THE *IN VITRO* ASSESSMENT OF THE BIOAVAILABILITY OF IRON IN NEW ZEALAND BEEF

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ABSTRACT

The bioavailability of iron in New Zealand beef either alone or as part of a 'typical' New Zealand meal was investigated. The solubility of iron and its *in vitro* absorption by mouse intestinal tissue were used to evaluate iron bioavailability.

The solubility of haem and/or non-haem iron in meat (beef longissimus muscle), vegetables and meat-plus-vegetables was investigated. Samples were cooked and then subjected to *in vitro* gastrointestinal digestion with pepsin followed by a combination of pancreatic enzymes and bile. Cooking at 65°C for 90 minutes reduced the soluble iron concentration in meat by 81% and reduced the haem iron concentration by 27%, which coincided with a 175% increase in non-haem iron concentrations. However, gastrointestinal digestion increased the solubility of iron in cooked meat (333%), vegetables (367%) and meat-plus-vegetables (167%). A proportion (35%) of the haem iron in the meat was broken down by the action of pancreatic enzymes leading to a 46% increase in non-haem iron concentrations, although this was not the case for the meat-plus-vegetables.

Validation studies showed that mouse intestinal segments mounted in Ussing chambers maintained integrity and viability, and were responsive to glucose, theophylline and carbachol. Intestinal tissue from iron deficient mice was then used in the Ussing chambers to investigate the absorption of iron from ferrous gluconate and the soluble fractions of meat, vegetables and meat-plus-vegetables after gastrointestinal digestion. Results indicated a trend towards a higher absorption of iron from meat and ferrous gluconate, compared to vegetables and meat-plus-vegetables. However, iron absorption results were difficult to interpret due to the wide variation in the data. This variation was possibly due to errors associated with the sample processing and the analysis of iron, which was by inductively coupled-mass spectroscopy.

Overall, the present study showed that before estimations can be made on the bioavailability of food iron, the effects of the cooking and gastrointestinal digestion processes must be considered. Further, the use of *in vitro* gastrointestinal digestion followed by the use of Ussing chambers to assess intestinal absorption is a potentially valuable system for assessing mineral bioavailability.

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%	percent
µAmps	microamperes
µAmps/cm^2	microamperes per square centimetre
µg	micrograms
µg/g	micrograms per gram
µg/g/min	micrograms per gram per minute
µm	micrometre
µl	microlitre
Ω	ohm
Ω/cm^2	ohms per square centimetre
AA	atomic absorption spectroscopy
ANOVA	Analysis of Variance
ATP	adenosine triphosphate
°C	degrees Celsius
$CaCl_2$	calcium chloride
Caco-2	human colonic adenocarcinoma cell line
cAMP	cyclic adenosine monophosphate
cm	centimetre
cm^2	square centimetre
CO_2	carbon dioxide
Cook	after cooking
CRM	Certified Reference Material
CV	coefficient of variation
DCT-1	divalent cation transporter-1
Dcyt b	duodenal cytochrome b
df	degrees of freedom
DMT-1	divalent metal transporter-1
DNA	deoxyribose nucleic acid
f	f statistic
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
$FeCl_2$	ferrous chloride
$FeCl_3$	ferric chloride
Fe glu	ferrous gluconate
[Fe-S]	iron-sulphur
fL	femtolitre
FP-1	ferroportin-1
g	grams
g	g force
g/L	grams per litre
g/kg	grams per kilogram
GLM	general least squares model
H	haem iron
ICP-MS	inductively coupled plasma-mass spectrometry
IRE	iron-responsive element

IREG-1	iron-regulating protein-1
IRP-1	iron-regulatory protein-1
IRP-2	iron-regulatory protein-2
K_2EDTA	potassium ethylenediaminetetraacetic acid
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
M	molar
MCV	mean cell volume
mg	milligrams
$MgCl_2$	magnesium chloride
mg/day	milligrams per day
mg/kg	milligrams per kilogram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mOsm/L	millliosmoles per litre
mRNA	messenger ribonucleic acid
MS	mean square
Mt	meat
Mt + Vg	meat-plus-vegetables
MTP-1	metal transport protein-1
MUAEC	Massey University Animal Ethics Committee
mV	millivolts
n	number
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	sodium phosphate dibasic
Na_2HPO_4	sodium phosphate monobasic
ng/g	nanograms per gram
NH	non-haem iron
nm	nanometre
Nramp2	natural resistance-associated macrophage protein 2
NS	not significant
O_2	oxygen
p	probability statistic
p.	page
Panc	after pepsin and pancreatin-bile digestion
PCV	packed cell volume
Pep	after pepsin digestion
USF-2	upstream stimulatory factor-2
UTR	untranslated region
r^2	coefficient of determination
RDI	Recommended Daily Intake
RNA	ribonucleic acid
rpm	revolutions per minute
RSD	residual standard deviation
SD	standard deviation
SEM	standard error of the mean

TCA	trichloroacetic acid
SS	sum of squares
III SS	type III sum of squares
Vg	vegetables

INTRODUCTION

Iron is the most abundant trace element in the body and is vital in the nutrition of all mammalian species. The ability of this transition metal to exist in two redox states makes it useful at the catalytic centre of many biochemical reactions, including DNA synthesis, transport of oxygen and respiration. However, the same properties that make iron useful also make it toxic. Free iron is able to catalyse the formation of highly toxic oxidative free radicals that damage many important biological components, such as lipids, proteins and DNA. Despite such toxic potential, there is no physiological regulatory mechanism for excretion of iron from the body. Thus, the body must 'sense' its internal iron load and respond appropriately by altering iron absorption and storage processes.

The body is economical in its handling of iron. The iron from senescent, damaged or malformed erythrocytes is recycled and reutilised by the body. Extra iron, which is utilised at times of increased iron requirements, can be stored by a specially designated protein (ferritin) and is transported within the body between sites of absorption, storage and utilisation by transferrin. Furthermore, iron absorption is regulated by the requirements of the body. In spite of the variety of mechanisms that control iron metabolism and participate in the conservation and recycling of iron, iron deficiency is the most common deficiency disorder in the world. In most cases, attempts to remedy iron deficiency have been by way of orally administered iron supplements. However, improving the diet may play a more important role in the prevention of iron deficiency.

One of the leading causes of iron deficiency is low bioavailability of dietary iron. Bioavailability is defined as the proportion of the total mineral intake that is potentially available for absorption and utilisation for normal body functions (Wienk *et al.*, 1999). Thus, when evaluating foods for nutritional significance to the human body, knowledge of iron intake alone is of little value without an understanding of iron bioavailability.

Meat is a good source of iron, which *in vivo* and *in vitro* studies suggest is highly bioavailable. Furthermore, meat has been consistently shown to enhance the bioavailability of iron from other foods by increasing the solubility and intestinal absorption of iron. However, the effects of cooking and gastrointestinal digestion on the bioavailability of meat iron have not yet been fully investigated. Thus, the main aim of the research reported in this Thesis was to study the bioavailability of iron in New Zealand beef either alone or as part of a 'typical' New Zealand meal. Iron bioavailability was evaluated in two phases: firstly, by measuring the solubility of iron after cooking and gastrointestinal digestion and secondly, by measuring the *in vitro* absorption of iron by mouse intestinal tissue. Specifically, the objectives of the present study were:

1. To validate the use of total, haem and non-haem iron assays to determine these iron forms in the soluble and insoluble fractions of meat, vegetables and a combination of meat-plus-vegetables ('typical' New Zealand meal) (Chapter Two).
2. To investigate the effects of pepsin and pancreatin-bile digestion on the solubility of haem and/or non-haem iron in meat, vegetables and meat-plus-vegetables (Chapter Two).
3. To validate an *in vitro* technique using Ussing chambers for measuring iron absorption (Chapter Three).
4. To use the Ussing chamber model to investigate the absorption of iron, by mouse intestinal tissue, from ferrous gluconate and the soluble fractions of meat, vegetables and meat-plus-vegetables after gastrointestinal digestion (Chapter Four).

CHAPTER ONE

Literature review

Iron deficiency is the most prevalent single mineral deficiency worldwide. To help combat iron deficiency, food combinations and meals need to be assessed not only for total iron content but also for the bioavailability of iron, and the information used to develop diets that can be used to help prevent or treat iron deficiency. The following review focuses on mammalian metabolism of iron, including its transport, storage and absorption and the maintenance of iron homeostasis. The concept of bioavailability of iron is introduced followed by a description of *in vitro* methods for measuring iron bioavailability. Finally, the suitability of the Ussing chamber model as a means of investigating the bioavailability of iron from different food sources is discussed.

1.1 Physiological importance of iron

Iron is the most abundant trace element in the body. It is vital in the nutrition of all mammalian species. Iron is part of the haem porphyrin complex of haemoglobin in red blood cells, where it is responsible for the transport of oxygen to cells of the body, and of myoglobin, which stores oxygen in muscles for use during muscle contraction. Iron is also a component of several tissue enzymes that are critical for energy production and the immune system (Table 1.1).

Iron is a transitional element that exists mainly in two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), which can donate or accept electrons, respectively. The interconversion between these two oxidation states allows iron to reversibly bind ligands including oxygen, nitrogen or sulphur atoms. Cells exploit this ability of iron for the oxidative production of cellular energy as adenosine triphosphate (ATP). Energy generating cellular processes involve proteins that contain iron-sulphur [Fe-S] clusters and/or haem groups (Table 1.1), where the iron atoms cycle between ferrous iron (reduced) or ferric iron (oxidised) states with the donation and acceptance of electrons.

Table 1.1: Major iron-containing proteins and their functions in the body. Adapted from Stryer (1995).

Protein	Form of iron	Function
Haemoglobin	4 haem groups	Oxygen transport
Myoglobin	1 haem group	Storage of oxygen in tissues
NADH-Q reductase	3[4Fe-4S] + 3[2Fe-2S]	Mitochondrial electron transport chain
Cytochrome reductase	[2Fe-2S] + 3 haem groups	
Cytochrome oxidase	2 haem groups	
Cytochrome c	1 haem group	
Aconitase	[4Fe-4S]	Citric acid cycle enzyme
Succinate dehydrogenase	[2Fe-2S] + [3Fe-3S] + [4Fe-4S] + 1 haem group	
Cytochrome P_{450}s	1 haem group	Detoxification and degradation of aromatic hydrocarbons, xenobiotics and endogenous substrates
Catalases	4 haem groups	Protects the cell from the toxic effect of hydrogen peroxide
Perioxidases	4 haem groups	Bacterial killing (within neutrophils)
Ribonucleotide reductase	$2[2Fe\text{-}O_2]$	DNA synthesis

1.1.1 Iron requirements

Table 1.2 shows the recommended daily intake (RDI) of iron for different population groups, as suggested by the Nutrition Taskforce (1991).

Table 1.2: Recommended total daily dietary intake of iron adopted by New Zealand.

Population group	RDI (mg/day)
Infants (7 to 12 months)	9
Children (1 to 11 years)	6 - 8
Teenage (12 to 18 years)	10 - 13
Females	
Women (19 to 54 years)	12 - 16
Postmenopausal (54+ years)	5 - 7
Pregnancy	22 - 36
Men (19+ years)	7

As reviewed by Hallberg (2001), a number of factors increase the amount of iron required by the body. Growth and development increase the body's need for iron and hence, iron requirements are high for infants, children and adolescents. Females of childbearing age have higher iron requirements than males, due to loss

of iron during menstruation, until menopause when iron requirements drop to match those of males. Pregnancy increases iron requirements, especially in the last two trimesters, due to the demands of the rapidly growing foetus. Often during pregnancy iron need cannot be met by diet alone and supplementary iron must be provided.

1.1.2 Iron deficiency

Iron deficiency is a progressive condition that occurs when the body's iron stores become depleted. There are several reasons why this may occur, including inadequate dietary iron intake and/or low bioavailability of dietary iron, loss of blood (e.g. menstruation), impaired absorption or an increase in the body's requirement for iron (e.g. pregnancy, and times of increased growth in childhood and adolescence). As iron deficiency continues, synthesis of iron-containing proteins, such as haemoglobin, becomes compromised. Finally, when iron depletion becomes severe and haemoglobin concentration in blood falls below a specified value, iron deficiency has progressed to iron deficiency anaemia (Hallberg, 2001).

Iron deficiency anaemia is a major nutritional problem in both developed and developing countries. An estimated 30% of the world's population have anaemia, with many more having depleted iron stores and are at risk of developing anaemia. In 1998, the World Health Organization estimated that approximately 50% of children, 42% of women and 26% of men are affected by iron deficiency anaemia in developing countries (Turner, 2002). In the United States alone, 9% (~700,000) of toddlers aged 1 to 2 years and 9 to 11% (~7.8 million) of adolescent girls and women of childbearing age were found to be iron deficient; of these latter two groups, 3% (~240,000) and 2 to 3% (~3.3 million), respectively, have iron deficiency with anaemia (Looker *et al.*, 1997).

Within New Zealand, the estimated prevalence of inadequate iron intake for males is low (1%), but higher in females at 26% (Russell *et al.*, 1999). Among females, the percentage with inadequate intake is 45%, 39% and 42% for those in the age groups 15 to 18, 19 to 24 and 25 to 44, respectively, compared to 2 to 3% for

those over the age of 45. Between 7 to 13% of females in the New Zealand population have low iron stores (Ferguson *et al.*, 2001), with a prevalence of 6 to 7% for both iron deficiency and iron deficiency anaemia (Russell *et al.*, 1999). This prevalence corresponds to approximately 210,000 New Zealand women with iron deficiency and a similar statistic for iron deficiency anaemia. Furthermore, a study of New Zealand children aged 9 to 23 months admitted to Starship Children's Hospital during 1997 revealed 29% had iron deficiency anaemia (Wilson *et al.*, 1999).

The consequences of iron deficiency are well recognised. In infancy and childhood, iron deficiency with anaemia has a negative impact on growth and development and is associated with impaired cognitive function (Lozoff *et al.*, 1987; Walter *et al.*, 1989). These effects may not be reversed with iron therapy (Deinard *et al.*, 1986; Lozoff *et al.*, 1987, 1996). In adults, iron deficiency with or without anaemia is commonly associated with disturbances in work capacities (Woodson *et al.*, 1978; Celsing *et al.*, 1986; Li *et al.*, 1994; Zhu and Haas, 1997, 1998) and poor pregnancy outcomes (Murphy *et al.*, 1986; Steer *et al.*, 1995), as indicated by low birth weight, premature delivery and overall poor infant health. It is therefore important to ensure dietary iron intake is sufficient to meet daily requirements, especially for women and during childhood and adolescence when the risk of iron deficiency and development of anaemia is high.

1.2 Distribution and turnover of body iron

The human body contains about 2 to 4g of iron. Over 60% is present in the red blood cells as haemoglobin, up to 10% is present in myoglobin in muscle and 1 to 5% is contained in haem and non-haem iron-dependent enzymes; the remaining 25% is found in storage (Table 1.3).

Table 1.3: Approximate distribution of iron in adult males and females. From Groff and Gropper (1999, p. 404).

	Male (mg/kg body weight)	Female (mg/kg body weight)
Haemoglobin	31	28
Myoglobin	4	3
Haem enzymes	1	1
Non-haem enzymes	1	1
Transferrin iron	0.05	0.05
Ferritin	9	4
Hemosiderin	4	1
Total iron	50.05	38.05

1.2.1 Iron storage

Ferritin is the principal protein for iron sequestration, storage and detoxification (Harrison and Arosio, 1996). Almost all cells contain ferritin, which functions both as a safe storage molecule for iron and as a readily accessible store of iron acquired by the cell in excess of its immediate metabolic needs (Sheth and Brittenham, 2000). Mammalian ferritin consists of a protein shell (450kDa), which is composed of 24 oblong subunits (Hoare *et al.*, 1975) that are designated as heavy (H) and light (L). Each ferritin molecule is able to store up to 4,500 atoms of iron (Fischbach and Anderegg, 1965) in a ferric hydroxyphosphate polynuclear core (Harrison *et al.*, 1977).

Haemosiderin is a degraded form of ferritin in which the protein shells have partly disintegrated, allowing the iron cores to aggregate (Richter, 1984). It is usually found in lysozymes and as storage iron accumulates the proportion present as haemosiderin increases (Shoden *et al.*, 1953; Morgan and Walters, 1963).

1.2.2 Iron transport

Iron is transported between the sites of absorption, storage and utilisation by the plasma glycoprotein, transferrin (Aisen and Brown, 1977). Transferrin has a molecular weight of approximately 80kDa and consists of two homologous domains, each of which contains one high affinity ferric iron-binding site (Aisen *et al.*, 1966). Transferrin is recognised by specific membrane receptors that are 'gatekeepers' responsible for the physiological acquisition of iron by most cell types (Ponka, 1999). The iron-transferrin complex binds to the transferrin receptor and

the complex is internalised into the cell through endocytosis. Iron subsequently dissociates from transferrin in a pH-dependent fashion in an acidic, intracellular vesicular structure, known as an endosome (van Renswoude *et al.*, 1982; Rao *et al.*, 1983), and the receptor returns the apotransferrin to the cell surface, where it is released into the extracellular environment (Klausner *et al.*, 1983).

1.2.3 Recycling of body iron

Iron turnover and redistribution is a physiologically significant means of recycling iron within the body. For example, in a 70kg individual with a normal iron status, 35mg/day of iron is turned over in the plasma (Finch *et al.*, 1970). About 80% of the normal plasma iron turnover is accounted for by the release of iron from senescent, damaged or malformed erythrocytes, with approximately 0.8% of red blood cells being turned over each day in a normal individual (Adamson, 1999). Each millilitre of red blood cells contains 1mg of elemental iron, thus the need for iron for new haemoglobin synthesis amounts to 16 to 20mg of iron per day (Adamson, 1999). About 80 to 85% of iron recovered from senescent red blood cells is immediately returned to the circulation bound to transferrin and transported to erythroid marrow for haemoglobin synthesis, while some is targeted to tissues for local needs (Aisen, 1990). A small fraction is retained by the erythrophagocytosing macrophages and incorporated into the storage proteins ferritin and haemosiderin, where it may be released over the course of days or may remain indefinitely (Finch *et al.*, 1970).

1.2.4 Loss of body iron

For a healthy adult male, 1mg of total body iron enters and leaves the body on a daily basis (Table 1.4). Females have basal iron losses that total approximately 0.8mg/day, because of smaller body size and surface area. However, menstrual losses of iron in women are significant and highly variable. When averaged over a normal menstrual cycle, losses can amount to an additional 0.4 to 0.8mg/day. Therefore, the loss of iron due to menstrual and basal losses normally ranges from 1.2 to 1.6mg/day.

Table 1.4: Losses of iron from a healthy adult male. Adapted from Bothwell *et al.* (1979).

Loss from body	Amount of iron lost (mg/day)
Faecal losses Blood losses Bile and sloughed mucosal cells	 0.45 0.15
Skin losses	0.3
Urinary loss	0.1
Total iron loss	1mg/day

With more than 60% of the total body iron supply found in the red blood cells, blood loss is the most effective way for the body to lose iron. However, other than bleeding, the most important type of loss is through the sloughing of cells from the absorptive mucosa of the small intestine. Of these, duodenal enterocytes are probably most significant, because they are normally involved in iron absorption and are likely to contain significant amounts of stored iron when they end their functional life span and are released into the gut lumen (Andrews, 2000). Thus, in the absence of blood loss, losses by the body are minimal and absorbed iron is largely retained within the body. However, iron losses by the body are balanced with the precise control of intestinal iron absorption.

1.3 Iron absorption

Iron is acquired from the diet by absorption across the enterocytes of the proximal small intestine. Within the diet two kinds of iron are present: non-haem iron, which is mainly found in cereals, fruits, vegetables and meat; and haem iron, which is part of the porphyrin ring of haemoglobin and myoglobin in meat. According to Cook and Monsen (1976), haem iron represents 30 to 40% of the iron in pork and fish and 50 to 60% of the iron in beef, lamb and chicken.

The proportion of dietary iron absorbed usually ranges between 3 to 15% (Pirzio-Biroli *et al.*, 1958). Non-haem iron forms the main part of dietary iron intake by humans (Monsen, 1988), with haem iron usually constituting only 10 to 15% of the total iron intake (Hallberg, 1981). These two iron forms are transported across the enterocytes by independent pathways, described in the following sections.

1.3.1 Non-haem iron: the DMT-1 pathway

In the alkaline environment of the small intestine, most of the non-haem iron is present in the ferric state. Conversion of ferric iron to the more soluble ferrous state by a membrane-bound ferrireductase has been shown to be a prerequisite for intestinal iron absorption in mice (Raja *et al.*, 1992) and humans (Nunez *et al.*, 1994; Han *et al.*, 1995; Riedel *et al.*, 1995). Recently, a gene that appears to encode the ferrireductase has been cloned from the mouse (McKie *et al.*, 2001). The gene encodes a duodenal haem containing *b*-type cytochrome and was thus named duodenal cytochrome *b* (Dcyt *b*) (Figure 1.1).

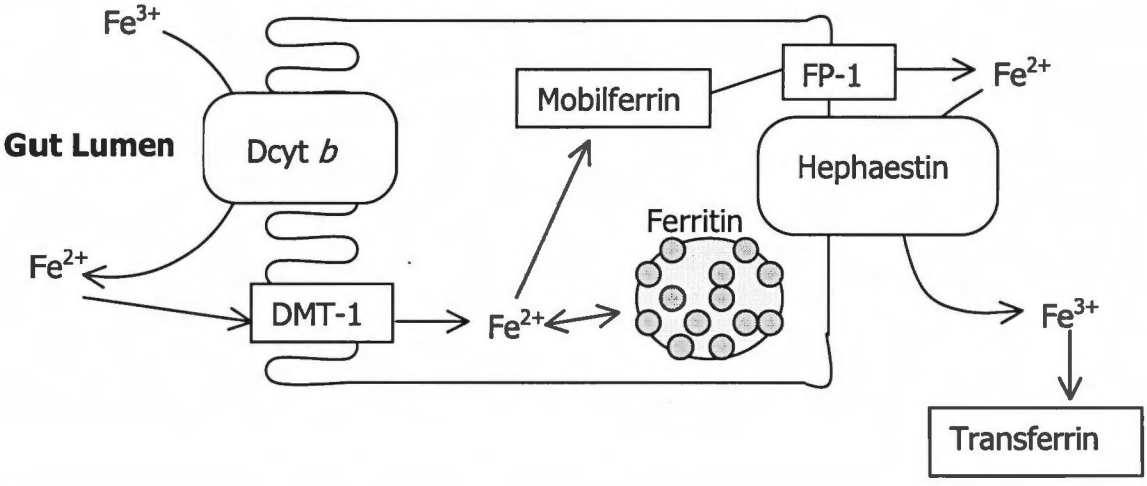


Figure 1.1: Model of non-haem iron absorption across enterocytes of the small intestine. DMT-1= divalent metal transporter-1; Dcyt *b*=duodenal cytochrome *b*; FP-1=ferroportin-1; Fe^{2+}=ferrous iron; Fe^{3+}=ferric iron.

Ferrous iron is then transported across the brush-border membrane by a transmembrane pump protein called divalent metal transporter-1 (DMT-1), which is also known as divalent cation transporter-1 (DCT-1) and natural resistance-associated macrophage protein 2 (Nramp2) (Fleming *et al.*, 1997; Gunshin *et al.*, 1997). Once in the enterocyte, the ferrous iron can follow one of two pathways depending on systemic iron load. In an iron replete state, iron is stored within the enterocyte by incorporation into ferritin, whereas in an iron depleted state, iron is transported to the basolateral membrane bound to an iron-carrying protein called mobilferrin (Conrad *et al.*, 1990).

At the basolateral membrane, ferroportin-1 (FP-1) and hephaestin are thought to be involved in the export of iron from the enterocyte (Figure 1.1). Hephaestin, a

membrane bound, copper-containing ferroxidase (Vulpe *et al.*, 1999) and FP-1, also known as iron-regulating protein-1 (IREG-1) or metal transport protein-1 (MTP-1), are similar to DMT-1 in that they are transmembrane iron transporters (McKie *et al.*, 2000; Abboud and Haile, 2000; Donovan *et al.*, 2000). After exiting the enterocyte, via FP-1, ferrous iron is converted to ferric iron by the action of hephaestin. The ferric iron immediately binds to transferrin, an iron-carrying protein that transports iron throughout the body.

DMT-1 is currently recognised as the protein for the transport of non-haem iron from the intestinal lumen into the enterocyte. However, convincing evidence from a study by Conrad *et al.* (2000) has led to the suggestion that two separate pathways exist for the transport of ferric and ferrous iron. The uptake of ferrous iron involves the DMT-1 pathway previously described (Figure 1.1), whereas ferric iron is transported by the integrin-mobilferrin pathway (Figure 1.2).

1.3.2 Non-haem iron: the integrin-mobilferrin pathway

Most dietary iron is in the ferric state which, unlike ferrous iron, is not soluble at a near-neutral pH. Thus, ferric iron must be chelated to remain soluble above pH 3.0. In the small intestine, chelators such as mucin (Conrad *et al.*, 1991) and ascorbic acid are available.

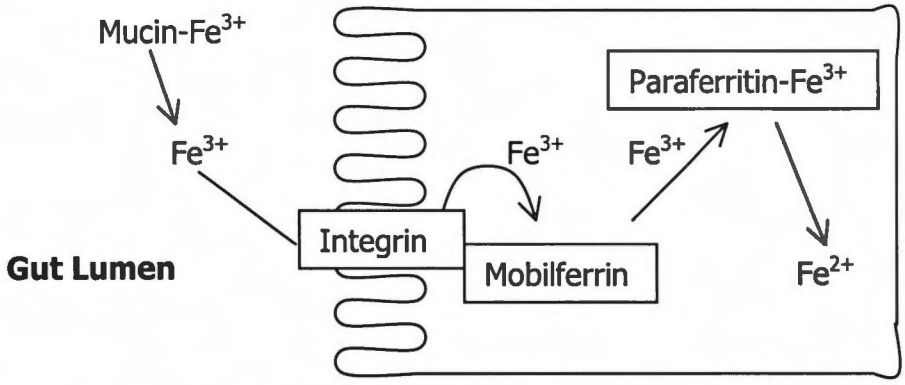


Figure 1.2: Model of the integrin-mobilferrin pathway for ferric iron absorption. Fe^{2+}=ferrous iron; Fe^{3+}=ferric iron.

Ferric iron enters the enterocyte by binding to a cell surface β_3-integrin (Conrad *et al.*, 1993) and is transferred to a calreticulin-like chaperone protein called mobilferrin (Conrad *et al.*, 1990). Before the iron can be used for cellular functions, it must be converted to the ferrous state. A cytoplasmic protein complex

called paraferritin, containing mobilferrin, β_3-integrin, flavin monoxygenase (Umbreit *et al.*, 1996) and the DMT-1 protein (Umbreit *et al.*, 2002), uses an electron-transport chain to perform this intracellular ferrireductase activity (Umbreit *et al.*, 1996). The iron is then available for use within the cell or for export via FP-1.

1.3.3 Haem iron

Within the gastrointestinal tract, haem is freed from the globin portion of myoglobin and haemoglobin by the combined actions of gastric acid and gastrointestinal enzymes. The degradation products of globin are important for maintaining haem in a soluble, readily absorbed state (Conrad *et al.*, 1966a, 1967). Haem is then transported from the intestinal lumen into the enterocyte as an intact metalloporphyrin (Conrad *et al.*, 1966b, 1967). However, the mechanism responsible for haem uptake by the enterocytes is not fully understood. Some studies indicate the haem is internalised by a haem binding protein, which has been demonstrated in pig enterocytes (Grasbeck *et al.*, 1979, 1982; Tenhunen *et al.*, 1980) as well as certain cell lines (Galbraith *et al.*, 1985; Majuri and Grasbeck, 1987; Majuri, 1989; Worthington *et al.*, 2001). However, other work suggests that haem iron is internalised by pinocytosis (Wyllie and Kaufman, 1982).

Despite controversy concerning the mechanism of haem iron uptake, once within the enterocyte the porphyrin ring is bound by an intracellular enzyme called haem oxygenase (Raffin *et al.*, 1974). The ferrous iron is then released by the oxidative cleavage of the haem molecule. Once released, the iron then enters the same intracellular pool as non-haem iron, where it can then be stored as ferritin or exported from the enterocyte via FP-1.

1.4 Maintenance of iron homeostasis

Iron is an essential nutrient for all living cells, yet it is toxic when present in excess. Free iron is able to catalyse the formation of highly toxic oxidative free radicals that damage many important biological components, such as lipids, proteins and DNA (Henle and Linn, 1997). Despite such toxic potential, there is no physiological regulatory mechanism for excretion of iron from the body. Thus, intestinal iron

absorption is balanced against minor losses due to blood loss and shedding of superficial cells.

Research during the early 1930s revealed that increasing levels of oral iron administration did not increase urinary excretion (McCance and Widdowson, 1937, 1938). Instead, there was an accumulation of iron in the epithelial cells of the small intestine. These observations lead to the conclusion that the control of iron balance in the body is regulated by the control of iron absorption, rather than excretion (McCance and Widdowson, 1937, 1938). This is now a widely accepted aspect of body iron homeostasis. Thus, the primary focal point for maintenance of whole body iron homeostasis is the regulation of intestinal iron absorption in response to alterations in body iron status.

1.4.1 Cellular control of iron homeostasis

In the past 20 years, evidence has been obtained that intracellular iron levels are controlled by a post-transcriptional mechanism that correlates the expression of key proteins involved in iron metabolism. This is accomplished by the interaction of cytosolic 'iron-sensing' proteins, iron-regulatory proteins (IRPs), with iron-responsive elements (IREs), which are stem-loop structures located in the untranslated regions of the respective mRNAs.

1.4.1.1 Iron-responsive elements

Iron-responsive elements (IRE) were first identified in the 5′-untranslated regions (UTR) of ferritin H- and L-chain mRNAs (Aziz and Munro, 1987; Hentze *et al.*, 1987a, b) and were documented to mediate inhibition of ferritin mRNA translation in iron-deprived cells. Five similar IRE motifs were later identified in the 3′-UTR of human transferrin receptor mRNA (Casey *et al.*, 1988). These IREs modulate the stability of transferrin receptor mRNA in the cytoplasm as a function of cellular iron levels (Mullner and Kuhn, 1988).

The IREs are *cis*-acting nucleotide sequences that form a stem-loop structure which includes a 6-nucleotide loop at the end of an RNA helix. These hairpin structures are specifically recognised by *trans*-acting cytosolic RNA-binding proteins

(Leibold and Munro, 1988; Rouault *et al.*, 1988), known as iron-regulatory proteins (IRPs), which control the rate of mRNA translation or stability.

1.4.1.2 Iron-regulatory proteins

Two iron-regulatory proteins (IRP), designated IRP-1 and IRP-2, have been characterised for a variety of mammalian tissues and cells (Leibold and Munro, 1988; Rouault *et al.*, 1988, 1990; Mullner *et al.*, 1989; Walden *et al.*, 1989; Henderson *et al.*, 1993; Guo *et al.*, 1994, 1995b). IRP-1 shares homology with mitochondrial aconitase (Rouault *et al.*, 1990, 1991), an [Fe-S] protein of the citric acid cycle. In iron-replete cells, IRP-1 contains a [4Fe-4S] cluster and, in this form, possesses aconitase activity and binds RNA with low affinity (Haile *et al.*, 1992; Kennedy *et al.*, 1992). In contrast, when iron is scarce IRP-1 lacks a [4Fe-4S] cluster, and associated aconitase activity, and binds RNA with high affinity (Haile *et al.*, 1992). The transition between the aconitase and RNA-binding form of IRP-1 is regulated post-translationally and occurs without changes in IRP-1 protein levels (Yu *et al.*, 1992; Tang *et al.*, 1992).

IRP-2, which shares 61% overall amino acid sequence with IRP-1 (Guo *et al.*, 1995b), also binds to IREs with similar affinities as IRP-1 and upon binding represses translation of IRE-containing mRNAs (Guo *et al.*, 1994; Kim *et al.*, 1995). However, in contrast to IRP-1, IRP-2 functions solely as an RNA-binding protein and does not contain an [Fe-S] cluster (Guo *et al.*, 1994; Samaniego *et al.*, 1994) but is regulated by degradation of protein levels (Henderson and Kuhn, 1995), possibly by a polypeptide insertion that confers susceptibility to proteasome-mediated proteolysis when iron is plentiful (Guo *et al.*, 1995a). Thus, IRP-2 is stabilised under low cellular iron concentrations.

1.4.1.3 Interaction of IRPs with IREs

When cellular iron becomes limiting, IRP-1 is recruited into the high-affinity RNA binding state and IRP-2 is stabilised. The binding of IRPs to the IRE in the 5′-UTR of the ferritin mRNA represses the translation of ferritin, thereby preventing the synthesis of ferritin and hence the storage of iron when cellular iron levels are low. Association of IRPs with IREs in the 3′-UTR of transferrin receptor mRNA stabilises

this transcript delaying its degradation. This maintains the transferrin receptor on the cell surface, allowing the cell to acquire iron to replenish low cellular iron pools. On the other hand, expansion of the intracellular labile iron pool inactivates IRP-1 as an RNA-binding protein and leads to the degradation of IRP-2. This results in efficient translation of ferritin mRNA and rapid degradation of transferrin receptor mRNA, thus, preventing further iron uptake by the cell via the transferrin receptor and stimulating storage through ferritin synthesis.

1.4.2 Iron homeostasis: communication with the small intestine

During times of iron deficiency, a marked elevation in iron absorption occurs, whereas increases in iron body stores lead to lower absorption efficiency (Bothwell *et al.*, 1958). However, the precise mechanisms regulating intestinal iron absorption have not been clearly determined, although it is thought the enterocyte responds to regulators released in response to a decrease in iron stores and an increase in body iron requirements.

Two regulators, namely the stores regulator and the erythroid regulator, may be involved in the control of iron absorption. When the amount of iron in tissues, such as the liver, skeletal muscle and blood drops below a critical level, a 'stores regulator' increases iron uptake until the reserves are replete again. Indeed, several studies (Bothwell *et al.*, 1958; Pirzio-Biroli *et al.*, 1958; Pirzio-Biroli and Finch, 1960; Gavin *et al.*, 1994) have demonstrated absorption to be a function of the amount of storage iron in the body. The erythropoietic regulator is believed to communicate the erythropoietic demand of the body, with iron absorption being up-regulated as the iron requirement for erythropoiesis increases. In support of this hypothesis, studies have demonstrated an enhancement of absorption when erythropoiesis was stimulated, for example, by bleeding or erythropoietin administration (Bothwell *et al.*, 1958; Skikne and Cook, 1992), and diminished when erythropoiesis is inhibited, for example, by hypertransfusion of red blood cells (Bothwell *et al.*, 1958; Pirzio-Biroli *et al.*, 1958; Weintraub *et al.*, 1965).

Both the stores regulator and erythropoietic regulator are hypothesised to be soluble components of the plasma (Roy and Enns, 2000), able to communicate

between sites of iron utilisation and mobilisation and the intestinal cells. However, the signalling pathway and the molecular components involved in the regulation of iron absorption through these two regulators have remained elusive. Possible candidate molecules have included serum ferritin (Taylor *et al.*, 1988), circulating transferrin (Taylor *et al.*, 1988; Raja *et al.*, 1999) and serum transferrin receptor (Cook *et al.*, 1990; Feelders *et al.*, 1999). Recent studies (Nicolas *et al.*, 2001; Pigeon *et al.*, 2001) have implicated the small circulating peptide hepcidin as a new candidate for the long-postulated regulator involved in modulating dietary iron absorption in a manner that reflects body iron status.

Hepcidin is a disulfide-bounded peptide that has been purified from human plasma (Krause *et al.*, 2000) and urine (Park *et al.*, 2001) and has been reported to have antifungal and bacteriocidal activity. The expression of the gene encoding hepcidin is predominately in the liver (Krause *et al.*, 2000; Park *et al.*, 2001; Pigeon *et al.*, 2001). A potential role for hepcidin in iron metabolism emerged when Pigeon *et al.* (2001) discovered that hepcidin mRNA was overexpressed in the livers of experimentally iron loaded mice. Further evidence was provided by Nicolas *et al.* (2001, 2002) using genetically modified mouse models. Mice with a disruption in the gene encoding the transcription factor upstream stimulatory factor-2 (USF-2) failed to produce hepcidin mRNA and developed spontaneous visceral iron overload (Nicolas *et al.*, 2001). The USF-2 gene is located immediately upstream of the mouse hepcidin genes (Pigeon *et al.*, 2001). Thus, the absence of hepcidin expression was thought to be caused by an alteration of the hepcidin gene due to USF-2 gene disruption (Nicolas *et al.*, 2001), and to be responsible for the progressive iron overload. In contrast, mice genetically engineered to overexpress hepcidin in the liver have decreased body iron levels and at birth exhibit severe iron deficiency anaemia (Nicolas *et al.*, 2002). Furthermore, changing rats and mice from an iron replete diet to an iron deficient diet caused an increase in iron absorption with a precise inverse correlation observed between the expression of hepcidin in the liver and the duodenal expression of Dcyt *b*, DMT-1 and FP-1 mRNA (Anderson *et al.*, 2002; Frazer *et al.*, 2002). These results strongly suggest that hepcidin plays an important role in maintaining iron homeostasis by limiting iron absorption.

Intestinal regulation of iron homeostasis is proposed to begin in the crypt cells, which take up iron from the blood and may be the body iron sensors/responders. Within the crypts of the intestine are immature proliferating cells (crypt cells), which take approximately 5 to 6 days in human small intestine to migrate up the villi (Lipkin *et al.*, 1962, 1963; MacDonald *et al.*, 1964), differentiating as they move, into enterocytes (Hocker and Weidenmann, 1998) that are capable of transporting iron. The current hypothesis is that in response to high iron conditions, increased uptake of transferrin-bound iron into the liver occurs via transferrin receptor 2, a recently identified homologue of transferrin receptor 1 (Kawabata *et al.*, 1999). In response, the production and secretion of hepcidin into the blood increases (Nicolas *et al.*, 2001). Hepcidin then interacts with transferrin receptors on intestinal crypt cells to modulate the iron set-point for absorption relative to body iron demands. Crypt cells may then be 'programmed' to up- or down-regulate expression of the DMT-1 transporter and/or the basolateral iron export protein FP-1 as they mature to become villus cells (Roy and Enns, 2000). This programming is thought to involve changes in the labile iron pool of the crypt cells and thus, as part of this regulatory mechanism, DMT-1 and FP-1 expression may be controlled post-transcriptionally by the IRE/IRP system.

1.4.3 Intestinal iron transporter proteins and the IRE/IRP system

Two spliced transcripts have been identified for DMT-1 gene (Fleming *et al.*, 1997; Gunshin *et al.*, 1997; Lee *et al.*, 1998). One transcript contains a sequence that resembles IRE within its 3′-UTR, whereas the alternatively spliced counterpart lacks this sequence (Gunshin *et al.*, 1997; Lee *et al.*, 1998). The IRE-like sequence of DMT-1 mRNA is predicted to fold into a stem-loop structure, which resembles the IRE present in transferrin receptor and ferritin mRNAs.

A recent study by Gunshin *et al.* (2001) revealed that both IRP-1 and IRP-2 interact with the IRE present on the DMT-1 mRNA, although with a lower affinity than ferritin mRNA. These authors reported that the expression of the non-IRE form in a Caco-2 cell line was not altered by iron treatment, whereas the IRE form was highly responsive to iron depletion and overload. From these observations, they proposed that regulation of DMT-1 mRNA involves mRNA stabilisation through

the binding of IRP-1 and/or IRP-2 to the putative IRE in DMT-1, in analogy to transferrin receptor mRNA. Thus, cellular accumulation of iron leads to dissociation of IRPs from the DMT-1 mRNA resulting in degradation of its mRNA to cease intestinal iron absorption.

An IRE-like sequence was also found in the 5′-UTR of mRNA encoding FP-1 (Abboud and Haile, 2000; Donovan *et al.*, 2000; McKie *et al.*, 2000). Thus, the IREs in the mRNAs of DMT-1 and FP-1 may play a crucial role in maintaining transepithelial iron transport in the intestine. FP-1 mRNA may be on translational or transcriptional arrest until DMT-1 delivers iron into the enterocytes, which trigger an increase in FP-1 expression and localisation to the basolateral membrane to facilitate the transfer of iron into the blood (Gunshin *et al.*, 2001).

This theory is supported by a recent study from Martini *et al.* (2002), who demonstrated a 57% and 52% decrease in the levels of DMT-1 and FP-1 mRNA, respectively, in a human intestinal cell line after exposure to high levels of iron. Further studies in rats (Gunshin *et al.*, 1997; Canonne-Hergaux *et al.*, 1999) have revealed that the DMT-1 mRNA and its protein are strongly (50- to 100-fold) up-regulated in the duodenum when kept on an iron deficient diet for 3 weeks. In humans, duodenal expression of DMT-1 mRNA is inversely correlated to body iron stores, as indicated by serum ferritin levels. Recently, Zoller *et al.* (2001) reported that duodenal expression of DMT-1 and FP-1 mRNAs and their respective proteins were significantly higher in iron deficient subjects compared to others with normal iron status. The levels of mRNA were unidirectionally up- or down-regulated, thus, increased duodenal iron uptake, as indicated by high DMT-1 expression, was associated with increased iron export from enterocytes into the circulation as reflected by high FP-1 mRNA levels and vice versa.

1.5 Bioavailability

In terms of nutritional science, bioavailability of any nutrient is defined as the proportion of the total intake that can be absorbed and utilised for normal body functions (Wienk *et al.*, 1999). Thus, when evaluating a meal for its nutritional

significance, knowledge of intake alone is of little value without an understanding of bioavailability.

1.5.1 Solubility and bioavailability

The bioavailability of iron is highly dependent upon its solubility, since, it is generally accepted that only soluble iron is absorbed from the intestinal digesta (Wienk *et al.*, 1999). The hydrolysis of iron, or the formation of iron hydroxides, renders the iron insoluble. This may be prevented by factors within food that reduce ferric iron and/or form soluble iron complexes. Ferric iron is rapidly hydrolysed at a pH >1.0, whereas ferrous iron does not hydrolyse at a pH below 7.0 (Bothwell *et al.*, 1979; Wienk *et al.*, 1999). However, ferrous iron is readily oxidised to ferric iron.

1.5.2 Nutrient impact on iron bioavailability

The bioavailability of dietary iron is also affected by the composition of the meal. In the small intestine, food components bind with iron and either enhance or inhibit its absorption (Table 1.5).

Table 1.5: Dietary constituents that affect the absorption of non-haem iron.

Enhancers	Inhibitors
Ascorbic acid	Phytates
Animal tissues	Polyphenols
β-carotene and Vitamin A	Calcium (?)
Other organic acids (e.g. citric, malic, tartaric and lactic)	Other elements (e.g. manganese, zinc)

Inhibitors form large, insoluble polymers with iron, thereby making it unavailable for absorption. Enhancers form soluble, monomeric complexes with iron and prevent its precipitation and polymerisation, thereby promoting its uptake (Conrad and Schade, 1968). Most of these dietary enhancers and inhibitors affect only the bioavailability of non-haem iron. Haem iron bioavailability is relatively unaffected by other dietary factors due to the absorption of the intact iron-porphyrin chelate (Hallberg and Solvell, 1967) and its relatively high solubility in the alkaline environment of the small intestine (Figure 1.3) (Conrad and Umbreit, 2000). As a

result, haem iron is highly bioavailable, compared to non-haem iron (Monsen, 1988), with 15 to 35% of total haem iron being absorbed from the diet by humans, compared to 2 to 20% for non-haem iron (Monsen *et al.*, 1978; Monsen, 1988; Hunt and Roughead, 2000).

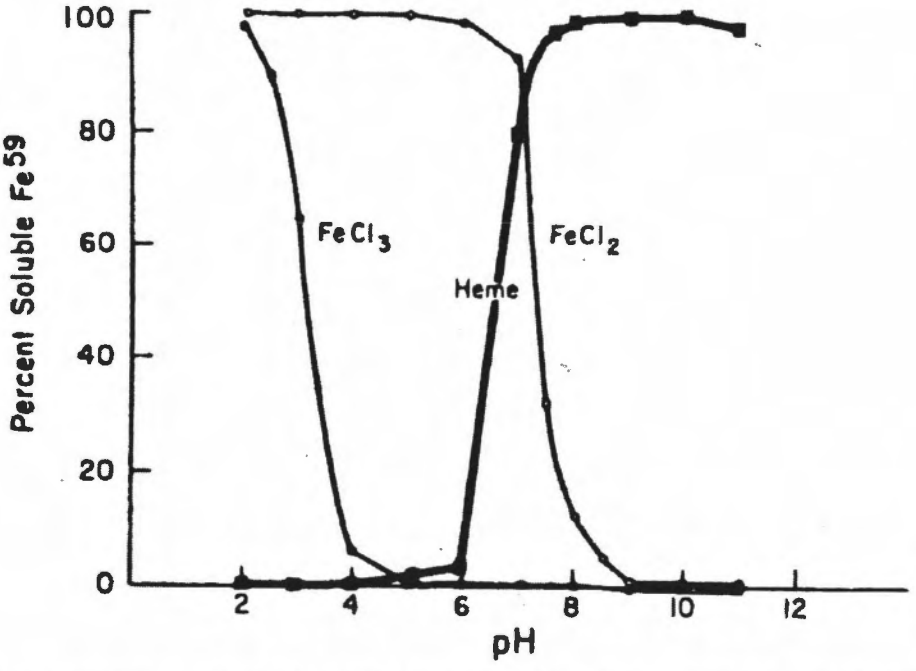


Figure 1.3: Changes in the solubility of ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), and haem iron with increasing pH. From Conrad and Umbreit (2000, p. 289).

1.5.2.1 Ascorbic acid

Ascorbic acid is a potent promoter of non-haem iron absorption. It may act by reducing ferric iron to the more soluble ferrous iron, or by forming a chelate with ferric iron, thereby maintaining its solubility in the alkaline environment of the small intestine (Conrad and Schade, 1968). The latter effect allows iron to pass into the small intestine from the stomach as an iron-ascorbate complex, which reduces the influence of inhibitory ligands that bind iron in the more alkaline pH of the duodenum.

Numerous studies with human subjects have demonstrated the beneficial effect of ascorbic acid on iron absorption. Studies with maize meals showed a six-fold increase in iron absorption after addition of 65mg and 100mg of ascorbic acid (Layrisse *et al.*, 1974) and a nine-fold increase with 50mg or 100mg of ascorbic acid (Derman *et al.*, 1977). Increasing the amount of ascorbic acid from 25 to 200mg led to an increase in iron absorption from a maize meal (Bjorn-Rassmussen

and Hallberg, 1974). Iron absorption from a semisynthetic meal increased from 0.8% of total iron to 1.9% and 3.2% after the addition of 50mg and 100mg of ascorbic acid, respectively (Cook and Monsen, 1977). The enhancing effect of ascorbic acid on iron absorption is dose-related and is directly proportional to the amount of ascorbic acid added over the range from 25 to 1000mg (Bjorn-Rasmussen and Hallberg, 1974; Cook and Monsen, 1977).

Ascorbic acid can improve iron absorption even in the presence of inhibiting factors, such as phytates, soy and polyphenols (Derman *et al.*, 1977; Rossander *et al.*, 1979; Morck *et al.*, 1982; Hallberg, 1987; Hallberg *et al.*, 1989; Siegenberg *et al.*, 1991). The addition of 100mg of ascorbic acid to a meal containing isolated soy protein increased mean iron absorption from 0.6 to 3.2% (Morck *et al.*, 1982). Similarly, the addition of ascorbic acid (50mg or 100mg) counteracted the inhibitory effect of 25mg of phytate added to wheat rolls and increased iron absorption two- to three-fold (Hallberg *et al.*, 1989). The inhibitory effect of polyphenols in tea, consumed with a maize meal by iron deficient Indian women, was counteracted by large amounts (250mg or 500mg) of ascorbic acid (Derman *et al.*, 1977).

1.5.2.2 Meat factor

It is well established that the bioavailability of iron from foods is enhanced by the presence of animal tissues (Layrisse *et al.*, 1968, 1969; Cook and Monsen, 1975; Bjorn-Rasmusen and Hallberg, 1979). The meat components responsible for the enhancement of iron absorption has come to be termed the 'meat factor', due to this effect occurring following the ingestion of a variety of meats, including beef, chicken, pork and fish. However, not all animal products appear to have the same effect; casein, cheese and egg albumin either inhibit or have no effect on iron absorption (Bjorn-Rassmussen and Hallberg, 1979; Cook and Monsen, 1976; Monsen and Cook, 1979; Hurrell *et al.*, 1988). Despite extensive studies demonstrating the enhancing effect of meat on iron absorption, the mechanism of the effect remains controversial.

The most popular explanation for the meat factor effect is that the products of proteolytic digestion complex with non-haem iron and increase iron solubility and, hence, absorption. Most evidence in support of this theory has been derived from *in vitro* studies showing that gastrointestinal digestion of meat or meat proteins increase iron solubility (Schricker *et al.*, 1981; Kane and Miller, 1984; Politz and Clydesdale, 1988; Slatkavitz and Clydesdale, 1988; Perez-Llamas *et al.*, 1996, 1997; Chiplonkar *et al.*, 1999; Seth *et al.*, 1999) and uptake of both ferrous and ferric iron by intestinal cell lines (Garcia *et al.*, 1996; Glahn *et al.*, 1996; Au and Reddy, 2000; Swain *et al.*, 2002).

Some studies have shown that peptides (Kane and Miller, 1984; Slatkavitz and Clydesdale, 1988; Kapsokefalou and Miller, 1991; Seth *et al.*, 1999) arising from the proteolytic digestion of meat have this effect. By chelating iron and possibly reducing ferric iron to the more soluble ferrous iron, these proteolytic digestion products may prevent the precipitation of iron in the gut lumen, by acting as ligands in the formation of soluble iron complexes.

Other studies have indicated that certain amino acids may play a role in the meat factor effect (Kroe *et al.*, 1963; Van Campen and Gross, 1969; Martinez-Torres and Layrisse, 1970; Martinez-Torres *et al.*, 1981; Layrisse *et al.*, 1984; Taylor *et al.*, 1986; Glahn and Van Campen, 1997). Kroe *et al.* (1963) reported that nine amino acids, including histidine, glutamine, glutamic acid and methionine increased ferrous iron uptake from ligated rat intestinal segments *in vivo*. However, in similar experiments using ferric iron, only histidine, cysteine, and to a lesser extent, lysine were effective (Van Campen and Gross, 1969; Van Campen, 1972, 1973). In another study, however, Vaghefi *et al.* (1998) observed no effect of histidine on iron uptake by rat intestinal segments mounted in Ussing chambers. In human iron absorption studies, cysteine was shown to be the only amino acid that enhances iron absorption from extrinsically and intrinsically labelled foods (Martinez-Torres and Layrisse, 1970; Martinez-Torres *et al.*, 1981; Layrisse *et al.*, 1984); histidine had no effect on iron absorption in human subjects (Layrisse *et al.*, 1984).

Digestion of proteins in the intestinal lumen yields mostly small peptides rather than free amino acids (Glahn and Van Campen, 1997), so it could be argued that peptides are more likely to be involved in binding and solubilisation of iron. Several studies have investigated the role of peptides in the meat factor effect with the amino acids cysteine (Layrisse *et al.*, 1984; Taylor *et al.*, 1986; Glahn and Van Campen, 1997; Garcia *et al.*, 1996) and/or histidine (Seth and Mahoney, 2000; Swain *et al.*, 2002) thought to be promising peptide residues.

1.5.2.3 Phytates

Phytates are a storage form of phosphates and minerals in cereals, seeds, nuts, vegetables and fruit (Torre *et al.*, 1991) and have been shown to strongly inhibit iron absorption (Gillooly *et al.*, 1983, 1984; Hallberg *et al.*, 1989; Reddy *et al.*, 1996). The mechanism involved has not been well characterised. However, the formation of insoluble and/or indigestible complexes between the phosphate groups of phytate and iron may be involved (Torre *et al.*, 1991; Brune *et al.*, 1992). Formation of a monoferric phytate complex is not inhibitory (Morris and Ellis, 1976; Simpson *et al.*, 1981), however, the formation of diferric and tetraferric phytate complexes in the gastrointestinal tract *in vivo* has a negative effect on the availability of iron for absorption (Morris and Ellis, 1982; Torre *et al.*, 1991).

A dose-dependent inhibitory effect of phytate on iron absorption has been demonstrated by the addition of varying amounts of phytate to wheat rolls (Hallberg *et al.*, 1989). Iron absorption was inhibited by 18% after the addition of 2mg of phytate, 64% by 24mg and 82% by 250mg. The inhibitory effect of phytate on iron absorption is proportionately more pronounced when dietary phytate is low and less when phytate is high (Hallberg *et al.*, 1989; Brune *et al.*, 1992; Hurrell *et al.*, 1992). In another study by Reddy *et al.* (1996), a drink containing maltodextrose and corn oil was used as a control meal to which sufficient sodium phytate was added to provide 300mg phytic acid. After addition of the sodium phytate, there was a 83 to 90% reduction in iron absorption.

1.5.2.4 Polyphenols

Phenolic compounds are common in the human diet as tannins in tea, coffee and vegetables. They are released from the food or beverage during gastrointestinal digestion and are thought to act through the formation of insoluble complexes between hydroxyl groups and iron. A reduction in iron absorption by approximately 60 to 70% (Disler *et al.*, 1975; Hallberg and Rossander, 1982; Morck *et al.*, 1983) and 45% (Derman *et al.*, 1977), was demonstrated when food with one cup (200 to 250ml) of normal-strength black tea was consumed. Iron absorption from bread was reduced to one-third and from vegetable soup to a quarter when served with tea, compared with water (Disler *et al.*, 1975). Further, iron absorption from a Western-type breakfast was reduced by about 60% following consumption of 150ml of tea (Rossander *et al.*, 1979).

The inhibitory effect of coffee on iron absorption in humans is less than that of tea. A cup of coffee reduced iron absorption from a hamburger meal by 30 to 40%, compared to a 60 to 65% decrease with tea (Hallberg and Rossander, 1982; Morck *et al.*, 1983). The polyphenols in vegetables also have a strong inhibitory effect on iron absorption. Vegetables, such as eggplant and spinach, have a high polyphenol content and low iron bioavailability (Gillooly *et al.*, 1983). Other beverages such as herbal and green teas (Hurrell *et al.*, 1999; Samman *et al.*, 2001) and red wine (Cook *et al.*, 1995) also contain phenolic compounds and have been reported to inhibit iron absorption.

1.5.2.5 Calcium

While it is often stated that calcium reduces iron absorption, the inconsistent results between experiments suggest that calcium-iron interactions are complex. Calcium may change the physiochemical state of iron making it less available for absorption, or compete for iron-binding sites on mobilferrin (Conrad *et al.*, 1993), or prevent movement of iron from the enterocyte into the circulation (Barton *et al.*, 1983).

The addition of calcium phosphate reduced the absorption of non-haem iron from a semisynthetic meal by 50%, however, addition of either calcium or phosphate

alone had no effect (Monsen and Cook, 1976). Hallberg *et al.* (1991) demonstrated that the addition of increasing amounts of calcium chloride (between 40 and 600mg) caused a dose-related reduction in non-haem iron absorption from a meal of wheat rolls. The inhibitory effect of calcium appears to be dose-related up to 300mg calcium, after which there is little additional inhibition. In contrast to calcium citrate and calcium phosphate, calcium carbonate (300 or 600mg calcium) did not inhibit the absorption of iron from ferrous sulfate supplements taken without food (Cook *et al.*, 1991). However, when taken with a hamburger meal all three calcium supplements were inhibitory. Furthermore, the addition of calcium to a meal of wheat rolls reduced percentage of haem iron absorption by 52 to 76% of control (Hallberg *et al.*, 1991, 1993).

1.6 Assessment of food iron bioavailability

Both *in vivo* and *in vitro* techniques can be performed to assess the bioavailability of iron from foods consumed by humans and animals.

1.6.1 *In vivo* versus *in vitro* techniques

In vivo (live, whole animal) experiments are the 'gold standard' for determining iron bioavailability. However, when involving humans, they are expensive and time consuming. This limits their use in screening large numbers of food materials and meals and also in exploring the effects of specific dietary components. They are also statistically complex because of wide inter- and intra-individual variations in iron absorption, due to differences in iron status between experimental subjects, and also day-to-day variations in iron absorption in the same individual (Van Campen, 1983). Due to these difficulties, attempts have been made to develop reliable and inexpensive alternative methods for the estimation of iron bioavailability using *in vitro* techniques to model *in vivo* events of iron absorption.

In vitro studies generally involve experiments within the laboratory that simulate selected processes that occur in the intact animal or human. They may include the use of isolated organs, tissues or cells which are maintained in an artificial environment that allows continuation of the biological processes that would normally be displayed *in vivo*. *In vitro* models have several advantages over *in*

vivo methods: they are more suited for rapid screening and analysis; they are relatively easy to use and have low ethical and financial cost. They also allow precise control over the conditions of the experiment (Wienk *et al.*, 1999). An additional advantage is the absence of possible disturbing effects on iron absorption, such as individual differences in iron status, resulting in lower variability of *in vitro* methods, compared to *in vivo* methods. *In vitro* methods also allow for the investigation of individual aspects of iron absorption.

1.6.2 *In vitro* methods used to investigate iron bioavailability

Various well-established *in vitro* techniques are available to investigate the bioavailability and absorption of iron from meals. These include measurements of food iron solubility and iron dialysability, absorption by everted gut sacs and the use of isolated epithelial cells and brush border/basolateral membrane vesicles.

1.6.2.1 Food iron solubility

Early *in vitro* methods involved measuring soluble iron by reacting aqueous food extracts with an iron-specific chromagen to produce colour. Initially, the technique was applied to undigested food using the ferrous chromagen α-α′ dipyridyl (Shackleton and McCance, 1936). However, these methods only measured ferrous iron.

A more physiological approach was reported by Jacobs and Greenman (1969). Their method was based on the amount of iron released from food treated with pepsin-hydrochloric acid solution, which represented the amount of iron released from food following gastric digestion. However, since most of the dietary iron is absorbed from the small intestine and not from the stomach, this method does not determine the true bioavailability of iron, especially when considering the pH change from the stomach to the small intestine.

Narasinga Rao and Prabhavathi (1978) extended the method of Jacobs and Greenman (1969) by subjecting the foods to treatment with pepsin at pH 1.35 and then subsequent adjustment of the pH to 7.5 and addition of pancreatic enzymes. This method more accurately measured the iron available for absorption by the

small intestine. In their study, Narasinga Rao and Prabhavathi (1978) revealed the addition of ascorbic acid at varying molar ratios (0 to 20) to iron progressively increased the amount of soluble iron in meals, with a maximum of a 20% increase when the molar ratio was 1:10. Likewise, meat extract progressively increased soluble iron in a rice meal up to 15% when added at a 1:2 ratio (rice:meat extract). The addition of 24mg of phytate and 200mg of tannin decreased soluble iron by 14% and 9%, respectively. Other studies using this technique have demonstrated an increase in iron solubility at neutral pH after the addition of digested meat or meat proteins (Politz and Clydesdale, 1988; Slatkavitz and Clydesdale, 1988; Seth *et al.*, 1999). In addition, Crews *et al.* (1985a, b) demonstrated that pepsin and pancreatin-bile digestion increased the solubility of total iron within beef and crab meat. However, a major limitation of food iron solubility studies for predicting iron bioavailability is their inability to simulate subsequent absorption after gastrointestinal digestion (Wienk *et al.*, 1999).

1.6.2.2 Iron dialysability

Miller *et al.* (1981) established a new approach for *in vitro* estimation of iron bioavailability by introducing iron dialysability. After pepsin digestion at low pH, meal aliquots were placed into beakers containing a dialysis bag filled with a sodium bicarbonate solution. Over time, the sodium bicarbonate diffuses into the digest, gradually increasing the pH from gastric to intestinal levels. This was thought to closely parallel the change in pH that occurs during the passage of food from the stomach into the duodenum. When the pH reached 5.0, a mixture of pancreatic enzymes and bile were added and the incubation continued. The dialysis bag was then removed and the iron that passed into the dialysis bag (dialysable iron) determined and used as an indicator of bioavailable iron.

Compared to iron solubility techniques, studies measuring dialysable iron are preferred as estimates of iron bioavailability and have been consistent with results from *in vivo* experiments (Hurrell *et al.*, 1988; Schricker *et al.*, 1981; Forbes *et al.*, 1989; Chiplonkar *et al.*, 1999). Studies using this technique have demonstrated a higher concentration of dialysable iron from digested meat than 'non-meat' sources (Perez-Llamas *et al.*, 1996, 1997; Kapsokefalou and Miller, 1991). For example,

Perez-Llamas *et al.* (1997) investigated the effects of digested beef, pork and isolated soy proteins on endogenous and exogenous dialysable iron. The amount of dialysable endogenous iron was higher for beef and pork (45% of initial total iron concentration) than from soy protein (10%). Additionally, beef (55%) and pork (40%) had a higher effect on the dialysability of exogenous iron, compared to soy protein (30%) (Perez-Llamas *et al.*, 1996). Furthermore, the addition of beef to a semi-synthetic meal yielded 12% dialysable iron, compared to only 4% for egg albumin (Mulvihill and Morrissey, 1998).

Addition of ascorbic acid (5mM) to ferric chloride (0.5mM) increased dialysable iron 12-fold, compared to a ferric chloride control (Kapsokefalou and Miller, 1991). Hazell and Johnson (1987) demonstrated the amount of dialysable iron from phytate-containing foods, such as cereals and legumes, was very low (2.1%), compared to that from fruits and vegetables (13.7%). Further, a decrease in dialysable iron was also observed with soy protein and egg white or egg albumin (Schricker *et al.*, 1982a; Kane and Miller, 1984; Hurrell *et al.*, 1988; Perez-Llamas *et al.*, 1996, 1997; Mulvihill and Morrissey, 1998).

Miller and Berner (1989) noted that although soluble, high molecular weight iron-peptide complexes formed during digestion might improve the solubility of dietary iron, the complexes are probably too large to be absorbed. Soluble, low molecular weight iron (dialysable iron) may better predict *in vivo* absorption, because low molecular weight peptides are more able to reach the brush border. Thus, unlike iron solubility, which measures all soluble iron as bioavailable, the use of a dialysis bag, with a molecular weight cut-off, ensures that only soluble, low molecular weight iron is measured in the estimation of bioavailable iron. However, despite advantages over iron solubility, iron dialysability experiments have their limitations. Movement of solute into a dialysis bag is dependent upon solutes passively moving down their concentration gradient into the dialysate. This is not physiological, since active transport is not simulated and concentration and osmotic gradients are considered to play little or no part in iron absorption by the small intestine.

1.6.2.3 Isolated cell lines

Isolated cell lines are increasingly being used to study the uptake and transport of iron. One of the most utilised cell lines for iron absorption research has been the Caco-2 cell. This human colonic adenocarcinoma cell line is capable of spontaneous differentiation into bipolar enterocytes and displays many structural and functional properties of mature human intestinal epithelial cells (Pinto *et al.*, 1983; Grasset *et al.*, 1984). The cells form a highly polarised monolayer exhibiting tight junctions, apical microvilli with a brush border membrane and express enzymes specific to the enterocyte (Chantret *et al.*, 1988).

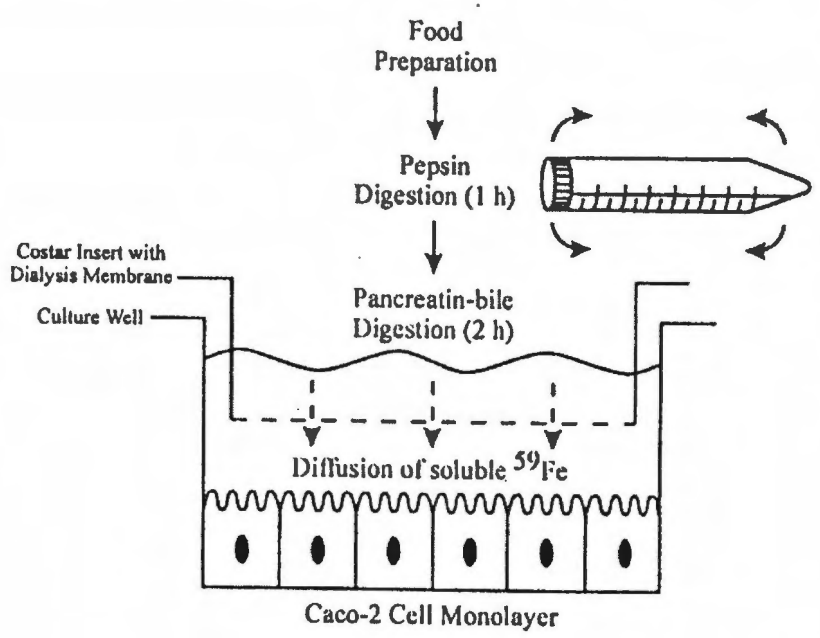


Figure 1.4: Diagram of digestion/Caco-2 cell culture model. From Glahn *et al.* (1996, p. 334).

Glahn *et al.* (1996) developed a model for assessing iron bioavailability from foods which combines the Miller *et al.* (1981) model of *in vitro* pepsin and pancreatin-bile digestion with the measurement of iron uptake by Caco-2 cell monolayers. The digestion was carried out in the upper chamber of a two-chamber system. Radioactive iron in the digest diffuses into the lower compartment through a dialysis bag to be taken up by the Caco-2 cells (Figure 1.4).

Since its development, this system has been used to estimate bioavailability of iron from various foods and meals (Glahn *et al.*, 1996; Au and Reddy, 2000). Studies using this model showed an enhancing effect of digested meat on both ferrous and

ferric iron uptake by the Caco-2 cells (Garcia *et al.*, 1996; Glahn *et al.*, 1996; Swain *et al.*, 2002) as well as a significant inhibitory influence of phytate and polyphenols (Glahn *et al.*, 2002a). This technique has also been used to determine iron bioavailability from several varieties of rice grains (Glahn *et al.*, 2002b), spirulina (Puyfoulhoux *et al.*, 2001) and infant formulae (Glahn *et al.*, 1998; Jovani *et al.*, 2001).

One of the major limitations of *in vitro* methods is the uncertainty associated with extrapolating the results from animal models to humans. The Caco-2 cell model may have an advantage in this respect, as they are of human origin and absorb iron in a manner similar to that which is believed to occur in the enterocytes of the human small intestine (Alvarez-Hernandez *et al.*, 1991, 1998; Han *et al.*, 1995; Gangloff *et al.*, 1996; Au and Reddy, 2000; Follett *et al.*, 2002). However, due to the transformed nature of these cells, the extent to which normal cellular metabolic processes are maintained remains questionable (Wienk *et al.*, 1999). Being a colonic tumour cell line, Caco-2 cells may mimic other tumour cells in having an increased avidity for iron because of the increased iron requirements necessary for cell turnover and growth (Goddard *et al.*, 1997). Furthermore, Caco-2 cells do not have a mucin layer, which may play a significant role in the intestinal absorption of iron (Conrad *et al.*, 1991).

1.6.2.4 Membrane vesicles

Membrane vesicles have been used to study epithelial transport processes. After isolation under appropriate conditions, plasma membranes are allowed to aggregate to form well-sealed vesicles. The transport of solutes into isolated membrane vesicles is then studied by incubating the vesicles with the radiolabelled solute. The vesicles are collected by filtration, then the radioactivity specifically bound to the vesicles measured and used to calculate the uptake of the solute in question.

Brush border membrane vesicles have been isolated from mouse duodenum and distal ileum (Simpson *et al.*, 1985), rabbit duodenum (Marx and Aisen, 1981; Simpson and Peters, 1986; Simpson *et al.*, 1988) and guinea pig duodenum and

jejunum (Chowrimootoo *et al.*, 1992) for investigation of iron transport mechanisms. However, their use for iron bioavailability studies is limited (Wienk *et al.*, 1999).

The use of membrane vesicles for investigation of transport processes has several advantages over other methods. The preparation is free of interference from metabolic enzymes and intracellular organelles and the paracellular pathway does not contribute to the transport system (Acra and Ghishan, 1991). However, vesicles are often contaminated so the purity must be assessed and the vesicular membranes may leak creating an 'artificial' efflux (Murer and Kinne, 1980; Acra and Ghishan, 1991). Nutrient transport may also be affected by possible damage to membrane-bound proteins during the preparation of the vesicles (Hidalgo *et al.*, 1989).

1.6.2.5 Everted gut sacs

Wilson and Wiseman (1954) first developed the everted gut sac technique. The sacs are prepared by everting intestinal segments over a glass rod (Figure 1.5), then the sacs are filled with oxygenated buffer solution. The sac is then tied and hung in a well-oxygenated and stirred incubation solution containing the desired solute, and the accumulation of the solute in the sac measured.

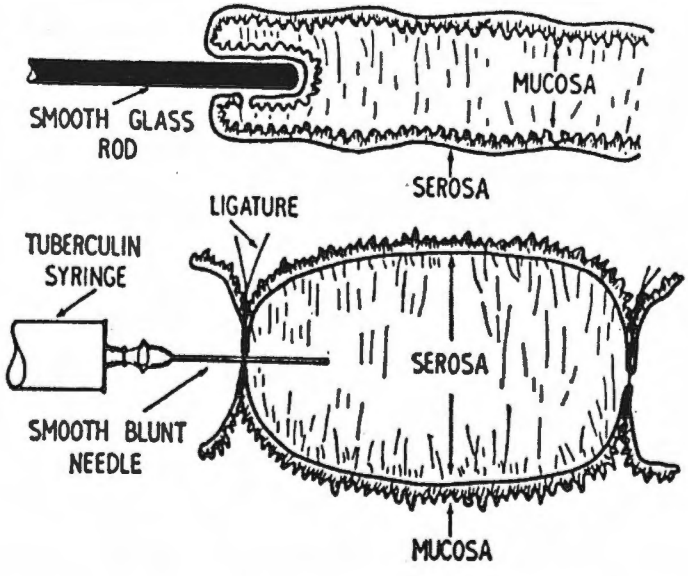


Figure 1.5: Diagram of the everted gut sac technique. Everting of intestine over glass rod (top), and filling of the sac using a blunt needle (bottom). From Wiseman (1961, p. 289).

Early studies with the everted sac technique, indicated iron in the ferrous state was absorbed across the intestinal mucosa against a concentration gradient, with the largest amount absorbed in the proximal duodenum (Dowdle *et al.*, 1960). The effect of prior dosing with iron was also studied using sacs removed from rats (Manis and Schachter, 1964). Mucosal iron uptake was reduced in the rats dosed with iron compared to rats given no dose with transport into the serosa decreased to an even greater extent. Furthermore, observations using everted sacs led Manis and Schachter (1964) to conclude that iron absorption is an active metabolic process and not merely a passive process governed by concentration gradients.

Everted gut sacs retain the full thickness of the intestinal mucosa, including mucous and muscle layers. However, *in vivo* iron does not cross the muscle layers of the intestine but enters the capillaries between the epithelia and the muscle layers. As this blood supply is not present *in vitro*, iron may build up between the epithelia and the muscle layers creating an unphysiological concentration gradient across the tissue (Manis and Schacter, 1962). Thus, measurements may be affected by the back flux of iron into the mucosal solution, or by fluxes across the paracellular pathway (Acra and Ghishan, 1991). Furthermore, the movement of iron across the walls of the everted sac does not duplicate *in vivo* absorption; the rate of iron transfer was slower in the sac studies than in the intact rat and the amount of iron moving across the sac wall did not continue to increase when more iron was added to the mucosal medium (Manis and Schacter, 1964; Jacobs *et al.*, 1966).

1.6.3 An *in vitro* method using Ussing chambers

Ussing and Zerahn (1951) first introduced the Ussing chambers to study the active transport of sodium as the source of an electric current in the short-circuited isolated frog skin. Since then, the Ussing chambers have been used to study transport across many types of membranes.

In this procedure, segments of intestine are mounted between two half chambers. While mounted in the Ussing chambers, mucosal and serosal surfaces of the intestine are bathed independently in Ringer's solution, which provides the

necessary electrolytes and nutrients to maintain the intestinal tissue. Both Ringer's solutions are oxygenated and circulated with carbogen gas (95% O_2 and 5% CO_2) throughout the experiment, while the temperature is maintained at 37°C by water-jacketed reservoirs (Figure 1.6).

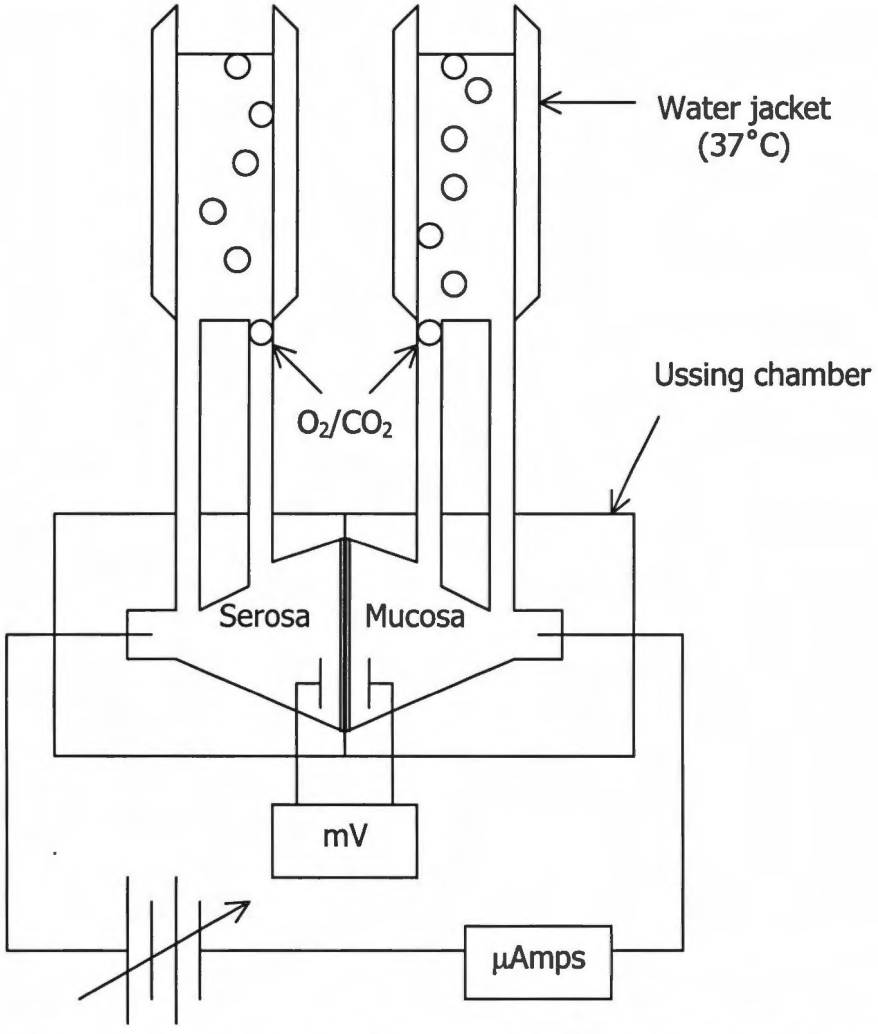


Figure 1.6: Diagram of the Ussing chamber apparatus with intestinal tissue mounted between the two half chambers.

Salt-agar bridges situated close to the intestinal epithelium connect to calomel reference electrodes, which measure the potential difference (mV) across the tissue (Figure 1.6). This potential difference arises due to the active transport of electrically charged substances across the intestine. This will affect the transport efficiency of the intestine and the tissue must therefore be short-circuited by applying a current that opposes this natural tissue current. This current (µAmps) is applied through silver chloride electrodes that are in contact with the serosal and mucosal Ringers via a second pair of salt-agar bridges (Figure 1.6). The potential

difference across the membrane and the magnitude of the current is taken and the resistance of the tissue is measured. The tissue resistance is an indication of the viability of the tissues with a decrease in tissue resistance indicating a loss of integrity of the tissue and viability of the preparation.

The Ussing chamber provides a major advantage over isolated cell lines in that it allows for the study of intact intestinal epithelium that is taken fresh from a euthanased animal. The tissue possesses most of the physiological processes that occur *in vivo*, including the presence of an adherent mucin layer. In addition, Ussing chambers have numerous advantages over the everted gut sac technique and membrane vesicles. When connected to a recording apparatus Ussing chambers allow constant assessment of tissue viability. Further, they offer the opportunity to control the electrochemical potential difference across the intestinal epithelium. However, as the mucosal and serosal membranes are studied together, it has been suggested that the presence of intact muscle and serosal layers can cause the measured unidirectional transmural fluxes to differ considerably from the actual transepithelial fluxes (Acra and Ghishan, 1991).

Previous studies have used the Ussing chamber system for measuring iron uptake by both rat (Helbock and Saltman, 1967; Vaghefi *et al.*, 1998, 2000a, b) and mouse (Costa *et al.*, 2000) intestinal tissue. Absorption of both haem and non-haem iron by intestinal tissue mounted in the Ussing chamber was observed in these studies. Further, the relative and absolute rates of iron absorption by intestinal segments mounted in Ussing chambers were in agreement with the rates seen *in vivo* by ligated intestinal segments (Helbrock and Saltman, 1967). These results support the use of the Ussing chamber model as an alternative *in vitro* method useful for iron absorption measurements in rat and mouse intestinal tissue.

1.7 Conclusions

Iron deficiency anaemia is a major nutritional problem in both developed and developing countries. In most cases, attempts to remedy iron deficiency have been by way of orally administered iron supplements. However, improving the diet may play a more important role in the prevention of iron deficiency.

There are several reasons why iron deficiency may occur, with one of the most important being low bioavailability of dietary iron. The bioavailability of iron is influenced by the solubility of iron and food components, which can enhance or inhibit the availability of iron for intestinal absorption. Further, some foods contain iron with high bioavailability (meat), while others with low bioavailability (cereals and some vegetal foods). Thus, to help combat iron deficiency, food combinations and meals need to be assessed not only for total iron content but also for the bioavailability of iron and the information used to develop diets that can be used to help prevent or treat iron deficiency.

Meat has been the focus of numerous iron bioavailability and absorption studies with the 'meat factor' effect consistently shown to enhance the bioavailability of iron from other foods. However, the effects of cooking and gastrointestinal digestion on the bioavailability of meat iron have not yet been fully investigated. Thus, the experiments described in Chapter Two were designed to examine the effects of cooking as well as pepsin and pancreatin-bile digestion on the solubility of haem and non-haem iron in meat either alone or as part of a meal. Solubility was used to estimate the amount of iron that would be available for intestinal absorption.

In vitro techniques have been used to study the bioavailability of iron in meat and other foods. Dialysability studies have suggested that meat iron is highly bioavailable, however, this technique does not accurately replicate active transport processes in the intestine. The use of Caco-2 cells has this advantage over dialysability measurements, however, due to their transformed nature, the extent to which normal cellular metabolic processes are maintained remains questionable. The use of an intact intestinal tissue provides advantages over the measurement of iron dialysability and absorption by Caco-2 cells to estimate the amount of iron available for absorption. Thus, the experiments described in Chapter Three are designed to validate the use of mouse intestinal tissue mounted in the Ussing chamber for iron absorption measurements. The Ussing chamber is then used in the experiments described in Chapter Four to investigate the absorption of iron from meat, vegetables and meat-plus-vegetables after gastrointestinal digestion.

CHAPTER TWO

Iron forms and solubility in foods: effects of cooking and *in vitro* pepsin and pancreatin-bile digestion

2.1 Introduction

Meat is a very important source of iron in the New Zealand diet, providing approximately 26% of total iron intake (Russell *et al.*, 1999). However, most studies of the iron content of meat have considered only the total amount of iron and have not distinguished between the haem and non-haem fractions. Even the New Zealand Food Composition Tables (Athar *et al.*, 1999) contain analysis of only the total iron content of meats. As discussed in Section 1.5, non-haem iron forms the main part of dietary iron intake in humans but it has a low bioavailability ranging from 2 to 20% (Monsen *et al.*, 1978; Hunt and Roughead, 2000). On the other hand, haem iron has a higher bioavailability, ranging from 15 to 35% (Monsen *et al.*, 1978; Hunt and Roughead, 2000). The difference in bioavailability between non-haem and haem iron illustrates the importance of differentiating between these two iron forms. The relative quantities of non-haem and haem iron must be known to accurately evaluate the total amount of bioavailable iron from foods. Also, the solubility of the haem and non-haem iron is important, as high solubility is generally associated with high bioavailability (Conrad and Umbreit, 2000).

There are many factors influencing the bioavailability of iron. Cooking reduces the overall soluble iron concentration of meat and degrades between 45% and 62% of haem iron, with a concomitant increase in the non-haem iron concentration (Schricker *et al.*, 1982b; Schricker and Miller, 1983; Buchowski *et al.*, 1988; Ahn *et al.*, 1993; Han *et al.*, 1993; Carpenter and Clark, 1995; Kristensen and Purslow, 2001). As food passes along the gastrointestinal tract it is subjected to digestion by gastric, pancreatic and intestinal enzymes and a change in pH as it moves from the acidic environment of the stomach to the alkaline pH of the small intestine. In addition, interactions between food constituents in the gastrointestinal tract have the potential to affect the bioavailability of iron by enhancing or inhibiting its

absorption. Although the absorption of haem iron is unaffected by the composition of the diet, non-haem iron absorption is inhibited by polyphenols and phytates, which form large, insoluble polymers with iron, thereby rendering iron unavailable for absorption. Conversely, ascorbic acid and proteolytic digestion products of meat form soluble, monomeric iron complexes and prevent the precipitation and polymerisation of iron, thereby promoting its uptake (Conrad and Schade, 1968). The effects of cooking, gastrointestinal digestion, pH and nutrient interactions emphasise the need to consider the solubility of haem and non-haem iron in food before and after cooking as well as throughout the gastrointestinal digestion process when determining the bioavailability of iron.

Hornsey (1956) was the first to determine haem iron concentrations in meat, by the 'total pigment analysis' method. Acid was used to extract the haem molecule from proteins and then acetone was added to solubilise the haem for spectrophotometric analysis. This is still the accepted method for determining haem iron concentrations in meat and has been used to measure haem iron concentrations in beef (Carpenter and Clark, 1995; Purchas *et al.*, 2003), lamb (Purchas *et al.*, 2003) and chicken (Clark *et al.*, 1997).

Non-haem iron concentrations in meat have also been determined spectrophotometrically using the iron-specific chromagens Ferrozine (Carter, 1971) or bathophenanthroline disulfonate (Schricker *et al.*, 1982b). There has been some debate regarding which chromagen measures non-haem iron more accurately. Ahn *et al.* (1993) concluded that the Ferrozine method of Carter (1971) was less prone to interference from haem iron than the bathophenanthroline methods used by Schricker *et al.* (1982b) and Rhee and Ziprin (1987). Since beef contains a high proportion of haem iron (Buchowski *et al.*, 1988; Carpenter and Clark, 1995), the Ferrozine method appears more suitable for determining non-haem iron levels in beef samples.

There have been studies investigating the effects of pepsin and pancreatin-bile digestion on the solubility of iron from cooked beef (Crews *et al.*, 1985a, b). These studies showed that pancreatin-bile digestion increased total iron solubility

compared to pepsin digestion, but the effects of pepsin digestion are unclear. Similarly, the solubility of iron prior to gastrointestinal digestion was not reported in these studies and iron was not separated into haem and non-haem fractions. Thus, it is not clear whether haem or non-haem iron was solubilised by the gastrointestinal digestion process.

There has been no attempt to determine the effect of cooking and the gastrointestinal digestion process on the concentrations and solubility of the different forms of iron in food. Therefore, the aim of Experiment One was to validate haem iron, non-haem iron and total iron assays for determining the concentrations of the different iron fractions in cooked meat, vegetables and meat-plus-vegetables. The sum of the haem and non-haem iron concentrations were compared to the total iron concentration determined by atomic absorption spectroscopy. In addition, the total, haem and non-haem iron assays were validated using standard solutions, certified reference materials and percent recoveries of haem and non-haem iron. Using these iron assay methods, Experiment Two was designed to determine the concentrations of haem and non-haem iron in the water-soluble and insoluble fractions of raw and cooked New Zealand beef and the effect of pepsin and pancreatin-bile digestion on the solubility of iron in meat, vegetables and meat-plus-vegetables. To simulate the gastrointestinal digestion process the *in vitro* method of Miller *et al.* (1981) was used. This method consists of a pepsin digestion step at pH 2.0, and subsequent adjustment to pH 7.0 for an intestinal digestion step with a mixture of pancreatic enzymes and bile extract.

2.2 Materials and methods

All glassware was soaked overnight in 5% nitric acid and rinsed thoroughly with deionised water before use.

2.2.1 Experiment One

Experiment One was conducted to determine whether the haem and non-haem iron assays were suitable for measuring these forms of iron in cooked meat, vegetables and meat-plus-vegetables.

2.2.1.1 Preparation of meat, vegetables and meat-plus-vegetables

A piece of beef rump steak (gluteus medius muscle) approximately 15mm thick and 130mm long was purchased from a local supermarket (Woolworths, New Zealand), as were the potatoes, carrots and frozen green peas. The meat was cooked by grilling at 180°C for 7½ minutes on each side. The vegetables were cooked by boiling in separate stainless steel saucepans over an electric element. The potatoes were cooked for 30 minutes, the carrots for 15 minutes, and the frozen green peas for 10 minutes.

The vegetable homogenate was prepared by combining 265g cooked potatoes, 105g cooked carrots and 75g of cooked frozen green peas in a Magimix Cuisine 5100 food processor (Magimix, Godalming, UK) and homogenised for 2 minutes. A meat-plus-vegetable homogenate was prepared by combining 215g of cooked meat with 425g of the cooked vegetable homogenate, which was then further homogenised with 500ml deionised water for 2 minutes. The meat-plus-vegetable homogenate was adjusted to pH 2.0 with 6M hydrochloric acid and two 100g aliquots were subjected to *in vitro* gastrointestinal digestion as described in Section 2.2.1.3. A second batch of 100g of potatoes, 100g of carrots and 100g of frozen green peas were collected and cooked as described above.

2.2.1.2 Sample collection

The following samples (25g) of cooked meat and vegetables were collected and stored frozen (-20°C) for later iron analysis:

1. Meat
2. Vegetable homogenate
3. Meat-plus-vegetables prior to the addition of water
4. Meat-plus-vegetables at pH 2.0
5. Meat-plus-vegetables after pepsin digestion
6. Meat-plus-vegetables after pepsin and pancreatin-bile digestion
7. Separate samples of potatoes, carrots and frozen green peas

A portion of each sample was analysed for total iron concentration as described in Section 2.2.1.5. The remaining sample in each case was then separated into

soluble and insoluble fractions as described in Section 2.2.1.4. The soluble and insoluble fractions were analysed for haem and non-haem iron concentrations as described in Sections 2.2.1.6 and 2.2.1.7, respectively. Total iron concentration was not analysed in the samples of cooked potatoes, carrots and frozen green peas.

2.2.1.3 *In vitro* gastrointestinal digestion

The following enzyme solutions were used for the *in vitro* digestion:

PEPSIN SOLUTION: 0.5g pepsin (Sigma, St Louis, USA) in 3.2ml 0.1M hydrochloric acid.

PANCREATIN-BILE MIXTURE: 0.6g pancreatin (Sigma, St Louis, USA) and 3.75g bile extract (Sigma, St Louis, USA) in 250ml 0.1M sodium bicarbonate.

Aliquots (100g) of the frozen homogenates were thawed and the temperature raised to 37°C in a shaking water bath (Heto Lab Equipment, Birkerod, Denmark). Pepsin solution (3.2ml) was added to each of the aliquots, which were then incubated in the shaking water bath (90rpm at 37°C) for 2 hours to simulate gastric digestion. After incubation, each aliquot was subdivided into five 20g samples. To one of these samples, 5ml of pancreatin-bile mixture was added and the pH was slowly raised to 7.0 by the drop-wise addition of 1M sodium bicarbonate. The added volume of sodium bicarbonate was recorded and this aliquot was then discarded. To the remaining aliquots, the recorded volume of 1M sodium bicarbonate was added in a drop-wise fashion and then placed in the shaking water bath (90rpm at 37°C). After 30 minutes, 5ml pancreatin-bile mixture was added to each 20g aliquot and the incubation was continued for a further 2 hours, to simulate intestinal digestion. The samples were then removed from the water bath and placed on ice to stop further enzymatic activity.

2.2.1.4 Separation of soluble and insoluble iron fractions

Approximately 20g of the sample was homogenised with three volumes (60g) of deionised water for 2 minutes in a Waring blender, centrifuged at 3000 x *g* for 20 minutes and the supernatant removed. The remaining pellet was washed and centrifuged twice more with the same volumes of deionised water as in the first

step. The supernatants were pooled and filtered (No. 6 filter paper, Whatman Maidstone, England) to remove fat and both the supernatant and pellet, which comprised the soluble and the insoluble fraction of the sample, respectively, were freeze-dried and ground using a mortar and pestle.

The weights for each of the soluble and insoluble samples were recorded before and after freeze-drying and the difference in weight was used to convert the iron concentrations from the freeze-dried to the non-freeze-dried sample (the 'freeze-dry-to-fresh factor').

2.2.1.5 Determination of total iron concentration

Total iron concentration was measured according to the method described by Clark *et al.* (1997). Weighed samples (0.40 to 0.80g) were digested in 25-ml Erlenmeyer flasks (Kimax, BioLab Scientific, Auckland, New Zealand) for 12 hours in 10ml of concentrated nitric acid (AnalaR, BDH Chemicals, Poole, UK). The resulting solution was heated to 105°C on a hotplate (Selby Ratec Co., UK). After approximately three hours, when all brown nitrous oxide fumes had dissipated, the temperature was raised to 120°C to remove the nitric acid. The remaining ash was dissolved in 3ml of 30% hydrogen peroxide (AnalaR, BDH Chemicals, Poole, UK) and heated to 100°C, to oxidise any fat present in the sample. Washing and heating with hydrogen peroxide was repeated until only a white ash remained. The white ash was then dissolved in 5ml of 2M nitric acid. Iron concentrations were determined using a GBC 933AA flame atomic absorption spectrometer and a standard curve was generated from iron standard solutions. Standards were made by dilutions of a 1000 ± 1μg/g commercial iron standard solution (SpectrosoL, BDH Chemicals, Poole, UK) to concentrations of 1, 2, 5 and 10μg/g in 2M nitric acid. Deionised water was used as the reagent blank. All assays were performed in triplicate.

2.2.1.6 Determination of haem iron concentration

Haem iron concentrations in both the soluble and insoluble fractions were determined using the method described by Hornsey (1956). Briefly, 0.25g of freeze-dried sample was dissolved in 2ml of deionised water, 0.25ml of

concentrated hydrochloric acid (AnalaR, BDH Chemicals, Poole, UK) and 10ml of acetone (AnalaR, BDH Chemicals, Poole, UK). The solution was centrifuged at 3000 x *g* for 20 minutes and the supernatant was filtered (GF/A filter paper, Whatman Maidstone, England). The absorbance of the filtrate was measured at 640nm against a reagent blank.

Hornsey (1956) calculated a conversion factor of 680 to convert absorbance into μg haematin/g meat, which was calculated for 10g of meat in a total volume of 50ml (acid, acetone, meat and water). This conversion factor corresponds to 136μg haematin/g meat/ml [680 x (10g ÷ 50ml)]. In this study, a quarter of the volume was used (12.5ml), thus absorbance was multiplied by 136μg haematin/g meat/ml then by 12.5ml and divided by the freeze-dried sample weight to give the concentration of total haem pigments as μg haematin/g freeze-dried sample. To convert the haem iron concentration from freeze-dried to non-freeze-dried material, the result was multiplied by the 'freeze-dry-to-fresh factor'. The iron concentration (μg/g) in the haem pigment was then calculated by multiplying by the molecular weight of iron (55.847) then dividing by the molecular weight of haematin (652). All assays were performed in duplicate.

2.2.1.7 Determination of non-haem iron concentration

The following solutions were used for the non-haem iron assay method:

CITRATE PHOSPHATE BUFFER: 0.906g of citric acid monohydrate (Sigma, St Louis, USA) and 1.615g of sodium phosphate dibasic (Sigma, St Louis, USA) in 100ml of deionised water (pH 5.5).

ASCORBIC ACID SOLUTION: 2% L-ascorbic acid (BDH Chemicals, Poole, UK) in 0.2M hydrochloric acid. Stored at 4°C and discarded after 3 days.

TRICHLOROACETIC ACID (TCA) SOLUTION: 11.3% trichloroacetic acid (BDH Chemicals, Poole, UK) in deionised water.

AMMONIUM ACETATE SOLUTION: 10% ammonium acetate (BDH Chemicals, Poole, UK) in deionised water.

FERROZINE REAGENT: 75mg Ferrozine (Sigma, St Louis, USA) and 75mg neocuproine (Sigma, St Louis, USA) in 25ml of deionised water containing 1 drop of concentrated hydrochloric acid (BDH Chemicals, Poole, UK). Stored at 4°C.

Non-haem iron was analysed by the Ferrozine method as described by Ahn *et al.* (1993). Briefly, 0.10 to 0.50g of freeze-dried sample was dissolved in 3ml of citrate phosphate buffer and 1ml of ascorbic acid solution. The solution was left to stand at room temperature for 15 minutes before adding 2ml TCA solution and centrifuging at 3000 x *g* for 10 minutes. To 2ml of the clear supernatant, 0.8ml of ammonium acetate solution and 0.2ml of Ferrozine reagent were added and the absorbance measured at 562nm against a reagent blank. Standards were made by dilution of the 1000 ± 1μg/g commercial iron standard solution to concentrations of 0, 5, 10 and 20μg/g iron in citrate phosphate buffer. The non-haem iron concentration of the sample was divided by weight of the freeze-dried sample analysed and converted from freeze-dried to non-freeze-dried material by multiplying the 'freeze-dry-to-fresh factor'. All samples were assayed in duplicate.

2.2.1.8 Validation of the assay for the total iron concentration

The assay method for total iron was validated by measuring the iron concentration of a Certified Reference Material (CRM) (International Atomic Energy Agency, animal muscle sample, H4), certified to have an iron concentration of 49μg/g. The CRM was analysed on four separate occasions (twice in duplicate and twice in triplicate) according to the method described in Section 2.2.1.5. The standard curves from these four assays were used to determine the goodness-of-fit of the predicted model to the actual standards and the range of iron concentrations at which the assay is most sensitive. The four estimates of the total iron concentration of the CRM were used to determine precision, bias and assay variation. Deionised water was used to assess the lowest detection limit of the assay.

2.2.1.9 Validation of the assay for the haem iron concentration

The haem iron assay was validated by assaying solutions containing known concentrations of porcine haematin (Sigma, St Louis, USA). The assayed haem iron concentration was then compared to the calculated concentration, which was determined as follows. Porcine haematin has a molecular weight of 652 and contains one iron atom (55.847); therefore, for a known weight of haematin, the iron content will be 8.6% (55.847 ÷ 652 x 100). Thus, the iron concentration for a

solution of haematin can be calculated by multiplying the concentration of haematin by 0.086. Standard solutions of haematin containing calculated concentrations of 3.75, 7.50 and 15μg/g haematin iron were prepared in 0.1M sodium hydroxide and assayed by the haem iron method.

To assess the ability of the haem iron assay to accurately measure the haem iron concentration from a protein solution, the iron concentration of two haemoglobin (Affco Labs, USA) solutions was measured using the haem iron and total iron assay and the results compared. Both haemoglobin solutions were assayed four times. Since all iron in the haemoglobin solutions is assumed to be in the haem form, both the total and haem iron assay should give the same result. The results from these assays were used to determine bias and precision and the reagent blank used to assess the lowest detection limit of the haem iron assay.

To determine assay variation, the haem iron concentration of a single freeze-dried sample of the soluble fraction of a raw meat homogenate was measured for each haem iron assay. To determine percent recovery of haem iron from meat, haematin (0.50ml of 102μg/g solution) was added to a soluble raw meat homogenate, with a measured haem iron concentration of 8.2μg/g, and the assayed result was compared to the expected result.

2.2.1.10 Validation of the assay for non-haem iron concentration

A standard curve was produced by measuring the iron concentration of the 1000 ± 1μg/g commercial iron standard solution at concentrations of 0, 5, 10 and 20μg/g. The standard curves from four test assays were used to determine the goodness-of-fit of the predicted model to the actual standards, and the range of iron concentrations at which the assay is most sensitive. The reagent blanks from these assays were used to assess the lowest detection limit of the non-haem iron assay. Precision was determined from four assays of a 10μg/g solution of ferric chloride. To determine assay variation, the non-haem iron concentration of a single freeze-dried sample of soluble raw meat homogenate was measured with each non-haem iron assay. To determine percent recovery of non-haem iron, ferric chloride (0.50ml of 102μg/g solution) was added to a soluble raw meat

homogenate, with a known non-haem iron concentration of 1.30μg/g, and the assayed result was compared to the expected result.

2.2.1.11 Haem and non-haem iron concentrations in pepsin, pancreatin and bile

To test whether pepsin, pancreatin and bile contributed iron to the digested food solutions, they were subjected to analysis by the haem and non-haem iron assays as described in Sections 2.2.1.6 and 2.2.1.7, respectively.

2.2.1.12 Statistical analysis

The standard curve for the total iron assay was generated using a least squares non-linear regression model with GBCAA System Management Software (GBC Software Ltd.). For the non-haem iron assay, the standard curve was generated using a linear regression model using GraphPad Prism 3.0 (GraphPad Software Inc.). The lowest detection limit for each assay was set as the mean absorbance of the assay reagent blank plus three standard deviations. The precision of each assay was determined by the coefficient of variation (CV) for the test samples. The section of the standard curve with the greatest slope determined the range of greatest sensitivity. Bias was determined using a Chi-squared analysis. A Chi-squared statistic was generated from standard deviations from precision analysis, and then compared to the 95% critical Chi-squared statistic. Assay variation was determined using one-way ANOVA (GraphPad Prism 3.0) to determine within- and between-assay mean squares. Within-assay variation was calculated by converting the within-assay mean square, which is an estimate of the within-assay variance, to a standard deviation and was then expressed as a percentage of the overall mean. The between-assay mean square equals the sum of the within-assay mean square and the between-assay variance, multiplied by the number of assays. Therefore, to calculate the between-assay variance, the within-assay mean square was subtracted from the between-assay mean square and the result divided by the number of assays. The between-assay variance was then converted to a standard deviation and then expressed as a percentage of the overall mean.

2.2.2 Experiment Two

Using the iron assay methods in Section 2.2.1, the concentrations of soluble haem and non-haem iron in raw and cooked meat and digests of cooked meat and meat-plus-vegetables after pepsin and pancreatin-bile digestion were determined. In addition, the concentrations of soluble non-haem iron in cooked vegetables and digests were measured.

2.2.2.1 Meat

A beef striploin cut (longissimus lumborum muscle) from a prime carcass (2 to 2½ years old) was purchased within 24 hours of slaughter from a local freezing works (AFFCO Manawatu, Feilding, New Zealand), vacuum packed and stored at 0 to 1°C. Two days after purchase, the meat was cut into 130mm long, 25mm thick slices and all visible fat and connective tissue removed. An average sample weight was approximately 190g. Each sample was placed in an individual plastic bag and suspended for 90 minutes in a water bath maintained at a constant 65°C. After cooking, the steaks were removed from the bags and the cooking juices were discarded.

2.2.2.2 Vegetables

The vegetables chosen (potatoes, carrots and frozen green peas) were those most frequently consumed in New Zealand as detailed in the 1997/98 New Zealand Total Diet Survey (Vannoort *et al.*, 2000). All vegetables were purchased from a local supermarket (Woolworths, New Zealand) on the day of cooking. The potatoes and carrots were peeled and sliced before cooking. The vegetables were cooked by boiling in separate stainless steel saucepans over an electric element. The potatoes were cooked for 30 minutes, the carrots for 15 minutes, and the frozen green peas for 10 minutes.

2.2.2.3 Preparation of the homogenates

After cooking, known weights of the meat, vegetables or a mixture of meat and vegetables were homogenised in the food processor with deionised water in proportions given in Table 2.1. Deionised water was added to each homogenate to achieve a liquid consistency.

Table 2.1: Weights of the cooked meat (Mt) and cooked vegetables (Vg) prepared as homogenates and the amount of water added to achieve a liquid consistency.

	Weight (g) of food constituents				Water added (g)
	Meat	Potatoes	Carrots	Peas	
Mt (6)	150	-	-	-	400
Vg (3)	-	175	120	100	300
Mt + Vg (3)	150	175	120	100	500

Number of replicates shown in parentheses.

Each homogenate was adjusted to pH 2.0 with 6M hydrochloric acid, divided into 100g aliquots and stored frozen at -20°C. Each 100g aliquot was then subjected to *in vitro* gastrointestinal digestion as described in Section 2.2.1.3.

2.2.2.4 Sample collection

The following samples (25g) were collected and stored frozen (-20°C) for later iron analysis:

1. Raw meat
2. Cooked meat
3. Samples of the cooked meat, vegetables and meat-plus-vegetables at pH 2.0 before pepsin digestion
4. Samples of the cooked meat, vegetables and meat-plus-vegetables after pepsin digestion
5. Samples of the cooked meat, vegetables and meat-plus-vegetables after pepsin and pancreatin-bile digestion

Each sample was separated into soluble and insoluble fractions as described in Section 2.2.1.4. The soluble and insoluble fractions were analysed for haem and non-haem iron concentrations as described in Sections 2.2.1.6 and 2.2.1.7, respectively.

2.2.2.5 Statistical analysis

For soluble haem, insoluble haem, soluble non-haem and insoluble non-haem, total haem iron, total non-haem iron, total soluble iron and total insoluble iron, the significance of the differences between raw and cooked meat were determined using independent-samples 2-tailed t tests with SPSS version 11.0 (SPSS Inc.).

Iron concentrations in the four iron fractions, total iron and total haem and non-haem iron were analysed as absolute values (μg/g) and as a percentage of the total iron concentration using a general least squares models (GLM) within the GLM procedure of SAS (SAS Inst. Inc.). Multiple comparisons were made using Tukey-Kramer *post hoc* tests. Separate analyses were run for soluble haem, insoluble haem, soluble non-haem, insoluble non-haem, total, total haem and total non-haem iron concentrations in the meat and meat-plus-vegetables after cooking, after pepsin digestion and after pepsin and pancreatin-bile digestion. The model included terms for the presence of vegetables (vegetable effect), a replicate effect of the same meat sample for the digestion treatments (replicate effect), a treatment effect (after cooking, after pepsin digestion and after pancreatin-bile digestion), and the interaction between the treatment and vegetable effect (treatment by vegetable interaction). The effectiveness of the model was shown by r^2 (coefficient of determination) and RSD (residual standard deviation). The vegetable effect was tested against the replicate effect, while the treatment and interaction effects were tested against the overall error in a repeat-measures-like analysis (see Appendix One for sample Analysis of Variance). Iron concentrations in the vegetables were analysed as absolute values (μg/g) and as a percentage of the total iron concentration using a one-way Analysis of Variance (ANOVA) using SPSS version 11.0. Multiple comparisons were made using Tukey's HSD *post hoc* tests. Separate analyses were run for soluble non-haem and insoluble non-haem in the cooked, pepsin and pancreatin-bile digested samples. Comparisons between the total soluble iron in the meat, vegetables and meat-plus-vegetables after cooking, after pepsin digestion and after pepsin and pancreatin-bile digestion were analysed as a percentage of the total iron concentration with a one-way ANOVA using SPSS version 11.0. Multiple comparisons were made using Tukey's HSD *post hoc* tests.

2.3 Results

2.3.1 Experiment One

2.3.1.1 Validation of the iron assay methods

Statistical data for the goodness-of-fit, sensitivity and variability of the total, haem and non-haem iron assays are presented in Table 2.2. Although the lowest

detection for each assay was below 1μg/g, the range of greatest sensitivity was at iron concentrations greater than 1μg/g. Precision for each assay was similar but assay variation was higher for the total iron assay.

Table 2.2: Statistical data for the goodness-of-fit, sensitivity and variability of the total iron, haem iron and non-haem iron assays.

	Total iron	Haem iron	Non-haem iron
Goodness-of-fit of the standard curve (r^2)	0.98 - 0.99	-	0.99 - 1.00
Lowest detectable concentration (μg/g)	0.1	0.6	0.2 - 0.5
Precision (CV)	≤ 10%	≤ 10%	≤ 10%
Range of greatest sensitivity (μg/g)	1 - 5	-	1 - 20
Bias	None	None	None
Recovery of spiked iron	-	93%	87%
Assay variation Within assay Between assay	 7.5% 5.7%	 6.0% 3.6%	 2.7% 5.5%

r^2=coefficient of determination; CV=coefficient of variation.

The mean for the total concentration of iron in the CRM, determined on four separate occasions using the total iron assay, was 52μg/g (Table 2.3) with a 95% confidence interval of 6.8%. This was comparable to the certified iron concentration of 49μg/g with a 95% confidence interval of 4.2%.

Table 2.3: The iron concentration in the Certified Reference Material (CRM) as determined by the total iron assay method. The CRM is certified to have a total iron concentration of 49μg/g.

Assay number	Mean ± SD
1	49.8 ± 4.7 (3)
2	53.7 ± 3.3 (3)
3	55.5 ± 1.3 (2)
4	46.5 ± 4.9 (2)
Overall mean	51.7 ± 4.7 (10)

The number of samples shown in parentheses.

The assayed concentration of haem iron in three separate solutions of haematin differed by no more than 2.0% from the calculated concentration (Table 2.4). Furthermore, the total and haem iron assay produced similar results for the iron concentration of two haemoglobin solutions demonstrating the haem iron assay could measure all of the haem iron from protein solutions (Table 2.5).

Table 2.4: Comparison of the calculated and assayed haem iron concentrations in three haematin solutions.

Haem iron concentration (μg/g)		% Difference
Calculated	Assayed	
3.75	3.8 (2)	1.3
7.50	7.5 (2)	0.0
15.00	14.9 (2)	0.7

The number of samples shown in parentheses.

Table 2.5: Means (± SEM) for the total and haem iron concentrations in two haemoglobin samples.

Total iron concentration (μg/g)	Haem iron concentration (μg/g)	% Difference
12.4 ± 0.3 (4)	13.9 ± 0.8 (4)	12.0
23.7 ± 1.4 (4)	25.4 ± 1.1 (4)	7.0

The number of samples shown in parentheses.

2.3.1.2 Determination of total, haem and non-haem iron concentrations in undigested and digested meat, vegetables and meat-plus-vegetables

The total iron concentration and the concentrations of soluble and insoluble haem and non-haem iron in separate samples of cooked, but undigested meat, vegetables and meat-plus-vegetables and the cooked meat-plus-vegetables after the three stages of simulated gastrointestinal digestion are presented in Table 2.6.

Table 2.6: Haem (H) and non-haem (NH) iron concentrations in cooked, but undigested meat (Mt), vegetables (Vg) and meat-plus-vegetables (Mt + Vg), and digested Mt + Vg.

	Undigested			Digested Mt + Vg		
	Mt	Vg	Mt + Vg	pH 2.0	Pep	Panc
Total iron (μg/g)	31.1	6.6	22.4	12.8	11.4	8.4
Soluble H (μg/g)	4.0	1.5	1.0	0.7	0.7	0.9
Insoluble H (μg/g)	18.3	2.2	13.3	4.8	4.4	2.3
Soluble NH (μg/g)	0.6	3.9	1.7	2.9	2.4	5.5
Insoluble NH (μg/g)	10.2	1.6	6.1	2.9	3.9	0.8
Total NH (μg/g)	10.8	5.4	7.7	5.8	6.3	6.3
Total H (μg/g)	22.3	3.7	14.2	5.6	5.1	3.2
NH + H (μg/g)	33.1	9.1	22.0	11.4	11.4	9.5
NH + H (% of total iron)	107	138	98	89	99	113

Note: Iron concentrations for digested Mt + Vg have not been corrected for the addition of deionised water, hydrochloric acid (pH 2.0), pepsin (Pep), bicarbonate and pancreatin-bile (Panc) solutions.

Compared to the undigested meat-plus-vegetables the digested samples had lower concentrations of iron, due to the addition of deionised water, hydrochloric acid, sodium bicarbonate and enzyme solutions. The pancreatin and bile extracts both contained haem iron, however, at the concentrations used for the digestion process, the concentration of iron contributed by the pancreatin-bile mix was negligible (less than 0.1μg/g).

For the meat and meat-plus-vegetables, the sums of the respective non-haem and haem iron concentrations were approximately equal to the corresponding total iron concentrations (Table 2.6). However, for the vegetables the sum of the non-haem and haem iron concentrations was 38% higher than the total iron concentration. This was due to the apparent presence of haem iron in the vegetable mix. Separate determination of haem iron concentrations in the potatoes, carrots and frozen green peas showed that most of the apparent haem iron came from the potatoes and peas (Table 2.7).

Table 2.7: Percent contribution of the potatoes, carrots and peas to the soluble and insoluble haem iron fraction of the vegetable mix.

	Potatoes	**Carrots**	**Peas**
Soluble haem (%)	80	8	12
Insoluble haem (%)	<0.1	5	95

The contribution of the potatoes, carrots and peas to the non-haem iron concentration of the vegetable mix is shown in Table 2.8.

Table 2.8: Non-haem iron concentrations in the potatoes, carrots and peas.

	Vegetable constituents			**Total iron (μg/g)**
	Potatoes	Carrots	Peas	
Soluble non-haem (μg/g)	1.5	0.3	1.0	2.8
Insoluble non-haem (μg/g)	1.8	0.2	0.4	2.3
Total non-haem iron (μg/g)	3.3	0.5	1.4	5.1

Although the peas had a high concentration of non-haem iron, the greatest contribution was from the potatoes, which formed the highest proportion of the vegetable mix (see Section 2.2.2.3). The total non-haem iron concentration of

5.1μg/g from the potatoes, carrots and peas closely agrees with the 5.4μg/g total non-haem iron in the vegetable mix (Table 2.6).

2.3.2 Experiment Two

All results presented in this section have been corrected for dilution due to the addition of water, enzyme solutions and acid and bicarbonate solutions during the preparation and digestion processes. Thus, all iron concentrations are per unit weight of cooked sample, except for the concentration of iron in the raw meat.

Table 2.9 shows the total, haem and non-haem iron concentrations in the cooked meat, vegetables and meat-plus-vegetables.

Table 2.9: Means (± SEM) for the total iron and total haem and non-haem iron concentrations in cooked, but undigested meat (Mt), vegetables (Vg) and meat-plus-vegetables (Mt + Vg).

	Iron concentration (μg/g)		
	Non-haem	Haem	Total
Mt + Vg (3)	6.3 ± 0.3	7.4 ± 1.0	13.8 ± 0.8
Vg (3)	5.0 ± 0.3	-	5.0 ± 0.3
Mt (6)	13.2 ± 0.8	12.7 ± 1.2	25.9 ± 1.6

The number of samples shown in parentheses.

2.3.2.1 Effect of cooking on meat haem and non-haem iron

The total iron concentration in meat was not affected ($p>0.05$) by cooking (Table 2.10). However, cooking significantly ($p<0.05$) reduced the haem iron concentration in the meat by 27% and increased ($p<0.001$) the non-haem iron concentration by 175%.

Table 2.10: Means (± SEM) for the haem and non-haem iron concentrations and soluble and insoluble iron concentrations in raw (n=4) and cooked (n=6) meat.

	Raw	Cooked
Haem iron		
Concentration (μg/g)	17.3 ± 0.7	12.7 ± 1.2*
% Total iron concentration	78.4 ± 2.7	48.9 ± 2.5***
Non-haem iron		
Concentration (μg/g)	4.8 ± 0.6	13.2 ± 0.8***
% Total iron concentration	21.64 ± 2.7	51.14 ± 2.5***
Soluble iron (% Total iron concentration)	80.6 ± 1.3	15.0 ± 2.9***
Insoluble iron (% Total iron concentration)	19.4 ± 1.3	85.0 ± 2.9***
Total iron concentration (μg/g)	22.1 ± 0.3	25.9 ± 1.6

*p<0.05, ***p<0.001 compared to raw iron concentrations.

Cooking also affected the solubility of iron; it significantly (p<0.001) reduced the concentration of total soluble iron by 81% and increased insoluble iron by 338% (Table 2.10). This was due to a reduction in the solubility of haem iron, but not non-haem iron, and was accompanied by an increase in the percentage concentrations of insoluble haem and non-haem iron (Figure 2.1).

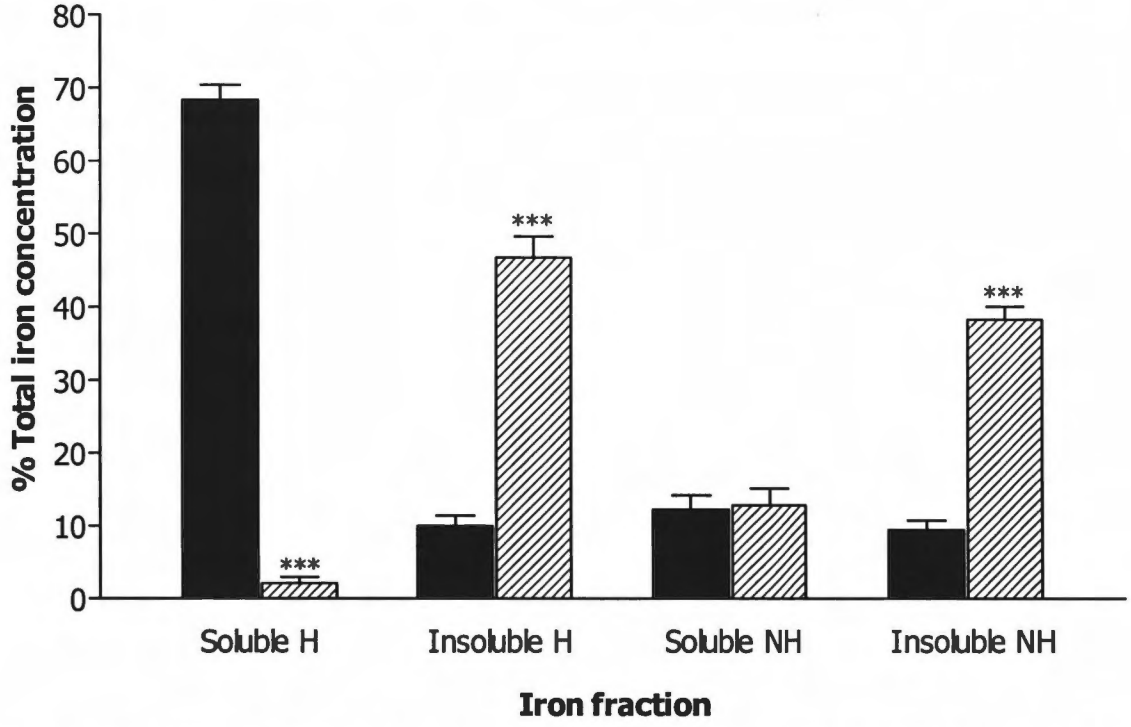


Figure 2.1: Mean (+ SEM) percentage iron concentrations in the soluble and insoluble fractions of haem (H) and non-haem (NH) iron in raw meat (▬) (n=4) and cooked meat (▨) (n=6). Within each iron fraction, ***p<0.001 between the raw and cooked values.

2.3.2.2 Effect of gastrointestinal digestion on iron solubility

Tables 2.11 and 2.12 show the levels of statistical significance of the vegetable effect, replicate effect, treatment effect and treatment by vegetable interaction for the GLM procedures. Comparisons were made using absolute (μg/g) (Table 2.11) and percentage (Table 2.12) values for the iron concentration. The absolute values are shown in Appendix Two.

Table 2.11: General linear model results for the absolute values (μg/g) of haem and non-haem iron.

	Haem iron		Non-haem iron	
	Soluble	Insoluble	Soluble	Insoluble
Vegetable effect[1]	*	**	***	**
Replicate effect[2]	NS	***	NS	NS
Treatment effect[3]	***	***	***	***
Treatment by vegetable interaction[4]	**	**	***	*
r^2 (RSD)[5]	93% (0.77)	96% (1.27)	96% (1.28)	89% (1.28)

[1] Vegetable effect refers to the overall differences between the meat and meat-plus-vegetables.
[2] Replicate effect compares the meat iron concentrations in the replicate meat samples across the three treatments (cooking, pepsin digestion and pancreatin-bile digestion).
[3] Treatment effect tests for differences due to cooking, pepsin digestion and pepsin and pancreatin-bile digestion.
[4] Treatment by vegetable interaction tests whether cooking, pepsin digestion and pepsin and pancreatin-bile digestion were different for the meat and meat-plus-vegetables.
[5] r^2 (coefficient of determination) and RSD (residual standard deviation) shows the fit of the data to the model.
Levels indicating the differences are NS=not significant, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

Table 2.12: General linear model results for haem and non-haem iron concentrations expressed as a percentage of the total iron concentration.

	Haem iron		Non-haem iron	
	Soluble	Insoluble	Soluble	Insoluble
Vegetable effect[1]	***	*	*	**
Replicate effect[2]	NS	NS	NS	NS
Treatment effect[3]	***	***	***	***
Treatment by vegetable interaction[4]	***	NS	NS	***
r^2 (RSD)[5]	94% (4.32)	93% (6.01)	94% (5.73)	91% (5.99)

[12345] Footnotes the same as for Table 2.11.
Levels indicating the differences are NS=not significant, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

In general, there was an overall difference in the solubility of iron between meat and meat-plus-vegetables (vegetable effect). Overall, gastrointestinal digestion

(treatment effect) had an effect on the solubility of both haem and non-haem iron, and there were differences in these effects between the meat and meat-plus-vegetables (treatment by vegetable interaction).

2.3.2.2.1 Meat

Figure 2.2 shows the effects of gastrointestinal digestion on the solubility of haem and non-haem iron in cooked meat. The cooked and pepsin digested meat had high percentage concentrations of insoluble iron. However, pancreatin-bile digestion greatly reduced the percentage concentrations of insoluble iron and increased the percentage concentrations of soluble iron, especially soluble non-haem iron. After pancreatin-bile digestion, most of the iron (53%) was present as soluble non-haem iron.

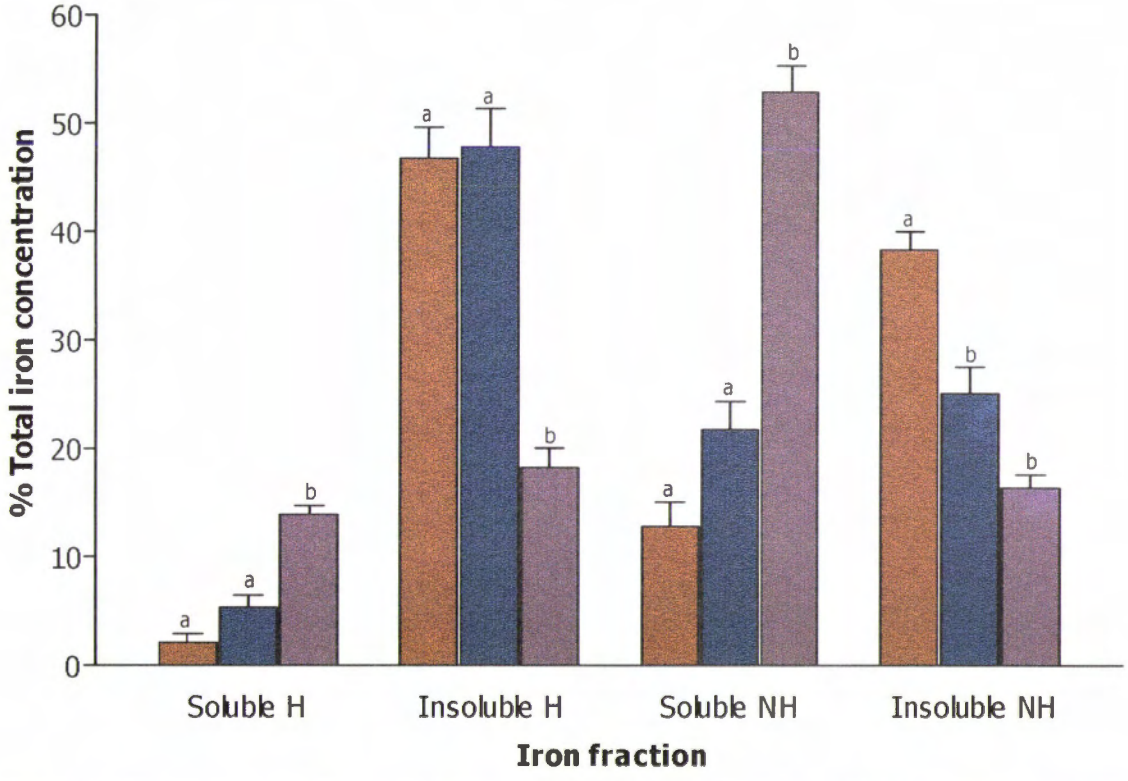


Figure 2.2: Mean (+ SEM) percentage iron concentrations as soluble haem, insoluble haem, soluble non-haem and insoluble non-haem in meat (n=6) after cooking (▬), after pepsin digestion (▬), and after pepsin and pancreatin-bile digestion (▬). Within each iron fraction, means do not differ significantly ($p>0.05$) if they have a common letter above them. H=haem iron; NH=non-haem iron.

Table 2.13 shows the means for the absolute values for soluble and insoluble haem and non-haem iron in meat after cooking, after pepsin digestion and after pepsin

and pancreatin-bile digestion, and the significance of the differences between the means.

Table 2.13: Mean (± SEM) concentrations of soluble and insoluble haem and non-haem iron in meat (n=6) after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	Cook	Pep	Panc
Soluble haem (μg/g)	0.6 ± 0.2^a	1.3 ± 0.3^a	3.7 ± 0.2^b
Insoluble haem (μg/g)	12.2 ± 1.2^a	12.1 ± 1.1^a	5.0 ± 0.8^b
Soluble non-haem (μg/g)	3.2 ± 1.3^a	5.4 ± 0.6^a	14.0 ± 0.6^b
Insoluble non-haem (μg/g)	9.9 ± 0.9^a	7.1 ± 0.7^b	4.4 ± 0.4^b

Within each row, means do not differ significantly ($p>0.05$) if they have a common letter beside them.

Pepsin digestion significantly ($p<0.01$) decreased the concentration of insoluble non-haem iron and also increased the concentrations of soluble non-haem and soluble haem iron, but these changes were not significant ($p>0.05$).

Compared to pepsin digestion, pancreatin-bile digestion significantly ($p<0.01$) increased soluble haem iron and decreased ($p<0.001$) insoluble haem iron concentrations in meat. There was also a significant ($p<0.001$) increase in soluble non-haem iron, which was greater than the decrease in the concentration of insoluble non-haem iron (Table 2.13). This coincided with an overall significant ($p<0.001$) decrease in the total haem iron concentration and a significant ($p<0.001$) increase in the concentration of non-haem iron after pancreatin-bile digestion (Table 2.14).

Table 2.14: Means (± SEM) for the total haem and non-haem iron concentrations in meat (n=6) after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	Cook	Pep	Panc
Haem iron (μg/g)	12.7 ± 1.2^a	13.5 ± 0.9^a	8.6 ± 0.9^b
Non-haem iron (μg/g)	13.2 ± 0.8^a	11.8 ± 0.8^a	18.3 ± 0.8^b
Total iron (μg/g)	25.9 ± 1.6	25.3 ± 1.1	26.6 ± 1.5

Within each row, means do not differ significantly ($p>0.05$) if they have a common letter or no letter beside them.

2.3.2.2.2 Meat-plus-vegetables

Figure 2.3 shows the effects of gastrointestinal digestion on the solubility of haem and non-haem iron in meat-plus-vegetables. As with the meat, both the cooked and pepsin digested meat-plus-vegetables contained higher percentage concentrations of insoluble than soluble iron. Pancreatin-bile digestion increased the percentage concentrations of soluble iron, with concomitant decreases in insoluble iron.

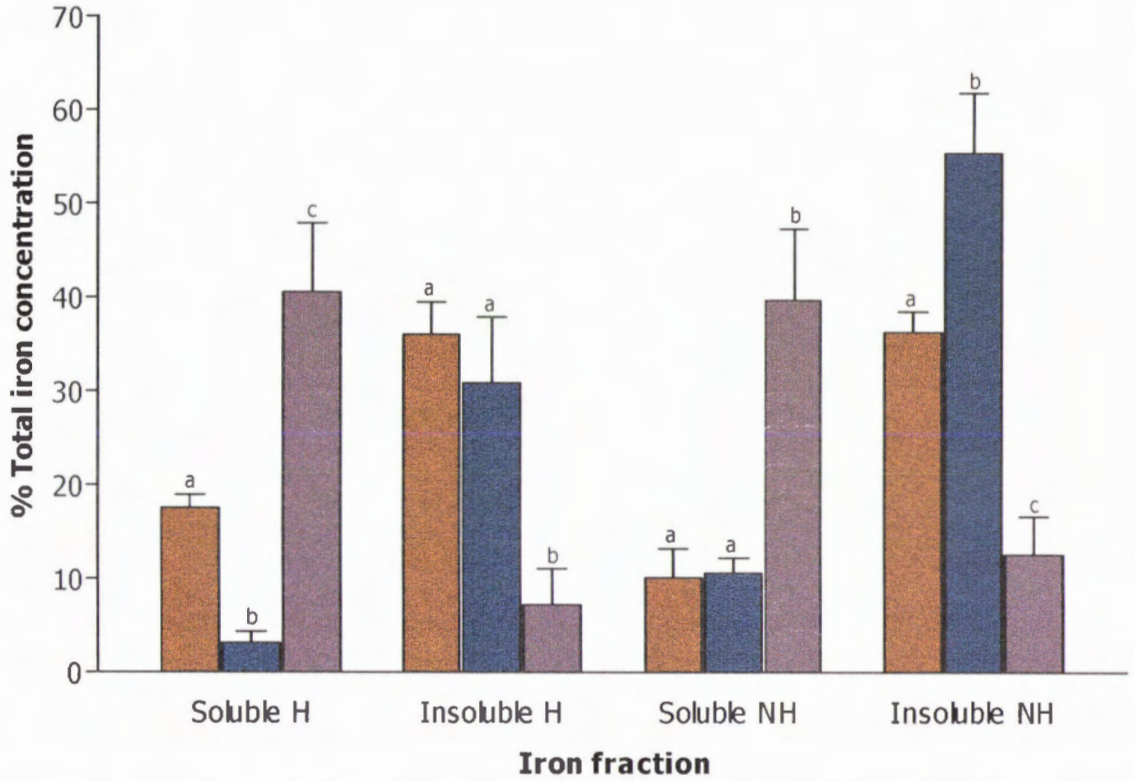


Figure 2.3: Mean (+ SEM) percentage iron concentrations as soluble haem, insoluble haem, soluble non-haem and insoluble non-haem in meat-plus-vegetables (n=3) after cooking (▬), after pepsin digestion (▬), and after pepsin and pancreatin-bile digestion (▬). Within each iron fraction, means do not differ significantly ($p>0.05$) if they have a common letter above them. H=haem iron; NH=non-haem iron.

Table 2.15 shows the means for the absolute values for soluble and insoluble haem and non-haem iron in the meat-plus-vegetables after cooking, after pepsin digestion and after pepsin and pancreatin-bile digestion, and the significance of the differences between the means.

Table 2.15: Mean (± SEM) concentrations of soluble and insoluble haem and non-haem iron in meat-plus-vegetables (n=3) after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	Cook	Pep	Panc
Soluble haem (μg/g)	2.4 ± 0.3^{a}	0.3 ± 0.1^{b}	6.1 ± 1.3^{c}
Insoluble haem (μg/g)	5.0 ± 0.7^{a}	3.3 ± 1.1^{ab}	1.0 ± 0.5^{b}
Soluble non-haem (μg/g)	1.3 ± 0.3^{a}	1.1 ± 0.2^{a}	5.8 ± 0.9^{b}
Insoluble non-haem (μg/g)	5.0 ± 0.2^{a}	5.5 ± 0.3^{a}	1.9 ± 0.7^{b}

Within each row, means do not differ significantly ($p>0.05$) if they have a common letter beside them.

Pepsin digestion significantly ($p<0.05$) reduced the concentration of soluble haem iron and increased the concentration of insoluble non-haem iron; this latter effect was only significant ($p<0.05$) when the values were expressed as percentage concentrations (Figure 2.3). The apparent loss of haem iron due to pepsin digestion was not accompanied by an increase in the concentration of insoluble haem iron or of soluble or insoluble non-haem iron (Table 2.15), but was accompanied by a 30% drop in the total iron concentration (Table 2.16).

Table 2.16: Means (± SEM) for the total haem and non-haem iron concentrations in meat-plus-vegetables (n=3) after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	Cook	Pep	Panc
Haem iron (μg/g)	7.4 ± 1.0^{a}	3.6 ± 1.0^{b}	7.1 ± 0.9^{a}
Non-haem iron (μg/g)	6.3 ± 0.3	6.6 ± 0.1	7.7 ± 0.6
Total iron (μg/g)	13.8 ± 0.8^{ab}	10.2 ± 0.9^{a}	14.8 ± 1.1^{b}

Within each row, means do not differ significantly ($p>0.05$) if they have a common letter or no letter beside them.

The soluble haem iron, apparently lost after pepsin digestion, 'reappeared' after pancreatin-bile digestion (Table 2.15), and was accompanied by a significant ($p<0.05$) increase in total iron concentration to approximately that measured before pepsin digestion (Table 2.16). Pancreatin-bile digestion also significantly ($p<0.01$) increased the concentrations of soluble haem and soluble non-haem iron and caused significant ($p<0.05$) decreases in the concentrations of insoluble non-haem and insoluble haem iron; this latter effect, on insoluble haem iron, was only significantly ($p<0.01$) different to the concentration after pepsin digestion when the values were expressed as percentage concentrations (Figure 2.3).

2.3.2.2.3 Comparison of meat and meat-plus-vegetable haem and non-haem iron concentrations

Since the total iron concentrations were lower in the meat-plus-vegetables than in the meat (Table 2.9), statistical comparisons between the concentrations of iron in the four iron fractions of meat and meat-plus-vegetables were performed using the concentrations expressed as a percentage of the total iron concentration. Overall, there were no statistically significant ($p>0.05$) differences between the meat and meat-plus-vegetables (treatment by vegetable interaction) for insoluble haem iron or soluble non-haem iron (Table 2.12). Therefore, results are presented only for the percentage concentrations of soluble haem and insoluble non-haem iron (Table 2.17).

Table 2.17: Statistical comparisons between meat (Mt) and meat-plus-vegetables (Mt + Vg) for concentrations of soluble haem and insoluble non-haem iron after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	% Total iron concentration					
	Soluble haem iron			Insoluble non-haem iron		
	Mt	Mt + Vg	p	Mt	Mt + Vg	p
Cook	2.1 ± 0.8	17.5 ± 1.5	**	38.3 ± 1.8	36.4 ± 2.2	NS
Pep	5.3 ± 1.1	3.2 ± 1.2	NS	25.1 ± 2.5	55.4 ± 6.4	***
Panc	13.8 ± 0.9	40.5 ± 7.4	***	16.1 ± 1.2	12.5 ± 4.1	NS

Levels indicating the differences between the meat and meat-plus-vegetables are NS=not significant, $^{**}p<0.01$ and $^{***}p<0.001$.

As shown in Table 2.17, compared to the meat, the percentage concentration of soluble haem iron was significantly greater in the meat-plus-vegetables after cooking and after pancreatin-bile digestion, but was similar after pepsin digestion. The absence of any significant difference after pepsin digestion may be due to the loss of haem iron in the meat-plus-vegetables after pepsin digestion (Table 2.16). However, despite the greater concentrations of haem iron in the meat-plus-vegetables, a higher proportion of meat haem iron was solubilised by digestion: 2 to 14% in meat (Figure 2.2) and 18 to 41% in meat-plus-vegetables (Figure 2.3). The difference in insoluble non-haem iron between meat and meat-plus-vegetables after pepsin digestion may be due to the apparent loss of haem iron (Table 2.16). This led to a reduction in the total iron concentration, thus affecting the non-haem iron concentration when expressed as a percentage of the total iron concentration.

2.3.2.2.4 Vegetables

Figure 2.4 shows the effects of gastrointestinal digestion on the solubility of non-haem iron in the vegetables. The solubility of non-haem iron was significantly ($p<0.05$) increased by pepsin digestion and by pancreatin-bile digestion (see also Table 2.18). These changes were accompanied by significant ($p<0.05$) decreases in insoluble non-haem iron.

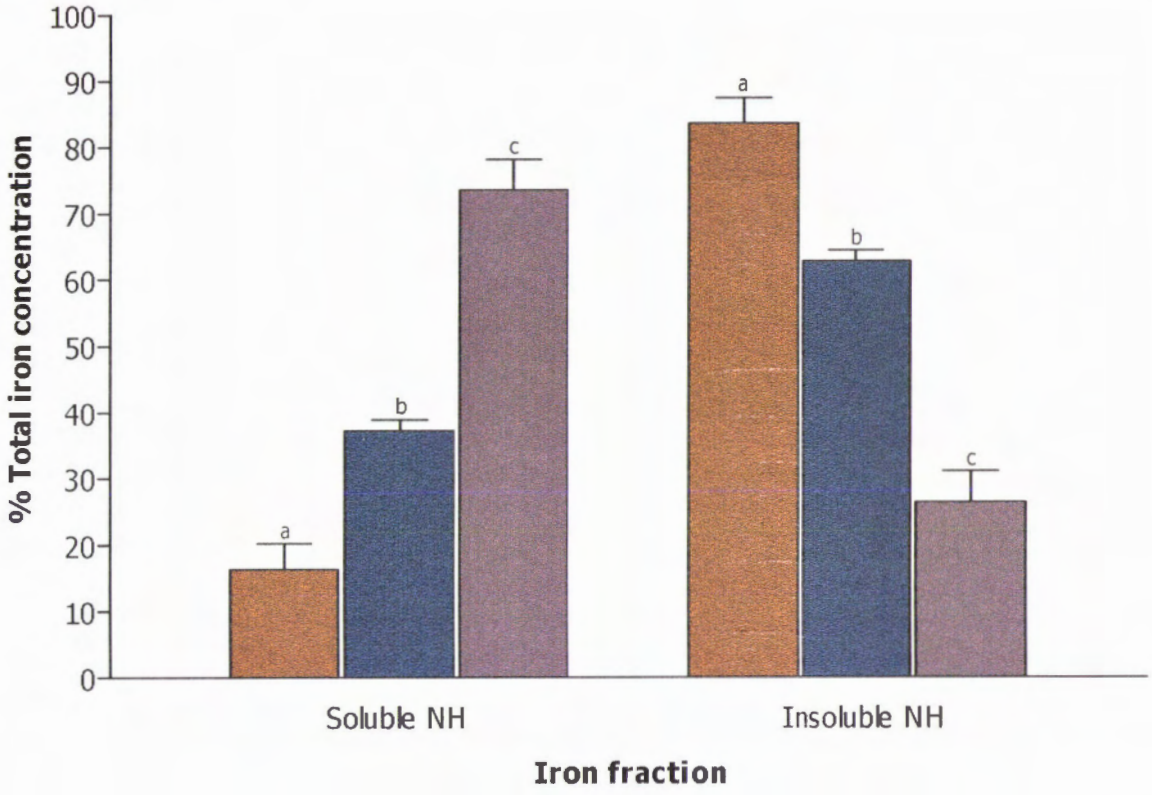


Figure 2.4: Mean (+ SEM) percentage iron concentrations as soluble non-haem and insoluble non-haem in vegetables (n=3) after cooking (▬), after pepsin digestion (▬), and after pepsin and pancreatin-bile digestion (▬). Within each iron fraction, means do not differ significantly ($p>0.05$) if they have a common letter above them. NH=non-haem iron.

Table 2.18: Mean (± SEM) concentrations of soluble and insoluble non-haem iron in vegetables (n=3) after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	Cook	Pep	Panc
Soluble non-haem (μg/g)	0.6 ± 0.3^a	1.7 ± 0.1^b	3.8 ± 0.3^c
Insoluble non-haem (μg/g)	4.2 ± 0.3^a	2.9 ± 0.1^b	1.4 ± 0.2^c

Within each row, means do not differ significantly ($p>0.05$) if they have a common letter beside them.

2.3.2.2.5 Total soluble iron

Figure 2.5 shows the percentage concentration of soluble iron in the meat, vegetables and meat-plus-vegetables as gastrointestinal digestion progressed. Overall, there was an increase in the solubility of iron between the three stages of digestion. The meat-plus-vegetables appeared to contain a higher percentage of soluble iron after cooking and after pancreatin-bile digestion. The lower concentration of soluble iron in the meat-plus-vegetables after pepsin digestion may be due to the loss of soluble haem iron from this sample (Table 2.15).

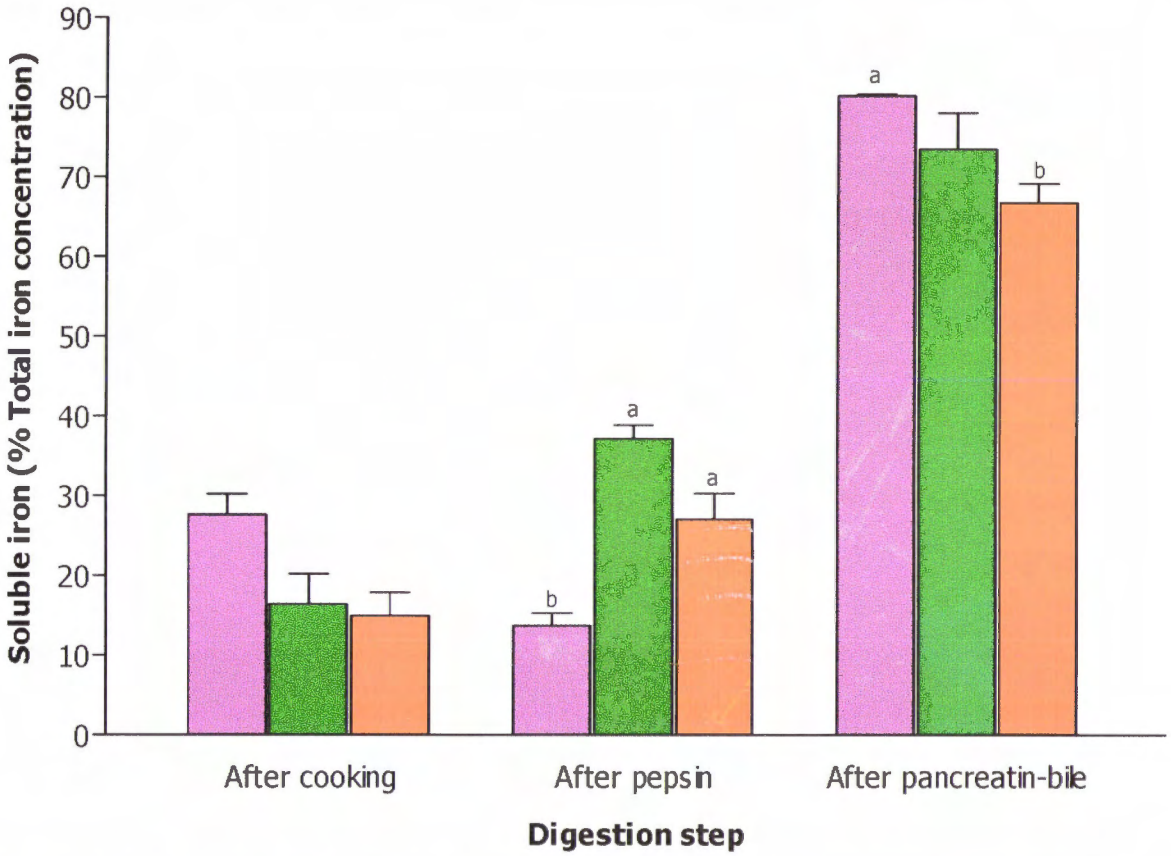


Figure 2.5: The effect of pepsin and pancreatin-bile digestion on the soluble iron in meat-plus-vegetables (▬), vegetables (▬) and meat (▬). Values are means + SEM. With each digestion step, means do not differ significantly ($p>0.05$) if they have a common letter or no letter above them.

2.4 Discussion

2.4.1 Experiment One

Validation studies showed that the total, haem and non-haem iron assays were accurate in measuring iron in the μg/g range and thus were adequate for assessing these iron forms in cooked and digested meat, vegetables and meat-plus-vegetables (Table 2.2). Further, replicate CVs of less than 10% were achieved and

the sensitivity and accuracy of all assays were similar to those reported previously (Hornsey, 1956; Carter, 1971; Ahn *et al.*, 1993; Carpenter and Clark, 1995; Clark *et al.*, 1997). Over 90% of haem and non-haem iron spikes were recovered from the soluble raw meat homogenate. The sums of the haem and non-haem iron concentrations for cooked meat and meat-plus-vegetables and the digested meat-plus-vegetables were comparable to the total iron concentrations determined by the total iron assay (Table 2.6). This demonstrates that the sum of the haem and non-haem iron concentrations provide an accurate estimate of the total iron concentrations in these types of samples.

It was not expected that any haem iron would be present in the vegetables. As the sum of the soluble and insoluble non-haem iron was comparable to the iron concentration determined by the total iron assay (Table 2.6 and 2.8), it seems likely that the apparent haem iron concentration is due to interference within the haem iron assay. The source of this interference in peas may be chlorophyll *a*, which is also extracted in 80% acetone and has an absorbance peak at 645nm (Arnon, 1949). Thus, the absorbance at 640nm by the vegetables was thought not to be due to the presence of haem iron. As a result, determination of haem iron concentration using the Hornsey (1956) method is not possible for the vegetables due to interference, possibly by chlorophyll *a*. Interestingly, this haem iron interference was not observed in the meat-plus-vegetables; the reason for this is unknown.

2.4.2 Experiment Two

The aim of the Experiment Two was to examine the effects of pepsin and pancreatin-bile digestion on the solubility and forms of iron in cooked meat, either alone or as part of a 'meal' with vegetables. The gastrointestinal digestion procedure and iron assay methods were validated in Experiment One (Section 2.3.1). Results for Experiment Two showed that cooking reduced the solubility of iron in meat, but pepsin and pancreatin-bile digestion increased the solubility of both haem and non-haem iron.

2.4.2.1 Iron concentrations in beef

As shown in Table 2.10, the mean for the haem iron concentration expressed as a percentage of total iron was 78% for raw beef. This is higher than some published data (Schricker *et al.*, 1982b; Jansuittivechakul *et al.*, 1985; Kalpalathika *et al.*, 1991), but similar to others (Buchowski *et al.*, 1988; Carpenter and Clark, 1995). Cook and Monsen (1976) reported that haem iron ranges from 50 to 60% of total iron in beef. Similarly, Schricker *et al.* (1982b) found the mean haem iron concentration of beef to be 62%. However, in studies by Buchowski *et al.* (1988) and Carpenter and Clark (1995) haem iron concentrations of 71% and 78%, respectively, were reported. Furthermore, higher concentrations of haem iron in ground beef, approximately 90% of total iron, have been reported (Chen *et al.*, 1984). The variation in haem iron concentration between studies may be due to differences in the muscle type analysed. Schricker *et al.* (1982b) reported ranges for haem iron of 59 to 63% of total iron concentrations in longissimus dorsi, biceps femoris, gluteus medius and triceps brachii muscles. This was similar to findings in a recent study (Purchas *et al.*, 2003) where haem iron concentrations differed by as much as 5% between three beef muscles (longissimus, triceps brachii and semitendinosus). Further, the lower haem iron percentage reported by Cook and Monsen (1976) and Schricker *et al.* (1982b) could be due to their non-haem iron assay method, which has been shown to also measure haem iron (Ahn *et al.*, 1993). Despite differences in percentage concentrations of haem iron, the total iron concentration of 22.1μg/g (Table 2.10) is comparable to other published data for various beef muscles (Hazell, 1982; Schricker *et al.*, 1982b; Chen *et al.*, 1984; Buchowski *et al.*, 1988; Han *et al.*, 1993; Carpenter and Clark, 1995; Purchas *et al.*, 2003).

2.4.2.2 Cooking effects

When meat is cooked using conventional methods, such as frying or grilling, the temperature within the meat will not be the same at all sites. Thus, for the current study described here, the variation in temperature was minimised by cooking the meat within plastic bags, which were suspended in water maintained at a constant temperature of 65°C for 90 minutes.

2.4.2.2.1 Haem and non-haem iron

Cooking had no effect on the total iron concentration of beef, however, the haem iron concentration was reduced after cooking (Table 2.10). This was accompanied by an increase in non-haem iron concentrations. These results are in agreement with those from other groups (Schricker *et al.*, 1982b; Schricker and Miller, 1983; Chen *et al.*, 1984; Jansuittivechakul *et al.*, 1985; Buchowski *et al.*, 1988; Kristensen and Purslow, 2001). It is thought that an increase in the non-haem iron concentrations after cooking results from the release of iron from the haem complex of myoglobin and haemoglobin (Schricker and Miller, 1983). Heating may cause oxidative cleavage of the porphyrin ring of haem, leading to the release of iron (Schricker *et al.*, 1982b; Schricker and Miller, 1983). Indeed, it is generally accepted that *in vivo* degradation of haem in humans and animals involves oxidation, by molecular oxygen, of the porphyrin ring producing ring cleavage and an unstable iron complex, which subsequently releases the iron (Elder, 1980). The observation of Igene *et al.* (1979) that hydrogen peroxide, a strong oxidant, released 60% of the total iron in unheated meat haem pigments supports the hypothesis that conversion of some of the haem iron to non-haem iron by cooking meat involves oxidation of the porphyrin ring.

2.4.2.2.2 Iron solubility

Few studies have included measures of the effect of cooking on iron in the soluble and insoluble fractions of meat. One such study, by Han *et al.* (1993), showed that the iron concentration in a water-soluble fraction decreased, while that of the insoluble fraction increased, in beef cooked to internal temperatures of 55, 70, 85 and 100°C. Compared with raw beef, iron in the water-soluble fraction decreased by 52%, while that in the insoluble fraction increased by 103% after cooking at 70°C. By contrast, in the present study cooking at 65°C reduced soluble iron by 81% and increased insoluble iron by 338% (Table 2.10). The reason for the difference between these results is unclear, but may be due to differing preparation and heating methods. Han *et al.* (1993) prepared meat slurries, which were heated in glass beakers by a steam water bath until the desired internal temperature was obtained, whereas in this study steak-sized pieces of meat were cooked in a water-bath maintained at a constant 65°C. A limitation of the Han *et*

al. (1993) study is that it is unknown whether the reduction in soluble iron was due to a decrease in the haem or non-haem iron concentration.

Water-soluble iron in raw meat includes low molecular weight iron, haem iron as myoglobin or haemoglobin and ferritin iron (Torrance *et al.*, 1968; Hazell, 1982). In cooked meat, soluble iron would only include low molecular weight iron and haem iron as undenatured or intact myoglobin, haemoglobin and ferritin iron. All iron that cannot be extracted by water is considered insoluble and includes iron in denatured myoglobin, haemoglobin and ferritin (Han *et al.*, 1993).

In the present study, cooking caused a considerable loss of soluble haem iron (Figure 2.1). After 90 minutes at 65°C, the percentage of soluble haem iron decreased from 68 to 2% of the total iron concentration. There are three possible explanations for this. The first is the formation of insoluble haem iron within the meat as denatured myoglobin, haemoglobin and ferritin; secondly, as outlined above, conversion of haem iron to soluble or insoluble non-haem iron within the meat by the oxidative release of the iron from the porphyrin ring; and thirdly, the loss of iron, either soluble or insoluble, in the cooking juices.

As shown in Figure 2.1, the decrease in soluble haem iron with cooking was accompanied by an increase in both insoluble haem and insoluble non-haem iron. Compared to raw meat concentrations, cooking increased insoluble haem iron by 370% and insoluble non-haem iron by 320%. Since there was no difference in the concentration of soluble non-haem iron between the raw and cooked meat (Figure 2.1), the 'lost' soluble haem iron must have been converted to insoluble haem and/or non-haem iron. There is also a possibility that soluble haem iron leached into the cooking juices, however, iron concentrations in the cooking juices were not evaluated in this study.

The changes in soluble haem iron concentrations due to cooking could reduce the amount of bioavailable iron. As discussed in Section 1.5.2, non-haem iron availability for intestinal absorption is influenced by a variety of enhancing, such as ascorbic acid, and inhibiting, such as polyphenols and phytates, substances

consumed in the diet (Layrisse *et al.*, 1968). Availability of haem iron, on the other hand, is not affected by the composition of the diet. As a result, non-haem iron has a lower bioavailability compared to that of haem iron (Monsen *et al.*, 1978; Hunt and Roughead, 2000). Thus, by destroying the porphyrin ring and releasing iron into the non-haem iron pool, cooking presumably decreases the amount of highly bioavailable haem iron that is contributed by meat. Furthermore, it is generally accepted that only soluble iron can be absorbed by the enterocytes of the small intestine (Wienk *et al.*, 1999). As shown in Table 2.10, cooking significantly reduced the concentration of soluble iron in meat, thereby decreasing the amount of iron that is in a form readily absorbed by the intestine.

2.4.2.3 Effects of gastrointestinal digestion on iron solubility

The *in vitro* digestion procedure used in this study was intended to simulate the effects of the gastric and intestinal environments and enzymes on food as it passes through the gastrointestinal tract. By its very nature, it cannot accurately reflect the complexity of the *in vivo* situation due to, for instance, the variation in gastric emptying rate due to the size and consistency of the meal (Davenport, 1982), but can provide information regarding the effects of enzymes on iron solubility.

Overall, pepsin and pancreatin-bile digestion increased the solubility of iron in meat, vegetables and meat-plus-vegetables. However, the effects of gastrointestinal digestion on iron solubility differed between the food samples. For instance, the action of pepsin at pH 2.0 had little effect on the solubility of iron in meat and meat-plus-vegetables, but significantly increased soluble non-haem iron in the vegetables. Despite this, the greatest increase in soluble iron for the meat, vegetables and meat-plus-vegetables was brought about by the action of pancreatin and bile at pH 7.0.

2.4.2.3.1 Meat

After cooking, the majority of the iron was in an insoluble form (Figure 2.2 and Table 2.13). Pepsin digestion had no significant effect on the solubility of haem and non-haem iron. Pancreatin-bile digestion caused the greatest increase in the solubility of meat haem and non-haem iron, with the majority of iron present as

soluble non-haem iron (Figure 2.2). The percentage concentration of total soluble iron in meat after pepsin digestion was 30% and after pancreatin-bile digestion was 70% (Figure 2.5), which is similar to levels reported previously (Crews *et al.*, 1985a, b).

As shown in Table 2.14, pancreatin-bile digestion of meat produced a net decrease in the total concentration of haem iron. Compared to 13μg/g and 14μg/g after cooking and after pepsin digestion, respectively, the haem iron concentration after pancreatin-bile digestion was significantly reduced to 9μg/g. Since there was no change in the total iron concentration after cooking, after pepsin digestion and after pancreatin-bile digestion, the decrease in haem iron concentration was not due to a loss of iron. Instead, the iron was recovered in the non-haem iron fraction, which significantly increased from 12μg/g following pepsin digestion to 18μg/g after pancreatin-bile digestion. Thus, the results reported in this study suggest that a proportion of the haem porphyrin complexes were broken down by the action of pancreatic enzymes. Conrad *et al.* (1966a) also reported a small quantity of haem degradation within the duodenal lumen, releasing non-haem iron.

The solubilisation of meat haem and non-haem iron is likely to involve two phases:

1. Lysis of the insoluble complexes binding haem and non-haem iron. Insoluble iron is likely to be bound to denatured proteins (Section 2.4.2.2.2).
2. Interaction of haem and non-haem iron with compounds that will maintain them in a soluble form.

The release of iron from the insoluble fraction of cooked meat has not been well studied. There have, however, been several studies (Conrad *et al.*, 1966a, b, 1967; Conrad and Schade, 1968) on the digestion of haemoglobin by pepsin and pancreatic enzymes. These studies show that haemoglobin is degraded to haem and globin degradation products in the small intestine. The globin degradation products, which include amino acids, amides and polypeptides, maintain the haem molecule in a soluble state, thus in a form available for absorption. Indeed, early absorption studies in both humans and animals indicate greater absorption of iron

from oral doses of haemoglobin than from comparable doses of haem alone (Conrad *et al.*, 1966a, 1967). Additionally, as discussed in Section 1.5.2.2, gastrointestinal digestion of meat releases a 'meat factor', which enhances the solubility of non-haem iron. This meat factor is thought to comprise peptides (Kane and Miller, 1984; Statkavitz and Clydesdale, 1988; Kapsokefalou and Miller, 1991; Seth *et al.*, 1999) and/or amino acids, such as cysteine (Layrisse *et al.*, 1984; Taylor *et al.*, 1986; Glahn and Van Campen, 1997; Garcia *et al.*, 1996) and histidine (Seth and Mahoney, 2000; Swain *et al.*, 2002).

2.4.2.3.2 Vegetables

Unlike for the meat and meat-plus-vegetables, pepsin digestion significantly increased the solubility of iron in the vegetables (Table 2.18). However, there was no difference in the solubility of non-haem iron between meat and meat-plus-vegetables, thus the presence of vegetables had no effect on meat non-haem iron solubility. The percentage concentration of total soluble iron in vegetables after pancreatin-bile digestion was 75% (Figure 2.5), which is the same as that reported for a previous study (Crews *et al.*, 1983).

2.4.2.3.3 Meat-plus-vegetables

Like the meat, the majority of the iron in the meat-plus-vegetables after cooking was present in an insoluble form (Figure 2.3 and Table 2.15). Pancreatin-bile digestion also caused the greatest increase in the solubility of the meat-plus-vegetables haem and non-haem iron, with a higher proportion of iron present as soluble non-haem and, unlike meat, also as soluble haem iron (Figure 2.3). Previous studies (Crews *et al.*, 1985a) have also reported that the solubility of iron in beef (and crab meat) was altered by the presence of other food constituents. For example, the addition of a 10% soya mix to a beef burger patty decreased the solubility of iron after pepsin digestion, but increased iron solubility after pancreatin-bile digestion.

Pepsin digestion of the meat-plus-vegetables reduced the total haem iron concentration (Table 2.16). The lost haem iron was not recovered in the non-haem iron fraction. Therefore, the sum of the haem and non-haem iron

concentrations, i.e. the total iron concentration, after pepsin digestion was less than that after cooking. However, the haem iron lost during pepsin digestion 'reappeared' as soluble haem iron after pancreatin-bile digestion (Table 2.15). The cause of the lower recovery of haem iron after pepsin digestion is unclear, although it suggests that haem iron, in both the soluble and insoluble fractions, was converted to a state in which it could not be detected by the haem iron assay. Since this did not occur in the pepsin digested meat, possible interactions between the meat and vegetable components may be involved.

Unlike the meat, haem iron was not degraded in the meat-plus-vegetables after pancreatin-bile digestion. Thus, along with a higher proportion of soluble haem iron in the meat-plus-vegetables after cooking (Table 2.17), the lack of haem degradation led to a higher percentage of soluble haem iron in the meat-plus-vegetables, than in meat, after pancreatin-bile digestion (Table 2.17). This may be due to compounds in the vegetables, which interact with meat, resulting in the protection of the porphyrin ring from degradation.

An overall increase in the solubility of iron, combined with the protection of haem iron from degradation, suggests that the meat iron present with vegetables may have a higher bioavailability than meat iron alone. Along with a meat factor, vegetable components released during digestion also have the potential to affect the solubility of iron. However, whereas proteolytic digestion products of meat enhance iron bioavailability, the release of vegetable components, such as ascorbic acid and polyphenols, can enhance and inhibit iron bioavailability, respectively. These affect the solubility and availability of iron for intestinal absorption by forming complexes with iron; inhibitors form large, insoluble polymers, whereas enhancers form soluble, monomeric complexes (Conrad and Schade, 1968).

2.4.2.3.4 Comparison between the soluble iron in the meat, vegetables and meat-plus-vegetables

Although statistical analysis was conducted on the data presented in Figure 2.5, comparisons between the soluble iron in the meat, vegetables and meat-plus-vegetables cannot be reliably made due to the following factors:

- The degradation of haem iron to non-haem iron in the meat after pancreatin-bile digestion (Table 2.14).
- The loss of soluble haem iron in the meat-plus-vegetables after pepsin digestion (Table 2.15).
- The fact that only non-haem iron contributed to the soluble iron fraction in vegetables, whereas in the meat and meat-plus-vegetables haem and non-haem iron were present.

2.5 Conclusions

- Cooking at 65°C for 90 minutes reduced the soluble iron concentration in beef longissimus muscle.
- Cooking also caused a reduction in the haem iron concentration of beef, which was accompanied by an increase in non-haem iron concentrations.
- Gastrointestinal digestion increased the solubility of iron in the meat, vegetables and meat-plus-vegetables. Pepsin digestion had little effect on the solubility of haem and non-haem iron in the meat and meat-plus-vegetables, but significantly increased non-haem iron solubility in the vegetables. The greatest increases in soluble iron in the meat, vegetables and meat-plus-vegetables were seen after digestion with pancreatin-bile.
- A proportion of the haem iron in the meat was broken down by the action of pancreatic enzymes leading to an increase in non-haem iron concentrations. This was not seen for the meat-plus-vegetables.
- Overall, the solubility of iron in cooked food was low, but is increased by the actions of pepsin and pancreatin-bile. Thus, when estimating the bioavailability of iron from foods, changes in the solubility of haem and non-haem iron in food before and after cooking as well as throughout the gastrointestinal digestion process must be considered.

The present study focussed on the effects of pepsin and pancreatin-bile digestion on a meat sample from one muscle type from one animal. Future studies should include a range of meat samples from various muscle cuts and also animals of different ages and sex. Furthermore, it was assumed that all haem and non-haem iron in the soluble form was available for absorption by the enterocytes of the small

intestine. However, there is a possibility that soluble iron may not be in a form that can be absorbed by the intestinal mucosa (Miller and Berner, 1989). Therefore, in the following Chapters, experiments are described in which the absorption of iron from digested meat, vegetables and meat-plus-vegetables is investigated using live intestinal tissues.

CHAPTER THREE

Validation of an *in vitro* system using Ussing chambers for the assessment of iron bioavailability

3.1 Introduction

Since its development in 1951 by Ussing and Zerahn (1951), the Ussing chamber has been used extensively to study epithelial transport mechanisms *in vitro* (Field *et al.*, 1971; Powell *et al.*, 1972; Binder and Rawlins, 1973; Schwartz *et al.*, 1974; Kaufman *et al.*, 1980; Heyman *et al.*, 1980; Clauss *et al.*, 1985; Sheldon *et al.*, 1989; Schroder *et al.*, 1995; Madsen *et al.*, 1996). However, when using any *in vitro* technique, it is necessary to first establish that the tissues remain functionally viable throughout the experiment. Traditionally, functional viability of tissues in the Ussing chamber has been determined by measuring the changes in transepithelial potential difference and/or short-circuit current in response to specific absorptive or secretory stimuli.

Table 3.1: Effects of theophylline, glucose and carbachol on the secretion and/or absorption of charged ions by intestinal epithelium.

	Effect on ion secretion/absorption by epithelium
Theophylline	Increases intracellular cAMP secretion, which causes chloride ion channels to open on the mucosal side of the enterocytes. This results in the secretion of negative chloride ions causing an increase in short-circuit current.
Glucose	Glucose absorption is linked to sodium ion uptake. Addition of glucose to the mucosal medium causes absorption of glucose, and the associated influx of positive sodium ions into the enterocytes. This causes an increase in short-circuit current.
Carbachol	Increases intracellular calcium ion levels, which causes chloride ion channels to open on the apical side of the enterocytes. This results in a net efflux of negative chloride ions causing an increase in short-circuit current.

In the present study, theophylline, glucose and carbamylcholine chloride (carbachol) have been used to validate the physiological viability of mouse intestinal tissue mounted in the Ussing chamber. These test reagents were chosen due to their stimulatory effects on the absorption or secretion of charged ions by the intestinal epithelium (Table 3.1). To provide further evidence that tissue

integrity is maintained, the tissues were examined histologically after they had been removed from the Ussing chambers. Along with integrity and biological viability, assessment of iron absorption by the intestinal tissue is also vital to ensure suitability of this technique for iron absorption measurements. Ussing chambers have been used in previous studies of iron absorption in rats (Helbock and Saltman, 1967, Vaghefi *et al.*, 1998, 2000a, b) and mice (Costa *et al.*, 2000). These studies reported absorption of both haem and non-haem iron by intestinal tissue mounted in the Ussing chambers. In the present study, the removal of inorganic iron by mouse intestinal tissue was investigated.

3.2 Materials and Methods

The experiments described in this Chapter were approved by the Massey University Animal Ethics Committee (MUAEC #01/41).

3.2.1 Animals

Twenty male, Balb/c mice aged between 6 and 12 weeks were obtained from the Massey University Small Animal Production Unit (Palmerston North, New Zealand). They were housed in groups of no more than six on wood shavings in plastic cages with stainless steel covers, and were provided free access to tap water in plastic bottles equipped with stainless steel spouts. The mice were fed Rodent Diet 83, a pelleted diet prepared by the Food Processing Unit at Massey University. The composition of the diet is given in Table 3.2.

Table 3.2: Composition of Rodent Diet 83.

Ingredient	**%**
Wheat	40.35
Barley	30
Broll	5
Lucerne	5
Meat and bone	6
Fishmeal	7
Skim milk powder	5
Soya bean oil	1
Methionine	0.1
Salt	0.05
Vitamin/mineral premix	0.5

The pellets were placed on top of the cage and were available *ad libitum* to all mice throughout the experimental period. Room temperature and humidity were maintained at 22 ± 1°C and 55 ± 5%, respectively, with lighting set at a 12 hour light and dark cycle, with dawn and dusk transitional periods of approximately 10 to 15 minutes.

3.2.2 Tissue preparation

The mice were euthanased by cervical dislocation, and the duodenum and jejunum were removed and carefully washed with Ringer's solution (Table 3.3) at room temperature (pH 7.4; 290mOsm/L water).

Table 3.3: Composition of the Ringer's solution (A.G. Butt, personal communication).

Electrolytes and substrates	**Concentration (mM)**
Sodium chloride (NaCl)[1]	120
Potassium chloride (KCl)[1]	5
Calcium chloride ($CaCl_2$)[1]	2
Magnesium chloride ($MgCl_2$)[1]	2
Sodium phosphate dibasic (NaH_2PO_4)[2]	1.8
Sodium phosphate monobasic (Na_2HPO_4)[2]	0.2
Sodium bicarbonate ($NaHCO_3$)[1]	25
Pyruvate[2]	2
Glutamine[2]	2

[1]BDH Chemicals, Poole, UK; [2]Sigma Chemicals, St Louis, USA.

Four individual segments (2cm each) of the proximal small intestine comprising the duodenum (segments 1 and 2) and jejunum (segments 3 and 4) were studied from each animal. Each segment was opened longitudinally along the mesenteric border and mounted as an intact, flat sheet between siliconised Ussing half chambers (World Precision Instruments, Sarasota, Florida), with an exposed tissue surface area of 0.67cm^2.

3.2.3 Ussing chamber experiments

The Ussing chamber, with a segment of intestinal tissue in place is shown in Figure 3.1. The epithelium was bathed on both mucosal and serosal sides independently with 10ml of Ringer's solution. Solutions were oxygenated and circulated with

carbogen gas (95% O_2 and 5% CO_2) (BOC Gases, Wellington, New Zealand), which was first bubbled through water to minimise evaporative water loss. The temperature of the Ringer's solution was maintained at 37°C by water-jacketed reservoirs (World Precision Instruments, Sarasota, Florida).

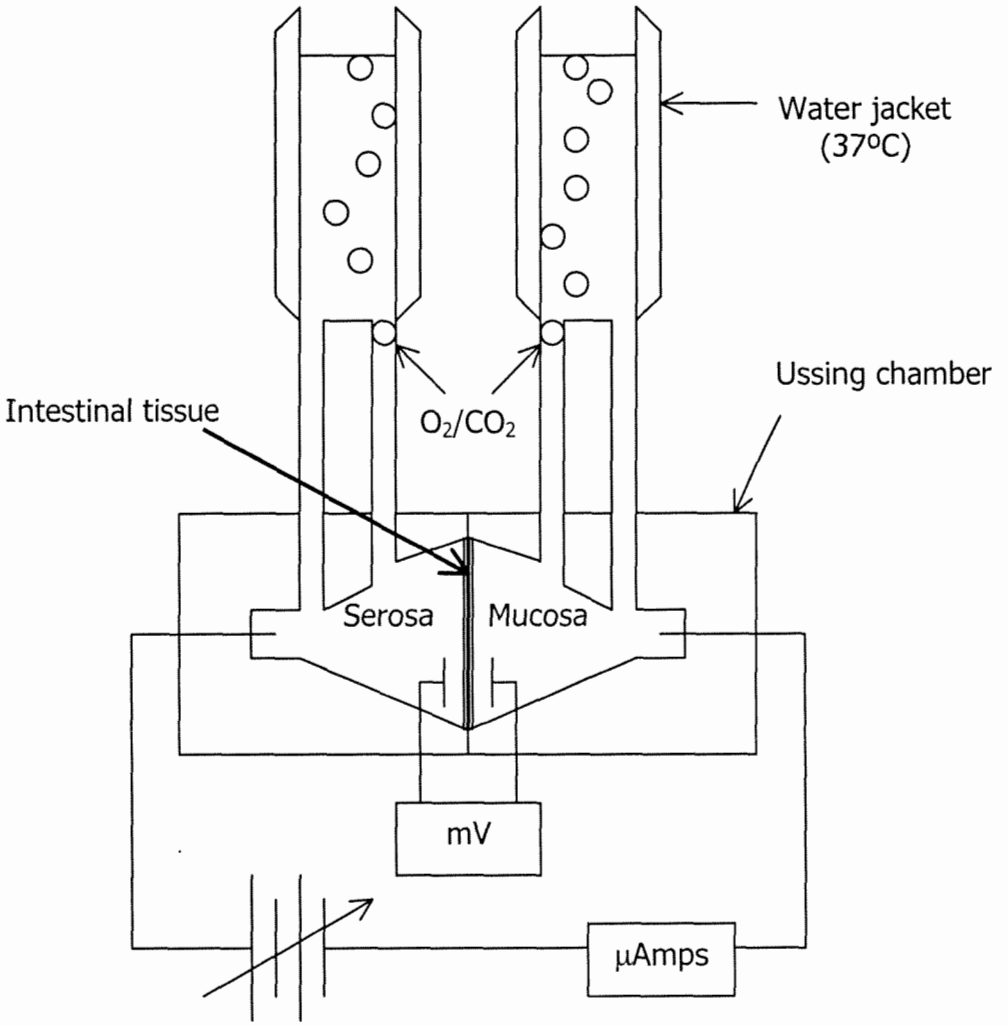


Figure 3.1: Diagram of the Ussing chamber apparatus with intestinal tissue mounted between the two half chambers.

The mucosal and serosal solutions were connected via two salt-agar bridges (3.5% agar, 3M potassium chloride) situated close to the epithelium, to separate calomel reference electrodes (Radiometer Pacific, Copenhagen, Denmark) immersed in saturated potassium chloride, for measurement of the potential difference across the tissue. In order to provide short-circuited conditions, a second pair of salt-agar bridges positioned at either end of each half chamber were connected to silver chloride electrodes immersed in saturated potassium chloride, which passed the

current through the tissue using an automatic voltage clamp unit (Biodesign, South Campus Electronics, University of Otago, New Zealand).

The tissues were short-circuited at all times by the clamping units. Tissue resistance was determined by imposing a potential difference of 5mV for 0.5 seconds across the tissue every 5 minutes. Using Ohm's Law, the clamp units then automatically divided the applied potential difference by the recorded change in current at the end of the pulse. The clamping units automatically compensated for fluid resistance, which had been measured previously using the clamp units in the absence of tissue. Short-circuit current and tissue resistance were recorded with a PowerLab data acquisition system (ADInstruments, Castle Hill, Australia) using Chart for Windows, version 4.0.1 (ADInstruments, Castle Hill, Australia). Values of short-circuit current and tissue resistance were divided by the surface area of the tissue ($0.67cm^2$) and reported as $\mu Amps/cm^2$ and Ω/cm^2, respectively.

Values less than 25Ω for tissue resistance indicated loss of integrity of the tissue, whereas values close to $0\mu Amps$ for short-circuit current indicated the tissue was not actively secreting or absorbing and therefore no longer viable. Such tissues were discarded from the experiment.

3.2.4 Test reagents

Solutions of theophylline, glucose and carbachol were prepared as follows:

GLUCOSE: 1.8g glucose (BDH Chemicals, Poole, UK) in 10ml deionised water; 0.1ml added to mucosal Ringer's solution.

THEOPHYLLINE: 0.18g theophylline (Sigma, St Louis, USA) in 10ml 0.1M sodium hydroxide; 0.1ml added to mucosal Ringer's solution. The 0.1M sodium hydroxide vehicle produced no alteration in basal short-circuit current or tissue resistance.

CARBACHOL: 0.0018g carbachol (Sigma, St Louis, USA) in 10ml deionised water; 0.1ml added simultaneously to mucosal and serosal Ringer's solutions.

These solutions were added to the Ussing chambers to provide the final mucosal and/or serosal concentrations shown in Table 3.4.

Table 3.4: Test reagent concentrations and final bath concentrations in 10ml Ringer's solution.

Test reagent	Test reagent concentration	Volume (ml) added to 10ml	Final bath concentration
Glucose	1.0M	0.1	10mM
Theophylline	0.1M	0.1	1mM
Carbachol	1.0mM	0.1	0.01mM

3.2.5 Histological examination of intestinal tissues

Eight segments of intestinal tissue from the duodenum and jejunum were removed from the chambers after a 2-hour incubation and immediately immersed in 10% formalin for 12 hours. The tissues were then impregnated into paraffin using an automatic tissue processor (Leica Jung TP105) and mounted in paraffin blocks. Sections (6μm thick) were cut with a rotary microtone (Leitz Wetzlar) and placed on glass slides.

Table 3.5: Method for staining intestinal sections for histological analysis.

Process	Reagent	Time
Deparaffinising	Xylene Absolute ethanol 70% ethanol Tap water	2 changes x 7 minutes 3 - 5 seconds 3 - 5 seconds 3 - 5 seconds
Staining	Mayer's haemalum Tap water Scott's tap water Tap water 1% aqueous eosin Tap water	10 minutes 3 - 5 seconds 2 minutes 3 - 5 seconds 2 minutes 3 - 5 seconds
Differentiate and dehydrate	70% ethanol Absolute ethanol Xylene	3 - 5 seconds 2 changes x 3 - 5 seconds 2 changes x 3 - 5 seconds

The slides were stained using the procedure outlined in Table 3.5 before mounting with DPX mountant (BDH Chemicals, Poole, UK) and sealed with a cover slip. The sections were examined using an Olympus CHS microscope (Olympus Inc., Japan), with the photomicrographs taken using a Nikon Coolpix 4500 digital camera (Nikon Instruments, Japan).

3.2.6 Iron absorption experiments

Removal of iron from mucosal solution by mouse intestinal tissue in Ussing chambers was assessed using the following protocol.

3.2.6.1 Iron preparation

Ferrous gluconate (Aldrich Chemical Company Inc., Milwakee, USA) was used for all experiments. A 5500μg/g solution was prepared by dissolving 0.4822g in 10ml of warm deionised water. Then, 0.1ml of this iron solution was added to the 10ml mucosal Ringer's solution giving a final bath concentration of 55μg/g.

3.2.6.2 Sample collection

After addition of the iron solution, sequential 500μl samples of the mucosal and serosal Ringer's solution were taken at 0, 15, 30, 60, and 90 minutes.

3.2.6.3 Iron analysis

Before analysis, all mucosal solutions were diluted 10-fold with deionised water to bring the concentration of iron within the detection range of the assay. Iron assays were performed on a Roche Cobas Fara II clinical chemistry analyser (Hoffman La Roche, Basel, Switzerland) using a diagnostic kit for iron (Hoffman La Roche, Basel, Switzerland). The kit was based on the Ferrozine method without deproteinisation (Siedel *et al.*, 1984) and was used according to the manufacturer's instructions.

3.2.7 Statistical analysis

For short-circuit current and tissue resistance, the statistical significance of the differences between baseline and maximum/minimum values reached were determined by paired-samples t tests using SPSS version 11.0 (SPSS Inc.). For the removal of iron from the mucosal solution, a linear regression line was generated using the linear regression model in GraphPad Prism 3.0 (GraphPad Software Inc.).

3.3 Results

3.3.1 Tissue viability

3.3.1.1 Basal bioelectric measurements

Immediately after being mounted, the intestine preparations exhibited an initial decrease in short-circuit current. Therefore, all basal values and additions of test reagents were performed after the short-circuit current had reached a stable value, usually 30 minutes after mounting.

About 10% of mouse intestinal tissues exhibited spontaneous oscillations in the basal short-circuit current (Figure 3.2) and potential difference. The magnitude of these spontaneous oscillations was approximately 10 to 15μAmps/cm^2 for short-circuit current and normally lasted 10 to 20 minutes after mounting.

Figure 3.2: A typical recording of basal short-circuit current in mouse proximal small intestinal tissue exhibiting spontaneous oscillations.

Basal values of short-circuit current, tissue resistance and potential difference are shown in Table 3.6, and were able to be maintained for at least 4 hours.

Table 3.6: Baseline bioelectric measurements for mouse proximal small intestinal tissue mounted in Ussing chambers. Basal responses were taken 30 minutes after mounting.

Bioelectric parameter	Mean ± SD
Short-circuit current (μAmps/cm^2)	55.1 ± 45.4 (75)
Tissue resistance (Ω/cm^2)	76.0 ± 30.2 (75)
Potential difference (mV)	2.6 ± 2.2 (75)

The number of intestinal segments shown in parentheses.

3.3.1.2 Tissue response to the test reagents

Table 3.7 shows the changes in bioelectric parameters after addition of glucose, theophylline or carbachol.

Table 3.7: Means (± SEM) for short-circuit current of mouse proximal small intestinal tissue mounted in Ussing chambers and exposed to theophylline, glucose or carbachol.

	Short-circuit current (μAmps/cm^2)		
	Baseline	Maximum reached	Change
Glucose 10mM (13)	44.96 ± 7.73	81.85 ± 9.94***	36.88 ± 5.76
Theophylline 1mM (19)	61.31 ± 8.79	96.73 ± 11.11***	35.43 ± 4.10
Carbachol 0.01mM (9)	48.36 ± 12.99	118.07 ± 20.95***	69.73 ± 9.35

The number of intestinal segments shown in parentheses. ***$p<0.001$ compared to baseline values.

Carbachol, a stimulant of chloride ion secretion, had the greatest effect; glucose and theophylline also produced changes in short-circuit current. Each of the test reagents produced a small increase ($p<0.05$) in tissue resistance of approximately 1 to 2Ω/cm^2 (data not shown). The tissues were responsive to repeated stimulation by glucose (10mM) over a 4-hour period and responded to theophylline after a 4-hour incubation in the Ussing chambers (data not shown).

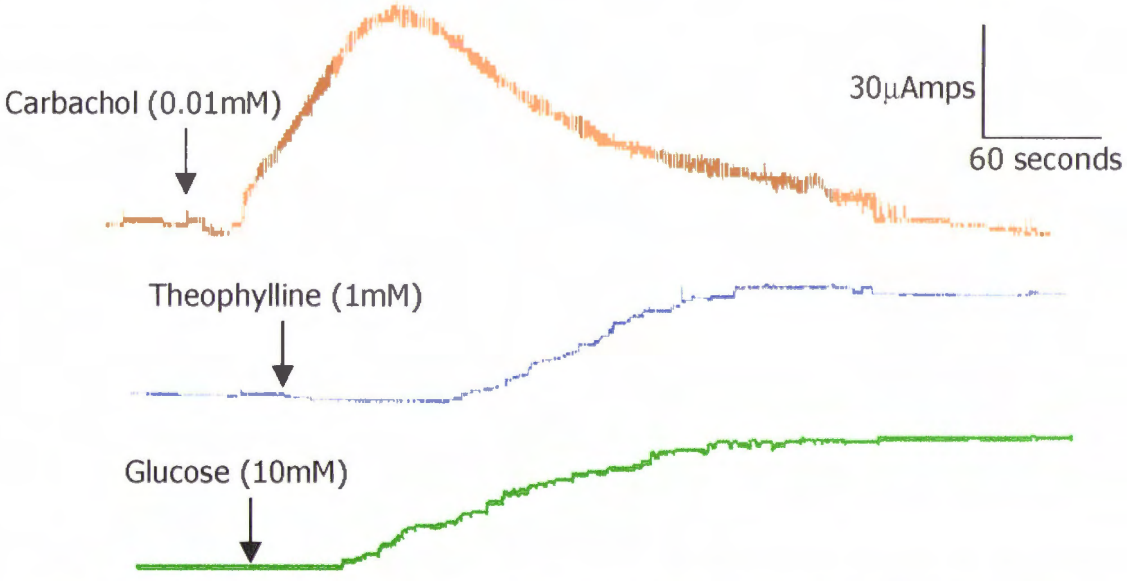
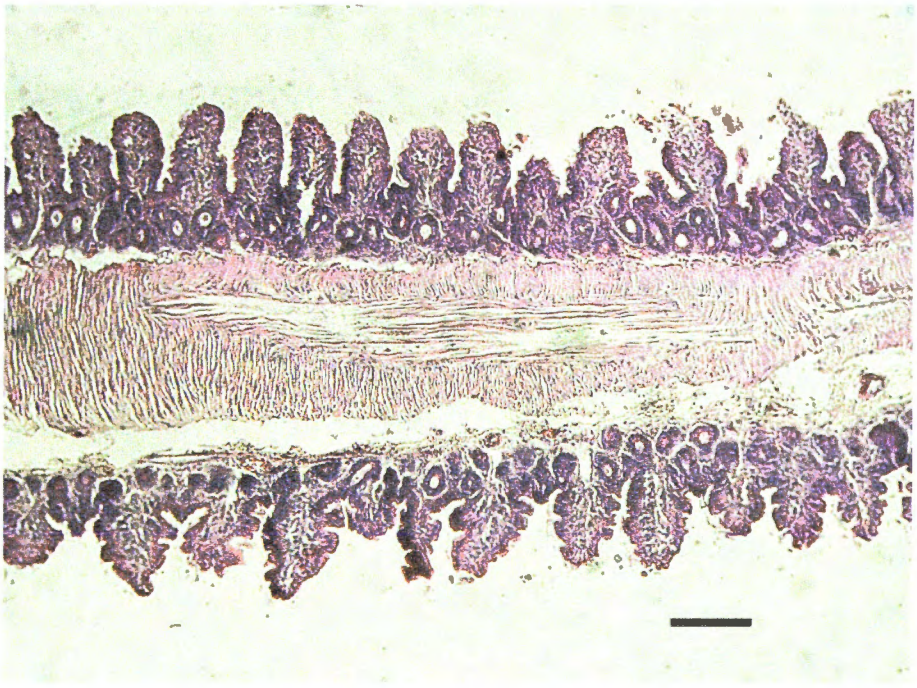


Figure 3.3: Typical recording of the change in short-circuit current in response to carbachol, theophylline or glucose to mouse proximal small intestinal tissue mounted in Ussing chambers.

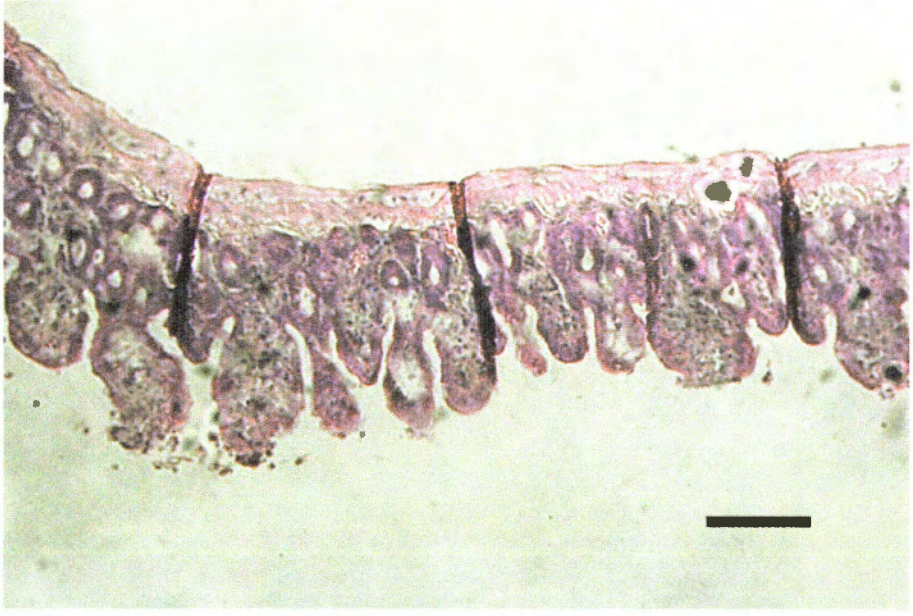
Figure 3.3 shows the effect on short-circuit current produced when theophyline, glucose or carbachol were added to the mucosal and/or serosal Ringer's solutions of mouse small intestinal tissue. From these traces it can be seen that the addition of each the test reagents caused an increase in short-circuit current. However, unlike glucose and theophylline, the short-circuit current in response to carbachol returning to near baseline values after approximately 5 minutes.

3.3.1.3 Histology of intestinal tissues

Histological examination of jejunal segments mounted in the Ussing chamber for two hours demonstrated that the tissue remained intact, with normal morphological characteristics (Figure 3.4). The villi were still present and the enterocytes appeared to still have intact cell membranes.



Scalebar = 250μm



Scalebar = 100μm

Figure 3.4: Photomicrographs of a cross section of mouse jejunum after being mounted in an Ussing chamber. Note: tissue is folded onto itself (top) and vertical dark lines are where the tissue is folded (bottom).

3.3.2 Iron absorption measurements

After the addition of ferrous gluconate to the mucosal side of the Ussing chambers, there was no change in short-circuit current or tissue resistance (data not shown). Table 3.8 presents the results for the analysis of iron in the mucosal solution. No iron was detected in the serosal medium (data not shown).

Table 3.8: Percentage of iron as ferrous gluconate removed from the mucosal solution (starting iron concentration of 55μg/g) in Ussing chambers containing mouse proximal small intestinal tissue.

Time (min) after addition of the iron source	% decrease from the initial iron concentration	Rate of change from the previous value (μg/g/min)
15	3.83 ± 1.05 (6)	0.14
30	9.20 ± 3.15 (6)	0.20
60	27.95 ± 2.78 (6)	0.35
90	33.37 ± 3.09 (6)	0.10
120	40.30 ± 3.38 (3)	0.13

Values are mean ± SEM with the number of intestinal segments shown in parentheses.

It appeared that the rate of iron removed from the mucosal solution increased to a peak between the 30 and 60-minute sample times (Table 3.8). However, the data conformed to a linear regression model (Figure 3.5), with an r^2 value of 0.85.

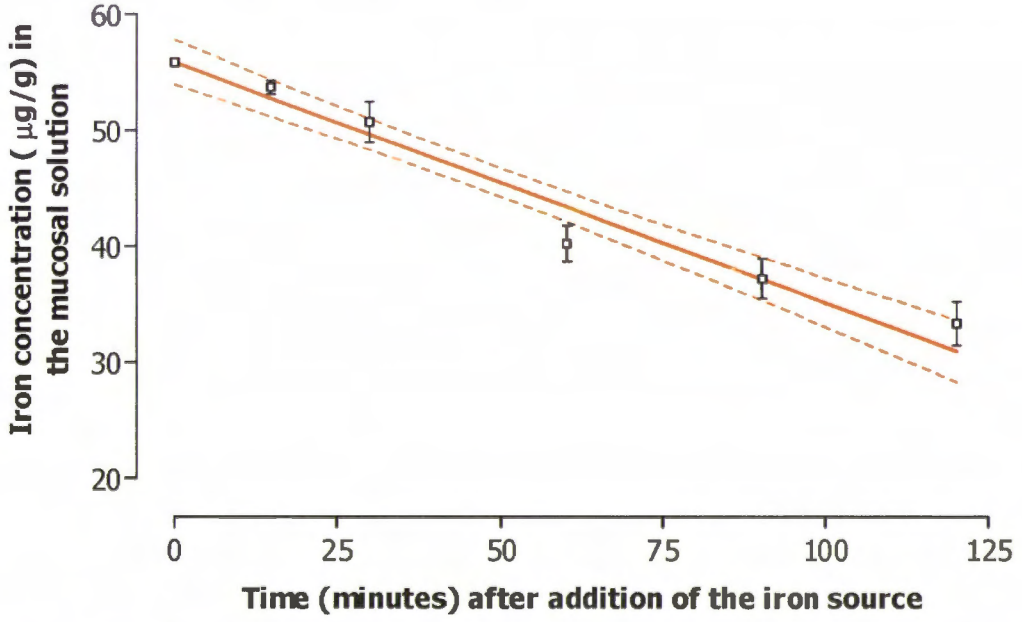


Figure 3.5: Linear regression line (± 95% confidence intervals) for the removal of iron from the mucosal solution of Ussing chambers containing proximal mouse intestinal tissue. Iron was added at time zero at a concentration of 55μg/g iron as ferrous gluconate. Data points are means ± SEM.

3.4 Discussion

The experiments described in this Chapter were designed to demonstrate the viability of mouse intestinal tissues under the conditions in which future iron absorption studies would be conducted. Further, they described preliminary observations of iron absorption by mouse small intestinal tissues in Ussing chambers.

3.4.1 Tissue viability

The functional viability of the tissues in the Ussing chambers was demonstrated by changes in transepithelial potential difference and/or short-circuit current in response to specific absorptive or secretory stimuli. Concentrations of glucose, theophylline and carbachol were prepared to provide final bath concentrations as used by other groups (Kaufman *et al.*, 1980; Sheldon *et al.*, 1989). The addition of these test reagents to the mucosal and/or serosal Ringer's solutions caused significant responses in the short-circuit current of the intestinal tissues, which corresponds to the net flow of charged ions across the epithelium. This provides a qualitative assessment of tissue viability, since ATP is required for these active transport processes.

The observed changes in short-circuit current of 70 and 37μAmps/cm^2 for carbachol and glucose, respectively, are comparable to published results (Field *et al.*, 1971; Sheldon *et al.*, 1989; Kaufman *et al.*, 1980; Grubb, 1995). Increases in short-circuit current of 23μAmps/cm^2 for rabbit ileum (Field *et al.*, 1971) and 42μAmps/cm^2 (Kaufman *et al.*, 1980) and 33μAmps/cm^2 (Grubb, 1995) for mouse jejunum after the addition of 10mM glucose have been reported. Sheldon *et al.* (1989) reported an increase of 80μAmps/cm^2 in short-circuit current for mouse jejunum after the addition of carbachol (0.01mM). As observed in the present study, Sheldon *et al.* (1989) also reported that the addition of carbachol evoked an increase in short-circuit current, which returned to baseline after 5 to 10 minutes. The increase of 35μAmps/cm^2 observed after the addition of theophylline (1mM) was lower than the 81μAmps/cm^2 increase reported by Sheldon *et al.* (1989). The final bath concentrations of theophylline were identical for the two studies, thus the reason for the difference in responses was unknown. However, the results in

this study indicate mouse intestinal tissues remain viable within the Ussing chambers and respond to absorptive and secretory stimuli.

After a 2-hour incubation in the chambers, the intestinal tissues were examined histologically to determine the morphological characteristics of the intestine. Figure 3.4 clearly shows that the intestinal tissues exhibited normal morphological characteristics, including intact submucosa, villi and enterocytes. This provides visual proof that epithelial integrity can be maintained while intestinal tissue is mounted in the Ussing chamber.

3.4.2 Spontaneous oscillations

In the present study, the segments of mouse intestinal tissue exhibited spontaneous oscillations in short-circuit current. Such oscillations in short-circuit current and potential difference have been reported by others for both small and large intestine from a number of species, including humans (Read *et al.*, 1977; Baxter *et al.*, 1989), dogs (Read *et al.*, 1977; Rangachari and McWade, 1986), rats (Andres *et al.*, 1985) and mice (Sheldon *et al.*, 1989), for *in vitro* as well as *in vivo* preparations. The origin of these oscillations is not fully understood. It has been suggested that they are due to the contractile activity of the smooth muscle layers present in the tissue (Read *et al.*, 1977; Sheldon *et al.*, 1989). Indeed, the addition of the neuronal conduction blocker, tetrodotoxin, has been shown to abolish the oscillations in short-circuit current (Andres *et al.*, 1985; Rangachari and McWade, 1986; Sheldon *et al.*, 1989). However, in these last studies it was noted that jejunal tissue stripped of muscle layers still displayed oscillations (Andres *et al.*, 1985; Rangachari and McWade, 1986; Sheldon *et al.*, 1989). In another study, Grubb (1995) investigated the presence of spontaneous oscillations in the absence of certain ions in the Ringer's solution. They concluded that the secretion of chloride ions are involved in the generation and maintenance of these oscillations.

3.4.3 Iron absorption

To investigate iron absorption, ferrous gluconate, a highly bioavailable source of inorganic iron, was used at an iron concentration of 55μg/g, which is similar to that used in a previous Ussing chamber study (Vaghefi *et al.*, 1998). Although it

appeared that the absorption of iron increased to a peak between the 30 and 60-minute sample times, overall absorption conformed to a linear regression model. Thus, suggesting that the rate of iron absorption was constant over time.

Iron was not detected in the serosal solution. Thus, iron lost from the mucosal solution was assumed to be bound to the mucus layer and/or stored within the enterocytes. Throughout the small intestine, a layer of mucus covers the luminal surface. The main constituent is mucin, which is a complex, high-molecular weight glycoprotein secreted by surface epithelial cells, goblet cells and Brunner's glands. The secreted mucins hydrate and gel, forming a blanket of mucus that covers and protects the intestinal epithelium from chemical and physical damage (Ganong, 1999). This layer also acts as a lubricant and aids in the host-defence mechanism against potential pathogens.

Mucin has been implicated in the iron absorption process (Conrad *et al.*, 1991). It has been shown to bind iron at acidic pH and maintain iron in a form suitable for absorption in the alkaline environment of the small intestine (Conrad *et al.*, 1991). Iron chelates of ascorbate, fructose and histidine also donate iron to mucin at neutral pH (Conrad *et al.*, 1991). Thus, intestinal mucin may deliver inorganic iron to intestinal absorptive cells in a form suitable for absorption. In the present study, a proportion of the iron lost from the mucosal Ringer's solution may have been bound to the mucus layer of the intestine rather than being fully absorbed into the enterocytes.

The release of iron into the serosal medium after absorption by the intestinal tissue was not expected. Under *in vivo* conditions, iron is released by the enterocyte into the blood, where it is immediately bound by transferrin for transport around the body (Aisen and Brown, 1977). Since, in the present study, there is no blood flow through the capillaries to present transferrin to the basolateral membrane of the enterocytes, iron that is absorbed is most likely to accumulate within the enterocytes, where it is stored within ferritin. Additionally, the absorption of iron from the intestinal lumen and its transfer across the basolateral membrane are two separate steps. Each step requires independent transport processes and occurs

over different time scales (Manis and Schachter, 1962; Wheby *et al.*, 1964) with the transfer of iron across the basolateral membrane slower than the uptake of iron across the apical membrane. Thus, the length of the experiment may be a confounding factor to the release of iron from the enterocytes. Furthermore, not all iron that enters the enterocytes from the intestinal lumen is necessarily transferred to blood. The export, as well as absorption, of iron is dependent on the iron load of the body (see Section 1.4). In an iron deficient state, nearly all iron entering the enterocytes will be transferred into plasma, whereas in an iron abundant state, iron will be incorporated into ferritin within the enterocyte for storage.

Vaghefi *et al.* (1998, 2000a) demonstrated a transfer of iron to the serosal medium after a 2-hour incubation with ferrous gluconate and without the addition of an iron acceptor. This difference may be due to the use of rat intestine, which requires stripping of the intestinal muscle layers before mounting. Due to the thickness of rat intestinal tissue, stripping of these layers is required, to allow adequate diffusion of oxygen to the epithelium. Stripping of the muscle layers may expose the capillaries or allow diffusion of iron, providing an easier access for iron to the serosal medium after absorption.

In order to determine whether all the iron added to the system was accounted for, total recovery should be determined. It was concluded that washing the inside of the Ussing chambers and glass reservoirs with 5% nitric acid along with a careful wash of the intestinal tissue, to remove attached mucus, followed by determination of iron within the intestinal segment would be necessary to allow accurate determination of iron absorption.

3.5 Conclusions

- The electrophysiological measurements made, show that the mouse small intestinal tissues when maintained under the conditions described were able to generate a spontaneous and stable electrical potential difference.
- The addition of the test reagents, which stimulate the secretion and/or absorption of charged ions, produced significant changes in short-circuit

current, indicating that tissues remained viable, while mounted in the Ussing chamber.

- Preliminary observations indicate iron, as ferrous gluconate, is absorbed *in vitro* by mouse intestinal tissues.

In the following Chapter, experiments are described which use the Ussing chambers to determine the concentration of iron absorbed by mouse intestinal tissue from the soluble fractions of meat, vegetables and meat-plus-vegetable after gastrointestinal digestion.

CHAPTER FOUR

Estimation of the iron bioavailability of foods using Ussing chambers

4.1 Introduction

Ussing chambers have been used previously to study iron absorption with either rat (Helbock and Saltman, 1967; Vaghefi *et al.*, 1998, 2000a, b) or mouse (Costa *et al.*, 2000) small intestinal tissues.

Helbock and Saltman (1967) showed that low molecular weight chelating agents solubilise iron and maintain it in a form available for absorption. In addition, the relative and absolute rates of iron uptake by *in vitro* methods were in good agreement with the rates seen *in vivo*. Vaghefi and colleagues reported that rat small intestinal tissues absorbed haem iron from pepsin digested haemoglobin as well as non-haem iron as ferrous gluconate (Vaghefi *et al.*, 1998). They also showed that the addition of the amino acid cysteine, but not histidine, to the Ussing chamber increased the absorption of both haem and non-haem iron absorption (Vaghefi *et al.*, 2000a). Furthermore, increasing the level of haemoglobin hydrolysis, defined as the ratio of peptide bonds cleaved to the total number of peptide bonds, was associated with an enhanced uptake of iron, which ranged between 5 to 6% of the starting iron concentration (Vaghefi *et al.*, 2000b).

Costa *et al.* (2000) investigated the use of the radioisotope ^{55}Fe to measure the unidirectional flux of iron across intestinal tissues from iron-loaded mice and untreated controls for 'normal' iron status. They reported that, compared to controls, intestinal segments from parenterally iron-loaded mice had decreased iron absorption. They concluded that the Ussing chamber technique was able to reproduce the adaptive responses to changes in body iron requirements obtained *in vivo*. Further, iron transfer from the mucosal to serosal side of the epithelium was increased in acidic (pH 5.5), compared to neutral pH (pH 7.4) Ringer's solution.

These studies demonstrate that iron, from both non-haem (Helbock and Saltman, 1967; Vaghefi *et al.*, 1998, 2000a; Costa *et al.*, 2000) and haem (Vaghefi *et al.*, 1998, 2000a, b) sources, was absorbed by rodent intestinal tissues mounted in Ussing chambers. Further, relative and absolute rates of iron uptake by intestinal segments mounted in Ussing chambers were in agreement with *in vivo* absorption (Helbrock and Saltman, 1967). These results support the use of the Ussing chamber model as an alternative *in vitro* method useful for iron absorption measurements in rat and mouse intestinal tissue.

In the present study, Ussing chambers with mouse small intestinal tissue have been used to compare the absorption of iron from the soluble fractions of meat, vegetables and meat-plus-vegetables after pepsin and pancreatin-bile digestion.

4.2 Materials and Methods

All experiments described in this Chapter were approved by the Massey University Animal Ethics Committee (MUAEC #01/41). All glassware and the Ussing chambers were soaked overnight in 5% nitric acid and rinsed thoroughly with deionised water before use.

4.2.1 Animals

Male, Swiss-Webster mice obtained from the Massey University Small Animal Production Unit (Palmerston North, New Zealand) were allocated at random into two groups at weaning. The first group (iron deficient) were fed a powdered low iron diet to induce iron deficiency, while the second group (iron replete) were fed their normal pelleted diet.

The iron deficient mice were used for iron absorption experiments. Sufficient mice were used for six replicate experiments for each of the digested meat, vegetable and meat-plus-vegetable solutions and ferrous gluconate solutions. Segments of the small intestine from a further six iron deficient mice were mounted in the Ussing chambers and bathed with Ringer's solution for 90 minutes for determination of baseline intestinal iron concentrations. The mice in the iron

replete group were used to obtain 'normal' haematological indices and intestinal and liver iron storage levels.

4.2.1.1 Iron deficient mice

Thirty-five male, Swiss-Webster mice weighing between 12 to 17g were fed a low iron diet (13mg/kg) from weaning at 21 days of age for 6 weeks. The mice were housed in groups of six on stainless steel wire screens in plastic cages with stainless steel covers and were provided free access to deionised water in plastic bottles equipped with stainless steel spouts.

Table 4.1: Composition of the powdered low iron diet.

Ingredient	**%**
Casein	20
Methionine	0.3
Corn starch	34.3
Sucrose	34.5
Cellulose	5
Soya bean oil	5
Citrate	0.4
Vitamin/mineral premix*	0.5

*See Appendix Three for details of the vitamin/mineral premix.

The powdered low iron diet was placed in stainless steel feeders inside the cage and was available *ad libitum* to all mice throughout the 6 weeks. The composition of the low iron diet (Table 4.1) met all the nutrient requirements for a growing mouse with the exception of iron (American Institute of Nutrition, 1977). Room temperature and humidity were maintained at 22 ± 1°C and 55 ± 5%, respectively, with lighting set at a 12 hour light and 12 hour dark cycle, with dawn and dusk transitional periods of approximately 10 to 15 minutes.

4.2.1.2 Iron replete mice

Twenty male, Swiss-Webster mice were kept as outlined in Section 3.2.1. They were fed a normal pelleted diet (Rodent Diet 83, Food Processing Unit, Massey University, composition shown in Table 3.2), which contained 235mg/kg iron, from weaning at 21 days of age for 6 weeks.

4.2.2 Haematology

The mice were weighed before being anaesthetised with 5% Halothane (Vetpharm NZ Ltd., Auckland, New Zealand) in oxygen. While anaesthetised, blood (250 to 500µl) was collected by cardiac puncture into 500µl K_2EDTA microtainers (Becton Dickinson, Franklin Lakes, USA) for haematology analysis. The blood was analysed fresh for haemoglobin, packed cell volume (PCV) and mean cell volume (MCV) using an ADVIA120 haematology system (Bayer, Germany).

4.2.3 Tissue preparation

While under Halothane anaesthesia, the mice were euthanased by cervical dislocation and the liver, duodenum and jejunum removed. The liver was removed for analysis of iron content as described in Sections 4.2.5 and 4.2.6. The duodenum (segment 1 and 2) and jejunum (segment 3 and 4) were rinsed in Ringer's solution at room temperature and mounted in the Ussing chambers as described in Sections 3.2.2 and 3.2.3.

4.2.4 Iron solutions

The following solutions were added to the mucosal side of the intestinal tissues mounted in the Ussing chambers.

4.2.4.1 Digested meat, vegetable and meat-plus-vegetable solutions

Digested meat, vegetables and meat-plus-vegetables (Section 2.2.2.3) were separated into soluble and insoluble fractions using a method similar to that described in Section 2.2.1.4. Briefly, after pancreatin-bile digestion the meat, vegetables and meat-plus-vegetables were centrifuged at 2500 x *g* (4°C) for 15 minutes. Any visible fat was skimmed and the supernatants were collected and freeze-dried. Total iron concentrations in the freeze-dried samples were determined by the atomic absorption spectroscopy, as described in Section 2.2.1.5.

Immediately prior to each experiment, freeze-dried samples of the soluble fractions of digested meat, vegetables or meat-plus-vegetables were dissolved in deionised water. A final iron concentration of approximately 3.5µg/g was chosen based on the results of preliminary experiments (see Appendix Four). The pH of each

solution was adjusted to 6.5 by addition of 0.1M hydrochloric acid and made up to a final volume of 80ml with deionised water. At the start of each experiment, after the tissues had equilibrated for 30 minutes, the mucosal Ringer's solution was replaced by the digested meat, vegetables or meat-plus-vegetable solutions, and then incubated with intestinal tissue for 90 minutes.

The effects of acids, enzymes or bile solutions added during the *in vitro* gastrointestinal digestion process on short-circuit current and tissue resistance were also investigated. Deionised water (100g) was subjected to the gastrointestinal digestion procedure as described in Section 2.2.1.3 and added to the mucosal side of the Ussing chambers containing segments of intestinal tissue (n=8) from two iron replete mice (Section 4.2.1.2).

4.2.4.2 Ferrous gluconate

A 40μg/g stock solution of iron was prepared by adding 34.5mg of ferrous gluconate (Aldrich Chemical Company Inc., Milwakee, USA) to 100ml of warm deionised water. One ml of the stock solution was added to 9ml of mucosal Ringer's solution to provide a mucosal solution iron concentration of 4μg/g, and then incubated with intestinal tissue for 90 minutes.

4.2.5 Sample collection

Approximately 30 seconds after adding the iron solution to the mucosal chamber, a 500μl sample of the mucosal solution was taken. After the 90-minute incubation, the remaining mucosal solution was collected for analysis.

Duplicate samples (7 to 10mg) of each mouse liver were weighed in tared, capped glass tubes (Kimax, BioLab Scientific, Auckland, New Zealand) and were dried in an oven (DeLongi Airstream Convection Oven) for 16 hours at 105°C. The intestinal tissue was removed from each chamber and trimmed to leave only the tissue that had been exposed to the mucosal and serosal solutions. The tissue sample was washed in 0.1% nitric acid to remove attached mucus and prevent iron loss from the tissue (after Vaghefi *et al.*, 1998). The intestinal tissues were then weighed in tared, capped glass tubes and dried in the oven for 16 hours at 105°C.

A 5% nitric acid rinse of each of the glass reservoirs and chambers was collected in 25-ml Erlenmeyer flasks (Kimax, BioLab Scientific, Auckland, New Zealand). This wash sample was combined with the acid wash from the corresponding intestinal segment.

4.2.6 Sample processing and iron analysis

4.2.6.1 Acid digestion of mucosal solutions and tissue samples

Samples (250µl) of the start and end mucosal solutions were weighed in tared, capped glass tubes. The mucosal solution samples and the dried intestine and liver samples were left for 16 hours in 1ml of concentrated nitric acid (ARISTAR, BDH Chemicals, Poole, United Kingdom). Following the acid digestion, the tubes were placed into stainless steel blocks, which were heated to 120°C with a hotplate (Selby Ratec Co., UK) for approximately 48 hours, or until the volume was reduced to below 0.6ml. After cooling, the samples were transferred to plastic tubes (Biotek, Auckland, New Zealand) and a weighed volume of deionised water added to give a final sample weight of 12g.

4.2.6.2 Wash samples

The Erlenmeyer flasks containing the wash samples were placed on the hotplate for 16 hours at 100°C to reduce fluid volume. Five ml of concentrated nitric acid was then added to each of the samples, which were then left to incubate at room temperature for 16 hours. The solution was then heated to 110°C until all liquid was evaporated. The remaining ash was then dissolved in 2ml of 30% hydrogen peroxide (AnalaR, BDH Chemicals, Poole, UK) and the samples were placed back on the hotplate until all peroxide was evaporated and a white ash remained. The ash was made up to 10ml with a weighed volume of 2% nitric acid then transferred to plastic tubes.

4.2.6.3 Iron analysis

Iron analysis was conducted using an Elan 6100 DRC+ inductively coupled plasma-mass spectrometer (ICP-MS), with dynamic reaction cell (Perkin Elmer/SCIEX). The sensitivity and accuracy of this method was better than atomic absorption spectroscopy for concentrations of iron in the ng/g range (see Appendix Five).

The variation in the data due to acid digestion of the samples (Section 4.2.6.1), and analysis by ICP-MS was determined by coefficient of variation (CV) values. The starting iron concentrations for each experimental day were repeated samples of the same iron solution, thus, they were treated as replicates and mean, standard deviation and CV values were determined. Furthermore, the variation in ICP-MS analysis was determined by reading three of the acid digested mucosal samples twice.

4.2.7 Baseline iron levels

Six iron deficient mice (Section 4.2.1.1) were used to determine baseline intestinal iron levels. These mice were euthanased as described in Section 4.2.3 and the intestine was mounted in the Ussing chamber as described in Section 3.2.2 and 3.2.3. The intestinal segments were incubated in Ringer's solution for 90 minutes and analysed for iron concentration as described in Section 4.2.6.

Six iron replete mice (Section 4.2.1.2) were used to determine 'normal' haematological parameters and levels of intestinal and liver iron stores. Mice were weighed and haematology was conducted as described in Section 4.2.2. The liver and intestine were removed as described in Section 4.2.3. The intestine was mounted in the Ussing chambers as described in Section 3.2.2 and 3.2.3 and incubated in Ringer's solution for 90 minutes. Iron concentration in the intestinal segments and liver were determined as described in Section 4.2.6.

4.2.8 Calculations

Percent iron removed (Fe%removed) from the mucosal solution was calculated using the following equation:

$$Fe\%removed = \frac{(Fe_{start} \times V_{start}) - [(Fe_{end} \times V_{end}) + (Fe_{start} \times 0.5ml)]}{(Fe_{start} \times V_{start}) - (Fe_{start} \times 0.5ml)} \times \frac{100}{1}$$

Where:

Fe_{start} = concentration of iron (μg/g) in the mucosal solution 30 seconds after the start of incubation.

V_{start} = volume (ml) of the mucosal solution at the start of the experiment.

Fe_{end} = concentration of iron (μg/g) in the mucosal solution 90 minutes after the start of incubation.

V_{end} = volume (ml) of the mucosal solution at the end of the experiment.

Apparent absorption (Appabs%) of iron by mouse intestinal tissue from the mucosal solution was calculated using the following equation:

$$Appabs\% = \frac{(Fe_{start} \times V_{start}) - [(Fe_{end} \times V_{end}) + (Fe_{start} \times 0.5ml) + Fe_{wash}]}{(Fe_{start} \times V_{start}) - (Fe_{start} \times 0.5ml)} \times \frac{100}{1}$$

Where:

Fe_{wash} = total amount of iron (μg) in the wash sample.

Total recovery (Totrec%) of the iron added to the Ussing chamber experiment was calculated using the following equation:

$$Totrec\% = \frac{(Fe_{start} \times V_{start}) - [(Fe_{end} \times V_{end}) + (Fe_{start} \times 0.5ml) + Fe_{wash} + (Fe_{tissue} - Fe_{base})]}{(Fe_{start} \times V_{start}) - (Fe_{start} \times 0.5ml)} \times \frac{100}{1}$$

Where:

Fe_{tissue} = total amount of iron (μg) in the intestinal segment mounted in the Ussing chamber.

Fe_{base} = mean, total amount of iron (μg) in the intestinal segments that were mounted in the Ussing chambers but not incubated with iron solutions.

4.2.9 Statistical analysis

All statistical analyses were conducted using SPSS version 11.0 (SPSS Inc.). For body weight, PCV, haemoglobin, MCV, intestinal iron, liver iron and liver weight, the statistical significance of the differences between normal and iron deficient mice were determined using independent-samples 2-tailed t tests. For short-circuit current and tissue resistance, the differences between baseline and after the addition of the digested meat, vegetable and meat-plus-vegetable solutions were

determined using paired-samples t tests. For the changes in short-circuit current and tissue resistance, the statistical significance of the difference between the digested meat, vegetables and meat-plus-vegetables were determined using one-way ANOVAs. Differences between the resistance of the digested meat, vegetable or meat-plus-vegetable solutions and Ringer's solution were determined using a one-way ANOVA.

As there was an unexpected wide variation in the data for the iron concentrations in the mucosal solutions of each treatment group, outliers were identified using box and whisker plots generated by SPSS version 11.0. Outliers were defined as values over 1.5 box lengths from the upper or lower edge of the box; the box is the interquartile range from the 25^{th} percentile to the 75^{th} percentile. The removal of such values was performed only once and further outliers appearing after this cut remained in the data set. No more than 10% of the total number of values was removed from any data set.

For the percentage of iron removed from the mucosal solution and the apparent absorption, the statistical significance of the differences between ferrous gluconate and digested meat, vegetables and meat-plus-vegetables for the four intestinal segments were determined using a 4 (4 treatments) x 4 (4 intestinal segments) ANOVA. Differences in intestinal iron concentrations between tissues incubated with digested meat, vegetables or meat-plus-vegetables, ferrous gluconate or Ringer's solution (baseline) were determined using a one-way ANOVA. The statistical significance of the differences between the intestinal regions (duodenum and jejunum) within each iron solution were determined using independent-samples 2-tailed t tests. For this analysis, the experimental unit was each intestinal segment. To determine whether the iron absorbed from each iron solution was different from zero, staring iron amounts were compared to the respective end iron amounts using paired-samples t tests for individual intestinal segments, intestinal regions and all tissue segments.

For all ANOVA tests, Levene's Homogeneity of Variance tests were run. When variances were equal, Tukey's HSD *post hoc* tests were used to determine

differences between samples. When variances were unequal, Tamhane's *post hoc* tests were used.

4.3 Results

4.3.1 Body weight and iron status

At weaning, there was no significant ($p>0.05$) difference between the body weights for the mice starting the normal pelleted diet or the low iron diet (Table 4.2). However, 6 weeks after weaning, the body weights of the mice kept on the low iron diet were significantly ($p<0.05$) lower than those of the mice fed the pelleted diet. Compared to an average increase of 25g in body weight for iron replete mice, mice maintained on the low iron diet gained on average only 7g in body weight.

Table 4.2: Means (± SEM) for the haematological indices, tissue iron concentrations and body weights of iron replete and iron deficient mice.

	Iron status	
	Replete	Deficient
Body weight (g)		
At weaning (3 weeks old)	13.5 ± 0.2 (6)	14.3 ± 0.3 (35)
At experiment (9 weeks old)	38.6 ± 2.8 (6)	21.2 ± 0.9* (35)
Packed cell volume (%)	40.2 ± 1.6 (5)	41.8 ± 1.8 (15)
Blood haemoglobin (g/L)	123.4 ± 4.9 (5)	135.1 ± 3.1 (15)
Mean cell volume (fL)	49.2 ± 0.5 (5)	45.0 ± 1.4 (15)
Baseline intestinal iron		
Iron concentration (μg/g dry weight)	237.2 ± 15.1 (23)	191.9 ± 12.2* (23)
Iron amount (μg)	1.5 ± 0.09 (23)	1.0 ± 0.05*** (23)
Liver		
Iron concentration (μg/g dry weight)	527.8 ± 42.5 (6)	299.8 ± 22.6* (28)
Total wet weight (g)	1.5 ± 0.1 (6)	0.7 ± 0.04* (34)
Relative weight (% body weight)	3.8 ± 0.2 (6)	3.5 ± 0.2 (34)

Number of mice or intestinal segments (for baseline intestinal iron) shown in parentheses. *$p<0.05$, ***$p<0.001$ compared to normal iron status values.

There were no significant differences ($p>0.05$) between groups for PCV, haemoglobin concentration or MCV. However, liver iron concentrations for the iron deficient mice were significantly ($p<0.05$) lower than those for the iron replete mice (Table 4.2). Iron stores within the liver of mice maintained on the low iron diet were depleted 43%, compared to those for the iron replete mice.

4.3.2 Basal bioelectric parameters

Table 4.3 shows the mean baseline bioelectric parameters for all mouse intestinal segments after 30 minutes mounted in the Ussing chambers.

Table 4.3: The baseline bioelectric measurements for mouse duodenum and jejunum mounted in Ussing chambers.

Bioelectric parameter	**Mean ± SD**
Short-circuit current (μAmps/cm^2)	49.9 ± 29.0 (154)
Tissue resistance (Ω/cm^2)	156.1 ± 48.2 (133)
Potential difference (mV)	4.7 ± 2.4 (132)

Number of intestinal segments shown in parentheses.

4.3.3 Changes in bioelectric measurements in response to the digested meat, vegetable and meat-plus-vegetable solutions

Addition of gastrointestinal digested water to the Ussing chambers containing duodenal (n=4) or jejunal (n=4) intestinal segments had no effect on short-circuit current or tissue resistance (data not shown). Further, the addition of ferrous gluconate to the mucosal Ringer's solution had no effect on short-circuit current or tissue resistance.

Table 4.4: Means (± SEM) for changes to short-circuit current of mouse small intestinal tissue after the replacement of the mucosal Ringer's solution with digested meat (Mt), vegetable (Vg) or meat-plus-vegetable (Mt + Vg) solutions.

	Short-circuit current (μAmps/cm^2)		
	Baseline	Maximum reached	Change
Mt (19)	41.4 ± 4.7	133.8 ± 14.3***	91.4 ± 10.7
Vg (20)	42.6 ± 5.2	151.3 ± 22.1***	93.0 ± 13.3
Mt + Vg (23)	58.4 ± 9.5	140.0 ± 13.9***	85.2 ± 11.1

Number of intestinal segments shown in parentheses. $^{***}p<0.001$ compared to baseline values.

In response to the replacement of the mucosal Ringer's solution with the digested meat, vegetable or meat-plus-vegetable solutions, mouse duodenum and jejunum exhibited significant ($p<0.001$) changes in short-circuit current (Table 4.4) and tissue resistance (Table 4.5).

Table 4.5: Means (± SEM) for changes to tissue resistance of mouse small intestinal tissue after the replacement of the mucosal Ringer's solution with digested meat (Mt), vegetable (Vg) or meat-plus-vegetable (Mt + Vg) solutions.

	Tissue resistance (Ω/cm^2)		
	Baseline	Minimum reached	Change
Mt (26)	169.1 ± 8.1	49.6 ± 6.8***	120.0 ± 7.4
Vg (21)	146.0 ± 7.0	69.1 ± 7.8***	80.3 ± 6.7
Mt + Vg (23)	164.2 ± 12.8	75.6 ± 7.9***	86.7 ± 8.4

Number of intestinal segments shown in parentheses. ***$p < 0.001$ compared to baseline values.

The digested meat and vegetables tended to cause a greater increase in short-circuit current than did the meat-plus-vegetables, however, the differences between the digested solutions were not significant ($p > 0.05$). For tissue resistance, meat had a significantly ($p < 0.05$) greater effect, compared to the digested vegetables or meat-plus-vegetables. The effects of digested meat, vegetables and meat-plus-vegetables on short-circuit current are shown in Figure 4.1.

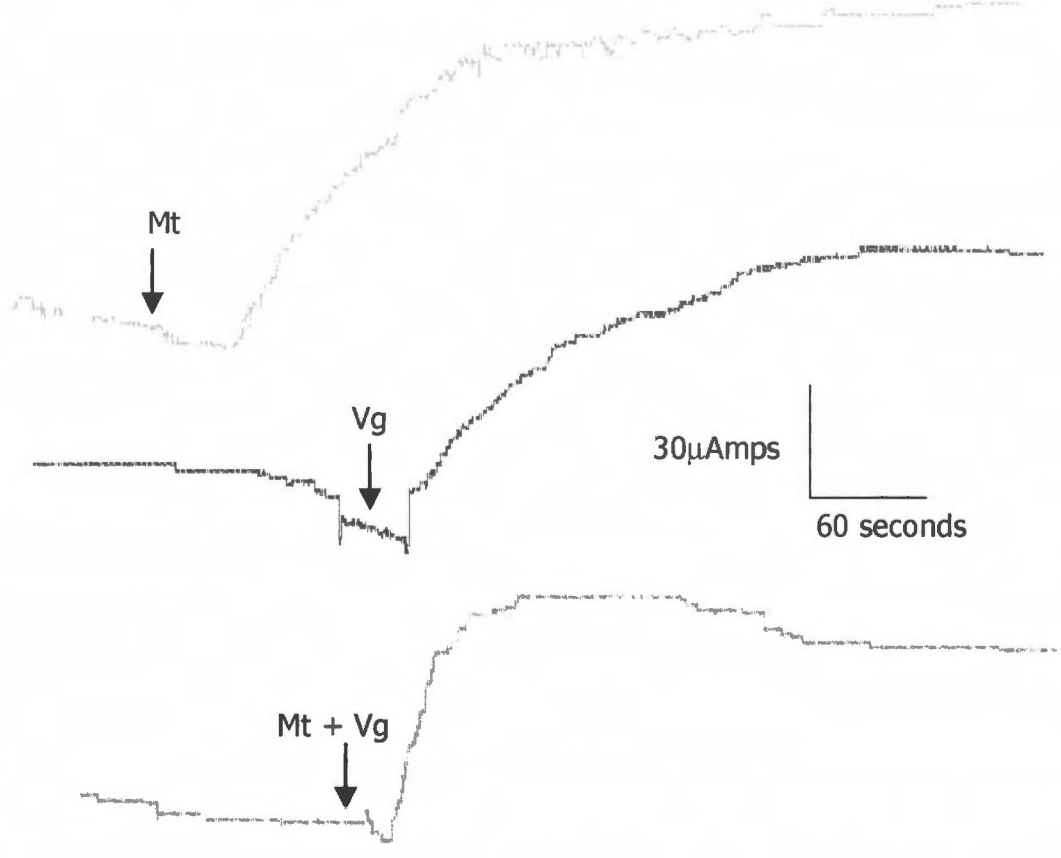


Figure 4.1: Typical recordings of the changes in short-circuit current after the addition of digested meat (Mt), vegetable (Vg) or meat-plus-vegetable (Mt + Vg) solutions to mouse small intestinal tissues mounted in Ussing chambers.

4.3.4 Resistance of the mucosal solution

Along with changes in short-circuit current and tissue resistance, replacing the mucosal Ringer's solution with digested meat, vegetables or meat-plus-vegetable solutions significantly ($p<0.001$) decreased the resistance of the mucosal solution (Table 4.6). Furthermore, the resistance of the digested meat solution was significantly ($p<0.001$) lower than that of the digested vegetable or meat-plus-vegetable solutions.

Table 4.6: Means (± SEM) for the resistance (Ω) of the Ringer's solution and the digested meat, vegetable and meat-plus-vegetable solutions.

Iron solution	Resistance (Ω)
Meat	54.8 ± 2.5^{b} (8)
Vegetables	68.4 ± 3.1^{a} (8)
Meat-plus-vegetables	76.5 ± 2.8^{a} (8)
Ringer's solution	96.4 ± 3.7^{c} (8)

Number of samples shown in parentheses. Means do not differ significantly ($p>0.05$) between the solutions if they have a common letter beside them.

4.3.5 Iron absorption

There was no significant effect of intestinal segments (1 to 4) or intestinal region (duodenum and jejunum) on the concentration of iron removed from the mucosal solution. Thus, data for all intestinal segments for the ferrous gluconate, and digested meat, vegetables and meat-plus-vegetables were grouped together for further analysis.

4.3.5.1 Iron removed from the mucosal solution

Data for the percent iron removed from the mucosal solution and apparent absorption from the digested meat, vegetables and meat-plus-vegetables and ferrous gluconate are presented in Table 4.7.

Table 4.7: Means (± SEM) for the percent iron removed from the mucosal solution and apparent absorption of iron from ferrous gluconate (Fe glu) or digested meat (Mt), vegetables (Vg) or meat-plus-vegetables (Mt + Vg).

	Mt	Vg	Mt + Vg	Fe glu
Start iron amount (μg)	47.0 ± 7.0	37.2 ± 3.3	29.4 ± 3.4	86.0 ± 7.6
End iron amount (μg)	37.7 ± 5.3	36.0 ± 2.4	23.1 ± 0.7	68.0 ± 5.5
Iron removed from mucosal solution (%)	14.5 ± 7.1	-1.0 ± 6.5	9.6 ± 2.8	20.1 ± 6.6
Iron in wash sample (μg)	3.9 ± 0.5	7.1 ± 1.0	3.0 ± 0.5	11.6 ± 1.8
Apparent absorption (%)	10.0 ± 6.3[a]	-25.7 ± 8.0[b]	-0.4 ± 3.6[a]	6.4 ± 6.8[a]
n (outliers)				
Iron removed	23(2)	23(1)	21(2)	18(2)
Apparent absorption	22(3)	24(0)	22(1)	18(2)

Within the iron removed and apparent absorption rows only, means do not differ significantly ($p>0.05$) if they have a common letter or no letter beside them.

There were no significant differences between the iron solutions for the percentages of iron removed from the mucosal solution after 90 minutes. Nevertheless, there appeared to be a loss of iron from the digested meat, meat-plus-vegetable solutions and ferrous gluconate solutions. However, after addition of the iron recovered in the wash sample (apparent absorption), the percentages decreased (Table 4.7). Despite an initial negative loss of -1.0% of iron from the digested vegetable solution, addition of the iron present in the wash sample decreased this percentage further, to -26%. Although there appeared to be absorption of iron from meat and ferrous gluconate, paired-samples t tests showed there were no significant ($p>0.05$) differences between the start and end iron amounts for any of the iron solutions (data not shown).

There were no significant differences ($p>0.05$) between the baseline intestinal iron concentrations, and the concentrations of iron in the intestinal tissues after incubation with ferrous gluconate or digested meat, vegetables or meat-plus-vegetables (Table 4.8). Furthermore, there were no significant differences ($p>0.05$) in intestinal iron concentrations between tissues for each of the iron solutions.

Table 4.8: Means (± SEM) for the iron concentrations (μg/g dry weight) in intestinal segments after incubation with Ringer's solution (baseline), ferrous gluconate (Fe glu) or digested meat (Mt), vegetables (Vg) or meat-plus-vegetables (Mt + Vg). Tissues were separated into individual intestinal segments (1 to 4), duodenum (segments 1 and 2) and jejunum (segments 3 and 4).

	Intestinal iron concentration (μg/g dry weight)				
	Baseline	Mt	Vg	Mt + Vg	Fe glu
Segment 1	215.4 ± 38.2 (6)	162.3 ± 23.9 (8)	217.9 ± 45.2 (6)	144.0 ± 12.2 (6)	200.4 ± 32.6 (5)
Segment 2	223.0 ± 41.3 (6)	138.0 ± 14.1 (4)	151.3 ± 14.8 (6)	318.2 ± 110 (5)	227.2 ± 40.6 (4)
Segment 3	172.4 ± 16.6 (6)	144.7 ± 6.6 (6)	171.9 ± 29.0 (6)	201.4 ± 42.0 (6)	196.5 ± 52.2 (5)
Segment 4	192.9 ± 16.3 (6)	253.2 ± 51.8 (6)	199.9 ± 38.8 (5)	176.0 ± 13.0 (6)	134.9 ± 10.0 (5)
Duodenum	219.2 ± 26.8 (12)	154.2 ± 16.5 (12)	184.6 ± 24.8 (12)	223.2 ± 54.7 (11)	212.3 ± 24.3 (9)
Jejunum	182.7 ± 11.5 (12)	199.0 ± 29.8 (12)	187.1 ± 24.1 (11)	188.7 ± 21.3 (12)	165.7 ± 27.0 (10)
Total mean	300 ± 22.8	205 ± 28.0	186 ± 17.0	177 ± 17.3	228 ± 43.4

Number of intestinal segments shown in parentheses. Within each column and row, means do not differ significantly ($p>0.05$) if they have a common letter or no letter beside them.

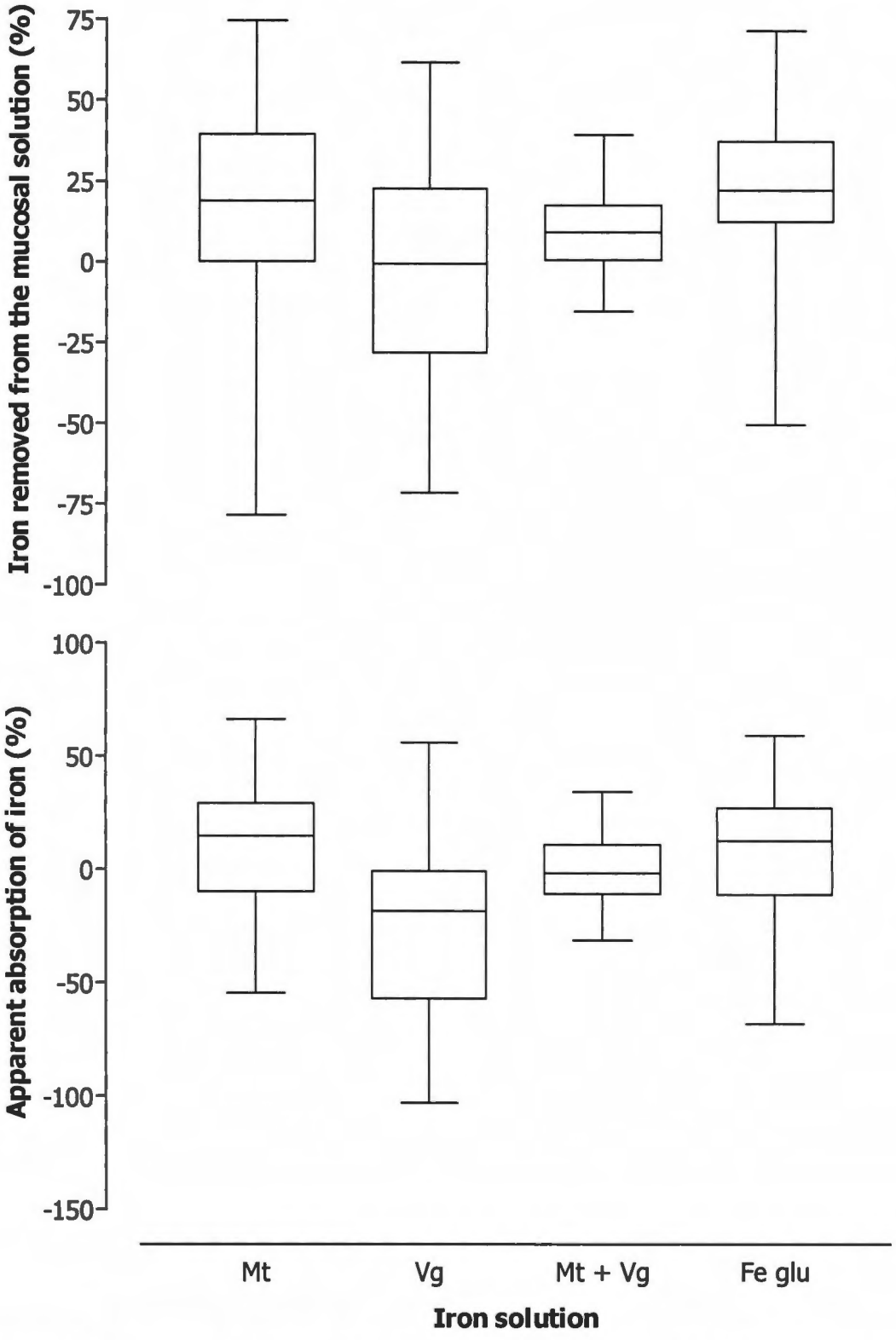


Figure 4.2: Box and whisker plots for the percent iron removed from the mucosal solution (top) and apparent absorption (bottom) after mouse duodenal and jejunal tissues were incubated for 90 minutes with ferrous gluconate (Fe glu) or digested meat (Mt), vegetables (Vg) or meat-plus-vegetables (Mt + Vg). Box and whisker plots present the spread and quartiles of the data. The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile). The whiskers extend above and below the box to show the highest and lowest values, respectively. Note: n values are given in Table 4.7.

4.3.5.2 Data variation

There was an unexpected wide variation in the data obtained for each of the iron solutions. As shown in Figure 4.2, the spread of the data for the digested meat-plus-vegetables was relatively small. The spreads for the meat and ferrous gluconate data were generally similar, while that for the vegetable data was the greatest. The variation was evident even though outliers had been removed from the data sets.

Data for the starting mucosal iron concentrations on each experimental day are shown in Table 4.9. In general, CV values were very high for all iron solutions with the exception of the digested meat-plus-vegetables, for which most of the CV values were within the desired 5% or less.

Table 4.9: Means, standard deviations (SD) and coefficient of variation (CV) for the starting iron concentrations in the ferrous gluconate solutions and digested meat, vegetable or meat-plus-vegetable solutions.

Starting solution	Mean iron concentration (μg/g)	SD	CV (%)
Meat			
1	6.4 (7)	4.9	76
2	6.3 (4)	5.6	88
3	4.1 (4)	1.1	28
4	3.6 (7)	0.8	23
5	3.1 (3)	0.4	13
Vegetables			
6	3.8 (6)	1.9	50
7	4.4 (8)	2.5	56
8	4.0 (4)	0.7	17
9	2.9 (3)	0.6	16
10	3.2 (3)	0.8	25
Meat-plus-vegetables			
11	2.3 (8)	0.1	5
12	2.8 (8)	0.2	8
13	2.8 (2)	0.1	5
14	6.3 (2)	5.4	86
15	3.0 (2)	0.02	0.6
Ferrous gluconate			
16	10.2 (8)	3.8	37
17	9.2 (4)	6.4	69
18	7.6 (8)	0.9	11

Numbers of replicates shown in parentheses.

Repeated ICP-MS analysis of the iron concentration in three acid digested mucosal samples is presented in Table 4.10. For both meat and vegetables, there was up to a 17% variation between readings.

Table 4.10: Repeat ICP-MS reading of three acid digested mucosal samples.

Sample	Iron concentration (μg/g)		% Difference
	First reading	Repeat reading	
Vegetable 1	10.4	11.1	6.8
Vegetable 2	10.7	12.5	16.5
Meat	10.6	12.2	15.1

4.3.6 Total recovery

Table 4.11 presents the mean values for total recovery of iron, expressed as a percentage of starting amounts. While the values for meat-plus-vegetables and ferrous gluconate are close to the expected 100%, the vegetables and meat recoveries are high.

Table 4.11: Means (± SD) for the total recovery of iron (%) for ferrous gluconate solutions or digested meat, vegetable or meat-plus-vegetable solutions.

Iron solution	Total recovery (%)
Meat	112.2 ± 60.8 (25)
Vegetables	129.2 ± 42.9 (24)
Meat-plus-vegetables	99.5 ± 23.4 (23)
Ferrous gluconate	106.9 ± 49.6 (20)

Number of intestinal segments shown in parentheses.

4.4 Discussion

Mice were fed a low iron diet to induce iron deficiency, and small intestinal tissues from these mice were used to study the absorption of iron from a ferrous gluconate solution and the soluble fractions of meat, vegetables and meat-plus-vegetables after gastrointestinal digestion. While the low iron diet successfully depleted tissue iron stores, iron absorption results were difficult to interpret due to the wide variation in the data.

4.4.1 Iron deficient diet

An iron deficient diet based on that used by Tsuchita *et al.* (1991) was used in this study to induce a state of iron deficiency in mice. The diet was shown by Tsuchita *et al.* (1991) to induce anaemia in rats after 4 weeks, reducing haemoglobin levels from normal, at approximately 170g/L, to 60g/L. Likewise, Manabe *et al.* (2000), using the same diet, induced severe anaemia in mice after 6 weeks. Other studies have reported a reduction in haemoglobin levels (Beard *et al.*, 1995; Rao and Jagadeesan, 1995) as well as a reduction in PCV for mice and rats (Hamilton *et al.*, 1978; Kuvibidila *et al.*, 1983, 2001) maintained for 3 to 4 weeks on similar iron deficient diets.

Despite following the recipe of Tsuchita *et al.* (1991), the iron deficient diet in this study contained 13mg/kg. This was much higher than the 5mg/kg iron concentration reported by Tsuchita *et al.* (1991), and also higher than other iron deficient diets used by previous groups to induce anaemia (Hamilton *et al.*, 1978; Rothenbacher and Sherman, 1980; Flanagan *et al.*, 1984; Beard *et al.*, 1995; Kuvibidila *et al.*, 1983, 2001). However, the 13mg/kg iron concentration is less than 40% of the recommended iron concentration (35mg/kg) for a normal mouse diet (American Institute of Nutrition, 1977). Thus, although the diet contained too high a concentration of iron to produce anaemia, it induced iron deficiency in mice fed the diet for a period of 6 weeks.

4.4.2 Iron status of the mice

Haematological indices of mice maintained on the low iron diet were not indicative of the presence of iron deficiency or iron deficiency anaemia; they were not significantly different from those for iron replete mice. However, analysis of liver iron concentrations revealed a 43% depletion of liver iron stores, compared to those of iron replete mice. This indicates the diet was deficient in iron and mobilisation of liver iron stores was required to meet the iron requirements for growth and development. As their iron stores were not fully depleted, haematological indices were not likely to differ from 'normal' values as the iron absorbed from the diet and liver storage iron was sufficient to support erythropoiesis. If kept on the low iron diet for longer, the liver iron stores of mice

would have become fully depleted, decreasing the availability of iron to support erythropoiesis. Haemoglobin and PCV values would then be compromised and expected to fall below 'normal' values.

Interestingly, intestinal iron concentrations were also significantly higher for iron replete than iron deficient mice in this study. This has been reported by other groups (Savin and Cook, 1980; Topham *et al.*, 1992; Pountney *et al.*, 1999), who have demonstrated decreased iron and ferritin content in duodenal enterocytes of mice and rats fed low iron diets. Presumably, the amount of iron stored within the intestinal cell was limited, as ferritin iron stores within the enterocytes are mobilised for export and all iron absorbed is shunted across the basolateral membrane to the circulation. Indeed, a study by Topham and Eads (1991) demonstrated a six-fold reduction in the incorporation of radioactive iron into mucosal ferritin of rats fed a low iron diet. Thus, all available iron appears to be mobilised to the blood to support daily iron requirements when the intake of dietary iron is low.

In the present study, the mean body weight of mice fed the low iron diet for 6 weeks was significantly lower than that for mice fed the normal pellet diet. The low weight gain of mice fed the low iron diet is consistent with results from other studies using iron deficient mice (Kuvibidila *et al.*, 1983, 2001; Morse *et al.*, 1999) or rats (Nalder *et al.*, 1972; Rothenbacher and Sherman, 1980; Rao and Jagadeesan, 1995). Studies by Beard and colleagues (Beard, 1987; Beard *et al.*, 1995) indicated that the poor growth rates in iron deficient animals are related to poor feed utilisation efficiency (calories retained/calories absorbed) rather than anorexia. Indeed, a study by Rao and Jagadeesan (1995) reported a 65% and 80% reduction in feed utilisation efficiency, compared to control rats, in Fischer 344 and Wistar rats fed an iron deficient diet. However, the reason for the low weight gain and growth in iron deficiency has not been established, although iron is required for RNA synthesis and functioning of many enzymes and proteins (see Section 1.1).

In an iron deficient state, mammals increase the expression of the intestinal iron transporter proteins, DMT-1 and FP-1, as outlined in Section 1.4. Indeed, several groups (Hamilton *et al.*, 1978; Flanagan *et al.*, 1984; Rabie *et al.*, 1995; Santos *et al.*, 1997; Pountney *et al.*, 1999) have demonstrated increased iron absorption in iron deficient mice, compared to iron replete mice. Thus, intestinal tissue from iron depleted mice should absorb greater amounts of iron when mounted in the Ussing chambers.

4.4.3 Bioelectric parameters

Short-circuit current and tissue resistance measurements are indicative of tissue integrity and viability while mounted in the Ussing chamber. As outlined in Section 3.2.3, tissues are regarded as having lost their integrity when the resistance falls below 25Ω and/or values for short-circuit current are close to 0μAmps. For the experiments described here, intestinal tissues generated a mean basal short-circuit current of 50μAmps/cm^2 and tissue resistance of 156Ω/cm^2. This indicated that the intestinal tissues maintained integrity and viability while in the chambers.

As shown in Table 4.4, replacing the mucosal solution with digested meat, vegetable or meat-plus-vegetable solutions caused a significant increase in basal short-circuit current. In contrast, as shown in Table 4.5, tissue resistance decreased significantly from basal values after addition of the digested meat, vegetable or meat-plus-vegetable solutions. However, these changes in tissue resistance may have been due to an effect on total resistance rather than a direct effect on the tissue.

Tissue resistance was determined as the difference between the total resistance and the fluid resistance. The resistance of the Ringer's solution (fluid resistance) was measured before the tissues are mounted in the Ussing chambers. As shown in Table 4.6, compared to Ringer's solution, the digested meat, vegetables and meat-plus-vegetable solutions had significantly lower resistances. Since the clamp units store the original fluid resistance as a constant value, decreasing the resistance of the mucosal side, by the addition of the digested meat, vegetable or meat-plus-vegetable solutions, will cause an apparent decrease in the tissue

resistance. Furthermore, the digested meat solution had a significantly lower fluid resistance compared to that for digested vegetable and meat-plus-vegetable solutions and this was reflected in the significantly greater decrease in tissue resistance observed after the addition of the digested meat solution (Table 4.5).

Gastrointestinal digested water had no effect on short-circuit current or tissue resistance of both duodenal or jejunal intestinal segments (data not shown). Thus, the changes in short-circuit current were due to the movement of charged substances present in the digested solutions into the enterocytes. Possible substances producing the response in short-circuit current include the absorption of charged ions, or nutrients, such as glucose and amino acids coupled to the co-transport of sodium ions (see Schultz and Curran, 1970).

Indeed, the addition of the amino acids glutamine, alanine, proline (20mM) and leucine (10mM) to the mucosal solution caused an increase of 50, 26, 20 and 19μAmps/cm^2, respectively, in short-circuit current of pig small intestine (Grondahl and Skadhauge, 1997). Rhoads *et al.* (1990) demonstrated similar changes after the addition of glutamine. As observed in Chapter Three (Table 3.7), glucose (10mM) produced an increase in short-circuit current of 37μAmps/cm^2. Similar changes in short-circuit current have been demonstrated by other groups (Field *et al.*, 1971; Kaufman *et al.*, 1980; Grubb, 1995). The observed increases in response to digested meat, vegetables and meat-plus-vegetables (Table 4.4) are greater than the responses observed by our and other groups after the addition of glucose or amino acids. However, this may be due to a dose-dependent effect on short-circuit current. The concentration of glucose and/or amino acids may be higher in the digested solutions than that used in the previous studies (Field *et al.*, 1971; Kaufman *et al.*, 1980; Rhoads *et al.*, 1990; Grubb, 1995; Grondahl and Skadhauge, 1997), which may have caused a greater increase in short-circuit current. Alternatively, the greater change in short-circuit current may have been due to the summation of responses to the many nutrients present in the digested meat, vegetable and meat-plus-vegetable solutions. It is equally plausible, that the increase in short-circuit current may have been due to the secretion of negatively

charged ions, such as chloride and bicarbonate (Ussing and Zehran, 1951), from the enterocytes.

4.4.4 Iron absorption

Iron absorption was analysed in two sequential steps. The first was to establish the amount of iron removed from the mucosal solution as a percentage of the starting iron amount. As shown in Table 4.7, iron was removed from the mucosal solutions of meat, meat-plus-vegetables and ferrous gluconate, but not from the vegetables. Although the percentage of iron removed from these solutions appeared to be different, the differences were not significant. However, the majority of iron removed from the mucosal solution was recovered in the wash sample. The wash sample included the iron attached to the mucus of the intestinal segment as well as that adhering to the glass reservoirs and Ussing chambers. With the addition of the wash samples, the apparent absorption (Table 4.7) appeared to be small for meat and ferrous gluconate, negligible for meat-plus-vegetables and negative for vegetables. Overall, t tests revealed no apparent absorption for any of the iron solutions.

It is possible that the iron present in the wash sample was in the process of being absorbed. In previous studies with Ussing chambers assembled with no intestinal tissue, iron present in Ringer's solution as ferrous gluconate did not adhere to the reservoirs and chambers (S. Edmunds, personal communication). Therefore, the recovery of iron in the wash sample was due to a tissue effect, such as the presence of mucus. Assuming that the iron in the digested meat, vegetable and meat-plus-vegetable solutions did not adhere to the apparatus, all iron present in the wash sample must have been attached to the mucus of the intestinal segment. As discussed in Section 3.4.3, mucus covers the luminal surface of the small intestine and its main constituent, mucin, has been implicated in the iron absorption process (Conrad *et al.*, 1991). Mucin has been shown to bind iron and maintain it in a form suitable for absorption in the alkaline environment of the small intestine (Conrad *et al.*, 1991). Thus, the iron absorption process may begin with the binding of iron to mucin, which presents it in a form suitable for absorption by the enterocytes.

Although the apparent absorption was not significant, the results in Table 4.7 show that there appeared to be iron absorbed from meat and ferrous gluconate. However, it is not possible to draw clear conclusions due to the wide variation in the data and the presence of negative values indicating a net secretion of iron into the mucosal solutions during the experiment.

In contrast to meat, ferrous gluconate or meat-plus-vegetables, the percentage of the iron removed from the digested vegetables was negative (-1.0%). This value became more negative (-25.7%) when the iron concentration in the wash was included. Within the vegetable data, approximately 40% of the values representing the difference between the amount of iron (μg) in the start and end mucosal solutions were between -5μg and -20μg, with extreme values of -39μg and -37μg. These negative values indicate a net secretion of iron into the mucosal solution during the vegetable experiments. The only other source of iron within the Ussing chamber system is that which is stored as ferritin in the enterocytes of the intestinal segment. Indeed, sloughing of enterocytes from the absorptive mucosa of the small intestine occurs *in vivo* (Ganong, 1999) and may occur under the conditions of this experiment. However, analysis of intestinal segments of mice maintained on the low iron diet revealed that the total amount of iron in the tissue in the chamber averaged 1.0μg (Table 4.2); a value far less than the large differences observed between the start and end solutions. Thus, the loss of iron from the enterocytes cannot account for the apparent secretion of iron by the intestinal tissues during the experiments with digested vegetable solutions.

To determine whether the amount of iron added to the Ussing chamber system at the start of the experiment could be accounted for at the end of the experiment, total recovery was determined. Total recovery of the iron at the end of the experiment was expressed as a percentage of the starting iron amount, and thus should equal approximately 100%. As can be seen from Table 4.11, the iron from the digested meat-plus-vegetable solution was completely recovered at the end of the experiment, with a mean total recovery of 100%. Total recovery for meat (112%) and ferrous gluconate (107%) were acceptably close to 100%, but at 129% the total recovery for digested vegetables was very high. The source of this

extra iron is unclear and, as noted in the previous paragraph, cannot be accounted for by the small amount of iron in the intestinal segment. Instead, the high total recovery for the vegetable solution was a further indication of the variation and error associated with the results.

For all treatments, the SEMs for percent iron absorbed were large (Table 4.7), indicating a large variation in the data. Figure 4.2 shows the spread and range of the percent iron removed and apparent absorption of iron from each of the iron solutions. The spread of data for the meat-plus-vegetables was relatively small, compared with the other iron solutions, with a median relatively close to zero. This indicates 50% of the values showed positive (absorption) values, while 50% showed negative (secretion) values. The meat and ferrous gluconate data showed a greater spread, with over 50% of the values above zero. The data for the digested vegetables had the greatest spread among the iron solutions. The top of the box was at approximately zero for the iron removed from the mucosal solution, indicating 75% of values are negative, whereas for the apparent absorption data the median was relatively close to zero. Thus, for all iron solutions the box extended below zero indicating all data sets have absorption values that are negative. Since, as discussed above, it is physiologically impossible to have negative percentage absorption values, the variation must be due to the processing of the samples by acid digestion and/or error associated with the analysis by ICP-MS.

The variation in the data due to sample processing and analysis error was estimated using CV for starting iron concentrations. On each experimental day, a fresh stock solution of ferrous gluconate or digested meat, vegetables or meat-plus-vegetables were prepared. Thus, starting iron concentrations within each experimental day were samples of the same solution, and were treated as replicates. Previous studies using Ussing chambers demonstrated a 13% (Vaghefi *et al.*, 1998) and 5.2% (Vaghefi *et al.*, 2000a) absorption of ferrous gluconate (0.1mM) by rat small intestine after 2 hours. Thus, to allow accurate detection of at least a 5% change in iron concentration between the start and end mucosal solutions, the variation associated with the sample processing and ICP-MS analysis

must be less than 5%. CV values for the starting mucosal iron concentration on each experimental day are shown in Table 4.9. The CV measures the amount of variation or spread in the values and is the result of expressing the standard deviation as a percentage of the mean.

Over the 18 experimental days, the values for the CV ranged from 0.6 to 88%. The starting concentrations for only three experimental days exhibited a CV of less than 5%; these were values for the digested meat-plus-vegetable solution. The CV for the meat, vegetable and ferrous gluconate data were variable, with meat showing the highest values at 76% and 88%. These inconsistent CV results suggest error was associated with the processing of the samples by acid digestion and/or iron analysis by ICP-MS.

The origin of the error associated with the sample analysis is unclear, although possible sources of the variation could be the following:

- Contamination of the samples with external sources of iron.
- Interference from other substances present in the digested meat, vegetables or meat-plus-vegetables.
- Error in the analysis of iron by ICP-MS.
- The large dilution factor associated with the acid digestion method.

The likelihood of sample contamination from the nitric acid and glassware is small. All tubes and glassware were soaked overnight in 5% nitric acid, rinsed in deionised water and dried in a dust-free oven the day before use. Nitric acid is used as it solubilises contaminant iron by forming iron nitrate (Kneen *et al.*, 1972), which can then be removed by rinsing with deionised water. Additionally, the concentrated nitric acid used in the assay method has a maximum limit of impurity of 0.02μg/g for iron. To lower contamination of the nitric acid, small bottles (500ml) were purchased to reduce the number of times the acid was subsampled. This should reduce contamination from atmospheric iron or dust particles. However, the sample tubes were uncapped on the heating block for 48 hours during acid digestion, thus leading to the possibility of sample contamination.

There are many substances within food that can bind iron and form soluble and insoluble complexes. Fatty acids have been shown by various groups to bind iron and enhance its uptake (Simpson and Peters, 1987; Simpson *et al.*, 1988; Qian and Eaton, 1991). Fat is likely to be present in the digested food solutions, particularly the meat and meat-plus-vegetables, even though any visible fat was skimmed from the digested solutions prior to freeze-drying. Fat is notoriously difficult to digest and cooking of the meat may also form compounds that are hard to digest (Carpenter and Clark, 1995). The addition of a hydrogen peroxide step has been used successfully to remove fat from meat samples during a nitric acid digestion (W. Johnson and D. Simcock, personal communication) and was included in the sample preparation for the total iron assay as described in Section 2.2.1.5. However, it was not thought necessary to include hydrogen peroxide in the acid digestion method described in this study, as visible fat was removed and concentrated nitric acid was able to completely digest the samples, as indicated by the solution turning colourless. Alternatively, the presence of iron-binding substances may produce variation in iron concentrations by hindering an even distribution of iron within the solution. For instance, one sample may contain a high proportion of an iron-binding substance, while another sample of the same digested solution may contain less. This may cause variation in the concentration of iron between samples, especially if the substance binds multiple iron molecules. However, since variation was not just present in the results from the digested solutions but also in the ferrous gluconate samples (Figure 4.2), interference from food substances was unlikely to have been a source of variation. Furthermore, this variation was not observed in initial samples analysed using the ICP-MS method (see Appendix Five).

To investigate whether analysis by ICP-MS produced variation in the iron concentrations, three mucosal samples, with high iron concentrations, were read twice. As shown in Table 4.10, the repeated readings of the vegetable and meat samples were increased from the original value. Although repeat analysis was only conducted on three samples, these increases in iron concentration suggest the variation in the ICP-MS analysis is reasonably high. This error may occur within the same samples and/or between the days of analysis.

Another possible contributor to the variation in results may include the large dilution factor associated with the acid digestion method. In the development of the acid digestion method (see Appendix Five), a large dilution factor was required to reduce the interference from other ions in the digested solutions and Ringer's solutions. The ICP-MS machine is sensitive to interference from high concentrations of salt. Thus, to reduce the salt content to within an acceptable range for ICP-MS analysis, all mucosal samples were diluted 50-fold (S. Knowles, personal communication). With such a large dilution factor, the iron concentrations of the samples are reduced to the ng/g range. A small error in the analysis of iron at these concentrations can become quite large when the 50-fold dilution factor is accounted for. However, initial results suggested that an effect of the dilution factor was not significant (see Appendix Five), but would compound any other errors in the acid digestion method.

4.5 Conclusions

- Haematological indices for mice fed a low iron diet were within the 'normal' range suggesting iron deficiency anaemia was not present. However, liver iron stores were depleted 43%, compared to those of normal mice, indicating the presence of iron deficiency.
- Replacing the Ringer's mucosal solution with digested solutions of meat, vegetables or meat-plus-vegetables significantly increased short-circuit current.
- Iron absorption results indicated a trend towards higher absorption of iron from ferrous gluconate and meat, compared to vegetables and meat-plus-vegetables, although they were difficult to interpret due to the wide variation in the data.
- The source of the wide variation in the data is unknown, however, errors associated with the sample processing and analysis of iron by ICP-MS were probably involved.

CHAPTER FIVE

General discussion

The main aim of the research reported in this Thesis was to study the bioavailability of iron in New Zealand beef either alone or as part of a 'typical' New Zealand meal. Bioavailability is defined as the proportion of the total iron intake that can be absorbed and utilised for normal body functions (Wienk *et al.*, 1999). Initially, the solubility of haem and non-haem iron was used to estimate the amount of iron that would be available for intestinal absorption (Chapter Two). However, although it is generally accepted that only the soluble iron in food is absorbed by the small intestine (Wienk *et al.*, 1999), only a proportion of this soluble iron may be in a form that is available for absorption. Thus, an *in vitro* technique, using a living intestinal tissue, was used to measure iron absorption in an attempt to assess food iron bioavailability (Chapter Three and Four). Using these techniques, the specific objectives of the experiments described in this Thesis were:

1. To validate the use of total, haem and non-haem iron assays to determine these iron forms in the soluble and insoluble fractions of meat, vegetables and a combination of meat-plus-vegetables ('typical' New Zealand meal).
2. To investigate the effects of pepsin and pancreatin-bile digestion on the solubility of haem and/or non-haem iron in meat, vegetables and meat-plus-vegetables.
3. To validate an *in vitro* technique using Ussing chambers for measuring iron absorption.
4. To use the Ussing chamber model to investigate the absorption of iron, by mouse intestinal tissue, from ferrous gluconate and the soluble fractions of meat, vegetables and meat-plus-vegetables after gastrointestinal digestion.

5.1 Iron solubility

The solubility of iron has been used previously to indicate the amount of absorbable or bioavailable iron within foods (Narasinga Rao and Prabhavathi, 1978; Miller *et al.*, 1981; Kane and Miller, 1984; Politz and Clydesdale, 1988; Slatkavitz

and Clydesdale, 1988; Perez-Llamas *et al.*, 1996, 1997; Seth *et al.*, 1999). Thus, in Chapter Two, solubility was used as an indicator of bioavailable iron.

Within raw beef longissimus muscle, the concentration of soluble iron was 80% in the present study. This is comparable to reported concentrations of 74% of soluble iron for beef semitendinosus muscles (Purchas *et al.*, 2003), but slightly higher than 69% reported for beef longissimus muscle (Han *et al.*, 1993). However, despite the high concentration of soluble iron in raw meat, cooking led to considerable losses of soluble iron (both haem and non-haem), corresponding with marked increases in insoluble iron (Table 2.10 and Figure 2.1) (Han *et al.*, 1993; Purchas *et al.*, 2003). Furthermore, increasing the cooking time (Purchas *et al.*, 2003) and temperature (Han *et al.*, 1993; Purchas *et al.*, 2003) generally led to greater losses of soluble iron. Additionally, as reported in the present study (Table 2.10), cooking has been shown to cause a loss of haem iron and a corresponding increase in non-haem iron concentrations in meat (Schricker *et al.*, 1982; Schricker and Miller, 1983; Chen *et al.*, 1984; Jansuittivechakul *et al.*, 1985; Buchowski *et al.*, 1988; Kristensen and Purslow, 2001).

The use of solubility measurements to assess the bioavailability of iron can be taken a step further by investigating food iron solubility within the gastrointestinal tract. By using an *in vitro* method, the conditions food iron encounters in the gastrointestinal tract can be simulated. Miller *et al.* (1981) developed an *in vitro* method to simulate the gastrointestinal digestion process, which consisted of pepsin digestion at pH 2.0 and subsequent adjustment to pH 7.0 for digestion with a mixture of pancreatic enzymes and bile extract. This same method, or with slight modifications, has been used extensively by others (Kane and Miller, 1984; Crews *et al.*, 1983, 1985a, b; Politz and Clydesdale, 1988; Slatkavitz and Clydesdale, 1988; Perez-Llamas *et al.*, 1996, 1997; Seth *et al.*, 1999) and was thus adopted in the present study. This *in vitro* digestion procedure was intended to simulate the effects of the gastric and intestinal environments and enzymes on food as it passes through the gastrointestinal tract. By its very nature, it cannot precisely replicate the complexity of the *in vivo* situation, for instance, the variation in gastric emptying rate due to the size and consistency of the meal (Davenport, 1982), but

can provide indicative information regarding the effects of enzymes on iron solubility.

Several groups have demonstrated that *in vitro* pepsin and pancreatin digested meat increases the solubility of extrinsic iron at neutral pH *in vitro* (Politz and Clydesdale, 1988; Slatkavitz and Clydesdale, 1988; Seth *et al.*, 1999). Further, the percentage of dialysable intrinsic iron from *in vitro* pepsin and pancreatin-bile digested meat was higher than 'non-meat' sources in some studies (Perez-Llamas *et al.*, 1996, 1997; Kapsokefalou and Miller, 1991), although not in others (Kane and Miller, 1984) and the addition of beef to a semi-synthetic meal yielded 12% dialysable iron, compared to 4% for egg albumin (Mulvihill and Morrissey, 1998). Thus, the intrinsic iron in meat would appear to be more soluble than iron in 'non-meat' sources after gastrointestinal digestion. However, these studies used raw meat samples, which contain a high proportion of soluble iron prior to digestion (Table 2.10) (Han *et al.*, 1993; Purchas *et al.*, 2003) and it is thus unclear whether digestion would increase the solubility of iron in cooked meat. Furthermore, no distinction between haem and non-haem iron fractions was made, despite the fact they have different absorption processes and bioavailability (see Sections 1.3 and 1.5.2). In addition, the separate effects of pepsin and pancreatin-bile digestion on the solubility of meat haem and non-haem iron were not investigated.

In Chapter Two, the results of investigations into haem and non-haem iron concentrations in the water-soluble and water-insoluble fractions of meat, vegetables and meat-plus-vegetables are reported and discussed. Validation of total, haem and non-haem iron assays were carried out before they were used to investigate the effects of cooking and pepsin and pancreatin-bile digestion on the solubility of haem and/or non-haem iron in meat, vegetables and meat-plus-vegetables. The main findings from these experiments were:

- Haem and/or non-haem iron assay methods accurately determined these iron forms in meat, vegetables and meat-plus-vegetables.

- Cooking at 65°C for 90 minutes reduced the haem iron concentration in meat by 27% of total iron. This coincided with a 175% increase in the non-haem iron concentration.
- Cooking reduced the soluble iron concentration in meat by 81%.
- Pepsin and pancreatin-bile digestion increased the solubility of iron in meat (333%), vegetables (367%) and meat-plus-vegetables (167%).
- A proportion (35%) of the haem iron in the meat was broken down by the action of pancreatic enzymes leading to a 46% increase in non-haem iron concentrations.

The pancreatin-bile mixture degraded the haem porphyrin complex in meat (Table 2.14), however, this effect was not observed when meat was combined with vegetables (Table 2.16). This may be due to compounds in the vegetables, which interacted with meat resulting in the protection of the porphyrin ring from degradation. In turn, this may have enhanced the bioavailability of meat iron as the bioavailability of haem iron is higher than non-haem iron (Monsen *et al.*, 1978; Monsen, 1988; Hunt and Roughead, 2000), thereby suggesting that other food constituents in a meal may have an effect on meat iron bioavailability. Thus, distinguishing between the haem and non-haem iron forms is important to our understanding of the contribution of these iron forms to the bioavailable iron 'pool'.

Overall, the experiments described in Chapter Two demonstrate that the solubility of iron in foods after cooking is low. Using solubility as an indicator of bioavailable iron, it appears that cooking could reduce the bioavailability of iron in meat by decreasing its solubility and to a lesser extent degrading haem iron to non-haem iron. However, the reduction in iron solubility was partially reversed by the actions of pepsin and pancreatin-bile, which increased the concentrations of soluble haem and non-haem iron. Thus, before *in vitro* estimations can be made on the bioavailability of food iron, the effects of both the cooking and the gastrointestinal digestion processes must be considered.

It is generally accepted that only soluble iron is absorbed by the enterocytes of the small intestine (Wienk *et al.*, 1999). Thus, in this study, as with previous studies

solubility was used as an indication of bioavailable iron (Narasinga Rao and Prabhavathi, 1978; Miller *et al.*, 1981; Kane and Miller, 1984; Politz and Clydesdale, 1988; Slatkavitz and Clydesdale, 1988; Perez-Llamas *et al.*, 1996, 1997; Seth *et al.*, 1999),. However, iron solubility may not correlate closely with iron absorption (Sato *et al.*, 1987; Kapsokefalou and Miller, 1995) as only a proportion of soluble iron is in a form that is available for intestinal absorption, according to Miller and Berner (1989). Furthermore, solubility may not be an accurate indicator of bioavailable iron as raw meat was slightly less effective in repletion of iron stores in anaemic piglets than cooked meat, despite the lower iron solubility in cooked meat (P. Morel, unpublished data). In addition, overall iron absorption may also vary according to the proportions of haem and non-haem iron present (Seligman *et al.*, 2000) and the presence of the 'meat factor'. Thus, in the work described in Chapters Three and Four, the estimation of the bioavailability of iron in foods was taken a step further by validating and using an *in vitro* technique to investigate iron absorption by live, intact intestinal segments in Ussing chambers.

5.2 Estimation of food iron bioavailability: the use of Ussing chambers

Previous groups have used Ussing chambers for iron absorption studies with rat Helbock and Saltman, 1967; Vaghefi *et al.*, 1998, 2000a, b) and mouse (Costa *et al.*, 2000) small intestinal tissue. These studies demonstrated iron, from both non-haem and haem iron sources, is absorbed by intestinal tissue mounted in Ussing chambers. Compared to other *in vitro* techniques, the advantage of using the Ussing chamber model for iron absorption and bioavailability studies is that it allows for the study of intact intestinal epithelium, which is taken fresh from a euthanased animal. The tissue possesses most of the features that occur *in vivo*, including the presence of an adherent mucin layer. When connected to recording apparatus, Ussing chambers allow constant assessment of the tissue viability. Further, they offer the opportunity to control the electrochemical potential difference across the intestinal epithelium.

The majority of studies investigating the mechanisms of intestinal transport and metabolism of iron have used intact animals and intestinal segments and cells

derived from laboratory rodents. However, a study by Reddy and Cook (1991) have challenged the validity of extrapolating results from animals to humans due to the influence of dietary factors on iron absorption. In rats, iron absorption is less affected by dietary factors, such as meat and ascorbic acid, than in humans (Reddy and Cook, 1991). Furthermore, unlike humans, rats absorb haem iron poorly and to a lesser extent than non-haem iron (Wheby *et al.*, 1970). However, the same mechanisms of haem iron absorption and its regulation, depending on the dose and on iron status, are known to occur in rodents and humans. For instance, in both the rodent and human, up-regulation of haem iron absorption by iron deficiency is less pronounced than non-haem iron (Wheby *et al.*, 1970; Raffin *et al.*, 1974; Roberts *et al.*, 1993). Additionally, the absorption of haem iron was observed in preliminary studies using mouse duodenum (see Appendix Six) and in previous Ussing chamber studies using rat duodenum (Vaghefi *et al.*, 1998; 2000a, b). Thus, although a great deal of caution is required when extrapolating results from animal models to humans, the Ussing chamber model, using rodent tissue, can be used to investigate trends in human responses (Vaghefi *et al.*, 2000a).

In Chapter Three, the results of preliminary experiments using mouse intestinal tissues mounted in the Ussing chamber system to measure iron absorption are presented. The intestinal epithelium under the conditions described was able to generate a spontaneous and stable electrical potential difference. Furthermore, when glucose, theophylline or carbachol, which have stimulatory effects on the absorption or secretion of charged ions by the intestinal epithelium, were added to the mucosal and/or serosal side of the tissues, short-circuit current increased in ways similar to that reported by other groups (Field *et al.*, 1971; Sheldon *et al.*, 1989; Kaufman *et al.*, 1980; Grubb, 1995). This indicated that mouse intestinal tissue maintained its integrity and viability while mounted in the Ussing chambers, and that it responded to absorptive and secretory stimuli. Finally, after the addition of an iron source to the mucosal side of intestinal tissue mounted in the Ussing chambers, the disappearance of iron from the mucosal solution was observed.

Using the validated Ussing chamber system, the absorption of iron, by mouse intestinal tissue, from ferrous gluconate and from the soluble fractions of meat, vegetables and meat-plus-vegetables after gastrointestinal digestion was investigated. A significant increase in short-circuit current was observed after the replacement of the mucosal Ringer's solution with the digested meat, vegetable or meat-plus-vegetable solutions. However, the iron absorption data were difficult to interpret due to the wide variation. The source of the variation is unknown, although it is thought that errors associated with the sample processing and analysis of iron by ICP-MS were probably involved.

Despite the above errors in sample processing, the data indicated a trend towards higher absorption of iron from ferrous gluconate and meat, compared to vegetables and meat-plus-vegetables. The mean percentages for the apparent absorption of iron for meat and ferrous gluconate (10.0% ± 6.3% and 6.4% ± 6.8%, respectively) were within a similar range for those reported by Vaghefi *et al.* (1998, 2000a) for rat intestinal tissue in Ussing chambers, and for overall iron absorption *in vivo* from a meal containing meat by humans (Cook and Monsen, 1975, 1976), piglets (P. Morel, unpublished data) and Caco-2 cells (Garcia *et al.*, 1996; Glahn *et al.*, 1996). Thus, if the variation associated with the assaying of the iron can be reduced, it is most likely that the Ussing chamber model would be a valid, relatively easy and inexpensive technique for simulating *in vivo* iron absorption. The model could be used to rapidly screen large numbers of food samples for the assessment of iron bioavailability.

5.3 Future directions

There are several aspects of this research that warrant further investigation. Experiments reported in Chapter Two focussed on the effects of pepsin and pancreatin-bile digestion on a meat sample from one muscle type from one animal. Future studies into the effects of digestion on cooked meat should include a range of meat samples from various muscle cuts and also animals of different ages and sex. Further, the effects of different food combinations on the solubility of meat iron should be studied.

For the studies into absorption of iron from meat, vegetables and meat-plus-vegetables by *in vitro* intestinal tissue (Chapter Four), there were a number of complications associated with the processing of the samples which, if remedied, should produce more accurate measures of iron absorption. However, if increased assay accuracy does not reveal more clear patterns of absorption of iron by intestinal segments, then further remedies may be necessary, such as using a radioactive iron tracer for iron absorption; the use of an exogenous iron source was not used in the present study as the aim was to investigate the absorption of intrinsic iron by intestinal tissue.

Radioactive iron tracers ($^{59}FeCl_3$) have been used previously (Miller *et al.*, 1981; Glahn *et al.*, 1996) to determine the amount of soluble iron as an estimation of the bioavailability of iron from foods. One advantage of using a radioactive tracer would be the elimination of any error associated with the acid digestion and iron analysis process. However, the addition of an extrinsic iron source may affect estimations on the bioavailability of the intrinsic iron. Furthermore, although it is likely that some of the intrinsic non-haem iron of a food sample is freely exchangeable with the extrinsic radiolabeled pool, it is unclear how well the intrinsic iron of a food equilibrates with an extrinsic radiolabel and there remains no precise way to determine this equilibrium (Consaul and Lee, 1984; Glahn *et al.*, 1996). Furthermore, in a study by Costa *et al.* (2000) where radioactive iron was used to measure the unidirectional flux of iron across mouse intestinal epithelium mounted in Ussing chambers, it was reported that a high proportion of the radioactive iron was strongly bound to the Perspex walls of the Ussing chambers as well as to the agar-salt bridges. Thus, the non-specific binding of iron isotopes needs to be fully investigated before they can be used for *in vitro* studies of iron absorption. In addition, experiments could be conducted to determine the relative contribution of the absorption of haem and non-haem iron to the total iron absorption from a meal.

Another question that needs considering is whether the results for the concentrations of haem and/or non-haem iron in the soluble and insoluble fractions of food following *in vitro* gastrointestinal digestion are reproducible in the *in vivo*

situation. The solubility of iron throughout the gastrointestinal tract could be studied by feeding various meals to rats and/or pigs and then the contents of the stomach and at various lengths along the intestine could be collected for analysis of the soluble and insoluble concentrations of haem and non-haem iron.

Overall, this Thesis shows that before estimations can be made on the bioavailability of food iron, the effects of the cooking and gastrointestinal digestion processes must be considered. Further, the use of *in vitro* gastrointestinal digestion followed by the use of Ussing chambers to assess intestinal absorption could be a potentially valuable system for assessing mineral bioavailability.

APPENDIX ONE

Sample Analysis of Variance (ANOVA)

The following tables present the ANOVA results for the soluble haem iron concentration (μg/g) in the meat and meat-plus-vegetables.

Source	df	SS	MS	f	p
Model	12	97.61	8.13	13.87	***
Error	14	8.21	0.59		
Corrected total	26	105.82			

***p<0.001.

Source	df	III SS	MS	f	p
Vegetable effect	1	6.98	6.98	11.91	**
Replicate effect	7	7.72	1.10	1.88	NS
Treatment effect	2	75.86	37.93	64.68	***
Treatment by vegetable interaction	2	13.47	6.74	11.49	**
Error	1	6.98	6.98	6.33	*

NS=not significant, *p<0.05, **p<0.01 ***p<0.001.

Test of the vegetable effect using the type III MS for the replicate effect as an error term.

Source	df	III SS	MS	f	p
Vegetable effect	1	6.98	6.98	6.33	*

*p<0.05.

APPENDIX TWO

Absolute values for the iron concentrations in meat, vegetables and meat-plus-vegetables

Table A2.1: The concentrations of haem iron in the insoluble and soluble fractions of meat (Mt) and meat-plus-vegetables (Mt + Vg) after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	Haem iron concentration (μg/g)					
	Cook		**Pep**		**Panc**	
	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
Mt + Vg A	2.20	5.22	0.45	2.34	6.34	1.14
Mt + Vg B	2.02	3.66	0.36	2.06	3.64	1.77
Mt + Vg C	3.05	6.16	0.09	5.38	8.23	0.09
Mt B	1.51	9.38	2.33	9.51	3.81	3.92
Mt C	0.34	15.28	0.67	14.87	3.94	4.72
Mt D	0.76	15.01	1.21	14.08	4.23	8.38
Mt F	0.13	11.06	1.12	12.09	2.84	4.00
Mt G	0.28	8.50	1.80	8.21	3.94	3.20
Mt H	0.33	13.68	0.78	14.10	3.32	5.54

Table A2.2: The concentrations of non-haem iron in the insoluble and soluble fractions of meat (Mt), vegetables (Vg) and meat-plus-vegetables (Mt + Vg) after cooking (Cook), after pepsin digestion (Pep) and after pepsin and pancreatin-bile digestion (Panc).

	Non-haem iron concentration (μg/g)					
	Cook		**Pep**		**Panc**	
	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
Mt + Vg A	1.07	5.42	0.72	6.10	6.82	2.11
Mt + Vg B	2.01	4.71	1.14	5.32	6.54	0.68
Mt + Vg C	0.95	4.80	1.37	5.13	3.92	2.96
Vg A	0.25	3.75	1.54	2.91	4.13	0.93
Vg B	0.51	4.85	1.78	3.12	4.03	1.48
Vg C	1.17	3.97	1.79	2.64	3.26	1.69
Mt B	5.48	9.05	4.56	9.50	12.83	5.64
Mt C	1.76	13.23	5.12	7.37	14.75	3.51
Mt D	3.44	11.56	6.54	6.42	15.30	5.57
Mt F	2.64	9.45	6.60	5.01	15.81	4.32
Mt G	3.51	8.64	6.53	8.64	12.17	3.62
Mt H	2.50	7.60	3.23	5.54	12.87	3.49

APPENDIX THREE

Vitamin/mineral premix for mouse iron deficient diet

The vitamin/mineral premix provided the following (g/kg premix):

Potassium iodate	0.4
Cobalt sulphate heptahydrate	0.8
Selenium premix	4.0
Amecodox D	0.08
Choline chloride	115.2
Zinc oxide	12.4
Manganese oxide	16.0
Copper sulphate pentahydrate	4.0
Calcium carbonate	421.4
Calcium pantothenate	5.6
Magnesium oxide	196.0
Potassium chloride	190.8
Vitamin A	2.8
Vitamin B_1	0.8
Vitamin B_2	1.2
Vitamin B_6	1.6
Vitamin B_{12}	0.4
Vitamin D_3	0.4
Vitamin E	20.0
Vitamin K_3	0.8
Biotin	0.8
Niacin	4.0
Folic acid	0.4

APPENDIX FOUR

Osmolarity of the digested meat, vegetable and meat-plus-vegetable solutions

During the course of preliminary experiments, the possible effects of osmolarity on the viability of the tissues were investigated. Six experiments with digested meat, vegetables and meat-plus-vegetables were conducted. The mice used were fed a normal pelleted diet and housed as described in Section 4.2.1.2. The Ussing chamber experiments were conducted as described in Section 4.2.3 and 4.2.4.1, except the digested meat, vegetable and meat-plus-vegetable solutions had a final iron concentration of 5μg/g, rather than 3.5μg/g; based on the iron concentrations after pancreatin-bile digestion (Appendix Two), 5μg/g was thought to be in the physiological range for iron in the intestine. However, the addition of the meat and meat-plus-vegetable solutions to the Ussing chambers resulted in rupturing of the intestinal segments. This occurred in over 50% of tissues after the addition of the digested meat solution and in 25% of tissues for the meat-plus-vegetable solution, but did not occur after the addition of the vegetable solution. It was postulated that rupturing may be due to osmotic pressure and thus the osmolarity of the mucosal solutions were examined. The results are shown in Table A4.1.

Table A4.1: Osmolarity of the Ringer's solution and the digested meat, vegetable and meat-plus-vegetable solutions at an iron concentration of 5μg/g. Each digest solution replaces the mucosal Ringer's solution.

Mucosal solution	Approximate osmolarity range (mOsm/L)
Meat	1000 - 1200
Vegetables	600 - 700
Meat-plus-vegetables	700 - 800
Ringer's solution	290 - 300

In vivo, the osmolarity of plasma, interstitial fluid and intracellular fluid are each approximately 300mOsm/L (Ganong, 1999). Thus, the osmolarity of the mucosal and serosal solutions used in Ussing chamber experiments should be, ideally, close to 300mOsm/L. At a lower osmolarity water will enter the cells causing them to

swell, while a higher osmolarity will cause water to pass by osmosis out of the cells, causing the cells to shrink. To reduce osmolarity, the digested meat, vegetable and meat-plus-vegetable solutions were diluted by 30% with deionised water so that that the final iron concentration was approximately 3.5μg/g. This reduced the osmolarity of the solutions to approximately 400 to 600mOsm/L, which is comparable to that in the small intestine of pigs (Harpur and Popkin, 1965).

In addition, closer observation revealed that some of the tissues were rupturing under the pressure associated with replacement of mucosal Ringer's solution with solutions of digested meat, vegetables and meat-plus-vegetables. To strengthen the tissue, the removal of the intestine from the euthanased mouse was revised. Originally, the intestine was 'stripped' from the mouse by carefully pulling the small intestine from the proximal end. This process removed the mesentery and fat associated with the intestinal tissue and part of the muscularis externa and thus, may have weakened the tissue. Instead, the intestine was carefully dissected from the mouse leaving the muscle layers intact. Additionally, to reduce tearing the tissues during the mounting process the pins of the chambers were sharpened.

After the above modifications, a further two experiments were conducted, one each with digested meat and meat-plus-vegetables. The mice used were fed a normal pelleted diet and housed as described in Section 4.2.1.2. The Ussing chamber experiments were conducted as described in Section 4.2.3 and 4.2.4.1. Using the revised concentration of iron (3.5μg/g) in the digested solutions and tissue dissection methods, no rupturing of the intestinal tissues occurred.

APPENDIX FIVE

Description of inductively coupled plasma-mass spectrometer analysis and comparison to atomic absorption spectroscopy

Table A5.1 presents the statistical data for the goodness-of-fit, sensitivity and variability of the analysis of total iron concentrations by atomic absorption spectroscopy (AA) and ICP-MS. Compared to AA, ICP-MS has a lower detection limit with the range of greatest sensitivity being between 0.001 and 0.1μg/g. Precision and 'goodness-of-fit' of the standard curve for ICP-MS improved on AA. However, analysis of a Certified Reference Material (CRM) and variation between and within assays was similar between AA and ICP-MS.

Table A5.1: Comparison of the statistical data for the goodness-of-fit, sensitivity and variability of analysis of total iron concentration by atomic absorption spectroscopy (AA) and inductively coupled plasma-mass spectroscopy (ICP-MS).

	ICP-MS	**AA**
'Goodness-of-fit' of the standard curve (r^2)	1.00	0.98 to 0.99
Lowest detectable concentration (μg/g)	0.001	0.10
Precision (CV)	≤ 5%	≤ 10%
Range of greatest sensitivity (μg/g)	0.001 - 0.1	1 - 5
Bias	None	None
CRM iron concentration Certified (μg/g ± 95% confidence interval) Observed (μg/g ± 95% confidence interval)	 0.103 ± 0.005 0.099 ± 0.008	 49.0 ± 4.2 51.7 ± 6.8
Assay variation Within assay Between assay	Typically less than 10% but usually closer to 5%	 7.5% 5.7%

r^2=coefficient of determination; CV=coefficient of variation.

As with AA, ICP-MS is sensitive to interference from high concentrations of salts and acids. The digested solutions and Ringer's solution had high salt content (data not shown) and the digestion process required high concentrations of acid. To reduce the salt concentration, an ICP-MS digestion method was developed. This method used only 250μl of mucosal solution, which was then digested in 1ml of concentrated nitric acid in 12ml glass tubes. The tubes were heated at 110 to 120°C in heating blocks until the sample volume was reduced to below 0.6ml, after

which the remaining nitric acid was diluted to a final volume of 12ml with deionised water. This process diluted the acid concentration to below 5% and diluted the original mucosal solution 50-fold so that both acid and salt concentration were within a 'workable' range for ICP-MS analysis (S. Knowles, personal communication).

Variation in iron concentration between subsamples, the efficacy of acid digestion versus just acid dilution, and whether there was a food 'matrix' effect using the ICP-MS digestion method, was examined. Eight replicate samples (800μl) of the digested vegetable solution (single experiment) and the digested meat-plus-vegetable solution (single experiment) were taken while they circulated within the Ussing chamber apparatus without tissue. Before analysis by ICP-MS, the samples were either acid digested according to the ICP-MS method or simply diluted 50-fold with weak nitric acid. The results are shown in Table A5.2.

Table A5.2: Iron concentrations (μg/g) in replicate samples of digested vegetable (Vg) and meat-plus-vegetable (Mt + Vg) solutions after dilution or digestion with nitric acid. Means, standard deviations (SD) and coefficient of variation (CV) values are given for each analysis.

	Vg iron concentration (μg/g)		**Mt + Vg iron concentration (μg/g)**	
	Dilution	Digestion	Dilution	Digestion
	2.17	2.38	2.29	2.42
	2.28	2.42	2.28	2.56
	2.31	2.17	2.28	2.38
	2.27	3.02	2.27	2.52
Mean	2.26	2.50	2.28	2.47
SD	0.061	0.365	0.008	0.084
CV	2.7%	14.6%	0.35%	3.4%

Overall, acid dilution produced the least amount of variation between repeated samples. However, digestion with nitric acid leads to a greater yield of iron from both the digested vegetable and meat-plus-vegetable solutions. This could be due to an inability of acid dilution to release all iron bound up by food components. Thus, digestion with nitric acid, using the procedure as outlined above, was used to determine the total iron concentration in the digested meat, vegetable and meat-plus-vegetable solutions. Previous studies using rat intestinal tissue mounted in Ussing chambers have demonstrated absorption between 5% and 13% using ferrous gluconate (Vaghefi *et al.*, 1998, 2000a). Thus, ideally the variation

associated with the sample processing and analysis by ICP-MS must be less than 5%. While the CV for the vegetable sample in this test was high, overall, the ICP-MS method would appear to have less variation than the AA method; with CV's less than 5% generally possible as opposed to approximately 10% for the AA method. Thus overall, the ICP-MS method is preferred for analysis of iron absorption from digested meat, vegetable and meat-plus-vegetable solutions by intestinal tissue in the Ussing chamber.

APPENDIX SIX

Apparent absorption of haem and non-haem iron by mouse duodenal and jejunal tissue

To further validate the use of mouse intestinal tissue in Ussing chambers for investigating iron absorption, the absorption of haem and non-haem iron was investigated. In two experiments the absorption of haem iron from a digested haemoglobin solution was studied, while in three experiments the absorption of non-haem iron in the form of ferrous gluconate was investigated. The mice used in these experiments were fed and housed as described in Section 4.2.1.2 and the Ussing chamber experiments were conducted as described in Sections 4.2.3 and 4.2.5, with the exception that 2ml of mucosal solution was removed from the chambers for the analysis of iron concentrations in the start mucosal solution. Mucosal samples were analysed for iron concentration using the method described in Section 2.2.1.5. Wash and tissue samples were processed as described in Section 4.2.6.2, although the iron concentration was determined using atomic absorption spectroscopy. Apparent absorption and total recovery were calculated as described in 4.2.7, with the exception that baseline intestinal tissue iron levels were not subtracted from total recovery results.

Absorption of haem iron

A digested haemoglobin solution was prepared by dissolving 2g of haemoglobin in 15ml of deionised water and 5ml of 1M hydrochloric acid. This solution was then digested with 0.2ml of pepsin solution (described in Section 2.2.1.3) at 37°C for 24 hours and then adjusted to pH 7.0 with 5ml 0.1M sodium hydroxide. Using the method described in Section 2.2.1.5, the total iron concentration of the solution was 160μg/g. A volume of 250μl of digested haemoglobin solution was added to 9.75ml Ringer's solution to provide a final mucosal haem iron concentration of 4μg/g. The apparent absorption and total recovery of the haem iron are shown in Table A6.1.

Table A6.1: Means (± SEM) for the apparent absorption and total recovery of haem iron from a digested haemoglobin solution.

Tissue Piece	Apparent absorption (%)	Total recovery (%)
1 (duodenum)	9.6 ± 1.2	109.8 ± 5.4
2 (duodenum)	5.1 ± 2.9	105.8 ± 3.5
3 (jejunum)	-3.6 ± 1.5	125.0 ± 2.0
4 (jejunum)	1.8 ± 0.7	111.1 ± 2.3
Overall mean	3.2 ± 1.1	112.9 ± 6.1

Statistical analysis, using paired-samples t tests, revealed that there were no significant ($p>0.05$) differences between the haem iron concentration in the start and end mucosal solutions. However, when duodenal and jejunal tissue pieces were treated separately, the haem iron concentration in the mucosal start and end solutions were significantly ($p<0.05$) different for the duodenum. Thus, suggesting that haem iron absorption occurs in the duodenum of mouse intestine.

Absorption of non-haem iron

A stock solution of 100μg/g of iron as ferrous gluconate was prepared by adding 170mg of ferrous gluconate to 100ml of deionised water. In one experiment, 1ml of this stock solution was added to 9ml of mucosal Ringer's solution to provide a final mucosal iron concentration of 10μg/g. In the other two experiments, 0.5ml of the ferrous gluconate stock solution was added to 9.5ml of Ringer's solution to provide a final mucosal iron concentration of 5μg/g. The apparent absorption and total recovery are shown in Table A6.2.

Table A6.2: Means (± SEM) for the apparent absorption and total recovery of non-haem iron from the mucosal Ringer's solution containing ferrous gluconate.

Tissue Piece	Apparent absorption (%)	Total recovery (%)
1 (duodenum)	-3.1 ± 7.4	112.3 ± 6.6
2 (duodenum)	6.2 ± 9.0	102.7 ± 9.6
3 (jejunum)	11.4 ± 4.7	94.1 ± 3.8
4 (jejunum)	9.9 ± 6.1	95.7 ± 6.0
Overall mean	8.9 ± 9.7	101.2 ± 8.9

Overall, there were no significant ($p>0.05$) differences in the apparent absorption or total recovery with the 5 and 10μg/g ferrous gluconate solutions. Paired-samples t tests revealed that, when all data was pooled, there were significant

($p<0.05$) differences between start and end mucosal solutions. However, when duodenal and jejunal tissue pieces were treated separately, mucosal start and end solutions were only significantly ($p<0.05$) different for the jejunal tissue pieces. Thus it would appear that non-haem iron absorption occurs in the jejunum in the mouse intestine.

In the experiments in which 5μg/g iron as ferrous gluconate and 4μg/g iron as digested haemoglobin, the atomic absorption spectroscopy readings are at the lower end of the sensitivity range of the assay. Further, there are high degrees of error in the ferrous gluconate samples. Overall, these experiments need repeating to provide definitive results, however, the data, as presented, indicate that both haem and non-haem iron absorption occurs in the mouse small intestine.

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