THE USE OF THE USSING CHAMBER SYSTEM TO INVESTIGATE IRON ABSORPTION BY THE DUODENUM, JEJUNUM AND ILEUM IN THE MOUSE.

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ABSTRACT

Iron deficiency anaemia is found in approximately 30% of the worlds population and is particularly prevalent in developing countries. The majority of these deficiencies are due to insufficient absorption of iron from the diet. Iron is absorbed primarily by the proximal small intestine, however, there is evidence for a gradient of absorption along the full length of the small intestine. In 1951 Ussing and Zerahn developed a bicameral method for studying iron transport by *in vitro* epithelia. This method has been used previously to investigate iron transport mechanisms in the proximal small intestine.

In the present study Ussing chambers were used to investigate iron absorption by the full length of the mouse small intestine. Consistently high levels of iron were removed from the mucosal compartment by all regions of the small intestine. This iron removal was due to the physiological actions of the tissue and was not caused by iron adhering to the interior of the Ussing chamber apparatus. There was no change in iron uptake when large intestine or caecum was used in place of small intestine.

Ferrous gluconate was chosen as the reference test chemical as it is a readily bioavailable form of iron which has been used previously to investigate iron absorption with the Ussing chamber model. There was a consistently high level of iron uptake when 27.9 mg/L or 9.3 mg/L was added to the mucosal compartment, with no significant differences between results for either concentration.

When 9.15 mg/L manganese sulphate was combined with 9.3 mg/L ferrous gluconate in the mucosal compartment, iron removal was significantly lower in the proximal than the mid small intestine. This was presumably due to competition between the iron and the manganese for transport by the DCT1 protein.

When 200 mg/L calcium chloride and 9.3 mg/L ferrous gluconate were added to the mucosal compartment, there was no significant difference to results compared to ferrous gluconate alone.

The addition of glucose to the intestinal lumen has been shown previously to increase the passive transport of solutes across the intestinal mucosa. However, in the present

experiments when glucose was added to the mucosal Ringer's solution in place of mannitol there was a significant decrease in iron removed from the mucosal compartment by all intestinal regions.

There was evidence that the gluconate portion of ferrous gluconate increased iron absorption in the distal small intestine. This was supported by a significant decrease in iron uptake by the distal small intestine when ferrous sulphate was used in place of ferrous gluconate.

Ferric chloride was unsuitable for use in this system as it precipitated out of the Ringer's solution.

Histological examination of jejunal samples after a typical Ussing chamber experiment found there was no damage to the tissue and the epithelial layer remained intact.

There were significant levels of iron found in both the intestinal tissue and secreted mucus for all intestinal segments. The binding of iron to secreted mucus appears to involve a significant proportion of iron and should be measured in all future Ussing chamber studies.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGMENTS iv

TABLE OF CONTENTS v

LIST OF ILLUSTRATIONS viii

LIST OF TABLES x

ABBREVIATIONS xii

1. INTRODUCTION 1

2. LITERATURE REVIEW 3

2.1 INTRODUCTION 3

2.2 PHYSIOLOGICAL IMPORTANCE OF IRON 3

2.2.1 Requirements 3

2.3 BIOAVAILABILITY OF IRON 6

2.3.1 Factors Affecting Iron Bioavailability 6

2.4 ABSORPTION OF IRON 7

2.4.1 Regulation of Iron Absorption 7

2.4.2 Iron Transport Across Intestinal Mucosa 8

2.4.2.1 Paracellular Absorption of Iron 8

2.4.2.2 Transcellular Absorption of Iron 9

2.4.3 Haem Iron Movement Into the Enterocyte 10

2.4.4 Non-Haem Iron Movement into the Enterocyte 11

2.4.4.1 Solubility in the Intestinal Lumen 11

2.4.4.2 Transport of Inorganic Iron Across the Apical Membrane 12

2.4.5 Ferritin 14

2.4.6 Cytosolic Processing and Exit 14

2.4.7 Mucus 15

2.5 METHODS FOR MEASURING IRON ABSORPTION 15

2.5.1 Solubility Studies 16

2.5.2 Whole Animal Based Methods 17

2.5.2.1 Iron Balance Studies 18

2.5.2.2 Dual Isotope Studies 18

2.5.2.3 Tracking Haematological Parameters 19

2.5.3 *In Situ* Methods 19

2.5.3.1 Perfused Intestinal Segments 20

2.5.4 *In Vitro* Tissues 21
2.5.4.1 Everted Sacs 21
2.5.4.2 Membrane Vesicles 22
2.5.4.3 Isolated Enterocytes in Solution 23
2.5.4.4 Cultured Cell Lines 24
2.5.5 Ussing Chambers 25
2.5.5.1 Apparatus 26
2.5.5.2 Electrophysiology 28
2.5.5.3 Tissue viability 28
2.6 SUMMARY AND CONCLUSIONS 29

3 METHODS 30
3.1 ANIMALS 30
3.2 RINGER'S SOLUTION FOR THE EXPERIMENTS 30
3.3 EXPERIMENTAL PROCEDURE 31
3.3.1 Fluid Resistance 31
3.3.2 Tissue Mounting 34
3.3.3 Experimental Procedure 35
3.3.3.1 Test Chemical Combinations 36
3.3.4 Total Recovery Experiments 38
3.3.5 Control Experiments 38
3.3.6 Sample Analysis 39
3.3.7 Histological Examination of Intestinal Tissue 39
3.3.8 Preparation and Care of Equipment 41
3.4 DATA ANALYSIS AND PRESENTATION 42

4. RESULTS 43
4.1 ELECTROPHYSIOLOGY 43
4.2 FERROUS GLUCONATE 44
4.3 FERROUS GLUCONATE PLUS MANGANESE SULPHATE 48
4.4 FERROUS GLUCONATE PLUS CALCIUM CHLORIDE 49
4.5 FERROUS GLUCONATE PLUS GLUCOSE 50
4.7 FERRIC CHLORIDE 52
4.3 REGIONAL COMPARISONS 53
4.4 NON-ABSORBING TISSUE 55
4.5 TISSUE STAINING 55
4.6 TOTAL IRON RECOVERY EXPERIMENTS 56
4.7 BASELINE INTESTINAL IRON CONTENTS 56

5. DISCUSSION **61**
5.1 EXPERIMENTAL PROCEDURE 61
5.2 FERROUS GLUCONATE 63
5.3 ADDITION OF MANGANESE SULPHATE 66
5.4 ADDITION OF CALCIUM CHLORIDE 68
5.5 INCREASING PASSIVE ABSORPTION 70
5.6 OTHER FORMS OF IRON 72
5.6.1 Ferrous Sulphate 72
5.6.2 Ferric Chloride 73
5.7 HISTOLOGICAL EXAMINATION OF INTESTINAL TISSUE 74
5.8 NON-ABSORBING TISSUE 75
5.9 COLLECTING MUCUS AND MEASURING MUCOSAL IRON LEVELS 76

6. CONCLUSIONS **79**

7. APPENDICIES **81**
APPENDIX A: TEST CHEMICAL STOCK SOLUTIONS 81
APPENDIX B: EXPERIMENTAL RESULTS AND RAW DATA 82
APPENDIX C: ELECTROPHYSIOLOGICAL DATA 110
APPENDIX D: ICP INTESTINE ANALYSIS 113

8. BIBLIOGRAPHY **114**

LIST OF ILLUSTRATIONS

Figure 2.1: Paracellular and transcellular transport routes across the intestinal epithelia. 8

Figure 2.2: The transcellular movement of iron across the intestinal mucosa. 10

Figure 2.3: Artificial digestion for *in vitro* iron analysis. 16

Figure 2.4: Preparation of apical membrane vesicles. 22

Figure 2.5: The Ussing chamber apparatus as assembled during an experiment. 27

Figure 3.1: Mounting face of half cell. 31

Figure 3.2: The Ussing chamber apparatus ready for fluid resistance recording. 32

Figure 3.3: Consecutive intestinal segments as used in experiments, grouped into regions. 42

Figure 4.1: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 27.9 mg/L ferrous gluconate was added to the mucosal compartment. 44

Figure 4.2: Chart recording of I_{sc} and Rt for a typical experiment with 10 mM mannitol added to the mucosal Ringer's solution. 45

Figure 4.3: Chart recording of I_{sc} and Rt for a typical experiment with 10 mM glucose added to the mucosal Ringer's solution. 46

Figure 4.4: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferrous gluconate was added to the mucosal compartment. 47

Figure 4.5: Percentage iron removed from the mucosal Ringer's solution when 9.3 mg/L ferrous gluconate and 9.15 mg/L manganese sulphate were added to the mucosal compartment. 49

Figure 4.6: Percentage iron removed from the mucosal Ringer's solution when 9.3 mg/L ferrous gluconate and 200 mg/L calcium chloride were added to the mucosal compartment. 50

Figure 4.7: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferrous gluconate and 10 mM glucose were added to the mucosal compartment. 51

Figure 4.8: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferrous sulphate was added to the mucosal compartment..... 51

Figure 4.9: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferric chloride was added to the mucosal compartment..... 52

Figure 4.10: Average percent iron removed from the mucosal compartment, grouped by test chemical and intestinal region..... 54

Figure 4.11: Photomicrograph of a cross section of mouse jejunum after being used in an Ussing chamber experiment, stained with H&E..... 58

Figure 4.12: Photomicrograph of a cross section of mouse jejunum which has not been used in an Ussing chamber experiment, stained with H&E. 58

Figure 4.13: Photomicrograph of a cross section of mouse jejunum after use in an Ussing chamber experiment with no iron added, stained with Perl's Prussian blue reaction and neutral red..... 59

Figure 4.14: Photomicrograph of a cross section of mouse jejunum after use in an Ussing chamber experiment with iron added, stained with Perl's Prussian blue reaction and neutral red..... 59

Figure 4.15: Photomicrograph of spleen, stained with Perl's Prussian blue reaction..... 60

Figure 4.16: The iron content of intestinal segments taken from small intestinal tissue which was not used in an Ussing chamber experiment. 60

LIST OF TABLES

Table 2.1: Recommended total daily intake of iron per population group. 3

Table 2.2: Dietary factors affecting iron absorption. 7

Table 2.3: Methods for studying iron absorption. 16

Table 3.1: Composition of the Ringer's solution. 30

Table 3.2: Experimental procedures which were used to investigate iron absorption by the mouse intestine. 37

Table 3.3: Method for staining sections with haematoxylin and eosin. 40

Table 3.4: Method for staining sections with Perl's Prussian blue reaction. 41

Table 4.1: Summary of electrophysiological readings for all experiments. 43

Table 4.2: Change in iron concentration after 90 minutes during the control experiments in which 27.9 mg/L ferrous gluconate was added to the Ringer's solution. 44

Table 4.3: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous gluconate was added to the Ringer's solution. 47

Table 4.4: Change in iron concentration per intestinal segment after 90 minutes when 9.3 mg/L ferrous gluconate and 91.5 mg/L manganese sulphate were added to the Ringer's solution. 48

Table 4.5: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous gluconate and 9.15 mg/L manganese sulphate were added to the Ringer's solution. 49

Table 4.6: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous gluconate and 200 mg/L calcium chloride were added to the Ringer's solution. 50

Table 4.7: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous sulphate was added to the Ringer's solution. 52

Table 4.8: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferric chloride was added to the Ringer's solution. 53

Table 5.1: Comparison of I_{sc} for three Ussing chamber studies using mouse small intestine with the muscularis externa attached. 62

ABBREVIATIONS

ANOVA	Analysis of Variance
$CaCl_2$	calcium chloride
Caco2	human colon carcinoma epithelial cells
Cd	cadmium
Co	cobalt
Cu	copper
°C	degrees Celsius
DCT1	divalent cation transporter one
DMT1	divalent metal transporter one
FAAS	flame atomic absorption spectrophotometer
Fe	iron
$FeCl_3$	ferric chloride
$FeSO_4$	ferrous sulphate
g	gram
HCl	hydrochloric acid
HNO_3	nitric acid
ICP	inductively coupled plasma emission spectrophotometry
IRE	iron response element
IRP	iron regulatory protein
I_{sc}	short circuit current
HFE	major histocompatibility complex class I-like molecule
K	potassium
KCl	potassium chloride
kg	kilogram
L	Litre
M	molar
mg	milligram
mg/L	micrograms per litre
$MgCl_2.6H_2O$	manganese chloride
mL	millilitre
mM	millimolar
Mn	manganese

$MnSO_4$	manganese sulphate
n	number
Na	sodium
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NaH_2PO_4	sodium phosphate monobasic
$NaHCO_3$	sodium bicarbonate
Ni	nickel
nramp2	natural-resistance-associated macrophage protein 2
%	percent
P	probability
p.d.	potential difference
Pb	lead
Rf	fluid resistance
Rt	tissue resistance
se	standard error
SGLT1	Na^+/glucose co-transport protein
Zn	zinc
$\bar{x}$	mean
μAmps	microamperes
μM	micromolar
μg	microgram
Ω	ohm

ERRATA

p ii line 6	"… iron …" should read: "… ion …"
p xii 5th to last line	"… micrograms …" should read: "… milligrams …"
p 3 line 9	"… Ussing Chamber …" should read: "… Ussing chamber …"
p 6 line 7	"… call I-like …" should read: "… class I-like …"
p 13 last line	"… a effective …" should read: "… an effective …"
p 30 Table 3.1	"… Weight (mM) …" should read: "… Concentration (mM) …"
p 30 line 12	"… began …" should read: "… begun …"
p 30 paragraph 3 line 1	"… was …" should read: "… were …"
p 31 paragraph 5 line 4	"… Figure 4.2 …" should read: "… Figure 3.2 …"
p 35 paragraph 3 line 3	"… (Table 4.2) …" should read: "… (Table 3.2) …"
p 36 line 2	"… Table 3.3 …" should read: "… Table 3.2 …"
p 42 paragraph 2 line 4	"… preformed …" should read: "… performed …"
p 43 last line	"… Figure 4.1 …" should read: "… Figure 4.2 …"
p 44 line 1	"… Figure 4.2 …" should read: "… Figure 4.3 …"
p 44 paragraph 2 line 3	"… was no significant variation …" should read: "… were no significant differences …"
p 44 paragraph 2 line 3	"… Figure 4.3 …" should read: "… Figure 4.1 …"
p 46	"… Figure 4.2 …" should read: "… Figure 4.3 …"
p 47 line 6	"… was no significant variation …" should read: "… were no significant differences …"
p 48 last line	"… was no significant variation …" should read: "… were no significant differences …"

p 53 paragraph 3 line 1 "… was no significant variation …" should read: "… were no significant differences …"

p 56 line 1 "… was also significant variation …" should read: "… were also significant differences …"

p 57 line 2 "… was no significant variation …" should read: "… were no significant differences …"

p 58 Figure 4.11 "… x200 .." should read: "… x100 …"

p 61 paragraph 3 line 3 "… proir …" should read: "… prior …"

p 63 3rd to last line "… added to …" should read: "… present in …"

p 63 penultimate line "… thesnon …" should read: "… the …"

p 64 penultimate line "… affects …" should read: "… effects …"

p 64 last line "… in Ussing …" should read: "in an Ussing …"

p 65 line 9 "… solutuion …" should read: "solution …"

p 69 paragraph 2 line 1 "… was no significant variation …" should read: "… were no significant differences …"

p 73 paragraph 4 line 1 "… ere …" should read: "… were …"

p 76 paragraph 2 line 6 "… rom …" should read: "… from …"

p 76 paragraph 5 line 2 "… was no significant variation …" should read: "… were no significant differences …"

p 79 number 5 "… than absorbed …" should read: "… than being absorbed …"

p 79 point 7 "… added the …" should read: "… added to the …"

p 119 reference 6 "… Coning …" should read "… Cloning …"

p 121 line 1 "… 210(), 694-. …"should read: "… 210, 694-700. …"

p 124 reference "… Scricker …" should read: "… Schricker …"

CORRIGENDA

p ii paragraph 2, the final sentence should read:-

"There was no change in iron uptake when the small intestine was replaced with large intestine or caecum."

p 7-8, Section 2.4.1 should read:-

"As iron is not excreted as a waste product, physiological losses are small and iron homeostasis is maintained by regulation of absorption from the diet (McCance and Widdowson, 1938, cited in Hallberg, 2001). The purpose of the regulatory process is to limit iron absorption to the amount needed to cover losses. Regulation of iron uptake is a complex process whereby the iron status of the animal and iron content of the enterocytes affect iron uptake (Conrad, Weintraub and Crosby, 1964; Bothwell et al., 1958). This process is not yet fully understood.

The synthesis of a number of the proteins responsible for iron absorption is controlled by iron regulatory proteins (IRPs). These bind to specific sections of mRNA called iron response elements (IREs) when the body iron content is low (Eisenstein, 2000; Leibold and Guo, 1992) causing the translation of mRNA to be either increased or decreased depending on which protein is being synthesised (Eisenstein, 2000; Leibold and Guo, 1992). IREs are present in the mRNA of many proteins involved in the luminal uptake (e.g. Divalent Cation Transporter 1 (DCT1), see Section 2.4.4.2), intracellular storage (e.g. ferritin, see Section 2.4.5) and serosal release (e.g. transferrin, see Section 2.4.6) of iron (Eisenstein, 2000) indicating that these also play a role in the regulation of iron absorption."

p 18 end of first paragraph, add the following sentence:-

"Small amounts of endogenous iron are also excreted into the urine with values estimated as being up to 0.3 mg/day (Beard et al., 1996)."

p 20 paragraph four, the first sentence should read:-

"Everted intestinal sacs are an *in vitro* method frequently used in the study of iron absorption."

p37 Table 3.2:-

"Final Concentration (mg/L)" should read: "*Initial Test Chemical Concentration in the Mucosal Ringers Solution (mg/L)*"

p 39 third paragraph, the last sentence should read:-

"Before analysis all samples were collected and digested were necessary as described in Sections 3.3.3 and 3.3.4, then diluted as necessary to ensure the iron concentration was within this range."

p 39 penultimate line:-

"... removed from the chambers and immediately fixed ..." should read: "... removed from the chambers, rinsed in 1% HNO_3, and immediately fixed ..."

p 43 Table 3.1, the final sentence of the title should read:-

"All values are mean ± se (number of intestinal segments) and are expressed per square centimetre of tissue."

p47 paragraph one, the last sentence should read:-

"Individual Student's T tests showed that this was caused by the average percentage of iron removed by the first intestinal segment being significantly ($P<0.05$) lower than the average percentage removed by all other segments; there was no significant differences between segments 2 to 8."

p50 paragraph 1, the last sentence should read:

"These control experiments showed a significant ($P=0.01$) change in iron concentration after the 90 minute experimental period which could not be accounted for by measurement error (Table 4.6) ."

p 54 first paragraph, the final sentence should read:-

"Data for each test chemical were then grouped into three intestinal regions; proximal, mid and distal; representing the duodenal, jejunal and ileal sections of the small intestine"

p 55 paragraph 3, the last sentence should read:-

"This was within the range of iron removed from the mucosal solution by small intestinal tissues, and was not significantly different to iron uptake averaged over all regions of the small intestine."

p56 paragraph four, the third sentence should read:-

"Although iron concentration tended to be higher in the proximal region of the small intestine, a two way ANOVA showed no significant difference between the individual intestinal segments ($P=0.4$)."

p 64 paragraph 7 should read:-

"3) There was no significant difference between the percentage iron removed when starting concentrations of either 27.9 mg/L or 9.3 mg/L ferrous gluconate were present in the mucosal Ringer's solution."

p 70 third paragraph, the second sentence should read:-

"This could explain why there was a decrease in iron concentration after the control experiments containing both iron and calcium but no the tissue-mounted experiments showed no significant difference between the percentage of iron absorbed with or without calcium added to the mucosal Ringer's solution."

p 79 number 7:-

"... was removed ..." should read "... may have been removed ..."

p80 number 11, the second sentence should read:-

"However, a qualitative difference in iron staining between tissue which had or had not been exposed to iron in the mucosal Ringer's solution could not be demonstrated with Perl's Prussian blue reaction."

p 82, add the following paragraphs:-

"Where the mucosal Ringer's samples were diluted to obtain iron concentrations within the detection limits of the FAAS, the percentage iron removed from the mucosal Ringer's solution was calculated as follows:

$$\% \text{ removed} = \left[\frac{\text{start} - (\text{end}*\text{dil})}{\text{start}}\right] * 100$$

start = concentration of iron in the mucosal solution at the start of the experimental period

end = concentration of iron measured in the mucosal solution at the end of the experimental period

dil = dilution factor

The percentage iron removed from the mucosal solution for all other samples was calculated as follows:

$$\% \text{ removed} = \left[\frac{\text{start} - \text{end}}{\text{start}} \right] * 100$$

start = concentration of iron in the mucosal solution at the start of the experimental period

end = concentration of iron in the mucosal solution at the end of the experimental period"

p 124 reference 7 should read:-

"Schachter, D., Rosen, S. M. (1959). Active Transport of $_{45}$Ca by the Small Intestine and its Dependence on Vitamin D. American Journal of Physiology, 196, 357- 362."

1. INTRODUCTION

Iron deficiency is found in approximately 30% of the worlds population and is particularly prevalent in developing countries (DeMaeyer and Adiel-Tegman, 1985). The majority of these deficiencies occur when iron absorption from the diet is insufficient to compensate for any physiological iron losses and fulfil the body's metabolic requirements for iron (Hallberg, 2001; Baynes and Bothwell, 1990).

As iron is not actively excreted from the body, iron homeostasis is maintained by the regulation of iron absorption from the diet (McCance and Widdowson, 1937, cited in Hallberg, 2001). However iron absorption is a complex process influenced by a number of factors. For example, the iron status of the individual may affect iron uptake, with anaemia increasing total iron absorption (Bothwell, Pirzio-Biroli and Finch, 1958). An important factor influencing iron uptake is the bioavailability of iron in the diet. One measurement of iron bioavailability is how readily the iron is absorbed by the small intestine. This is influenced by both dietary constituents, which may interact with the iron in the intestinal lumen, and the chemical forms of iron present (Wienk, Marx and Beynen, 1999; Lynch, 1997). In order to prevent the development of iron deficiency it is important to ensure adequate levels of bioavailable iron are present in the diet (DeMaeyer and Adiel-Tegman, 1985). However iron bioavailability and uptake are difficult to predict. Further understanding of the absorption process will aid this prediction.

The majority of iron absorption occurs in the duodenum and many iron absorption studies focus on this region (Rucker, Lonnerdal and Keen, 1994). However there is evidence of iron absorption in the full length of the intestine, with a gradient of absorption from high uptake in the duodenum to low uptake in the terminal ileum (Chowrimootoo, Debnam, Srai and Epstein, 1992). Therefore investigation of absorption in all regions is worthwhile.

In 1951, Ussing and Zerahn developed a method for studying nutrient transport using *in vitro* tissues mounted in a bicameral chamber apparatus. This system has been modified over time and has been used to successfully investigate iron transport in the proximal small intestine (Costa, da Costa and de Sousa, 2000; Vaghefi, Nedjaoum, Guillochon, Bureau, Arhan and Bougle, 2000; Vaghefi, Guillochon, Bureau, Neuvill, Jacob, Arhan

and Bougle, 1998; Helbock and Saltman, 1967). It allows the investigation of specific iron transport processes in different intestinal regions while retaining the physiological processes present in the tissue. Therefore the Ussing chamber apparatus has been used in the following experiments to investigate iron absorption along the full length of the mouse small intestine.

2. LITERATURE REVIEW

2.1 Introduction

Iron is absorbed from the diet by the small intestine. It is a complex process involving several transport mechanisms located on the apical and basolateral membranes of the enterocytes, and the cell cytoplasm. There are a number of methods available for the investigation of these transport mechanisms at the molecular, cellular, organ and whole animal level. This literature review discusses the transport mechanisms by which iron is absorbed and some of the methods by which they may be investigated. Particular emphasis is given to the Ussing Chamber system used in the experiments presented in this thesis.

2.2 Physiological Importance Of Iron

Iron is an essential trace element. It is required for oxygen storage and transport, electron transfer and as a cofactor to many enzymes (Rucker et al., 1994). It's main role is in the transport of oxygen, with two-thirds of the body's total iron contained in haemoglobin (Hoffbrand and Pettit, 1993; Cook, Baynes and Skikne, 1992).

2.2.1 Requirements

The recommended daily intake of iron for different population groups is outlined in Table 2.1.

Table 2.1: Recommended total daily intake of iron per population group.

Population group	*Recommended total daily iron intake (mg)*
Children	10
Adolescent females	15
Adolescent males	12
Adult females (pre-menopause)	15
Pregnant females	30
Adult males and post-menopausal females	10

Data from Baynes and Bothwell, 1990.

Iron requirements increase during periods of growth (Hallberg 2001). Hence, adolescents have a high iron requirement as they are growing rapidly (Hallberg 2001). Females of child-bearing age have higher iron requirements than do males due to iron loss during menstruation, until menopause when their iron requirement drops to match that of adult males (Hallberg 2001). Pregnant females have the highest iron requirements due to the rapid growth of the foetus, particularly in the second and third trimesters when their iron need can not be met from diet alone (Hallberg 2001).

2.2.2 Iron Deficiency

A review by DeMaeyer and Adiels-Tegman (1985) has estimated that at least 30% of the world's population are anaemic, however, they do not provide information on how they defined anaemia. While the highest incidence of anaemia occurs in developing countries, there are significant levels present in developed countries (Baynes and Bothwell, 1990; DeMaeyer and Adiels-Tegman, 1985; Expert Scientific Working Group, 1985). Iron deficiency leading to anaemia generally occurs because the uptake of iron from the diet is insufficient to fulfil the body's iron requirements (DeMaeyer and Adiels-Tegman, 1985). There are several reasons why this may occur, including loss of blood (e.g., menstruation, gastrointestinal bleeding), increased need for iron (e.g., during pregnancy and growth), iron malabsorption or disease (e.g., thalassemia, sickle-cell anaemia) (Hoffbrand and Pettit, 1993; DeMaeyer and Adiels-Tegman, 1985), and low dietary intake of iron (e.g., cereal based diet) (DeMaeyer and Adiels-Tegman, 1985).

The onset of iron deficiency and anaemia is a gradual process that can be divided into three stages: iron depletion, iron deficient erythropoiesis and iron deficiency anaemia (Baynes and Bothwell, 1990; Expert Scientific Working Group, 1985). In the first stage, ferritin iron concentrations fall as iron is moved from storage into the general circulation to maintain circulating levels (Fairweather-Tait, 1993). Once the stores are depleted, plasma iron concentration drops and there is less available for normal metabolism (Baynes and Bothwell, 1990; Expert Scientific Working Group, 1985). The erythropoietic cells in the marrow are the site of haemoglobin synthesis and red cell formation. When plasma iron levels are reduced the supply of iron to the marrow decreases, haemoglobin formation is impaired and iron deficient erythropoiesis occurs (Baynes and Bothwell, 1990; Expert Scientific Working Group, 1985). This is the

second stage of deficiency and is characterised by a drop in transferrin saturation and reduced haemoglobin content of new red blood cells (Baynes and Bothwell, 1990). As iron levels drop still further, anaemia, the third stage of deficiency, begins. Circulating red blood cells become microcytic and hypochromic and the oxygen carrying capacity of the blood is decreased (Fairweather-Tait, 1993; Baynes and Bothwell, 1990). By this stage iron levels throughout the body are reduced, affecting all iron-containing enzymes (Hoffbrand and Pettit, 1993). Symptoms of anaemia include headache, fatigue, angular stomatitis, glossitis and koilonychia (ridged or spooned nails) (Beard, Dawson and Pinero, 1996). Iron deficiency, both with and without anaemia, has been associated with attention span deficits and learning difficulties in children and with reduced work capacity and lowered endurance in adults (Hallberg, 2001; Beard et al., 1996; Cook and Lynch, 1986).

Iron deficiency is corrected by increasing the amount of iron available for absorption (DeMaeyer and Adiels-Tegman, 1985). This can be achieved by either long term dietary fortification or short term daily supplementation (Baynes and Bothwell, 1990; DeMaeyer and Adiels-Tegman, 1985). Dietary fortification is achieved by the addition of extra iron to individual food items (e.g., flour), thus increasing overall iron levels in the diet (Baynes and Bothwell, 1990). Fortification is useful for improving and maintaining the iron status of a population on a long term basis (Baynes and Bothwell, 1990). By fortifying a staple food which is widely consumed, the majority of the people will benefit without extra effort on their part (Baynes and Bothwell, 1990).

Daily supplementation involves the oral administration of iron in the form of a pill or liquid (DeMaeyer and Adiels-Tegman, 1985). It is usually given to an already iron deficient individual in order to correct the deficiency (Baynes and Bothwell, 1990). For daily supplementation to be effective it is important the iron being administered can be readily absorbed (DeMaeyer and Adiels-Tegman, 1985). For this reason ferrous sulphate or ferrous gluconate are often used because both are highly soluble and can be readily absorbed from the small intestine (Hoffbrand and Pettit, 1993).

2.2.3 Excess Iron

Excess iron in the body is toxic due to iron's ability to oxidise various cellular components and promote the formation of free radicals (Eisenstein 2000, Nichols,

Pearce, Alverez, Bibb, Nichols, Alfred and Glass, 1992; Octave, Schneider, Trouet and Crichton, 1983). When too much iron builds up in the body, it causes tissue damage resulting in cirrhosis and fibrosis of the liver, diabetes, arthritis, cardiac failure and arrhythmia (Cook et al., 1992). The main cause of iron overload is hereditary haemochromatosis. This is a genetic disorder which causes an inability to regulate iron uptake from the small intestine due to the absence of HFE (a major histocompatibility complex call I-like molecule) (Cook et al., 1992). The faulty gene is homozygous in 1 out of 300 to 400 individuals of European descent (Cook et al., 1992). This disorder is treated by regular bleeding to lower iron levels in the body (Cook et al., 1992). Excessive ingestion of iron does not induce haemochromatosis or cause iron overload in a normal person. However, increased iron levels in the diet will cause excessive iron levels in individuals with haemochromatosis (Cook et al., 1992).

2.3 Bioavailability of Iron

The term 'bioavailability' is often used in nutritional studies, however it's exact definition is not always clear and may vary from study to study (Wienk et al., 1999). Fairweather-Tait (1987) has defined bioavailable iron as all iron that is digested, absorbed and metabolised. However, during *in vitro* studies it is not possible to quantify metabolisable iron. A more practical definition of iron bioavailability for *in vitro* studies is to define bioavailable iron as all iron that is taken up by the enterocyte. This is the definition used in this thesis. Any iron removed from the mucosal solution and moved either into or through the intestinal tissue is considered to have been absorbed.

2.3.1 Factors Affecting Iron Bioavailability

The bioavailability of the iron in any given meal is dependent on the composition of the meal and the forms of iron present (haem vs. non-haem iron and ferrous vs. ferric iron) (Lynch, 1997; Beard et al., 1996). Haem iron is bound to a porphyrin haem ring (Weintraub, Weinstein, Huser and Rafal, 1968). In humans a greater proportion of available haem iron is absorbed compared to that of non-haem, with the absorption rate of haem from a meal being about three times that of non-haem (Costa et al., 2000). Haem iron absorption is generally not affected by other constituents of the diet (Weintraub, Conrad and Crosby, 1965; Turnball, Cleton and Finch, 1962). Non-haem iron is inorganic iron in either the ferrous (Fe^{2+}) or ferric (Fe^{3+}) state. Ferrous iron is readily absorbed whereas ferric is not (Forth and Rummel, 1973).

Other dietary constituents have a considerable effect on the solubility and bioavailability of iron in a meal, particularly on the non-haem iron (Lynch, 1997; Beard et al., 1996). The major dietary factors influencing uptake are listed in Table 2.2. The effects of each of these factors are described in Section 2.4.

Table 2.2: Dietary factors affecting iron absorption.

Promotes iron absorption	*Inhibits iron absorption*
Ascorbic acid	Phytate
Citrate and other organic acids	Polyphenols e.g., tannins found in tea
Meat	Divalent cations (e.g., manganese, calcium)

2.4 Absorption of Iron

Iron absorption occurs primarily in the small intestine (Rucker et al., 1994). There is a gradient of absorption along the small intestine, with the highest rate of absorption occurring in the duodenum and the lowest in the ileum (Chowrimootoo et al., 1992; Conrad, Permley and Osterlot, 1987; Muir and Hopfer, 1985; Cox and O'Donnell, 1981). Because of this, the majority of experimental investigations into iron absorption have focused solely on the duodenum.

Iron is not absorbed from the mouth, oesophagus or stomach (Rucker et al., 1994). There is a small amount of passive iron uptake in the caecum and colon, particularly in iron deficient animals (Campos, Gomez-Ayala, Lopez-Aliaga, Pallares, Hartiti, Pharm, Alferez, Barrionuevo, Rodriguez-Matas and Lisbona, 1996), however this is considered too small to be nutritionally significant.

2.4.1 Regulation of Iron Absorption

As iron is not excreted as a waste product, physiological losses are small and iron homeostasis is maintained by regulation of absorption from the diet (McCance and Widdowson, 1938, cited in Hallberg, 2001). The purpose of the regulatory processes is to limit iron absorption to the amount needed to cover losses. Regulation of iron uptake is a complex process whereby the iron status of the animal and iron content of the enterocytes affect iron uptake (Conrad, Weintraub and Crosby, 1964; Bothwell et al.,

1958). The synthesis of these proteins is controlled by iron regulatory proteins (IRPs) which bind to specific sections of mRNA called iron response elements (IREs) when the body iron content is low (Eisenstein, 2000; Leibold and Guo, 1992). IREs are present in the mRNA of many proteins implicated with iron absorption including those involved with the luminal uptake (e.g. Divalent Cation Transporter 1 (DCT1), see Section 2.4.4.2), intracellular storage (e.g. ferritin, see Section 2.4.5) and serosal release (e.g. transferrin, see Section 2.4.6) of iron (Eisenstein, 2000). When an IRP binds to an IRE the translation of the mRNA is either increased or decreased depending on which protein is being synthesised (Eisenstein, 2000; Leibold and Guo, 1992).

2.4.2 Iron Transport Across Intestinal Mucosa

Iron transport across the small intestine is carried out by the enterocytes. Each enterocyte is attached to the adjacent cells by tight junctions (Madara, 1998). Tight junctions are multi-protein complexes associated with the cell's acto-myosin cytoskeleton, which hold the cells together to form a continuous layer through which the absorbed nutrient must pass (Madara, 1998). This occurs via one of two routes, the transcellular route or the paracellular route (Figure 2.1).

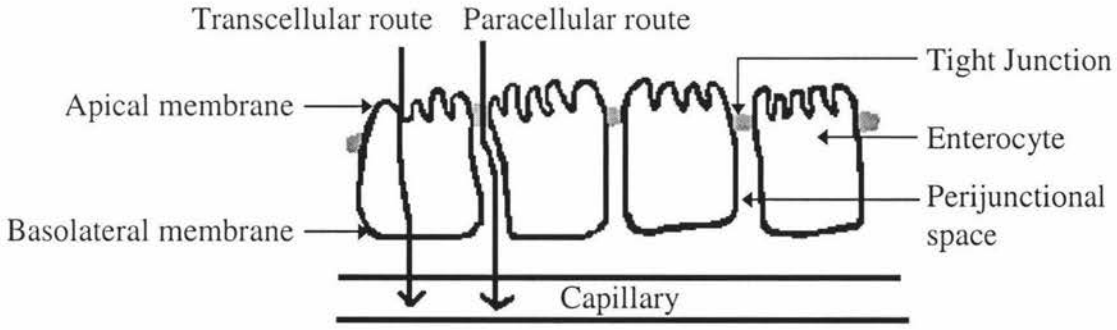

Figure 2.1: Paracellular and transcellular transport routes across the intestinal epithelia.

2.4.2.1 Paracellular Absorption of Iron

The paracellular route refers to the movement of nutrients through the tight junctions, into the perijunctional space and thence into the capillaries. This occurs because although the tight junctions are generally impermeable to large molecules, they allow water and small molecules to pass through (Madara, 1998). Movement through the tight junctions is a passive, electrochemical-gradient driven process, so it is likely that this pathway is only used when iron levels in the lumen are relatively high (Madara, 1998;

Beard et al., 1996; Pearson and Reich, 1965). It is not known if passive paracellular transport of iron is a nutritionally significant mechanism.

The permeability of tight junctions is not fixed and can be affected by solutes within the intestinal lumen (Berglund, Riegler, Zolotarevsky, Wenzi and Turner, 2001; Madara, 1998; Sadowski and Meddings, 1993). For example, high levels of glucose cause the acto-myosin molecules to contract thus opening the tight junction (Madara, 1998; Perez, Baber and Ponz, 1996). It has been shown that increased absorption of glucose from the intestinal lumen in turn increases absorption of other solutes including charged ions such as ferrocyanide (Sadowski and Meddings, 1993), uncharged hydrophilic solutes such as L-xylose (Fine, Santa Ana, Porte and Fordtran, 1994) or D-mannitol (Perez et al., 1996) and possibly iron (Manis and Schachter, 1962). This may allow a greater level of iron movement through the paracellular pathway and across the intestinal epithelium (Powell, Whitehead, Lee and Thompson, 1994).

2.4.2.2 Transcellular Absorption of Iron

The transcellular route passes through the enterocytes. The cell membrane of the enterocyte consists of two regions, the apical and the basolateral regions, which are separated by the tight junctions (Madara, 1998). As the tight junctions block the movement of transmembrane bound proteins each region has a distinct set of transport proteins (Bronk, 1999). Transcellular transport across the enterocyte must cross three separate barriers: the apical membrane on the mucosal side of the cell, the cytoplasm and the basolateral membrane on the serosal side of the cell (Perewusnyk and Funk, 1997; Nichols et al., 1992; Manis and Schachter, 1962).

Figure 2.2 shows the main iron transport pathways through the enterocyte. Haem and non-haem iron are absorbed into the enterocyte via separate pathways (Weintraub et al., 1968; Turnbull et al., 1962). Once inside the cell, haem iron is released from it's porphyrin ring and enters a common cytosolic iron pool (Beard et al., 1996). From there the iron is either used by the cell, added to the storage protein ferritin or transported across the basolateral membrane out of the cell (Beard et al., 1996). The mechanisms are described in more detail in the following sections.

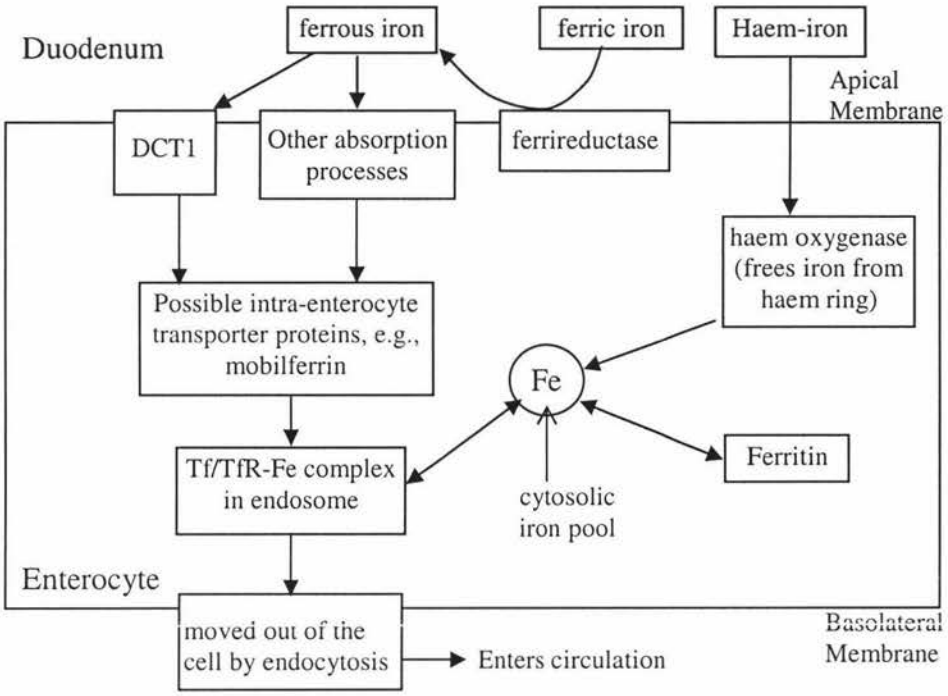

Figure 2.2: The transcellular movement of iron across the intestinal mucosa. Tf = transferrin, TfR = transferrin receptor, DCT1 = divalent cation transporter one.

2.4.3 Haem Iron Movement Into the Enterocyte

Haem iron is produced when haemoglobin, myoglobin and cytochromes are degraded in the gut by the action of digestive enzymes in gastrointestinal secretions. The entire haem complex is absorbed intact into the enterocyte via one of two mechanisms (Turnball et al., 1962). The first is by passive diffusion through the apical membrane. Haem iron is highly lipophilic and soluble in an alkaline environment, so it moves easily through the apical membrane and into the cytoplasm (Rucker et al., 1994). The second is transport by an apical membrane-bound haem-receptor protein. This protein selectively binds haem iron and transports it across the apical membrane and into the cell (Rucker et al., 1994).

Once inside the enterocyte, haem-oxygenase releases the iron via oxidative cleavage (Weintraub et al., 1968). This cleavage must occur for the iron to be further absorbed by the body (Weintraub et al., 1968). There is evidence that the activity of haem-oxygenase is the rate limiting step for haem iron movement into the enterocyte (Rucker et al., 1994). Haem-oxygenase activity is regulated by cytosolic concentrations of iron in the enterocyte, which in turn are affected by overall iron levels in the body, thus providing a link between whole body iron status and haem iron uptake (Wessling-Resnick 2000;

Weintraub et al., 1968). Once released from haem the iron is in an inorganic form and enters the common iron pool in the cytoplasm (Wessling-Resnick, 2000; Weintraub et al., 1968).

2.4.4 Non-Haem Iron Movement into the Enterocyte

The movement of non-haem iron into the enterocyte is the first step in non-haem iron absorption and is affected by several factors, including the composition of the luminal contents and the iron status of the animal.

2.4.4.1 Solubility in the Intestinal Lumen

The solubility of iron in the intestinal lumen is an important determinant of iron bioavailability since insoluble iron is not absorbed (Wienk et al., 1999). Ferric iron is very insoluble in the duodenum as the near neutral pH of the duodenal contents causes it to become hydrolysed and form insoluble chelates (Wienk et al., 1999; Garcia-Casal, Layrusse, Solano and Baron, 1998; Miller, Schricker, Rasmussen and Van Campen, 1981). Ferrous iron, however, stays soluble in the duodenum and is available for absorption (Wood and Han, 1998; Rucker et al., 1994). There is a ferrireductase mechanism active in the mucosa which reduces ferric iron to ferrous iron thus making it more available for uptake. This has been found in both the mouse and the human duodenum (Riedal, Remus, Fitscher and Stremmel, 1995; Raja, Simpson and Peters, 1992). Blocking duodenal ferrireductase activity lowers non-haem uptake from the lumen indicating that it is an important step in the uptake process (Wessling-Resnick, 2000; Wood and Han, 1998). Once reduced, ferrous iron will readily oxidise back to the less-soluble ferric form (Wood and Han, 1998). To prevent this, inorganic iron is complexed with luminal non-haem iron binding proteins (Leibold and Guo, 1992; Helbock and Saltman, 1967). These protect the ferrous iron from oxidation and prevent the formation of insoluble iron hydroxides (Nichols et al., 1992). There are a number of possible candidates for this iron binding role but the exact proteins and glycoproteins involved and their part in the absorption process have not been determined.

Ascorbic acid, of all the food constituents, has the greatest enhancing effect on iron uptake (Baynes and Bothwell, 1990). It has been shown to increase iron solubility and thus availability by both reducing ferric iron to ferrous and chelating with iron forming a soluble iron-ligand complex (Han, Failla, Hill, Morris and Smith, 1995; Nunez,

Alvarez, Smith, Tapia and Glass, 1994; Baynes and Bothwell, 1990). Other organic acids such as citric acid also chelate iron, thus keeping it soluble, however they do not reduce ferric iron to the ferrous form (Rucker et al., 1994). Meat has a large enhancing effect on iron absorption. This is thought to be due to cysteine-containing peptides, which are released as the meat is broken down (Glahn and Van Campen, 1997; Lynch, 1997; Baynes and Bothwell, 1990). These bind readily to inorganic iron forming soluble iron-ligand complexes, thus making the iron available for absorption (Lynch, 1997).

Phytate and polyphenols have strong inhibitory effects on iron absorption. Both substances have free hydroxyl groups which strongly bind with inorganic iron to form insoluble complexes which can not be absorbed (Lynch, 1997; Baynes and Bothwell, 1990).

2.4.4.2 Transport of Inorganic Iron Across the Apical Membrane

Once the iron has been reduced and complexed it is transported across the apical membrane and into the enterocyte. A study by Pearson and Reich in 1965 indicated that there is likely to be more than one mechanism operating at any time, with a mixture of passive and active transport processes responsible for moving iron into the enterocyte. However, the majority of non-haem transport across the apical membrane is a saturable, protein-mediated, active transport mechanism (Muir, Hopfer and King, 1984). This works against a concentration gradient thus ensuring uptake of the necessary iron even when levels in the lumen are low (Helbock and Saltman, 1967).

One of the earliest mechanisms proposed for active transport of iron into the cell involves transferrin as a transport protein. Transferrin was thought to bind to free iron in the intestinal lumen and form a transferrin-iron complex which is then transported into the cell via membrane-bound transferrin receptors (Huebers, Huebers, Csiba, Rummel and Finch, 1983). However Bannerjee, Flanagan, Cluett and Valberg (1986) did not find significant numbers of transferrin receptors in the apical membrane. Instead these receptors are localised on the basolateral membrane indicating that transferrin does not contribute to luminal iron uptake into the cell (Wolf, 1994; Banerjee, Flanagan, Cluett and Valberg, 1986).

In 1997 Gunshin, Mackenzie, Berger, Gunshin, Romero, Boron, Nussberger, Gollan and Hediger discovered the divalent cation transporter 1 (DCT1) protein in the apical membrane. This protein, also known as divalent metal transporter 1 (DMT1) or natural-resistance-associated macrophage protein 2 (Nramp2), is a transmembrane protein which uses proton-coupled active transport to move divalent cations across the apical membrane (Gunshin et al., 1997). The cations which DCT1 has been shown to transport (in order of specificity) are: Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} (Wessling-Resnick, 2000; Gunshin et al., 1997). The ability of DCT1 to bind divalent cations other than iron explains why manganese, zinc, copper and cadmium when added to the diet lower the active uptake of ferrous iron (Pollack, George, Reba, Kaufman and Crosby, 1965). It is thought that this inhibition is due to competition between divalent ions for the DCT1 protein (Wood and Han, 1998; Goddard, Coupland, Smith and Long, 1997; Powell et al., 1994).

Although DCT1 is expressed in many tissues throughout the body, it is found mainly in the apical membrane of the proximal intestine with decreasing expression along the distal axis (Canonne-Hergaux, Gruenheid, Ponka and Gros, 1999; Gunshin et al., 1997). Expression of this protein is increased in the duodenum during iron deficiency (Canonne-Hergaux, et al., 1999; Gunshin et al., 1997). Gene knockout animals missing the DCT1 protein (e.g., mk/mk mice and b/b rats) exhibit hypochromic, microcytic anaemia due to reduced uptake of iron by enterocytes (Wessling-Resnick, 2000; Wood and Han, 1998). However, these animals survive despite their anaemia indicating there are likely to be other iron absorption pathways into the enterocyte (Wessling-Resnick, 2000; Wood and Han, 1998).

Many studies have shown that a high level of calcium in the diet inhibits the uptake of iron (reviewed in Barton, Conrad and Parmley, 1983). However it is not yet fully understood why and under what circumstances this inhibition occurs as many of the results are conflicting (Lynch, 1997; Barton et al., 1983). The inhibitory effects of calcium are highest when administered in a whole meal (Cook, Dassenko and Whittaker, 1991). Calcium has been shown to protect phytate and prevent its degradation during cooking and digestion, thus increasing the phytate levels in the intestinal lumen. This decreases iron uptake as phytate is a effective inhibitor of iron

absorption (Lynch, 1997; Hallberg, Brune, Erlandsson, Sandberg and Rossander-Hulten, 1991).

Calcium has also been shown to decrease iron absorption in the absence of phytate. There is evidence that this inhibition occurs at the basolateral membrane to stop iron from exiting the enterocyte (Barton et al., 1983; Manis and Schachter, 1962). Both haem and non-haem iron absorption is lowered equally by excess calcium despite having separate pathways into the enterocyte (Hallberg et al., 1991). A study by Manis and Schachter (1962) showed that calcium inhibited iron transfer across the basolateral membrane and out of the cell much more than iron transfer across the apical membrane into the cell. Calcium may be competing for proteins involved in metal ion transport either within the cytoplasm or through the basolateral membrane, however the exact mechanism is not yet known (Hallberg et al., 1991; Barton et al., 1983; Manis and Schachter, 1962).

2.4.5 Ferritin

Ferritin, along with its derivative hemosiderin, is the main protein involved in storage of excess iron (Beard et al., 1996). When there is excess iron available in the enterocyte ferritin acts as an iron sink by binding the iron in an inert form (Leibold and Guo, 1992). Ferritin-bound iron may be released back into the cytoplasm and subsequently absorbed into the body, or it may be lost from the body when the enterocyte is sloughed from the mucosal surface (Beard et al., 1996; Conrad et al., 1964). Thus ferritin plays an important role in the prevention of excess iron entering the body (Beard et al., 1996).

2.4.6 Cytosolic Processing and Exit

Once inside the enterocyte iron joins a cytosolic inorganic iron pool. All iron in this pool is treated the same whether it is haem or non-haem in origin. Iron is transported within the cytoplasm via a protein facilitated mechanism, either to ferritin for storage or to the basolateral membrane for export out of the cell. One candidate for this role is mobilferrin, a cytosolic iron binding protein localised in the enterocytes of the duodenum (Conrad, Umbreit, Moore and Rodning, 1992; Conrad, Umbreit, Moore, Peterson and Jones, 1990). At the basolateral membrane the iron is bound to transferrin and moved across the membrane and into circulation (Nunez and Tapia, 1999). Transferrin function is suppressed by the presence of HFE reducing the release of iron

from the enterocyte (Eisenstein, 2000). There are a number of other proteins which may also be involved in the export of iron from the cell including ceruloplasmin, hepaestin and ferroportin 1 (Eisenstein, 2000; Beard et al., 1996). However the exact roles and interactions of these proteins have not been determined. The synthesis of these proteins may be regulated by IRP, as described in Section 2.4.1, providing a link between iron content of the body and the release of iron from the enterocyte (Eisenstein, 2000).

2.4.7 Mucus

The luminal surface of the small intestine is covered by a layer of insoluble mucus which consists of a mixture of secretions and exfoliated epithelial cells (Neutra and Forstner, 1987). It's main constituent is mucin, a high molecular weight glycoprotein secreted by the goblet cells (Conrad, Umbreit and Moore, 1991). The mucus layer forms a physiological barrier between the intestinal mucosa and the contents of the intestinal lumen, protecting the intestinal epithelia from chemical and physical damage (Neutra and Forstner, 1987). It also acts as a lubricant and a host-defence mechanism against many viruses and bacteria (Neutra and Forstner, 1987).

Insoluble mucin has been implicated in the iron absorption process as it has been shown to bind iron in the intestinal lumen (Conrad et al., 1991). Mucin binds to both ferrous and ferric forms of iron in the acidic conditions of the stomach and keeps it soluble as it moves into the neutral pH of the duodenum (Conrad et al., 1991). This binding is reversible, thus iron bound by mucin is able to be absorbed by the enterocytes. Removal of the mucus layer has been shown to reduce iron uptake supporting its role in this process (Conrad et al., 1991). Therefore mucin may play an important role in keeping inorganic iron soluble in the intestinal lumen and thus available for absorption.

2.5 Methods for Measuring Iron Absorption

There are a variety of laboratory models commonly used for investigating the absorption of iron. These are summarised in Table 2.3 and described in the following sections.

Table 2.3: Methods for studying iron absorption.

Description	*Reference*
In vitro solubility studies, non-animal based.	Miller et al., 1981; Narasinga Rao and Prabhavathi, 1978.
In vivo iron balance studies	Forrester, Conrad and Crosby, 1962; Dern and Hart, 1961A; Dern and Hart, 1961B.
In vivo blood parameters	Wienk and Beynen, 1996.
Doubly labelled extrinsic tags	Saylor and Finch, 1953.
Perfused intestinal segments *in situ*	Barton et al., 1983; Manis and Schachter, 1962.
Everted Intestinal Sacs, *in vitro*	Wilson and Wiseman, 1954.
Membrane Vesicles, *in vitro*	Kessler, Acuto, Stroelli, Murer, Muller and Semenza, 1978; Schmitz, Preiser, Maestracci, Ghosh, Cerda and Crane, 1973.
Enterocyte Suspensions, *in vitro*	Kimmich, 1970; Levine and Weintraub, 1970.
Artificial Cell Lines, *in vitro*	Nichols et al., 1992; Alvarez-Hernandez, Nichols and Glass, 1991; Hidalgo, Raub and Borchardt, 1989.
Ussing Chambers, *in vitro*	Ussing and Zerahn, 1951.

2.5.1 Solubility Studies

Solubility studies involve the laboratory analysis of a chosen meal to determine the concentration of soluble iron present. They do not use any animal tissue, therefore any predictions made about iron uptake do not consider differences in iron status of the animal, or species-specific factors (Miller et al., 1981).

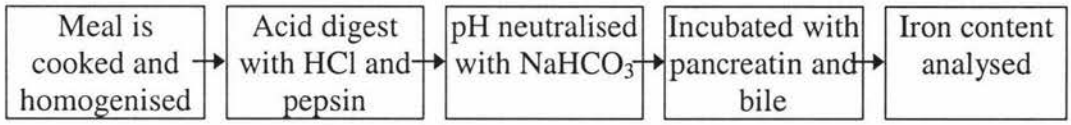

Figure 2.3: Artificial digestion for *in vitro* iron analysis (after Narasinga Rao and Prabhavathi, 1978).

Before analysis the meal is cooked and artificially digested following the steps outlined in Figure 2.3. The meal is cooked and homogenised in a blender. The homogenate is then mixed with pepsin and HCl at pH 1.3 and incubated at 37 °C (Narasinga Rao and

Prabhavathi, 1978; Sanford, 1960). This latter step is designed to simulate digestion in the stomach. The pH is then adjusted to 7.5 using $NaHCO_3$ and a pancreatin-bile mixture is added to simulate digestion in the small intestine (Narasinga Rao and Prabhavathi, 1978), followed by a further incubation step. The digested meal is then analysed to measure the iron concentrations (Narasinga Rao and Prabhavathi, 1978; Jacobs and Greenman, 1969; Sanford, 1960). As some of the iron in the resulting digest forms insoluble chelates which can not be absorbed, Miller et al. (1981) modified the procedure by using dialysis tubing to separate the soluble iron from the rest of the digested sample.

While these studies do not give any insight into the actual absorption mechanisms of iron, they are useful for predicting levels of soluble, and hence absorbable, iron in a meal. This allows the investigation of the effects of food preparation and cooking (Sanford, 1960); meal composition (Miller et al., 1981; Narasinga and Prabhavathi, 1978) and digestion (Miller et al., 1981) on iron bioavailability. These studies are cheap, fast and have no ethical cost, making them useful for screening a number of test meals to choose those suitable for further experimentation (Narasinga and Prabhavathi, 1978).

2.5.2 Whole Animal Based Methods

Methods for quantifying iron absorption in whole animals often involve the use of radioactive iron (e.g., Fe^{59}, Fe^{55}) to differentiate between endogenous and exogenous nutrient pools (Wienk et al., 1999). Whole animal studies can give accurate data on the overall iron uptake from a specific meal, however it is not always possible to investigate specific mechanisms or biochemical pathways of iron absorption using whole animals (Hallberg, 2001; Goddard et al., 1997).

In vivo studies of iron absorption have been preformed on a variety of animals including humans. The use of humans has a high ethical and monetary cost and there are limits on the procedures possible (Wienk et al., 1999; Latunde-Dada, Bianchi and de Oliveira, 1998). Therefore, other animals are often used to model the physiological processes occurring in humans. It is necessary to exercise caution when extrapolating from one species to another as there may be differences in the absorption processes present in each species (Wienk et al., 1999; Latunde-Dada et al., 1998; Reddy and Cook, 1991; Scricker, Miller, Rasmussen and Van Campen, 1981; Weintraub et al., 1965).

2.5.2.1 Iron Balance Studies

Iron balance studies aim to quantify the amount of iron absorbed from a test meal given to a live animal. This may be done in one of two ways. The first is to measure how much iron passes through the gastrointestinal tract without being absorbed. All waste products (e.g., faeces and urine) excreted during the experiment are collected and their iron content determined (Wienk et al., 1999; Bothwell et al., 1958). All iron retained by the animal is considered to have been absorbed by the animal. This method was used by McCance and Widdowson (1937) to determine that iron homeostasis was regulated by iron absorption by the small intestine (cited in Hallberg, 2001). However the indirect measure of iron absorption is not always accurate as a significant amount of iron may be retained in the enterocytes past the end of the experimental period and subsequently lost in the faeces (Cook, Layriss, Martinez-Torres, Walker, Monsen and Finch, 1972; Bothwell et al., 1957).

The second method is the direct measurement of the amount of iron retained by the animal at the end of the experimental time. Radio-iron is added to the meal and the concentration of iron retained by the animal is measured using either whole body scintillation counting to give a total-body radiation count (Forrester et al., 1962; Field, Seki, Mitchell and Chalmers, 1960) or scintillation counting of blood and tissue samples where any increase in radioactivity in the sample is considered to be caused by labelled iron from the test meal (Dern and Hart, 1961A; Dern and Hart, 1961B). Iron balance studies can give accurate results as they retain both the meal and animal based factors affecting iron absorption.

2.5.2.2 Dual Isotope Studies

One of the earliest uses of dual isotopes was in 1953 by Saylor and Finch to correct for tissue partitioning of iron in whole animal studies. Previous absorption studies measured the increase in radioactive iron in the blood to demonstrate iron absorption. However a proportion of the iron absorbed is partitioned into other parts of the body. This proportion varies depending on the body's need for iron and can have a significant effect on results from these studies (Saylor and Finch, 1953). Therefore Saylor and Finch (1953) devised a method where a known amount of Fe^{55} is given orally and a known amount of Fe^{59} is simultaneously injected intravenously. The injected sample is a reference dose which gives a direct measure of iron partitioning. The animal is left for

a pre-determined experimental period to allow for absorption and utilisation of the iron, and then a blood sample is taken and analysed (Saylor and Finch, 1953). The ratio of Fe^{55} to Fe^{59} is used to calculate the correct absorption of Fe^{55} (Saylor and Finch, 1953).

The dual isotope method has been used by Cook and Monson (1975) to compare iron absorption from semisynthetic and normal meals. Test meals labelled with different isotopes of iron were administered to the subject on successive days. Then 14 days after the administration of the final meal, blood samples were taken and the ratio of both isotopes determined. Alternatively, a single meal may be prepared containing both isotopes of iron, each one attached to a different form of iron. The experimental procedure is carried out as described above, with the exception that only one meal is administered. This technique was used by Bjorn-Rasmussen, Hallberg and Walker, (1972) and Cook et al. (1972) to compare the absorption of extrinsically labelled iron with that of intrinsically labelled iron in the same meal. The use of dual isotopes in this manner does not give absolute amounts of iron absorption due to the partitioning factors described by Saylor and Finch (1953). However, it does allow the direct comparison of the absorption of two forms of iron.

2.5.2.3 Tracking Haematological Parameters

The measurement of haematological parameters to monitor iron status can be used to indirectly measure iron absorption (Wienk et al., 1999; Wienk and Beynen, 1996). This is done by taking blood samples both before and after the administration of iron to measure haemoglobin concentration, total iron-binding capacity, transferrin concentration and saturation and serum ferritin concentration (reviewed in Fairweather-Tait, 1993 and Cook et al., 1992). Iron deficient subjects may be used as they absorb iron more efficiently than iron replete subjects (Wienk et al., 1999). This method is commonly used to determine the efficiency of a treatment for improving iron status of the animal and is useful for comparing possible iron fortification or supplementation schemes (Wienk et al., 1999; Wienk and Beynen, 1996).

2.5.3 *In Situ* Methods

Iron absorption may be studied using tissues or organs which have been surgically modified but left *in situ* (Manis and Schachter, 1962; Fisher and Parsons, 1949). These studies can be difficult to carry out as the animal must be kept alive both during and

after the surgical procedure (Manis and Schachter, 1962; Fisher and Parsons, 1949). There are also ethical considerations when experimenting on live animals which may reduce the types of experiments possible. However, these studies allow the investigation of tissue or organ level iron transport mechanisms without the removal of the physiological effects from the whole animal (Wienk et al., 1999).

2.5.3.1 Perfused Intestinal Segments

An *in situ* technique which has been used to investigate iron absorption involves perfusion of an isolated intestinal segment which retains its normal nerve and blood supply. The animal is anaesthetised, the intestine is exposed and a segment isolated. The isolated segment is then tied at both ends, cannulated and the lumen perfused with an oxygenated physiological buffer containing the nutrients to be investigated (Manis and Schachter, 1962; Fisher and Parsons, 1949). At the end of the experimental period the animal is euthanased, the intestinal loop removed, and the buffer and the intestinal tissues are analysed to determine iron uptake (Manis and Schachter, 1962; Fisher and Parsons, 1949). It is also possible to take blood samples from the venous drainage of the isolated segment to directly measure iron crossing the intestinal mucosa (Hidalgo et al., 1989).

This technique was used Manis and Schachter (1962) to demonstrate their two-step model of iron absorption. They found that iron uptake at the mucosal surface was more rapid than the subsequent transfer of iron to the blood stream, indicating that these are discrete steps in the iron absorption mechanism.

An alternative technique for measuring iron absorption from an *in situ* intestinal segment involves the addition of radio-labelled iron to the perfusion buffer. Immediately after the perfusion of the physiological buffer into the intestinal segment total-body radioactivity is measured using a whole body scintillation counter (Forrester, 1960). At the end of the experimental period the animal is euthanased, the intestinal segment removed and total-body radioactivity measured again to determine the concentration of radio-labelled iron which has been absorbed. This technique was used by Barton et al. (1983) to demonstrate calcium inhibition of iron absorption. By isolating intestinal segments from different parts of the intestine they were able to

localise calcium inhibition to the duodenum and jejunum, which would not be possible when using an intact animal.

The use of *in situ* intestinal segments provides physiological conditions very similar to those found *in vivo*, however it is possible that the anaesthetic and surgery may effect nutrient uptake compared to that seen in intact, conscious animals. (Manis and Schachter, 1962).

2.5.4 *In Vitro* Tissues

In vitro studies involve the use of tissue freshly removed from an animal. The cellular processes remain active throughout the experiment allowing the direct investigation of nutrient movement through the intestinal mucosa (Fisher and Parsons, 1949). The results from many *in vitro* studies have been shown to be similar to those found *in vivo*, and they can provide useful information about iron absorption mechanisms (Miller et al., 1981).

2.5.4.1 Everted Sacs

A frequently used *in vitro* method for the study of iron absorption is everted intestinal sacs. Segments of the small intestine are removed from the animal and everted to bring the mucosal surface to the outside (Wilson and Wiseman, 1954). The inside of the sac is filled with buffer and the ends tied off (Wilson and Wiseman, 1954). A bubble of oxygen is included inside the sac to oxygenate the tissue (Wilson and Wiseman, 1954). The sac is then immersed in a physiological buffer containing iron and which is continuously oxygenated and mixed to prevent an unstirred water layer forming next to the tissue (Wilson and Wiseman, 1954). After the experimental period the buffer inside the sac is removed and analysed to determine the amount of iron absorbed (Dowdle, Schachter and Schenker, 1960). Alternatively, the insertion of a glass cannula at one end of the everted sac allows for repeated sampling throughout the experimental period (Crane and Wilson, 1958).

This method has been used to investigate the active transport mechanisms involved in iron absorption (Pearson and Reich, 1965; Manis and Schachter, 1962; Dowdle et al., 1960; Brown and Justus, 1958) and played an important role in the development of the two-step model of iron absorption (Manis and Schachter, 1962).

Everted sacs retain the full thickness of intestinal mucosa, including the mucus layer and the muscularis externa. However, in the whole animal iron does not cross the muscularis externa per se, instead it enters the capillaries between the epithelia and the muscularis externa. As this blood supply is not present *in vitro*, iron may build up between the epithelia and the attached muscle layer, creating an unphysiological concentration gradient across the tissue (Manis and Schachter, 1962). This may not only prevent the passive transport of iron from the intestinal lumen but may also promote an unmeasured back flux of iron in the serosal to luminal direction (Manis and Schachter, 1962). When the concentrations of iron absorbed by *in situ* intestinal segments and *in vitro* everted sacs were compared, the transfer of iron across the basolateral membrane and out of the enterocyte was consistently lower in the *in vitro* preparations (Forth and Rummel, 1973; Manis and Schachter, 1962), supporting the possibility that a concentration gradient develops across the tissue.

2.5.4.2 Membrane Vesicles

Iron transport into the enterocyte can be investigated using vesicles made of apical membrane which has been separated from the rest of the intestinal mucosa. Apical membrane vesicles are prepared by homogenising and centrifuging intestinal mucosa to break up the enterocytes and release the apical membrane, as shown in Figure 2.4 (Kessler et al., 1978; Schmitz et al., 1973). This apical membrane then forms spherical vesicles (Kessler et al., 1978; Schmitz et al., 1973).

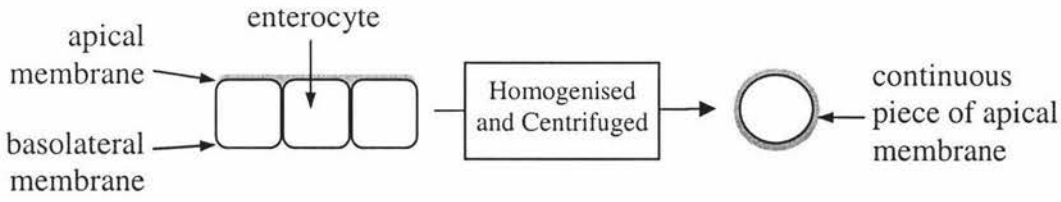

Figure 2.4: Preparation of apical membrane vesicles.

During a typical experiment the membrane vesicles are suspended in a physiological buffer containing iron and any other nutrients to be measured. After an incubation period absorption is stopped by washing the vesicles in ice cold buffer, the suspension filtered or centrifuged to separate the vesicles from the buffer and the iron content of the vesicles measured (Greenberger, Balcerzak and Ackerman, 1969).

As the vesicles which form have no basolateral membrane or intracellular components they can be used to investigate iron transport mechanisms specific to the apical membrane (Teichman and Stremmel, 1990). They have been used to investigate both the regional specificity (Chowrimootoo, 1991; Muir and Hopfer, 1985; Cox and O'Donnell, 1981) and effects of iron status (Muir and Hopfer, 1985; Muir et al., 1984; Greenberger et al., 1969) on iron binding and uptake by the apical membrane. This has lead to the characterisation of iron-specific binding proteins in the apical membrane (Wood and Han, 1998; Teichman and Stremmel, 1990; Muir et al., 1984)

The lack of intracellular processes may affect iron uptake across the apical membrane and should be taken into account when extrapolating results to the intact animal (Hidalgo et al., 1989). There is also a possibility that membrane-bound proteins may be damaged during the preparation of the vesicles, thus affecting nutrient transport (Hidalgo et al., 1989).

2.5.4.3 Isolated Enterocytes in Solution

Enterocytes that have been isolated from fresh intestinal tissue and suspended in solution allow the investigation of iron movement into the enterocyte while keeping the intracellular contents intact (Halliday and Powell, 1973). Fresh pieces of small intestine are cut into pieces and are placed into an incubation media where they are agitated to loosen the cells (Kimmich, 1970; Levine and Weintraub, 1970). The resulting cell suspension is washed and centrifuged and the cell pellet suspended in fresh oxygenated growth media containing iron (Kimmich, 1970; Levine and Weintraub, 1970). After the experimental period the cells are removed and the amount of iron taken up by the cells measured (Halliday and Powell, 1973).

As these cells are no longer joined together by tight junctions and are dispersed in solution it is not possible to measure vectorial transport of iron across the mucosa (Goddard et al., 1997). Instead, these cell suspensions are used to measure the movement of iron across the apical membrane from the suspension buffer into the enterocyte (Halliday and Powell, 1973). Halliday and Powell (1973) have demonstrated that due to the time lapse between iron entering the cell via the apical membrane and leaving via the basolateral membrane (Manis and Schachter, 1962), it is possible to stop the experiment after a short period of time and thus measure only the initial transport

across the apical membrane. This demonstrates the same transport processes as the apical membrane vesicles described in Section 2.5.4.2, with the added advantage of retaining the intracellular processes intact. However, the exposure of all parts of the cell to the suspension buffer may allow nutrients to be absorbed across the basolateral membrane, which does not occur *in vivo* (Goddard et al., 1997).

2.5.4.4 Cultured Cell Lines

A human colon adenocarcinoma cell line (Caco-2) has been developed which reproducibly displays properties characteristic of differentiated intestinal cells (Hidalgo et al., 1989). They spontaneously form a polarised monolayer with tight intracellular junctions between cells, abundant microvilli and enzymatic secretion typical of human small intestine enterocytes (Hidalgo et al., 1989). By growing the cells in bicameral chambers it is possible to separate the growth medium on the apical side from that on the basolateral side thus allowing the measurement of vectorial transport of iron through the monolayer (Alvarez-Hernandez et al., 1991).

Studies with Caco-2 cells have been conducted which show these cells displaying characteristics of human intestinal cells as expected from previous *in vivo* iron absorption studies. These characteristics include the synthesis of transferrin and ferritin (Halleux and Schneider, 1991); the expression of DCT1 (Tallkvist, Bowlus and Lonnerdal, 2000); the reduction of ferric iron to ferrous before absorption (Han et al., 1995); preferential absorption of ferrous iron over ferric (Alvarez-Hernandez et al., 1991); and changes in iron uptake depending on the iron status of the cell (Gangloff, Lai, Van Campen, Miller, Norvell and Glahn, 1996). Au and Reddy (2000) carried out a direct comparison between iron absorption from the same meal with Caco-2 cell lines *in vitro* and human iron absorption studies *in vivo*. They found a close correlation between the levels of iron absorption demonstrated using both methods indicating that the quantitative levels of iron absorption seen in Caco-2 cells reflect those seen *in vivo*.

Any artificial cell line, including Caco-2, are made up entirely of one cell type. However, *in vivo* the intestinal mucosa contains a mixture of cells including crypt cells and mucus-secreting goblet cells. Therefore the morphology of the Caco-2 monolayer may be different to that found *in vivo*, which in turn may affect nutrient uptake (Hidalgo et al., 1989). There is also a possibility that the Caco-2 cell line's development from

oncogenic cells may predispose them to a higher rate of proliferation than found *in vivo*, although this is yet to be seen.

Another intestinal cell line which has been shown to absorb iron is IEC-6 (Thomas and Oates, 2002; Nichols et al., 1992). These cells originate from the rat small intestine and have a finite life span (Wienk et al., 1999; Nichols et al., 1992). Therefore, while they avoid possible side effects due to an oncogenic origin, they introduce species specific differences in iron absorption when extrapolating the results to humans (Nichols et al., 1992)

2.5.5 Ussing Chambers

The Ussing chamber system was developed in 1951 by Ussing and Zerahn as a method for studying sodium transport across frog skin. Since then Ussing chambers have been widely used in the study of nutrient transport across many types of tissue including intestinal epithelia. Studies in which Ussing chambers were used to investigate iron absorption by the small intestine have been conducted by Costa et al. (2000), Vaghefi et al. (2000), Vaghefi et al. (1998) and Helbrock and Saltman, (1967). All these studies focussed on the duodenum and did not investigate iron absorption further along the small intestine.

Helbrock and Saltman (1967) postulated that a low molecular weight chelate was necessary to solubilise the iron and make it available for absorption and that the chemical form of this chelate effected the amount of iron absorbed. The net charge of each iron-ligand compound was different depending on the chelate involved. Therefore, any unphysiological electrochemical gradient across the tissue could interfere with the transport of these compounds. The removal of potential difference (p.d.) across the tissue as carried out in the Ussing chamber (see Section 2.5.5.2) prevents the build up of these electrochemical gradients. This study also included experiments using *in situ* intestinal segments to give a direct comparison of absorption *in vitro* and *in vivo*. Similar concentrations of iron were absorbed with both methods, supporting the use of the Ussing chamber system to model iron absorption seen *in vivo*.

In 1998, Vaghefi et al. investigated the effects of cysteine and histidine on iron absorption. Their study comprised two parts; firstly, the absorption of ferrous gluconate

and haem iron at two different concentrations was compared, secondly, cysteine or histidine was added and changes in absorption measured. They found that the proportions of ferrous gluconate and haem absorbed at the lower concentration were not significantly different, but more ferrous gluconate than haem was absorbed at the higher concentration. Histidine did not enhance absorption for either form of iron whereas cysteine did. The investigation of the effects of amino acids on iron absorption was continued by Vaghefi et al. in 2000. Haemoglobin hydrosylates were prepared with increasing levels of hydrolysis and the absorption of iron from each hydrolsylate was compared. They demonstrated that increased hydrolysis of haemoglobin was associated with enhanced iron uptake.

Costa et al. (2000) investigated the use of radio-labelled iron to measure iron flux across the small intestine in Ussing chambers. They found significant levels of iron transferred from the serosal to the mucosal solution, however this did not account for the full loss of activity from the serosal solution. Further investigation found a percentage of the radio-iron was lost through non-specific adherence to the internal walls of the Ussing chamber apparatus. They also compared iron absorption at pH 7.4 and pH 5.5, demonstrating a significant increase in absorption at the acidic pH.

The muscularis externa attached to the intestinal tissue may affect iron transport in Ussing chamber experiments in the same manner as described for the everted sac method (Section 2.5.4.1). However, unlike the earlier method, the muscularis externa may be removed from the tissue directly before mounting in the Ussing chamber, allowing iron to move directly from the intestinal mucosa into the serosal solution. This technique has been used previously by Vaghefi et al. (1998) and Costa et al. (2000). It may be very difficult to remove the muscularis externa from intestinal tissue taken from some species of animal (e.g. mouse) due to the small size and fragility of the tissue.

2.5.5.1 Apparatus

The Ussing chamber consists of two acrylic half-cells clamped together with a sheet of intestinal tissue between them to form serosal and mucosal compartments. Solutes must pass through the tissue to get from one compartment to the other. The equipment is assembled as shown in Figure 2.5.

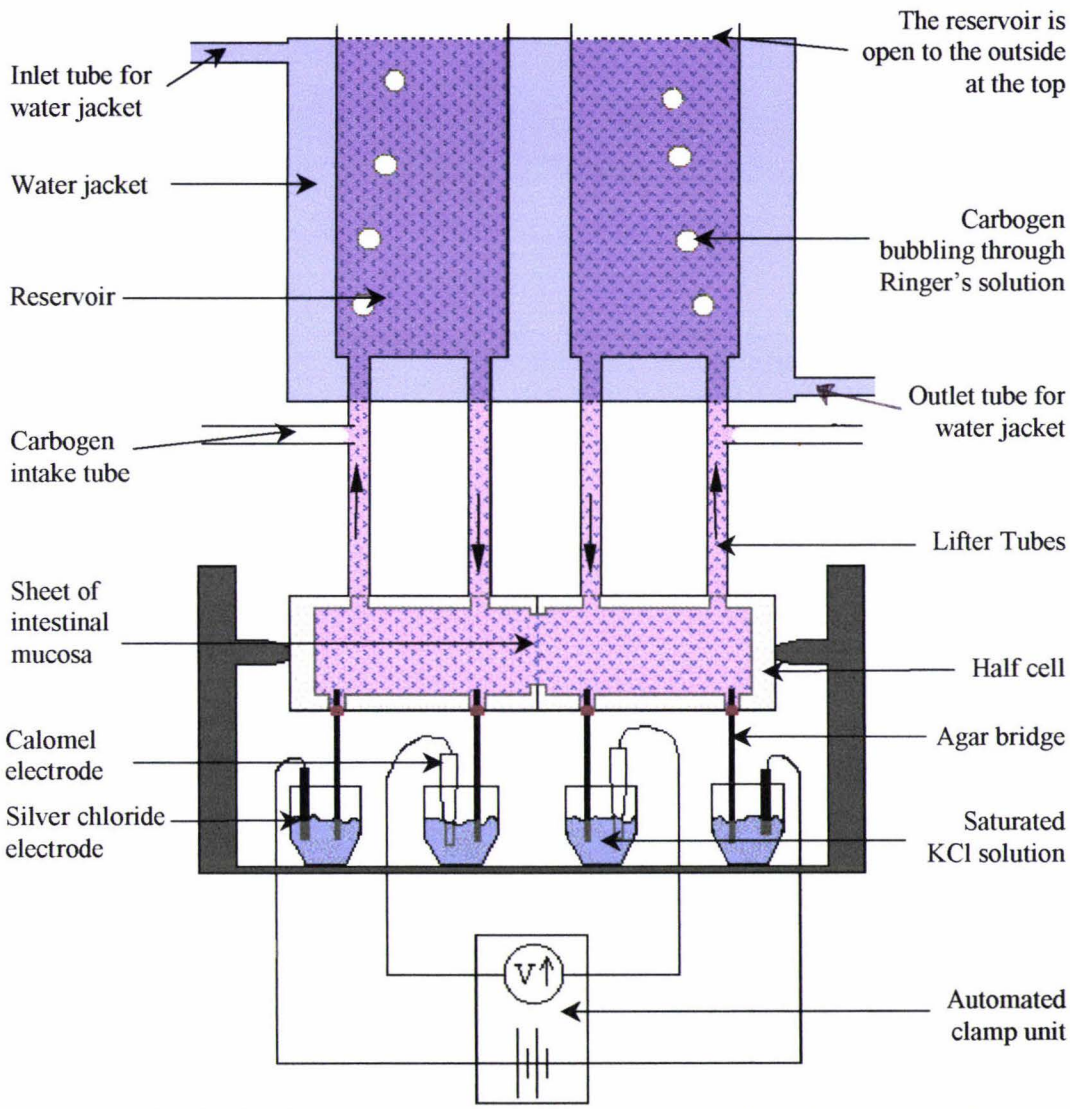

Figure 2.5: The Ussing chamber apparatus as assembled during an experiment.

Each compartment is filled with Ringer's solution; a water jacket around the reservoirs maintains the Ringer's solution at the normal body temperature of the animal from which the tissue is taken. Carbogen (95% O_2, 5% CO_2) is bubbled through each reservoir. This creates a gas lift to circulate the liquid, as well as providing oxygen for the tissue and CO_2 maintaining the pH of the bicarbonate buffered Ringer's solution.

2.5.5.2 Electrophysiology

When the chambers are assembled with intestinal tissue mounted in them, a serosal-side positive p.d. develops across the tissue (Asano, 1964). This is caused by the active movement of ions such as Na^+ across the tissue (Costa et al., 2000; Grubb, 1995). This p.d. does not develop *in vivo* as ions are removed by the venous drainage of the tissue. However, as there is no blood supply *in vitro*, electrochemical gradients may build up to unphysiological levels and interfere with the transport of charged solutes across the tissue (Ussing and Zerahn, 1951). Therefore the p.d. is removed using a method developed by Ussing and Zerahn (1951) whereby electrical current is added to each side of the chamber as necessary to maintain the p.d. across the tissue at 0 mV. This method involves two sets of electrodes, a pair of calomel electrodes and a pair of silver chloride electrodes, which are connected to an automatic clamping unit (Figure 2.4). The calomel electrodes are used to continuously measure the potential across the tissue, allowing the clamp unit to calculate how much current needs to be added via the silver chloride electrodes to each side to keep the p.d. at zero. When the tissue is short circuited in this way there is no net passive transport of ions due to electrochemical gradients (Ussing and Zerahn, 1951).

2.5.5.3 Tissue viability

The electrophysiological readings taken during the experiment give an indirect measure of viability and intactness of the tissue without interfering with the tissue or bathing solutions. It has been shown that I_{sc} is caused by the active transport of ions by the tissue (Ussing and Zerahn, 1951; Costa et al., 2000). As this transport is dependent on metabolic energy provided by the tissue, a high I_{sc} demonstrates that the tissue is viable. Therefore this parameter can be used in Ussing chamber experiments to confirm tissue viability during the experiment. Transepithelial resistance (Rt) is related to paracellular permeability of intestinal epithelia and may be used as a measure of tissue integrity

(Madara, 1998). Therefore bi-directional pulses of current are administered periodically to the tissue throughout the experiment and the Rt calculated.

Tissue viability and intactness may also be measured directly after the experiment is concluded. Sheldon, Malarchik, Fox, Burks and Porreca (1989) tested tissue viability after the experimental period by the addition of 1mM theophylline, a compound which is known to increase I_{sc}, to the Ringer's solution. Tissues which did not react to this compound were no longer metabolically active and thus discarded. Histological examination of intestinal segments allows direct observation of tissue intactness and may involve staining of tissue segments to investigate changes in morphology (Vaghefi et al., 2000; Vaghefi et al., 1998).

2.6 Summary and Conclusions

The main objective of this project is to investigate the validity of the Ussing chamber method for measuring iron absorption along the length of the mouse small intestine. Although it has been accepted that iron absorption only occurs in the proximal small intestine there is evidence that iron may be absorbed further along the intestine. Therefore the first goal of this project was to determine in what regions of the small intestine iron absorption occurs in the mouse. Previous studies have indicated that there are both active and passive uptake mechanisms involved in iron absorption by the small intestine. The second goal of this project was to investigate which mechanisms are involved in iron absorption in the mouse. The third goal of this project was to demonstrate that any absorption measured in the above experiments was physiological in nature rather than an experimental artefact.

3 METHODS

3.1 Animals

A total of 51 adult Balb/c mice (6-12 weeks old) and 3 adult Swiss mice (6-8 weeks old) were used for these experiments. The mice were obtained from the Small Animal Production Unit at Massey University. They were housed in groups of no more than six to a cage and kept in a room with controlled temperature (22 ± 1 °C), humidity (55 ± 5%) and lighting (12 hour light and dark cycles with dawn and dusk transitional periods). The mice were fed a balanced diet (Rodent Diet 83) prepared by the Food Processing Unit at Massy University and were given access to both food and demineralised water *ad libitum* until the day of the experiment. The protocols for all experiments described in this thesis were approved by the Massey University Animal Ethics Committee before experimentation begun.

3.2 Ringer's Solution for the experiments

All chemicals used for these experiments were analytical grade unless otherwise specified.

Before each experiment two litres of Ringer's solution was prepared according to Sheldon et al. (1989) (Table 3.1) volumetrically with reverse osmosis (RO) water. The calcium chloride was added after all other chemicals were dissolved to prevent precipitation.

Table 3.1: Composition of the Ringer's solution.

Chemical	*Weight (mM)*	*Supplier*
NaCl	114	BDH Laboratory Supplies, Poole, England
KCl	5	BDH Laboratory Supplies, Poole, England
$MgCl_2 \cdot 6H_2O$	1.1	Sigma Chemical Company, St Louis, USA
Na_2HPO_4	1.65	Sigma Chemical Company, St Louis, USA
NaH_2PO_4	0.3	Sigma Chemical Company, St Louis, USA
$NaHCO_3$	25	BDH Laboratory Supplies, Poole, England
$CaCl_2$	1.25	BDH Laboratory Supplies, Poole, England

After mixing, the solution was divided into two portions of one litre each, one for use in the serosal compartment and the other for use in the mucosal compartment. D-Mannitol (10 mM, Riedal-de Haen, Seelze, Germany) was added to the mucosal Ringer's solution and the pH adjusted to 6.5 using hydrochloric acid (HCl). D-glucose (10 mM, BDH

Laboratory Supplies, Poole, England) was added to the serosal Ringer's solution and the pH adjusted to 7.4 with HCl.

All Ringer's solution was stored at 5 °C when not being used and was discarded after 48 hours. The temperature of the solution was adjusted to room temperature before use to prevent cold shock to the tissue.

3.3 Experimental Procedure

Iron absorption was measured using an Ussing chamber system as described in Section 2.5.5. This system comprises the Ussing chamber per se where the tissue is mounted, lifter tubes connecting the chambers with the reservoirs and glass reservoirs with built-in water jacket. The entire system was purchased from World Precision Instruments Incorporated, Sarasota, USA. The acrylic half cells comprising each Ussing chamber (model number CHM6) had a 0.67 cm^2 aperture and an internal volume of 0.8mL. The three components had a combined internal volume on each side of the tissue of 10 mL.

3.3.1 Fluid Resistance

Before starting each experiment the resistance of the Ringer's solution (fluid resistance, Rf) in each chamber was measured as follows:

A thin layer of silicone gel was applied to the mounting face of each chamber before assembly to prevent fluid leakage from the join between the two half cells (Figure 3.1). The half cells were then brought together and the Ussing chamber, lifters and reservoirs assembled as shown in Figure 3.2.

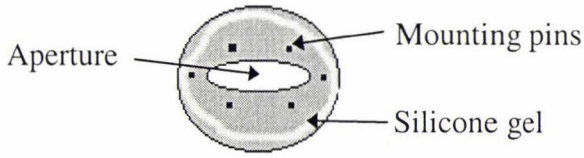

Figure 3.1: Mounting face of half cell.

Four electrodes (two calomel electrodes and two silver wire electrodes) were connected to the chambers via agar bridges and saturated KCl solution as shown in Figure 4.2. Cut-down pipette tips were used to ensure a tight fit between the agar bridge and the chambers. The agar bridges were made by dissolving first 1.725 g agar (Sigma

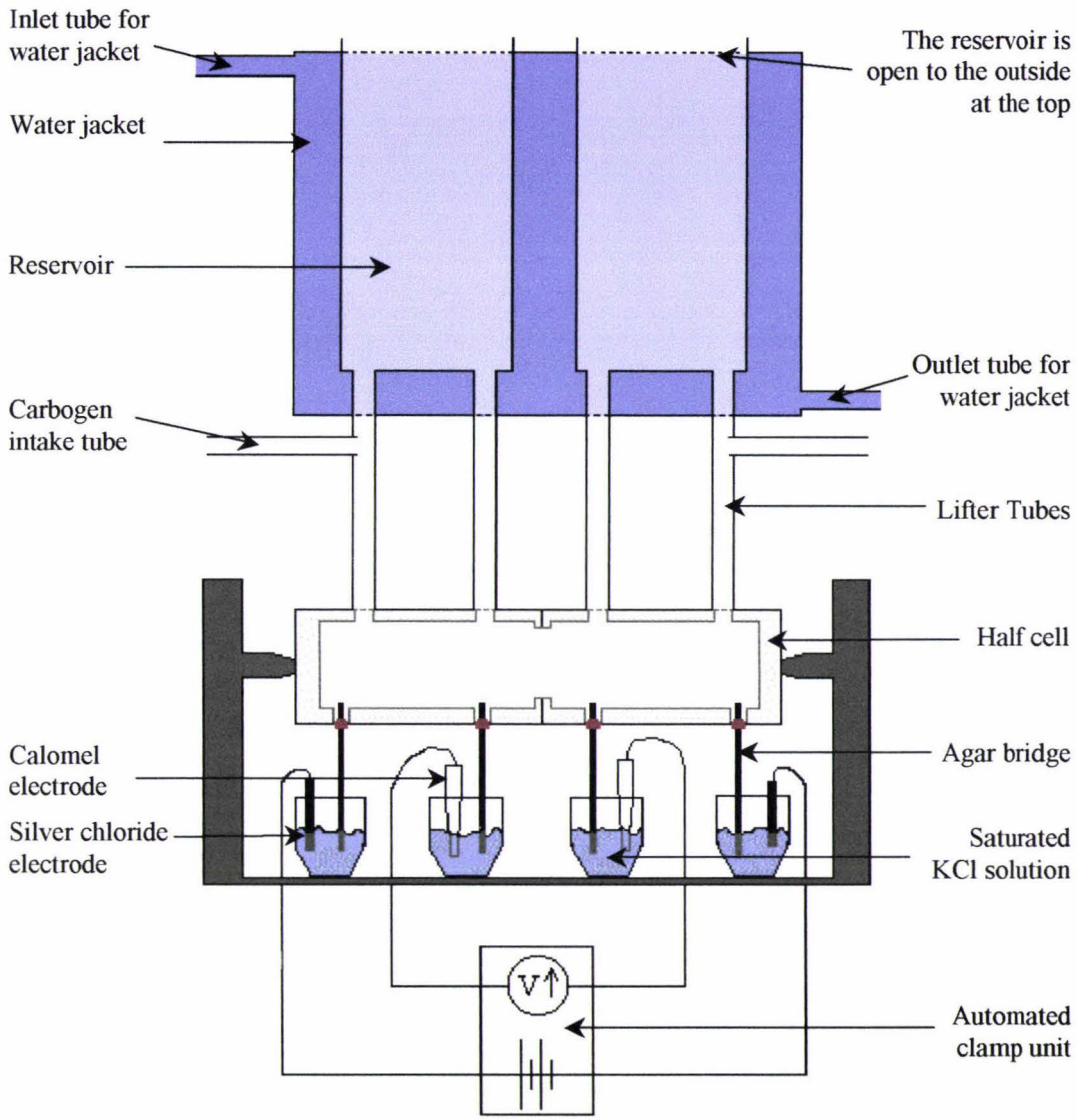

Figure 3.2: The Ussing chamber apparatus ready for fluid resistance recording.

Chemical Company, St Louis, USA) then 11.1825 g KCl (BDH Laboratory Supplies, Poole, England) in 50 mL of RO water heated to 80 °C. The agar/KCl mixture was then drawn into 18 centimetre lengths of polyethylene tubing (2 mm inner diameter, 3 mm outer diameter) using a syringe and allowed to set. The agar bridges were stored until use in a closed container with a small amount of 3M KCl to prevent them from drying out. Saturated potassium chloride solution was prepared by dissolving 42.6 g KCl (BDH Laboratory Supplies, Poole, England) per 100 mL RO water at 50 °C and allowed to cool.

The electrodes for each chamber were connected to an automatic clamp unit (South Campus Electronics, Dunedin). The clamp units were attached to a Maclab/8s recording unit (ADInstruments Pty Ltd, Melbourne, Australia) which recorded both the short circuit current and tissue resistance using Chart for Widows, version 4.0.1 (ADInstruments Pty Ltd, Melbourne, Australia).

Once the apparatus was assembled, 20 mL of serosal Ringer's solution was added to each apparatus. As there was no tissue dividing the chamber, the Ringer's solution circulated freely on both sides. The Ringer's solutions were mixed and aerated by bubbling carbogen (BOC Gas, Auckland, New Zealand) through each reservoir and were maintained at 37 °C via a water jacket around each chamber. Any air bubbles trapped inside the chamber or lifters were removed, all connections were checked to ensure there were no leaks, and an Rf reading taken by the clamp unit for each apparatus.

Fluid resistance readings outside the expected range of 60 - 150 Ω were considered indicative of a problem with the experimental equipment. Incorrect readings were usually caused by an interruption in the electrical circuit between the Ringer's solution and the clamp unit due to an air bubble in an agar bridge or the Ringer's solution, dilution of the saturated KCl with spilt Ringer's solution or a faulty connection between an electrode and the clamp unit. This interruption was identified and corrected before the experiment continued. Once an acceptable Rf measurement was obtained the apparatus was left for 15-30 minutes to ensure the electrical properties were stable.

3.3.2 Tissue Mounting

At the end of this stabilisation period a new Rf was recorded, the lifter tubes were clamped to prevent the loss of Ringer's solution from the reservoirs and the chambers removed ready for tissue mounting. While the chamber was out of the apparatus the serosal Ringer's solution in the mucosal reservoir was replaced with mucosal Ringer's solution.

A mouse was killed by cervical dislocation just before the tissue was to be mounted in the chambers. The abdomen was opened by a midline incision and the small intestine dissected out. The intestine was then emersed in serosal Ringer's solution at room temperature. The attached muscularis externa was not removed from the intestine. Two-centimetre long sections were cut from the required parts of the intestine and mounted individually in the chambers. Each piece was laid along the half-cell with the mounting pins uppermost and opened longitudinally along the mesenteric border. It was then spread out over the opening and attached to the mounting pins. Care was taken to ensure that the entire opening was covered by tissue and there were no holes. The matching half cell was joined to seal the chamber and the complete chamber reattached to the lifter tubes. The agar bridges were inserted and the Ringer's solution released back into the cells. Care was taken to release both sides at once and to keep the volume of solution equal on both sides to prevent hydrostatic pressure across the tissue.

Once the tissue was mounted and the chamber in place, the p.d. across the tissue was set to zero and tissue resistance (Rt) and short circuit current (I_{sc}) readings were taken. A p.d. of exactly zero was not always possible due to electrically unbalanced calomel electrodes. A p.d. of 0.0 ± 0.5 mV was considered acceptable. During the experiments each automatic clamp unit was set to reject all I_{sc} and Rt readings outside pre-set limits and return an error message. The pre-set limits for these experiments were I_{sc}: 0 to -150 μAmps, Rt: 20 to 200 Ω. The majority of readings outside these limits were caused by problems with the apparatus as described in Section 3.3.1 and were resolved by adjustments to the equipment. However in some cases there was a problem with either the integrity or viability of the tissue. In particular, a Rt below 20 Ω indicated loss of integrity of the intestinal tissue, generally caused by tissue damage during mounting,

while a low I_{sc} indicated that the tissue was no longer actively secreting and therefore no longer viable. In these cases the tissue was discarded.

Once the tissue was successfully mounted and short circuited, it was washed by changing the Ringer's solution on both sides of the tissue three times, taking care to avoid hydrostatic pressure differences across the tissue. This removed any luminal contents adhering to the tissue segments when they were mounted. The chambers were then filled to a pre-measured mark to give exactly 10 mL of solution on each side of the apparatus and left for 45 minutes while the electrophysiological readings stabilised. At the end of this time, both I_{sc} and Rt were recalculated and recorded immediately prior to the addition of the test chemical(s).

3.3.3 Experimental Procedure

All test chemicals were added to the mucosal side of the chamber by replacing a known volume of the mucosal solution with an equal volume of the appropriate nutrient stock solution (Table 4.2). Timing started as soon as the nutrient was added. Approximately three minutes later a 3 mL sample was taken from each mucosal chamber and placed into acid washed 10 mL sample tubes. These were later used to measure the starting concentration of iron. An equal volume of solution was removed from the serosal chamber at the same time and discarded.

The experiment was conducted over a 90 minute period. During that time the tissue was continuously short circuited by the automatic voltage clamp device. Tissue resistance, p.d. and I_{sc} were monitored throughout the experiment and did not deviate from the pre-set limits at any time. During the experiment the equipment was continuously observed and the lifter tubes periodically checked for leaks and to ensure that the Ringer's solution was still circulating.

At the end of the 90 minute period, the Ringer's solution from each reservoir was collected into separate sample tubes and stored at -20 °C for later analysis. Before use all sample tubes were soaked in 5% nitric acid for at least four hours, rinsed three times with RO water and dried for 20 minutes at 30 °C in a DeLongi Airstream Convection Toaster Oven.

3.3.3.1 Test Chemical Combinations

A number of iron salts, sugars and divalent cations were added to the mucosal compartment as shown in Table 3.3. Ferrous gluconate was chosen as the reference test chemical as it is a highly bioavailable form of iron which is absorbed via both active and passive mechanisms (Wessling-Resnick, 2000). It has also been used successfully to demonstrate iron absorption in a previous Ussing chamber experiment (Vaghefi et al., 1998).

Manganese sulphate was added with ferrous gluconate as it has been shown to reduce the active transport of iron via competitive inhibition of the DCT1 protein (Goddard et al., 1997). Calcium chloride has also been shown to inhibit iron uptake, although the exact mechanism is unclear (Barton et al., 1983). Therefore calcium chloride was added with ferrous gluconate to investigate this inhibition. As there was a possibility that calcium would be insoluble in the Ringer's solution, 200 mg/L calcium carbonate was added to 20 mL mucosal Ringer's solution without iron and aerated for 90 minutes with carbogen to investigate the possibility of calcium precipitation during the experimental procedure.

The addition of glucose to the luminal side of intestinal tissue increases the passive transport of many solutes (Fine et al., 1994). To investigate whether glucose also increases the passive absorption of iron the D-mannitol in the mucosal Ringer's solution was replaced with an equal concentration of D-glucose.

Since the gluconate portion of ferrous gluconate may interact with the tight junctions between the cells to increase passive absorption along the paracellular pathway (Madara, 1998), ferrous gluconate absorption was compared with equal concentrations of ferrous sulphate. Ferric iron may exhibit lower levels of absorption than ferrous iron due to it's reduced solubility at the near neutral pH used in these experiment. Therefore ferrous gluconate was replaced with an equal concentration of ferric chloride.

To investigate non-specific tissue effects the small intestine was replaced with caecum and large intestine, as these latter tissues are known not to absorb significant amounts of iron *in vivo*.

Table 3.2: Experimental procedures which were used to investigate iron absorption by the mouse intestine.

Test Chemical(s)	*Final Concentration (mg/L)*	*Stock Solution (mg/L)* *	*Number of Mice*	*Number of tissue pieces per mouse*	*Number of Control Experiments*	*Other modifications*
ferrous gluconate	27.9	558	11	8 consecutive pieces along the length of the small intestine	5	
ferrous gluconate	9.3	186	7	8 consecutive pieces along the length of the small intestine	5	
ferrous gluconate + manganese sulphate	9.3 91.5	186 1830	1	8 consecutive pieces along the length of the small intestine		
ferrous gluconate + manganese sulphate	9.3 9.15	186 183	7	8 consecutive pieces along the length of the small intestine	6	
ferrous gluconate + calcium chloride	9.3 200	186 4000	6	8 consecutive pieces along the length of the small intestine	4	
ferrous gluconate	9.3	186	6	8 consecutive pieces along the length of the small intestine		1.8 g D-glucose added to mucosal Ringers in place of 1.8 g D-mannitol
ferrous sulphate	9.3	186	5	8 consecutive pieces along the length of the small intestine	4	
ferric chloride	9.3	186	6	8 consecutive pieces along the length of the small intestine	7	
ferrous gluconate	9.3	186	2	1 pieces of caecum and 3 pieces of large intestine		
ferrous gluconate	9.3	186	1	4 jejunum		after experiment, tissue was fixed and stained
ferrous gluconate	9.3	186	3	8 consecutive pieces along the length of the small intestine		intestine and mucus was collected, digested and analysed

* 0.5 mL added to the mucosal reservoir. Chemical suppliers listed in Appendix A

3.3.4 Total Recovery Experiments

Mucus is secreted by the intestine and has been found to bind iron (Conrad et al., 1991). Therefore iron may be binding to secreted mucus instead of being absorbed by the intestinal tissue. To investigate the proportions of iron found in the mucus and the intestinal tissue, a series of experiments was conducted where the intestinal tissue and secreted mucus were collected for analysis after the experiment along with the usual Ringer's solution samples. During these experiments 9.3 mg/L ferrous gluconate was added to the mucosal compartment and intestinal tissue from Swiss mice was mounted in the chambers instead of tissue from Balb/c mice. The experimental procedure was carried out as described in Section 3.3.3 and then the intestinal segments and secreted mucus collected as follows.

The segments of intestinal tissue were removed from each chamber and trimmed, leaving only the tissue that had been exposed to the Ringer's solution. The tissue was washed in 5% HNO_3 to remove the mucus and prevent iron loss, dried overnight at 50 °C in a DeLongi Airstream Convection Toaster Oven, and then stored at -20 °C until assayed. Before the iron content of the tissue was measured, each piece of tissue was thawed and then digested by boiling the tissue with 69% HNO_3 (BDH Laboratory Supplies, Poole, England) on a hot plate (Selby-Ratec, Britain) placed in a fume cupboard until the acid evaporated leaving a yellow or white ash. The ash was then made up to 5 mL with concentrated nitric acid ready for analysis.

The mucus was collected by washing both the segment of intestinal tissue and the interior of the apparatus with 15 mL of 5% HNO_3 and digested following the same procedure as for the intestinal tissue.

3.3.5 Control Experiments

Control experiments during which there was no tissue mounted in the chambers were carried out for all test chemical combinations. The chambers and reservoirs were assembled and fluid resistance readings taken as described in Section 3.3.1. The test chemical was added to each chamber as described in Section 3.3.3. However, as there was no tissue dividing the chamber the test chemical circulated through both compartments. Therefore twice as much stock solution was added to give the same overall concentration in the Ringer's solution. The experiment was conducted over

ninety minutes, and start and end samples collected and analysed as outlined in Sections 3.3.3 and 3.3.6.

During the course of this study, opportunity arose to measure baseline iron levels in mouse small intestine by inductively coupled plasma emission spectrophotometry (ICP) at AgResearch, Palmerston North, New Zealand. The small intestine was removed from the mouse and cut into two centimetre long segments. Each segment was rinsed in 5% nitric acid to removed the mucus and mounted in an Ussing chamber to match tissue size to aperture size. After being left in the chamber for five minutes to allow the tissue edges to be compressed, the tissue was removed and excess trimmed off as it would be after a normal experiment. Each piece was dried at 50 °C in a DeLongi Airstream Convection toaster oven until the dry weight was stable. Each piece was dissolved in 0.5 mL concentrated nitric acid (Aristar, Merck, 69%) and left overnight. The following day they were heated to 60 °C ± 6 °C for four hours in a convection oven (Delonghi #95 FLC) within a fume cupboard. The resulting ash was allowed to cool for 1 hour, then dissolved in 5.5 mL deionised water and iron content determined using a Varian VISTA CCD Simultaneous Induced Coupled Plasma Optical Emission Spectrometer.

3.3.6 Sample Analysis

The iron content of all intestinal tissue, secreted mucus and Ringer's solution samples was determined by flame atomic absorption spectrophotometry using a GBC 933AA Flame Atomic Absorption Spectrophotometer (FAAS). The detection limits of the spectrophotometer were 0.5 - 10.5 mg/L. All measurements contained a percentage error taken from the FAAS calibration graph. Before analysis all samples were diluted as necessary to ensure the iron concentration was within this range.

3.3.7 Histological Examination of Intestinal Tissue

Histological examination of the tissue allows the direct investigation of tissue integrity. An experiment was conducted using four pieces of jejunum from one mouse. Two chambers had 9.3 mg/L ferrous gluconate added to the mucosal compartment as the test chemical and the other two did not have a test chemical added. The experimental procedure was carried out as described in Section 3.3.3. At the end of the experimental period the intestinal segments were removed from the chambers and immediately fixed for 12 hours in 10 % formal saline. The tissue were then impregnated with paraffin

using an automatic tissue processor (Leica Jung TP105) and mounted in paraffin blocks. Sections 6 μm thick were cut with a rotary microtone (Leitz Wetzlar) and placed on slides. These were stained with either Perl's Prussian blue reaction or haematoxylin and eosin, mounted with DPX mountant (BDH Ltd, Poole, England) and cover slipped. The sections were then studied using an Olympus CHS microscope.

Sections prepared from jejunal segments which had not been exposed to iron were stained using haematoxylin and eosin (Culling, 1985; see Table 3.3) and examined to assess tissue integrity.

Table 3.3: Method for staining sections with haematoxylin and eosin.

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

Sections taken from all jejunal segments were stained using Perl's Prussian blue reaction (Sheehan and Hrapchak, 1987; see Table 3.4). This reacts with storage iron such as hemosiderin to demonstrate iron within the tissue and may be used to localise iron which has been absorbed by the enterocytes (Culling, 1985). A sample of spleen was also stained as a positive control, as this tissue contains large concentrations of iron due to the macrophage digestion of the red blood cells within the red pulp.

Table 3.4: Method for staining sections with Perl's Prussian blue reaction (Lillie's technic for Turnbull's blue reaction - Sheehan and Hrapchak, 1987).

Process	*Reagent*	*Time*
Deparaffinising	xylene	2 changes x 7 minutes
	absolute ethanol	3-5 seconds
	70% ethanol	3-5 seconds
	tap water	3-5 seconds
Staining	400 mg potassium ferricyanide dissolved in 40 mL of 0.06M HCl	60 minutes
	1% acetic acid or 0.01M HCl	3-5 seconds
	neutral red	5-10 minutes
	distilled water	3-5 seconds
Differentiate and dehydrate	70% ethanol	3-5 seconds
	absolute ethanol	2 changes x 3-5 seconds
	xylene	2 changes x 3-5 seconds

3.3.8 Preparation and Care of Equipment

If more than one experiment was to be carried out on the same day the chambers, lifter tubes and reservoirs were rinsed between experiments with 5% nitric acid followed by RO water.

At the end of each day, the half cells were soaked overnight in Pyroneg detergent (DiverseyLever, Auckland, New Zealand), rinsed thoroughly in RO water and stored until needed. The inner surface of the reservoirs and lifter tubes were washed with 20 mL Pyroneg detergent (DiverseyLever, Auckland, New Zealand) then rinsed with RO water until all traces of detergent were gone. They were then rinsed three times with 5% nitric acid followed by three times with RO water. The carbogen intake tubes were also rinsed with 1% nitric acid followed by RO water.

Between experiments each pair of calomel electrodes was immersed in saturated KCl solution and short circuited to prevent a p.d. from building up between them. The silver wire electrodes were re-chlorided by soaking overnight in sodium hypochlorite solution (Janola, SaraLee, Auckland, New Zealand).

3.4 Data analysis and presentation

Analysis of intestinal and acid wash samples was carried out in the total recovery experiments, otherwise only the Ringer's solutions were analysed. The data for each test chemical are presented as a graph of the average amount removed from the mucosal chamber for each intestinal segment. The segments extend consecutively from the pyloric sphincter to the ileum and are numbered according to their location along the intestine (Figure 3.3). Data for each test chemical were then grouped into three intestinal regions, proximal, mid and distal, representing the physiological sections along the length of the intestine.

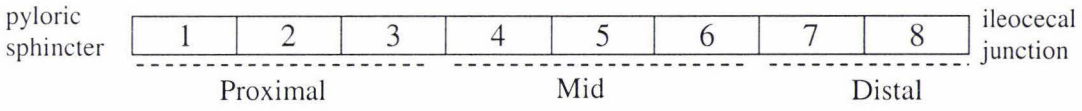

Figure 3.3: Consecutive intestinal segments used in experiments, grouped into regions.

Two way Analysis of Variance (ANOVA) was used to compare the results for each test chemical with those for each intestinal region. Variation was measured both within the results for each intestinal region and within the results for each test chemical. This analysis was preformed using SAS Statistical Analysis Package and significance was set at $P \leq 0.1$. Unpaired two-tailed Student's T-tests were used to measure the variation between average iron removed per intestinal region for 9.3 mg/L ferrous gluconate and all other test chemicals in turn. Unpaired two-tailed Student's T-tests were also used to measure variation between iron removed with each intestinal segment for all test chemicals. One way ANOVA was used to look for differences in iron uptake by each intestinal segment and within each intestinal region for all test chemicals. These analyses were performed using Microsoft Excel 97 (Microsoft Corporation, Washington, USA) and significance was set at $P \leq 0.05$.

4. RESULTS

Three hundred and twenty five intestinal segments were used for these experiments, representing duodenum, jejunum and ileum. A reduction of the concentration of iron in the mucosal Ringer's solution occurred in all experiments. However no iron was detected in the serosal compartments. Control experiments, without tissue in the chambers, were carried out for each combination of test chemicals and demonstrated that the iron lost from the mucosal solutions when tissue was mounted in the chambers was not due to iron binding to the chambers or lifters, but was absorbed, or bound, by the intestinal tissue. Raw data obtained is presented in Appendix B.

4.1 Electrophysiology

Fluid resistance averaged 112.3 Ω (n=341, se=0.8), and ranged from 79.9 - 148.3 Ω. The p.d. across the tissue was maintained at 0.0 ± 0.5 mV throughout the experiment. After the tissue was mounted and washed, both I_{sc} and Rt exhibited an initial drop and stabilised after 30-40 minutes. Starting values for both I_{sc} and Rt were recorded after this stabilisation period and immediately before the addition of the test chemicals at the start of the experimental period, and were monitored throughout the experiment (Table 4.1).

Table 4.1: Summary of electrophysiological readings for all experiments. Starting values were measured immediately before the addition of the test chemical(s). Change in I_{sc} was measured continuously over the 90 minute experimental period. All values are mean ± se (number of intestinal segments).

Added to the mucosal compartment	*Starting I_{sc} ($\mu Amp.cm^{-2}$)*	*Change in I_{sc} ($\Delta \mu Amp.cm^{-2}$)*	*Rt ($\Omega.cm^{-2}$)*
mannitol	-47 ± 3.5 (156)	1 ± 0.5	90 ± 3.5 (166)
glucose	-59 ± 7.0 (16)	23 ± 5.9 (16)	81 ± 1.5 (16)

The starting values for both I_{sc} and Rt were similar for all experiments. There was no significant change in Rt during the 90 minute experimental period. The I_{sc} recorded during experiments in which mannitol was added to the mucosal solution did not change significantly. By contrast tissues that had glucose added to the mucosal compartment exhibited a continuous increase in I_{sc} throughout the experimental period. This difference can be clearly seen when comparing the electrophysiological recording for two experiments with 9.3 mg/L ferrous gluconate during which mannitol (Figure 4.1) or

glucose (Figure 4.2) was present in the mucosal solution. All electrophysiological readings are presented in Appendix C.

4.2 Ferrous Gluconate

When 27.9 mg/L ferrous gluconate was added to the mucosal compartment the average amount of iron removed by each intestinal segment ranged from 45% to 56% of the starting value (Figure 4.3). There was no significant variation between results for individual segments (P=0.49).

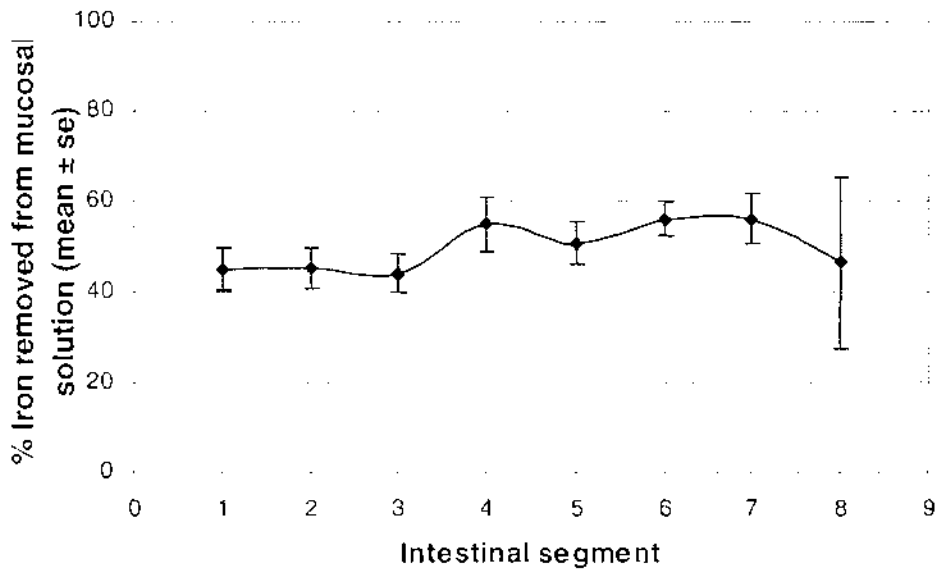

Figure 4.1: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 27.9 mg/L ferrous gluconate was added to the mucosal compartment (each point is the mean for 2-11 observations).

Control experiments (no tissue in the chambers) during which 27.9 mg/L ferrous gluconate was added to the Ringer's solution showed there was no significant change in iron concentration after the 90 minute experimental period (Table 4.2).

Table 4.2: Change in iron concentration after 90 minutes during the control experiments in which 27.9 mg/L ferrous gluconate was added to the Ringer's solution. Measurement errors were calculated from the calibration graph of the FAAS.

Chamber Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*	*Measurement Error (%)*
1	27.9	28.7	2.9	12
2	27.9	28.6	2.5	12
3	27.9	25.4	-9.0	12
4	27.9	25.5	-8.6	12
5	27.9	25.3	-9.3	12
mean ± se	27.9 ±0.0	26.7 ± 0.8	-4.3 ± 2.9	12

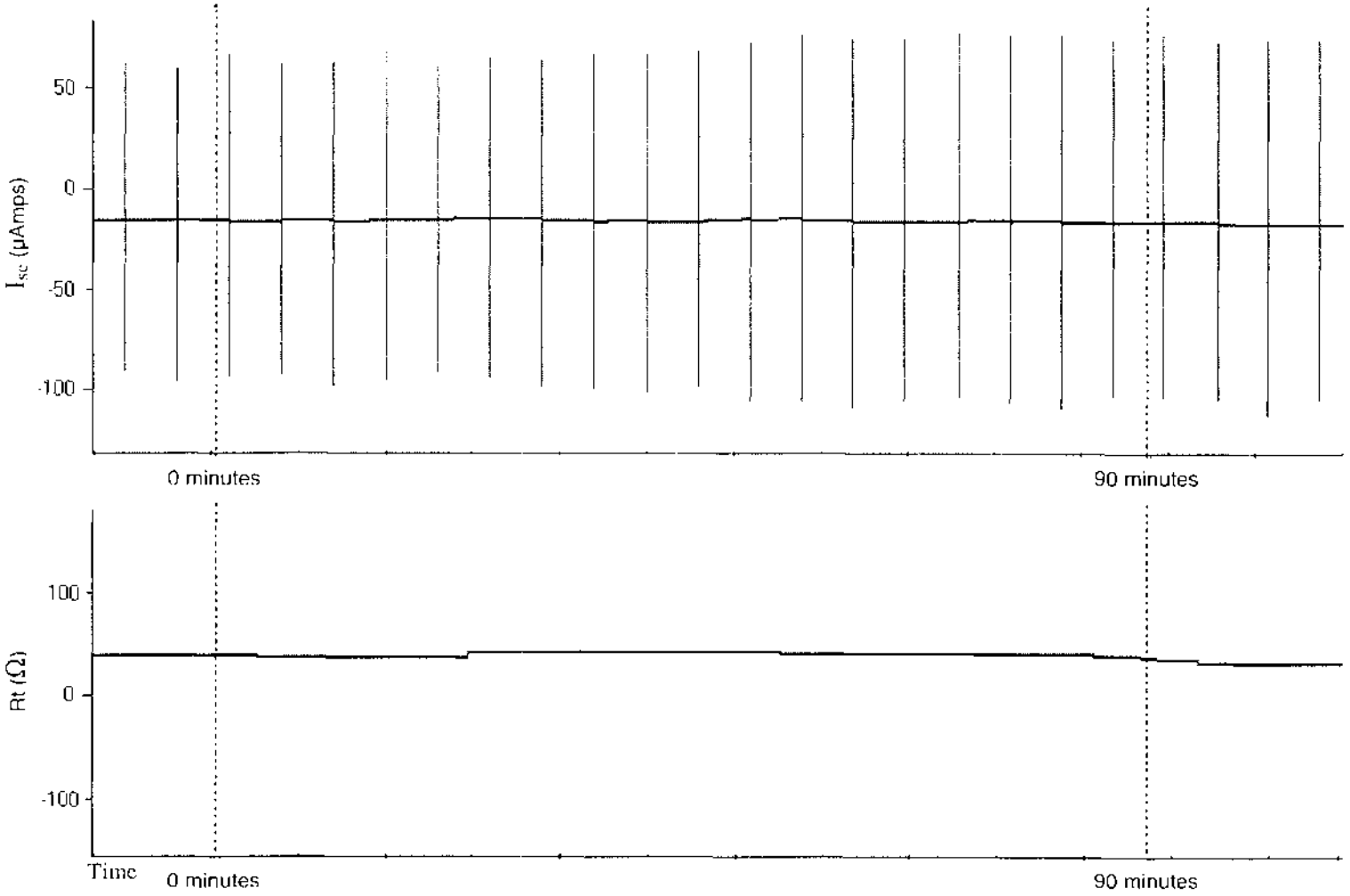

Figure 4.2: Chart recording of I_{sc} and Rt for a typical experiment with mannitol (10 mM) added to the mucosal Ringer's solution. 9.3 mg/L ferrous gluconate was added at time 0 and the experiment ended after 90 minutes. The vertical lines on the I_{sc} trace are bi-directional pulses of current passed through the chamber to calculate tissue resistance.

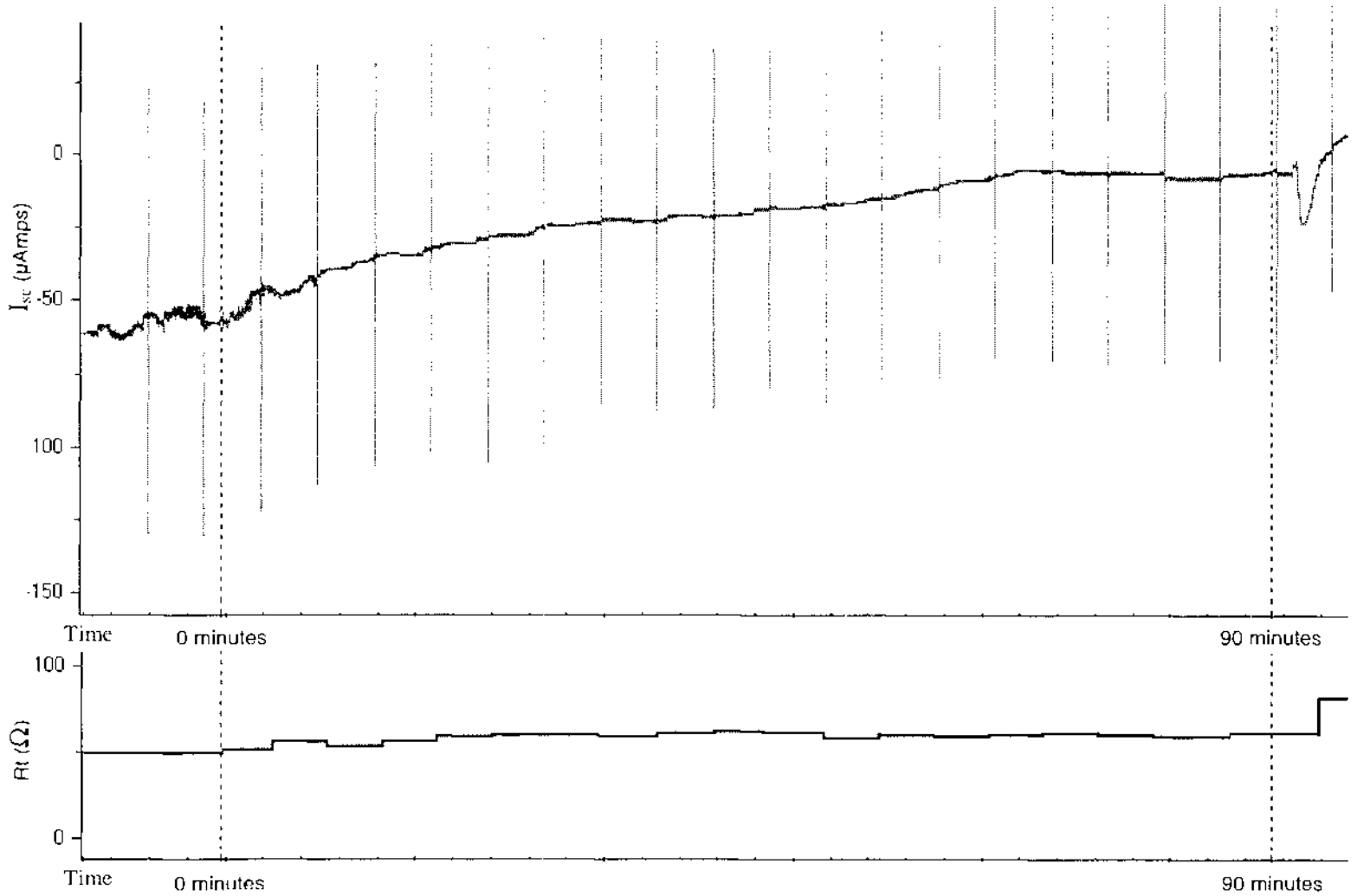

Figure 4.2: Chart recording of I_{sc} and Rt for a typical experiment with glucose (10 mM) added to the mucosal Ringer's solution. 9.3 mg/L ferrous gluconate was added at time 0 and the experiment ended after 90 minutes. The vertical lines on the I_{sc} trace are bi-directional pulses of current passed through the chamber to calculate tissue resistance.

When 9.3 mg/L ferrous gluconate was added to the mucosal compartment the percent iron removed from the mucosal compartment remained high, with averages between 39% and 64% (Figure 4.4). There was a small but significant variation between results for individual intestinal segments (P=0.04). This was caused by the average percentage of iron removed by the first intestinal segment being significantly (P<0.05) lower than the average percentage removed by all other segments; there was no significant variation between segments 2 to 8.

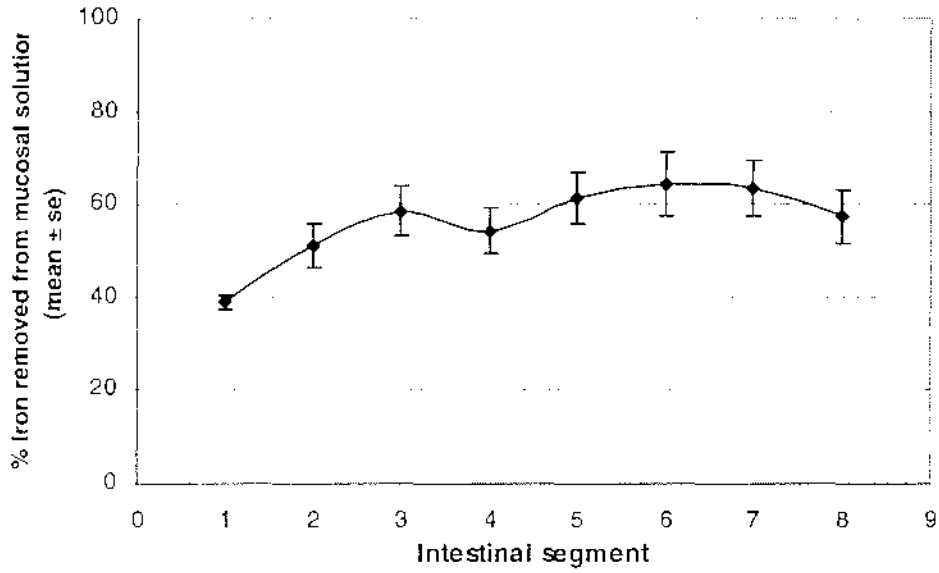

Figure 4.4: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferrous gluconate was added to the mucosal compartment (each point is the mean of 3-7 observations).

Control experiments during which 9.3 mg/L ferrous gluconate was added to the Ringer's solution showed there was no change in the iron concentration after the 90 minute experimental period (Table 4.3).

Table 4.3: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous gluconate was added to the Ringer's solution. Measurement errors were calculated from the calibration graph of the FAAS.

Chamber Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*	*Measurement error (%)*
1	9.2	9.0	-1.3	8
2	7.8	7.6	-2.5	8
3	8.0	8.0	-0.1	8
4	8.8	8.5	-3.6	8
5	8.2	8.0	-2.1	8
mean ± se	8.4 ± 0.3	8.2 ± 0.2	-1.9 ± 0.6	8

4.3 Ferrous Gluconate plus Manganese Sulphate

When a tenfold excess of manganese sulphate (91.5 mg/L) was added to the mucosal compartment together with the ferrous gluconate (9.3 mg/L), there was a wide variation in the percent iron removed from the mucosal compartment (Table 4.4). This was due to the formation of a powdery precipitate visible in the mucosal compartment and in the mucosal Ringer's samples collected for analysis. This precipitate could be seen coating the intestinal tissue and preventing circulation of Ringer's solution through the lifter tubes. No further experiments were carried out with this combination of trace elements.

Table 4.4: Change in iron concentration per intestinal segment after 90 minutes when 9.3 mg/L ferrous gluconate and 91.5 mg/L manganese sulphate were added to the Ringer's solution. Measurement errors were calculated from the calibration graph of the FAAS.

Intestinal Segment	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*	*Measurement error (%)*
2	5.9	5.9	0	2
3	6.0	4.9	- 18.5	2
4	6.6	5.0	- 24.7	2
5	5.4	2.3	- 58.5	2
6	6.7	4.3	- 35.3	2
mean ± se	6.1 ± 0.2	4.5 ± 0.6	-27.4 ± 9.7	2

There was no precipitation present when the concentration of manganese sulphate was lowered to 9.15 mg/L and added with 9.3 mg/L ferrous gluconate to the mucosal compartment. The percent iron removed from the mucosal compartment ranged between 31% and 60% (Figure 4.5). The amount of iron removed by the proximal part of small intestine tended to be lower than that removed by the mid or distal segments, however there was no significant variation between results for each intestinal segments (P=0.16).

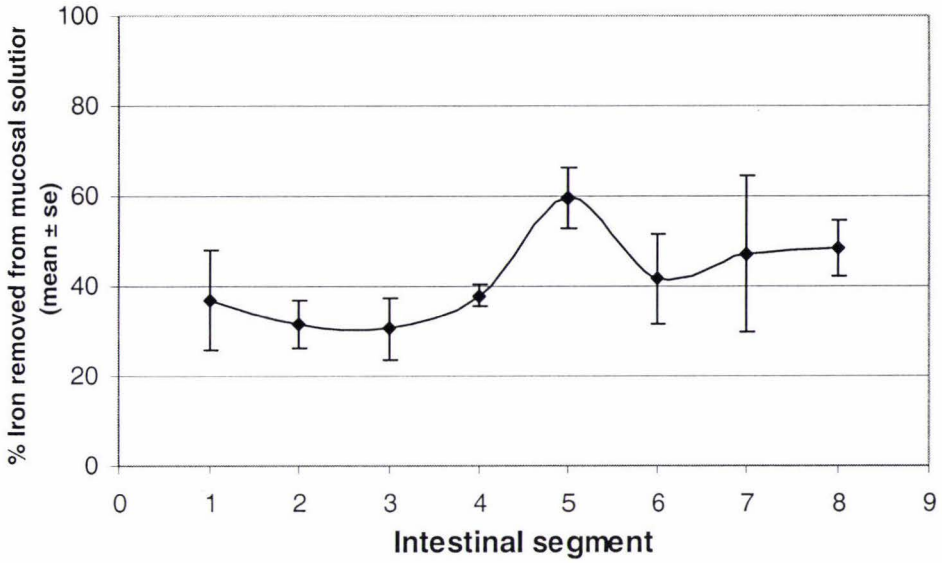

Figure 4.5: Percentage iron removed from the mucosal Ringer's solution when 9.3 mg/L ferrous gluconate and 9.15 mg/L manganese sulphate were added to the mucosal compartment (each point is the mean of 2-7 observations).

Control experiments with 9.15 mg/L manganese sulphate and 9.3 ferrous gluconate added to the Ringer's solution showed no change in iron concentration after the 90 minute experimental period (Table 4.5).

Table 4.5: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous gluconate and 9.15 mg/L manganese sulphate were added to the Ringer's solution. Measurement errors were calculated from the calibration graph of the FAAS.

Chamber Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*	*Measurement error (%)*
1	7.1	6.8	-4.3	6
2	7.9	7.6	-4.2	6
3	8.9	9.0	0.6	6
4	7.4	7.3	-1.1	6
5	8.4	8.3	-1.2	6
mean ± se	7.9 ± 0.3	7.8 ± 0.4	-2.0 ± 1.0	6

4.4 Ferrous Gluconate plus Calcium Chloride

The percentage of iron removed from the mucosal compartment when 200 mg/L calcium chloride and 9.3 mg/L ferrous gluconate were added to the mucosal Ringer's solution ranged between 52% and 69% (Figure 4.6), with no significant variation between results for individual intestinal segments (P=0.99).

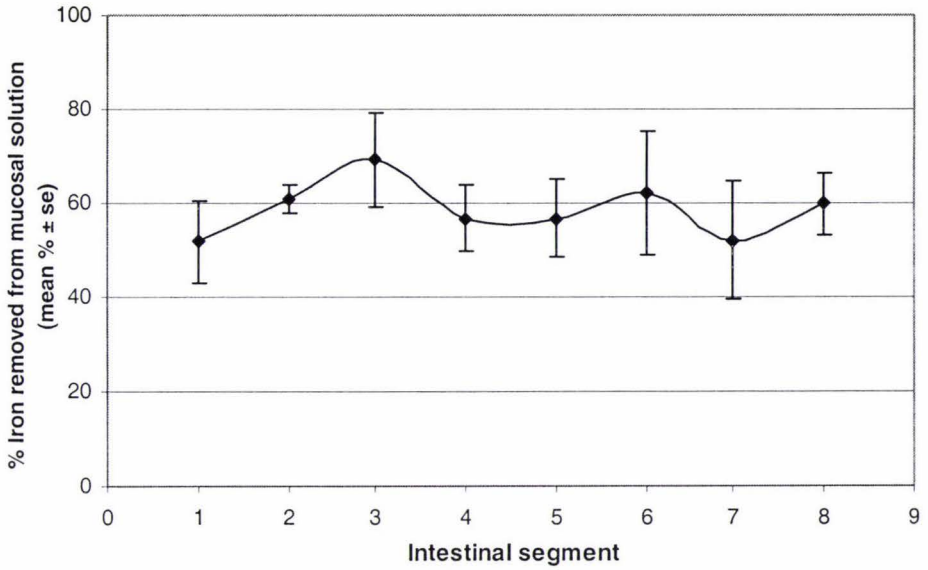

Figure 4.6: Percentage iron removed from the mucosal Ringer's solution when 9.3 mg/L ferrous gluconate and 200 mg/L calcium chloride were added to the mucosal compartment (each point is the mean of 2-6 observations).

A small amount of white precipitate formed in the mucosal compartment during the experiment and was present in the mucosal Ringer's samples at the end of the experiment. This precipitate also formed during control experiments when 9.3 mg/L ferrous gluconate and 200 mg/L calcium chloride was added to the Ringer's solution. These control experiments showed a change in iron concentration after the 90 minute experimental period which could not be accounted for by measurement error (Table 4.6).

Table 4.6: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous gluconate and 200 mg/L calcium chloride were added to the Ringer's solution. Measurement errors were calculated from the calibration graph of the FAAS.

Chamber Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*	*Measurement error (%)*
1	6.2	5.9	-5.6	6
2	6.7	4.7	-29.1	6
3	6.1	3.6	-41.2	6
4	6.4	4.4	-31.8	6
mean ± se	6.4 ± 0.1	4.7 ± 0.5	-26.9 ± 7.6	6

4.5 Ferrous Gluconate plus Glucose

When the mannitol in the mucosal Ringer's solution was replaced with an equal concentration of glucose (10 mM) and 9.3 mg/L ferrous gluconate was added to the

mucosal compartment, the percent iron removed from the mucosal compartment ranged from 33% and 50% (Figure 4.7). There was no significant variation between results for individual intestinal segments (P=0.75).

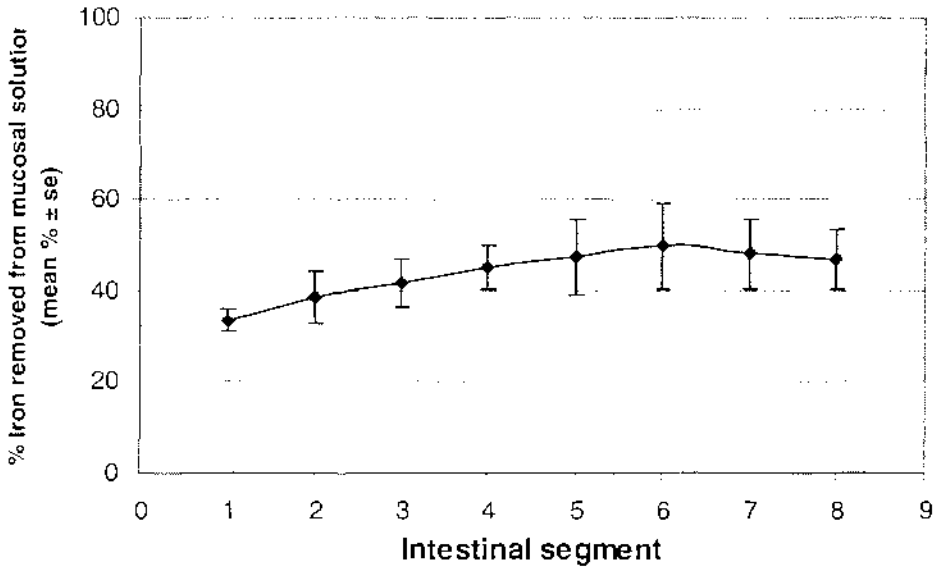

Figure 4.7: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferrous gluconate and 10 mM glucose were added to the mucosal compartment (each point is the mean of 4-6 observations).

4.6 Ferrous Sulphate

Although there appeared to be a wide variation in the average percentage of iron removed from solution by each intestinal segment when 9.3 mg/L ferrous sulphate was added to the mucosal reservoir, with averages ranging between 29% and 64% (Figure 4.8), there was no significant difference (P=0.30) between the results for each intestinal

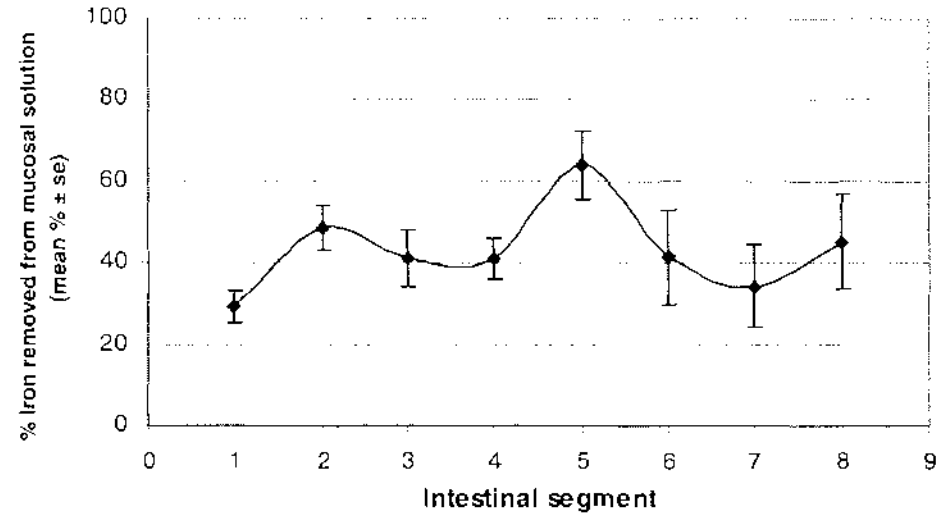

Figure 4.8: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferrous sulphate was added to the mucosal compartment (each point is the mean of 3-5 observations).

segment. There was also no change in iron concentration over the 90 minutes experimental period of the control experiments when 9.3 mg/L ferrous sulphate was added to the Ringer's solution (Table 4.7).

Table 4.7: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferrous sulphate was added to the Ringer's solution. Measurement errors were calculated from the calibration graph of the FAAS.

Chamber number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*	*Measurement error (%)*
1	8.8	8.8	0.0	6
2	8.3	7.9	-4.8	6
3	8.9	8.7	-2.2	6
4	8.4	8.1	-2.7	6
mean ± se	8.6 ± 0.1	8.4 ± 0.2	-2.4 ± 1.0	6

4.7 Ferric Chloride

When 9.3 mg/L ferric chloride was added to the mucosal reservoir in place of ferrous gluconate, there was a large variation in percent iron removed from the mucosal compartment, with averages ranging between 35% and 55% (Figure 4.9).

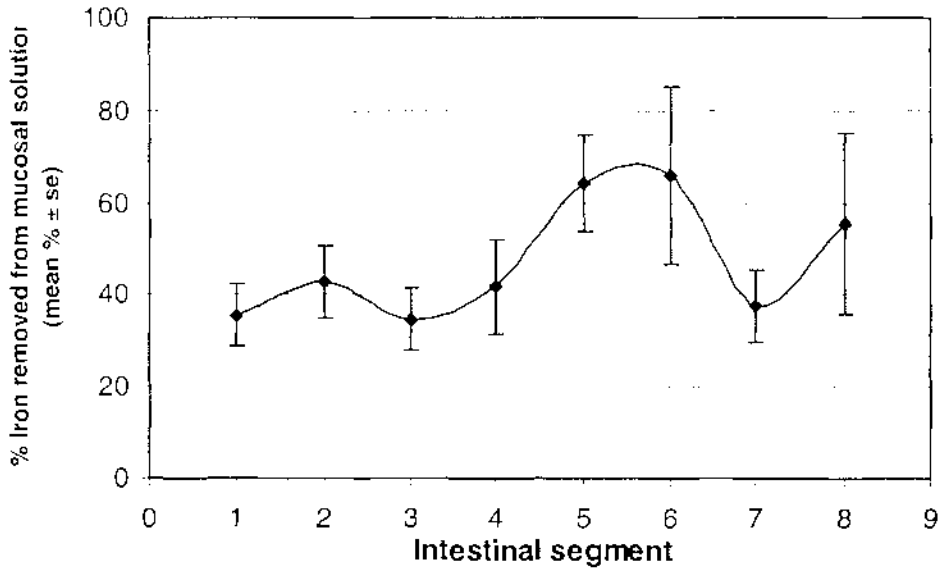

Figure 4.9: Percentage iron removed from the mucosal Ringer's solution by each intestinal segment when 9.3 mg/L ferric chloride was added to the mucosal compartment (each point is the mean of 4-6 observations).

These averages had unusually high standard errors, from 7 to 20%. The iron concentrations in samples taken at the start of the experiment were inconsistent, ranging from 3.8 - 6.7 mg/L instead of the expected 9.3 mg/L. Examination of the samples

before analysis showed yellow crystalline precipitation in some of the sample tubes. It is likely that the precipitate was composed of insoluble ferric iron.

There were high levels of iron loss during control experiments with 9.3 mg/L ferric chloride added to the Ringer's solution (Table 4.8). This variation could not be accounted for by measurement errors of the FAAS.

Table 4.8: Change in iron concentration after 90 minutes during the control experiments in which 9.3 mg/L ferric chloride was added to the Ringer's solution. Measurement errors were calculated from the calibration graph of the FAAS.

Chamber number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*	*Measurement error (%)*
1	10.4	6.8	-34.5	6
2	4.0	5.1	27.1	6
3	6.1	3.1	-50.0	6
4	3.3	3.4	0.9	6
5	3.5	3.1	-11.7	6
6	2.5	4.3	70.1	6
7	3.4	4.3	26.8	6
mean ± se	4.7 ± 1.0	4.3 ± 0.5	4.11 ± 5.5	6

4.3 Regional Comparisons

Because there was no significant variation between the results for the different intestinal segments within each trace element/glucose combination it is possible to group these data according to intestinal region (proximal, mid or distal small intestine) (Figure 4.10).

There was significant variation (P=0.08) in the average percent iron removed per intestinal region when 9.3 mg/L ferrous gluconate was added to the mucosal chamber. This was due to iron absorption by the proximal region being significantly lower than that by both the mid (P=0.015) and distal (P=0.02) regions. Although there was no overall significant difference between regions when 27.9 mg/L ferrous gluconate was added to the mucosal difference between regions when 27.9 mg/L ferrous gluconate was added to the mucosal solution, a paired Student's T-test showed a significant difference between the proximal region and the mid region (P=0.035) with this concentration of iron.

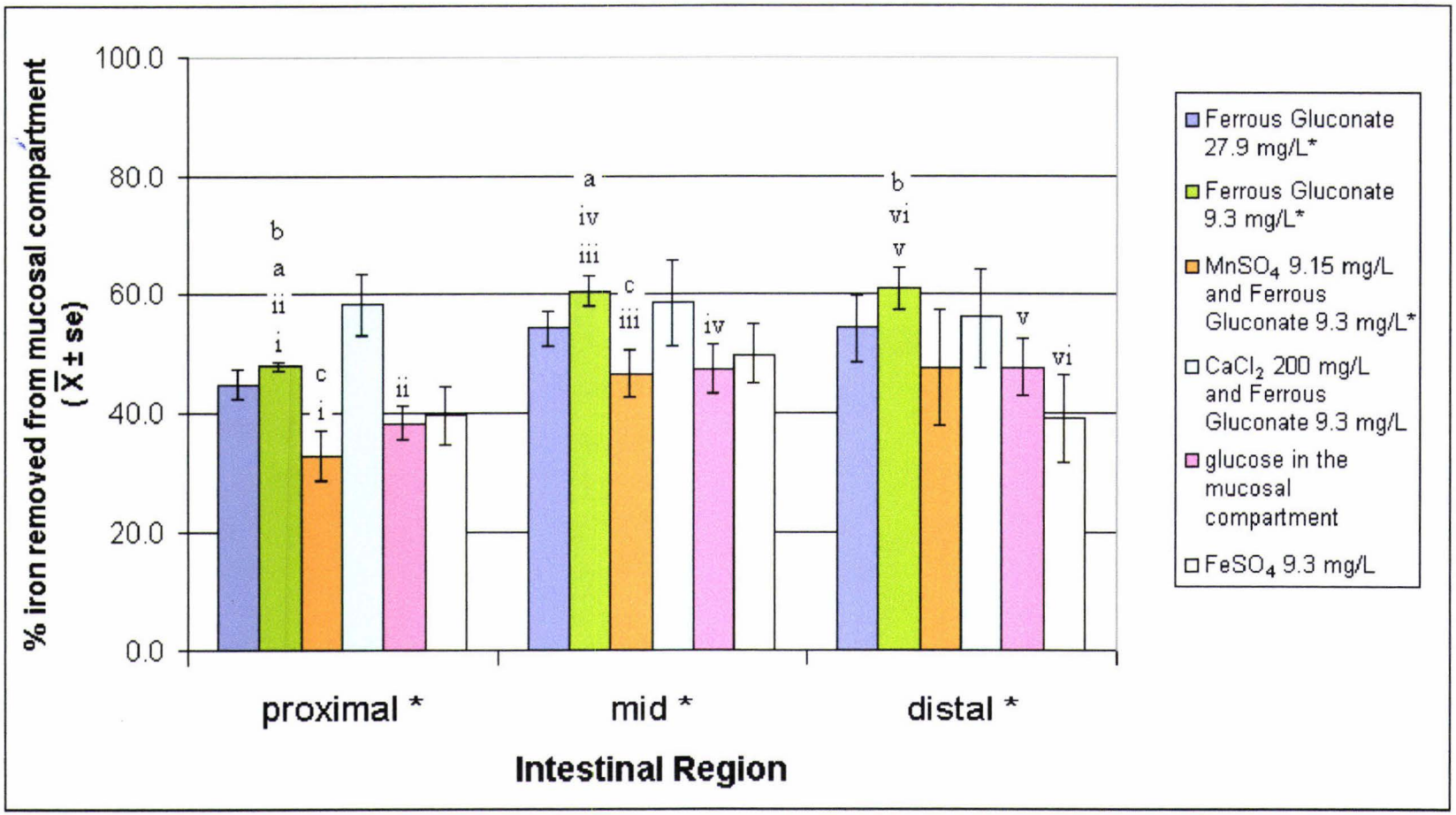

Figure 4.10: Average percent iron removed from the mucosal compartment, grouped by test chemical and intestinal region. Pairs with the same superscript are significantly different. Within intestinal region: i: P=0.006, ii: P=0.03, iii: P=0.02, iv: P=0.03, v: P=0.02. Within test chemical: a: P=0.01, b: P=0.02, c: P=0.02, d: P=0.035.

There was also significant variation (P=0.04) in the average percent iron removed per intestinal region when 9.15 mg/L manganese sulphate plus 9.3 mg/L ferrous gluconate were added to the mucosal compartment. This was due to iron removal being significantly lower in the proximal region than the mid (P=0.02) but not the distal (P=0.12) regions. There was a significant drop in the average percentage iron removed by the proximal (P=0.006) and mid (P=0.02) regions with this trace element combination when compared to 9.3 mg/L ferrous gluconate alone.

The average percentage iron removed by the proximal (P=0.03) and mid (P=0.03) intestinal regions was significantly lower with glucose added to the mucosal Ringer's solution than with mannitol. There was also a weakly significant (P=0.06) decrease in iron absorbed by the distal intestinal region when glucose was added.

4.4 Non-Absorbing Tissue

Iron was removed from the mucosal Ringer's solution when tissue from the caecum or large intestine was mounted in the chambers, and 9.3 mg/L ferrous gluconate added to the mucosal compartment. The mean percentage of iron removed by the caecum was 43% ± 1% (n=6) by the large intestine was 38% ± 5% (n=2). This was within the range of iron removed by small intestinal tissues.

4.5 Tissue Staining

Histological examination of segments of jejunum removed from the chamber after a typical 90 minute experimental procedure (as described in Section 3.3.7) showed that the tissue remained intact and with normal morphological characteristics (Figure 4.11) and comparison with jejunal tissue which had not been used in an experiment (Figure 4.12) showed no significant differences. The villi and underlying submucosa were present and enterocytes appear to have intact cell membranes. While there was some tissue damage, the cell fragments were still present on the slide indicating this damage occurred during tissue processing rather than during the experiment. The muscularis externa was intact and attached to the tissue.

Jejunal segments were also stained using Perl's Prussian blue reaction, however, there was very little iron staining present. There was no qualitative difference in staining between tissue which had been exposed to iron during the experimental procedure

(Figure 4.13) and tissue which had not (Figure 4.14). A sample of splenic tissue was stained using Perl's Prussian blue reaction as a positive control to demonstrate the ferrous iron binding action of this process (Figure 4.15). Large areas of blue were present where the stain had bound to iron released from erythrocytes by the macrophages in the red pulp.

4.6 Total Iron Recovery Experiments

Mucus was secreted by the intestinal tissue into the mucosal Ringer's solution during all experiments and could be seen floating in solution, adhering to the interior of the chambers, lifters and reservoirs and coating the tissue's mucosal surface. After the experiment, the mucus was collected by rinsing the intestinal tissue and the interior of the apparatus with 1% nitric acid. The secreted mucus, intestinal tissue, mucosal Ringer's solution and serosal Ringer's solution were analysed to determine iron content.

The average percentage of iron added to the mucosal solution found in each of these samples after the experimental period were: nitric acid with secreted mucus 17.4% ± 2.0% (n=18); intestinal tissue 5.7% ± 0.8% (n=17); end mucosal solution 74.7% ± 2.3% (n=18) and end serosal solution 2.3% ± 0.3% (n=18). The concentration of iron detected in the wash samples was significantly higher than the concentration in the intestinal tissue sample ($P<0.01$). The average percentage of iron removed from the mucosal compartment by all intestinal segments in these experiments was 33.6% ± 4.6% (n=17). The average recovery of the iron which had been added to the mucosal chamber at the start of the experiment was 88.3% ± 4.4% (n=18).

4.7 Baseline Intestinal Iron Contents

When control pieces of small intestine were analysed using ICP, the average iron content per segment ranged from 125.8 µg/g to 371.0 µg/g of dry tissue (Figure 4.15), with an overall average iron concentration per sample of 1.11×10^{-05} mg/L. As this was below the detection limits of the FAAS it did not contribute significantly to the intestinal iron concentrations found in experimental samples. Although iron concentration tended to be higher in the proximal region of the small intestine, there was no significant difference between individual intestinal segments ($P=0.4$). However,

when the data were grouped by physiological region (proximal, mid and distal small intestine), the proximal small intestine contained significantly more iron than the mid (P=0.007) or distal (P=0.009) regions. Raw data from ICP analysis are presented in Appendix D.

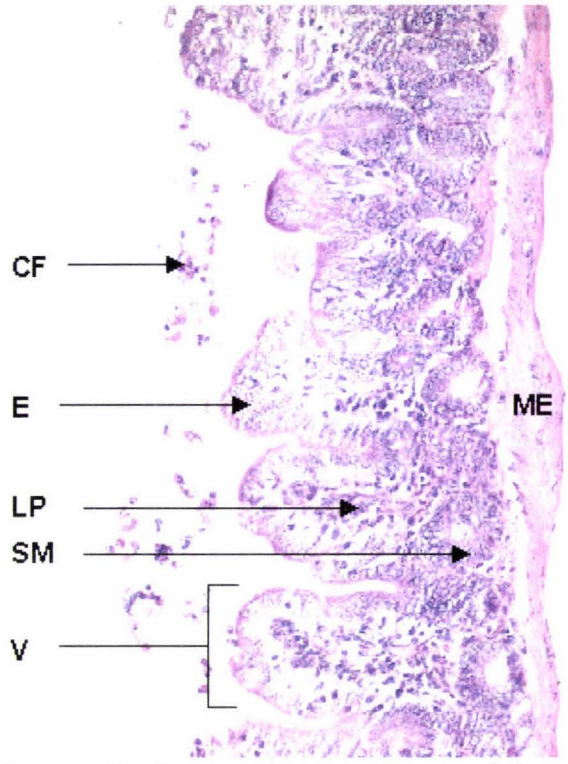

Figure 4.11: Photomicrograph of a cross section of mouse jejunum after being used in an Ussing chamber experiment, stained with H&E, magnification x200. CF = cell fragments; E = epithelia; LP = lamina propria; SM = submucosa; V = villi; ME = muscularis externa.

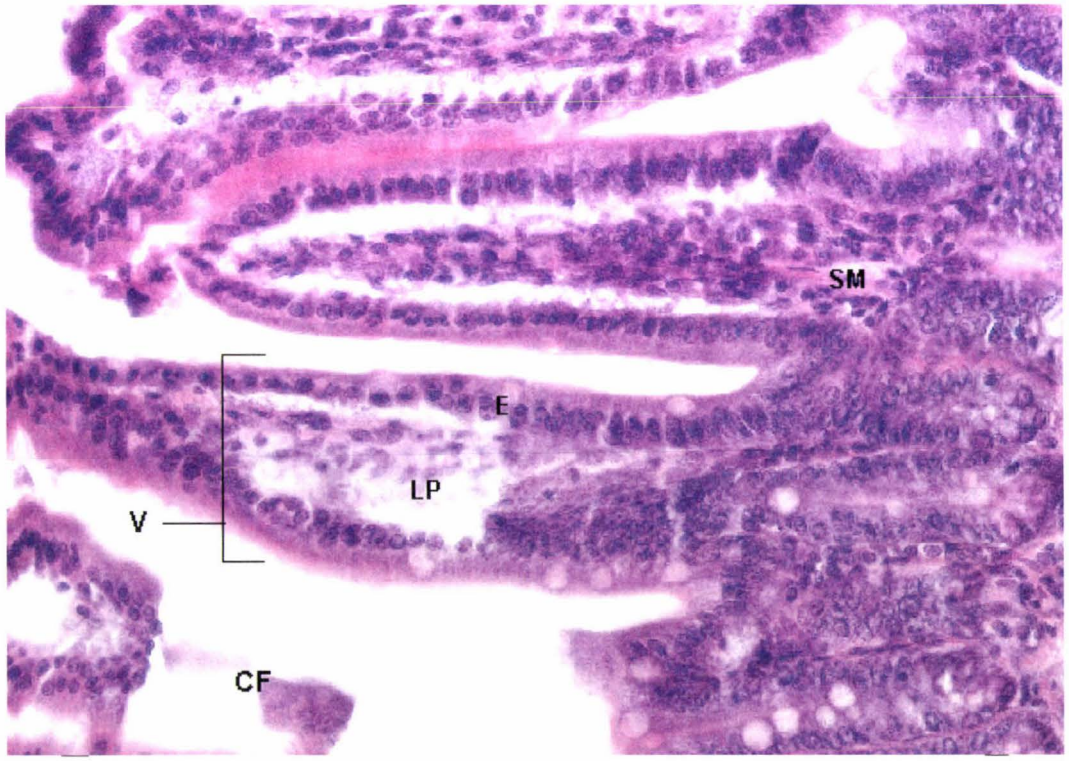

Figure 4.12: Photomicrograph of a cross section of mouse jejunum, not used in an Ussing chamber experiment, stained with H&E, magnification x200. CF = cell fragments; E = epithelia; LP = lamina propria; SM = submucosa; V = villi; ME = muscularis externa.

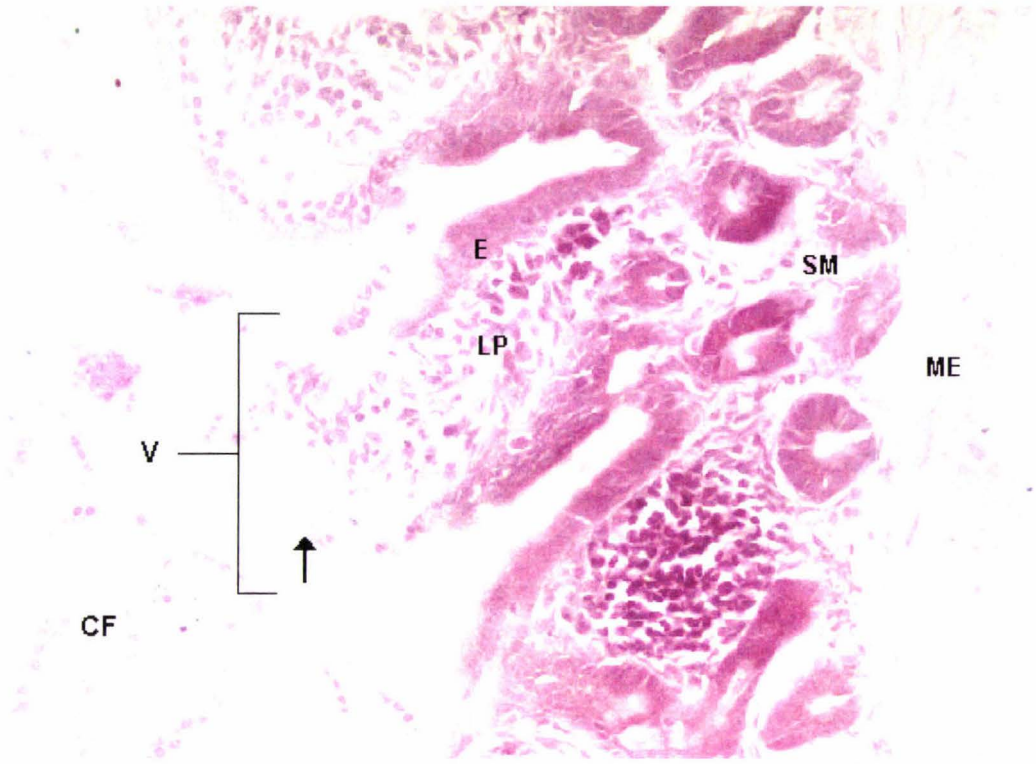

Figure 4.13: Photomicrograph of a cross section of mouse jejunum after being used in an Ussing chamber experiment with no iron added, stained with Perl's Prussian blue reaction and neutral red, magnification x200. CF = cell fragments; E = epithelia; LP = lamina propria; SM = submucosa; V = villi; ME = muscularis externa; arrows = iron.

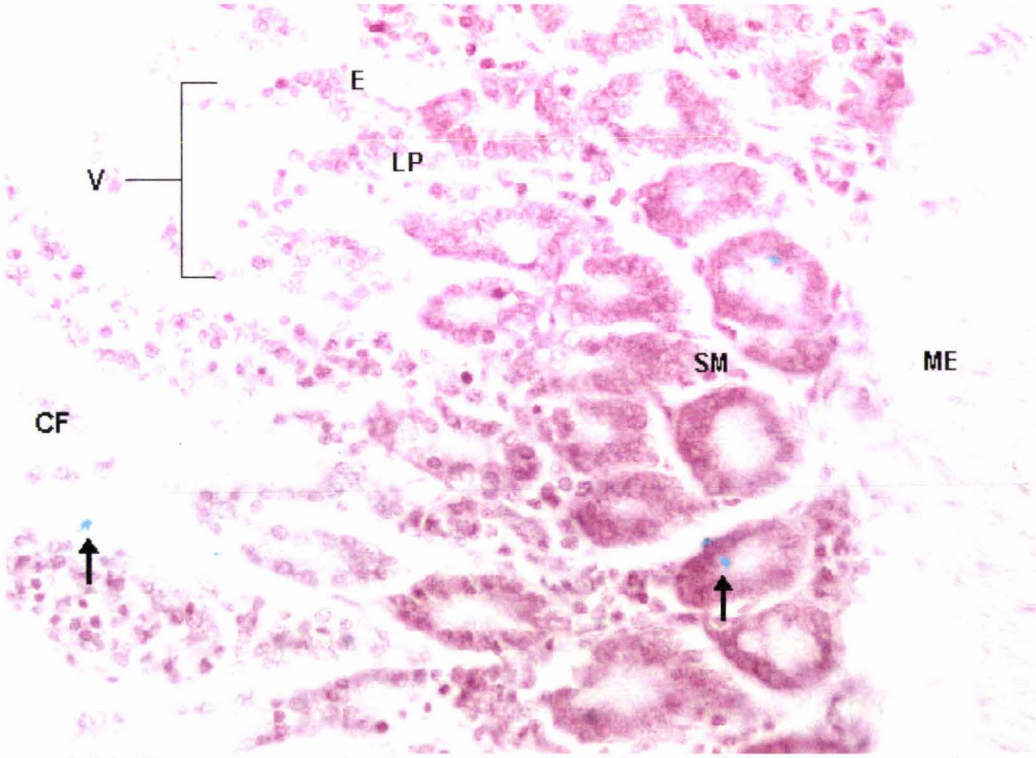

Figure 4.14: Photomicrograph of a cross section of mouse jejunum after use in an Ussing chamber experiment with iron added, stained with Perl's Prussian blue reaction and neutral red, magnification x200. CF = cell fragments; E = epithelia; LP = lamina propria; SM = submucosa; V = villi; ME = muscularis externa; arrows = iron.

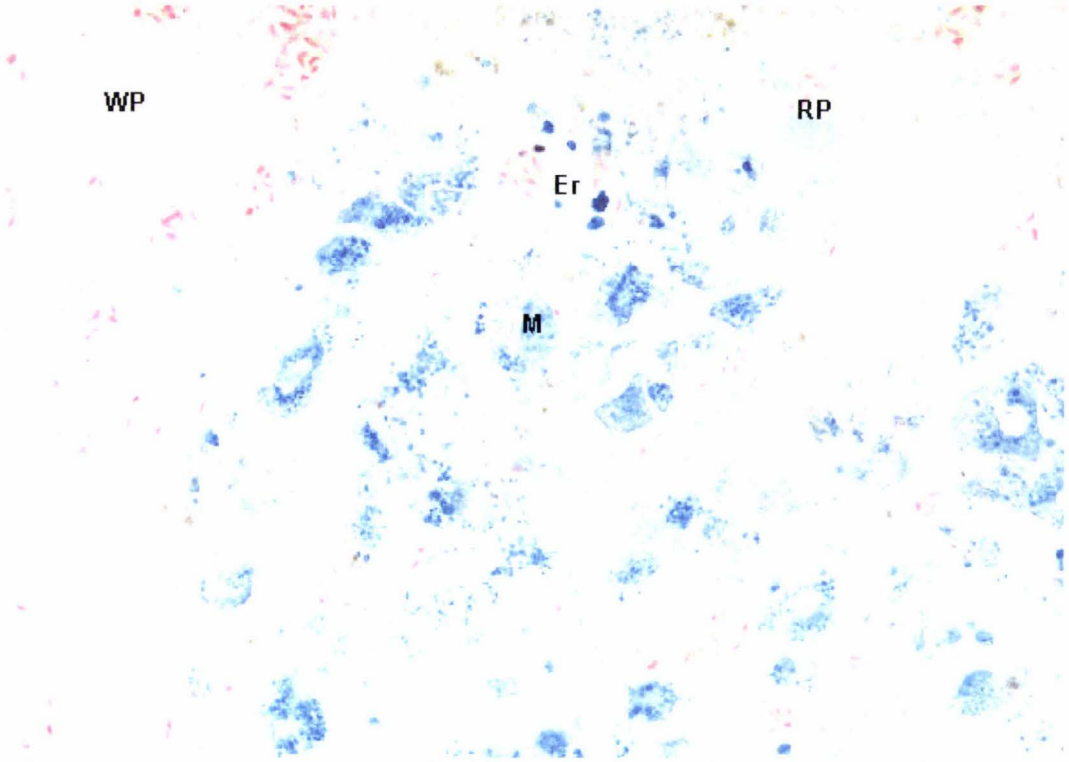

Figure 4.15: Photomicrograph of spleen, stained with Perl's Prussian blue reaction, magnification x400. WP = white pulp; RP = red pulp; M = macrophage, Er = erythrocytes.

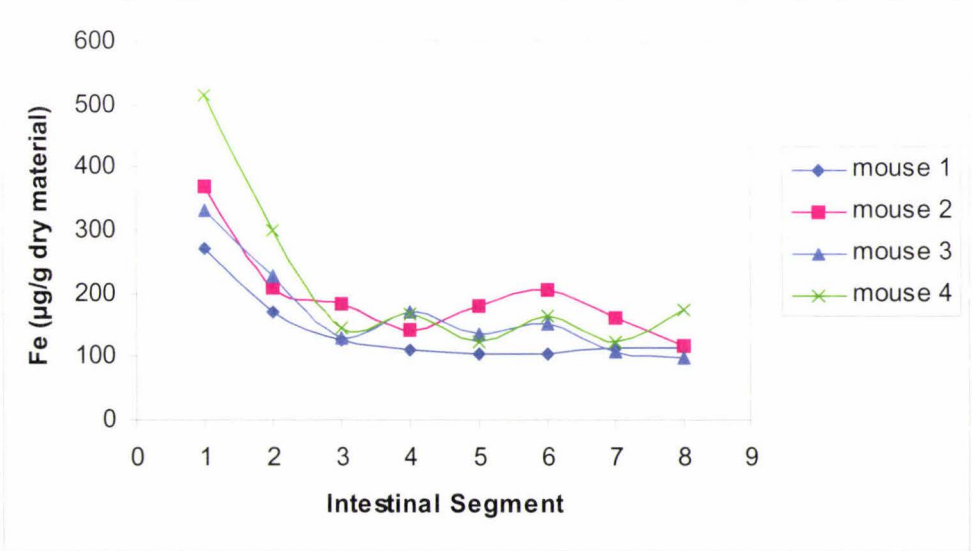

Figure 4.16: The iron content of intestinal segments taken from small intestinal tissue which was not used in an Ussing chamber experiment (µg/g dry material)

5. DISCUSSION

The results reported in this thesis show that Ussing chambers can be used to study iron absorption by mouse small intestine. This conclusion is supported by previous Ussing chamber studies of iron absorption by the small intestine (Costa et al., 2000; Vaghefi et al., 2000; Vaghefi et al., 1998; Helbrock and Saltman, 1967). While iron absorption has been reported to occur in all regions of the small intestine, it is higher in the proximal compared to the distal regions (Chowrimootoo et al., 1992; Conrad et al., 1987; Muir and Hopfer, 1985; Cox and O'Donnell, 1981). It is not surprising, therefore, that these previous Ussing chamber studies focussed on the duodenum and did not measure iron absorption by other regions of the small intestine (Costa et al., 2000; Vaghefi et al., 2000; Vaghefi et al., 1998; Helbrock and Saltman, 1967). The results presented here show consistently high percentages of iron removed from the mucosal compartment along the full length of the small intestine.

There is evidence that the iron removal recorded here was due to physiological absorption processes. While there was some iron loss due to precipitation, there was no unexplained iron loss from the Ringer's solution during any of the control experiments without tissue mounted in the chambers. This demonstrated that iron was not removed from the Ringer's solution by adherence to the interior of the chambers, lifters or reservoirs, or by diffusion into the salt agar bridges. Both I_{sc} and Rt were monitored throughout each experiment and did not deviate from predetermined limits. This indicates that the tissue remained viable and intact throughout the experimental period.

5.1 Experimental Procedure

In the present study the muscularis externa was not removed from the intestinal tissue mounted in the chambers due to the small size of the tissue. Some previous Ussing chamber workers using mouse small intestine removed the attached muscle layer proir to mounting the tissue in the chamber (Costa et al., 2000), while some did not (Grubb, 1995; Sheldon et al., 1989). The average values for I_{sc} presented here were similar to those found in two previous studies using unstripped mouse small intestine (see Table 5.1). Sheldon et al. (1989) compared stripped and unstripped mouse small intestine and found no significant difference in the I_{sc} of either tissue. They also

found that unstripped tissue transported ions and responded to other chemicals, despite having a significantly lower conductance than stripped tissue.

Table 5.1: Comparison of I_{sc} for three Ussing chamber studies using mouse small intestine with the muscularis externa attached. All values are mean ± se (number of intestinal segments).

Study	I_{sc} *($\mu Amp.cm^{-2}$)*
Present experiments (mannitol in the mucosal Ringer's solution)	-47 ± 3.5 (156)
Present experiments (glucose in the mucosal Ringer's solution)	-59 ± 7.0 (16)
Sheldon et al., (1989) (mannitol in the mucosal Ringer's solution)	-69.3 ± 6.1
Grubb, (1995) (mannitol in the mucosal Ringer's solution)	-43.2 ± 5.9 (10)

There was no iron detected in the serosal Ringer's solution after any of the experiments presented in this thesis. This was likely to be due to the accumulation of iron within the submucosa. Iron does not cross the submucosa and muscularis externa *in vivo* per se, and is instead transported into the capillaries in the centre of each villi, where it then binds to apotransferrin and enters the venous circulation, as described in Section 2.4.6. Removal of iron from the basolateral membrane by the capillaries *in vivo* forms an iron sink, thus creating a mucosal-to-serosal concentration gradient and encouraging iron transport out of the cell (Manis and Scachter, 1962). However, as this blood supply is not present in *in vitro* tissue preparations, iron remains in the submucosa.

Alternatively, the absence of iron in the serosal solution may be due to the length of the experimental period. The transport of iron across the epithelia via the transcellular route involves two sequential steps, transport across the apical membrane into the enterocyte and exit from the enterocyte via the basolateral membrane (see Section 2.4.2.2). Each of these steps is a separate process involving different transport mechanisms and occurring over different time scales (Halliday and Powell, 1973; Manis and Schachter, 1962). Manis and Schachter (1962) demonstrated that the transfer of iron across the basolateral membrane is slower than transport across the apical membrane and may not occur directly after iron has entered the enterocyte. Therefore, if the experimental period is too short then iron will not have time to cross the basolateral membrane before the experiment is ended. While the experimental period could be extended to investigate the

effects of experimental length on iron absorption, there are limits to how long the intestinal tissue remains viable while mounted in the Ussing chambers. Soderholm, Hedman, Olaison, Artusson, Frazen, Larsson, Permet and Pantzar (1998) found that tissue damage increased after 120 minutes and suggested that the experimental period be limited to 90 minutes to ensure tissue viability.

The Ringer's solution used to maintain tissues *in vitro* is usually adjusted to pH 7.4 to mimic the conditions seen *in vivo*. Thus, in the present experiments the SEROSAL Ringer's solution was adjusted to pH 7.4. However, the MUCOSAL Ringer's solution was kept slightly acidic at pH 6.5, as this is similar to the pH of the luminal contents of the duodenum *in vivo* (Powell et al., 1994) and has been shown to increase iron solubility (Forth and Rummel, 1973) thus increasing iron absorption (Costa et al., 2000; Jacobs, Bothwell and Charlton, 1966).

The initial concentration of iron measured in the mucosal solution in many of the present experiments differed from what was expected due to variations in the volume of Ringer's solution in the mucosal reservoir. It was difficult to add the correct volume of solution to the reservoir due to the air bubbles moving through the reservoir. Once this variation was discovered, samples of mucosal Ringer's solution were taken at the start of the experimental period so the exact iron concentration could be measured. However, starting samples were not taken for the early experiments, and the initial concentration was calculated according to the concentration of stock solution added to the mucosal Ringer's solution. This may have introduced an extra experimental error to these results.

5.2 Ferrous Gluconate

Ferrous gluconate was chosen as the reference test chemical as it is a non-toxic, readily bioavailable form of iron (Hurrel, 2002) and has been used previously to investigate iron absorption by intestinal tissues mounted in an Ussing chamber (Vaghefi et al., 1998).

The concentration of ferrous gluconate added to the mucosal compartment in the initial experiments was 27.9 mg/L and was chosen as it fell within thesnon range of iron concentrations used in previous Ussing chamber iron absorption studies (Costa et al.,

2000; Vaghefi et al., 1998). In the present study, the results obtained using this concentration of ferrous gluconate showed that:

1) Consistently high levels of iron were removed from the mucosal compartment by all intestinal segments, with average percentages removed ranging between 45% and 55%

2) There was significantly less iron uptake by the proximal small intestine than the mid or distal regions of the small intestine.

As analysis of a typical meal showed an iron concentration of 8 mg/L (Walker, L., unpublished data., November 2001), 27.9 mg/L ferrous gluconate was greater than would be expected in the intestinal lumen *in vivo*. This increased iron concentration may create an unphysiological iron concentration gradient across the tissue, thus increasing passive absorption of iron. Therefore the concentration of ferrous gluconate added to the mucosal compartment was lowered to 9.3 mg/L. The results obtained using this latter concentration showed that:

1) The average percentages of iron removed from the mucosal compartment remained high, ranging between 39% and 64%.

2) The percentage of iron removed by the proximal small intestine was significantly lower than that removed by the mid or distal regions of the small intestine.

3) There was no significant difference between the percentage iron removed by any segment with either 27.9 mg/L or 9.3 mg/L ferrous gluconate.

The average percentages of iron removed from the mucosal compartment with both concentrations of ferrous gluconate (27.9 mg/L and 9.3 mg/L) was greater than expected from previous iron absorption studies. Absorption of non-haem iron from the diet has been measured at 5% to 20% during *in vivo* studies using humans (Beard et al., 1996; Cook and Monsen, 1975; Bothwell et al., 1958). Iron absorption in the present study may be greater than this due to the use of iron salts rather than a whole meal, thus removing the overall inhibitory affects of other food constituents (Lynch, 1991). However, in Ussing chamber study of iron absorption, Vaghefi et al. (1998) also used

ferrous gluconate instead of a whole meal. They demonstrated iron losses from the mucosal compartment of between 5% and 14%, markedly lower than the percentage of iron removed in the present experiments. There may be a species specific difference between these results as the previous study used rat small intestine as opposed to the mouse small intestine used here. Also, the experimemts presented here used a slightly acidic mucosal Ringer's solution whereas those presented by Vaghefi et al., (1998) did not. Both Costa et al., (2000) and Jacobs et al., (1966) showed increased iron absorption at acidic pH's, with Jacobs et al., (1966) reporting an increase from between 2% and 6.5% of iron removed from the mucosal solutuion at neutral pH to between 32% and 45% iron removed when the pH of the mucosal Ringer's solution was lowered with HCl. Increased iron solubility due to the acidic pH may have increased iron absorption.

The low iron absorption by the proximal small intestine recorded for both iron concentrations does not agree with the pattern of iron absorption seen *in vivo*, where iron transport is highest in the proximal small intestine (see Section 2.4). The control experiments without tissue mounted in the chamber demonstrated no change in iron concentration when either 27.9 mg/L or 9.3 mg/L was added to the mucosal compartment, indicating that the change in iron concentration seen when tissue was mounted in the chambers was due to physiological processes.

As the first intestinal segment removed significantly less iron from the mucosal solution than the rest of the intestinal segments, the low absorption by the proximal region when the results were grouped was due to the results from the first segment alone. There may be an unknown experimental artefact or bias affecting just this intestinal segment. During each experiment the segments were mounted sequentially in the chambers in the same order, so the first intestinal segment was always mounted in the first apparatus. Therefore there may be an equipment factor involved, e.g. the volume of Ringer's solution in the first reservoir may have been miscalibrated to give a lower starting concentration of iron, thus skewing results for the first segment. As no starting samples were taken when 27.9 mg/l or 9.3 mg/L ferrous gluconate was used this can not be confirmed. All further experiments in this thesis included the analysis of mucosal Ringer's solution taken at the start of the experiment and there was no significant difference in absorption by the first intestinal segment, suggesting that this bias was removed. Previous Ussing chamber iron absorption studies (Costa et al., 2000; Vaghefi

et al., 2000; Vaghefi et al., 1998; Helbrock and Saltman, 1967) only investigated absorption by the duodenum, therefore any difference in absorption by the duodenum compared to the jejunum or ileum was not measured.

As there was no significant difference between the amounts of iron removed when either 27.9 mg/L or 9.3 mg/l ferrous gluconate were added to the mucosal solution it is likely that the same iron transport processes were operating in both sets of experiments. Previous studies (Wessling-Resnick, 2000; Pearson and Reich, 1965) have indicated that there are a number of different transport processes involved in iron absorption by the small intestine. These include both active and passive transport processes and are described in Section 2.4.2. Therefore further experiments were conducted to investigate which transport mechanisms are involved in the removal of iron from the mucosal compartment recorded here. Understanding the processes involved will allow greater understanding of why the results presented here differ from those seen in previous experiments.

5.3 Addition of Manganese Sulphate

Ferrous iron is actively transported across the apical membrane into the enterocyte by the DCT1 transport protein (see Section 2.4.4.2), forming an important part of the iron absorption process (Gunshin et al., 1997). DCT1 transports a number of divalent cations including Mn^{2+} and the presence of this cation in the intestinal lumen has been shown to competitively inhibit iron transport by DCT1 (Goddard et al., 1997; Pollack et al., 1965). Therefore the addition of Mn^{2+} and iron to the mucosal solution is expected to reduce DCT1-mediate iron transport (Goddard et al., 1997).

Goddard et al. (1997) demonstrated that the combination of a ten-fold excess of manganese sulphate with ferrous ascorbate significantly decreased iron uptake. Therefore 91.5mg/L MnSO4 and 9.3mg/L ferrous gluconate was added to the mucosal compartment in a single experiment. The results showed that:

1) A large amount of off-white precipitate formed in the mucosal Ringer's solution during the experimental period.

2) There was a wide variation in the average percentages of iron removed from the mucosal Ringer's solution by each intestinal segment, ranging from 0% to 58%.

Due to the large amounts of precipitation seen, it is likely that the excess manganese has precipitated out of solution. It is also possible for manganese and iron to form insoluble complexes (Goddard et al., 1997). This may account for some of the precipitation here. Any iron bound by these complexes would be unavailable for absorption by the tissue and would not be detected by the FAAS. Therefore, iron may have been removed from the mucosal solution by reaction with the excess manganese rather than by transport into the intestinal tissue.

By the end of the experimental period the precipitate had blocked the lifter tubes, preventing circulation of the Ringer's solution. There was also a layer of precipitate coating the mucosal surface of the intestinal tissue. It is likely that iron in the mucosal Ringer's solution was unable to reach the apical membrane to be absorbed. The precipitate present in the mucosal Ringer's solution during analysis caused blockages in the intake of the FAAS, reducing the flow of the sample into the machine and interfering with the results of the analysis (Alkemade and Herrman, 1979).

While there may have been iron removal due to physiological absorption by the intestinal tissue, this could not be distinguished from that due to precipitation. Therefore the results are not considered valid.

To reduce the amount of precipitate formed in the mucosal Ringer's solution, the concentration of manganese was lowered to give close to equimolar concentrations of manganese sulphate (9.15 mg/L) and ferrous gluconate (9.3 mg/L). The results showed that:

1) Iron uptake was significantly lower in the proximal than in the mid region of the small intestine.

2) The average percentage of iron removed from the mucosal solution was significantly lower in the proximal and mid regions of the small intestine with manganese sulphate plus ferrous gluconate than with ferrous gluconate alone. There was no difference in

iron removed by the distal region of the small intestine with or without manganese sulphate.

3) There were no changes in iron concentration after the control experiments (no tissue mounted) using this mineral combination.

The reduction in iron uptake by the proximal and mid small intestine was presumably due to competitive inhibition of DCT1-mediated iron transport by the manganese. This is in agreement with previous studies which have found that manganese directly inhibits iron transport (Goddard et al., 1997; Pollack et al., 1965). The involvement of DCT1 is supported by the significant decrease in iron uptake by the proximal, but not distal, small intestine; DCT1 is principally expressed in the proximal small intestine (Canonne-Hergaux et al., 1999; Guinshin et al., 1997). It would be interesting to carry out further experiments using intestinal tissue from mk/mk gene knockout mice to confirm DCT1 involvement. As these mice do not express the DCT1 protein (Canonne-Hergaux et al., 1999) overall iron absorption should be reduced and the addition of manganese should not decrease absorption.

These results indicate that in experiments using tissue from the proximal or mid regions of the small intestine, at least 15% of the iron added to the mucosal Ringer's solution was actively transported across the apical membrane by the DCT1 protein. As the manganese added to solution may not saturate the binding sites on DCT1 to fully inhibit iron transport, the exact proportion of iron removal due to this protein may be higher.

Although there was a small amount of off-white precipitate present in the mucosal Ringer's samples with equimolar concentrations of ferrous gluconate and manganese sulphate, there was no significant changes in iron concentration in the control experiments indicating that any change in iron concentration caused by the precipitation was too small to be detected by the FAAS.

5.4 Addition of Calcium Chloride

Calcium has been shown to inhibit iron absorption *in vivo* (see Section 2.4.4.2), although the exact inhibitory mechanisms are unclear (Lynch, 1997; Barton et al., 1983). Cook et al. (1991) found evidence that calcium's inhibitory effect on iron was

greater when administered with a whole meal then when using metal salts alone. Barton et al. (1983) demonstrated a significant decrease in iron absorption when $CaCl_2$ in concentrations of 10 mM and 100 mM were combined with 1mM of $FeCl_3$, but not when 1 mM or less of $CaCl_2$ was used. Therefore 2.5 mM calcium chloride (200 mg/L) and 0.17 mM ferrous gluconate (9.3 mg/L) were added to the mucosal compartment to investigate the effect of calcium on iron uptake without the complication of other dietary factors. The results showed:

1) There was no significant variation in iron uptake by each intestinal region, with the averages removed per intestinal segment ranging between 52% and 69%.

2) There was no difference between the average percentage of iron removed from the mucosal Ringer's solution with both $CaCl_2$ and ferrous gluconate than with ferrous gluconate alone.

3) There was some precipitate present in the mucosal Ringer's solution after the experimental period.

4) There was an average decrease of 26.9% ± 7.6% in the iron concentration after the experimental period in control experiments without tissue mounted in the chambers. This was greater than the experimental error calculated from the calibration graph of the FAAS.

In the present experiments the addition of calcium chloride did not appear to affect iron absorption. This was unexpected since previous studies have shown the addition of calcium inhibits iron uptake (Cook et al., 1991; Barton et al., 1983; Manis and Schachter, 1962). It has been postulated that the majority of calcium's inhibitory effect on iron absorption *in vivo* occurs at the basolateral membrane where iron exits the enterocyte (Barton et al., 1983; Manis and Schachter, 1962). As iron did not reach the serosal Ringer's solution in these experiments (see Section 5.1) iron transport across the basolateral membrane can not be measured. Therefore, while calcium may in fact be inhibiting iron transport across the basolateral membrane, this could not be confirmed in the present experiments.

There is evidence that calcium also inhibits iron transport across the apical membrane. Greenberger et al. (1969) demonstrated that the addition of calcium significantly decreased iron absorption in apical membrane vesicles. These findings were later confirmed by Barton et al. (1983) using intestinal loops. This decrease may be due to competition between calcium and iron for transport by an apical membrane transport protein, possibly DCT1 (Barton et al., 1983; Greenberger et al., 1969). Therefore some decrease in iron removal from the mucosal compartment was expected in these results.

While there was no obvious precipitate circulating in the Ringer's solution during the experiments, there was a small amount of white precipitation present in the Ringer's solution collected at the end of the experiment. Control experiments with no tissue mounted and 200 mg/L calcium chloride (without iron) showed no precipitate, whereas control experiments with 200 mg/L calcium chloride plus 9.3 mg/L ferrous gluconate contained small amounts of white precipitate and significant decreases in iron concentration, averaging 26.9% ± 7.6%. Monsen and Cook (1976) found evidence that insoluble complexes of calcium-phosphate-iron may form when these minerals are present, thus lowering the concentration of soluble iron in solution. Analysis of digesta by Wienk et al. in 1996 found the solubility of iron decreased when calcium was present in the intestinal lumen. Therefore it is likely that the precipitate seen here is due to interactions between the iron and calcium in solution.

It is possible that a decrease in iron removal from the mucosal Ringer's solution due to calcium's inhibition of iron transport was accompanied by a corresponding increase in iron removal from solution due to the formation of insoluble calcium-iron complexes. This would explain why there was a decrease in iron concentration after the control experiments but not in the tissue-mounted experiments. Therefore this precipitation needs to be prevented before the effects of calcium on physiological iron transport can be fully investigated.

5.5 Increasing Passive Absorption

A proportion of the absorption recorded here may have been due to the passive transport of iron through the tight junctions and along the paracellular pathway (Fine et al., 1994; Sadowski and Meddings, 1993; Pearson and Reich, 1965). The addition of glucose to the mucosal solution has been shown to increase the passive transport of solutes across

the intestinal mucosa, as described in Section 2.4.2.1. While the direct effect of glucose on passive transport of iron has not been investigated, Manis and Schachter (1962) showed that metabolisable hexoses such as glucose increased the transport of iron across the intestinal mucosa. They postulated that this was due to an increase in active transport of iron caused by stimulation of the SGLT1 protein, however, passive absorption may also have been involved. Therefore the mannitol in the mucosal Ringer's solution was replaced with an equal concentration of glucose in an attempt to increase the passive absorption of iron. When 10mM glucose and 9.3 mg/L ferrous gluconate were added to the mucosal Ringer's solution the results showed:

1) There was a significant decrease in the percentage iron removed from the mucosal solution by all intestinal regions with glucose in the mucosal Ringer's solution compared to mannitol.

2) The I_{sc} increased steadily throughout the 90 minute experimental period when glucose was present in the mucosal Ringer's solution. This did not occur in any experiment where the mucosal Ringer's solution contained mannitol.

3) The Rt remained stable throughout the experimental period.

The decrease in iron uptake was the opposite to what was expected based on previous studies (Perez 1996; Fine et al., 1994; Sadowski and Meddings, 1993; Manis and Schachter, 1962). The accumulation of iron in the submucosa due to the lack of blood supply (see Section 5.1) may create an unphysiological serosal-to-mucosal concentration gradient across the tissue thus reducing or removing paracellular absorption of iron (Forth and Rummel, 1973; Manis and Schachter, 1962). The absence of paracellular transport would explain why glucose did not increase iron absorption. Inert tracer compounds such as D-mannitol, L-xylose, polyethylene glycol or dextran may be added to the mucosal solution to directly measure paracellular transport (Fine et al., 1994; Sadowski and Meddings, 1993). As active transport mechanisms for these compounds have not been found it is expected that they cross the epithelia through the paracellular pathway (Sadowski and Meddings, 1993). It would be useful to conduct a series of experiments with glucose added to the mucosal Ringer's solution using one of

these tracer compounds to quantify the paracellular absorption present in these experiments.

The increase in I_{sc} is consistent with the results found in previous experiments (Berglund et al., 2001; Grubb, 1995) and is due to the active co-transport of glucose and Na^+ by the SGLT1 protein. Although Berglund et al. (2001) found that increased I_{sc} due to SGLT1 stimulation was accompanied by a decrease in the Rt, indicating a corresponding increase in tissue permeability, there was no change in Rt in the present experiments and iron absorption was not increased. Therefore it is unlikely that tissue permeability was affected by the addition of glucose to the mucosal Ringer's solution.

5.6 Other Forms of Iron

A variety of iron salts have been used in previous *in vitro* experiments to investigate iron absorption (Latunde-Dada et al., 1998; Forth and Rummel, 1973). As each iron salt has different chemical characteristics the choice of salt may have an effect on iron uptake by the tissue.

5.6.1 Ferrous Sulphate

It is possible that the gluconate portion of ferrous gluconate interacts with the tight junctions between the enterocytes by the same mechanism as glucose (Madara, 1998), thus increasing passive absorption. Therefore the ferrous gluconate was replaced with an equal concentration of ferrous sulphate (9.3 mg/L) to investigate what effect the gluconate portion has on iron absorption. Ferrous sulphate is a highly bioavailable form of iron and has been used previously in iron absorption studies *in vitro* (Hurrel, 2001; Costa et al., 2000; Manis and Schachter, 1962). The results showed that:

1) There was no significant difference in iron uptake by each intestinal region, with the average percentage iron removed per intestinal segment ranging between 29% and 64%.

2) There was a significantly lower concentration of iron removed by the distal region with 9.3 mg/L ferrous sulphate compared to 9.3 mg/L ferrous gluconate.

3) There were no changes in iron concentration after the control experiments (no tissue mounted) using this ferrous sulphate combination.

The absence of variation in iron absorption between intestinal regions does not fit the absorption pattern demonstrated by previous studies, where iron absorption was higher in the proximal small intestine than in the mid or distal intestine (See Section 2.4). Iron absorption by the distal small intestine is significantly lower with ferrous sulphate than with ferrous gluconate and is assumed to be due to the replacement of gluconate with sulphate. This provides evidence that the passive absorption of iron from ferrous gluconate by the distal small intestine was increased by interactions between gluconate and the tight junctions between the enterocytes. The increase in passive absorption may be confirmed by the addition of a marker compound to the mucosal Ringer's solution to measure paracellular transport, as discussed in Section 5.4.

5.6.2 Ferric Chloride

Ferric iron has been used in many previous *in vitro* studies of iron absorption including an Ussing chamber study by Helbrock and Saltman (1967). Ferric iron has also been used as a radio-labelled iron tracer in iron absorption studies (Costa et al., 2000; Goddard et al., 1997; Perewusnyk and Funk, 1997). However, the ferric form of iron is less soluble than the ferrous, particularly at neutral pHs (Forth and Rummel, 1973). This reduced solubility can affect iron uptake as described in Section 2.4.4.1. To investigate any differences in iron removal of the two forms, the ferrous gluconate added to the mucosal Ringer's solution was replaced by an equal amount of ferric chloride (9.3 mg/L). The results showed that:

1) There was a wide variation in the percentage iron removed from the mucosal solution by each intestinal segments, ranging between 35% and 55%.

2) Many of the starting concentrations measured by FAAS ere much lower than those calculated from the amount of iron added, indicating a rapid loss of iron from the solution.

3) Control experiments with no tissue mounted in the chambers also showed a high variation in iron concentration, ranging between an increase of 70% to a decrease of 50% after the experimental period.

4) There was a large amount of crystalline precipitation present in the mucosal Ringer's solution for all experiments including the no tissue controls.

It is likely that the crystalline precipitate present in these experiments consisted of ferric ions which precipitated out of solution at the slightly acidic pH of the mucosal Ringer's solution (Forth and Rummel, 1973). As the FAAS can only detect soluble iron this insolubility would explain the low iron concentrations recorded for both the control experiments and for the samples taken at the start of the experimental period before absorption by the intestinal tissue. As ferric iron has been used previously to successfully demonstrate iron absorption (Costa et al., 2000; Goddard et al., 1997; Perewusnyk and Funk, 1997; Helbrock and Saltman, 1967) it is likely that at least some of the iron removed from the mucosal Ringer's solution present in the results presented here was due to physiological absorption by the intestinal tissue. The amount of iron which precipitated from the Ringer's solution appeared to vary between experiments and thus could not be corrected for to give a value for the amount of iron removed from solution due to physiological absorption by the intestinal tissue. Therefore these results were not considered valid. It may be possible to solubilise the ferric iron by the addition of ascorbic acid, as this has been shown to chelate with both ferrous and ferric iron to increase solubility and keep the iron available for absorption (Baynes and Bothwell, 1990). This would remove the problem of precipitation and allow direct comparison of ferric versus ferrous iron absorption using the Ussing chamber system.

5.7 Histological Examination of Intestinal Tissue

Histological examination of jejunal samples after a typical Ussing chamber experiment showed there was no damage to the tissue, the epithelial layer remained intact and overall morphology was similar to that of jejunal samples taken straight from the animal and stained following the same method. While there were some cell fragments present on the slides, these were likely to be due to mechanical damage of the tissue during the fixing and staining process. If the damage occurred before the tissue was mounted then these fragments would not be present. The intactness of the tissue reduces the possibility that iron left the mucosal solution through a hole in the intestinal epithelia, thus providing further evidence that the iron absorption seen in the present experiments was due to physiological iron transport mechanisms. It also demonstrates that rinsing

the tissue in nitric acid after the experiment to collect the mucus attached to the tissue (as carried out in the present experiments) does not damage the cell membrane releasing iron from within the enterocytes.

Jejunal samples taken from two Ussing chamber experiments, one with 9.3 mg/L ferrous gluconate added to the mucosal solution and one without, were stained using Perl's Prussian blue reaction. This reaction has been shown to bind intracellular iron to give a distinctive blue stain (Culling, 1985). However there was very little staining present in either jejunal sample, there was no qualitative difference between the iron staining present in either jejunal sample and it was not possible to localise iron to any part of the intestinal tissue. A splenic sample stained by the same procedure as the jejunal tissue had large amounts of staining due to the presence of iron released by the degradation of erythrocytes in the red pulp. This indicates that the low levels of staining seen in the jejunal samples was due to the low iron content of those tissues rather than a problem with the staining procedure itself. As Perl's Prussian blue reaction is specific for storage forms of iron such as hemosiderin (Culling, 1985) the iron present in the jejunal samples may not react with this stain. Staining with Perl's Prussian blue reaction did not provide any meaningful information about the iron absorption in the present experiments.

5.8 Non-Absorbing Tissue

In the present experiments iron may have been removed from the mucosal Ringer's solution by non-specific processes such as adherence to the outside surface of the tissue (Glahn, Gangloff, Van Campen, Miller, Wien and Norvell, 1995). Therefore segments of large intestine or caecum were mounted in the chambers in place of small intestine to investigate these non-specific processes. Iron absorption in these regions is very low in the whole animal (Rucker et al., 1994) and was expected to be negligible in the present experiments. However, the average percentage of iron removed from the mucosal compartment by both large intestine and caecum remained high and was not significantly different from iron removal by any region of the small intestine. This indicates that iron was removed from the mucosal solution by processes which are not restricted to the small intestine. However, as iron transport by the large intestine and caecum has been demonstrated in previous experiments (Campos et al., 1996), iron-specific mechanisms can not be ruled out. It would be interesting to repeat these

experiments using tissue which does not contain any physiological iron transport mechanisms such as mouse oesophagus or trachea, however, these tissues are difficult to mount in the chambers used in these experiments.

5.9 Collecting Mucus and Measuring Mucosal Iron Levels

Insoluble mucus was secreted by both small and large intestine into the mucosal Ringer's solution during the experimental period of all the experiments presented in this thesis. While some of the mucus remained free-floating in solution, mucus could be seen adhering to the interior of the chambers, lifters and reservoirs as well as on the mucosal surface of the intestinal tissue. As mucus has been shown to bind iron in the intestinal lumen (see Section 2.4.7) it may be contributing to the removal of iron rom the mucosal Ringer's solution recorded in these experiments. To investigate the role of mucus in iron absorption, experiments with 9.3 mg/L ferrous gluconate added to the mucosal Ringer's solution were repeated with the additional steps of collecting and analysing the secreted mucus and the intestinal tissue at the end of the experimental period to determine their iron content. The results showed:

1) There were high levels of iron found in both the intestinal tissue and secreted mucus for all intestinal segments.

2) The iron concentration of the mucus was, on average, four times that of the intestinal tissue.

3) The average recovery of the iron added to the mucosal Ringer's solution was 88%. There was no significant variation in the percent recovered for each intestinal segment.

4) The average percentage of iron removed from the mucosal solution by all intestinal segments was 33.6%. This appeared to be below the range of iron removal seen in the earlier experiments using 9.3 mg/L ferrous gluconate, although replicate numbers were too low to determine significance.

The majority of the iron removed from the mucosal solution during these total recovery experiments was bound to secreted mucus rather than transported into the intestinal tissue. As the concentration of iron found bound to mucus was high, it is expected that

this binding was responsible for a significant proportion of the iron uptake demonstrated in all the experiments reported in this thesis. As the iron content of the mucus and the intestinal tissue was only measured in the total recovery experiments, further experimentation is necessary to confirm this involvement.

In the whole animal mucus tends to adhere to the apical membrane of the epithelia due to the unstirred water layer (Neutra and Forstner, 1987). However, during these experiments the circulation of the Ringer's solution moved the mucus away from the apical membrane and it could be seen free floating in the mucosal solution. This may have allowed the mucus to come into contact with and bind more iron than it would *in vivo*, explaining the unexpectedly high concentrations of iron uptake recorded in the results presented here when compared to iron absorption *in vivo* (see Section 5.2). Also, as mucus was secreted by all intestinal segments, high levels of mucus iron binding may explain the similarity in iron absorption found in all intestinal regions, as opposed to the absorption gradient found *in vivo* (see Section 5.2).

Conrad et al. (1991) demonstrated that lead, cobalt and zinc compete for binding to the mucus. It is possible that manganese also competes with iron for mucus binding. The reduction in iron absorption demonstrated when 9.15 mg/L MnSO4 and 9.3 mg/L ferrous gluconate were added to the mucosal Ringer's solution may have been due to competition for binding to the mucus rather than for DCT1 transport as discussed in Section 5.3. However, this is unlikely as mucus was secreted by all intestinal segments but absorption was only reduced in the proximal small intestine.

As iron binding to mucus is a reversible process which keeps the iron in a soluble form the iron is available for absorption by the intestinal tissue (Conrad et al., 1991). Over time the mucus-bound iron may leave the mucus and enter the intestinal epithelia (Conrad et al., 1991). Therefore the proportion of iron found in the mucus as opposed to within the intestinal tissue may be affected by the length of the experimental period. An increase in experimental time may allow the mucus bound iron to be transported into the enterocytes thus increasing the proportion of iron within the intestinal tissue.

It is likely that the binding of iron to mucus was present in previous Ussing chamber studies although it was not always measured. Costa et al. (2000) and Helbrock and

Saltman (1967) did not measure the iron content of the intestinal mucus. Both Vaghefi et al. (2000) and Vaghefi et al. (1998) washed the interior of the chambers and the exterior of the intestinal tissue with nitric acid after the experimental period to remove adsorbed iron. This washing should collect the secreted mucus as described in Section 3.3.4, however, it is unclear if the nitric acid was collected and analysed or if any iron it contained was included in the results. It is important that future *in vitro* iron absorption studies using mucus-secreting tissue include the collection and analysis of both the secreted mucus and intestinal tissue so the concentration of iron bound to the secreted mucus can be measured.

The mean recovery of iron added to the mucosal Ringer's solution was high but did not reach 100%, leaving some iron unaccounted for. This was most likely to be caused by incomplete collection of mucus from the interior of the apparatus.

The iron removal from the mucosal compartment seen in the total recovery experiments appeared to be slightly lower than in previous experiments using ferrous gluconate 9.3 mg/L. This may be due to a breed effect as the total recovery experiments were carried out using Swiss mice as opposed to the Balb/c mice used earlier. However this can not be confirmed as replicate numbers for the total recovery experiments were too low to determine if this difference is significant.

There may be iron present in the intestinal mucosa before removal from the animal, thus increasing the iron content of the tissue samples analysed in the present experiments. Therefore pieces of intestinal tissue taken from Balb/c mice were analysed using ICP to measure iron levels naturally occurring in mouse small intestine. As this endogenous iron was well below the levels detected by the FAAS, it was not included in the analysis of intestinal tissue in present experiments and may be discounted.

6. CONCLUSIONS

Experiments described in this thesis have shown that:

1) The Ussing chamber system can be used to successfully demonstrate iron absorption by all regions of the mouse small intestine.

2) The electrophysiological parameters (I_{sc} and Rt) of the tissue remained within physiological limits at all times, indicating that the tissue remained intact and viable throughout the experimental procedure.

3) Iron did not adhere to the interior of the chamber, lifters and reservoirs and was not removed from the Ringer's solution by experimental artefact.

4) Iron uptake was not specific to the duodenum; similar amounts of iron absorption appeared to be absorbed by distal regions of the small intestine.

5) A significant proportion of iron removed from the mucosal Ringer's solution was bound to the mucus secreted by the intestinal tissue during the experimental procedure rather than absorbed by the intestinal tissue itself.

6) The percentage of iron removed from the mucosal Ringer's solution was higher than expected from previous *in vivo* and *in vitro* iron absorption studies. This was most likely due to the slightly acidic pH of the mucosal Ringer's solution used in these experiments, although iron binding to the intestinal mucus may also have been involved.

7) In the proximal or mid regions of the small intestine at least 15% of the iron added the mucosal Ringer's solution was removed by active transport of iron across the apical membrane by the DCT1 protein.

8) The addition of calcium chloride and ferrous gluconate to the mucosal Ringer's solution did not appear to affect iron absorption although a precipitate which formed in the mucosal solution during these experiments may have interfered with the results obtained.

9) The addition of glucose to the mucosal Ringer's solution in place of mannitol decreased iron absorption by all regions of the mouse small intestine. This was unexpected as previous studies of iron absorption found increased iron absorption with the addition of glucose (Manis and Schachter, 1962), the reasons for this decrease are unknown.

10) Ferric chloride was not suitable as a test chemical for this system due to it's insolubility in the Ringer's solution.

11) Histological examination of the intestinal tissue after the experimental period showed that the tissue remained intact and undamaged. However, iron could not be demonstrated within the tissue with Perl's Prussian blue reaction.

12) The experiments described in this thesis did not confirm paracellular absorption of iron by any region of the mouse small intestine.

7. APPENDICIES

Appendix A: Test Chemical Stock solutions

Stock solutions used in Ussing chamber experiments.

Metal Salt	*Weight* (g)*	*Concentration (mg/L)*	*Supplier*
ferrous gluconate	0.48212	558	Aldrich Chemical Company Inc., Milwaukee, USA
ferrous gluconate	0.1607	186	Aldrich Chemical Company Inc., Milwaukee, USA
$FeCl_3 \cdot 6H_2O$	0.0901	186	Riedal-de Haen, Seelze, Germany
$MnSO_4 \cdot 4H_2O$	0.744	1830	BDH Laboratory Supplies, Poole, England
$MnSO_4 \cdot 4H_2O$	0.0744	183	BDH Laboratory Supplies, Poole, England
$FeSO_4 \cdot 7H_2O$	0.0927	186	BDH Laboratory Supplies, Poole, England
$CaCl_2$	0.5545	4000	BDH Laboratory Supplies, Poole, England

* made up to 100 mL volumetrically with RO water

Appendix B: Experimental Results and Raw Data

The following abbreviations are used in Appendix B: m = mucosal solution, s = serosal solution. Missing results indicate the intestinal segment was abandoned, as described in section 3.3.2.

Ferrous Gluconate 27.9 mg/L

28 May 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	9.6	19.2	31.2
2s	<0.5		
2m	7.4	22.1	20.8
3s	<0.5		
3m	10.2	20.4	26.8
4s	<0.5		
4m	9.0	18.0	35.6
5s			
5m			
6s	<0.5		
6m	4.7	9.5	66.1
7s	<0.5		
7m	7.4	14.9	46.7

calibration error: 3%

5 June 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	10.0	20.0	28.4
2s			
2m			
3s			
3m			
4s	0.5		
4m	7.4	14.8	46.9
5s	<0.5		
5m	6.8	13.6	51.4
6s	<0.5		
6m	5.5	16.6	40.4
7s			
7m			

calibration error: 3%

Ferrous Gluconate 27.9 mg/L

5 June 2001s

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	3.3	10.0	64.1
2s			
2m			
3s	<0.5		
3m	10.6	21.1	24.2
4s			
4m			
5s	<0.5		
5m	4.4	13.3	52.5
6s	1.5		
6m	5.3	10.7	61.7
7s	<0.5		
7m	8.5	17.0	39.1
8s	<0.5		
8m	10.2	20.3	27.1

calibration error: 3%

2 July 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	4.3	12.9	53.9
2s	<0.5		
2m	5.5	16.6	40.5
3s	<0.5		
3m	3.9	11.8	57.8
4s	<0.5		
4m	2.1	6.3	77.6
5s	<0.5		
5m	3.3	9.9	64.5
6s	<0.5		
6m	9.2	18.5	33.7
7s			
7m			
8s			
8m			

calibration error: 1%

Ferrous Gluconate 27.9 mg/L

23 July 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s	<0.5		
2m	7.6	15.3	45.2
3s	<0.5		
3m	7.5	15.0	46.3
4s	<0.5		
4m	8.1	16.2	42.1
5s	<0.5		
5m	6.9	13.8	50.6
6s	<0.5		
6m	8.1	16.2	41.9
7s	<0.5		
7m	6.9	13.8	50.5
8s			
8m			

calibration error: 1%

16 July 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	9.4	18.8	32.7
2s			
2m	8.1	16.2	41.8
3s	<0.5		
3m	7.4	14.9	46.7
4s	<0.5		
4m	5.4	10.8	61.4
5s			
5m			
6s	<0.5		
6m	5.3	10.7	61.8
7s			
7m			
8s			
8m			

calibration error: 7%

Ferrous Gluconate 27.9 mg/L

6 August 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	6.3	12.6	54.8
2s	<0.5		
2m	7.2	14.4	48.2
3s	<0.5		
3m	6.3	12.6	54.9
4s	<0.5		
4m	2.8	5.7	79.6
5s	<0.5		
5m	4.2	8.3	70.1
6s	<0.5		
6m	5.1	10.2	63.5
7s	<0.5		
7m	5.0	9.9	64.4
8s			
8m			

calibration error: 4%

13 August 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	5.4	10.9	61.0
2s			
2m			
3s	<0.5		
3m	6.6	13.2	52.8
4s			
4m			
5s	<0.5		
5m	7.7	15.4	44.8
6s	<0.5		
6m	5.8	11.6	58.6
7s	<0.5		
7m	2.4	4.7	83.1
8s	0.7		
8m	4.8	9.5	65.8

calibration error: 4%

Ferrous Gluconate 27.9 mg/L

20 August 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	4.6	13.8	50.7
2s	<0.5		
2m	4.0	11.9	57.4
3s	<0.5		
3m	3.4	10.3	63.2
4s	<0.5		
4m	4.2	12.6	54.8
5s			
5m			
6s	<0.5		
6m	2.5	7.4	73.5
7s	<0.5		
7m	3.1	9.2	66.9
8s			
8m			

calibration error: 4%

27 August 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	<0.5		
1m	6.5	13.0	53.3
2s	<0.5		
2m	7.4	14.9	46.7
3s	<0.5		
3m	9.9	19.8	29.0
4s	<0.5		
4m	8.1	16.1	42.2
5s	<0.5		
5m	5.3	15.8	43.2
6s	<0.5		
6m	5.9	11.9	57.5
7s	2.1		
7m	8.8	17.6	36.8
8s			
8m			

calibration error: 6%

Ferrous Gluconate 27.9 mg/L

3 September 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*End iron concentration adjusted for dilution (mg/L)*	*Change in iron concentration (%)*
1s	0.0		
1m	7.0	21.1	24.3
2s	0.0		
2m	5.3	10.6	61.9
3s	0.0		
3m	8.3	16.6	40.4
4s			
4m			
5s	<0.5		
5m	10.0	20.1	28.1
6s	<0.5		
6m	5.7	11.4	59.2
7s	<0.5		
7m	5.4	10.8	61.5
8s			
8m			

calibration error: 4%

Ferrous gluconate 9.3 mg/L

19 November 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	1.1	
1m	6.9	26.1
2s	<0.5	
2m	5.0	46.0
3s		
3m		
4s	<0.5	
4m	5.8	38.2
5s	<0.5	
5m	4.6	50.9
6s	<0.5	
6m	5.7	39.2
7s	<0.5	
7m	2.2	76.2
8s	<0.5	
8m	2.8	70.4

calibration error: 7%

21 November 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	5.6	39.8
2s	<0.5	
2m	3.6	61.2
3s	<0.5	
3m	3.3	64.0
4s		
4m		
5s	<0.5	
5m	3.7	60.4
6s		
6m		
7s		
7m		
8s		
8m		

calibration error: 7%

Ferrous gluconate 9.3 mg/L

22 November 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	5.6	39.6
2s	<0.5	
2m	5.2	43.6
3s		
3m		
4s	<0.5	
4m	2.7	71.2
5s	<0.5	
5m	2.4	74.6
6s	<0.5	
6m	2.2	76.6
7s	<0.5	
7m	4.0	56.7
8s		
8m		

calibration error: 7%

28 November 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	5.5	41.2
2s	<0.5	
2m	2.7	70.8
3s	<0.5	
3m	3.5	62.1
4s	0.5	
4m	4.3	53.8
5s	<0.5	
5m	3.3	65.0
6s		
6m		
7s	<0.5	
7m	3.7	60.1
8s	<0.5	
8m	3.8	59.6

calibration error: 8%

Ferrous gluconate 9.3 mg/L

29 November 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		
1m		
2s	<0.5	
2m	6.2	33.1
3s		
3m		
4s	<0.5	
4m	5.0	46.0
5s	<0.5	
5m	1.6	82.4
6s	<0.5	
6m	1.9	80.0
7s	<0.5	
7m	2.0	78.4
8s		
8m		

calibration error: 8%

30 November 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	5.7	38.8
2s	<0.5	
2m	4.8	48.9
3s	<0.5	
3m	4.7	49.1
4s	<0.5	
4m	4.3	53.4
5s		
5m	0.5	
6s	<0.5	
6m	5.0	46.3
7s		
7m		
8s		
8m		

calibration error: 8%

Ferrous gluconate 9.3 mg/L

3 December 2001

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	4.8	47.9
2s	<0.5	
2m	4.2	54.8
3s		
3m		
4s	0.9	
4m	3.4	62.9
5s	<0.5	
5m	5.0	46.1
6s	0.5	
6m	1.9	79.4
7s	<0.5	
7m	5.0	46.2
8s	<0.5	
8m	5.6	39.5

calibration error: 8%

Ferrous gluconate 9.3 mg/L plus manganese sulphate 91.5 mg/L

12 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s		<0.5	
2m	5.9	5.9	-0.1
3s		<0.5	
3m	6.0	4.9	18.5
4s		<0.5	
4m	6.6	5.0	24.7
5s		<0.5	
5m	5.4	2.3	58.5
6s		<0.5	
6m	6.7	4.3	35.3
7s			
7m			
8s			
8m			

calibration error: 2%

Ferrous gluconate 9.3 mg/L plus manganese sulphate 9.15 mg/L

30 January 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s		<0.5	
2m	6.8	6.0	11.4
3s		<0.5	
3m	6.4	5.9	7.3
4s		<0.5	
4m	7.2	4.9	32.1
5s		<0.5	
5m	7.7	5.0	34.8
6s		<0.5	
6m	7.2	2.3	68.7
7s		<0.5	
7m	6.9	4.3	37.0
8s			
8m			

calibration error: 6%

Ferrous gluconate 9.3 mg/L plus manganese sulphate 9.15 mg/L

4 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		0.0	
1m	9.0	7.4	17.9
2s		0.0	
2m	9.5	5.2	45.4
3s		0.1	
3m	8.9	3.4	61.2
4s		0.1	
4m	9.2	6.2	31.9
5s			
5m			
6s			
6m			
7s			
7m			
8s		0.2	
8m	8.9	4.0	54.8

calibration error: 6%

5 February 2002

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	6.5	18.5
2s	<0.5	
2m	5.8	27.2
3s	2.2	
3m	3.9	51.8
4s	<0.5	
4m	4.2	47.3
5s	<0.5	
5m	2.8	64.6
6s	<0.5	
6m	6.1	23.3
7s		
7m		
8s		
8m		

calibration error: 6%

Ferrous gluconate 9.3 mg/L plus manganese sulphate 9.15 mg/L

8 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	6.9	3.4	50.1
2s		<0.5	
2m	8.2	5.4	34.1
3s		<0.5	
3m	6.8	5.2	24.1
4s		<0.5	
4m	8.2	5.4	34.6
5s		<0.5	
5m	7.2	1.7	76.0
6s		<0.5	
6m	8.5	5.3	37.2
7s		<0.5	
7m	8.3	5.3	36.5
8s			
8m			

calibration error: 5%

11 February 2002

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	6.5	dummy
1m		
2s	<0.5	
2m	4.4	45.2
3s	<0.5	
3m	5.5	31.7
4s	<0.5	
4m	5.2	35.5
5s	<0.5	
5m	2.9	63.8
6s	<0.5	
6m	3.6	55.4
7s	<0.5	
7m	1.5	80.7
8s	<0.5	
8m	4.6	42.1

calibration error: 2%

Ferrous gluconate 9.3 mg/L plus manganese sulphate 9.15 mg/L

12 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	7.2	2.8	61.7
2s		<0.5	
2m	7.8	5.7	26.6
3s		<0.5	
3m	6.9	4.9	29.2
4s		<0.5	
4m	7.5	4.0	45.9
5s		<0.5	
5m	5.9	2.4	58.5
6s			
6m			
7s			
7m			
8s			
8m			

calibration error: 2%

17 June 2002 /1

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s			
2m			
3s		<0.5	
3m	5.5	4.7	14.2
4s		<0.5	
4m	6.8	4.2	37.9
5s			
5m			
6s			
6m			
7s		<0.5	
7m	6.0	4.6	24.1
8s			
8m			

calibration error: 5%

Ferrous gluconate 9.3 mg/L and calcium chloride 200 mg/L

6 March 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	4.9	1.8	62.5
2s		<0.5	
2m	5.4	2.1	60.8
3s		<0.5	
3m	5.0	1.0	79.3
4s		<0.5	
4m	5.1	0.7	85.6
5s		<0.5	
5m	5.2	0.5	89.6
6s		<0.5	
6m	5.5	0.5	90.7
7s		<0.5	
7m	4.6	0.6	87.2
8s		<0.5	
8m	5.7	0.9	85.1

calibration error: 6%

7 March 2002 /1

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	4.3	3.0	30.2
2s			
2m			
3s			
3m			
4s		<0.5	
4m	3.8	1.8	52.0
5s		<0.5	
5m	5.2	1.1	79.2
6s		<0.5	
6m	5.7	1.0	81.6
7s		<0.5	
7m	3.8	3.2	16.1
8s		<0.5	
8m	4.5	2.3	48.8

calibration error: 6%

Ferrous gluconate 9.3 mg/L and calcium chloride 200 mg/L

20 March 2002 /1

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s		<0.5	
2m	7.1	2.0	72.2
3s			
3m	7.1		
4s			
4m			
5s		<0.5	
5m	7.1	1.9	73.2
6s		<0.5	
6m	7.2	4.3	40.1
7s		<0.5	
7m	7.2	3.2	56.2
8s		<0.5	
8m	7.0	1.1	84.2

calibration error: 5%

20 March 2002 /2

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	6.2	1.4	77.2
2s		<0.5	
2m	5.8	1.8	69.4
3s		<0.5	
3m	5.6	2.3	59.0
4s		<0.5	
4m	5.9	2.4	59.1
5s		<0.5	
5m	5.9	2.2	63.3
6s		<0.5	
6m	3.2		
7s		<0.5	
7m	6.0	1.8	70.4
8s		<0.5	
8m	7.2	3.3	54.7

calibration error: 5%

Ferrous gluconate 9.3 mg/L and calcium chloride 200 mg/L

22 March 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	7.6	3.6	52.9
2s			
2m			
3s			
3m			
4s		<0.5	
4m	8.0	1.8	77.2
5s		<0.5	
5m	7.7	4.1	46.6
6s		<0.5	
6m	7.9	5.9	25.3
7s		<0.5	
7m	7.2	1.0	86.0
8s		<0.5	
8m	7.2	1.0	86.2

calibration error: 5%

19 June 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	9.1	5.9	35.7
2s		<0.5	
2m	8.1	4.8	41.3
3s			
3m			
4s		<0.5	
4m	5.9	5.4	9.6
5s		<0.5	
5m	6.4	6.1	4.9
6s		2.5	
6m	5.9	3.5	40.0
7s		<0.5	
7m	5.5	4.8	12.4
8s		<0.5	
8m	6.8	5.5	19.3

calibration error: 5%

Glucose in the mucosal chamber (with ferrous gluconate 9.3 mg/L)

18 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	7.1	4.9	30.4
2s		<0.5	
2m	8.1	4.7	41.9
3s		<0.5	
3m	7.0	3.9	44.7
4s		<0.5	
4m	8.0	4.5	44.3
5s		<0.5	
5m	7.7	3.1	59.5
6s		<0.5	
6m	7.5	2.6	66.1
7s		<0.5	
7m	7.6	5.9	22.4
8s		<0.5	
8m	8.9	6.7	24.8

calibration error: 2%

19 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	7.7	5.4	29.6
2s		<0.5	
2m	8.7	7.1	18.3
3s			
3m			
4s		<0.5	
4m	8.1	5.4	33.0
5s		<0.5	
5m	8.7	5.8	33.7
6s		<0.5	
6m	7.6	2.8	64.0
7s		<0.5	
7m	7.3	3.0	58.7
8s		<0.5	
8m	7.8	2.5	68.0

calibration error: 2%

Glucose in the mucosal chamber (with ferrous gluconate 9.3 mg/L)

20 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s		<0.5	
2m	8.2	5.1	37.2
3s		<0.5	
3m	8.2	3.3	60.1
4s		<0.5	
4m	6.0	2.6	56.5
5s		<0.5	
5m	5.4	3.1	41.6
6s		<0.5	
6m	6.4	2.7	58.3
7s		<0.5	
7m	9.8	4.4	55.4
8s		<0.5	
8m	9.9	5.6	42.9

calibration error: 2%

21 February 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	8.2	4.9	39.7
2s		<0.5	
2m	8.7	5.1	42.2
3s		<0.5	
3m	7.9	5.2	34.2
4s		<0.5	
4m	8.1	4.2	48.4
5s		<0.5	
5m	7.8	5.5	29.2
6s		<0.5	
6m	6.7	5.7	13.7
7s		<0.5	
7m	8.1	4.9	39.9
8s		<0.5	
8m	7.8	5.2	34.1

calibration error: 2%

Glucose in the mucosal chamber (with ferrous gluconate 9.3 mg/L)

28 February 2002 /1

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s		<0.5	
2m	8.5	5.8	31.6
3s		<0.5	
3m	9.2	5.6	39.0
4s		<0.5	
4m	9.0	3.6	59.4
5s		<0.5	
5m	8.7	2.3	73.5
6s		<0.5	
6m	8.4	6.0	28.4
7s			
7m			
8s		3.6	
8m	7.8	3.6	54.5

calibration error: 2%

28 February 2002 /2

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	7.7	5.1	34.2
2s		<0.5	
2m	9.0	3.5	60.9
3s		<0.5	
3m	8.4	5.9	29.9
4s		<0.5	
4m	8.4	5.9	29.6
5s			
5m			
6s		<0.5	
6m	7.7	2.4	68.9
7s		<0.5	
7m	7.2	2.6	64.0
8s		<0.5	
8m	7.6	3.1	59.8

calibration error: 2%

Ferrous sulphate 9.3 mg/L

11 March 2002 /1

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	4.6	3.5	25.4
2s		<0.5	
2m	5.6	2.8	49.7
3s		<0.5	
3m	4.5	1.6	65.3
4s		<0.5	
4m	4.9	3.3	32.9
5s		<0.5	
5m	5.2	1.6	70.0
6s		0.1	
6m	10.2	8.0	21.4
7s			
7m			
8s		<0.5	
8m	4.0	2.7	32.7

calibration error: 6%

12 March 2002 /2

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	5.7	3.6	37.1
2s		<0.5	
2m	5.8	2.5	57.2
3s		<0.5	
3m	5.9	3.2	45.9
4s		<0.5	
4m	5.3	3.6	31.6
5s		<0.5	
5m	4.3	1.5	66.1
6s		<0.5	
6m	4.9	2.2	54.5
7s		<0.5	
7m	5.4	1.6	69.3
8s		<0.5	
8m	9.0	7.3	18.9

calibration error: 6%

Ferrous sulphate 9.3 mg/L

18 March 2002 /1

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	6.1	4.5	25.2
2s		<0.5	
2m	6.6	4.0	38.6
3s			
3m			
4s		<0.5	
4m	6.4	3.2	50.5
5s		<0.5	
5m	6.2	2.3	63.0
6s		<0.5	
6m	6.1	2.3	63.3
7s		<0.5	
7m	5.7	5.1	11.6
8s		<0.5	
8m	5.6	2.2	60.3

calibration error: 6%

18 March 2002 /2

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m	6.1		
2s			
2m			
3s		<0.5	
3m	5.1	3.2	36.7
4s			
4m	6.2		
5s		<0.5	
5m	10.8	1.5	86.5
6s		<0.5	
6m	6.3	4.0	37.7
7s		<0.5	
7m	5.2	2.8	45.9
8s		<0.5	
8m	5.2	1.6	68.8

calibration error: 6%

Ferrous sulphate 9.3 mg/L

16 April 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s		<0.5	
2m	10.5	8.9	15.2
3s		<0.5	
3m	10.2	5.1	49.5
4s		<0.5	
4m	10.3	6.7	34.5
5s		<0.5	
5m	9.8	8.8	10.4
6s		<0.5	
6m	10.2	7.8	23.9
7s			
7m			
8s			
8m			

calibration error: 6%

Ferric chloride 9.3 mg/L

10 January 2002

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		
1m		
2s	<0.5	
2m	3.6	40.5
3s	<0.5	
3m	2.6	56.6
4s	<0.5	
4m	2.1	65.2
5s	<0.5	
5m	1.3	78.1
6s	<0.5	
6m	0.9	85.6
7s	<0.5	
7m	4.0	33.3
8s	1.0	
8m	1.4	76.4

calibration error: 8%

11 January 2002

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	4.0	33.3
2s	<0.5	
2m	3.3	44.5
3s	<0.5	
3m	3.2	46.9
4s	<0.5	
4m	2.6	56.8
5s	<0.5	
5m	1.3	78.5
6s	<0.5	
6m	0.9	84.7
7s	<0.5	
7m	2.1	64.7
8s		
8m		

calibration error: 8%

Ferric chloride 9.3 mg/L

15 January 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s			
1m			
2s			
2m			
3s		0.9	
3m	6.6	3.7	44.5
4s		<0.5	
4m	4.7	2.7	41.2
5s		<0.5	
5m	4.5	1.2	72.5
6s		<0.5	
6m	5.4	3.6	32.9
7s			
7m			
8s		<0.5	
8m	3.9	1.0	74.1

calibration error: 4%

16 January 2002

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	2.7	54.5
2s	<0.5	
2m	1.9	69.0
3s	<0.5	
3m	4.7	21.9
4s		
4m		
5s		
5m		
6s		
6m		
7s		
7m		
8s		
8m		

calibration error: 8%

Ferric chloride 9.3 mg/L

17 January 2002

Intestinal Segment Number	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s	<0.5	
1m	4.2	30.5
2s	<0.5	
2m	4.8	19.3
3s	<0.5	
3m	5.0	17.4
4s	<0.5	
4m	3.6	39.2
5s	<0.5	
5m	1.8	69.6
6s	<0.5	
6m	4.3	27.7
7s	<0.5	
7m	4.6	23.2
8s		
8m		

calibration error: 8%

22 March 2002

Intestinal Segment Number	*Starting iron concentration (mg/L)*	*End iron concentration (mg/L)*	*Change in iron concentration (%)*
1s		<0.5	
1m	6.5	4.9	23.8
2s		<0.5	
2m	6.5	3.8	41.3
3s		<0.5	
3m	6.6	5.3	20.6
4s		<0.5	
4m	6.5	6.1	6.3
5s		<0.5	
5m	6.7	5.2	23.1
6s			
6m			
7s		<0.5	
7m	6.3	4.2	33.2
8s		<0.5	
8m	7.2	6.0	16.4

calibration error: 6%

Total Iron Recovery - summary of all results

Tissue Number	*Iron recovered in mucus (%)*	*Iron recovered in intestine (%)*	*Iron recovered in mucosal Ringer's solution (%)*	*Iron recovered in serosal Ringer's solution (%)*
1	28.66	1.00	66.35	3.98
2	20.51	9.67	66.42	3.41
3	32.48	4.77	61.33	1.42
4	28.33	7.79	60.21	3.67
5	21.89	8.63	66.81	2.67
6	22.77	11.91	61.73	3.60
7	14.62	8.10	70.67	6.61
8	8.03	4.69	85.63	1.65
9	3.62	4.13	89.93	2.31
10	9.09	4.64	84.90	1.37
11	10.01	0.00	88.15	1.84
12	9.60	10.36	77.89	2.14
13	8.54	3.65	86.93	0.88
14	14.17	7.66	75.90	2.27
15	17.71	2.23	79.35	0.72
16	28.98	3.14	67.07	0.81
17	20.12	3.91	75.19	0.78
18	13.41	6.35	79.33	0.90
average	17.4	5.7	74.7	2.3
standard error	2.0	0.8	2.3	0.3

calibration error: 1%

Tissue Number	*Starting iron concentration (mg/L)*	*Mucus iron concentration (mg/L)*	*Intestinal iron concentration (mg/L)*	*Mucosal Ringer's solution iron concentration (mg/L)*	*Serosal Ringer's solution iron concentration (mg/L)*	*Total iron recovered (mg/L)*	*% recovered*
1	8.19	1.64	0.06	3.80	0.23	5.73	69.9
2	8.20	1.34	0.63	4.33	0.22	6.52	79.5
3	7.68	2.22	0.33	4.19	0.10	6.83	88.9
4	6.28	1.55	0.43	3.29	0.20	5.47	87.1
5	6.81	1.00	0.39	3.05	0.12	4.56	67.0
6	5.70	0.98	0.51	2.66	0.16	4.31	75.6
7	7.79	1.02	0.57	4.93	0.46	6.98	89.5
8	8.50	0.53	0.31	5.66	0.11	6.61	77.8
9	9.67	0.37	0.42	9.14	0.24	10.16	105.1
10	7.58	0.64	0.33	6.02	0.10	7.09	93.5
11	6.74	0.44	-	3.84	0.08	4.35	64.6
12	6.28	0.38	0.41	3.09	0.09	3.97	63.2
13	7.51	0.81	0.35	8.22	0.08	9.46	125.9
14	6.07	0.72	0.39	3.85	0.12	5.07	83.5
15	7.78	1.36	0.17	6.10	0.06	7.68	98.7
16	5.69	1.97	0.21	4.57	0.06	6.81	119.6
17	6.61	1.58	0.31	5.89	0.06	7.83	118.5
18	6.82	0.74	0.35	4.39	0.05	5.54	81.2
average	6.8	1.1	0.4	4.8	0.1	6.4	88.3
standard error	0.2	0.1	0.03	0.4	0.02	0.4	4.4

Appendix C: Electrophysiological Data

Fluid Resistance - values taken from a sample of experiments

Average (Ω)	*Standard Error (Ω)*	*Maximum (Ω)*	*Minimum (Ω)*
112.3	0.8	148.3	79.9

Raw Data (Ω):

133.6	121.3	125.0	114.0	127.5	143.5	134.8	108.1	122.6
148.3	104.4	125.0	136.2	109.8	131.4	123.5	115.4	118.8
111.1	92.4	108.0	144.2	106.2	98.7	108.8	114.4	127.0
119.7	106.9	109.5	115.7	109.5	125.5	129.2	117.9	125.6
90.3	129.2	123.9	143.5	137.6	142.7	135.5	105.8	81.7
86.2	101.8	133.0	140.8	143.5	133.6	119.1	109.2	121.2
87.9	102.9	103.6	129.0	119.1	106.4	136.4	118.9	121.1
81.6	104.9	103.6	117.2	113.4	112.9	136.2	114.2	137.3
88.1	116.5	131.2	125.5	125.5	132.4	113.4	112.8	116.5
91.5	108.5	114.8	101.0	105.7	127.9	117.9	83.6	107.4
101.8	123.4	115.2	98.3	114.6	127.8	103.7	101.6	118.3
84.3	102.8	123.4	104.9	85.3	126.7	113.3	112.3	112.4
85.3	105.7	123.9	107.8	115.7	105.8	109.4	107.7	130.2
79.9	109.1	119.0	111.9	105.5	131.9	121.6	112.7	114.8
82.2	105.8	109.2	103.6	110.3	128.1	90.0	87.5	114.7
83.1	90.0	126.1	84.6	122.5	86.2	126.7	118.4	113.1
100.0	116.1	119.8	112.0	91.2	135.3	131.2	112.8	112.4
88.3	109.4	122.3	126.1	111.1	119.7	132.7	118.8	113.6
89.3	102.2	118.1	140.2	114.6	130.8	126.0	108.8	113.7
84.8	109.9	121.8	115.7	115.1	129.0	135.1	127.6	122.0
114.9	102.2	118.9	101.3	112.8	105.4	129.7	85.3	126.0
86.2	120.0	89.3	115.2	102.0	137.8	119.5	111.6	116.5
81.4	124.5	117.4	116.9	108.9	104.1	112.0	106.9	125.5
86.2	104.9	120.5	104.5	100.0	117.6	130.3	112.0	100.7
87.2	110.2	121.5	109.0	112.8	117.6	126.5	100.7	103.9
80.5	107.3	117.4	112.9	109.8	121.3	115.8	108.2	104.5
88.6	113.1	113.3	113.7	117.9	125.5	135.7	112.8	107.3
81.4	107.8	132.7	112.5	122.9	125.0	122.8	84.8	109.8
85.6	108.6	116.5	106.5	102.6	120.2	134.5	82.6	116.1
84.1	105.8	121.1	122.0	107.7	124.5	128.2	102.6	105.5
82.8	104.1	122.4	104.1	118.4	114.6	124.5	126.0	110.3
86.7	104.5	110.2	112.4	108.1	128.2	111.5	124.0	82.1
89.3	119.2	118.8	97.9	105.1	112.8	104.7	117.9	83.1
115.5	104.9	112.5	112.0	85.3	107.7	118.9	112.0	115.8
85.1	107.9	120.6	114.2	109.8	123.0	122.4	121.0	120.6
84.6	103.2	112.0	104.9	101.8	116.5	125.5	119.5	117.9
88.3	109.8	120.5	112.8	110.3	124.5	114.6	80.0	112.4
85.3	104.5	108.6	113.4	121.5	110.7	114.1	119.7	

Short Circuit Current - values taken from a sample of experiments.

Average ($\mu Amp.cm^{-2}$)	*Standard Error* ($\mu Amp.cm^{-2}$)	*Maximum* ($\mu Amp.cm^{-2}$)	*Minimum* ($\mu Amp.cm^{-2}$)
33.0	2.1	138.0	0.4

Raw Data ($\mu Amp.cm^{-2}$):

47	66	22	17	84	56	46	32	30
62	41	0.7	7	2	36	50	28	20
1.5	19	0.4	13	2	42	2	10	24
32	35	17	13	46	34	32	74	36
50	138	30	22	20	10	32	20	50
58	56	40	24	2	26	54	32	18
18	66	38	8	92	72	38	16	62
20	80	32	30	30	12	6	12	14
20	40	4	38	10	15	66	27	34
30	28	22	18	50	32	15	2	64
56	8	12	14	14	40	38	27	14
38	40	32	16	32	76	32	70	38
26	26	14	16	40	86	36	27	16
18	70	24	12	72	54	1.4	34	34
24	16	1.4	2	26	54	34	27	18
10	20	74	112	66	38	58	14	26

Tissue Resistance - values taken from a sample of experiments.

Average ($\Omega.cm^{-2}$)	*Standard Error* ($\Omega.cm^{-2}$)	*Maximum* ($\Omega.cm^{-2}$)	*Minimum* ($\Omega.cm^{-2}$)
63.5	2.2	158.0	8.0

Raw Data ($\Omega.cm^{-2}$):

12	44	153	34	100	88	50	80	66
38	43	55	43	46	56	44	44	48
69	24	37	31	36	68	66	66	42
104	48	58	50	40	80	42	60	40
54	8	16	28	40	70	23	60	40
74	54	42	26	46	66	60	34	84
72	120	46	64	158	57	50	58	44
68	106	66	114	72	91	56	88	64
62	60	52	36	70	70	38	58	51
62	60	52	36	128	51	54	53	48
99	81	80	71	43	32	70	79	37
38	104	44	60	65	125	46	138	126
60	102	68	46	68	35	25	76	107
154	118	80	58	88	90	114	114	60
56	24	48	54	64	80	100	44	24
98	82	122	80	42	56	60	62	70
44	66	22	34	70				

Starting Short Circuit Current - glucose in the mucosal chamber:

Average ($\mu Amp.cm^{-2}$)	*Standard Error* ($\mu Amp.cm^{-2}$)	*Maximum* ($\mu Amp.cm^{-2}$)	*Minimum* ($\mu Amp.cm^{-2}$)
35.4	4.2	64	13

Raw Data ($\mu Amp.cm^{-2}$):

32	49	19	29	21	49	25	26
58	28	64	28	62	17	47	13

Change In Short Circuit Current - glucose in the mucosal chamber:

Average ($\Delta \mu Amp.cm^{-2}$)	*Standard Error* ($\Delta \mu Amp.cm^{-2}$)
13.7	3.5

Raw Data ($\Delta \mu Amp.cm^{-2}$):

9	40	15	4	3	29	16	26
11	35	2	-6	-1	28	7	13

Tissue Resistance - glucose in the mucosal chamber:

Average (Ωcm^{-2})	*Standard Error* (Ωcm^{-2})	*Maximum* (Ωcm^{-2})	*Minimum* (Ωcm^{-2})
58.2	0.9	94.0	43.0

Raw Data (Ω.cm-2):

52	58	48	68	94	58	43	43
76	50	58	60	44	58	45	76

Appendix D: ICP Intestine Analysis

Intestinal segment	*mouse 1*	*mouse 2*	*mouse 3*	*mouse 4*	*average*
	Fe (ug/g dry material)				
1	272	368	331	513	371.0
2	171	209	228	301	227.3
3	127	182	130	146	146.3
4	110	144	170	168	148.0
5	104	179	135	123	135.3
6	105	205	150	164	156.0
7	114	163	107	123	126.8
8	114	116	98	175	125.8

Mouse number	*Intestinal Segment*	*Iron Content (ug/g)*	*Dry Weight (g)*	*Iron Concentration (mg/L)*
1	1	272	0.0056	8.40×10^{-06}
1	2	171	0.0135	1.26×10^{-05}
1	3	127	0.0119	8.28×10^{-06}
1	4	110	0.0119	7.23×10^{-06}
1	5	104	0.0110	6.27×10^{-06}
1	6	105	0.0103	5.93×10^{-06}
1	7	113	0.0063	3.92×10^{-06}
1	8	114	0.0109	6.78×10^{-06}
2	1	368	0.0157	3.17×10^{-05}
2	2	209	0.0114	1.30×10^{-05}
2	3	182	0.0073	7.29×10^{-06}
2	4	144	0.0064	5.02×10^{-06}
2	5	180	0.0070	6.93×10^{-06}
2	6	207	0.0030	3.40×10^{-06}
2	7	166	0.0038	3.44×10^{-06}
2	8	118	0.0078	5.09×10^{-06}
3	1	339	0.0117	2.17×10^{-05}
3	2	235	0.0090	1.16×10^{-05}
3	3	134	0.0124	9.15×10^{-06}
3	4	203	0.0053	5.92×10^{-06}
3	5	137	0.0086	6.47×10^{-06}
3	6	128	0.0047	3.34×10^{-06}
3	7	110	0.0060	3.60×10^{-06}
3	8	101	0.0101	5.56×10^{-06}
4	1	524	0.0115	3.31×10^{-05}
4	2	263	0.0063	9.06×10^{-06}
4	3	145	0.0063	5.02×10^{-06}
4	4	165	0.0051	4.61×10^{-06}
4	5	124	0.0139	9.52×10^{-06}
4	6	176	0.0082	7.96×10^{-06}
4	7	123	0.0081	5.48×10^{-06}
4	8	179	0.0099	9.74×10^{-06}
			average:	1.11×10^{-05}

8. BIBLIOGRAPHY

Alkemade C. T. J., Herrman R., (1979). Fundamentals of Analytical Flame Spectroscopy, Bristol: Adam Hilger Ltd, p 44-46.

Alvarez-Hernandez, X., Nichols, G. M., Glass, J. (1991). Caco-2 Cell Line: A System for Studying Intestinal Iron Transport across Epithelial Cell Monolayers. Biochimica et Biophysica Acta, 1070(1), 205-208.

Asano, T. (1964) Metabolic Disturbances and Short-Circuit Current Across Intestinal Wall of Rat. American Journal of Physiology, 207, 415-422.

Au, A. P., Reddy, M. B. (2000). Caco-2 Cells can be used to Assess Human Iron Bioavailability from a Semipurified Meal. The Journal of Nutrition, 130(5), 1329-1334.

Banerjee, D., Flanagan, P. R., Cluett, J., Valberg, L. S. (1986). Transferrin Receptors in the Human Gastrointestinal Tract: Relationship to Body Iron Stores. Gastroenterology, 91(4), 861-869.

Barton, J. C., Conrad, M. E., Parmley, R. T. (1983). Calcium Inhibition of Inorganic Iron Absorption in Rats. Gastroenterology, 84(1), 90-101.

Baynes, R. D., Bothwell, T. H. (1990). Iron Deficiency. Annual Review of Nutrition, 10, 133-148.

Beard, J. L., Dawson, H., Pinero, D. J. (1996). Iron Metabolism: A Comprehensive Review. Nutrition Reviews, 54(10), 295-317.

Berglund, J. J., Riegler, M., Zolotarevsky, Y., Wenzi, E., Turner, J. R. (2001). Regulation of Human Jejunal Transmucosal Resistance and MLC Phosphorylation by Na+-Glucose Cotransport. American Journal of Physiology, 281(6, part 1), G1487-G1493.

Bjorn-Rasmussen, E., Hallberg, L., Walker, R. B. (1972). Food Iron Absorption in Man. I. Isotopic Exchange Between Food Iron and Inorganic Iron Salt Added to Food: Studies on Maize, Wheat, and Eggs. The American Journal of Clinical Nutrition, 25(3), 317-323.

Bothwell, T. H., Pirzio-Biroli, G., Finch, C. A. (1958). Iron Absorption. I. Factors Influencing Absorption. The Journal of Laboratory and Clinical Medicine. 51(1), 24-36.

Bronk, J.R. (1999). Human Metabolism, Harlow: Addison Wesley Longman Limited.

Brown, E. B., Justus, B. W. (1958). *In Vitro* Absorption of Radioiron by Everted Pouches of Rat Intestine. American journal of Physiology, 194, 319.

Campos, M. S., Gomez-Ayala, A. E., Lopez-Aliaga, I., Pallares, I., Hartiti, S., Pharm, B., Alferez, M. J. M., Barrionuevo, M., Rodriguez-Matas, M. C., Lisbona, F. (1996). Role of the Proximal Colon in Mineral Absorption in Rats With and Without Ferropenic Anemia. Nutrition Research, 16(9), 1529-1543.

Canonne-Hargaux, F., Gruenheid, S., Ponka, P., Gros, P. (1999). Cellular and Subcellular Localization of the Nramp2 Iron Transporter in the Intestinal Brush Border and Regulation by Dietary Iron. Blood, 93(12), 4406-4417.

Chowrimootoo, G., Debnam, E. S., Srai, S. K. S., Epstein, O. (1992). Regional Characteristics of Intestinal Iron Absorption in the Guinea-Pig. Experimental Physiology, 77(1), 177-183.

Conrad, M. E., Parmley, R. T., Osterlot, K. (1987). Small Intestinal Regulation of Iron Absorption in the Rat. The Journal of Laboratory and Clinical Medicine, 110(4), 418-426.

Conrad, M. E., Umbreit, J. N., Moore, E. G. (1991). A Role for Mucin in the Absorption of Inorganic Iron and Other Metal Cations: A Study in Rats. Gastroenterology, 100(1), 129-136.

Conrad, M. E., Umbreit, J. N., Moore, E. G., Peterson, R. D. A., Jones, M. B. (1990). A Newly Identified Iron Binding Protein in Duodenal Mucosa of Rats. The Journal of Biological Chemistry, 265(9), 5273-5279.

Conrad, M. E., Umbreit, J. N., Moore, E. G., Rodning, C. R. (1992). Newly Identified Iron-Binding Protein in Human Duodenal Mucosa. Blood, 70(1), 244-247.

Conrad, M. E., Weintraub, L. R., Crosby, W. H. (1964). The Role of the Intestine in Iron Kinetics. The Journal of Clinical Investigation, 43(5), 963-974.

Cook, J. D., Baynes, R. D., Skikne, B. S. (1992). Iron Deficiency and the Measurement of Iron Status. Nutrition Research Reviews, 5, 189-202.

Cook, J. D., Dassenko, S. A., Whittaker, P. (1991). Calcium Supplementation: Effect on Iron Absorption. The American Journal of Clinical Nutrition, 53(1), 106-111.

Cook, J. D., Layrisse, M., Martinez-Torres, C., Walker, R., Monsen, E., Finch, C. A. (1972). Food Iron Absorption Measured by Extrinsic Tag. The Journal of Clinical Investigation, 51(4), 805-815.

Cook, J. D., Lynch, S. R. (1986). The Liabilities of Iron Deficiency. Blood, 68(4), 803-809.

Cook, J. D., Monsen, E. R. (1975). Food Iron Absorption. I. Use of a Semisynthetic Diet to Study Absorption on Nonheme Iron. The American Journal of Clinical Nutrition, 28(11), 1289-1295.

Costa, L. M. G., da Costa, A. R., de Sousa, M. (2000). Voltage-clamp: A Useful Approach to Study *In vitro* Duodenal Iron Transport in the Mouse. Biometals, 13, 169-178.

Cox, T. M., O'Donnell, M. W. (1981). Studies on the Binding of Iron by Rabbit Intestinal Microvillus Membranes. The Biochemical Journal - Cellular Aspects, 194(3), 753-759.

Crane, R. K., Wilson, T. H. (1958). *In Vitro* Method for the Study of the Rate of Intestinal Absorption of Sugars. Journal of Applied Physiology, 12, 145 .

Culling, C. F. A. (1985). Handbook of Histopathological and Histochemical Techniques. (4th Edition). London:Butterworths & Co. (Publishers) Ltd.

DeMaeyer, E., Adiels-Tegman, M. (1985). The Prevalence of Anaemia in the World. World Health Statistics Quarterly, 38, 302-316.

Dern, R. J., Hart, W. L. (1961A). Studies with Doubly Labeled Iron. I. Simultaneous Liquid Scintillation Counting of Isotopes Fe55 and Fe59 as Ferrous Perchlorate. The Journal of Laboratory and Clinical Medicine, 57(2), 322-330.

Dern, R. J., Hart, W. L. (1961B). Studies with Doubly Labeled Iron. II. Separation of Iron From Blood Samples and Preparation of Ferrous Perchlorate for Liquid Scintillation Counting. The Journal of Laboratory and Clinical Medicine, 57(3), 460-467.

Dowdle, E. R., Schachter, D., Schenker, H. (1960). Active Transport of Fe^{59} by Everted Segments of Rat Duodenum. American Journal of Physiology, 198(3), 609-613.

Eisenstein, R. S. (2000). Iron Regulatory Proteins and the Molecular Control of Mammalian Iron Metabolism. Annual Review of Nutrition, 20, 627-662.

Expert Scientific Working Group. (1985). Summary of a Report on Assessment of the Iron Nutritional Status of the United States Population. The American Journal of Clinical Nutrition, 42(6), 1318-1330.

Fairweather-Tait, S. (1993). Iron. International Journal for Vitamin and Nutrition Research, 63(4), 296-301.

Fairweather-Tait, S. (1987). The Concept of Bioavailability as it Relates to Iron Nutrition. Nutrition Research, 7, 319-325.

Field, M., Seki, M., Mitchell, M. L., Chalmers, T. C., (1960). Studies in Iron Absorption. I. Determination in Rats by Measurement of Total Body Radioactivity. The Journal of Laboratory and Clinical Medicine, 55(6), 929-935.

Fine, K. D., Santa Ana, C. A., Porter, J. L., Fordtran, J. S. (1994). Mechanism by Which Glucose Stimulates the Passive Absorption of Small Solutes by the Human Jejunum *In vivo*. Gastroenterology, 107(2), 389-395.

Fisher, R.B., Parson, D. D. (1949). A Preparation of Surviving Rat Small Intestine for the Study of Iron Absorption. The Journal of Clinical and Laboratory Medicine, 110, 36-46.

Forth, W., Rummel, W. (1973). Iron Absorption. Physiological Reviews, 53(3), 724-792.

Forrester, R. H., Conrad, M. E., Crosby, W. H. (1962). Measurement of Total Body $Iron^{59}$ in Animals Using Whole-Body Liquid Scintillation Detectors. Proceedings of the Society for Experimental Biology and Medicine, 111(1), 115-119.

Forstner, J. F., Forstner, G. G. (1975). Calcium Binding to Intestinal Goblet Cell Mucin. Biochimica et Biophysica Acta, 386, 283-292.

Gangloff, M. B., Lai, C., Van Campen, D. R., Miller, D. D., Norvell, W. A., Glahn, R. P. (1996). Ferrous Iron Uptake but Not Transfer is Down-Regulated in Caco-2 Cells Grown in High Iron Serum-Free Medium. The Journal of Nutrition, 126(12), 3118-3127.

Garcia-Casal, M. N., Layrusse, M., Solano, L., Baron, M. A. (1998). Vitamin A and Beta-Carotene can Improve Nonheme Iron Absorption from Rice, Wheat and Corn by Humans. The Journal of Nutrition, 128(3), 646-650.

Glahn, R. P., Gangloff, M. B., Van Campen, D. R., Miller, D. D., Wien, E. M., Norvell, W. A. (1995). Bathophenanthrolene Disulfic Acid and Sodium Dithionite Effectively Remove Surface-Bound Iron from Caco-2 Cell Monolayers. The Journal of Nutrition, 125(7), 1833-1840.

Glahn, R. P., Van Campen, D. R. (1997). Iron Uptake is Enhanced in Caco-2 Cell Monolayers by Cysteine and Reduced by Cysteinyl Glycine. The Journal of Nutrition, 127(4), 642-647.

Goddard, W. P., Coupland, K., Smith, J. A., Long, R. G. (1997). Iron Uptake by Isolated Human Enterocyte Suspensions *In Vitro* Dependent on Body Iron Stores and Inhibited by Other Metal Cations. The Journal of Nutrition, 127(1), 177-183.

Greenberger, N. J., Balcerzak, S. P., Ackerman, G. A. (1969). Iron Uptake by Isolated Intestinal Brush Borders: Changes Induced by Alterations in Iron Stores. The Journal of Laboratory and Clinical Medicine, 73(5), 711-721.

Grubb, B. R. (1995). Ion Transport Across the Jejunum in Normal and Cystic Fibrosis Mice. American Journal of Physiology, 268(31), G505-G513.

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L., Hediger, A. (1997). Coning and Characterization of a Mammalian Proton-Coupled Metal-Ion Transporter. Nature, 388, 482-488.

Hallberg, L. (2001). Perspectives on Nutritional Iron Deficiency. Annual Review of Nutrition, 21, 1-21.

Hallberg, L., Brune, M., Erlandsson, M., Sandberg, A., Rossander-Hulten, L. (1991). Calcium: Effect of Different Amounts on Nonheme- and Heme-Iron Absorption in Humans. The American Journal of Clinical Nutrition, 53(1), 112-119.

Halleux, C., Schneider, Y. (1991). Iron Absorption by Intestinal Epithelial Cells: 1. Caco-2 Cells Cultivated in Serum-Free Medium, on Polyethyleneterephthalate Microporous Membrane, as an *In Vitro* Model. *In Vitro* Cell and Developmental Biology, 27A, 293-302.

Halliday, J. W., Powell, L. W. (1973). The Use of Suspensions of Isolated Rat Mucosal Cells to Study Mechanisms or Iron Absorption. Clinica Chimica Acta, 43(2), 267-276.

Han, O., Failla, M. L., Hill, A. D., Morris, E. R., Smith, J. C. (1995). Reduction of Fe(III) is Required for Uptake by Caco-2 Cells. The Journal of Nutrition, 125(5), 1291-1299.

Helbrock, H. J., Saltman, P. (1967). The Transport of Iron by Rat Intestine. Biochimica et Biophysica Acta, 135, 979-990.

Hidalgo, I. J., Raub, T. J., Borchardt, R. T. (1989). Characterization of the Human Colon Carcinoma Cell Line (Caco-2) as a Model System for Intestinal Epithelial Permeability. Gastroenterology, 96(3), 736-749.

Hoffbrand, A. V., Pettit, J. E. (1993). Essential Haematology. (3rd Edition). Blackwell Scientific Productions: London.

Huebers, H. A., Huebers, E., Csiba, E., Rummel, W., Finch, C. A. (1983). The Significance of Transferrin for Intestinal Iron Absorption. Blood, 61(2), 283-290.

Hunt, J. R., Gallagher, S. K., Johnson, L. K., Lykken, G. I. (1995). High- Versus Low-Meat Diets: Effects on Zinc Absorption, Iron Status, and Calcium, Copper, Iron, Magnesium, Manganese, Nitrogen, Phosphorus, and Zinc Balance in Postmenopausal Women. The American Journal of Clinical Nutrition, 62(3), 621-632.

Hurrel, R. (2002). How to Ensure Adequate Iron Absorption from Iron-fortified Food. Nutrition Reviews, 60(7), S7-S15.

Jacobs, P., Bothwell, T. H., Charlton, R. W. (1966). Intestinal Iron Transport; Studies Using a Loop of Gut with an Artificial Circulation. American Journal of Physiology, 210(), 694-.

Jacobs, A., Greenman, D. A. (1969). Availability of Food Iron. British Medical Journal, 1(5645), 673-676.

Kessler, M., Acuto, O., Stroelli, C., Murer, H., Muller M., Semenza, G. (1978). A Modified Procedure for the Rapid Preparation of Efficiently Transporting Vesicles from Intestinal Brush Border. Biochimica et Biophysica Acta, 506, 136-154.

Kimmich, G. A. (1970). Preparation and Properties of Mucosal Epithelial Cells Isolated from Small Intestine of the Chicken. Biochemistry, 9(19), 3659-3668.

Latunde-Dada, G. O., Bianchi, M. L. P., de Oliveira, J. E. D. (1998). On the Methods for Studying the Mechanisms and Bioavailability of Iron. Nutrition Reviews, 56(3), 76-80.

Leibold, E. A., Guo, B. (1992). Iron-Dependent Regulation of Ferritin and Transferrin Receptor Expression by the Iron-Responsive Element Binding Protein. Annual Review of Nutrition, 12, 345-368.

Levine, P. H., Weintraub, L. R. (1970). Preparation of Suspensions of Small Bowel Mucosal Epithelial Cells. The Journal of Laboratory and Clinical Medicine, 75(6), 1026-1029.

Lynch, S. R. (1997). Interaction of Iron with Other Nutrients. Nutrition Reviews, 55(4), 102-110.

Madara, J. L. (1998). Regulation of the Movement of Solutes across Tight Junctions. Annual Review of Physiology, 60, 143-59.

Manis, J. G., Schachter, D. (1962). Active Transport of Iron by the Intestine: Features of a Two-Step Mechanism. American Journal of Physiology, 203(1), 73-80.

Miller, D. D., Schricker, B. R., Rasmussen, R. R., Van Campen, D. (1981). An *In vitro* Method for Estimation of Iron Availability from Meals. The American Journal of Clinical Nutrition, 34(10), 2248-2256.

Monsen, E. R., Cook, J. D. (1976). Food Iron Absorption in Human Subjects. IV. The Effects of Calcium and Phosphate Salts on the Absorption of Nonheme Iron. The American Journal of Clinical Nutrition, 29, 1142-1148.

Muir, A., Hopfer, U. (1985). Regional Specificity of Iron Uptake by Small Intestinal Brush-Border Membranes from Normal and Iron-Deficient Mice. American Journal of Physiology, 248(3), G376-G379.

Muir, A., Hopfer, U., King, M. (1984). Iron Transport across Brush-Border Membranes from Normal and Iron-Deficient Mouse Upper Small Intestine. The Journal of Biological Chemistry, 258(8), 4896-4903.

Narasinga Rao, B. S., Prabhavathi, T. (1978). An *In vitro* Method for Predicting the Bioavailability of Iron from Foods. The American Journal of Clinical Nutrition, 31(1), 169-175.

Neutra, M. R., Forstner, J. F. (1987). Gastrointestinal Mucus: Synthesis, Secretion and Function. In L.R. Johnson (Ed). Physiology of the Gastrointestinal Tract, (2nd edition) New York: Raven Press.

Nichols, G. M., Pearce, A. R., Alverez, X., Bibb, N. K., Nichols, K. Y., Alfred, C. B., Glass, J. (1992). The Mechanisms of Nonheme Iron Uptake Determined in IEC-6 Rat Intestinal Cells. The Journal of Nutrition, 122(4), 945-952.

Nunez, M. T., Alaverz, X., Smith, M., Tapia, V., Glass, J. (1994). Role of Redox Systems on Fe^{3+} Uptake by Transformed Human Intestinal Epithelial (Caco-2) Cells. American Journal of Physiology, 267(36), C1582-C1588.

Nunez, M. T., Tapia, V. (1999). Transferrin Stimulates Iron Absorption, Exocytosis, and Secretion in Cultured Intestinal Cells. American Journal of Physiology, 276(45), C1085-C1090.

Octave, J. N., Schneider, Y. J., Trouet, A., Crichton, R. R. (1983). Iron Uptake and Utilisation by Mammalian Cells. I. Cellular Uptake of Transferrin and Iron. Trends In Biochemical Sciences, 8(6), 217-220.

Pearson, W. N., Reich, M. (1965). *In vitro* Studies of ^{59}Fe Absorption by Everted Intestinal Sacs of the Rat. The Journal of Nutrition, 87, 117-124.

Perewusnyk, G., Funk, F. (1997). Iron Uptake by Rabbit Intestinal Brush Border Membrane Vesicles Involves Movement Through the Outer Surface, Membrane Interior, Inner Surface and Aqueous Interior. The Journal of Nutrition, 127(6), 1092-1098.

Perez, M., Barber, A., Ponz, F. (1996). Effect of Osmolarity on the Epithelial Paracellular Permeability in Rat Jejunum. Journal of Physiology and Biochemistry, 52(2), 103-112.

Pollack, S., George, J. N., Reba, R. C., Kaufman, R. M., Crosby, W. H. (1965). The Absorption of Nonferrous Metals in Iron Deficiency. The Journal of Clinical Investigation, 44(9), 1470-1473.

Powell, J. J., Whitehead, M. W., Lee, S., Thompson, R. P. H. (1994). Mechanisms of Gastrointestinal Absorption: Dietary Minerals and the Influence of Beverage Ingestion. Food Chemistry, 51(4), 381-388.

Raja, K. B., Simpson, R. J., Peters, T. J. (1992). Investigation of a Role for Reduction in Ferric Iron Uptake by Mouse Duodenum. Biochima et Biophysica Acta, 1135(2), 141-146.

Reddy, M. B., Cook, J. D. (1991). Assessment of Dietary Determinants of Nonheme Iron Absorption in Humans and Rats. The American Journal of Clinical Nutrition, 54(4), 723-728.

Riedal, H.D., Remus, A.J., Fitscher, B.A., Stremmel, W. (1995). Characterization And Partial Purification Of A Ferrireductase From Human Duodenal Microvillous Membrane. Biochemical Journal, 309, 745-748.

Rucker, R. B., Lonnerdal, B., Keen, C. L. (1994). Intestinal Absorption of Nutritionally Important Trace Elements. In L. R. Johnson (Ed.), Physiology of the Gastrointestinal Tract - 3rd edition (p. 2183-2201). New York: Raven Press.

Sadowski, D. C., Meddings, J. B. (1993). Luminal Nutrients Alter Tight-Junction Permeability in the Rat Jejunum: An *In Vivo* Perfusion Model. Canadian Journal of Physiology and Pharmacology, 71, 835-839.

Sanford, R. (1960). Release of Iron from Conjugates in Foods. Nature, 185(4712), 533-534.

Saylor, L., Finch, C. A. (1953). Determination of Iron Absorption Using Two Isotopes of Iron. The Journal of Clinical Investigation, 172, 372-376.

Schachter, D., Rosen, S. M. (1959). American Journal of Physiology, 196, 357- .

Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J. J., Crane, R. K. (1973). Purification of the Human Intestinal Brush Border Membrane. Journal of Biological Chemistry, 248, 25-33.

Scricker, B. R., Miller, D. D., Rasmussen, R. R., Van Campen, D. (1981). A Comparison of *In vivo* and *In vitro* Methods for Determining Availability of Iron from Meals. The American Journal of Clinical Nutrition, 34(10), 2257-2263.

Sheehan, D. C., Hrapchak, B. B. (1987) Theory and Practice of Histotechnology, Ohio:Battelle Press, 218.

Sheldon, R. J., Malarchik, M. E., Fox, D. A., Burks, T. F., Porreca, F. (1989). Pharmacological Characterization of Neural Mechanisms Regulating Mucosal Ion Transport in Mouse Jejunum. The Journal of Pharmacology and Experimental Therapeutics, 249(2), 572-582.

Soderholm, J. D., Hedman, L., Olaison, G., Artusson, P., Frazen, L., Larsson, J., Permet J., Pantzar N., (1998). Integrity and Metabolism of Human Ileal Mucosa *In vitro* in the Ussing Chamber. Acta Physiologica Scandinavica, 162(1), 47-57.

Tallkvist, J., Bowlus, C. L., Lonnerdal, B. (2000). Functional and Molecular Responses of Human Intestinal Caco-2 Cells to Iron Treatment. The American Journal of Clinical Nutrition, 72(3), 770-775.

Teichmann, R., Stremmel, W. (1990). Iron Uptake by Human Upper Small Intestine Microvillous Membrane Vesicles: Indication for a Facilitated Transport Mechanism Mediated by a Membrane Iron-Binding Protein. The Journal of Clinical Investigation, 86(6), 2145-2153.

Thomas, C., Oates, P. S., (2002). IEC-6 Cells Are an Appropriate Model of Intestinal Iron Absorption in Rats. The Journal of Nutrition, 132, 680-687.

Turnbull, A., Cleton, F., Finch, C. A. (1962). Iron Absorption. IV. The Absorption of Hemoglobin Iron. The Journal of Clinical Investigation, 41(10), 1897-1907.

Ussing, H. H., Zerahn, K. (1951). Active Transport of Sodium as the Source of Electric Current in the Short-circuited Isolated Frog Skin. Acta Physiologica Scandinavica, 23, 110-127.

Vaghefi, N., Guillochon, D., Bureau, F., Neuvill, D., Jacob, B., Arhan, P., Bougle, D. (1998). Effect of Stabilising Amino Acids on the Digestive Absorption of Heme and Non-heme Iron. Reproduction Nutrition Development, 38(5), 559-566.

Vaghefi, N., Nedjaoum, F., Guillochon, D., Bureau, F., Arhan, P., Bougle, D. (2000). Influence of the Extent of Haemoglobin Hydrolysis on the Digestive Absorption of Haem Iron in the Rat. An *In Vitro* Study. Experimental Physiology, 85(4), 379-385.

Weintraub, L. R., Conrad, M. E., Crosby, W. H. (1965). Absorption of Hemoglobin Iron by the Rat. Proceedings of the Society for Experimental Biology and Medicine, 120(3), 840-843.

Weintraub, L. R., Weinstein, M. B., Huser, H., Rafal, S. (1968). Absorption of Hemoglobin Iron: The Role of a Heme-Splitting Substance in the Intestinal Mucosa. The Journal of Clinical Investigation, 47(3), 531-539.

Wessling-Resnick, M. (2000). Iron Transport. Annual Review of Nutrition, 20, 129-151.

Wienk, K. J. H., Beynen, A. C. (1996). Efficacy of Iron Repletion in Anemic Rats After the Feeding of a Purified or Natural-Ingredient Diet with Ferrous Sulphate or Carbonyl Iron. Nutrition Research, 16(4), 615-626.

Wilson, T.H., Wiseman., G. (1954). The Use of Sacs of Everted Small Intestine for the Study of the Transference of Substances from the Mucosal to the Serosal Surface. Journal of Physiology, 123, 119-125.

Wienk, K. J. H., Marx, J. J. M., Beynen, A. C. (1999). The Concept of Iron Bioavailability and Its Assessment. European Journal of Nutrition, 38(2), 51-75.

Wolf, G. (1994). An Integrin-Mobilferrin Iron Transport Pathway in Intestine and Hematopoietic Cells. Nutrition Reviews, 52(11), 387-389.

Wood, R. J., Han, O. (1998). Recently Identified Molecular Aspects of Intestinal Iron Absorption. The Journal of Nutrition, 128(11), 1841-1844.