
<td><strong>Tubers, fruits, leafy vegetable, nuts</strong></td>
<td></td>
</tr>
<tr>
<td>Carrots, raw</td>
<td>0.09</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>1.91</td>
</tr>
<tr>
<td>Walnut</td>
<td>0.65-2.38</td>
</tr>
<tr>
<td>Almond</td>
<td>1.35-3.22</td>
</tr>
<tr>
<td>Cashew nut</td>
<td>0.63-1.97</td>
</tr>
</tbody>
</table>
Mean dietary intakes of PA vary widely depending on the country and the population group under study (REDDY and SATHE 2002). For example, British students have been reported to consume 670 mg PA/d compared to U.S. college students with a daily intake of 1293 mg PA. In contrast, Malawian preschool children had a daily intake of 1675-2010 mg PA (FERGUSON et al. 1989) and a study in Indian adolescent and adult vegetarians reported a daily PA consumption of 1565-2030 mg (KHOKHAR et al. 1994). The higher PA intake in the latter countries may be considered indicative of an extensive subsistence on plant foods, as is commonly reported for populations in the developing world (ZIMMERMANN et al. 2005; GIBSON et al. 2006).

Chemically, PA is myo-inositol 1,2,3,4,5,6-hexak-s dihydrogen phosphate – also referred to as IP6 – and is known to form poorly soluble ferric phytate complexes, especially at low pH (MAKOWER 1970; CHERYAN 1980). In a complete meal, the protein component may modulate complex precipitation as was shown by REDDY et al. (1996). Beef attenuated the inhibitory effect of 300 mg PA on iron absorption from a SSM, whereas soy protein and egg albumin caused a non-significant decrease. The authors suggested differences in the formation of insoluble protein/peptide-iron-phytate complexes as a possible explanation for these observations.

An early report using the chemical balance technique (WIDDOWSON and MCCANCE 1942) indicated an inhibitory effect of wheat bran on non-heme iron absorption, which was attributed to the high PA content of this foodstuff. Reduced absorption of food iron and iron salts in the presence of added sodium phytate lent support to this assumption (MCCANCE et al. 1943; TURNBULL et al. 1962). Later, the inhibitory properties of wheat bran were confirmed in a dual radioisotope study and a dose-dependent effect established (BJORN-RASMUSSEN 1974). Adding 3.3-10% wheat bran – corresponding after baking to 6-20 mg PA – to a wheat bread meal reduced iron absorption by 31-57%.

In an attempt to decipher the bran constituent(s) mediating the inhibition, SIMPSON et al. (1981) observed a similar reduction of 51-74% with 12 g (~4.4%) bran added to a muffin/milk shake meal and demonstrated total bran phosphate but not PA content to be the major determinant. However, incomplete phytate removal in the dephytinized bran samples may have led to inadequate appreciation of the inhibitory nature of PA. Likewise, the less pronounced effect of maize bran observed in another study...
(SIEGENBERG et al. 1991) was probably due to the 10 mg PA already contained in the control meal. HURRELL et al. (1992) reported a doubling in iron absorption from a SSM containing 30 g soy protein isolate (6.5-8.4 mg/g initial PA content) when removing 86-95% of the total PA by acid-washing. However, near complete PA removal (< 0.01 mg/g) with phytase treatment was necessary to obtain a 4.4-fold increase. Based on these observations, the authors calculated that a meal PA content < 10 mg had to be achieved for substantial absorption enhancement to occur.

A more in-depth analysis of the wheat bran fractions confirmed that PA contributed substantially to the markedly decreased iron absorption (HALLBERG et al. 1987); fiber was suggested as an additional source of inhibitors. Interestingly, soluble monoferric phytate added to a semisynthetic or standard hamburger meal was absorbed equally well as ferric chloride (SIMPSON et al. 1981), whereas a mixture of monoferric phytate with magnesium and potassium salts of phytate as present in bran decreased iron absorption from a hamburger/bread meal to the same extent as did bran itself (HALLBERG et al. 1987).

Soybeans and nuts are further PA-rich foods with a demonstrated detrimental effect on non-heme iron bioavailability (HALLBERG and ROSSANDER 1982c; MACFARLANE et al. 1988). They also contain large amounts of polyphenols (isoflavones), which presumably add to the inhibitory action although this has never been shown. Higher doses of absorption enhancers may be necessary to overcome or at least partially offset the inhibition by soybeans (GILLOOLY et al. 1984; DERMAN et al. 1987), as soy protein per se provides a third class of absorption inhibitors (see chapter 1.2.2.3.3). The lack of effect of coconuts observed by MACFARLANE et al. (1988) can not be explained by the lower levels of PA and polyphenols compared to the other nuts tested, as the administered dose would still be expected to decrease iron absorption. Accordingly, one might speculate on the presence of some unknown enhancing factor(s) that counteract the inhibitors.

Extrinsic sodium phytate inhibited iron absorption from bread rolls in a dose-dependent fashion (HALLBERG et al. 1989a). More marked levels of decrease were observed in the lower concentration range of 7-35 mg phytate and a maximum inhibition of 82% obtained at the highest dose of 878 mg phytate. However, the
sodium phytate was added to the rolls immediately before consumption, which may have lead to an overestimation of the inhibitory effect of native phytic acid as cautioned by CHERYAN (1980), since PA concentration and chemical form depends on the culinary preparation. Although the results from HALLBERG and colleagues (1989a) generally corresponded well with earlier reports (BJORN-RASMUSSEN 1974; HALLBERG et al. 1987), the lack of an anti-phytate effect of 50 g beef in the meal containing 88 mg phytate may have resulted from such influences.

Lower inositol phosphates (IP3, IP4, IP5) arising from partial phytate degradation have been shown to exert a similarly marked inhibitory action as PA on iron absorption from bread meals (BRUNE et al. 1992). Notably, no correlation between meal fiber content and iron absorption was observed, which contradicts a previous hypothesis of these authors (HALLBERG et al. 1987). Studies in Caco-2 cells paralleled the in vivo findings on PA (GLAHN et al. 2002) and lower inositol phosphates (HAN et al. 1995a; SKOGLUND et al. 1999), although IP3 failed to decrease iron uptake. The significantly lower uptake of hemoglobin iron in the presence of PA (GLAHN et al. 2002) is, however, not in line with human studies.

Aqueous extraction, heat treatment, and enzymatic degradation are means to decrease food PA content. Enzymatic degradation may be accomplished by addition of extrinsic phytase or by activation of intrinsic phytases through soaking, germination, or fermentation (EGLI 2001; HURRELL 2004). Dephytinization of complementary foods using an extrinsic phytase was demonstrated to significantly improve iron absorption in infants (DAVIDSSON et al. 1994a) and adults (DAVIDSSON et al. 2001; HURRELL et al. 2003). However, reducing the phytate content of a soy-based infant formula by 83% caused only a marginal, though significant increase from 5.5 to 6.8%, whereas complete removal enhanced absorption from 3.9 to 8.7% (DAVIDSSON et al. 1994a). Based on earlier findings (HALLBERG et al. 1989a; HURRELL et al. 1992), HURRELL (2004) has thus recommended that a PA:Fe ratio of < 1:1, and preferably < 0.4:1 be achieved for a relevant enhancement in iron bioavailability from iron-fortified foods like infant cereals to occur.

1.2.2.3.3 Soy proteins
Soy and soy-based foods are becoming increasingly important alternatives to animal protein sources like meat and milk (ERDMAN and FORDYCE 1989) and have been
Literature review

demonstrated to favorably affect blood lipid and cholesterol levels (Goldberg et al. 1982). Furthermore, epidemiological evidence suggests that a high soy intake may decrease the relative risks of osteoporosis (Zhang et al. 2005), hypertension (Yang et al. 2005), and certain cancers (Ho et al. 2006). On the other hand, compelling evidence exists for a strong inhibitory effect of soy on iron absorption (Cook et al. 1981; Hallberg and Rossander 1982c; Hurrell et al. 1992; Davidsson et al. 1994a).

Soybeans contain high amounts of PA (see Table 12), and dephytinization has been shown to improve iron bioavailability from soy-based foods (Hurrell et al. 1992; Davidsson et al. 1994a; Hurrell 2004). However, even after near-complete PA removal some inhibitory activity remained, which was potentially protein-related (Hurrell et al. 1992). Later on, the 7S conglycinin fraction in soy protein was found to be the source of inhibition (Lynch et al. 1994). It is noteworthy that soy conglycinin has also been reported to display poor digestibility (Astwood et al. 1996). The formation of high-molecular-weight iron-conglycinin complexes may thus account for the diminished bioavailability. This assumption is supported by studies on iron dialyzability from soy protein products. Soy flour as well as soy protein isolate demonstrated low levels of dialyzable iron (≤3.3%) compared to beef and bovine serum albumin with dialyzability percentages well above 10% (Schricker et al. 1982b; Kane and Miller 1984). After dephytinization, a pea protein isolate showed significantly higher iron bioavailability in healthy, young women (Davidsson et al. 2001) compared to soy protein isolate studied in infants (Davidsson et al. 1994a), which was explained by differences in legume protein composition. On this basis, pea protein isolates could represent a suitable alternative to soy products in weaning foods, where mineral bioavailability is critical.

Substituting isolated soy protein for egg albumin in a SSM dramatically decreased the amount of iron absorbed by 89% (Morck et al. 1982), but baking the isolated soy protein or whole soybeans was shown to moderately improve iron absorption (2-fold and 1.5-fold, respectively). This may have been due to partial degradation of the contained PA or the heat-labile trypsin inhibitor, which might otherwise interfere with protein digestion (Baynes et al. 1990). The statistically significant improvement could, however, not be confirmed when baked soy protein isolate was added to a liquid
infant formula (Derman et al. 1987), although a similar trend was observed. The limited usefulness of extensive heating of isolated soy protein is reinforced by the undesirable changes in soy functional properties like decreased water dispersibility (Mörck et al. 1982).

In a meat-containing diet, the negative effect of soy protein on non-heme iron absorption may be partially canceled out by its promoting action on heme iron absorption (Lynch et al. 1985) and its substantial contribution to total iron content (Hallberg and Rossander 1982c; 1984). This finding gains special importance in light of the practice of extending ground beef with soy protein, e.g. in American school lunch programs or by the U.S. military (Miles et al. 1987).

1.2.2.3.4 Milk proteins
The nutritive value of milk and milk products largely derives from the high protein quality and/or the sometimes substantial calcium content (Jackson and Lee 1992). Furthermore, bioactive peptides set free upon milk protein digestion may exert antihypertensive or antithrombotic effects (Chabance et al. 1995; Yamamoto 1997; Chabance et al. 1998). Anticariogenic and osteoprotective properties have also been hypothesized (Scholz-Ahrens and Schrezenmeir 2000) but convincing evidence is lacking. Regarding iron bioavailability, two lines of investigation have been followed to date. One suggests a beneficial action of certain caseinophosphopeptides (CPPs) as iron-binding and absorption-increasing agents, whereas the other describes a deleterious effect of milk proteins on iron nutrition. Within the scope of this review, emphasis is placed on the latter aspect.

In their review on the role of dairy products in iron nutrition, Jackson and Lee (1992) provided evidence that positive effects of milk proteins were largely restricted to animal studies. One human study (Hallberg and Rossander 1982b) could find no effect of replacing water with milk on iron absorption from a hamburger meal, whereas the majority of reports have documented varying degrees of inhibition. For example, human iron absorption from a reference dose solution containing ferrous sulfate and AA was reduced by 50% when it was followed by 200 ml milk instead of water (Disler et al. 1975a). Breakfast meal iron was ~3 times better absorbed when taken with a water drink instead of milk (Gleerup et al. 1993). Casein, milk, and cheese also markedly decreased iron absorption from semisynthetic and standard
American meals (Cook and Monsen 1976), as did whey, which was only tested in the semisynthetic meal (Hurrell et al. 1989). The 45% inhibition by casein in the latter study did not reach statistical significance, presumably owing to the small sample size and widely differing iron status of the subjects. One might speculate that the observed inhibition relates to the high calcium and phosphate content of these dairy products (see next chapter). However, Hurrell et al. (1989) showed acid and enzyme hydrolysis of casein or whey protein preparations to improve iron absorption, although non-significantly for whey. The lesser hydrolysis – 36% for whey vs. 84% for casein – may explain the differences in significance. Of note, the impact of hydrolysis was less pronounced than in parallel dialyzability studies, suggesting calcium to be the major inhibitor in dairy products. Low iron dialyzability (1%) from an iron-casein preparation was also reported by Kane and Miller (1984), contrasted by 13% and 19% dialyzability of beef iron and BSA iron, respectively.

A small dose of AA (8.4 mg) improved iron absorption 5-fold from an infant milk formula containing 1.5 g milk protein (Derman et al. 1987). Increasing the AA dose to 25 mg caused a further doubling in absorption. When soy protein was substituted for milk protein, absorption decreased substantially and remained much lower upon the addition of AA. Regarding iron nutrition, milk protein can thus be considered a better choice for infant formulas compared to soy protein, unless cow’s milk allergy forbids its use. This holds especially when AA is present in the formula.

The iron bioavailability from human milk of ~50% as reported in earlier studies (Saarinen et al. 1977; Hallberg et al. 1992) was more recently found to be closer to about 15% (Davidsson et al. 1994b; Domelof et al. 2002). Nevertheless, human milk seems to be a superior source of bioavailable iron compared to cow’s milk formula (reviewed in Lonnerdal 1997), which is probably related to marked differences in protein composition and a much higher mineral content (especially calcium phosphate) of cow’s milk. Lactoferrin is the major iron-binding protein in human milk, whereas cow’s milk mainly contains casein-iron complexes (Jovani et al. 2003). Iron from recombinant human lactoferrin has recently been reported to be equally well absorbed in healthy, young women as ferrous sulfate (Lonnerdal and Bryant 2006). In neonates, binding to lactoferrin receptors on the brush border membrane (Suzuki et al. 2001) may be specific for human lactoferrin (Kawakami and
LONNERDAL 1991), thus safeguarding the milk iron to its absorption site in the small intestine. This would explain the lack of effect observed with bovine lactoferrin in vivo (FAIRWEATHER-TAIT et al. 1987), which also displays low dialyzability in vitro (JOVANI et al. 2003). However, improved iron absorption by infants 2-10 mo of age from human breast milk made devoid of intrinsic lactoferrin (< 3% of initial) compared to untreated breast milk does not support a major role for this protein (DAVIDSSON et al. 1994b). Whether the lactoferrin removal procedure may have affected other milk compounds involved in iron absorption is open to question. The authors pointed out, though, that iron absorption in early infancy (< 3 mo) may still be positively affected by lactoferrin. Additionally, SERFASS and REDDY (2003) demonstrated LMW whey protein fractions (1-6 kDa) from human breast milk to efficiently solubilize extrinsic iron and improve its transfer across Caco-2 monolayers. Since these small proteins showed some resistance to in vitro digestion with pepsin and pancreatin, they may have a role in human iron absorption by providing absorbable iron to the small intestinal mucosa.

In recent years, increasing attention has been paid to bioactive peptides from milk and dairy products with a wide variety of hypothesized functional properties, e.g. antithrombotic or immunomodulating activity (FIAT et al. 1993), but evidence is only slowly accumulating. In terms of iron nutrition, CPP-iron complexes have been proposed as a potential source of highly bioavailable iron (CHAUD et al. 2002; BOUHALLAB and BOUGLE 2004). Rat studies showed CPP-iron – more precisely β-casein fragment 1-25, abbreviated β-CN(1-25) – to be comparable to or more effective than ferrous gluconate or ferrous sulfate in the hemoglobin repletion assay and duodenal loop absorption (AIT-OUKHATAR et al. 1999; AIT-OUKHATAR et al. 2002; BOUHALLAB et al. 2002); absorbed iron was favourably incorporated into liver and spleen. Furthermore, in vitro digestion experiments indicated extensive resistance to enzymatic hydrolysis of the CPP-iron complex (AIT-OUKHATAR et al. 2000). However, no significant difference in iron absorption from milk enriched with β-CN(1-25) or ferrous sulfate was detected in humans (AIT-OUKHATAR et al. 2002). This may be viewed as further support against the rat as a valid model of iron bioavailability in humans. In conclusion, evidence for a beneficial role of CPPs in human iron absorption is still lacking, and inhibition of iron absorption by similar peptides formed on milk digestion would explain the inhibitory nature of casein.
1.2.2.3.5 Calcium (Ca)
Calcium is an essential mineral for humans, contributing 1-2% of the total body weight (CASHMAN 2002). It is mainly involved in proper bone and tooth growth (> 99% of total body Ca), acts as a second messenger in intracellular signal transduction (RIZZUTO and POZZAN 2006), controls muscle contraction (RIOS 2006), and participates in the blood coagulation cascade (SPRONK et al. 2003). According to the Dietary Reference Intakes (INSTITUTE OF MEDICINE 2001a), acceptable Ca intake levels for individuals aged ≥ 9 y range from 1000 to 1300 mg/d. Dietary Ca is largely provided by milk and dairy products, fruits, and grains. Supplemental calcium intake may be substantial in certain population groups and displays an upward trend (MILLEN et al. 2004).

In the context of iron bioavailability, Ca has been found to have a dose-dependent inhibitory effect. In the range of 75-300 mg Ca (added as the chloride salt), iron absorption from wheat rolls significantly decreased by 16-56% (HALLBERG et al. 1991); raising the Ca content to 600 mg caused no further changes. MONSEN et al. (1976) observed a significant inhibitory effect of Ca from a SSM when it was administered with phosphate, but neither ion caused a significant decrease when added as calcium chloride or potassium phosphate, respectively. Iron may thus have been rendered insoluble by the formation of a calcium-iron-phosphate complex. Furthermore, supplemental calcium carbonate did not affect ferrous sulfate absorption from supplement sources at Ca:Fe weight ratios (mg) of 300:37 and 600:18, whereas calcium citrate (-49%) and calcium phosphate (-62%) did at the latter ratio (COOK et al. 1991b). Given with food, all supplements inhibited ferrous sulfate as well as dietary non-heme iron absorption. Presumably, complex interactions between food constituents and the supplements accounted for these differences.

Some studies (ROSSANDER et al. 1979; TURNLUND et al. 1990) failed to demonstrate an effect of Ca added as milk on non-heme iron absorption from iron-fortified cereal-based diets. Unfortunately, no information is given on total Ca in the experimental diets. If the diets without milk already contained ≥300 mg Ca, no further reduction in iron absorption would be expected (HALLBERG 1998). This is exemplified by a study showing no significant decrease in non-heme iron absorption from a diet containing
~800 mg Ca compared to a control meal with 224 mg Ca (GRINDER-PEDERSEN et al. 2004). In addition, the fecal monitoring approach used in one of the studies (TURNLUND et al. 1990) suffers from limited precision when only small amounts of analyte are absorbed or excreted. On the other hand, milk may have positively affected availability of the elemental iron used for fortification as suggested by *in vitro* data, thus canceling out the inhibition. The authors also point out a possible differential effect of milk on iron from a solution compared to iron added into the cereal portion of the meal.

Calcium is unique among the iron absorption inhibitors in that it also affects heme iron. HALLBERG and co-workers (1991) demonstrated similarly decreased bioavailability of heme iron as was shown for non-heme iron from a wheat roll meal containing graded doses of calcium chloride. In a follow-up study (HALLBERG et al. 1993), the mechanism of inhibition on heme iron absorption was further investigated in hamburger and wheat roll meals, demonstrating a direct action of Ca since the meat factor was unable to overcome the inhibition. The authors suggested the site of inhibition to lie within the absorptive enterocytes because heme and non-heme iron were affected to the same extent. However, additional inhibition of non-heme iron uptake at the enterocyte apical membrane may be operative. Calcium was shown to compete for iron-binding sites on mobilferrin, a component protein of the IMP pathway suggested to provide an alternative route for non-heme iron entry in the ferric form into the intestinal mucosa and red blood cells (CONRAD et al. 2000).

Given with an overall inhibitory meal containing milk, phytate and coffee, Ca further reduced iron absorption (COOK et al. 1991b), which indicates an additive effect of this mineral. Moreover, it was shown to interfere with phytate degradation when added to the dough (HALLBERG et al. 1991), possibly by rendering the phytate insoluble and thus unavailable for enzymatic breakdown. In this case, as little as 40 mg Ca led to a 40% reduction in iron bioavailability. If insoluble calcium phytate maintains the inhibitory potential of its constituents, this aspect might reach nutritional significance when milk and dairy products are used for bread-making. Separating Ca-rich meals from those high in iron improved non-heme iron absorption by 30-50% (GLEERUP et al. 1995). Whereas the population-wide feasibility of this approach is cast with doubt, longer-term studies provide an indication it may not even be necessary in iron-replete
subjects consuming Western-style diets. Supplemental Ca (500-1200 mg/d) given with meals for 3, 6, or 12 months showed no detrimental effect on blood indices of iron status (SOKOLL and DAWSON-HUGHES 1992; MINIHANE and FAIRWEATHER-TAIT 1998; MOLGAARD et al. 2005). The significantly decreased non-heme iron absorption from the Ca-supplemented diet in the study by MINIHANE and FAIRWEATHER-TAIT (1998) may, however, lead to iron depletion in the long run. In contrast, REDDY and COOK (1997) failed to observe any differences in non-heme iron absorption between self-selected diets and diets modified to be low or high in calcium, each given over a 5 d-period.

1.2.2.3.6 Oxalic acid (OA)
Oxalic acid (OA) is a regular constituent of plant foods like green leafy vegetables, beetroot, carrots, potatoes, cocoa, and tea (ZAREMBSKI and HODGKINSON 1962; CHAI and LIEBMAN 2005). Especially rich sources are rhubarb (HONOW and HESSE 2002), spinach, mangold, and purslane (CHAI and LIEBMAN 2005), which contain up to 1-2 g/100 g fresh weight (Table 13).

Table 13 Oxalic acid content of selected foods (data from (NOONAN and SAVAGE 1999; AL-WAHSH et al. 2005; CHAI and LIEBMAN 2005))

<table>
<thead>
<tr>
<th>Food</th>
<th>Oxalic acid mg/100 g fresh wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhubarb (Rheum rhabonticum)</td>
<td></td>
</tr>
<tr>
<td>Victoria, stewed</td>
<td>260</td>
</tr>
<tr>
<td>raw</td>
<td>275-1336</td>
</tr>
<tr>
<td>Beetroot (Beta vulgaris)</td>
<td>121-450</td>
</tr>
<tr>
<td>Purslane (Portulaca oleracea)</td>
<td>910-1679</td>
</tr>
<tr>
<td>Spinach (Spinacia oleracea)</td>
<td>320-1260</td>
</tr>
<tr>
<td>Swiss chard (Beta vulgaris var. cicla)</td>
<td></td>
</tr>
<tr>
<td>red, leaves</td>
<td>1167</td>
</tr>
<tr>
<td>green, leaves</td>
<td>964</td>
</tr>
<tr>
<td>Coffee (Coffea arabica)</td>
<td>50-150</td>
</tr>
<tr>
<td>Cocoa (Theobroma cacao)</td>
<td>500-900</td>
</tr>
<tr>
<td>Potato (Solanum tuberosum)</td>
<td>20-141</td>
</tr>
<tr>
<td>Tea (Thea sinensis)</td>
<td>300-2000</td>
</tr>
<tr>
<td>Parsley (Petroselinum sativum)</td>
<td>140-200</td>
</tr>
<tr>
<td>Soybean</td>
<td>23-54</td>
</tr>
<tr>
<td>Carrot (Daucus carota)</td>
<td></td>
</tr>
<tr>
<td>raw</td>
<td>44</td>
</tr>
<tr>
<td>boiled</td>
<td>18-23</td>
</tr>
</tbody>
</table>
OA may be present in foods as insoluble calcium oxalate crystals (FRANCESCHI and NAKATA 2005) or as soluble sodium or potassium oxalate; insoluble OA is usually calculated as the difference between total and soluble OA. Relative contributions of these two fractions to total OA seem to vary widely within and between plant species. HONOW and HESSE (2002) reported soluble and total OA contents in boiled spinach of 33-168 mg/100 g and 100-627 mg/100 g fresh weight, respectively. This is in line with values of soluble and total OA in boiled spinach of 107 mg/100 g and 460 mg/100 g fresh weight, respectively (CHAI and LIEBMAN 2005). Raw spinach was shown by the latter authors to contain 803 mg/100 g soluble OA and 1145 mg/100 g total OA. The extensive OA losses during boiling are most likely due to leaching of soluble OA into the cooking water, since steaming caused less reduction in soluble OA and insoluble OA was not affected by steaming or boiling. Soluble OA contributed 70% to total OA in raw spinach and carrots, whereas rhubarb stalks were found to contain 42% as soluble OA (CHAI and LIEBMAN 2005). Similar results have been reported for these foods grown in New Zealand, although total OA in raw spinach was substantially lower at 330 mg/100 g fresh weight (SAVAGE et al. 2000). ZAREMBSKI and HODGKINSON (1962) analyzed OA contents of 80 food items commonly used in English homes and hospitals and calculated the daily intake to be 118 mg in the household setting and to range between 70-150 mg in six hospital diets. This compares to results from a small study including five subjects, in which the mean daily OA intake was estimated to be 152 mg (range 44-351 mg) (HOLMES and KENNEDY 2000).

It is well known that OA inhibits calcium absorption in humans (HEANEY et al. 1988; HEANEY and WEAVER 1989), and absorption of magnesium (BOHN 2003) and zinc (KELSAAY and PRATHER 1983) may also be compromised. The most likely explanation is the formation of insoluble metal-oxalate complexes, since calcium and zinc oxalate solubility (Table 14) has been reported to be < 1 mg/100 mL (HODGKINSON and ZAREMBSKI 1968). This assumption is supported by the observation that addition of magnesium (BERG et al. 1986) or calcium (VON UNRUH et al. 2004) decreased OA absorption from diets.
Literature review

Table 14 Stability constants and solubility of different oxalate salts (from (HODGKINSON and ZAREMBSKI 1968))

<table>
<thead>
<tr>
<th>Oxalate salt</th>
<th>Stability constant$^1$ (log $K_S$)</th>
<th>Solubility (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium oxalate</td>
<td>3.0</td>
<td>0.67 (13°C)</td>
</tr>
<tr>
<td>Magnesium oxalate</td>
<td>2.76 (20°C)</td>
<td>70.0 (16°C)</td>
</tr>
<tr>
<td>Zinc oxalate</td>
<td>4.9</td>
<td>0.79 (18°C)</td>
</tr>
<tr>
<td>Ferrous oxalate</td>
<td>&gt;4.7</td>
<td>22.0</td>
</tr>
<tr>
<td>Ferric oxalate</td>
<td>9.4</td>
<td>very soluble</td>
</tr>
</tbody>
</table>

$^1$ T = 25°C, I = 0.1 unless stated otherwise.

Surprisingly little attention has been paid to the effect of OA on iron bioavailability. GILLOOLY et al. (1983) reported a 39% reduction in ferrous iron absorption from a vegetable soup in the presence of 1 g calcium oxalate, corresponding to a molar Fe:OA ratio of 1:144. However, this inhibition may have just as much been brought about by the contained calcium (~300 mg), which maximally inhibits non-heme iron absorption at this dose (HALLBERG et al. 1991). In contrast, iron absorption from a rice meal slightly increased when 100 g rhubarb (537 mg OA) were added (BALLOT et al. 1987). Yet rhubarb does not only contain OA but is also high in malic acid (1.7 g/100 g) and citric acid (410 mg/100 g), which have both yielded equivocal results with regard to iron absorption (GILLOOLY et al. 1983; HALLBERG and ROSSANDER 1984; BALLOT et al. 1987). Furthermore, iron absorption from three OA-rich vegetables was not correlated with their OA content. Spinach (230 mg OA) and beetroot greens (460 mg OA) inhibited iron absorption, whereas iron from beetroot (170 mg OA) was well absorbed. Data from rat studies showed a neutral to slightly enhancing effect of OA (VAN CAMPEN and WELCH 1980; GORDON and CHAO 1984), but the rat model has been shown to deliver unreliable results with regard to dietary factors influencing iron absorption (REDDY and COOK 1991). In Caco-2 cells, OA slightly reduced ferrous iron but significantly increased ferric iron uptake, with maximum stimulation at a molar Fe:OA ratio of ~1:80 (SALOVAARA et al. 2002). This might hint at a differential effect of OA on ferrous and ferric iron as suggested by solubility data (Table 14). However, our own observations from human absorption studies do not support such a differential effect and iron valence largely depends on the redox state of the food matrix within the gastrointestinal tract.


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Manuscript 1

IRON-BINDING PEPTIDES FROM MEAT

ACCEPTED FOR PUBLICATION IN JOURNAL OF FOOD SCIENCE
Iron-binding Properties, Amino Acid Composition and Structure of Muscle Tissue Peptides from In Vitro Digestion of Different Meat Sources

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Keywords: iron; meat factor; in vitro digestion; iron-binding peptides

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Abstract

Background: The enhancing effect of meat on non-heme iron bioavailability in humans is thought to be due to the release of low-molecular-weight (LMW) iron-binding peptides during digestion.

Objective: To better characterize LMW iron-binding peptides from meat digests.

Methods: Cooked beef, chicken, cod, lamb, and pork myofibrillar or sarcoplasmic protein extracts, casein, and egg albumin were digested in vitro with pepsin or pepsin/pancreatin. Ultrafiltrates were analyzed for N and iron and further characterized by gel filtration with added $^{59}\text{Fe}$, amino acid analysis, and LC-MS.

Results: 84-98% of total iron in enzymic digests was associated with soluble LMW peptides (< 10 kDa) of the myofibrillar proteins compared to only 2-20% in the corresponding sarcoplasmic protein digests. Pepsin digestion alone of the myobrillar proteins generated > 80% soluble LMW iron, compared to < 5% with casein and egg albumin. Iron-binding peptides from myofibrillar protein with an estimated 2 kDa molecular mass were separated by gel filtration. Peptides in this fraction were enriched in aspartic and glutamic acid residues and included potential peptide fragments of myosin.

Conclusion: LMW (< 10 kDa) peptides in enzyme digests of myofibrillar proteins were the major facilitators of iron solubility. Unlike with casein, egg albumin, and most sarcoplasmic proteins, these LMW peptides were generated on pepsin digestion. One group of iron-binding peptides had a mass of ~2 kDa and was enriched in glutamic and aspartic acids. Such early generation of a multitude of LMW iron-binding peptides could explain the enhancing effect of muscle tissue on iron absorption.
Introduction
Non-heme iron absorption in humans is influenced by a variety of dietary factors. Phytic acid and polyphenols are known absorption inhibitors that are abundant in plant-based diets (Siegenberg and others 1991). They have been suggested to strongly complex and/or precipitate non-heme iron in the gut lumen thereby rendering it unavailable for mucosal uptake (Disler and others 1975; Hurrell 2004). On the other hand, ascorbic acid (Reddy and Cook 1991; Siegenberg and others 1991; Cook and Reddy 2001) and muscle tissue (Martinez-Torres and Layrisse 1971; Martinez-Torres and others 1974; Hurrell and others 1988; Engelmann and others 1998), the so called “meat factor”, increase non-heme iron bioavailability by reducing and/or binding iron in soluble complexes thus preventing the reaction with inhibitors (Hallberg and others 1986; Carpenter and Mahoney 1989; Siegenberg and others 1991; Neale 1992). However, it is not animal source proteins per se that enhance iron bioavailability. Egg albumin and casein decrease iron absorption (Cook and others 1981; Hurrell and others 1989) with casein being the more inhibitory protein, and among the plant proteins, soy protein features prominently as an iron absorption inhibitor (Lynch and others 1994).

The “meat factor” has been previously investigated, but its chemical nature and the mechanism of action remain elusive. Water and protein constitute the major fractions of lean meat (Pearson and Young 1989). In addition, protein was the only macronutrient to affect non-heme iron absorption (Monsen and Cook 1979), which is why meat proteins and amino acids have become the focus of research on the “meat factor”. In human absorption studies, cysteine was the sole amino acid to enhance iron absorption (Martinez-Torres and Layrisse 1970; Martinez-Torres and others 1981; Layrisse and others 1984) making the cysteine-rich myofibrillar proteins myosin and actin likely candidates for the origin of the “meat factor”. Taylor and others (1986) demonstrated higher iron absorption from a meat broth with normal cysteine content compared to an oxidized broth low in cysteine and thus proposed cysteine-containing LMW peptides as the active principle, although it cannot be ruled out that other “meat factor” compounds were oxidized. Such peptides, released upon enzymic digestion of meat protein, may bind iron and/or reduce it from the ferric to the ferrous form. Consequently, ferric hydroxide precipitation and interaction with absorption inhibitors like phytic acid is minimized.
The idea of cysteine and cysteine-containing peptides as the “meat factor” was further investigated by others (Mulvihill and others 1998; Mulvihill and Morissey 1998; Mulvihill and Morrissey 1998; Diaz and others 2002) with the major finding that salt-soluble meat protein extracts containing mainly myofibrillar proteins exhibited enhanced \textit{in vitro} iron dialyzability. In addition, Caco-2 iron uptake increased in the presence of cysteine and reduced cysteinyl-glycine (Glahn and Van Campen 1997) and peptide sulphydryl content was related to iron solubility and reduction of ferrous iron (Vattem and others 2001). The heavy meromyosin, a fragment of the major myofibrillar protein myosin and very rich in cysteine, contributed largely to enhanced iron dialyzability (Mulvihill and others 1998). Moreover, histidine-containing peptides from meat digests were demonstrated to increase iron solubility (Seth and Mahoney 2000b) and iron uptake into Caco-2 cells (Swain and others 2002), two \textit{in vitro} surrogates of iron bioavailability. If the cysteine-containing peptides of muscle tissue are a major component of the “meat factor”, they must have a special nature since egg albumin which has a cysteine content similar to actin (6 cysteines per molecule, of which 2 form a disulfide bridge) does not enhance iron absorption or iron dialyzability.

The aim of this study was to obtain further \textit{in vitro} information on the “meat factor” from meat of different animals. To this end, water- and salt-soluble extracts from beef, chicken, cod, lamb, and pork were subjected to a simulated gastrointestinal digestion followed by ultrafiltration and gel filtration. Sodium caseinate, egg albumin, and wheat starch were included to assess the effect of non-meat animal source proteins and starch, respectively, on the production of soluble low-molecular-weight (LMW) iron. Gel filtration and immobilized-metal affinity chromatography were used as two different ways to isolate the iron-binding LMW peptides from digest ultrafiltrates and liquid chromatography-mass spectrometry (LC-MS) was employed to obtain information on peptide structure.

\textbf{Materials and Methods}

Unless otherwise stated, all chemicals were from Merck (Dietikon, Switzerland) and of analytical grade or higher. Water used throughout the experiments was of Nanopure (Barnstead NANOpure Cartridge System, Skan AG, Basel-Allschwil, Switzerland) or MilliQ quality (Millipore AG, Volketswil, Switzerland).
Protein extraction

The extraction of meat protein is shown diagrammatically in Fig. 1. Proteins were extracted from raw meat on the basis of their solubility in water or a 0.5 M NaCl solution, since the saline soluble myofibrillar proteins myosin and actin have been reported to potentially contain the meat factor (Mulvihill and others 1998). It was not possible to cook the meat prior to the extraction procedure as this would render much of the protein insoluble and prevent the desired fractionation. Seth and others (1999) reported no difference in iron solubility in digests of heated and unheated meat fractions.

Portions of ca. 150 g of raw meat (fresh beef, chicken, lamb, and pork or frozen cod fillets) from a local supermarket were trimmed of visible fat, minced in a food blender, and 100 g of this meat mince were transferred to precooled beakers. After homogenisation in 500 ml cold deionised water, each suspension was stirred for 2 h at 4°C. The resulting slurries were transferred to precooled centrifugation tubes and centrifuged at 5000 × g for 20 min at 4°C (Sorvall RC5B, Digitana AG, Horgen, Switzerland). The supernatants (termed water-soluble extracts), which contained mainly the sarcoplasmic proteins (for example myoglobin, glycolytic enzymes) were decanted and stored at -25°C until use.

The pellets were rehomogenized in 500 ml cold saline buffer (0.5 M NaCl, 0.01 M Na₄P₂O₇) and extracted and centrifuged again as described above. The supernatants (termed salt-soluble extracts), which contained mainly the myofibrillar proteins, myosin and actin, were decanted and stored at -25°C until use. The pellets – containing approximately 40% of the total meat protein – were discarded. This fraction would mainly contain insoluble proteins like titin and other components of the connective tissue. Protein content of water-soluble and 0.5 M NaCl extracts was determined by automated Kjeldahl analysis (Büchi AG, Flawil, Switzerland) and calculated as N x 6.25.

Commercially available sodium caseinate (Emmi Schweiz AG, Luzern, Switzerland) and egg albumin (Monark Egg Corp, Kansas City, Missouri, USA) were evaluated as examples of non-meat animal proteins.

Simulated cooking of sarcoplasmic and myofibrillar protein extracts

Aliquots of the protein extract solutions containing 2 g protein were weighed into 500 ml round-bottomed flasks and evaporated under vacuum at 37°C (Rotavapor RE
111, Büchi B-461, Vacuum control PVK 600, Büchi)) to reduce the volume to below 70 ml. The resulting suspensions were transferred to tared 250 ml beakers and made to 95 g with water. On the next day, the samples were boiled for 2 min in a microwave (Micromat Combi, AEG, Hamburg, Germany) with repeated stirring. Extensive coagulation and browning were judged indicative of protein denaturation. Iron content in a sub-sample of the uncooked preparations was determined by graphite-tube atomic absorption spectroscopy (GT-AAS, SpectrAA-400, Varian Technotron Pty Limited, Mulgrave, Victoria, Australia) after microwave mineralization (MLS-ETHOS with easyWAVE software version 3.5.4.0, Egrolyt Laborgeräte, Oberwil, Switzerland) in a mixture of conc. HNO₃/30% H₂O₂. Protein suspensions were sealed with Parafilm (Pechiney Plastic Packaging, VWR International, Dietikon, Switzerland) and stored overnight at 4°C.

**Enzymic digestion of sarcoplasmic and myofibrillar protein extracts**

The pH of the protein suspensions was first adjusted to ~2.5 with 6 M HCl and the iron content brought to 2 mg by adding a ferrous sulfate solution (FeSO₄·7 H₂O, Fluka; 1 mg/ml Fe²⁺ in 0.1 M HCl). The pH was then lowered to 2.0 with 6 M HCl and the final weight made to 100 g with 0.01 M HCl.

For the enzymic digestion, the method described by Miller and others (1981) was used with slight modifications. Both pepsin/pancreatin digests and pepsin-only digests were prepared. The pepsin digests were prepared by adding 5 ml of 0.1 M NaHCO₃ instead of pancreatin/bile mixture before the second incubation period. This was done for all proteins (except pork and lamb) to assess whether LMW iron-binding peptides are already set free upon pepsin digestion. At the end of the enzymic digestion, the samples were placed on ice until centrifugation. Aliquots (~40 g) of protein digest solutions were centrifuged (Centrikon H-401, Kontron Hermle, Gosheim, Germany) for 30 min at 8000 × g and 4°C and the supernatants filtered through filter paper circles (No. 589², Ø 55 mm, Schleicher&Schuell, Dassel, Germany). The filtered supernatants were stored overnight at 4°C when analyzed the following day or deep-frozen (-25°C) for later analysis.

A series of steps were then taken so as to characterize the iron-binding peptides in the digests. These steps are shown schematically in Fig. 2. After centrifugation, the supernatants from both the pepsin and pepsin/pancreatin digests (containing the soluble iron and peptides) were separated by ultrafiltration into those passing a
10 kDa filter and those passing both a 10 kDa and a 5 kDa filter. At different stages of the separation procedure analyses were made of iron, N, and amino acids so as to determine partitioning of these nutrients. Insoluble Fe/N was calculated as the difference between total digest Fe/N and supernatant Fe/N after centrifugation and filtration. Soluble Fe/N > 10 kDa was calculated as the difference between supernatant Fe/N and 10 kDa ultrafiltrate Fe/N. In addition, the 5 kDa ultrafiltrates from the pepsin/pancreatin digests were further separated by gel filtration with an added $^{59}$Fe radioiron tag. Selected digest and gel filtration peaks were subjected to amino acid (AA) analysis and selected gel filtration peaks were analyzed by LC-MS.

**Iron and nitrogen partitioning during centrifugation and ultrafiltration of enzyme digests**

Ultrafiltration was used to obtain information on the solubility and molecular size of the iron and peptides after digestion. Five milliliters of the enzyme digest filtrates were transferred to concentrator tubes of 5 kDa and 10 kDa molecular-weight cut-offs (Vivaspin 6, Sartorius AG, Göttingen, Germany). Ultrafiltration was performed at 1750 × g and 4°C (Heraeus Omnifuge 2.0, Kendro Laboratory Products AG, Zurich, Switzerland) until at least 4 ml ultrafiltrate were obtained (approx. 2.5 h). A control digest containing only iron and enzymes and a digest in which meat protein was replaced by wheat starch (2 g) were run to see how iron partitioning is influenced by the presence of the different protein sources and whether a starch source would also yield soluble low-molecular-weight iron. The nitrogen and iron content of uncentrifuged digests and ultrafiltrates were determined by an automated Kjeldahl method and GT-AAS, respectively. Iron and protein (N x 6.25) contents of the unfiltered digests were set at 100% and the residual iron and protein in the filtrates and ultrafiltrates calculated as a percentage of their respective unfiltered digests.

**Radioiron partitioning during gel filtration of 5 kDa ultrafiltrates of pepsin/pancreatin digests**

Gel filtration was used to further separate the iron-binding peptides identified during ultrafiltration by their molecular weight. In this experiment, $^{59}$Fe was first added to the 5 kDa ultrafiltrates of the pepsin/pancreatin digests and then gel filtration was performed to separate the peptides and the iron simultaneously. $^{59}$Iron was obtained from Amersham Biosciences (Buckinghamshire, UK) as 3.7 MBq $^{59}$FeCl$_3$ in 0.5 M HCl (IFS-1) with a specific activity of 239 MBq/ml. Stock activity was diluted with
0.1 M HCl to obtain an activity of 37 kBq in 10-30 µl of $^{59}$Fe solution throughout the experiments.

Gel filtration experiments were performed on a HiPrep™ Sephacryl™ S-100HR 26/60 column (Amersham Biosciences AG, Dübendorf, Switzerland) using 0.2 M sodium acetate buffer at pH 6.0 as the mobile phase. The molecular weight separation range of this column is 1-100 kDa. All buffers were filtered through 0.45 µm nylon filter membranes (filter-Ø 47 mm, Alltech Associates Inc., Socochim SA, Lausanne, Switzerland) and degassed by sonication (Bandelin Sonorex Super RK 510, Faust Laborbedarf AG, Schaffhausen, Switzerland) for 5 min.

Protein digest ultrafiltrates with a molecular weight below 5 kDa were filtered through disposable syringe filter holders with a pore size of 0.45 µm (Chromafil A-45/25, Macherey-Nagel, Düren, Germany). One milliliter of this solution was pipetted into a disposable microcentrifuge tube (Eppendorf, Hamburg, Germany) together with 800 µl 0.2 M acetate buffer, pH 6. After addition of 37 kBq $^{59}$Fe and 10 µl 1 M non-radiolabelled FeCl$_3$ in 0.1 M HCl the final volume was made to 2 ml with 0.1 M HCl (molar ratio of Fe:Peptide of ~1.33:1, based on an average molecular weight of the peptides of 2 kDa). This mixture was left to stand overnight at room temperature to allow for isotopic exchange between radioactive and native iron.

On the next morning, the solution was centrifuged at 14000 rpm for 15 s (Centrifuge 5415C, Eppendorf), the supernatant filtered through a disposable syringe filter (Macherey-Nagel) of pore size 0.45 µm, and 1 ml of this filtrate applied to the gel filtration column by manual injection (Once Syringe LUER U-100, 1 ml, Pharma-Plast A/S, Rødby, Denmark). The flow rate was set to 1.3 ml/min (IPC-08 V1.32 peristaltic pump, Ismatec Labortechnik-Analytik, Glattbrugg-Zurich, Switzerland) and fractions were collected at 7 min intervals for 315 min with an Ultronac 7000 fraction collector (LKB Bromma, Sweden). UV detection was performed at 280 nm (Single Path Monitor UV-1, Optical and Control Unit, Amersham Biosciences) and the signal integrated with a Shimadzu C-R1B Chromatopac integrator (Shimadzu Deutschland GmbH, Duisburg, Germany).

Aliquots (3 ml) of gel filtration fractions 21-45 were transferred to polystyrene tubes (Milian SA, Geneva, Switzerland) and subjected to $\gamma$-counting (COBRA II, Perkin Elmer Life Sciences, Zaventem, Belgium).
**Amino acid analysis**

Aliquots from uncentrifuged meat pepsin/pancreatin digests, digest supernatants, and 5 kDa ultrafiltrates containing approximately 1 mg protein were weighed into hydrolysis tubes, made to 1 ml with water, and 100 mg dithiothreitol (DTT) added as a reducing agent to allow for total cyst(e)ine determination. The solutions were diluted 1:1 with fuming HCl, 37%, and hydrolysed at 110°C for 24 h under argon atmosphere. Some peptide fractions from the gel filtration were prepared and hydrolyzed in the same way but without DTT. This was because cysteine contents were too low to be determined precisely and DTT may interfere with the determination of aspartic acid.

Hydrolyzed samples were evaporated to dryness (Rotavapor, Büchi) at ≤ 50°C and redissolved in 1 ml of loading buffer (0.16 M Li-acetate-buffer, pH 2.20; No. 5.403.047, Laborservice Onken GmbH, Gründau-Breitenborn, Germany). After filtration of the diluted samples through a 0.45 µm PVDF membrane, 50 µl of the solution were injected into an automated Biochrom30 Analyser (Laborservice Onken). Separation was achieved by ion exchange chromatography (Lithium High Resolution Column, 4.0x125mm, No. 5.503.611, Laborservice Onken) using a 5 buffer system. Following separation, amino acids were derivatized with ninhydrin and detected at 570 nm and 440 nm. Quantification was based on external standards (Cat-No. 20088ZZ, Pierce, Perbio Science Switzerland SA, Lausanne, Switzerland) consisting of a mixture of 5.0 nmol of each amino acid in 50 µl loading buffer. Results were compared to those of the respective uncentrifuged meat digests. Tryptophan was not determined.

**Immobilized-metal affinity chromatography (IMAC)**

IMAC can be used to selectively separate metal-binding peptides from a crude peptide mixture. Here, IMAC was performed according to the method of Watts and others (1994) with slight modifications. Briefly, a 5 ml HiTrap™ Chelating Column (Cat-No. 17-0409-01, Amersham) was loaded with Fe^{3+} by injecting 20 ml of a 30 mM ferric chloride solution in 0.1 M HCl into a 20 ml loop followed by 10 column volumes of 0.1 M acetic acid, pH 3 (washing buffer). Excess iron was eluted with 5 column volumes of 0.1% ammonium acetate, pH 8, containing 50 mM Na_{2}HPO_{4} (elution buffer). Meat peptide ultrafiltrate aliquots containing 15 mg protein were made to 1 ml with washing buffer, injected into the loop and loaded onto the column with washing
buffer at a flow rate of 2 ml/min. After the UV signal (254 nm) had returned to baseline for 5 min, 10 ml of elution buffer were injected and the eluted peptide fraction collected for LC-MS analysis. The column was regenerated for the next separation by injecting another 10 ml of elution buffer followed by 20 ml of washing buffer.

Identification of iron-binding peptides by liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry was used to identify the molecular structures of the iron-binding meat peptides. Gel filtration samples for LC-MS analysis were prepared on a Superdex™ Peptide 10/30 column (Amersham Biosciences) as in the gel filtration studies, in 50 mM ammonium acetate buffer at a flow rate of 0.75 ml/min. Iron-binding peptides were largely confined to the first major peptide peak.

Eluates from IMAC and gel filtration enriched in iron-binding peptides were diluted in 0.1% formic acid/2% acetonitrile and 25-50 µl injected first onto a trap column (0.3x5 mm, PepMap C18, Dionex) followed by separation on an analytical column (0.1x100 mm, Magic C18, Spectronex) installed directly in the nanospray interface.

The HPLC system, consisting of a Rheos 2000 pump with CPS-module (Flux Instruments, Basel, Switzerland) and a PAL HTC autosampler (CTC Analytics, Zwingen, Switzerland) was coupled to a LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, USA) equipped with a NanoESI source (ThermoFinnigan). Peptides were eluted with a linear gradient of 5-50% B in 30 min with solvent A consisting of 0.1% (v/v) formic acid/2% acetonitrile and solvent B consisting of 0.1% formic acid/80% acetonitrile. Full scan MS and MS/MS data acquisition and analysis was performed with Xcalibur software V1.3 (ThermoFinnigan), including Bioworks V3.0 software package for SEQUEST™ database searches using a SwissProt database (www.expasy.org/sprot, October 2004) subset of sequences from beef (bos), pork (sus), chicken (gallus), sheep (ovis) and cod (gadus).

Statistics

Statistical analyses were performed using Microsoft® Excel 2002 SP3 and the SPSS software package (SPSS 13.0 for Windows, SPSS Inc.). Ultrafiltration results were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test. Due to variance
inhomogeneity, the significance level was set at \( P < 0.01 \). Differences between pepsin and pepsin/pancreatin treatment were assessed using Student’s \( t \)-test for paired samples and the significance level set at \( P < 0.05 \).

**Results and Discussion**

*Nitrogen partitioning in enzyme digests after centrifugation and ultrafiltration*

Figures 3 and 4 show the solubility and molecular weight distribution of Kjeldahl nitrogen from pepsin and pepsin/pancreatin digests of myofibrillar and sarcoplasmic meat protein extracts, respectively. The solubility and molecular weight distribution of casein and egg albumin digests are included in Fig. 4 for comparison. Enzyme nitrogen largely (≥ 80%) passed through the 10 kDa membrane in the digests containing no added protein or wheat starch instead of protein (data not shown). In almost all cases, peptides < 10 kDa also passed through the 5 kDa membrane. However, to allow for full comparability, only the results from 10 kDa ultrafiltrates are presented.

**Myofibrillar proteins (Fig. 3).** The major salt-soluble (myofibrillar) proteins are myosin (50%) and actin (20%) (Pearson and Young 1989). Myofibrillar protein extracts of all meat sources digested by PP displayed a similar nitrogen distribution pattern and soluble nitrogen < 10 kDa constituted the largest fraction, varying between 66-79%. Pork PP digests contained significantly more LMW nitrogen (79%) than beef (66%, \( P = 0.006 \)), but not compared to chicken, cod or lamb. In contrast, of the PP digests beef was highest in insoluble nitrogen (23%), differing significantly from cod (9%, \( P = 0.005 \)), but not from chicken (17%), lamb (18%) or pork (15%). Soluble nitrogen > 10 kDa was significantly higher (\( P = 0.009 \)) in the PP digests of cod (19%) compared to pork (6%), but there was no significant difference with beef (11%), chicken (16%), or lamb (13%).

The digests of the myofibrillar proteins made with pepsin alone also produced a majority of LMW peptides < 10 kDa (55-65%) although the levels were slightly lower than the 66-79% produced by PP digestion. The P digest of myofibrillar cod protein, however, was the only P digest to contain a significantly lower amount of LMW soluble N (55%) than its PP digest (72%, \( P < 0.01 \)). Similarly, there were no significant differences in the levels of peptides > 10 kDa in the P and PP digests of beef, chicken, cod, lamb and pork, although there was
slightly more insoluble nitrogen \( (P < 0.05) \) in the P digest of myofibrillar beef and cod proteins as compared to the respective PP digests.

**Sarcoplasmic proteins (Fig. 4).** The PP digests of the sarcoplasmic protein extracts, as with the myofibrillar protein extracts, also contained predominantly LMW nitrogen \(< 10\, \text{kDa}\) which ranged between 67-89% of total nitrogen. Significant differences \( (P < 0.001) \) were observed between chicken (67%) and beef (89%), lamb (88%) and pork (87%). Insoluble nitrogen was very low (2-6%) in all five meat sources. Pepsin/pancreatin digests of chicken sarcoplasmic protein extract contained the highest amount of soluble nitrogen \( > 10\, \text{kDa}\), 26%, being significantly \( (P \leq 0.001) \) higher than for beef (9%), lamb (6%) and pork (8%), but not cod (13%, \( P = 0.016 \)). As compared to the PP digest of the myofibrillar extracts, the PP digests of the sarcoplasmic extracts contained equal or higher levels of LMW nitrogen reaching statistical significance \( (P < 0.001) \) in beef (89% vs 66%) and lamb (88% vs 69%). Insoluble nitrogen was significantly lower \( (P \leq 0.006) \) in PP digests of sarcoplasmic proteins than from myofibrillar proteins; beef (2% vs 23%), lamb (6% vs 18%) and pork (5% vs 15%). No significant differences in soluble nitrogen \( > 10\, \text{kDa}\) were observed between the PP digests of the different extracts.

The P digests of the sarcoplasmic protein extracts contained similar amounts of LMW peptides \( < 10\, \text{kDa}\) (76-88%) as the PP digests (67-89%), with also only small differences in the amounts of soluble N \( > 10\, \text{kDa}\) and insoluble N.

**Casein and egg albumin (Fig. 4).** LMW nitrogen \( < 10\, \text{kDa}\) was also relatively high in the PP digests of casein (73%) and egg albumin (60%). The level in the egg albumin digest, however, was significantly lower than in the PP digests of sarcoplasmic protein extracts of beef, lamb, pork and cod and the myofibrillar extract from pork \( (P < 0.01) \). The level in the casein digest was similarly significantly lower than in the PP digests of the sarcoplasmic proteins from beef, lamb, and pork \( (P < 0.005) \).

Looking at insoluble nitrogen, beef and lamb myofibrillar PP digests had higher contents than casein PP digests \( (P \leq 0.005) \), whereas only the beef myofibrillar PP digest was significantly different from egg albumin \( (P = 0.01) \). In relation to the fraction of soluble nitrogen \( > 10\, \text{kDa}\), the PP digest of egg albumin contained more of this fraction (34%) than did the PP digests of all other proteins \( (P < 0.004) \) except casein and the sarcoplasmic proteins from chicken. The soluble nitrogen \( > 10\, \text{kDa}\) in the PP digest of casein (22%) was higher than in the PP digests of the sarcoplasmic proteins of lamb and pork and the myofibrillar proteins of pork \( (P < 0.008) \).
The P digests of casein and egg albumin differed from those of the meat protein extracts in that they contained much less nitrogen < 10 kDa. The P digest of casein contained 44% LMW nitrogen and egg albumin 40%. This compares to a range of 55-65% in the P digests of myofibrillar proteins and 76-84% in the P digests of sarcoplasmic proteins, all being significantly different from casein and egg albumin (\(P \leq 0.01\)). It is tempting to speculate that meat proteins are digested to a larger extent in the stomach than other proteins and yield LMW iron-binding peptides which protect the food iron from interaction with absorption inhibitors. At the higher pH of the duodenum, where iron absorption capacity is highest (Wheby 1970), the iron may be released from its peptide complexes and subsequently taken up by the mucosa. A similar mechanism has been described for EDTA, which keeps iron complexed under acidic conditions in the stomach but releases it at the higher pH of the duodenum (Lynch and others 1993). Beef, chicken and cod all contained large amounts of LMW iron-binding peptides after pepsin digestion. Pork and lamb were not digested by pepsin alone but could be expected to behave in a similar way.

Iron partitioning in enzyme digests after centrifugation and ultrafiltration

**Myofibrillar proteins (Fig. 5).** The enzyme digests of salt-soluble extracts of proteins from beef, chicken, cod, lamb, and pork displayed similar iron partitioning. Ultrafiltrable iron (< 10 kDa) was > 80% (84-94%) in all PP and P digests, whereas soluble iron > 10 kDa ranged between 0-18%. There were no differences in the percentage of the added iron present in the LMW < 10 kDa fraction of the P or the PP digests of beef or cod. The amount of iron in the < 10 kDa fraction of the P digest of chicken was slightly lower (\(P < 0.05\)) than in the PP digest. The highest fraction of insoluble iron was found in the pepsin (19%) and pepsin/pancreatin (16%) digests of beef, differing significantly (\(P \leq 0.001\)) from the pepsin/pancreatin digests of chicken (0%), cod (3%) and pork (0%), but not from lamb (7%, \(P = 0.092\)). This observation parallels the higher amounts of insoluble nitrogen in the beef digests (Fig. 3).

**Sarcoplasmic proteins (Fig. 6).** In contrast to the myofibrillar proteins, sarcoplasmic meat proteins as well as casein and egg albumin did not show considerable amounts of soluble iron in the LMW fraction (< 10 kDa), even though it represented ca. 80% of the total nitrogen (Fig. 4). The same was true for “protein-free” digests containing only iron and enzymes or wheat starch instead of protein (data not shown).
Enzymic digests of the sarcoplasmic proteins of chicken, cod, lamb, and pork contained from 2-20% soluble LMW (< 10 kDa) iron. Beef was the exception with 40% after pepsin and 58% after pepsin/pancreatin digestion. Pepsin/pancreatin digests of sarcoplasmic proteins from all meat sources thus contained between 2-58% 10 kDa ultrafiltrable iron, which was significantly lower than in their corresponding myofibrillar proteins (P < 0.001). With respect to the insoluble iron in the digests of sarcoplasmic proteins of cod and chicken, it was considerably higher than in the corresponding digests of myofibrillar proteins. Pepsin/pancreatin digests of sarcoplasmic chicken proteins contained 85% insoluble iron, compared to zero in the corresponding myofibrillar protein digest (P < 0.001). For cod, the respective values were 93% and 3% (P < 0.001).

Finally, soluble iron > 10 kDa was very high in pepsin/pancreatin digests of sarcoplasmic proteins from lamb (73%) and pork (90%), intermediate in beef (35%) and low in chicken (10%) and cod (5%). These values were significantly different (P < 0.001) from the corresponding values in the pepsin/pancreatin digests of the myofibrillar proteins from beef, lamb, and pork.

**Casein and egg albumin (Fig. 6).** The enzymic digests of casein and egg albumin contained a maximum of 10% total iron in the LMW (< 10 kDa) fraction. In contrast to the P digests of the myofibrillar proteins, P digests of casein and egg albumin contained < 5% soluble iron in the LMW fraction. Most of the iron in the casein digests was in the soluble > 10 kDa fraction and most of the iron in the egg albumin digests was insoluble. As casein is inhibitory in terms of iron bioavailability (Hurrell and others 1989), it might be speculated that the solubilized iron is too strongly bound to be taken up by the mucosal cells or too large to reach them. The latter aspect is supported by data from Hurrell and others (1989) who demonstrated proportionately higher iron absorption from casein with increasing degree of hydrolysis. Generally, these results hint at the fact that iron solubility per se does not reliably predict iron bioavailability, at least when assessing the effect of dietary proteins (Miller and Berner 1989). The high level of insoluble (73%) and high-molecular-weight soluble (24%) iron in egg albumin digests is also in line with a relatively low iron dialyzability *in vitro* (Hurrell and others 1988, 1989) and absorption *in vivo* (Cook and Monsen 1976; Hurrell and others 1988, 1989) from egg albumin-rich meals.
Previous studies on the separation of peptides and iron in meat protein digests by ultrafiltration and gel filtration have shown some inconsistencies likely due to the different separation methods used and the form and time of the iron addition. There are likewise similarities and differences between the present study and the previously reported studies. As in the present study, Slatkavitz and Clydesdale (1988) showed that iron solubility in enzymic digests was significantly affected by the acid-insoluble fraction of chicken muscle but not the water-soluble or acid-soluble extracts. The acid-insoluble fraction would be expected to contain the myofibrillar proteins but also other insoluble proteins. Also in agreement with our findings, they reported that peptides < 10 kDa solubilized significantly more iron than peptides > 10 kDa and concluded that the influence of chicken muscle digests on iron solubility was related to the production of digestion intermediates which act as ligands in the formation of soluble complexes. The molecular weight range of the soluble iron complexes was subsequently reported to be 2500-6200 Da (Politz and Clydesdale 1988), however the maximum amount of iron solubilized after PP digestion was 15% compared to 5% after P digestion.

A major difference with the present study, which found 90% and more soluble iron in the < 10 kDa fraction of the PP digests of myofibrillar proteins from all meats, was that iron was added prior to digestion as ferric chloride and not ferrous sulfate. Ferric chloride is poorly soluble above pH 3 (Forth and Rummel 1973) and would thus require binding or reduction to the ferrous form to be maintained in solution. Ferrous sulfate, though prone to oxidation at near neutral pH, is much more soluble and could have remained so by binding to LMW peptides in the digest without the need for prior reduction.

Mahoney and coworkers (Seth and others 1999; Seth and Mahoney 2000b; Seth and Mahoney 2000a; Vattem and others 2001; Diaz and others 2002) investigated the iron binding properties of mainly myofibrillar chicken muscle peptides in a series of studies. The proteins were not soluble in 0.15 M NaCl. They reported that > 90% of the peptides were < 10 kDa and 45% were < 3.5 kDa (Seth and Mahoney 2000a) however, unlike in our studies, most of the iron was bound to the small quantity of soluble peptides > 10 kDa with only ca. 5% in the LMW < 10 kDa fraction. However, although they reported a 20-fold increase in soluble iron after PP digestion of myofibrillar chicken muscle protein compared to little or no increase in iron solubility on digestion of casein and egg albumin, the amount of soluble iron in the casein and
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ey egg albumin digests was 14-17 times higher than in the digests of the myofibrillar protein. The differences again could be explained by the addition of ferric chloride and not ferrous sulfate prior to enzyme digestion. It is difficult to predict in what form food iron is present in the gastrointestinal tract and whether ferrous or ferric iron would be more representative. The valency of iron will depend on the reducing ligands such as ascorbic acid in the meal and our results would certainly be representative of fortification iron added as ferrous sulfate. Seth and others (1999), although they added iron in the ferric state, found the majority of soluble iron complexed with the peptides of the myofibrillar protein in the ferrous form and they concluded that muscle protein digests solubilize iron by reduction and chelation of large > 10 kDa peptides. As the authors themselves point out however, the bioavailability of such iron is not clear. Terato and others (1973) have shown that high molecular weight chelates (> 10 kDa) could not be absorbed by the rat intestine and their size would slow diffusion through the intestinal mucus to the brush border membrane and the sites of iron uptake (Miller and Berner 1989). The possibility that these large peptides act as transporters of iron however cannot be ruled out.

It has been repeatedly demonstrated that dietary iron absorption by humans from low bioavailability meals is enhanced in the presence of meat (Cook and Monsen 1976; Hazell and others 1978; Kane and Miller 1984; Baech and others 2003a; Baech and others 2003b). The proposed mechanism is the production of LMW peptides during gastrointestinal protein breakdown which reduce and/or solubilize iron thereby rendering it more bioavailable (Taylor and others 1986; Swain and others 2002; Baech and others 2003a; Baech and others 2003b). The myofibrillar proteins of animal muscle tissue have been suggested to be responsible for the observed effect, because they are high in cysteine, which, in turn, is thought to mediate the reduction and binding via its sulphydryl group (Taylor and others 1986; Mulvihill and others 1998; Mulvihill and Morrissey 1998; Mulvihill and Morrissey 1998). Histidine-rich LMW peptides from beef have been demonstrated to enhance iron uptake in Caco-2 cells (Swain and others 2002).

The present findings on ultrafiltration of enzymic digests further substantiate this general hypothesis and extend it to include myofibrillar proteins from beef, pork, lamb, and cod as well as chicken. We have shown specifically that the LMW < 10 kDa peptides of myofibrillar proteins are the main iron-solubilizing peptides. Unlike with casein, egg albumin, and most sarcoplasmic proteins from meat, these
LMW soluble peptides from myofibrillar proteins were formed on pepsin digestion. We propose the extensive pepsin digestion of myofibrillar meat proteins is the “meat factor” and is responsible for solubilizing iron and enhancing iron absorption in humans. The iron-binding peptides formed in the stomach would bind iron before the entry to the duodenum and protect iron from reaction with hydroxide or inhibitory ligands such as phytate or polyphenols.

**Iron partitioning during gel filtration of enzyme digests**

Gel filtration at pH 6 of radioactively tagged 5 kDa ultrafiltrates of myofibrillar meat proteins was performed to identify and separate highly iron-binding fractions. Excess iron was added to the ultrafiltrates to find out at what time unbound iron might elute from the column. Furthermore, it was a means to visualize potential iron solubilization by the different peptides. Casein, egg albumin, and the water-soluble sarcoplasmic proteins from all meats did not show any soluble iron-binding peptides (data not shown), because extensive protein precipitation occurred on addition of excess iron. After centrifugation, radioactivity was almost entirely (> 99%) confined to the pellet in these samples. The results reported relate only to the myofibrillar peptide ultrafiltrates prepared by either P or PP digestion. Although some precipitation was also observed in these samples, much peptide-bound iron remained soluble. No soluble radioactivity was recovered from a buffer blank as the $^{59}$Fe precipitated immediately upon loading onto the column.

Table 1 shows the total recovery of added $^{59}$Fe and the amount in the first peak only of the gel filtrations performed with 5 kDa ultrafiltrates of P and PP digests of myofibrillar meat protein extracts. Selected chromatograms are shown in Fig. 7. Data are presented as percentage radioiron recovery per 10 mg protein. Radioactive iron always eluted in greater quantities at the time the first peptide peak eluted, and 50-75% of the recovered radioactivity was associated with this peptide fraction in all meat samples except lamb. When the first peptide peak was small, as with cod and lamb, a concomitant decrease in $^{59}$Fe recovery was observed.

Pepsin digests of beef and cod exhibited considerable iron-binding properties (43% and 30%, respectively of the total iron was recovered), whereas under the conditions of the gel filtration only 1% of the iron added to the chicken digests was recovered. However, chicken peptides displayed the same iron-binding capacity (26%) as beef peptides (25%) upon pepsin/pancreatin digestion. On the other hand, cod lost two
thirds of its iron-binding capacity, 10% compared to 30%, when subjected to the pepsin plus pancreatin digestion. Lamb and pork were not digested by pepsin alone. The recovery of radioactivity in the pepsin/pancreatin pork myofibrillar protein digest was 32% and similar to the corresponding ultrafiltrated beef protein digest.

The gel filtration chromatograms from the 5 kDa ultrafiltrates of the P and PP digests of beef and cod are shown in Fig. 7. The grey line indicates the peptide elution profile (AU$_{280nm}$), whereas the black line represents the iron distribution given as background-corrected counts per minute (ccpm). In all meat extracts, usually, five peptide peaks were observed: a leading peak of varying size, followed by a group of three smaller peaks, and a small solitary peak at the end of the chromatogram. The size of the peptides in the first peak was estimated to be ~2 kDa, based on a standard of insulin A-chain which has a molecular weight of 2.5 kDa and which showed a retention time of 160 minutes. This is in line with findings by Swain and others (2002) who report on LMW iron-binding peptides from beef in the range of 1-7 kDa. The solitary fifth peak probably represents free amino acids and small peptides interacting with the column.

The gel filtration results with added ferrous iron at pH 6 further confirm the strong iron-binding properties of the LMW pepsin digests of beef and cod, which bound more iron after P than after PP digestion. It is probable that the further digestion decreased the iron-binding capacity of the peptides as has been observed previously (Slatkavitz and Clydesdale 1988). Rather surprisingly, the P digests of the chicken myofibrillar proteins precipitated on addition of excess iron at pH 6 in the same way as the digests of casein, egg albumin, and the sarcoplasmic proteins. This does not of course preclude that LMW chicken peptides bind iron under physiological conditions, but indicates that peptides of chicken myofibrillar proteins are not the same as those from beef and cod. We did not make P digests of pork and lamb. Gel filtration of the ultrafiltrates of myofibrillar proteins of the different meats gave somewhat different results, however the basic gel filtration pattern was similar with the major iron-binding peak for each muscle tissue being consistent with peptides in the 2 kDa molecular weight range. With beef and cod, but not chicken, these peptides were higher after P digestion than after PP digestion. The maximum amount of iron recovery on gel filtration was only 43%. Most of the iron therefore precipitated with other LMW peptides in all the protein fractions. It is probable that at lower iron...
concentrations some of these peptides will remain soluble and will also play a role in enhancing iron absorption in vivo.

**Amino acid (AA) composition of enzyme digest fractions**

AA analyses of the unfiltered PP digests of the myofibrillar proteins, the supernatants obtained after centrifugation and filtration, and the 5 kDa ultrafiltrates revealed that only small compositional changes occurred during the separation process (data not shown). Thus, although the peptides in the 5 kDa ultrafiltrate bound ca. 90% of the iron, and represented some 60% of the total N, they were similar in amino acid composition to the original myofibrillar protein digest. There was no concentration in cysteine (Vattem and others 2001) or histidine residues (Seth and Mahoney 2000b) as has been previously reported for iron-binding peptides. This is presumably because the 5 kDa ultrafiltrates contain many peptides which do not bind iron as well as those peptides which do bind iron.

The AA composition of the 2 kDa mixture of peptides which bound iron during gel filtration is shown in Table 2. Compared to the total PP digests, these peptides with an average of about 18 amino acids were enriched in proline (45-127%), threonine (17-50%), glycine (31-67%), aspartic acid (29-51%), and glutamic acid (28-77%), although there were major differences in the peptide fractions from the different meats. The enrichment of iron in this fraction is most likely due to iron-binding to the free γ- and δ-carboxyl groups of aspartic and glutamic acid residues, respectively, although peptides and proteins can also interact with iron via the peptide bond (Chaud and others 2002). Both aspartic and glutamic acid are known to form very stable iron chelates with a putative tridentate structure (Perrin 1958). For the free amino acids, stable iron-glycine and iron-proline complexes have been described as well (Perrin 1958; Hertrampf and Olivares 2004). Proline induces structural bends in proteins (Yang and others 1998; Nelson and Cox 2004) and thus might aid peptides in assuming a conformation that favors iron-binding. In addition, proline-rich peptides preferentially resist digestion due to steric hindrance, which might further enhance complex stability. Only at the brush border are proline bonds cleaved by dipeptidyl peptidase IV and related enzymes (Yaron and Naider 1993).

Rather surprisingly, cysteine, the amino acid most often associated with the meat factor (Martinez-Torres and Layrisse 1970; Martinez-Torres and others 1981; Layrisse and others 1984; Taylor and others 1986; Mulvihill and others 1998;
Mulvihill and Morissey 1998; Mulvihill and Morissey 1998; Vattem and others 2001), was too low to be detected reliably in the selected gel filtration samples (GF peak I). In addition, the level of histidine was lower in the 2 kDa peptide fraction than in the total digest not supporting the *in vitro* data that point to histidine as part of the meat factor (Seth and Mahoney 2000b; Swain and others 2002). It is probable therefore that the 2 kDa iron-binding peptides which we have identified are only some of many low-molecular-weight peptides from muscle tissue which bind iron under physiological conditions. In our methodology, the addition of excess iron prior to gel filtration precipitated a portion of the peptides which unfortunately was not possible to quantify. It is likely therefore that these peptides could also bind iron in the gastrointestinal tract and possible that these are the peptides previously reported to be enriched in cysteine and histidine.

**Sequence analysis by LC-MS of iron-binding peptides**

LC-MS analysis showed the 2kDa fraction from gel filtration to be a complex mixture of peptides. In total, over 1000 peptide sequence matches were detected, of which about 80 showed a potential connection with muscle tissue and could be matched to the five animal species tested. The peptide sequences listed in column 1 of Table 3 matched with peptide masses detected by LC-MS analysis and represent fragments of the proteins that are likely to be found in myofibrillar meat protein extracts (column 2, Table 3). SwissProt codes (column 3) are given for follow-up on protein characteristics. Overall, surprisingly few peptide masses matched with fragments of myosin and none with actin. One or more of the listed fragments were identified in the 2 kDa fraction of each myofibrillar protein extract analyzed. It is possible that sufficient quantities of these peptides are present to bind most of the iron in the digests. In agreement with the AA analysis, most sequences are enriched in aspartic and/or glutamic acid. Note that the AA analysis did not distinguish between the carboxyl and amide forms of aspartic and glutamic acid. Although iron passed the gel filtration with the 2 kDa peptide fraction, it is likely that some but not all of the peptides in that fraction bind iron. Four of the peptide sequences detected are from myosin, and are thus potentially more important because this is the major myofibrillar protein. The other sequences were mainly from enzymes and proteoglycans. On the other hand, no histidine or cysteine appeared in the peptide sequences. Proline was present in some of the sequences and might
have a role in peptide stability and conformation. Another important structural feature revealed by LC-MS analysis was the phosphorylation of serine and tyrosine residues. Introducing negatively charged phosphate groups creates suitable binding sites for positively charged metals like iron, calcium, or zinc (Miquel and others 2005). Of note, some LC-MS peptide sequences matched with proteins of the proteoglycan type. Proteoglycan carbohydrate structures, the so-called glycosaminoglycans (GAGs), were recently demonstrated to enhance iron uptake in Caco-2 cells (Huh and others 2004). One could thus speculate that the identified peptides could be an indirect indication of iron-binding GAGs or that peptides and carbohydrates together increase iron bioavailability.

These data still cannot tell us if the “meat factor” is a single peptide or a well defined group of peptides contained in a mixture of iron-neutral peptides, or if it is due to a multitude of iron-binding peptides generated on pepsin digestion and which mostly retain their iron-binding capacity after pancreatin digestion. The low-molecular-weight iron-binding peptides, which we isolated by gel filtration were enriched in aspartic and glutamic acids. Interestingly, the peptides fragments identified as coming from myosin were high in one or both of these amino acids and could be among the iron-binding peptides in this fraction.

Conclusions
At the start of these studies we hypothesized that the “meat factor” was a single peptide or well characterized group of peptides enriched in cysteine and histidine. We postulated that these peptides could be isolated and characterized, and perhaps used as a food additive as an alternative to ascorbic acid to enhance iron absorption from foods. Our data, taken together with the published literature, however, indicate that the “meat factor” is less well defined and is co-migrating with a multitude of peptides, making it difficult if not impossible to isolate. Based on our findings, we propose that iron-binding peptides are produced by pepsin digestion and bind iron in a soluble form in the stomach preventing interaction with absorption inhibitors such as phytic acid and polyphenols. The iron remains complexed to the peptides in the duodenum and is not precipitated at the higher pH. It is thus presented to the iron transporters on the mucosal cell surface in a soluble, absorbable form. We detected one group of iron-binding peptides with a molecular mass of about 2 kDa. These peptides are enriched in aspartic and glutamic acid and possibly originate from
myosin. Other LMW iron-binding peptides doubtlessly exist, probably enriched in cysteine and histidine, but these peptides were not isolated in this study.
References


Yang WZ, Ko TP, Corselli L, Johnson RC, Yuan HS. 1998. Conversion of a {beta}-strand to an {alpha}-helix induced by a single-site mutation observed in the crystal structure of Fis mutant Pro(26) Ala. Protein Sci 7(9): 1875-1883.

Acknowledgments

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Table 1

Recovery of $^{59}$Fe from pepsin and pepsin/pancreatin digests of 5 kDa ultrafiltrates of myofibrillar meat proteins, casein, and egg albumin after gel filtration (GF peak I) and relative contents of $^{59}$Fe and protein in peak I (n = 2)

<table>
<thead>
<tr>
<th>Samples</th>
<th>$^{59}$Fe recovery $^{\text{mean±SD}}$</th>
<th>$^{59}$Fe in GF peak I$^{\dagger}$</th>
<th>Protein in GF peak I$^{\dagger}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pepsin digests</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef SS</td>
<td>43%±7%</td>
<td>75%±2%</td>
<td>66%±3%</td>
</tr>
<tr>
<td>Cod SS</td>
<td>30%±1%</td>
<td>67%±1%</td>
<td>59%±2%</td>
</tr>
<tr>
<td>Chicken SS</td>
<td>1%±1%</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>Pepsin/Pancreatin digests</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef SS</td>
<td>25%±8%</td>
<td>58%±17%</td>
<td>35%±13%</td>
</tr>
<tr>
<td>Cod SS</td>
<td>10%±2%</td>
<td>50%±25%</td>
<td>18%±4%</td>
</tr>
<tr>
<td>Chicken SS</td>
<td>26%±14%</td>
<td>54%±16%</td>
<td>34%±13%</td>
</tr>
<tr>
<td>Pork SS</td>
<td>32%±14%</td>
<td>62%±14%</td>
<td>45%±14%</td>
</tr>
<tr>
<td>Lamb SS</td>
<td>15%±5%</td>
<td>23%±1%</td>
<td>13%±2%</td>
</tr>
<tr>
<td>Casein</td>
<td>0%</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>0%</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^{\dagger}$ % fraction of total area under the curve
n.d. = not determined
<table>
<thead>
<tr>
<th></th>
<th>Pork</th>
<th>Chicken</th>
<th>Beef</th>
<th>Lamb</th>
<th>Cod</th>
<th>Average increase/decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>9.54 (51%)</td>
<td>9.78 (38%)</td>
<td>9.68 (24%)</td>
<td>9.65 (43%)</td>
<td>11.15 (29%)</td>
<td>37%</td>
</tr>
<tr>
<td>Thr</td>
<td>4.95 (22%)</td>
<td>4.87 (31%)</td>
<td>5.12 (34%)</td>
<td>5.23 (17%)</td>
<td>3.55 (50%)</td>
<td>31%</td>
</tr>
<tr>
<td>Ser</td>
<td>4.27 (24%)</td>
<td>4.36 (23%)</td>
<td>4.25 (33%)</td>
<td>4.43 (22%)</td>
<td>4.04 (38%)</td>
<td>28%</td>
</tr>
<tr>
<td>Glu</td>
<td>15.93 (77%)</td>
<td>15.94 (48%)</td>
<td>15.93 (31%)</td>
<td>15.52 (56%)</td>
<td>16.94 (44%)</td>
<td>51%</td>
</tr>
<tr>
<td>Pro</td>
<td>2.95 (108%)</td>
<td>2.89 (88%)</td>
<td>3.06 (38%)</td>
<td>3.14 (102%)</td>
<td>2.41 (127%)</td>
<td>93%</td>
</tr>
<tr>
<td>Gly</td>
<td>3.50 (67%)</td>
<td>3.73 (53%)</td>
<td>3.39 (55%)</td>
<td>3.67 (65%)</td>
<td>3.94 (31%)</td>
<td>54%</td>
</tr>
<tr>
<td>Ala</td>
<td>5.95 (22%)</td>
<td>6.05 (19%)</td>
<td>6.01 (19%)</td>
<td>5.99 (18%)</td>
<td>7.08 (2%)</td>
<td>15%</td>
</tr>
<tr>
<td>Val</td>
<td>5.34 (-20%)</td>
<td>5.43 (-7%)</td>
<td>5.19 (3%)</td>
<td>5.26 (-2%)</td>
<td>5.27 (-3%)</td>
<td>-6%</td>
</tr>
<tr>
<td>Met</td>
<td>3.46 (-98%)</td>
<td>3.48 (-84%)</td>
<td>3.28 (-93%)</td>
<td>3.32 (-79%)</td>
<td>3.40 (-78%)</td>
<td>-86%</td>
</tr>
<tr>
<td>Ile</td>
<td>4.83 (-8%)</td>
<td>4.96 (0%)</td>
<td>4.83 (-2%)</td>
<td>4.85 (-3%)</td>
<td>3.99 (9%)</td>
<td>-1%</td>
</tr>
<tr>
<td>Leu</td>
<td>8.97 (-42%)</td>
<td>8.99 (-33%)</td>
<td>8.90 (-19%)</td>
<td>8.88 (-41%)</td>
<td>8.84 (-30%)</td>
<td>-33%</td>
</tr>
<tr>
<td>Tyr</td>
<td>5.50 (-100%)</td>
<td>4.67 (-90%)</td>
<td>5.64 (-93%)</td>
<td>5.34 (-91%)</td>
<td>4.67 (-88%)</td>
<td>-92%</td>
</tr>
<tr>
<td>Phe</td>
<td>4.18 (-89%)</td>
<td>4.16 (-82%)</td>
<td>4.22 (-73%)</td>
<td>4.21 (-83%)</td>
<td>3.75 (-76%)</td>
<td>-81%</td>
</tr>
<tr>
<td>Lys</td>
<td>10.21 (-15%)</td>
<td>10.11 (-2%)</td>
<td>10.52 (13%)</td>
<td>10.28 (-13%)</td>
<td>12.12 (-21%)</td>
<td>-8%</td>
</tr>
<tr>
<td>His</td>
<td>3.05 (-55%)</td>
<td>3.12 (-38%)</td>
<td>2.64 (-8%)</td>
<td>2.80 (-47%)</td>
<td>1.38 (-12%)</td>
<td>-32%</td>
</tr>
<tr>
<td>Arg</td>
<td>7.39 (-77%)</td>
<td>7.43 (-69%)</td>
<td>7.35 (-46%)</td>
<td>7.42 (-73%)</td>
<td>7.02 (-67%)</td>
<td>-66%</td>
</tr>
<tr>
<td>Cys</td>
<td>0.91 n.d.</td>
<td>1.05 n.d.</td>
<td>1.00 n.d.</td>
<td>1.11 n.d.</td>
<td>n.d. n.d.</td>
<td>-</td>
</tr>
</tbody>
</table>

1 AA, amino acid (three-letter code)
n.d. = not determined
Table 3

Selected peptide sequence matches identified by comparison of LC-MS molecular mass data of myofibrillar meat peptides to a Swissprot database subset of the five animal species studied. Samples were prepared by gel filtration (GF peak I) of 5 kDa ultrafiltrates from pepsin/pancreatin-digested myofibrillar meat extracts.

<table>
<thead>
<tr>
<th>Peptide sequence(s)</th>
<th>Protein match</th>
<th>SwissProt code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp-Ala-Ala-Phe-Pro-Pro-Asp-Val-Ala-Gly-Asn</td>
<td>Myosin regulatory light chain 2, skeletal muscle isoform</td>
<td>MLRS_CHICK</td>
</tr>
<tr>
<td>Ile-Thr-Asn-Asn-Pro-Tyr-Tyr-Asp-Ala-Phe</td>
<td>Myosin heavy chain</td>
<td>MYH7_PIG</td>
</tr>
<tr>
<td>Ser-Val-MetOx-Asp-Leu-Glu-Asn-Asp</td>
<td>Myosin heavy chain</td>
<td>MYSC_CHICK</td>
</tr>
<tr>
<td>Gln-TyrP-Gln-Thr-Pro-Leu-Phe-Val-Trp-SerP-Val</td>
<td>Cytochrome c oxidase 1</td>
<td>COX1_BOVIN</td>
</tr>
<tr>
<td>Asn-Met-Asp-Ile-Lys-Asn-Thr</td>
<td>Hemoglobin beta chain</td>
<td>HBB_GADMO</td>
</tr>
<tr>
<td>Leu-Phe-Asp-Lys-Pro-Val-Ser-Pro-Leu-Leu</td>
<td>Creatine kinase B chain</td>
<td>KCRB_CHICK</td>
</tr>
<tr>
<td>Thr-Leu-Asn-Glu-Leu-Gly-Ile-SerP-Glu-Glu-Leu</td>
<td>Cytochrome c oxidase</td>
<td>COXA_BOVIN</td>
</tr>
<tr>
<td>Gln-Leu-Phe-Leu-Asp-Gln-Gln-Ser</td>
<td>Keratocan</td>
<td>KERA_CHICK</td>
</tr>
<tr>
<td>Ser-Gly-Phe-Pro-Asp-Ile-Ser-Gly-Glu-Thr</td>
<td>Aggrecan core protein</td>
<td>PGCA_BOVIN</td>
</tr>
<tr>
<td>Gly-Val-Phe-Phe-Pro-Gly-Gly-Leu-Gly-Val</td>
<td>Elastin</td>
<td>ELS_BOVIN</td>
</tr>
</tbody>
</table>

1 Amino acids in three-letter code. SerP, phosphoserine; TyrP, phosphotyrosine; MetOx, oxidized methionine
Figure 1
Schematic representation of the extraction of water-soluble (sarcoplasmic) and salt-soluble (myofibrillar) proteins from raw meat homogenates. Extract aliquots containing 2 g protein were taken for *in vitro* digestion.

Figure 2
Fractionation of water-soluble (sarcoplasmic) and salt-soluble (myofibrillar) meat proteins after simulated gastrointestinal digestion. Proteins were digested with either pepsin (P) or pepsin followed by pancreatin (PP). Digests were centrifuged and the resulting supernatants subjected to ultrafiltration through 10 kDa and 5 kDa molecular weight cut-off membranes. Iron and Kjeldahl-N determinations were performed on total digests, filtered supernatants and 10 kDa and 5 kDa ultrafiltrates.

Figure 3
Nitrogen solubility and molecular weight distribution of peptides in pepsin (P) and pepsin/pancreatin (PP) digests of salt-soluble beef, chicken, cod, lamb, and pork protein extract digests (n = 2-4). Fractionation was accomplished by sequential *in vitro* digestion, centrifugation and ultrafiltration through 10 kDa molecular weight cut-off membranes. Insoluble N was calculated as the difference between total N and supernatant N. Soluble N with a molecular weight > 10 kDa was calculated as the difference between supernatant N and 10 kDa ultrafiltrate N.

Figure 4
Nitrogen solubility and molecular weight distribution of peptides in pepsin (P) and pepsin/pancreatin (PP) digests of sodium caseinate, egg albumin, and water-soluble beef, chicken, cod, lamb, and pork protein extract digests (n = 2-4). Fractionation was accomplished by sequential *in vitro* digestion, centrifugation and ultrafiltration through 10 kDa molecular weight cut-off membranes. Insoluble N was calculated as the difference between total N and supernatant N. Soluble N with a molecular weight > 10 kDa was calculated as the difference between supernatant N and 10 kDa ultrafiltrate N.
**Figure 5**
Iron solubility and molecular weight distribution in pepsin (P) and pepsin/pancreatin (PP) digests of salt-soluble beef, chicken, cod, lamb, and pork protein extract digests (n = 2-4). Fractionation was accomplished by sequential *in vitro* digestion, centrifugation and ultrafiltration through 10 kDa molecular weight cut-off membranes. Insoluble Fe was calculated as the difference between total Fe and supernatant Fe. Soluble Fe with a molecular weight > 10 kDa was calculated as the difference between supernatant Fe and 10 kDa ultrafiltrate Fe.

**Figure 6**
Iron solubility and molecular weight distribution in pepsin (P) and pepsin/pancreatin (PP) digests of sodium caseinate, egg albumin, and water-soluble beef, chicken, cod, lamb, and pork protein extract digests (n = 2-4). Fractionation was accomplished by sequential *in vitro* digestion, centrifugation and ultrafiltration through 10 kDa molecular weight cut-off membranes. Insoluble Fe was calculated as the difference between total Fe and supernatant Fe. Soluble Fe with a molecular weight > 10 kDa was calculated as the difference between supernatant Fe and 10 kDa ultrafiltrate Fe.

**Figure 7**
Gel filtration chromatograms of 5 kDa ultrafiltrates from pepsin (a, c) and pepsin/pancreatin (b, d) digests of beef (a, b) and cod (c, d) myofibrillar protein extracts; black line = $^{59}$Fe, grey line = protein; ccppm, background-corrected counts per minute; AU, absorption units. Gel filtration was performed on a Sephacryl S-100HR 26/60 column with a 0.2 M sodium acetate buffer at pH 6.0 and a flow rate of 1.3 ml/min. Fractions for $^{59}$Fe determination by gamma-counting were collected in 7-min intervals and protein elution was recorded by UV absorption at a wavelength of 280 nm.
Procedure to isolate water-soluble and salt-soluble meat proteins

~150 g raw meat
- Trimmed of visible fat
- Cut up
- Homogenized in a food blender

100 g meat homogenate
- Blended with 500 g Nanopure water
- Stirred for 2 h at 4°C
- Centrifuged for 20 min at 9500 x g

Supernatant = water-soluble extract
⇒ predominantly sarcoplasmic proteins

Supernatant = salt-soluble extract
⇒ predominantly myofibrillar proteins

Pellet
- Rehomogenized in 500 g 0.5M NaCl/10 mM Na-pyrophosphate
- Stirred for 2 h at 4°C
- Centrifuged for 20 min at 9500 x g

Pellet discarded

Figure 1
Overview of procedures used to characterize iron-binding peptides in meat protein fractions

In vitro digestion of sarcoplasmic and myofibrillar protein extracts with pepsin (P) or pepsin/pancreatin (PP) (2 g protein, 2 mg Fe) → Digests analyzed for total iron and total Kjeldahl N → Centrifugation 10900 rpm, 4°C, 30 min → Pellet discarded

Supernatant ultrafiltered through 10kDa and 5 kDa membranes and ultrafiltrates analyzed for Fe and Kjeldahl N ➔ Supernatant filtered and analyzed for Fe and Kjeldahl N

1. Calculation of N and Fe partitioning
2. Gel filtration of 5 kDa ultrafiltrates from P- and PP-digests with 59Fe tag
3. Amino acid analysis of selected digests and gel filtration peaks
4. LC-MS analysis of selected gel filtration peaks

Figure 2

![Graph showing % of total N for different enzyme digests](image)

Figure 3
Figure 7
Appendix to Manuscript 1

APPENDIX TO MANUSCRIPT 1 – TECHNICAL REPORT

Introduction
Iron uptake in Caco-2 cells is used as an in vitro tool to determine its potential absorbability in the human gut (GLAHN et al. 1996; AU and REDDY 2000). Caco-2 iron uptake studies were performed with pepsin/pancreatin (PP) digests of whole meat and 5 kDa ultrafiltrates of PP digests prepared from myofibrillar muscle protein extracts. The procedure to obtain the different digests is described in Manuscript 1 of this thesis. Results were compared to a control digest made from infant cereal (see (FIDLER et al. 2003) for composition of the infant cereal).

Materials and Methods

Cell culture
Caco-2 cells (ATCC, Rockville, USA) at passage 28 were grown in 75 cm² cell culture flasks (BD Falcon, Basel, CH) using Dulbecco’s modified Eagle’s medium (DMEM, D-5671, Sigma, Fluka, Buchs, CH), pH 7.4, supplied with 20% fetal bovine serum (FBS, F-7524, Sigma), 1% non-essential amino acids (100x MEM, M-7145, Sigma), 100 IU/ml penicillin/streptomycin (P-0781, Sigma), 50 µg/ml gentamicin (Cat-No. 15710-049, Gibco), and 1.25 µg/ml fungizone (Cat-No. 15290-026, Gibco). The cells were maintained at 37 °C in an incubator with 95% air/5% CO₂ atmosphere. At 80-100% confluency, they were trypsinized (Trypsin-EDTA, T3924, Sigma) and reseeded in 75 cm² cell culture flasks (BD Falcon) at a density of $1.1 \times 10^4$ cells/cm² for continuing growth.

Iron uptake experiments
For iron uptake experiments, Caco-2 cells at passages 29-32 were seeded onto PET-membrane inserts (Ø 2.4 cm, 0.4 µm pore size, Cat-No. 353090, BD Falcon) in 6-well culture plates (BD Falcon) at a density of $5 \times 10^5$ cells/insert. The volume of DMEM was 1.5 ml in the upper and 2.5 ml in the lower compartment. Culture medium was changed every 2-3 days.

After 17-18 days, the cell monolayers were washed with Hanks' balanced salt solution (HBSS, H-6648, Sigma). The HBSS and DMEM used during the uptake experiments were made 50 mM in HEPES and treated with Chelex-100 (No. 142-2822, Bio-Rad, Reinach, CH) prior to use to render them free of iron and are hereafter designated as low-Fe HBSS and low-Fe DMEM, respectively. Iron
uptake was assessed at two pH values, pH 6.7 and 7.4. While the former rather mimics the in vivo duodenal pH in human subjects (FALLINGBORG 1999), the latter is most commonly used in cell culture studies and reflects the standard pH of the culture media used. The lower pH (6.7) was adjusted by adding 6 M HCl to low-Fe DMEM of pH 7.4. Furthermore, the influence of the valence of the non-heme iron species was evaluated by comparing incubation solutions containing either ferric (Fe$^{3+}$) or ferrous (Fe$^{2+}$) iron. Premixes of 1 mM FeCl$_3$ in 0.1 M HCl and 1 mM FeSO$_4$ in 0.1 M H$_2$SO$_4$, respectively, with their corresponding radioisotopes, $^{59}$FeCl$_3$ (IFS-1, Amersham Biosciences) and $^{59}$FeSO$_4$ (NEZ049, Perkin Elmer, Boston, MA, USA), were prepared to obtain concentrations of 10 µM Fe and 0.5 µCi/well in the final incubation mixtures. These premixes were left for 30 min at room temperature to allow for isotopic exchange. Ascorbic acid (AA, 200 mM) in MilliQ water was prepared freshly each day and added to the iron premixes used as positive controls (final molar ratio of AA:Fe was 20:1). Low-Fe DMEM containing meat protein-free digest (cereal + digestive enzymes) was used as the baseline control.

Whole meat PP digests and the myofibrillar LMW meat peptides < 5 kDa were dissolved in low-Fe DMEM, added to the iron or AA:Fe premixes to reach final concentrations of 0.1 or 1 g/l peptide (based on Kjeldahl-N), and their effect on Caco-2 iron uptake assessed. All samples contained the same amount of meat-protein free infant cereal digest supernatant. All incubation solutions were prepared freshly each day and all tubes and pipette tips used to prepare the incubation solutions were acid washed.

Transepithelial electrical resistance (TEER) was assessed (Millicell Voltohmmeter, Millipore, Volketswil, CH) at RT to check for monolayer integrity. Monolayers with a TEER value below 250 Ω • cm$^2$ at the beginning or 220 Ω • cm$^2$ at the end of an experiment were discarded. Incubation time was 2h at 37°C in a 10% CO$_2$/90% air atmosphere, after which the apical and basal fluids were collected, the monolayers washed with 0.5 ml ice-cold low-Fe HBSS (wash I), and surface-bound iron removed according to the method of GLAHN et al. (1995) (wash II) with slight modifications: i) bathophenanthroline and sodium dithionite were dissolved in low-Fe HBSS, and ii) incubation was 9 min at RT with 0.5 ml ice-cold removal solution. After another washing step with 0.5 ml ice-cold low-Fe HBSS (wash III) the three wash solutions were pooled. Finally, the cells were solubilized in 1 ml of 0.5 M NaOH and sonicated on ice with a Branson W-150 Sonifier™ (VWR International, Dietikon, CH).
Radioactivity was measured in 500 µl aliquots of the apical, basal, cell, and wash solutions by gamma-counting (Cobra-II, Packard Instruments). Surface-bound and apical radioactivity was combined to give total iron not taken up by the cells. Total cell protein was measured with the bicinchoninic assay (BCA Assay Kit, Cat-No. 23225, Pierce) according to the manufacturer’s instructions on 96-well microplates using BSA as the protein standard. Readings were performed on a Spectramax 250 (Molecular Devices, Bucher Biotec AG, Basel, CH) using the BCA assay settings included in the Softmax™ Pro software package.

Results and Discussion

Caco-2 cell monolayers were used as a model system to estimate whether the iron-solubilizing meat peptides separated by ultrafiltration would also influence iron uptake in small intestinal enterocytes. The Caco-2 cell line has been repeatedly demonstrated to indicate the correct trend of food iron bioavailability (GARCIA et al. 1996; GLAHN et al. 1996; AU and REDDY 2000). Meat peptide concentrations were chosen on the basis of literature reports (AU and REDDY 2000; SWAIN et al. 2002; SERFASS and REDDY 2003) and correspond to a molar N:Fe ratio of 45:1 (1 g/L) and 4.5:1 (0.1 g/L). Iron uptake was assessed at pH 7.4 (data not shown) and 6.7, because these are the most widely used for the study of iron absorption in Caco-2 cells. Results are expressed as percent radiolabel absorption (sum of intracellular and basolateral ^{59}\text{Fe}) and show the influence of infant cereal PP digests containing AA or meat compared to a meat peptide-free infant cereal digest (control).

Figure 1 demonstrates that AA at a molar AA:Fe ratio of ~20:1 significantly \((P < 0.001)\) increased iron uptake 6-7-fold over the control PP digest at pH 6.7. Such high AA:Fe ratios are hardly reached in the human diet, unless vitamin C supplements are taken with the meal. In Caco-2 cells however, this has been frequently used as a standard ratio (GLAHN et al. 1996; GLAHN and VAN CAMPEN 1997; SWAIN et al. 2002; SERFASS and REDDY 2003; ENGLE-STONE et al. 2005; YEUNG et al. 2005a). Nevertheless, AA:Fe ratios as found in human diets likewise enhanced iron uptake in Caco-2 cells (YUN et al. 2004), yet the response was greater than actually observed in humans. Uptake levels for the ferric and ferrous control as well as the ferrous ascorbate were in line with results observed by YEUNG et al. (2005a), although the incubation media and procedure differed. In comparison, ferrous sulfate absorption in adult humans from the same infant cereal used in our Caco-2
experiments was 2.6% (Fidler et al. 2004). Generally, a 2-4-fold increase in iron absorption may be expected from meals containing up to 100 mg ascorbic acid (Hallberg et al. 1989b).

Supernatants of infant cereal digests containing whole beef only slightly enhanced ferric iron bioavailability (~25%, \( P > 0.1 \)) but significantly increased ferrous iron uptake (~52%, \( P < 0.001 \)). This would suggest that the whole beef digest has little potential to reduce/solubilize ferric iron but prevents ferrous iron from oxidation and/or precipitation. Unexpectedly, beef 5 kDa ultrafiltrates of myofibrillar protein extracts decreased significantly the uptake of ferrous and ferric iron in a concentration-dependent fashion (\( P < 0.001 \)). Similar observations were made with chicken, cod, lamb, and pork (data not shown). This contradicts findings by Swain et al. (2002) who reported 1.5-fold and 2-fold higher iron uptake with LMW (1-7 kDa) beef peptides at concentrations of 1 and 2 g/L, respectively. However,
methodological variations may account for a large part of the differences as was recently concluded in a consensus statement from the HarvestPlus expert consultation (FAIRWEATHER-TAIT et al. 2005). In the present study, longer pancreatin digestion times were used and centrifugation, ultrafiltration, cell culture and uptake studies performed differently.

It is probable that our iron-binding peptides are only part of the “meat factor” and that other peptides not isolated by us also have iron-binding properties. Such peptides would be those histidine-containing peptides studied by SWAIN et al. (2002) or the cysteine-containing peptides studied by other authors (MULVIHILL et al. 1998; VATTEM et al. 2001). It would appear that the iron-binding properties of the peptides contained in the 5 kDa ultrafiltrates of PP-digested myofibrillar meat protein extracts studied in our experiments are stronger than of those studied by SWAIN et al. (2002). This is a likely reason for their inhibitory effect at similar concentrations. At lower concentration they may become enhancing to Caco-2 iron uptake. An analogy can be drawn with EDTA, which is enhancing at low concentration but inhibits at high concentration (INACG 1993). However, it is difficult to define an appropriate dose of isolated peptides that would resemble in vivo conditions and at the same time account for potential bioactivity changes due to separation from other peptide fractions.

Caco-2 cell experiments have frequently shown large variations in responsiveness (GLAHN et al. 1998; GLAHN et al. 2000; ETCHEVERRY et al. 2005), which prohibits the use of this model as a quantitative indicator of iron bioavailability. However, not even qualitatively correct data were obtained in the study of potential iron compounds for flour fortification (YEUNG et al. 2005b; HOPPE et al. 2006). Consequently, Caco-2 data demand cautious interpretation. More generally, rigid standardization of methodology and data reporting is needed to allow for better comparability of results (Sharp 2005).

Although viewed as a prerequisite for iron to be bioavailable, iron solubility alone is not a reliable predictor, especially when assessing the effect of dietary proteins (MILLER and BERNER 1989). This might partly explain the iron uptake results seen with LMW meat peptides demonstrated to solubilize large amounts of iron in a low-molecular weight form (Manuscript 1, Fig. 5). For example, phytic acid is equally capable of forming LMW complexes of high solubility and dialyzability (GLAHN et al. 2002) but poor bioavailability (HALLBERG et al. 1989a; HURRELL et al. 1992).
Conclusions

The LMW peptides from PP digests of myofibrillar protein extracts inhibited iron uptake into Caco-2 cells and thus confirmed their strong iron-binding properties. As the inhibition may be concentration-dependent, it is not possible to say whether they would inhibit iron absorption \textit{in vivo}.

References


Manuscript 2

GLYCOSAMINOGLYCANS AND IRON ABSORPTION

SUBMITTED FOR PUBLICATION TO THE JOURNAL OF NUTRITION
Non-heme iron absorption in humans is not influenced by purified sulfated and unsulfated glycosaminoglycans

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ABSTRACT

Meat is a well known enhancer of iron absorption, yet the molecular entity mediating the effect remains obscure. Recently published data indicated highly acidic sulfated glycosaminoglycans (GAG) from fish and chicken muscle as effective stimulants of iron uptake in Caco-2 cells. Two stable isotope studies were performed in a group of sixteen apparently healthy young women to assess the effect of purified sulfated and unsulfated GAG on human iron absorption. Iron absorption was measured on the basis of erythrocyte incorporation of $^{57}$Fe or $^{58}$Fe 14 d after the administration of labeled semisynthetic meals (SSM) based on egg albumin, corn oil, maltodextrin, and water. The meals were fed with or without added sodium hyaluronate (NaH, 300 mg) or chondroitin sulfate (CS, 360 mg) as representative unsulfated and sulfated GAG, respectively. The level of GAG added was three times (NaH) to about ten times (CS) the amount that would be expected to be present in 150 g beef muscle. Geometric mean iron absorption from SSM containing NaH (21.2%) or CS (19.4%) did not differ from that of SSM without GAG (19.5% and 20.3%, respectively). NaH and CS at the levels tested have no significant influence on human non-heme iron absorption.

Key words: iron absorption, glycosaminoglycans, meat factor, stable isotopes; semisynthetic meal
INTRODUCTION

Meat is a well-known enhancer of non-heme iron absorption (1-3), yet the molecular entity in meat responsible for this effect remains elusive. Until now, the meat factor was assumed to be a protein component (4-6) of cellular origin, however, recent in vitro data suggest that glycosaminoglycans (GAG) may also play a role as part of the meat factor. Huh et al (7) showed enhanced radioiron uptake by Caco-2 cells in the presence of a purified dilute acid-soluble fraction from haddock or chicken. Notably, the purified acid-soluble fraction was very low in protein, peptides, and amino acids (< 2.3 g/100 g extract after HPLC), but contained mainly acidic carbohydrate structures most probably derived from GAG in the extracellular matrix (7). Caco-2 cells exhibit a human small intestinal phenotype upon differentiation (8) and when compared with in vitro digestion and dialysis usually give the same direction of response to enhancers and inhibitors of iron absorption as do human subjects (9-12). GAG are part of the connective tissue between the muscle fibres and represent about 0.1% of muscle wet weight (13). Seven different types of GAG have been described (Table 1), the differences being related to disaccharide subunit composition and degree of sulfation. Contributions of the subclasses to the total GAG content of muscle tissue vary depending on factors like species, age, and tissue source (14, 15). In mouse muscle tissue, hyaluronic acid (HA) accounted for about 70% of the total GAG, whereas dermatan sulfate (chondroitin sulfate B), the major fraction among the sulfated GAG, contributed 20% (14). The remainder was made up by chondroitin sulfate (CS) and heparan sulfate. Likewise, chondroitin sulfate B was reported to be the major sulfated GAG in rabbit muscle (16) and porcine skeletal muscle epimysium (17), namely 80-90% of total sulfated GAG. However, others reported chondroitin sulfate A/C (i.e., 4- or 6-sulfated CS) to constitute 44% of the total hexuronic acid content in rabbit muscle (18). HA consists of repeating disaccharide units of N-acetyl-glucosamine and D-glucuronic acid. CS differs from HA in that N-acetyl-glucosamine is replaced by N-acetyl-galactosamine and the C4 or C6 of the galactosamine is sulfated. Whereas HA is the only unsulfated GAG, CS can be viewed as representative of sulfated GAG.

The present absorption studies were designed to evaluate the possibility that GAG are part of the meat factor. We assessed the influence on iron absorption of purified
NaH (the sodium salt of HA) and CS as model GAG. Interestingly, iron-chondroitin sulfate complexes (Condrofer®, Blutal®) have been used in the treatment of iron deficiency anemia (19, 20) and hyaluronic acid has been shown in vitro to bind iron (21). Iron absorption from a semi-synthetic meal (SSM6: hydrolyzed corn starch, chicken egg white, corn oil) served with NaH or CS was compared with iron absorption from the SSM alone. Absorption was measured based on the incorporation of the stable iron isotope labels into erythrocytes (22, 23).

SUBJECTS AND METHODS

Subjects

Sixteen apparently healthy women (aged 19-34 y; maximum body weight 60 kg) were recruited from the staff and student populations of ETH Zurich (Zurich, CH), University of Zurich and the University Hospital Zurich. The subjects participated in two studies, which were performed in a randomized, cross-over design. Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin or mineral supplements were allowed during the study. Women regularly taking vitamin or mineral supplements discontinued supplementation 2 wk before the start and during the study. No subjects were recruited who had donated blood within 4 months of the beginning of the study or who were planning to donate blood during the study period. The study protocol was reviewed and approved by the Ethical Committees of ETH Zurich and of the canton of Zurich. Subjects were informed orally and in writing about the aims and procedures of the study. Written informed consent was obtained from all study subjects.

Study design

Iron absorption was determined with the use of a double stable-isotope technique and based on erythrocyte incorporation of $^{57}$Fe or $^{58}$Fe 14 d after test meal administration. Each subject received a total of four test meals (2xA, 1xB, 1xC; see below). All test meals were fed between 7 and 9 am, after the subjects had fasted

6 Abbreviations: AA, amino acid; CS, chondroitin sulfate; GAG, glycosaminoglycan(s); HA, hyaluronic acid; NaH, sodium hyaluronate; SSM, semi-synthetic meal
overnight, under standardized conditions. Purified GAG were compared in a fully randomized cross-over design, with each subject acting as her own control. A few days prior to the first test meal administration (day 0), a baseline venous blood sample was drawn after an overnight fast for determination of iron status (plasma ferritin, hemoglobin) and subjects' height and weight were recorded. The first two labeled test meals were fed on two consecutive days (day 1 and 2). Fourteen days later (day 16), a second blood sample was drawn, followed by the administration of test meals 3 (day 17) and 4 (day 18). Another 14 d later (day 32), a final blood sample was drawn.

Test meals

The control meal (meal A) was a semisynthetic meal (SSM) consisting of 67 g hydrolyzed corn starch (CStar MD 01910, Cargill Cerestar, Blattmann AG, Wadenswil, CH), 35 g corn oil (Mazola®, Unilever, Rotterdam, NL), 35.2 g egg white (Monark Egg Corporation, Kansas City, MO, USA) corresponding to 30 g protein and 200 ml high-purity (18 MΩ) water. Pineapple flavor (#74185-33, Givaudan, Kemptthal, CH) was added at 0.05% (v/w). This flavor was chosen because it is devoid of carboxylic and phenolic groups likely to interact with iron. SSM shakes were prepared freshly the day before consumption. The test meal (meal B) in study I consisted of the SSM, to which 300 mg food-grade sodium hyaluronate (Freda Biochem, Shandong, CN) was added on the morning of meal administration. In study II (meal C) the NaH was replaced by 360 mg food-grade chondroitin sulfate (CS, from shark cartilage, Paul Bruns GmbH, Elmshorn, GER).

The amount of NaH was estimated to equal triple the daily consumption with an average meat intake of ~150 g/d (13, 24). For CS, the same amount on a molar basis, 360 mg, was added to allow for better comparison, although CS intake would most probably be closer to 20-30 mg/d. NaH had a molecular weight of 1000-1200 kDa and contained 97.03% hyaluronic acid according to the certificate of analysis. CS was of ~20 kDa molecular weight and contained 92.3% chondroitin sulfate (10% C4-sulfated and 90% C6-sulfated), the remainder being protein (< 6%), ash and water. High-purity water (230 g) was served as a drink.

With each control and test meal, a separate high-purity water drink (70 g) containing 4 mg of iron - added as isotopically labeled ferrous sulfate (57FeSO₄ or 58FeSO₄) on the morning of consumption - was served. Subjects were asked to start with the SSM
and to intermittently drink the water. No time restriction was imposed but all subjects finished their SSM within 5-25 min. Control and test meals were fed once per study. To alleviate any unpleasant aftertaste, the subjects received a peppermint-flavored Wrigley’s Eclipse® Flash strip after each meal.

**Stable-isotope labels**

Isotopically labeled $^{57}\text{FeSO}_4$ and $^{58}\text{FeSO}_4$ were prepared from isotopically enriched elemental iron (Chemgas, Boulogne, F) dissolved in 0.05 sulfuric acid. The solutions were stored in polytetrafluoroethylene containers flushed with argon to keep the iron in the 2+ oxidation state.

**Quantification of iron in isotope solutions**

Isotope dilution mass spectrometry was used to determine the concentration of the isotopic labels in solution. To aliquots taken from the prepared isotopic labels, an accurately known amount of iron of natural isotopic composition was added. The used iron standard was prepared gravimetrically from an isotopic reference material (IRM-014, EU Institute of Reference Materials, Geel, BE). Isotopic analysis was performed using negative thermal ionization-mass spectrometry (25). Iron concentrations in the isotopic labels were calculated on the basis of the observed shift in iron isotopic abundances, the determined isotopic abundances of the pure isotopic labels, and measured natural iron isotopic abundances (23).

**Iron status measurements**

Venous blood samples (7 ml) were drawn into EDTA-treated vacutainers (368453, Becton Dickinson, Milian SA, Meyrin, CH) a few days before the first test meal administration and again 14 d after the first and second set of test meals on day 16 and day 32 of the study, respectively. Samples were analyzed for iron status indexes (plasma ferritin, hemoglobin; day 0) and for the incorporation of $^{57}\text{Fe}$ and $^{58}\text{Fe}$ into erythrocytes (day 16, day 32). Hemoglobin was measured in fresh whole blood using the cyanmethemoglobin method (D5941, Sigma, Buchs, CH). Plasma was separated and stored frozen for later ferritin analysis with the use of an enzymatic immunoassay (Immulite®, DPC Bühlmann GmbH, Salzburg, AT). Commercial quality control materials for hemoglobin (Digitana AG, Horgen, CH) and ferritin (Immulite®) were run with each analysis.
Quantification of iron isotope in the blood

Each isotopically enriched blood sample was analyzed in duplicate for its iron isotopic composition as previously described by Walczyk et al (23). The blood samples were mineralized using a mixture of nitric acid and hydrogen peroxide and microwave digestion. The iron was separated from the matrix by anion-exchange chromatography and a solvent-solvent extraction step into diethylether. The isotopic analyses were performed by negative thermal ionization-mass spectrometry on a single-focusing magnetic sector field mass spectrometer (MAT 262, Finnigan MAT, Bremen, GER) (25).

Calculation of iron absorption

The amounts of $^{57}$Fe and $^{58}$Fe isotopic labels in blood 14 d after administration of the test meals were calculated on the basis of the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic (23). Circulating iron was calculated on the basis of blood volume and hemoglobin concentration (22). Blood volume calculations were based on height and weight according to Brown et al (26). For calculations of fractional absorption, 80% incorporation of the absorbed iron into erythrocytes was assumed (27).

Statistics

Student’s paired t test was used to evaluate study data. Values were logarithmically transformed before statistical analysis (EXCEL 2002 SP3, Microsoft Corporation, Redmond, WA). Results are presented as geometric means (-1 SD, +1 SD) and were considered statistically significant at $P < 0.05$. Post-hoc power calculations for paired t tests were performed using GraphPad StatMate for Windows, version 2.00 (GraphPad Software, San Diego, USA) with n = 14 in study I and n = 15 in study II. The same fourteen subjects in study I and study II were used for paired comparison between the NaH-containing SSM and the CS-containing SSM after normalization for mean isotope absorption from the GAG-free reference SSM in both studies.
RESULTS

Subjects

Of the sixteen subjects recruited into the two studies, fifteen completed the CS arm and fourteen completed the NaH arm. Reasons for dropout were taste aversion and accidental spilling of isotope label, respectively. Mean body weight was 54.6±3.6 kg and mean age was 24.1±4.2 y. One of the subjects had iron deficiency anemia (Hb: 112 g/L, plasma ferritin: < 12 µg/L), one had depleted iron stores (plasma ferritin: < 12 µg/L) and two displayed low hemoglobin (Hb: 111 and 112 g/L) with normal plasma ferritin (23.6 µg/L and 28.4 µg/L, respectively).

Iron absorption

The geometric mean absorption from the control meal was 19.5% in the NaH study and 20.3% in the CS study (Table 2). Iron absorption from the SSM containing GAG was 21.2% for NaH and 19.4% for CS. No significant differences in absorption from test meals compared to control (NaH: $P = 0.42$; CS: $P = 0.64$) were observed. After normalization for mean iron absorption from the GAG-free SSM (20.5%) in the fourteen subjects who participated in both studies, absorption from the GAG-containing meals did not differ significantly ($P = 0.50$) The study design would have allowed detecting a change in iron absorption of about 30% at 80% power.

DISCUSSION

We studied the effect of the purified glycosaminoglycans sodium hyaluronate and chondroitin sulfate as a possible part of the meat factor on non-heme iron absorption from a semisynthetic meal. This study was prompted by recent reports of acidic carbohydrate structures of the GAG type as enhancers of iron uptake in Caco-2 cells (7). In contrast to the 3-5-fold increased iron uptake in the in vitro data, we observed no enhancement of iron absorption in humans.

The SSM has been repeatedly employed as a standardized meal in iron absorption studies (5, 28-30). Its value lies in the highly defined chemical composition allowing for the study of inhibitors and enhancers of iron bioavailability alike. The level of absorption from the control meal in the present study (~20%) is substantially higher
than reported by others (5, 29, 30), which can be explained by the addition of calcium and phosphate in the earlier studies. Reddy et al (28), feeding the SSM without added calcium or phosphate, observed a percentage iron absorption of 9.67%. The lower bioavailability of the ferric chloride (31) used by Reddy et al (28) and the inclusion of male subjects could account for the differences to the data presented in this paper, which were obtained with ferrous sulfate in women with relatively poor iron status (mean serum ferritin: 30 µg/L). Nevertheless, mean percentage iron absorption from ferric chloride added to a similar SSM containing beef or chicken muscle was recently reported to be as high as 16-37% compared to 8.4% absorption from a reference SSM with egg albumin (32). A similarly pronounced effect of GAG would have been detectable in the present study.

Data on meat consumption from the European Prospective Investigation into Cancer and Nutrition (EPIC) study showed that total meat intake ranged between 46-127 g/d in women and 79-242 g/d in men (24). Assuming that the total glycosaminoglycan content in meat of different species does not vary greatly, the estimated GAG intake would thus amount to 46-127 mg/day in women and 79-242 mg/day in men. Unfortunately, data on GAG composition in meat used for human consumption is scarce. In mouse muscle tissue, the unsulfated GAG hyaluronic acid (HA) constitutes about 70% of total GAG (14), the remainder being sulfated GAG like chondroitin sulfate B (dermatan sulfate) or heparan sulfate. Thus, daily HA and sulfated GAG intakes probably range between 32-169 mg/d and 14-73 mg/d, respectively. The rationale for using 300 mg of NaH was to simulate an intake 2-3 times the daily consumption of non-vegetarians. To allow for direct comparison between NaH and CS, we fed the same molar amounts of CS (360 mg), although dietary CS intake would be only about one fifth of NaH intake. It is still possible but unlikely that it could be enhancing at lower levels.

It has been reported that soluble HA-iron complexes are obtained at a molar ratio of 3:1 (HA disaccharide:Fe) and that iron interacts specifically with the acetylated nitrogen of N-acetyl-glucosamine in aqueous solution (21). In the present study, a GAG disaccharide:Fe molar ratio of ~10:1 was employed. Molar ratio is a key issue in the study of enhancers and inhibitors of iron absorption. Usually, dietary factors influencing iron absorption show a more or less pronounced dose-response relationship (33-36). The only known exception is EDTA, which when added to meals of low bioavailability at a molar ratio of ≤1:1 acts as an enhancer (37, 38), whereas
higher EDTA:Fe ratios lead to decreased iron absorption (37). Thus, the lack of any effect at the rather high molar GAG:Fe ratio used in the present study makes it unlikely but not impossible that a lower molar GAG:Fe ratio would change dietary iron bioavailability. If larger amounts of GAG had a beneficial effect, however, they would have to be provided by dietary supplements.

It is known that during synthesis GAG can undergo several chemical modifications such as (de-)sulfation, (de-)acetylation or epimerization. Huh et al (7) report that preliminary characterization of their chicken and fish extracts suggested highly acidic oversulfated heparan sulfate moieties as major constituents of the fractions enhancing Caco-2 cell iron uptake and virtual absence of proteins, peptides and amino acids. It is still possible that this small specific fraction of muscle GAG could be an iron absorption enhancer. However, it should be stressed that these Caco-2 studies were made by adding the acid-soluble fractions onto Caco-2 cells directly without the addition of a meal. When other positive or negative ligands are present from partially digested food, the influence of the acid extract could be very different.

The high cost of commercial heparan sulfate prevented its use in the present study. Large scale production for human feeding trials of the fish extract used in the Caco-2 experiments was attempted but was unsuccessful. Whether the chemical modification of GAG substantially modulates their influence on iron bioavailability remains to be determined. Generally, differences between the different types of GAG largely relate to their sequence of disaccharide subunits so that fragments of low molecular weight become increasingly similar to one another. To what extent GAG are modified during digestion, i.e., gastric acid treatment as simulated by HCl incubation in the study by Huh et al (7), is difficult to tell. However, gastric digestion in humans would probably release low-molecular-weight oligosaccharides from larger GAG molecules in a similar fashion to that observed in the Caco-2 study.

Meat has repeatedly been shown to enhance iron absorption in humans (1, 2, 29, 39-42), the effect being more pronounced from single meals compared to complete diets. In a subsequent evaluation of the relative contribution of the three macronutrients carbohydrate, fat and protein, meat protein was shown to be the likely candidate for harboring the meat factor (5). This is not surprising, considering that lean meat is mainly composed of water and protein. Further evidence was reported recently which supports the enhancing properties of meat protein. Subjects fed a semisynthetic meal in which egg albumin was replaced by highly purified chicken or
beef meat protein extracts (94-98% protein) displayed 2-3-fold higher iron absorption than from the egg albumin control (32). The protein extract preparation procedure involved several precipitation and wash steps, thus minimizing the presence of other meat constituents like carbohydrates. However, the higher absorption observed with chicken protein isolate was suggested to perhaps relate to some non-protein component, which could be GAG.

In conclusion, the purified glycosaminoglycans sodium hyaluronate and chondroitin sulfate had no detectable effect on iron absorption from a semisynthetic meal. The GAG doses administered represented intake levels achievable with very high meat consumption or low dose supplement use. It is unlikely that these compounds constitute a major part of the meat factor. However, the potential role of chemical modifications to the GAG structure and the GAG:Fe molar ratio warrant further investigation.

ACKNOWLEDGMENTS

The help of Eberhard Denk, Ralf Biebinger, and Rita Wegmüller in performing the human studies is greatly appreciated. Special thanks go to Christophe Zeder for preparing the isotope solutions and helping with data evaluation, to Charlotte Züllig and Marlies Krähenbühl for taking blood samples, and to the test subjects for their willingness to participate in the studies.
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Table 1
Composition of repeating disaccharide units of glycosaminoglycans (GAG) (modified from (43))

<table>
<thead>
<tr>
<th>GAG</th>
<th>No. of disaccharide units</th>
<th>Repeating disaccharide (A-B), subscripts</th>
<th>Sulfates per disaccharide unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronic acid</td>
<td>50-10000</td>
<td>β-D-GlcUA, β-D-GlcNAc</td>
<td>0</td>
</tr>
<tr>
<td>Chondroitin 4-Sulfate</td>
<td>20-60</td>
<td>β-D-GlcUA, β-D-GalNAc</td>
<td>0.2-1.0</td>
</tr>
<tr>
<td>Chondroitin 6-Sulfate</td>
<td>20-60</td>
<td>β-D-GlcUA, β-D-GalNAc</td>
<td>0.2-2.3</td>
</tr>
<tr>
<td>Dermatan Sulfate(^a)</td>
<td>30-80</td>
<td>β-D-GlcUA, β-D-GalNAc</td>
<td>1.0-2.0</td>
</tr>
<tr>
<td>Heparan Sulfate (HS)(^a)</td>
<td>10-60</td>
<td>β-D-GicUA, α-D-GlcNAc</td>
<td>0.2-3.0 (HS)</td>
</tr>
<tr>
<td>and Heparin (H)(^a)</td>
<td>10-60</td>
<td>α-L-IdUA, α-D-GlcNAc</td>
<td>2.0-3.0 (H)</td>
</tr>
<tr>
<td>Keratan Sulfate</td>
<td>5-40</td>
<td>β-D-GlcNAc, β-D-Gal</td>
<td>0.9-1.8</td>
</tr>
</tbody>
</table>

\(^a\) these GAG may contain two different types of disaccharide units

GAG, glycosaminoglycan; GlcNac, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; IdUA, iduronic acid; GlcUA, glucuronic acid; Gal, galactose
Table 2
Iron absorption from a semisynthetic meal with and without added glycosaminoglycans\(^a\)

<table>
<thead>
<tr>
<th>Study/Meal</th>
<th>Plasma ferritin</th>
<th>Iron absorption</th>
<th>Absorption ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/L</td>
<td>% of dose</td>
<td>B:A</td>
</tr>
<tr>
<td>1 (n = 14F)</td>
<td>30 (9-51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (SSM)</td>
<td>19.49 (10.53; 36.05)</td>
<td>(0.74-1.61)</td>
<td></td>
</tr>
<tr>
<td>B (+ NaH(^b))</td>
<td>21.24 (12.18; 37.01)</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>2 (n = 15F)</td>
<td>29 (9-49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (SSM)</td>
<td>20.33 (9.59; 43.09)</td>
<td></td>
<td>(0.66-1.39)</td>
</tr>
<tr>
<td>C (+ CS(^c))</td>
<td>19.42 (8.73; 43.16)</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Geometric mean (-1 SD; +1 SD). Subjects aged 19-34 y. SSM, semisynthetic meal; NaH, sodium hyaluronate; CS, chondroitin sulfate

\(^b\) 300 mg sodium hyaluronate

\(^c\) 360 mg chondroitin sulfate
APPENDIX TO MANUSCRIPT 2 – TECHNICAL REPORT

Introduction

In parallel to the human studies on glycosaminoglycans (GAGs) and iron absorption described in Manuscript 2, Dr. Fuxia Jin and Dr. Raymond Glahn at Cornell University, USA, agreed to perform Caco-2 iron uptake experiments with those meals fed to the study subjects. This is a report on the outcome of the Caco-2 studies. The Materials and Methods as well as the Results section were kindly provided by Dr. Jin and Dr. Glahn.

Materials and Methods

Chemicals, enzymes, and hormones. Unless otherwise stated, all chemicals, enzymes and hormones were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental design. The objective of this experiment was to evaluate the effect of two GAGs, hyaluronic acid (HA) and chondroitin sulfate (CS), on non-heme Fe bioavailability from a semi-synthetic meal (SSM). Each sample digest was made to 15 mL with the addition of 1.0 g of semi-synthetic meal as a starting point for the basic calculations. Aliquots of 1.5 mL of these sample digests were put on the Caco-2 cell monolayer insert for iron uptake and six replicates of sample digests were prepared for uptake by six wells of Caco-2 cells. Each sample digest control was a 1.0 g of SSM with $^{58}$Fe-labeled FeSO$_4$ (i.e., 11.86 µg $^{58}$Fe) in a meal to $^{58}$Fe ratio equal to that used in the human study reported in Manuscript 2. Sodium hyaluronate (NaH) or chondroitin sulfate was added to the control in the same GAG to meal ratio as that in the human study (i.e., 0.847 mg of NaH or 1.02 mg of CS per 15 mL sample digest with 1.0 g of SSM and 11.86 µg of $^{58}$Fe).

Sample preparations. All meal samples were supplied by Stefan Storcksdieck genannt Bonsmann from the Human Nutrition Laboratory at ETH Zurich, Switzerland. A day prior to the cell experiment, meals were prepared in exactly the same manner as in the human study. To make the meal samples suitable for cell culture experiments, the ingredients of each meal were mixed together with a baby food processor.

Cell cultures. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17, and used in experiments at passage 29-32.
Cells were seeded at a density of 50000 cells/cm² in collagen-treated 6-well plates (Costar Corp., Cambridge, MA, USA). The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, GIBCO, Grand Island, NY, USA) with 10% v/v fetal bovine serum (FBS, GIBCO), 25 mmol/L HEPES. Cells were maintained at 37°C in an incubator with a 5% CO₂/95% air atmosphere with constant humidity, and the medium was replaced every 2 days. Cells for each study were used 14 d post-seeding. Under these conditions, the amount of cell protein measured in each well was found to be highly consistent from well to well within each culture plate.

**In vitro digestion/Caco-2 cell culture method.** In vitro digestion and enzyme preparation methods have been described in detail elsewhere (GLAHN et al. 1998). Briefly, samples were combined in 50 mL tubes as described in Experimental Design. To initiate the gastric phase of digestion, 7 mL of 140 mmol/L NaCl, 5 mmol/L KCl, pH 2 solution was added to each 50 mL tube. After pH adjustment to 2 with 1 N HCl, 0.5 mL of pepsin solution was added to each tube, and the mixtures were incubated for 1 h at 37°C on a rocking platform shaker (model RP-50, Laboratory Instruments, Rockville, MD, USA). After incubation, the pH was raised to 5.5-6.0 with 1 mol/L NaHCO₃ and 2.5 mL pancreatin/bile solution was added to each mixture. The pH was then adjusted to approximately 7.0, and the volume contained within each tube was adjusted by weight to 15 mL using a 140 mmol/L NaCl, 5 mmol/L KCl, pH 6.7 solution. At this point, the mixtures were referred to as “digests”.

To initiate the intestinal digestion period, a 1.5 mL aliquot of the digest was placed into the upper chamber of a two-chambered system formed by placing well inserts fitted with 15000 Da MWCO dialysis membranes into plate wells containing Caco-2 cell monolayers. Plates were covered and incubated at 37°C for 2 h on a rocking platform shaker (approximately 12 oscillations/minute). Following the 2 h incubation, the inserts were carefully removed and an additional 1 mL of MEM was added to each well. The cell plates were then replaced into the incubator to allow time for ferritin formation. Cells were harvested after 22 h (24 h from the start of the intestinal digestion). To minimize the possible artifact of surface adsorption, an additional wash of the cells with a removal solution of 1 mmol/L bathophenanthroline disulfonate and 5 mmol/L sodium hydrosulfite dissolved in a regular rinse/stop solution of 130 mmol/L NaCl, 5 mmol/L KCl and 5 mmol/L PIPES prepared freshly 20 min prior to cell harvest was performed to remove any surface bound iron.
Iron Assays. All glassware used in sample preparation and analyses was rinsed with 10% HCl and 18 MΩ deionized water before use. Caco-2 cell ferritin formation served as a marker of cell total Fe uptake. Caco-2 cell protein content of samples was measured, after solubilization in 0.5 mol/L NaOH, using a Bio-Rad DC protein assay kit, which is a commercial semi-micro-adaptation of the Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA). An immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Serum Ferritin Assay, RAMCO Laboratories, Houston, TX, USA). A 10 µL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL, was used for each ferritin measurement. Analyses of total Fe content of the experimental solutions, samples, and digests were conducted using an inductively coupled argon plasma emission spectrometer (ICP-AES) (ICAP Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, MA, USA) after wet-ashing with HNO₃ and HClO₄.

An aliquot of 1.3 mL of the cell suspension was transferred into a 2.0 mL Eppendorf tube for centrifugation at 10000 rpm for 15 min. One milliliter of the supernatant was transferred to a clean tube for ICP-MS analysis of total Fe and ⁵⁸Fe concentration in each cell sample. Cell supernatant was analyzed using an inductively coupled plasma mass spectrometer (Agilent 7500c ICP-MS, Agilent Technologies, USA).

Statistical analyses. Data were analyzed using the software package GraphPad Prism (GraphPad Software, San Diego, CA, USA). ANOVA with Tukey’s post-test was used to compare differences among means. Means with significantly different variances were logarithmically transformed prior to ANOVA analysis. Differences among means were considered significant at n = 6, P ≤ 0.05.

Results

The presence of two glycosaminoglycans sodium hyaluronate and chondroitin sulfate in the semi-synthetic control meals did not result in any significant difference on Caco-2 cell ferritin formation when compared to the control meal only (Fig. 1).
Caco-2 cell ferritin formation in response to in vitro digests of GAG study meals. SSM, SSM + HA, and SSM + CS represents semi-synthetic control meal, semi-synthetic control meal with sodium hyaluronate, and semi-synthetic control meal with chondroitin sulfate. Digests contained 1.0 g of the test meal and a constant $^{58}$Fe concentration of 13.63 µmol/L. Values are mean ± SEM. Bars with no letters in common are significantly different (n = 6, $P \leq 0.05$).

$^{58}$Fe uptake by Caco-2 cells was significantly higher when sodium hyaluronate was present in the semi-synthetic meal compared to either the semi-synthetic meal alone or the semi-synthetic meal with chondroitin sulfate added (Fig. 2).

$^{58}$Fe concentration in the supernatants of harvested Caco-2 cell suspensions in response to in vitro digests of GAG study meals. SSM, SSM + HA, and SSM + CS represents semi-synthetic control meal, semi-synthetic control meal with sodium hyaluronate, and semi-synthetic control meal with chondroitin sulfate. Digests contained 1.0 g of the test meal and a constant $^{58}$Fe concentration of 13.63 µmol/L. Values are mean ± SEM. Bars with no letters in common are significantly different (n = 6, $P \leq 0.05$).
The presence of chondroitin sulfate in the semi-synthetic meal did not change the cell uptake of $^{58}$Fe significantly as compared to the semi-synthetic meal (Fig. 2). Total Fe uptake from chondroitin sulfate meals seemed to be slightly lower than that from the control and SSM/HA meal owing to low variability of the data (Figure 3).

![Figure 3](image_url)  
**Figure 3** Total Fe concentration in the supernatants of harvested Caco-2 cell suspensions in response to *in vitro* digests of GAG study meals. SSM, SSM + HA, and SSM + CS represents semi-synthetic control meal, semi-synthetic control meal with sodium hyaluronate and semi-synthetic control meal with chondroitin sulfate. Digests contained 1.0 g of the test meal and a constant $^{58}$Fe concentration of 13.63 µmol/L. Values are mean ± SEM. Bars with no letters in common are significantly different ($n = 6$, $P \leq 0.05$).

**Discussion**

Our human absorption studies looking into GAGs as part of the “meat factor” (see Manuscript 2) had been prompted by Caco-2 data showing enhanced iron uptake in the presence of cooked fish extracts virtually devoid of proteins/amino acids but enriched in GAGs (HUH et al. 2004). We found no effect of sodium hyaluronate or chondroitin sulfate – chosen as representative of unsulfated and sulfated GAGs, respectively – on iron absorption from a semisynthetic meal. In agreement with our human study data, sodium hyaluronate and chondroitin sulfate did not affect Caco-2 iron uptake as measured by ferritin formation (Fig. 1). This would indicate that the tested compounds do not contribute to the “meat factor”. However, since the cooked fish extract in the study of HUH et al. contained mainly small fragments (<1 kDa) of oversulfated heparan sulfate, we cannot rule out that very specific GAG structures are necessary for enhanced iron absorption to occur. On the other hand, Caco-2 iron
uptake may not always predict iron absorption in vivo as has been reported for the absorption promoter ascorbic acid (Au and Reddy 2000). As a consequence, it remains possible that the extracted GAGs have no effect at all in humans. Absorption studies with GAGs prepared in the same way as in the study by HuH et al. would be necessary to obtain conclusive information on this point.

The statistically significantly lower total iron uptake from SSM digests containing chondroitin sulfate (Fig. 3) may simply relate to low data variability, and the increased $^{58}$Fe uptake observed with sodium hyaluronate (Fig. 2) may be viewed as a cell culture artifact since it does not match the human absorption data. Taking a different stance, higher uptake into enterocytes does not necessarily translate into higher iron bioavailability, since basolateral transfer of absorbed iron into the circulation is ultimately required for the iron to reach its functional compartments.

Conclusions

The purified glycosaminoglycans sodium hyaluronate and chondroitin sulfate do not affect iron absorption in humans, and Caco-2 ferritin formation as a marker of iron bioavailability parallels this finding. Whether highly specific GAG structures such as oversulfated heparan sulfate promote iron bioavailability remains to be demonstrated in human absorption studies.

References


Oxalic acid does not influence non-haem iron absorption in humans – a comparison of kale and spinach meals

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SSgB was involved in the planning of the study, carried out the study, measured iron status parameters, analysed the data and wrote the first draft of the manuscript. TW contributed to the study design, supervised the meal administration and revised the manuscript draft. SR performed the mass spectrometric iron isotope measurements. RFH supervised the project, contributed to the study design and critically revised the manuscript draft.

Running title: Oxalic acid and non-haem iron absorption
ABSTRACT

Objective: To evaluate the influence of OA on non-haem iron absorption in humans.
Design: Two randomised cross-over stable iron isotope absorption studies.
Setting: Zurich, Switzerland.
Subjects: Sixteen apparently healthy women (18-45 y, <60 kg body weight), recruited by poster advertising from the staff and student populations at ETH, University, and University Hospital of Zurich, Switzerland. Thirteen subjects completed both studies.
Methods: Iron absorption was measured based on erythrocyte incorporation of $^{57}\text{Fe}$ or $^{58}\text{Fe}$ 14 d after the administration of labeled meals. In study I, test meals consisted of two wheat bread rolls (100 g) and either 150 g spinach with a native OA content of 1.27 g (reference meal) or 150 g kale with a native OA content of 0.01 g. In study II, 150 g kale given with a potassium oxalate drink to obtain a total OA content of 1.27 g was compared to the spinach meal.
Results: After normalisation for the spinach reference meal absorption, geometric mean iron absorption from wheat bread rolls with kale (10.7%) did not differ significantly from wheat rolls with kale plus 1.26 g OA added as potassium oxalate (11.5%, $P = 0.86$). Spinach was significantly higher in calcium and polyphenols than kale and absorption from the spinach meal was 24% lower compared to the kale meal without added OA, but the difference did not reach statistical significance ($P > 0.16$).
Conclusion: Potassium oxalate did not influence iron absorption in humans from a kale meal and our findings strongly suggest that OA in fruits and vegetables is of minor relevance in iron nutrition.
Key words: iron absorption, oxalic acid, organic acids, stable isotopes
INTRODUCTION

Oxalic acid (OA) is a common constituent of plant foods such as green leafy vegetables, rhubarb, parsley, beetroot, carrots, potatoes, cocoa, and tea (Zarembski and Hodgkinson, 1962; Chai and Liebman, 2005). Concentrations vary depending on season, variety, age, soil conditions, maturity and part of the plant (Kasidas and Rose, 1980). The largest amounts, up to 1-2 g/100 g wet weight, are found in rhubarb (Honow and Hesse, 2002) and spinach, mangold, and purslane (Chai and Liebman, 2005). OA may be present in foods as insoluble calcium or magnesium oxalate crystals or as soluble sodium or potassium oxalate. Relative contributions of these two fractions to total OA seem to vary widely within and between plant species. Zarembski and Hodgkinson analysed OA contents of 80 food items commonly used in English homes and hospitals (Ait-Oukhatar et al., 2002) and calculated the average daily intake to be 118 mg in the household setting and to range between 70-150 mg in six hospital diets (Zarembski and Hodgkinson, 1962). This compares to results from a small study including five subjects, in which mean daily OA intake was estimated to be 152 mg (range 44-351 mg) (Holmes and Kennedy, 2000). Additionally, the human body is itself able to synthesise oxalic acid, with ascorbic acid and glycine being the major precursors (Hodgkinson and Zarembski, 1968).

OA is a well-known inhibitor of calcium absorption in humans (Heaney et al., 1988; Heaney and Weaver, 1989), and has been reported to decrease zinc (Kelsay and Prather, 1983) and magnesium (Bohn, 2003) absorption. Formation of insoluble oxalate complexes is the most likely explanation based on the observation that addition of calcium decreased OA absorption from the diet in a dose-dependent fashion (von Unruh et al., 2004). However, surprisingly little attention has been paid to the effect of OA on iron bioavailability. Gillooly et al. (1983) reported a 39% reduction in iron absorption from a vegetable soup in the presence of 1 g calcium oxalate (corresponding to a Fe:OA molar ratio of 1:144). However, it is impossible to discern whether this inhibition was caused by OA or the contained calcium (~300 mg), which maximally inhibits non-haem iron absorption at this dose (Hallberg et al., 1991). In contrast, iron absorption from a rice meal increased by 68% when 100 g rhubarb (537 mg OA) were added (Ballot et al., 1987), however, data interpretation is also difficult as rhubarb is high in malic acid (1.7 g/100 g), which may
have enhanced iron absorption although human studies have yielded equivocal results (Gillooly et al., 1983; Ballot et al., 1987). Results from rat studies showed a neutral to enhancing effect of adding purified OA to the diet (Van Campen and Welch, 1980; Gordon and Chao, 1984). In Caco-2 cells, OA reduced ferrous iron by 20% but increased ferric iron uptake fivefold, with maximum stimulation at a molar ratio of ~1:80 (Fe:OA) (Salovaara et al., 2002). The higher solubility of ferric oxalate (Hodgkinson and Zarembski, 1968) may explain the observed differences.

To better define the influence of dietary OA on iron bioavailability, we compared the absorption of non-haem iron from meals containing spinach (high OA), kale (low OA) and kale plus added OA in human volunteers. Both vegetables are reported to contain similar amounts of polyphenols (~100 mg/100 g wet weight) (Ninfali and Bacchiocca, 2003; Chun et al., 2004), an important class of inhibitors of iron absorption (Hurrell et al., 1999), but may vary in content. Ascorbic acid is a potent enhancer of iron absorption (Cook and Monsen, 1977; Hallberg et al., 1986) and kale has been reported to contain 105 mg/100 g compared to 52 mg/100 g in spinach (Souci et al., 1994). Iron absorption was measured based on the incorporation of stable iron isotope labels into erythrocytes (Kastenmayer et al., 1994; Walczyk et al., 1997).

SUBJECTS AND METHODS

Subjects

Sixteen apparently healthy women (aged 19-38 y; maximum body weight 60 kg) were recruited from the staff and student populations of ETH Zurich, University of Zurich and the University Hospital Zurich, Switzerland. The subjects participated in two studies, which were performed in a randomised, cross-over design. Exclusion criteria included pregnancy or lactation and known gastrointestinal or metabolic disorders. No medication (except oral contraceptives) or vitamin or mineral supplements were allowed during the study. Women regularly taking vitamin or mineral supplements discontinued supplementation 2 wk before the start of the study. No subjects were recruited who had donated blood within 4 months of the beginning of the study or who were planning to donate blood during the study period. The study protocol was reviewed and approved by the Ethical Committees of ETH Zurich and of the canton of Zurich. Subjects were informed orally and in writing about
the aims and procedures of the study. Written informed consent was obtained from all study subjects.

Test meals

The control meal (meal A) consisted of two wheat bread rolls (100 g) and 150 g spinach. The bread rolls were prepared in batches by mixing 1 kg low extraction wheat flour with high-purity water (18 MΩ, 600 g), salt (10 g), white sugar (32 g) and dry yeast (15 g). After fermentation for 5 h at room temperature, rolls were prepared from the dough, baked for 15 min at 200°C and stored at -25°C. Frozen spinach leaves were purchased at a local supermarket and heated in batches of 1 kg in steel pots until spinach leaves were thawed and started boiling. The spinach was left to boil for another 5 min, samples were pureed with a hand mixer, pooled, and 250 g of butter and 35 g of table salt (no added iodine or fluorine) added to improve taste. Samples of 150 g were weighed into aluminum trays and stored at -25°C until use.

Test meal B (study I) consisted of two wheat bread rolls and 150 g of kale. Fresh kale from a local supermarket was washed under cold tap water, trimmed of the midribs and boiled in tap water for 15 min. After removal of excess water, blanched leaves were pureed with a hand mixer, flavoured with 250 g of butter and 35 g of table salt, and boiled for another 10 min with repeated stirring. After pooling of individually prepared kale batches, samples of 150 g each were weighed into aluminum trays and stored at -25°C until use.

Test meal C (study II) was identical to meal B, except that subjects received an additional OA drink (20 ml, 1.26 g OA) made freshly each day by dissolving an appropriate amount of potassium oxalate monohydrate (Fluka, Buchs, CH) in high-purity water. Total iron content of all kale meals (1.38 mg) was balanced to spinach meal Fe levels (2.68 mg) by adding unlabeled ferrous sulphate (1.30 mg Fe). High-purity water (400 g; 380 g for kale plus OA meals) was served as a drink.

Subjects received a peppermint-flavoured Wrigley’s Eclipse® Flash strip after each meal to alleviate any unpleasant aftertaste.

Study design

Two separate studies were made. In study I, iron absorption from kale (meal B, 0.01 g OA) was compared with iron absorption from spinach (meal A, 1.27 g OA). In study 2, iron absorption from kale with 1.26 g added OA served as a drink (meal C,
1.27 g OA) was compared to spinach meal A. Comparisons were made between morning and lunch meals given over three consecutive days. This meal administration was necessary as we wanted the extrinsic tag to contribute as little iron as possible to total meal iron (Fairweather-Tait and Dainty, 2002). Each subject received a total of twelve test meals (study I: 3xA, 3xB; study II: 3xA, 3xC). One type of meal was administered in the morning and the other at lunch. A few days prior to the first test meal administration (day 0), a baseline venous blood sample was drawn after an overnight fast for determination of iron status (plasma ferritin, haemoglobin) and subjects’ height and weight was recorded. Iron absorption was determined with the use of a double stable-isotope technique and based on erythrocyte incorporation of $^{57}$Fe or $^{58}$Fe 14 d after test meal administration. The first pair of test meals (A/B or A/C) was fed on three consecutive days (day 1, 2, and 3) between 0700 and 0900, after the subjects had fasted overnight, and 4 h later under standardised conditions. No food or drink was allowed between breakfast and lunch meals and within 3 h after the lunch meals. Fourteen days later (day 17), a second blood sample was drawn and the second pair of test meals (A/C or A/B) was administered on three consecutive days (day 17, 18, and 19) under the same conditions. Another 14 d later (day 33), a final blood sample was drawn. Meals were compared in a fully randomised cross-over design, with each subject acting as her own control. By administering the dose of iron label over the course of three days, we achieved a contribution of the extrinsic tag of 31% to the total meal iron in the spinach meal and 61% in the kale meals (due to the lower iron content). The extrinsic tag has been shown to be a valid approach up to a level of 20% for dietary iron absorption studies using complete meals due to rapid isotopic exchange and the formation of a common non-haem iron pool, with a suggestion that higher levels are also acceptable (Cook et al., 1972; Bjorn-Rasmussen et al., 1973).

**Stable-isotope labels**

Isotopically labeled $^{57}$FeSO$_4$ and $^{58}$FeSO$_4$ were prepared from isotopically enriched elemental iron (Chemgas, Boulogne, F) dissolved in dilute sulphuric acid. The solutions were stored in polytetrafluoroethylene containers flushed with argon to keep the iron in the 2+ oxidation state.
Quantification of iron isotope labels

Isotope dilution mass spectrometry was used to determine the concentration of the isotopic labels in solution. To aliquots taken from the prepared isotopic labels, an accurately known amount of iron of natural isotopic composition was added. The used iron standard was prepared gravimetrically from an isotopic reference material (IRM-014, EU Institute of Reference Materials, Geel, BE). Isotopic analysis was performed using negative thermal ionisation-mass spectrometry (Walczyk, 1997). Iron concentrations in the isotopic labels were calculated on the basis of the shift in iron isotopic abundances, the determined isotopic abundances of the pure isotopic labels, and the natural iron isotopic abundances (Walczyk et al., 1997).

Iron status measurements

Venous blood samples (7 ml) were drawn into EDTA-treated vacutainers (368453, Becton Dickinson, Milian SA, Meyrin, CH) a few days before the first test meal administration and again 14 d after the first and second set of test meals on day 17 and day 33 of the study, respectively. Samples were analysed for iron status indexes (plasma ferritin, haemoglobin; day 0) and for the incorporation of $^{57}$Fe and $^{58}$Fe into erythrocytes (day 17, day 33). Haemoglobin was measured in fresh whole blood using the cyanmethaemoglobin method (D5941; Sigma, Buchs, CH). Plasma was separated and stored at -25°C for later ferritin analysis with the use of an enzyme immunoassay (Immulite®; DPC Bühlmann GmbH, Salzburg, AT). Commercial quality control materials for haemoglobin (Digitana AG, Horgen, CH) and ferritin (Immulite®) were run with each analysis.

Quantification of iron isotope in the blood

Each isotopically enriched blood sample was analysed in duplicate for its iron isotopic composition as previously described by Walczyk et al (1997). The blood samples were mineralised by using a mixture of nitric acid and hydrogen peroxide and microwave digestion. The iron was separated from the matrix by anion-exchange chromatography and a solvent-solvent extraction step into diethylether. The isotopic analyses were performed by negative thermal ionisation-mass spectrometry (Walczyk, 1997).
Calculation of iron absorption

The amounts of $^{57}$Fe and $^{58}$Fe isotopic labels in blood 14 d after administration of the test meals were calculated on the basis of the shift in iron isotope ratios and on the amount of iron circulating in the body. The calculations were based on the principles of isotope dilution and took into account that iron isotopic labels were not monoisotopic (Walczyk et al., 1997). Circulating iron was calculated on the basis of blood volume and haemoglobin concentration (Kastenmayer et al., 1994). Blood volume calculations were based on height and weight according to Brown et al (1962). For calculations of fractional absorption, 80% incorporation of the absorbed iron into erythrocytes was assumed (Hosain et al., 1967).

Food analysis

The iron and calcium contents of the wheat bread rolls, spinach, and kale were analysed by graphite tube- and flame-atomic absorption spectroscopy (SpectrAA 400; Varian, Mulgrave, AUS), respectively, after mineralisation by microwave digestion (MLS-ETHOS with easyWAVE software version 3.5.4.0; Egrolyt Laborgeräte, Oberwil, CH) in a mixture of HNO$_3$ and H$_2$O$_2$. Soluble and total oxalic acid were extracted with water and 2 M HCl, respectively, from cooked spinach and kale samples and measured with an enzymic assay (33-591C-1KT Oxalate Kit S, Trinity Biotech, Wicklow, IRL) according to the manufacturer’s instructions. Phytic acid was quantified spectrophotometrically (Makower, 1970) on an MRX microplate reader (Dynatech Laboratories, Guernsey, Channel Islands, UK), with the modification that iron was replaced by cerium in the precipitation step. Total polyphenol content of spinach and kale was determined using the Folin-Ciocalteu method (Singleton et al., 1999) and results are expressed as gallic acid equivalents (GAE). Ascorbic acid in spinach and kale was measured by RP-HPLC (Sapers et al., 1990; Nyyssönen et al., 1992).

Statistics

Student’s paired $t$-test was used to evaluate data within each study. Values were logarithmically transformed before statistical analysis (EXCEL 2002 SP3, Microsoft Corporation, Redmond, WA, USA). Results are presented as geometric means (-1 SD, +1 SD). Post-hoc power calculations for paired $t$-tests were performed using GraphPad StatMate for Windows, version 2.00 (GraphPad Software, San Diego,
USA). Student’s unpaired t-test was used for inter-study analysis and comparison of nutrient composition between the different meals. All results were considered significant at $P < 0.05$.

**RESULTS**

**Test meals**

The contents of iron, calcium, ascorbic acid, phytic acid, oxalic acid, and polyphenols in the different test meals are shown in Table 1. The OA content of cooked spinach was 848 mg/100 g, of which 271 mg were soluble oxalates. Cooked kale OA content amounted to 8 mg/100 g, with 3 mg being soluble. Iron and calcium concentrations were found to be 1.3 mg/100 g and 189 mg/100 g cooked spinach, respectively. In comparison, cooked kale contained 0.4 mg Fe/100 g and 14 mg Ca/100 g ($P < 0.0001$ for difference between spinach and kale calcium). The measured content of polyphenols in cooked spinach was 95 mg GAE/100 g, whereas cooked kale contained 48 mg GAE/100 g ($P < 0.0001$ for difference). Ascorbic acid content was 10 mg/100 g cooked spinach and 3 mg/100 g cooked kale, the difference between the vegetables being statistically significant ($P = 0.0012$). Phytic acid was below the limit of detection (< 0.0035%) in freeze-dried samples of cooked spinach, cooked kale, and bread rolls.

**Subjects**

Thirteen of the sixteen subjects initially recruited completed the two studies. The reason for dropout was taste aversion. Mean body weight was 55.8±5.4 kg and mean age was 25.1±4.7 y. One of the subjects displayed borderline haemoglobin with low iron stores (Hb: 119 g/l; plasma ferritin: 12 µg/l) and one had depleted iron stores (plasma ferritin: < 12 µg/l).

**Iron absorption**

The iron absorption data are shown in Tables 2 and 3. Geometric mean iron absorption in study I was 8.4% from spinach and 11.0% from kale (absorption ratio A/B: 0.76, $P = 0.19$). Geometric mean iron absorption in study II was 8.0% from spinach and 11.2% from kale plus OA (absorption ratio A/C: 0.72, $P = 0.16$). The average serum ferritin of the subjects in studies I and II was similar at 28 and
30 µg/L, respectively. Thus when compared to kale, spinach had a 24% lower absorption which failed to reach statistical significance. The addition of 1.26 g OA to kale did not change the absorption ratio, and again a 28% decrease in iron absorption was not significant. Two subjects in each study absorbed much less iron from kale than from spinach.

Absorption from kale plus OA (meal C, 11.2%) was similar to kale alone (meal B, 11.0%, \( P = 0.59 \)) and when these absorption values were normalised for the small differences in the respective spinach meal (meal A), the absorption values (11.5% vs 10.7%) were still not significant (\( P = 0.86 \)). The added OA (1.26 g) therefore did not influence iron absorption.

DISCUSSION

The present study clearly shows that OA added as 1.26 g soluble potassium oxalate to a kale meal does not influence iron absorption in humans. It would be expected therefore that soluble or insoluble oxalates in plant foods are of minor relevance in iron nutrition. This is in line with the report of no relationship between human iron absorption and the OA content of the three OA-rich vegetables, spinach, beetroot greens, and beetroot (Gillooly et al., 1983). The parallel finding by the same authors that 1 g of calcium oxalate added to a cabbage soup meal decreased iron absorption significantly from 32.0% to 19.5% (Gillooly et al., 1983) could be explained by the inhibitory nature of the added 300 mg calcium (Hallberg et al., 1991). Similarly, the slightly enhancing effect of 100 g rhubarb (537 mg OA) on a rice meal (200 g) (Ballot et al., 1987) can be explained by the malic acid (1.7 g/100 g) and citric acid (410 mg/100 g) it contains, as both these compounds at high levels can increase iron absorption (Gillooly et al., 1983).

The reason why oxalic acid has no influence on iron absorption but decreases absorption of calcium (Heaney et al., 1988; Heaney and Weaver, 1989), magnesium (Bohn, 2003), and zinc (Kelsay and Prather, 1983) is presumably related to the respective solubility and complex stability constants (Table 4). The poor water solubility of calcium and zinc oxalate (< 1 mg/100 ml) could explain the inhibitory effect of oxalate on zinc and calcium absorption. Magnesium oxalate is more soluble but the solubility is still only 70 mg/100 ml. Both ferrous and ferric iron form stable oxalate complexes (\( K_s > 4.7 \)), however ferrous oxalate is rather insoluble
(22 mg/100 ml) compared to ferric oxalate which is described as very soluble (Hodgkinson and Zarembski, 1968). Presumably most of the meal iron in the present study was in the ferric form in the gastric and duodenal phases of digestion. It is possible that when iron is in the ferrous form, as in meals high in ascorbic acid, oxalic acid may inhibit iron absorption by forming the more insoluble ferrous oxalate. It could also be speculated that in the present study, meal iron in the gastrointestinal tract was equally in the ferrous and ferric form and whereas OA increased the absorption of ferric iron, it reduced the absorption of ferrous iron, so the net effect would be no influence on iron absorption. Some support to this last theory is given by Caco-2 cell experiments, which have reported a fivefold enhancing effect of OA on the uptake of ferric iron but a 20% decrease in the uptake of ferrous iron, both given as the pure iron salts (Salovaara et al., 2002).

In our study, there was a 24% lower iron absorption from spinach than from kale which failed to reach statistical significance. The lack of statistical significance can be explained by the study design which aimed to detect a 30% difference at 80% power. We would like to emphasise that although most subjects had lower iron absorption from spinach than from kale, two subjects in each study had much lower iron absorption from kale than from spinach. Due to the higher variability in iron absorption values than we observe normally in comparable studies with comparable subject dropout numbers, we would have needed a 42-49% reduction in iron absorption from spinach compared to kale meals to reach statistical significance.

It would be expected from the literature that spinach inhibits iron absorption. In the study of Gillooly et al. (1983), iron absorption from spinach was extremely low (1.4%) when compared with other vegetables such as potatoes, carrots, cabbage, and broccoli, and Brune et al. (1989) showed that spinach inhibited iron absorption from a bread meal by 38%. It has always been assumed that the high polyphenol content was responsible for its inhibiting effect on iron absorption (Gillooly et al., 1983; Brune et al., 1989). Thus the apparently higher iron absorption from the kale meals relative to the spinach meals in our study could be due to the twofold higher polyphenol content in the spinach meal. Calcium, however, was also more than ten times higher in spinach than kale and the 300 mg present would also be expected to have an inhibitory effect on iron absorption (Hallberg et al., 1991).
Our results strongly suggest that OA in plant foods does not inhibit iron absorption, and that OA does not contribute to the reported inhibitory effect of spinach in iron absorption.

ACKNOWLEDGMENTS

The help of Eberhard Denk, Ralf Biebinger, and Ines Egli in performing the human studies is greatly appreciated. Special thanks go to Christophe Zeder for preparing the isotope solutions and helping with data analysis, to Karin Hotz for meal iron and calcium determination and to Charlotte Züllig and Marlies Krähenbühl for taking blood samples.
REFERENCES


### Table 1 Test meal composition

<table>
<thead>
<tr>
<th></th>
<th>Bread with spinach (A)</th>
<th>Bread with kale (B)</th>
<th>Bread with kale + OA (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Native Fe [mg]</td>
<td>2.68</td>
<td>0.13</td>
<td>1.38</td>
</tr>
<tr>
<td>added Fe(^1) [mg]</td>
<td></td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Calcium(^2) [mg]</td>
<td>284</td>
<td>0.9</td>
<td>25</td>
</tr>
<tr>
<td>Ascorbic acid(^3) [mg]</td>
<td>15.4</td>
<td>0.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Oxalic acid [mg]</td>
<td>1272</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>- total</td>
<td>407</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Oxalic acid added(^2) [mg]</td>
<td></td>
<td>1260</td>
<td></td>
</tr>
<tr>
<td>Phytic acid [%]</td>
<td>&lt;0.0035</td>
<td>&lt;0.0035</td>
<td>&lt;0.0035</td>
</tr>
<tr>
<td>Polyphenols(^3) [mg]</td>
<td>142</td>
<td>4</td>
<td>72</td>
</tr>
</tbody>
</table>

\(^1\) added as ferrous sulphate heptahydrate (2.6 mg Fe/ml) in 0.01 M HCl

\(^2\) served as an additional drink made from potassium oxalate monohydrate and containing 1.26 g/20 g oxalic acid

\(^3\) significantly different between kale and spinach meals, \(P < 0.002\) (Student’s t-test)
## Table 2: Iron absorption from wheat roll meals with spinach or kale

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Serum ferritin</th>
<th>Spinach (%)</th>
<th>Kale (%)</th>
<th>A:B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study I (13F)</td>
<td>µg/l</td>
<td>% of dose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>7.2</td>
<td>9.6</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>4.3</td>
<td>13.1</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>93</td>
<td>4.5</td>
<td>12.6</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>5.6</td>
<td>2.9</td>
<td>1.90</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>9.2</td>
<td>13.0</td>
<td>0.71</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>15.4</td>
<td>33.2</td>
<td>0.46</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>15.8</td>
<td>37.6</td>
<td>0.42</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>6.4</td>
<td>5.3</td>
<td>1.21</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>10.6</td>
<td>16.3</td>
<td>0.65</td>
</tr>
<tr>
<td>10</td>
<td>49</td>
<td>7.2</td>
<td>4.7</td>
<td>1.53</td>
</tr>
<tr>
<td>11</td>
<td>53</td>
<td>6.9</td>
<td>2.5</td>
<td>2.80</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>18.5</td>
<td>33.9</td>
<td>0.55</td>
</tr>
<tr>
<td>13</td>
<td>15</td>
<td>8.9</td>
<td>13.3</td>
<td>0.67</td>
</tr>
<tr>
<td>mean</td>
<td>28</td>
<td>8.4</td>
<td>11.0</td>
<td>0.76²</td>
</tr>
</tbody>
</table>

(-1SD; +1SD) (5; 51) (5.2; 13.3) (4.5; 26.8) (0.39; 1.48)

1 Geometric mean (-1 SD; +1 SD). Subjects aged 19-38 y. F, female
2 P = 0.16 (Student’s paired t-test)
Table 3 Iron absorption from wheat roll meals with spinach or kale plus added oxalic acid

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Serum ferritin µg/l</th>
<th>Spinach (A) % of dose</th>
<th>Kale + OA (C) % of dose</th>
<th>A:C Absorption ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study II (13F)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>10.6</td>
<td>14.7</td>
<td>0.72</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>2.7</td>
<td>8.4</td>
<td>0.32</td>
</tr>
<tr>
<td>3</td>
<td>93</td>
<td>6.9</td>
<td>9.4</td>
<td>0.74</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>2.9</td>
<td>8.6</td>
<td>0.34</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>7.6</td>
<td>14.1</td>
<td>0.54</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>11.4</td>
<td>11.5</td>
<td>1.00</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>18.5</td>
<td>40.4</td>
<td>0.46</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>18.5</td>
<td>15.6</td>
<td>1.19</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>2.1</td>
<td>9.5</td>
<td>0.22</td>
</tr>
<tr>
<td>10</td>
<td>53</td>
<td>8.2</td>
<td>2.0</td>
<td>4.18</td>
</tr>
<tr>
<td>11</td>
<td>63</td>
<td>8.2</td>
<td>2.2</td>
<td>3.64</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>23.8</td>
<td>42.5</td>
<td>0.56</td>
</tr>
<tr>
<td>13</td>
<td>15</td>
<td>9.7</td>
<td>24.0</td>
<td>0.40</td>
</tr>
<tr>
<td>mean</td>
<td>30</td>
<td>8.0</td>
<td>11.2</td>
<td>0.72 2</td>
</tr>
</tbody>
</table>

(-1SD; +1SD) (6; 55) (3.8; 17.0) (4.5; 27.9) (0.30; 1.73)

1 Geometric mean (-1 SD; +1 SD). Subjects aged 19-38 y. F, female; OA, oxalic acid; added OA (1.26 g) was included in the meal as a potassium oxalate solution
2 \( P = 0.19 \) (Student’s paired \( t \)-test)

Table 4 Stability constants and solubilities of some oxalic acid salts (modified from Hodgkinson and Zarembski, 1968)

<table>
<thead>
<tr>
<th>Oxalate salt</th>
<th>Stability constant 1 ( (\log K_\text{s}) )</th>
<th>Solubility ( (\text{mg/100 ml}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium oxalate</td>
<td>3</td>
<td>0.67 (13°C)</td>
</tr>
<tr>
<td>Magnesium oxalate</td>
<td>2.76 (20°C)</td>
<td>70.0 (16°C)</td>
</tr>
<tr>
<td>Zinc oxalate</td>
<td>4.9</td>
<td>0.79 (18°C)</td>
</tr>
<tr>
<td>Ferrous oxalate</td>
<td>&gt;4.7</td>
<td>22</td>
</tr>
<tr>
<td>Ferric oxalate</td>
<td>9.4</td>
<td>very soluble</td>
</tr>
</tbody>
</table>

1 \( T = 25°C, I = 0.1 \) unless stated otherwise.
Conclusions and Perspectives

The present thesis has looked into the relationship between two dietary constituents, meat and oxalic acid, and iron bioavailability. Equivocal results regarding a potential inhibitory effect of oxalic acid have been reported in the literature and these uncertainties were now clarified in a human stable isotope study. Excess oxalic acid showed no effect on iron absorption and fruits and vegetables high in oxalic acid would not be expected to influence iron absorption in humans.

With regard to meat, the initial purpose was to identify and isolate low-molecular-weight (LMW) iron-binding peptides from various meat sources (beef, chicken, cod, lamb, and pork) which would enhance iron absorption in humans. Once isolated, such peptides could be used as a more stable alternative to ascorbic acid in an industrial setting for improving the absorption of fortification iron added to infant foods or dietetic products. Strong evidence exists that LMW iron-binding meat peptides constitute the long sought “meat factor” or at least a vital part thereof. Previous literature reports showed cysteine (Taylor et al. 1986; Mulvihill and Morrissey 1998; Vattem et al. 2001) and histidine (Seth and Mahoney 2000; Swain et al. 2002) as important structural features. We, however, identified a new peptide fraction of 2 kDa molecular mass prepared from myofibrillar meat proteins and enriched in aspartic and glutamic acid that binds and solubilizes iron. Notably, pepsin digestion alone was sufficient to obtain these and other peptides. Whether the “meat factor” is due to the iron-binding nature of a few single peptides or due to a much larger set of LMW peptides we cannot tell from our data. However, the diverse amino acid and peptide structures that iron is capable of binding with would argue for more than one and probably a multitude of peptides.

Taken together with the published literature, our results suggest that iron is solubilized by the LMW meat peptides released during pepsin digestion in the stomach. Early binding in the stomach or proximal duodenum prevents interaction of the iron with absorption inhibitors such as phytic acid or polyphenols and delivers the iron in a soluble, LMW form to the absorptive sites as was postulated by Terato et al. (1973). Our experiments indicate that the “meat factor” is probably less well defined than initially hypothesized and co-migrates with a multitude of other peptides.
This makes isolation difficult if not impossible and questions any benefit for industrial exploration.

Although glycosaminoglycans (GAGs) present in muscle interconnective tissue were recently proposed as an additional meat-derived promoter of iron absorption (Huh et al. 2004), we could not demonstrate an influence of hyaluronic acid and chondroitin sulfate, which may be considered representative of unsulfated and sulfated GAGs respectively. From our findings, it seems unlikely that GAGs can improve iron absorption. However, isolation of the purified compounds may have changed their chemical structure and with it their biological activity. Additionally, we only tested two compounds out of a wide variety of possible structures. Caco-2 data indicated fragments of oversulfated heparan sulfate extracted from cooked fish and chicken as potent stimulants of iron uptake (Huh et al. 2004). Evaluation of such extracts in a human absorption study would be helpful to make more conclusive statements about the role of GAGs in iron nutrition.

References


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