Regulation of intestinal iron transport and storage proteins by body iron stores and the implications for the control of iron absorption

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ABSTRACT

This thesis sets out to examine how body iron stores may influence mucosal cell handling of dietary iron. It is proposed that transferrin receptors (TfR) located at the basolateral surface of the enterocytes serve to 'inform' mucosal cells regarding body iron stores. It is further postulated that mucosal crypt cells can only influence mucosal handling of dietary iron after they have migrated to their final destination at the terminal villus architecture: a 'lag period' of some 3-5 days. This concept of a 'lag period' stem from previous iron absorption studies where changes in body iron stores required a period of some 3-5 days before mucosal cells were able to 'respond' to changes in dietary iron.

A re-evaluation of the 'mucosal block' hypothesis was undertaken using recent molecular biological techniques with TfR at the basolacteral surface of enterocytes serving to 'inform' these cells regarding body iron stores. This information imparted to the enterocytes in turn sets up a specific L:H ferritin transcript ratio in the crypt cells. As these cells migrate to the terminal villus, ferritin mRNA translation will produce a specific heteroploymer protein that will either impede or facilitate iron transfer to the portal circulation based on ferritin L:H transcript ratios predetermined by body iron stores in crypt cells some 3 days earlier. To examine this hypothesis, an adaptation of a previously described method of separating crypt and villus cells was undertaken.

These studies revealed a reciprocal relationship between TfR and body iron stores such that when body iron stores were depleted, TfR expression was enhanced to facilitate iron entry into cells. However, when body iron stores become replete, TfR expression were downregulated to reduce iron entry to cells. Dietary manipulation revealed that mucosal cell ferritin transcripts responded to changes in luminal iron, but this response was observed in both crypt and villus cells within 24 hours of dietary manipulation and did not follow the proposed lag period of 3-5 days corresponding to crypt-villus cell migration.

Mucosal ferritin transcripts were also examined in subjects with primary iron overload i.e. genetic haemochromatosis (GH). A method of separating crypt and villus cells in the human intestinal mucosa had not been previously described and this technique was developed denovo. Mucosal ferritin expression were significantly lower in subjects with GH when compared to control subjects with normal iron stores. This observation is consistent with enhanced iron transfer to portal blood and probably does not indicate a defect in ferritin expression in subjects with GH.

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LIST OF ABBREVIATIONS

AU	arbitrary units
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CURL	compartment of uncoupling of receptor and ligand
DEPC	diethypyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothretol
FAO	Food Agriculture Organisation
IDA	iron deficiency anaemia
ip	intraperitoneal
IRE	iron responsive elements
IRE-BP	iron responsive element binding protein
IRP	iron regulatory protein
GII	genetic haemochromatosis
HBSS	hanks buffer salt solution
HMOX	haemoxygenase
MOPS	3-N-morpholino propanesulfonic acid
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
RE	reticuloendothelial system
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RTPCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TBE	tris-borate
TfR	transferrin receptors
TfN	transferrin
WHO	World Health Organisation
UTR	untranslated regions

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Publications

Abstracts

E Chua, Q Wang and Lombard MG.
Effect of iron overload (Carbonyl Fe) and iron deficiency on H- and L-ferritin mRNA expression in crypt and tip cells in the rat intestinal mucosa. Gastroenterology 108:A 720,1995

E Chua, Q. Wang and Lombard MG.
Ferritin mRNA expression in the crypt and villus cells in iron deficiency anaemia and genetic haemochromatosis
Gastroenterology, April 1996, A1171

Papers

Lombard MG, Chua E and O' Toole P.
Regulation of intestinal non-haem iron absorption. GUT 40:4;435-439, 1997

E Chua, Q Wang, O' Toole P and Lombard MG.
A method for examining differential mRNA expression along the crypt-villus axis of the human small intestine (submitted to Clinical Science)

E Chua, Q Wang, and Lombard MG.
Quantitative distribution transferrin receptor mRNA along the crypt- villus axis of the rat small intestine and its relationship to alterations in body iron stores (submitted to GUT).

E Chua, Q Wang and Lombard MG.
Effect of chronic dietary iron overload, iron deficiency and parenteral iron overload on ferritin gene expression the in crypt-villus axis of the rat intestinal mucosa: A re-evaluation of the mucosal block.
(submitted to Gastroenterology)

1.General Introduction

1.1 Iron chemistry

1.1.1 Biological properties

Iron (symbol Fe) is a transition metal with an atomic weight of 55.85 and an atomic number of 26. A transition element is one with a partially filled d or f orbital. Iron has an electronic configuration consisting of $1s^2$ $2s^2$ $2p^6$ $3s^2$ $3p^6$ $3d^6$ and $4s^2$ which thus enables valency states from 2^+ (ferrous), 3^+ (ferric) up to a possible 5^+. The net result of these valence numbers renders iron an important metal in human and animal physiology due its unique property to donate and accept electrons in its environment.

This transition metal is essential in biological systems mainly due its ability to bind reversibly with oxygen. It is incorporated into protoporphyrin to form haem in haemoglobin and myoglobin, a co-factor for several enzymes such as catalase, electron transport enzymes such as the cytochromes and many iron sulphur proteins such as aconitase, a key enzyme in the tricarboxylic acid cycle. In addition, iron is a co-factor for ribonucleotide reductase, the rate limiting enzyme in deoxyribonucleic acid (DNA) synthesis.

1.1.2 Natural occurrence and distribution

Iron is the fourth most abundant element in the earth's crust and the second most abundant metal (Crighton & Charloteaux-Wauters 1986). In nature, it exists exclusively in the ferric state, a state in which it is not biologically available (Horne 1978). Iron is also the most abundant transition metal in man and is mainly present in one of its oxidation states (Fe^{2+}, Fe^{3+}).

1.1.3 Iron bioavailability

Measurements of iron intake would be of limited value in assessing the nutritional value of diets if there was no indication on iron bioavailability. Iron bioavailability is

defined as the proportion of the total iron intake that is potentially available for absorption and normal body functions (Department of Health 1991). Methods to study iron availability have been reviewed by Fairweather-Tait (1992). Based on such studies, it is possible to divide foods into high, medium and low bioavailability as summarised in Table 1-1.

Diets too can be separated into three broad categories consisting of "low", "intermediate" and "high" bioavailability, (determined in individuals with very low iron stores but normal haemoglobin concentration) with mean iron absorption (a mixture of *haem* and *non-haem iron*) of approximately 5, 10 and 15% respectively (FAO/WHO 1988). Low bioavailable diets contain a preponderance of foods consisting of cereals and root vegetables that inhibit iron absorption and are dominant in many developing countries especially in the lower socio-economic groups. Intermediate bioavailability diets consist of similar foods as in the low bioavailability diets but with some meat and additional foods rich in ascorbic acid. High bioavailability diets contain generous amounts of meat, poultry and fish with high amounts of ascorbic acid and is the typical diet consumed by people in developed countries.

Of the two main forms in which iron can exist in foods, *haem* iron absorption appears to be relatively unaffected by dietary or physiological variables compared to *non-haem* iron (FAO/WHO 1988). The various mechanisms whereby dietary substances (enhancers and inhibitors) affect iron absorption will be discussed in *section 1.3.2.*

1.1.4 Iron toxicity

Iron poses two problems for biological systems. Firstly, in its ubiquitous state, iron is insoluble and inaccessible. Therefore, biological systems have had to evolve mechanisms to acquire iron. The absorptive mechanism(s) are not very efficient but this

Table 1-1

Relative bioavailability of *haem* and *non-haem* iron in food

Foods	Low (5%)	Medium (10%)	High (15%)
Cereals	Maize Oatmeal	Cornflour White flour	
Fruits	Apple Avocado Banana Peach Plum	Mango Pineapple	Guava Lemon Papaya Tomato
Vegetables	Aubergine Legumes Soy flour	Carrot Potato Pumpkin	Beetroot Broccoli Cabbage
Beverages	Tea	Red wine	Coffee
Nuts	Almond Peanut Walnut		
Animal proteins	Cheese Egg Milk		Fish Meat Poultry

Key: % is mean iron absorption

is overcome to some extent by the acquisition of efficient retaining mechanisms and the absence of a distinct excretory pathway for iron (McCance & Widdowson 1938). Secondly, iron is toxic in free solution due to its ability to promote the generation of hydroxyl radicals (Fenton 1894, Harber & Weiss 1934). A radical is an atom or molecule containing one or more electrons. The following reactions summarise the generation of the hydroxyl radicals:

$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$

(superoxide)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$ (Fenton reaction)

Combining the two reactions:

$O_2 + H_2O_2 \rightarrow O_2 + OH^- + OH^\bullet$ (Harber -Weiss reaction)

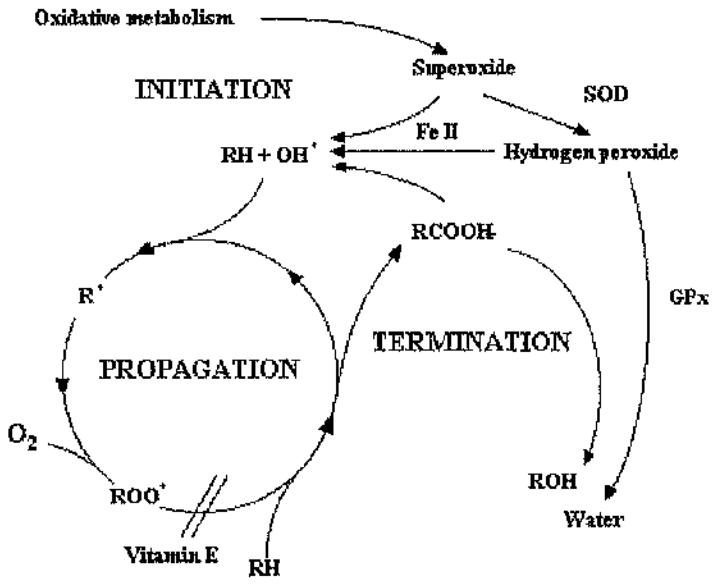
(hydroxyl radical)

Hydroxyl radicals cause disruption of biological membranes by lipid peroxidation. Peroxidation can be considered to occur in three phases namely initiation, propagation and termination. Initiation is brought about by the removal of a hydrogen atom from the methylene link (-CH2-) between two double bonds in a polyunsaturated fatty acid producing a carbon radical which reacts with oxygen to form a peroxy radical ($R\text{-}00^\bullet$). Peroxy radicals can then remove a hydrogen atom from another lipid molecule forming a lipid hydroperoxide (R-00H). This is described as propagation and it can be repeated many times within the lipid bilayer. Lipid hydroperoxides behave like hydrogen peroxide which can interact with transition metals to form alkoxyl radicals ($R0^\bullet$) and are also capable of initiating more lipid peroxidation. The propagation phase ends when two radicals interact or when a radical scavenger such as vitamin E reacts with the lipid radical. Various products result from lipid peroxidation, amongst them aldehyde products which can react with thiol and amino groups in biological membranes forming cross-links thus contributing to the declining function in radical damaged tissue. This autocatalytic chain of lipid peroxidation summarised in Figure 1-1 is the principle reason why iron has to be controlled in biological systems. This is achieved by binding to the polymeric protein, ferritin.

Figure 1-1

Autocatalytic cycle of lipid peroxidation, see text for discussion.

Key: SOD: superoxide dismutase, GPx: gluthiathione peroxidase, FeII: ferrous iron, $OH^{\bullet}$: hydroxy radicals; $RH^{\bullet}$: lipid or organic molecule, R: lipid radical, $ROO^{\bullet}$: peroxy lipid radical, ROOH: lipid hydroperoxide; RH: hydrogen donner.(Termination takes place by the interaction of vitamin E or via the action of GPx).



1.1.5 Evolution of protective mechanisms

There are several ways of limiting the entry of iron into the body in an attempt to limit iron toxicity. For instance, only a small amount of the dietary iron (10-15%) is normally absorbed but this amount can be increased to 30-40% in iron deficiency. In man, absorption is confined to the proximal small intestine with maximal absorption in the duodenum (Becker *et al* 1979, Johnson *et al* 1983, Muir & Hopfer 1985, Conrad *et al*

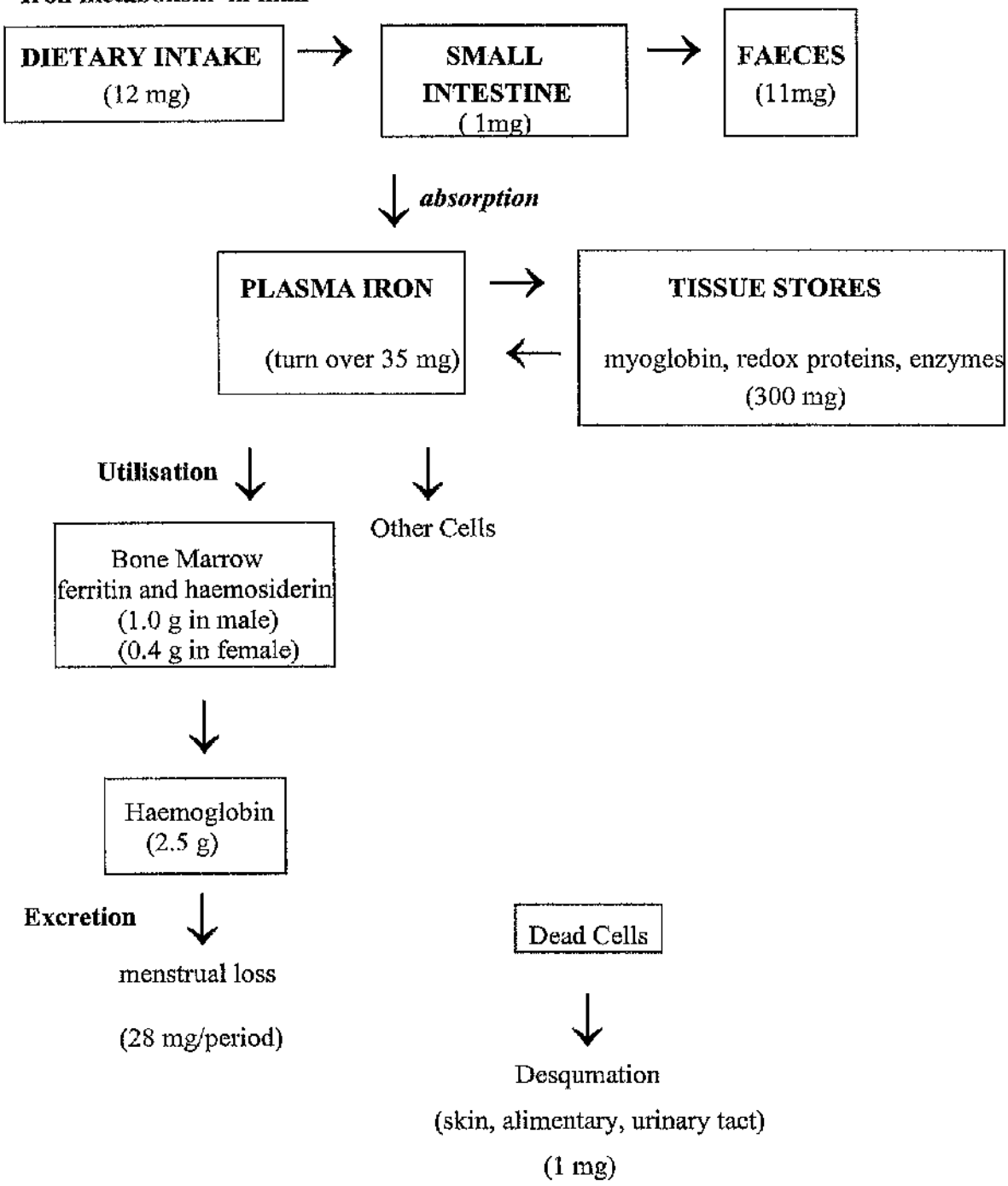
1987). The physical form of iron also affects absorption. Ionic iron or *non-haem iron* is more poorly absorbed (10-15%) than porphyrin or *haem iron* (25%) but the overall proportion that either form contributes is dependant on dietary factors. Luminal secretions of the gastrointestinal tract such as gastric juice (enhancing) and pancreatic secretions (inhibitory) also affect iron absorption (section 1.3.2iii). Once absorbed into the body, the complexation of iron within all cells requires an iron storage protein to maintain iron in a soluble, bioavailable and non-toxic form. These conditions are fulfilled by the iron storage protein ferritin and this will be discussed in Chapter 4.

1.2 Iron metabolism in man

Iron metabolism in man has been summarised in Figure 1-2. The amount of body iron in the healthy adult is between 3 to 4 g (Davidsion *et al* 1979) and is roughly distributed as follows: haemoglobin (2.5 g), tissue iron in myoglobin, redox proteins and iron enzymes (0.3 g) and in iron stores as ferritin and haemosiderin (1.0 g). However, non-specific iron excretory mechanisms do exist and result in losses of approximately 1 mg/day in the healthy person. This can occur in several ways; via the urinary tract, during menstruation, pregnancy and lactation or desquamation of skin cells and epithelial cells from the gastrointestinal tract. The latter is iron retained within the enterocytes being shed into the gut lumen at the end of their life cycle and can be potentially, a major iron loss (Green *et al* 1986). In addition, there is evidence that iron is also excreted via the globlet cells in the gastrointestinal tract (Refsum & Schreier 1980). Increased iron loss (up to 4 mg/day) has been reported in an iron loaded patient who had recovered from chronic anaemia (Crosby *et al* 1963) and more recently, iron-loaded thalassaemic patients after bone marrow transplantation show significant reduction in iron stores during a seven year follow up (Lucarelli *et al* 1993). In general, 1 mg of elemental iron (2 mg in women) is needed per day to replace iron losses.

Figure 1-2

Iron metabolism in man



Iron absorption has been extensively studied since McCance & Widdowson (1937,1938) suggested that regulation of absorption must be responsible for iron homeostasis as excretion was limited. However, despite intensive studies on iron metabolism, (Forth & Rummel 1973, Brock *et al* 1994), many aspects on iron absorption remain unclear, in particular, how the transfer of iron from the intestinal mucosa to the portal circulation takes place. Absorption of *non-haem iron* differs from *haem iron*. *Non-haem iron* is released from food by peptidases in the duodenum, the main site of absorption. On the other hand, *haem iron* in meat can be absorbed directly by interacting with specific haem receptors in mucosal cells where iron is released from the porphyrin ring by mucosal haem oxygenase.

Once iron is taken up by mucosal cells, it is rendered non-toxic by binding to proteins: either to mucosal transferrin (TfN) and then translocated across the enterocyte basement membrane to the portal circulation or retained within mucosal apoferritin. Iron bound to ferritin, sometimes referred to as the "ferritin curtain" (Chapter 4) remains within the enterocyte and is excreted when intestinal cells are shed into the gut lumen.

In plasma, iron is bound to its transport protein, TfN. Iron is transported either as diferric TfN (20%), monoferric TfN (40%) or apotransferrin (40%) (Young & Bomford 1984, Van der Huel *et al* 1984, Huebers *et al* 1983). The determinant factors for iron entry into cells is the presence of transferrin receptors (TfR) and the concentration and saturation of TfN with diferric TfN having the highest affinity for TfR. TfR are present on most types of cells. The majority of TfN bound iron is taken up by developing red blood cell precursors by receptor mediated endocytosis. TfR are embedded on the cell surface membranes and also as endocytic vesicles or endosomes (Ciechanover *et al* 1981, Dautry-Varsat *et al* 1983). The endocytic cycle of TfN is initiated by the binding of the charged TfN to the exposed receptor located at the bottom of the membrane pit which is coated with clathrin, a protein that provides a nidus for aggregation of receptors on cell surfaces (Pearse & Bretscher 1981). This clathrin-coated pit then folds into a vesicle

which is internalised into the cytoplasm, and, by means of a proton pump (Tyko & Maxfield 1982), the contents of the vesicle becomes acidic (approximately pH 5) thereby facilitating the dissociation of iron from TfN binding sites but leaving apotransferrin still bound to the receptor. Liberated iron equilibrates with ferritin (Klausner *et al* 1983, Bomford & Munro 1985). TfR bearing apotransferrin is recycled to the plasma membrane, where apotransferrin dissociates from the receptor. TfR mediated endocytosis for iron entry to cell has been summarised in Figure 1-3.

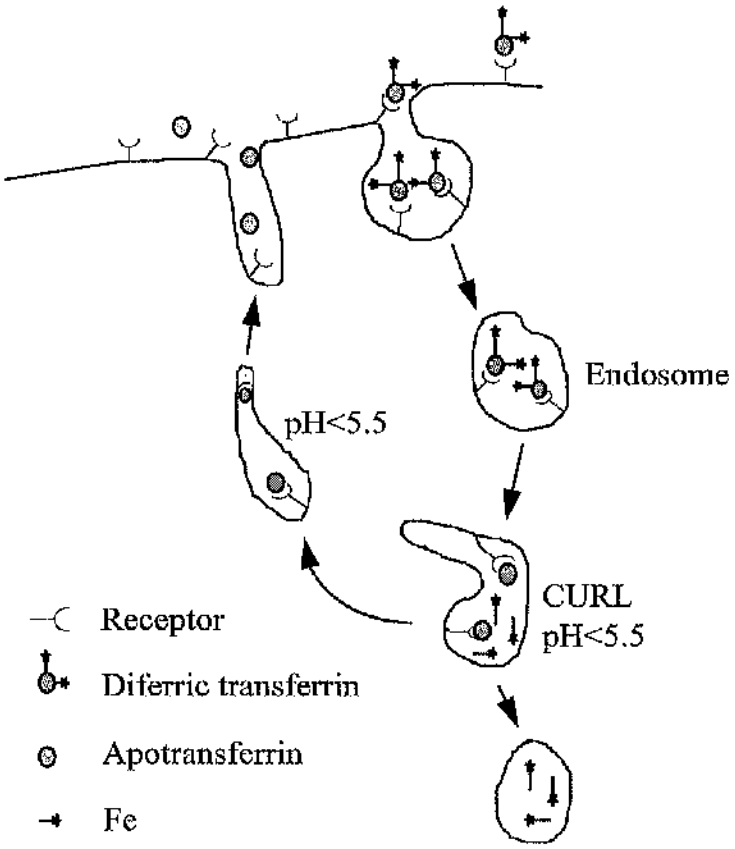
The liver accounts for most of the remaining TfN bound iron uptake but unlike erythroid cells, there is controversy about the mechanisms of iron uptake from TfN bound iron into hepatocytes. TfR and specific uptake of transferrin iron have been demonstrated in hepatocytes, albeit in small amounts (Young & Aisen 1981) but hepatocytes and Kupffer cells have additional mechanisms for iron uptake (Young *et al* 1983). A plasma membrane oxidoreductase mechanism has been described for hepatocytes (Thorstenson & Romslo 1988). Hepatocytes have receptors for circulating haemoglobin bound to haptoglobin and for haemin bound to haemopexin. Kupffer cells are capable of ingesting intact red blood cells (Munro & Linder 1978). A hepatocyte membrane receptor for ferritin has also been described in rats (Mack *et al* 1983) and in man (Adams *et al* 1988). Ferritin is also taken up via a receptor-mediated endocytosis route dependant on a microtubule process (Ramm *et al* 1990).

Once inside the cell, free iron in the cytoplasm is maintained within physiological limits by ferritin which incorporates ferrous iron into the apoprotein shell accompanied by its oxidation (Theil 1983). Ferritin has a half-life of 3 days in rats and one hypothesis suggests that the protein shell is degraded by both cytosolic and lysosomal proteolytic enzymes (Wixom *et al* 1980). Proteolytic removal of the ferritin shell within the lysosomal vesicles leaves the iron core which is recognised histologically as haemosiderin granules and demonstrated using Perls potassium ferricyanide stain on

Figure 1-3

Endocytic cycle of transferrin,

Key: CURL; compartment of uncoupling of ligand and receptor; See text for discussion



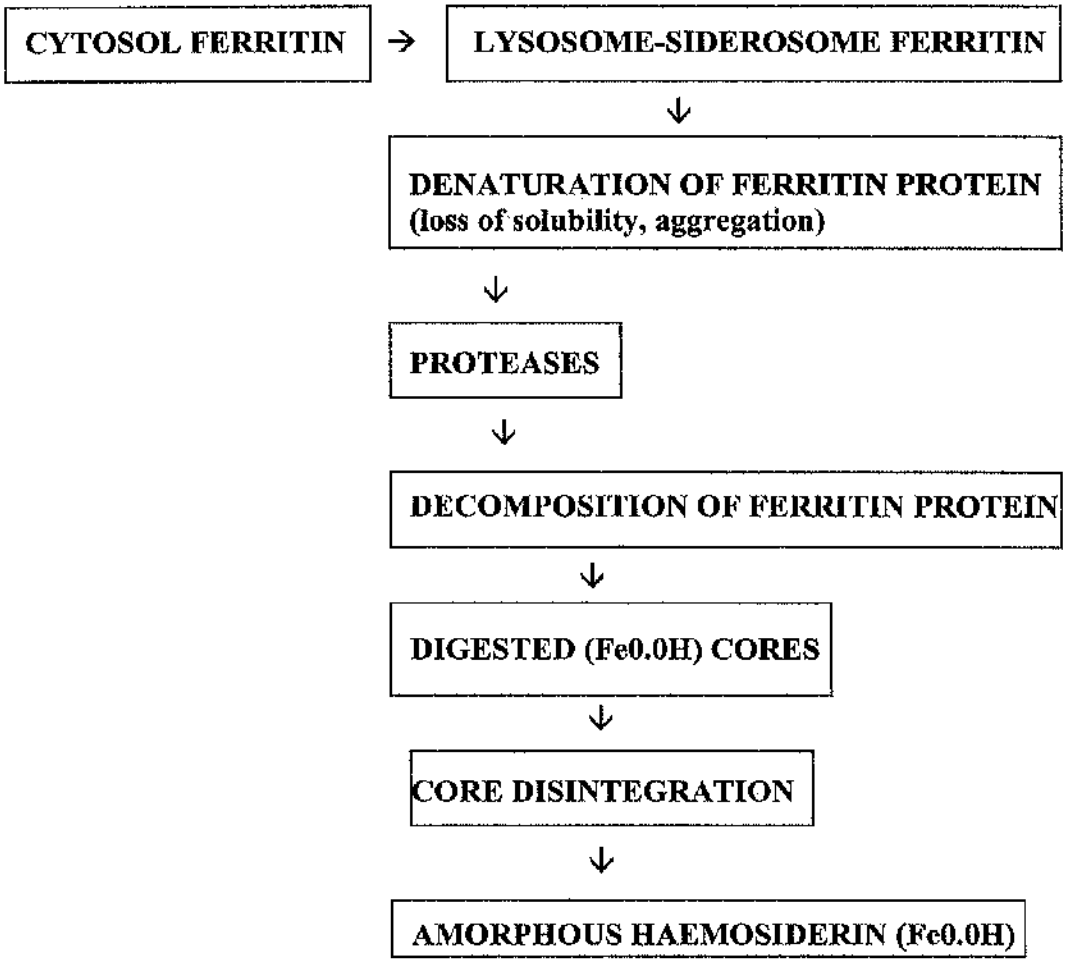
histological sections. By contrast, proteolytic removal of ferritin within the cytoplasm releases the ferric iron which is reduced by flavin mononucleotides and re-enters the ferrous iron pool. It has been proposed (Ritcher 1984) that soluble cytosol ferritin is progressively transferred to and accumulates within the lysosomes as haemosiderin in a sequence of events summarised in the scheme Table 1-2.

The role of haemosiderin is uncertain. Mossbauer spectroscopy studies have revealed significant differences in haemosiderin iron cores in different iron disease states.

In secondary iron over loaded states such as β-thalassaemia (an inherited disorder in haemoglobin synthesis requiring multiple blood transfusions), the haemosiderin iron core structures are those of the crystalline goethite structure (Mann *et al* 1988, Ward *et al* 1988). However, in primary iron overload such as genetic haemochromatosis (GH in section 3.4), the haemosiderin iron cores are similar to the ferrihydrite structure associated with liver ferritin. Goethite deposits are less labile and are thought to be less susceptible to chelation therapy than the ferrihydrite structure.

Table 1-2

Scheme for the formation of haemosiderin granules



1.3 Mechanisms of iron absorption

As there is no distinct excretory pathway for iron, regulation of iron stores must be at the site of absorption (McCance & Widdowson 1937, 1938). Absorption of *non-haem iron* differs from *haem iron* and is therefore considered separately. Although *haem iron* absorption is more efficient, the quantity of iron controlled by *non-haem iron* absorption is often much greater.

1.3.1 Non-haem iron absorption

Non-haem iron is released from food by peptidases in the duodenum. Only the ferrous form of *non-haem iron* can cross membranes and since most of the dietary *non-haem iron* is in the ferric state, it has to be reduced and made more soluble by the gastric juice containing hydrochloric acid or organic acids such as ascorbic acid. A previous study has also demonstrated that ascorbic acid is concentrated in the human stomach and may be secreted into the gastric juice (Sobala *et al* 1989). This may be a very important, unrecognised mechanism affecting iron absorption. Several stages are involved in the absorptive process. These include (i) binding of iron to the brush border (ii) iron uptake into the interior of the cell (iii) intracellular handling of iron (iv) transcellular transport and (v) release of mucosal iron to the portal circulation.

(i) Binding of iron and mucosal iron uptake

At least three mechanisms for *non-haem iron* binding to the brush border have been reported (Simpson *et al* 1989).

(a) a non-specific low affinity binding or paracellular uptake. This mechanism is non-saturable and non-regulated. However, it forms a small but significant basal uptake of iron and may explain the well described incomplete suppression of iron absorption in iron overload (Crosby *et al* 1966).

(b) a high affinity receptor binding which is well regulated and dependant on metabolic energy. The search for such a receptor has mainly been unrewarding. One of the specific membrane receptors identified for iron uptake was the TfR, a glycoprotein which is found on most cells in the body and particularly at high concentrations on cells with a high requirement for iron such as immature erythroid cells (Bomford & Munro 1985). Huebers *et al.*, (1983) proposed that mucosal apotransferrin was secreted into the gut lumen and takes up luminal iron and is then absorbed into the cell. Excess apotransferrin was recycled via the enterohepathic circulation. However, subsequent immunolocalisation techniques revealed that within the small intestine, TfR are predominantly found on the basolateral surfaces of enterocytes rather than in the apical microvillous brush borders (Levine & Woods 1990, Parmley *et al* 1985, Banerjee *et al* 1986, Osterloh *et al* 1988, Lombard *et al* 1990). Hence, TfR are unlikely to play a major role in mucosal iron uptake.

(c) binding to specific glycoproteins which presents iron to the mechanisms for shuttling iron into and across the enterocyte. Candidate proteins for this include an iron-binding protein described by Teichmann & Stremmel (1990) and mobileferrin (Conrad *et al* 1992 ,1993a &1993b). The former is an iron-binding protein isolated in the microvillus membrane vesicles of the proximal intestine; molecular weight 160 kDa with three 54 kDa monomeric peptides linked by disulphide bonds forming a transmembrane iron-transport protein. It is present in stomach and liver but not in the oesophagus and its concentration in the duodenum is increased in iron deficiency and reduced in secondary iron overload suggesting that it is responsive to iron stores. Mobilferrin appears to be localised in the apical cytoplasm of the proximal small bowel but not in other organs. It is a water soluble protein, molecular weight of 56 kDa which can bind reversibly to iron, in a molar ratio of 1:1 (Conrad *et al* 1990, 1992). Its location in the apical cytoplasm and its close association with integrin, another transmembrane protein consisting of two protein chains of 150 kDa and 90 kDa (Conrad *et al* 1993a & 1993b) suggest a possible role in

intracellular iron transport but further work is required to confirm and extend these observations.

(d) A variety of fatty acids in the brush border membrane vesicles such as oleic, stearic, phosphatidyl serine and phosphatidic acid also have iron-binding properties, with binding being pH dependant and may have a role in iron uptake into the enterocytes (Simpson & Peters 1987). Unlike binding in (b) and (c), this is a passive process and is only partially regulated (Simpson *et al* 1989).

(ii) Mucosal intracellular iron transport

Once iron is internalised within the enterocyte, it can be considered in two separate compartments (i) an iron storage compartment and (ii) an iron transfer compartment. Little is known about the iron transfer mechanism within the mucosa. Excess iron taken up is diverted to the storage compartment. Once in the storage compartment, it is thought that only about 10 % of the iron can re-enter the iron transfer compartment (Wheby & Umpierre 1964b).

The mechanism(s) for transcellular iron transport remain unknown. Several glycoproteins have been implicated namely TfN and ferritin. Mucosal TfN has been suggested but supportive evidence is lacking. Studies in man reveal that mucosal content of TfN is not different in iron deficient subjects compared to subjects with normal iron stores (Whittaker *et al* 1989). Hypotransferrinaemic mice (Buys *et al* 1991, Simpson *et al* 1991) and the rare human syndrome of congenital atransferrinaemia (Goya *et al* 1972) have enhanced iron absorption despite a lack of TfN in the small intestinal lumen, mucosa and plasma. In addition, the absence of TfN mRNA in the rat and human duodenal mucosal cells (Idzerda *et al* 1986, Pietrangelo *et al* 1992) suggests that TfN is not synthesised within the mucosal cell and is probably derived via TfR at the basolateral surface of enterocytes to supply iron for cellular growth and development (Chapter 3).

Ferritin, an iron storage protein has long been implicated as a repository for excess iron (Hahn *et al* 1943, Granick 1946). Absorption of radioactive iron in iron deficient dogs was increased compared to replete animals which absorbed little. Granick; (1946) proposed a "mucosal block" which prevented the absorption of un-needed iron and this block disappeared in iron deficiency. To explain the mechanism of this block, a "mucosal acceptor" for iron was postulated where physiological saturation of the acceptor prevented further entry of iron. This mucosal acceptor was thought to be ferritin where iron bound to it was lost when cells are shed from the intestinal mucosa. There is a good correlation between mucosal ferritin or ferritin mRNA with iron status, with low levels in iron deficient states and high levels in secondary iron overload in rodents and in man (Halliday *et al* 1978, Savin & Cook 1980, Whittaker *et al* 1989, Pietrangelo *et al* 1992). However, when it was shown that mucosal ferritin synthesis continued even in iron deficiency (Britten & Raval 1970), the concept of ferritin having a regulatory effect on iron absorption was largely discounted.

(iii) Mucosal iron transfer to portal circulation

This is the final step in the absorptive process but information regarding its mechanism is scanty. Iron may exit along the basal portion of the enterocyte or along the lateral spaces (Parmley *et al* 1978). It then has to traverse the interstitial spaces and the endothelial cell to enter the portal venous system. Expression of TfR only at the basolateral surface of enterocytes allows for the possibility that this may be the principal route for iron export to the portal circulation. However, several strands of evidence indicate that this is unlikely as discussed in section 3.4.3.

Most of the iron in the portal blood is in the ferrous state (Wollenberg *et al* 1990). This suggests that ferric iron is reduced to the ferrous state at the basolateral membrane prior to release. Ceruloplasmin, a copper binding protein may also be implicated in iron transfer since iron absorption is enhanced after intravenous ceruloplasmin administration.

It is postulated that when ferrous iron diffuses to the portal circulation, it is oxidised by ceruloplasmin upon entry to the capillary wall producing a ferric moiety for binding to plasma TfN (Wollenberg *et al* 1990).

1.3.2 Factors affecting non-haem iron absorption

Non-haem iron absorption is affected by several factors.

(i) Non-haem iron and bioavailability

Non-haem iron is found in foods from both plant and animal origin. In plants, there are at least three forms; metalloproteins e.g. plant ferritin (Sczekan & Joshi 1987), a soluble iron source in the sap of plants and an iron complex in the form of phytates (Hazell 1985). In animal products *non-haem iron* is found in many forms such as ferritin and haemosiderin in meat products, phosphoproteins in egg yolk and lactoferrin in milk. Dietary components especially proteins and exogenous ligands (enhancers and inhibitors) can also influence *non-haem iron* bioavailability.

(a) Enhancers of non-haem iron absorption

The main enhancers of *non-haem iron* absorption are proteins, ascorbic acid and organic acids. Bioavailability of iron in foods with significant amounts of ascorbic acid is high (Gillooly *et al* 1983, Ballot *et al* 1987). Ascorbic acid potentiates *non-haem* iron absorption by at least two mechanisms; by acting as a reducing agent in the stomach (ferrous iron better absorbed than ferric iron) and as a chelator in the stomach and the proximal small intestine by maintaining iron in a soluble state. However, ingestion of ascorbic acid on a long term basis can only affect iron status when the diet is poor in iron and when iron deficiency is present. The observation that ascorbic acid is concentrated and secreted in the gastric juice suggests that there may have been an important evolutionary mechanism to enhance iron absorption when diet was sub-optimal. Interestingly, in the replete state, supplemental dietary ascorbic acid does not appear to have any additional effect on iron assimilation (Looker *et al* 1988) and with further

adaptation at the enterocyte level to limit iron absorption (Cook 1990). Several other organic acids notably citric, malic and tartaric acid also have an enhancing effect on *non-haem* iron absorption whereas oxalic acid in foods such as spinach, rhubarb, chocolates and cocoa appears to have an inhibitory effect (Gillooly *et al* 1983).

(b) Inhibitors of non-haem iron absorption

The absorption inhibitors in natural foods include the phytates, polyphenols such as chlorogenic acid in coffee, phosphates and tannins in plant tissue and phosphoproteins in animal tissue. These reduce *non-haem iron* absorption by forming insoluble complexes (Morris & Ellis 1982). Calcium also, has an inhibitory effect on *non-haem iron* absorption but the exact inhibitory mechanism is not known. One postulated mechanism is that calcium inhibits the enzyme phytase present in cereals during baking, leading to increase amounts of phytate which in turn reduce *non-haem iron* absorption (Hallberg *et al* 1991). It is evident that enhancer-inhibitory interactions are just as important as iron-chelator interactions in influencing *non-haem iron* absorption.

(ii) Iron valency

Reduction of ferric to the ferrous state is thought to be essential before absorption can occur (Brise & Hallberg 1962). Once taken up by the enterocyte, iron is oxidised to be stored or shuttled across the enterocyte. Prior to release from the enterocyte to the interstitial space, iron is reduced, diffuses into the portal circulation and is reoxidised by ceruloplasmin (Wollenberg *et al* 1990) .

(iii) Luminal contents of the gastrointestinal tract

Patients with gastrectomy and achlorhydria develop iron deficiency due to diminished ability to absorb *non-haem* iron (Bothwell *et al* 1979). Gastric juice aids iron absorption by solubilising *non-haem* iron. The low pH of the gastric contents is neutralised within minutes on entering the duodenum. However, if iron is maintained in a

water soluble state e.g. in the presence of ascorbic acid, absorption continues despite the higher pH status. This may explain the enhancing effect of bile on iron absorption (Conrad & Schade 1968) although *invitro* studies suggest that this is due to the formation of mucoprotein ligands rather than iron-ascorbic acid complexes (Jacobs & Miles 1970). Iron can also be maintained in a water soluble chelate by taurocholate in bile further enhancing iron absorption (Sanyal *et al* 1990).

Pancreatic secretions tend to reduce *non-haem iron* absorption because bicarbonate ions raises the pH thereby decreasing solubility (Zemspky *et al* 1989). However, the overall effect of pancreatic juice may be to enhance *non-haem iron* absorption by releasing amino acids and polypeptides from foods which can act as absorption ligands.

Mucus and mucins lining the intestinal mucosa also play a role in promoting *non-haem iron* absorption (Conrad *et al* 1991, Wien & Van Campen 1991). It is suggested that mucins bind iron in the acidic gastric environment thus maintaining iron in solution despite the rise in pH in the duodenum, and, as iron is bound relatively weakly to mucins, it dissociates easily to be absorbed (Conrad *et al* 1991).

(iv) Internal factors: body iron status and erythropoeisis

(a) Body iron status

The inverse relationship between body iron stores and iron absorption is well established in both animals and humans (Cook *et al* 1974, Walters *et al* 1975, Magusson *et al* 1981, Baynes *et al* 1987, Cook 1990). However, despite many studies, the precise mechanism by which the enterocytes are informed of body iron status remains a mystery.

A humoral regulator has been suggested since infusion of plasma of pregnant rats to non pregnant rats enhanced iron absorption (Apte 1969). Increased iron absorption was

also observed in pregnant women (Whittaker *et al* 1991b). However, the search for humoral factors has generally been unrewarding.

Studies looking at the relationship between serum ferritin or serum TfN saturation and iron absorption have revealed two phases of regulation of absorption by body iron; a minor level of control responsible for fine adjustments at normal iron stores and a major level of control when iron stores are depleted. One of the more durable theories for internal control was a proposal by Crosby *et al.*, (Conrad & Crosby 1963) in that the intestinal mucosal cell may be 'conditioned' while in the crypts by the amount of iron supplied from plasma (section 1.4).

A liver transplant model in rats has been used to establish whether the liver has a regulatory function on iron absorption (Adams *et al* 1989). Rat livers were loaded with oral carbonyl iron (iron stored in hepatocytes) or parenteral iron dextran (iron stored in Kupffer cells) and then transplanted to isogenic rats with normal liver iron stores. Significant reduction in iron absorption (studied 10 days post transplant) was observed in these transplanted animals (20 fold and 0.5 fold respectively) suggesting that hepatocyte iron content may influence a putative messenger controlling intestinal absorption.

(b) Effect of erythropoeisis

Increased iron absorption associated with increased erythropoiesis is evident in patients with haematological disorders with ineffective erythropiesis such as thalassaemia major and sideroblastic anaemias where these patients develop significant iron overload (Bothwell *et al* 1979, Pippard *et al* 1979). By contrast, if erythropoiesis remains effective as in autoimmune haemolytic anaemias or hereditary spherocytosis, only modest increases in iron absorption are observed and it is unusual for such patients to develop significant iron overload. Disordered erythropoiesis thus produces conflicting signals to the intestinal mucosa resulting in the breakdown of the precise relationship between body iron reserves and intestinal absorption. Enhanced erythropoiesis may also be responsible

for the increase iron absorption in subjects transported to high altitudes (Reynaferje & Ramos 1961).

(v) Mucosal cell factors

The mucosal cell itself also influences iron absorption independent of body status. There were significant changes in iron absorption when small intestines from iron deficient rats and iron loaded rats were transplanted to isogenic iron deficient rats. Iron uptake by mucosal cells and iron transfer into carcass was significantly greater from intestines transplanted from iron deficient animals compared with the intestines from iron loaded animals (Adams *et al* 1991). Also, by raising mucosal cell iron concentration with a high iron containing meal led to a reduction in iron absorption from a test meal given subsequently(Fairweather-Tait & Wright 1984, Fairweather-Tait 1985).

The iron content of the mucosal cell may be self regulated in at least two ways; firstly, via TfR at the base of the enterocytes such that when mucosal cells have sufficient iron content, TfR are down regulated to reduce further entry of plasma transferrin iron. Secondly, mucosal ferritin mRNA levels are increased such that different amounts of mucosal appoferritin levels are produced to sequeste excess iron within the enterocyte (Pietrangelo *et al* 1992).

1.3.3 Haem iron absorption

Dietary *haem iron* is mainly derived from animal tissues with haemoglobin and myoglobin being the main precursor proteins. It is an important qualitative source of dietary iron because of its higher bioavailability (Martinez-Torres & Layrisse 1973). It is generally agreed that 20-30% of *haem iron* is absorbed and this is a constant figure being relatively unaffected by other dietary or physiological variables (FAO/WHO 1988). In industrialised countries, this accounts for 10-15% of the ingested iron but amounts to

one-third of the iron absorbed because of its higher bioavailability (Cook *et al* 1982, Bezwoda *et al* 1983).

1.3.4 Factors affecting haem iron absorption

Several factors are known to affect *haem-iron* absorption and these include:

(i) Intraluminal factors

Haem iron absorption is relatively independent of the components of the diet compared to *non-haem iron*. However, pure *haem iron* is poorly absorbed due to formation of polymers (Conrad *et al* 1966) but these are theoretical considerations since pure *haem iron* is not ingested.

(ii) Mucosal uptake

Uptake of *haem* is different compared to *non-haem iron*. As mentioned, the former involves interactions with specific *haem* receptors (Grasbeck *et al* 1979, 1982). *Haem iron* enters mucosal cells as microendocytic vesicles best observed at the base of the microvilli which are broken down by *haem* oxygenase within the enterocyte to release the iron (Raffin *et al* 1974) and two products biliverdin, a potent antioxidant (Tenhunen *et al* 1970, Kutty & Maines 1981, Stocker *et al* 1987) and carbon monoxide; a neurotransmitter (Verma *et al* 1993). This appears to be the rate limiting step in *haem* absorption (Wheby & Spyker 1981).

Haem oxygenase, molecular weight 33 kDa, is located in the endoplasmic reticulum, requires NADPH and molecular oxygen. This enzyme exists as two isoenzymes (Schacter 1988), with an inducible enzyme (HMOX1) located on chromosome 22 and the constitutive enzyme (HMOX2), located on chromosome 16 (Kutty *et al* 1994). In rat, activity of the enzyme is greatest in the spleen, bone marrow, liver, brain, kidney and lung with activity also demonstrated in the intestinal mucosa.

Once released from *haem*, iron enters the same intracellular pool as *non-haem iron*. Iron from lactoferrin, (molecular weight 80 kDa), is capable of binding 2 atoms of ferric iron per molecule and is thought to enter the mucosal cell by a similar way to *haem* iron as lactoferrin receptors have been isolated from the brush border membranes (Cox *et al* 1979). However, the role of lactoferrin in iron absorption remains unclear and lactoferrin is more generally understood to have a role in reducing bacterial overgrowth.

(iii) Internal factors: effect of iron status

There is also an inverse relationship between body iron stores and *haem iron* absorption although this relationship is less pronounced than for *non-haem iron* absorption (Lynch *et al* 1989a). At any serum ferritin level, *haem iron* absorption is greater than *non-haem iron* but at higher iron levels, inhibition of *haem iron* absorption is less pronounced than inhibition of *non-haem iron* absorption (Lynch *et al* 1989b).

1.3.5 Sites at which iron absorption can be regulated

From the discussion so far, the possible sites at which *non-haem iron* absorption can be regulated are (i) at the level of iron uptake by the enterocyte (ii) the mechanism(s) retaining iron within the enterocyte and (iii) the release of iron from the enterocyte to the portal circulation. All these sites can be influenced by body iron status and are important although it has been proposed that uptake by mucosal cell may be the main site of regulation (Nathanson & Mclaren 1987). By contrast, *haem iron* absorption is controlled largely at the site of mucosal transfer to the portal circulation although luminal uptake by the enterocyte can be increased in iron deficiency (Wheby & Spyker 1981). The different sites in the control of iron absorption may account for a greater sensitivity of *non-haem iron* absorption to changes in body iron stores. Iron absorption is a precisely regulated process but when this regulation becomes defective, it manifests in a condition called GH.

1.3.6 Aberrant iron absorption

Insights into the control of iron may be gleaned by studying aberrant iron absorption as it occurs in GH. The hereditary nature of the disease was first suggested by Sheldon (1927, 1935) but it was much later before its occurrence in association with the Major Histocompatability Complex (MHC) was described (Simon *et al* 1977a, 1977b) and that the mode of inheritance was autosomal recessive. The prevalence for GH is between 0.1 and 0.5 percent of the Caucasian adult population (Dadone *et al* 1982, Edwards *et al* 1988).

Occasionally, iron overload can be due to other causes such as excessive iron supplementation, alcohol, multiple blood transfusion, sideroblastic anaemia and these are referred to as secondary haemochromatosis. GH is due to an inborn error of iron metabolism resulting in an inappropriately high level of iron absorption from the diet in the face of increase iron stores. Although the precise defect is still unknown, much is known about the genetics and effective treatment is available. Recent studies in this condition provide some insight into the mechanisms regulating iron absorption.

Expression of this disorder may be influenced by dietary iron intake and other factors probably alcohol in particular. In countries where there are sufficient quantities of iron in the diet, the majority of patients homozygous for the defect will develop clinical and biochemical expression of the disease by the 5th decade of life. Excess iron deposition in liver, pancreas, reproductive system, joints and heart manifests as liver cirrhosis with an increased risk of hepatoma, diabetes mellitus, hypogonadotrophic hypogonadism, athropathy and cardiomyopathy. The disease is diagnosed by demonstrating an increase in iron stores in liver.

In theory, the metabolic defect in GH defect could be in the enterocyte or a more widespread disorder affecting the liver or the reticuloendothelial system. Several studies by (Boender & Verloop 1969, Powell 1970, Marx 1979) seem to suggest that the defect in iron absorption lies both in the mucosal uptake and the release of iron to the portal

circulation. However, mucosal iron kinetics studies (McLaren *et al* 1991) concluded that mucosal transfer was the major determining factor. In subjects with GH, intestinal mucosal H and L-ferritin (different forms of ferritin with potentially different metabolic properties, see Chapter 4) fail to rise in parallel with serum ferritin (Francazani *et al* 1989, Whittaker *et al* 1989) and steady state mRNA for both H and L-ferritin is also inappropriately low (Pietrangelo *et al* 1992). By contrast, TfR expression on the basal surface of mucosal cells in GH is increased (Banerjee *et al* 1986, Lombard *et al* 1990) as well as TfR mRNA (Pietrangelo *et al* 1992). This is in keeping with mucosal cells behaving as iron 'deficient' and their increase absorption in these circumstances would not be inappropriate. One possible explanation for this is that the enterocyte is not 'informed' about body iron stores. Alternatively, a primary defect within the intestinal mucosa could be caused by a failure to switch from neonatal to adult control of iron absorption (Srai *et al* 1984).

To explore how the enterocyte might be informed about body iron stores requires some understanding of cellular iron homeostasis.

1.3.7 Molecular regulation of ferritin and transferrin receptors

Iron enters cells via TfR and once inside cells, iron may be considered to be in one of three pools; (i) to be utilised for metabolic processes, (ii) sequestered as ferritin and (iii) a separate pool to regulate the iron regulatory element binding protein (IRE-BP) or IRP. In the 1970's Munro and co-workers (Zahringer *et al* 1976) postulated and subsequently demonstrated that control of ferritin expression was mainly regulated by translation (Munro & Linder 1978, Rogers & Munro 1987). Deletion analysis of ferritin mRNA indicated that the region for translation control reside within 30 nucleotides of the 212 nucleotides of 5'UTR on human H-ferritin mRNA (Hentze *et al* 1986, 1987b). This 30 nucleotide sequence could be folded to form a stem-loop structure. Similar stem-loop structures have also been demonstrated in rat ferritin L-chain mRNA (Aziz & Munro

1987, Leibold & Munro 1987,1988) and these stem-loops function as translational control elements and have been termed the iron regulatory or responsive element (IRE).

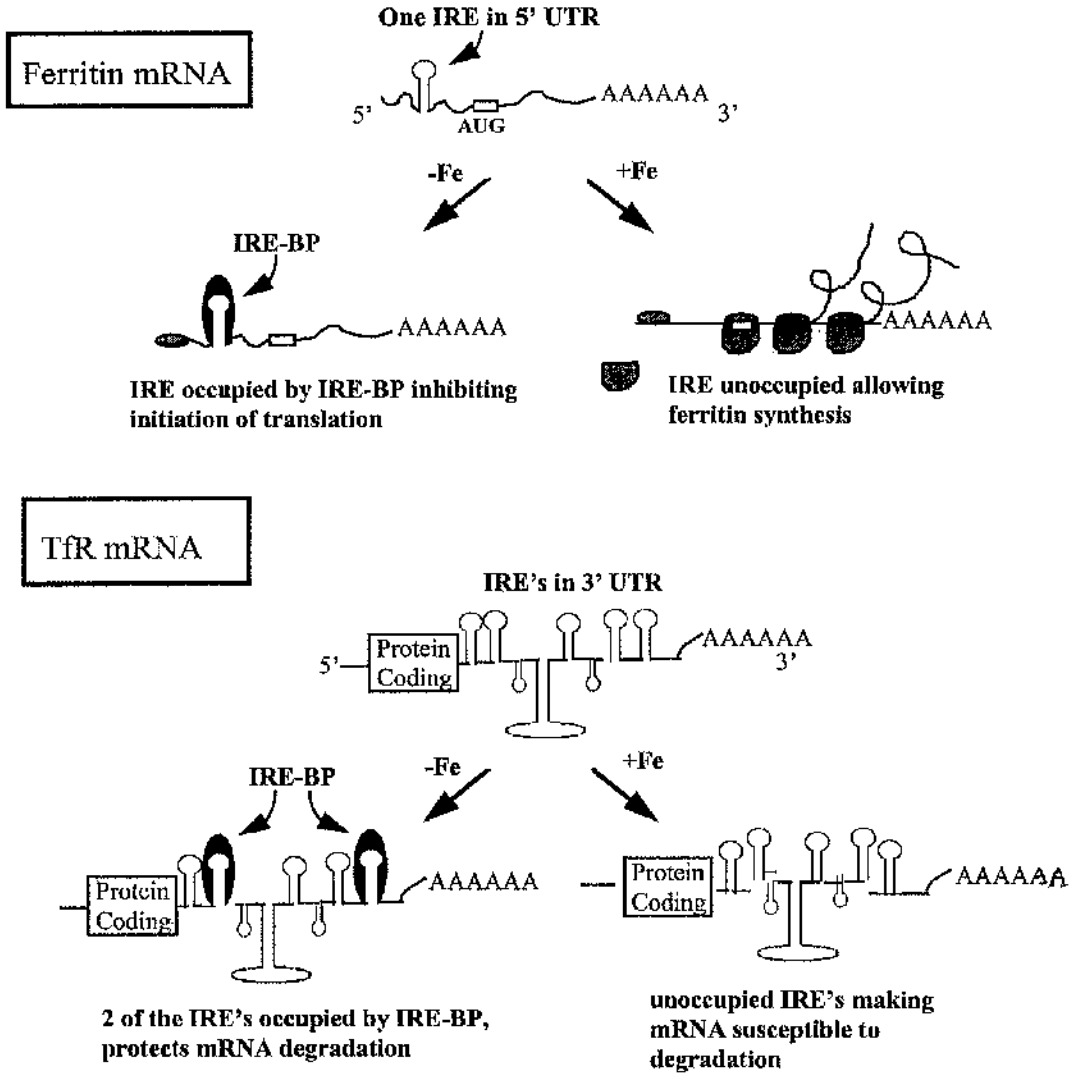
For TfR, immunoprecipitation studies (Mattia *et al* 1984, Rao *et al* 1985) indicate that the iron-dependent alterations in TfR biosynthesis were at the level of transcription. The locus of iron regulation of TfR expression is within a fragment of 678 nucleotides on the 3'UTR of TfR mRNA (Owen & Kuhn 1987, Casey *et al* 1988b). A total of 5 stem-loop structures have been recognised within the 3'UTR which are similar and contain homologue sequences to the IRE within the 5'UTR of ferritin mRNA (Casscy *et al* 1988a).

Several studies notably (Hentze *et al* 1987a, 1988, 1989b, Kuhn & Hentze 1992) have revealed the existence of an intriguing mechanism for the control of cellular iron homeostasis by modulating the translation of ferritin and TfR mRNA. Both IRE's on TfR and ferritin are capable of being bound by a 90 kDa iron regulatory protein (IRP1). Binding of IRP1 has the effect of stabilising TfR mRNA facilitating translation whereas binding of IRP1 to ferritin mRNA impedes translation by preventing ferritin mRNA interactions with ribosomes.

When iron stores are low, IRP1 has a high affinity for the 5'UTR of ferritin mRNA repressing ferritin translation. However, binding of IRP1 to 3'IRE TfR mRNA stabilises mRNA promoting translation. Enhanced TfR expression will facilitate iron entry into cells. However, when iron stores are high, affinity of IRP1 to IRE is reduced. TfR mRNA is destabilised, translation is attenuated and diminished TfR expression will reduce entry of iron into cells. Simultaneously, ferritin translation is derepressed culminating in ample ferritin protein to sequest excess iron. This reciprocal mechanism between ferritin and TfR mRNA translation by iron has been summarised in Figure 1-4.

Figure 1-4

Iron regulation of ferritin mRNA translation and TfR mRNA stability



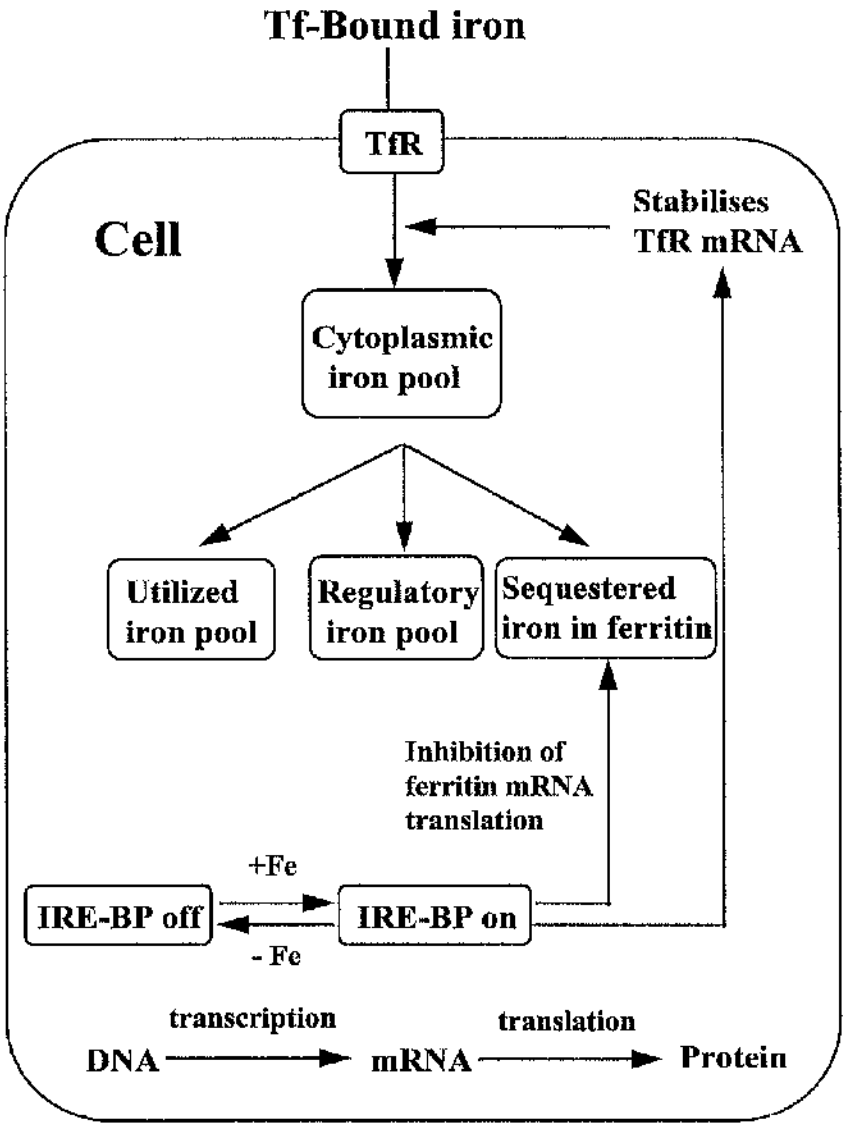
To date, there are at least two IRPs which bind specifically to the IRE's. IRP2 unlike IRP1 has a molecular mass of 105 kDa with the highest binding activity in the small intestine and brain. However, the functional significance of the different IRP's remain uncertain (Kim *et al* 1995).

Clues on how the IRP1 might sense changes in cellular iron status have come from observations that IRP1 is structurally very similar to the mitochondrial enzyme, aconitase (Hentze & Argos 1991, Rouault *et al* 1991) and possesses iron-dependant aconitase activity (Kaptain *et al* 1991, Haile *et al* 1992). This has been identified as cytosolic aconitase, the physiological 'iron sensor'. When cellular iron is low, IRP1 is seen as in a reduced state being an avid binder for the IREs (referred to as IRE-BP-**on** in Figure 1-5) thereby repressing ferritin mRNA translation and TfR mRNA degradation. Similarly when cellular iron is high, IRP1 is envisaged as in an oxidised state (referred to as IRE-BP **off** in Figure 1-5) with reduced avidity for the IRE thereby de-repressing ferritin mRNA translation and TfR mRNA degradation. The two forms of aconitase may not be interchangeable and *denovo* synthesis of either apoaconitase, depending on cellular iron availability may best explain its dual function.

To date, there is no evidence for a defect of this co-ordinate regulation in GH. Activity of IRP1 in TfR mRNA and ferritin mRNA appears to be increased in GH with mucosal ferritin translation in these patients being only slightly raised compared to control subjects but lower when compared to patients with secondary haemochromatosis (Pietrangelo *et al* 1995). Positional cloning have localised the gene for IRP1 to chromosome 9 (Hentze *et al* 1989a).

Figure 1-5

Regulation of cellular iron homeostasis through IRP-BP



1.4 Hypothesis

It is known that (i) induction of anaemia by blood loss in rats had no effect on iron absorption until about the 4th day (Crosby *et al* 1963). (ii) Induction of an acute iron overload was not accompanied by a reduction in iron absorption until at least 24 hours (Wheby *et al* 1964a). (iii) Parenteral administration of radioactive iron to rats resulted in the accumulation of radioactivity initially in the duodenal crypt cells and only after 3 days had lapsed were there any appreciable change in the location of the radiolabelled iron (Messier & Leblond 1958). (iv) This duration coincides approximately with the migration of cells from the crypts of Lieberkuhn onto the villus architecture in the rat intestinal mucosa (Leblond & Messier 1958). (v) *Non-haem iron* absorption is influenced by body iron stores (Baynes *et al* 1987, Cook 1990). (vi) Iron absorption correlates with mucosal ferritin content (Charlton *et al* 1963, 1965). (vii) TfR mRNA expression has an inverse relationship to ferritin mRNA in the enterocyte (Pietrangelo *et al* 1992) .

It has been proposed that mucosal cells in the intestinal mucosa may have been conditioned while in the crypts by body iron stores from plasma iron (as reflected by the percentage transferrin saturation) and these proliferating cells may influence iron absorption as they migrate the villus cellular architecture to the tip some 3 to 5 days later (Conrad & Crosby 1963). If this is correct, then changes in expression of TfR and ferritin along the crypts should follow a predictable pattern with changes in dietary iron.

I set out to test the hypothesis that body iron stores influence iron regulatory behaviour in the mucosal cells of the proximal small intestine by (i) conditioning the proliferating cells in the intestinal crypts via plasma TfN and TfR located on the basolateral surface of enterocytes (ii) this conditioning of the crypts sets up a specific L:H rich ferritin subunit transcript ratio referred to as the constitutive ratio and (iii) this constitutive ratio predetermined in the crypts should be consistent with the known pattern of iron absorption as crypt cells migrate to become functional villus cells at the tip some 3 to 5 days later.

1.5 Plan of investigations and outline of thesis

To test the first part of my hypothesis, I propose to show that L and H-ferritin rich subunit mRNA in the crypt and villus cells of the proximal intestine are different in differing states of iron repletion. This could be tested in human intestinal biopsies obtained endoscopically but would require a method of separating subpopulations of pure crypt and villus cells of the human intestinal mucosa. As such a method has not been described before, this will require the development of a new method and verification of the purity of the separated cell subpopulations. Furthermore, I would require a method of analysing mRNA expression in the separated cell subpopulations. This will be undertaken using Northern hybridisation techniques and I will also require human ferritin probes and gene cloning techniques to generate sufficient cDNA for examining ferritin transcripts in subjects with disordered iron metabolism (iron deficiency and GH).

To show that L and H-ferritin rich subunit mRNA in crypt and villus cells are responsive to acute alterations in body iron stores, I would have to use an animal model where iron stores in these animals could be manipulated by dietary means. I would also need to develop a method of separating these cells and also set up a method of analysing and quantifying mRNA in the separated cell subpopulations. However, to demonstrate that L and H-rich ferritin subunits in intestinal villus cells change progressively over a 3-5 day period in a way which can be predicted by alterations in L and H rich transcripts in crypt cells predetermined 3-5 days earlier, I would still be able to use the same animal model but to capture changes in regulatory behaviour in the intestinal mucosa, I would have to challenge the iron status 'acutely' in these animals by dietary means over the said period of 3-5 days after the animals had been initially conditioned to an iron deficient or an iron supplemented diet (carbonyl iron) for 5 weeks.

As a means of 'informing' the enterocytes regarding body iron stores and therefore programming regulatory behaviour, I postulated that this is mediated through TfR located on the basolateral surfaces of the enterocytes. I therefore propose to examine TfR mRNA

and protein expression in the same groups of animals. The latter was achieved by immunohistochemical techniques using monoclonal antibodies to rats and humans.

This thesis has been divided into four experimental sections. Chapter 2 describes the techniques used, the development/adaptation of methods to separate crypt and villus cells in the rat intestinal mucosa as well as the development of probes to examine mRNA transcripts (TfR and ferritin) to test my hypothesis. Chapter 3 describes experimental manipulation of body iron stores by diet using an animal model (rat) to study its effect on intestinal TfR mRNA expression in crypt and villus cells. By using immunohistochemical techniques, TfR protein expression was also studied in these animals. In Chapter 4, L and H ferritin mRNA expression was examined in the rat liver and also in crypt and villus cells of the rat intestine after body iron stores were manipulated by diet and parenterally. Chapter 5 examined L and H-rich ferritin subunit mRNA expression in crypt and villus cells (separated from endoscopic biopsies) in subjects with disordered iron metabolism. TfR protein expression was also examined in these subjects. General discussion, conclusions and potential for future work are covered in Chapter 6.

2. Development and adaptation of methods and techniques

2.1 Separation of intestinal crypt and villus cells in rat intestine

2.1.1 Introduction

The epithelium of the small intestine is maintained by a series of stem cells at the base of the crypts of Lieberkuhn. As these cells migrate up the crypt-villus axis they undergo functional and morphological differentiation giving rise to a series of terminally differentiated cells on the villus (Potten & Loeffler 1987, Trier & Madara 1981). This migration from the depths of the crypts to villus tip takes approximately 36-72 hours in rats (Messier & Leblond 1958) but 3-5 days in humans (Junqueira 1971) where these cells reach full maturity at the upper third of the villus architecture. This migration is accompanied by the development of the well-defined apical microvillar brush borders and the maturation of a number of apical (e.g. alkaline phosphatase and sucrase), basolateral (e.g Na^+/K^+-ATPase) and basal (e.g thymidine kinase) enzymes. Methods for separating crypt and villus cells in rodents have been described previously, the most notable is by the mechanical separation in chelation buffer (Weiser 1973). Initially, separation was undertaken at 37 °C which resulted in significant disruption of biochemical and molecular processes such as mRNA stability. Later, the separation technique was undertaken at 4 °C which preserved mRNA integrity (Flint *et al* 1990). I have modified the separation technique of Flint *et al* 1990 (section 2.1.2) to test my hypothesis in the rat model. To study isolated crypt and villus cells in human tissue in which tissue availability is limited, I have had to develop a method for separating these cell subpopulations from endoscopic samples (Chapter 5). Validation of these cell subpopulations were undertaken by histological acessment, assaying for specific enzymes such as alkaline phosphatase (villus cells) and 3H-thymidine incorporation (crypt cells). Below (Table 2-1), is a brief description of how I modified the Flint *et al* (1990) protocol of crypt-villus separation in the rat intestinal mucosa.

2.1.2 Materials and methods

(i) Reagent sources

All chemicals used in these studies were purchased from Sigma Chemical (Dorset, U.K.) unless stated in the text.

(ii) Animals and tissues

Eight to 10 weeks old, male Wistar rats (Charles Rivers, Ramsgate Kent, England) were housed individually in polythene cages and received a standard rodent diet (Special diet services, Essex, U.K.). At the end of the study, the animals were sacrificed by carbon dioxide inhalation and the proximal small intestine (25 cm. from the pyloric antrum) was removed for cell separation. This was essential in order to provide reproducible results (Schumann *et al* 1990). Throughout the study, the animals had unlimited access to water.

(iii) Protocol for crypt-villus separation in the rat intestinal mucosa

The proximal small intestine was removed and flushed with 20 mls of iced Hanks buffered salt solution (HBSS, low calcium and magnesium, Hyclone, UK) in 0.5 mM dithiothreitol. Tissues were everted using a blunt-ended spinal needle by a 'sleeve pull through' technique as shown in Figure 2-1. The everted bowel was divided into approximately 2 cm segments and in a sequential series of incubations and inversions at 4 ^{o}C, various sediment were removed to obtain cell fractions designated Vw, V1, V2, V3, C1 and C2. After separation, cells were concentrated by centrifugation (Mistral 6L Fission instruments) at 2000 rpm for 10 minutes at 4 ^{o}C. The pellet was resuspended in 0.5 mls chelation buffer and stored at -80 ^{o}C until RNA extraction or biochemical analysis were undertaken.

Figure 2-1

Eversion of the rat intestinal mucosa for separation of crypt-villus cells

Key: 1: spinal needle with end made blunt, 2: small intestine treaded through and one end secured, 3: small intestine 'pull' inside out 4: small intestine cut into appropriate size for cell separation

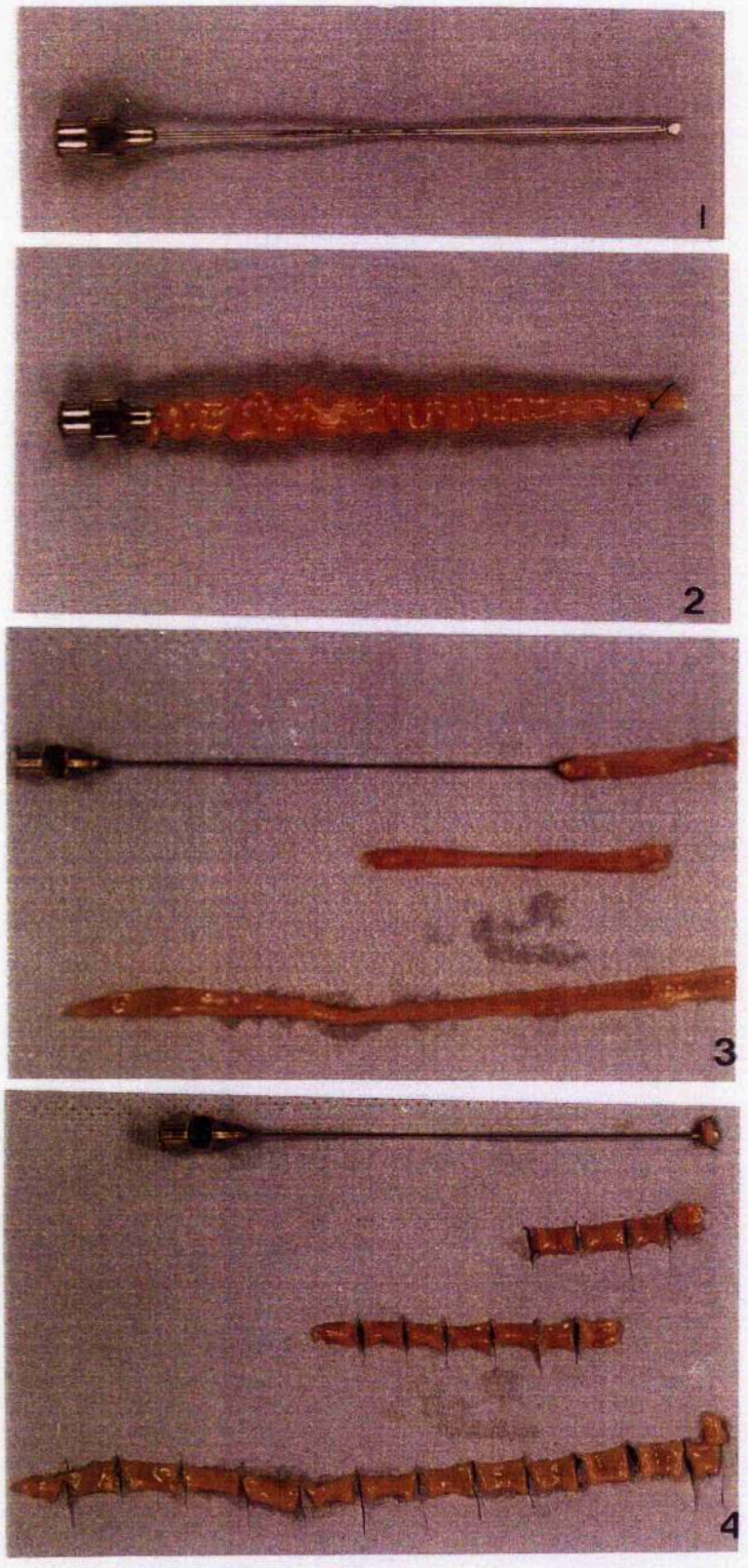


Table 2-1

Isolation of rat intestinal mucosa along the crypt-villus axis

Evert small intestinal mucosa

Cut specimens into 1-2 cm pieces. Incubate in 30 mls Hanks buffer with 0.5mM DTT at 4 °C for 5 minutes under constant stirring with a magnetic flea

Transfer small bowel tissues to 60 mls of *chelation buffer in a sterilin container

Incubate at 4 °C with constant stirring for 25 minutes

Obtain supernatant and pellet cells at 2000 g for 10 minutes

This fraction is labelled **Vw**

Transfer small bowel tissues to 60 mls of chelation buffer

Invert sterilin container gently by hand, approximately 30 inversions

Obtain supernatant and pellet cells at 2000 g for 10 minutes at 4 °C

Repeat twice in 60 mls of cold chelation buffer. Pellet cells as before

This gives fractions labelled V_1, V_2 and V_3

Transfer tissues to 60 mls chelation buffer

Incubate at 4 °C for 10 minutes with constant stirring

Discard suspension

Transfer small bowel tissues to 60 mls of chelation buffer

Invert sterilin container gently by hand (approximately 30 inversions)

Obtain supernatant and pellet cells as before

This fraction is labelled C_1

Repeat above until tissues float to the surface

This fraction is labelled C_2

**Details of chelation buffer can be obtained in the appendix at the end of this chapter*

(iv) Validation of cell subpopulations

The purity and character of the cell fractions obtained by this separation technique was assessed histologically, enzymatically (villus cells) and by 3H-thymidine incorporation (crypt cells).

(a) Alkaline phosphatase assay

Villus cells were identified by activity of constituent enzymes i.e. alkaline phophatase. For alkaline phosphatase measurements, cells were disrupted with a sonicator (MSE Soniprep 150) for 6 seconds at 10 μm wavelength. The suspension was centrifuged at 4 °C (Eppendof 5402R) at 10,000 g for 5 minutes. 200 μl of supernatant was then incubated at 37 °C with enzyme substrate (0.23 mM p-nitrophenylphosphate in 0.3 mM $ZnCl_2$ and 10 mM $MgCl_2$ in 0.5 M Tris-HCl, pH 7.4, total volume 1.1 mls) for 15 minutes and the reaction terminated with 0.5 mls of 0.5 M NaOH. Enzyme activity was determined by the release of p-nitrophenol detected spectrophotometrically at 420 nm. Results (Figure 2.3[top]) were expressed as the amount of p-nitrophenol per mg protein determined by the Lowry method (1951) as described below.

The Lowry protein assay utilised the following reagents

Solution A: Na_2C0_3 (20 g/l) in 0.1 M NaOH (4g /l)

Solution B: $CuS0_4$ $5H_20$ (0.5 g/100 ml) in 1% sodium citrate

Solution C: 50 mls of A + 1 mls of B

Folins reagent was diluted with distilled water (1:1).

The standard stock solution used is BSA (100 mg/100ml in 0.9% saline)

One ml of solution C was introduced to either 200 μl of test solution or standard solution. The mixture was vortexed and left to stand for 10 minutes at room temperature. Then 100 μl of diluted folin reagent was introduced and the mixture left at room temperature for a further 30 minutes. Absorption was read at 750 nm with saline as a blank and samples were analysed in duplicates.

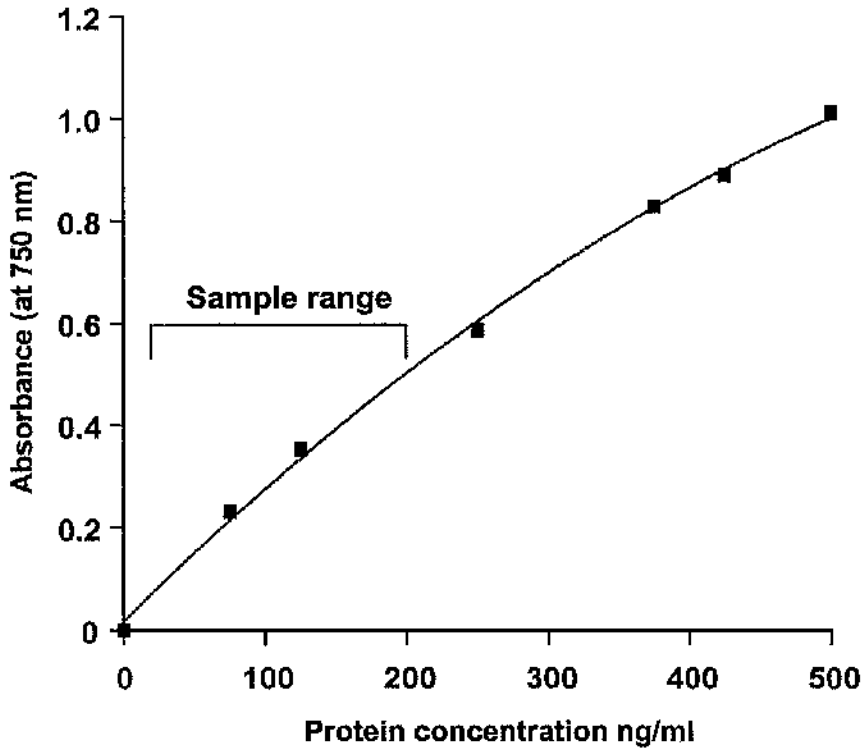
Construction of a standard curve

This was undertaken as described below (200 µl total volume)

	Blank	A	B	C	D	E	F
Conc (µg/ml)	0	50	125	250	500	749	1000
Stock (µl)	0	10	25	50	100	150	200
0.9% saline	200	190	175	150	100	50	0

Figure 2-2

Standard curve for protein assay



(b) Incorporation of 3H-thymidine

Crypt cells were identified by 3H-thymidine uptake (Leblond & Messier 1960). Male Wistar rats (100 g body weight) were injected intraperitoneally (ip) with methyl-3H-thymidine (Amersham, Buckinghamshire, U.K.) specific activity 79 Ci/mmol at 1 μCi/g 1 hour to label proliferative cells. At the end of the experiment, the animals were sacrificed by cervical dislocation. Isolation of cell subpopulation was undertaken as described previously in Table 2-1.

Two hundred μl of the resuspended cells was precipitated with 5 % trichloroacetic acid and the precipitate dissolved in 200 μl 0.1 mM NaOH and neutralised with an equal volume of 0.1 mM HCl. Radioactivity in the samples was determined by liquid-scintillation counting (10 ml of scintillant, LKB, Loughborough) on a LKB 1219 Rack Beta counter. Results (Figure 2.3[bottom]) were expressed as dpm per mg protein (Lowry *et al* 1951).

(c) Histological verification of separation technique

At each sequential stage of the isolation procedure, a small bowel remnant was removed and processed for histological examination after staining with haematoxylin and eosin (Figure 2-4).

2.1.3 Comment

The present study of alkaline phophatase activity and thymidine incorporation in the segregated cells reveal a gradient of 4 and 8 fold difference respectively of activity/incorporation between crypt and villus cells (Figure 2-3). This compares favourably (also a gradient of 4 and 8 fold difference respectively) to previous studies (Weiser 1973, Flint *et al* 1990). Histological assessment of cell loss suggest that serial separation contains proportionally more cells of one type than the other (Figure 2-4).

Figure 2-3

Alkaline phophatase activity (top) and 3H-thymidine incorporation (bottom) in segregated cell subpopulations following segregation of rat intestinal cell subpopulation. Note that enzyme activity is highest in villus cells (Vw) and lowest in crypt cells (C_2) and incorporation is highest in crypt cells (C_2) and lowest in villus cells (Vw)

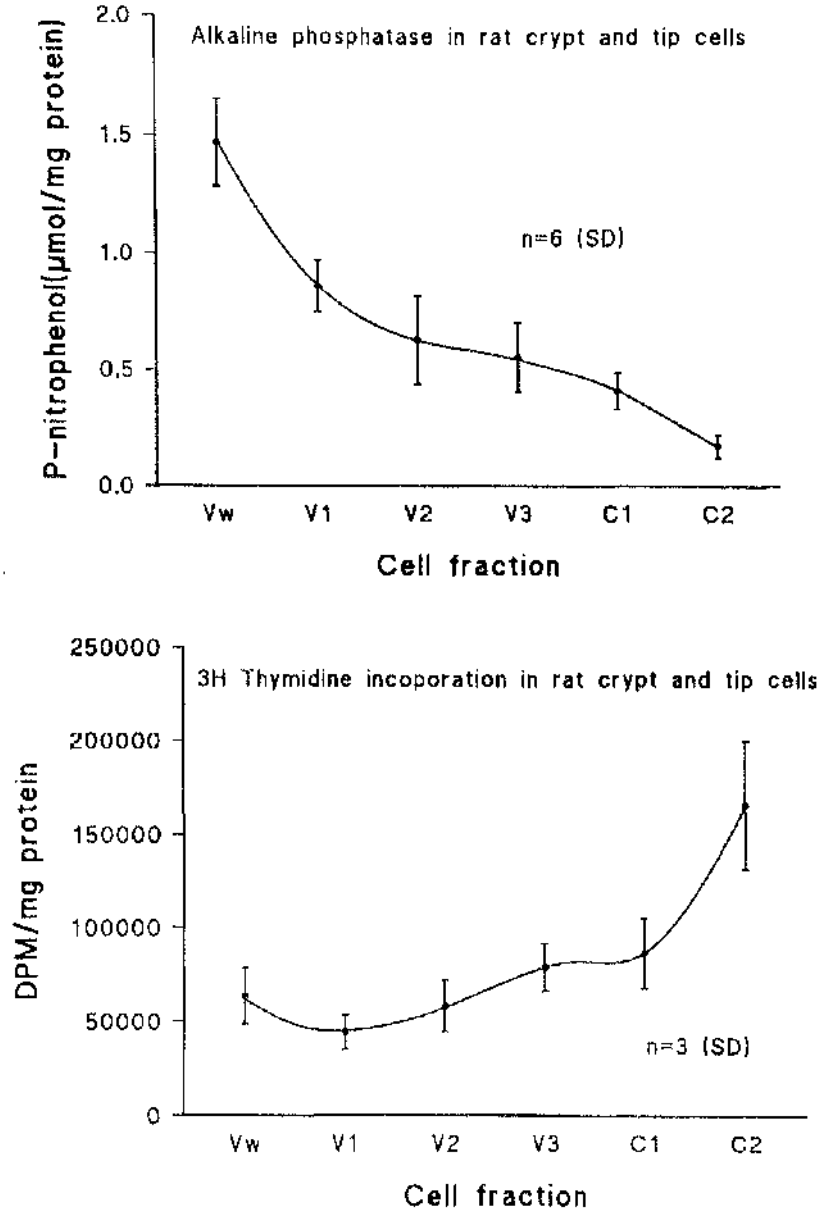
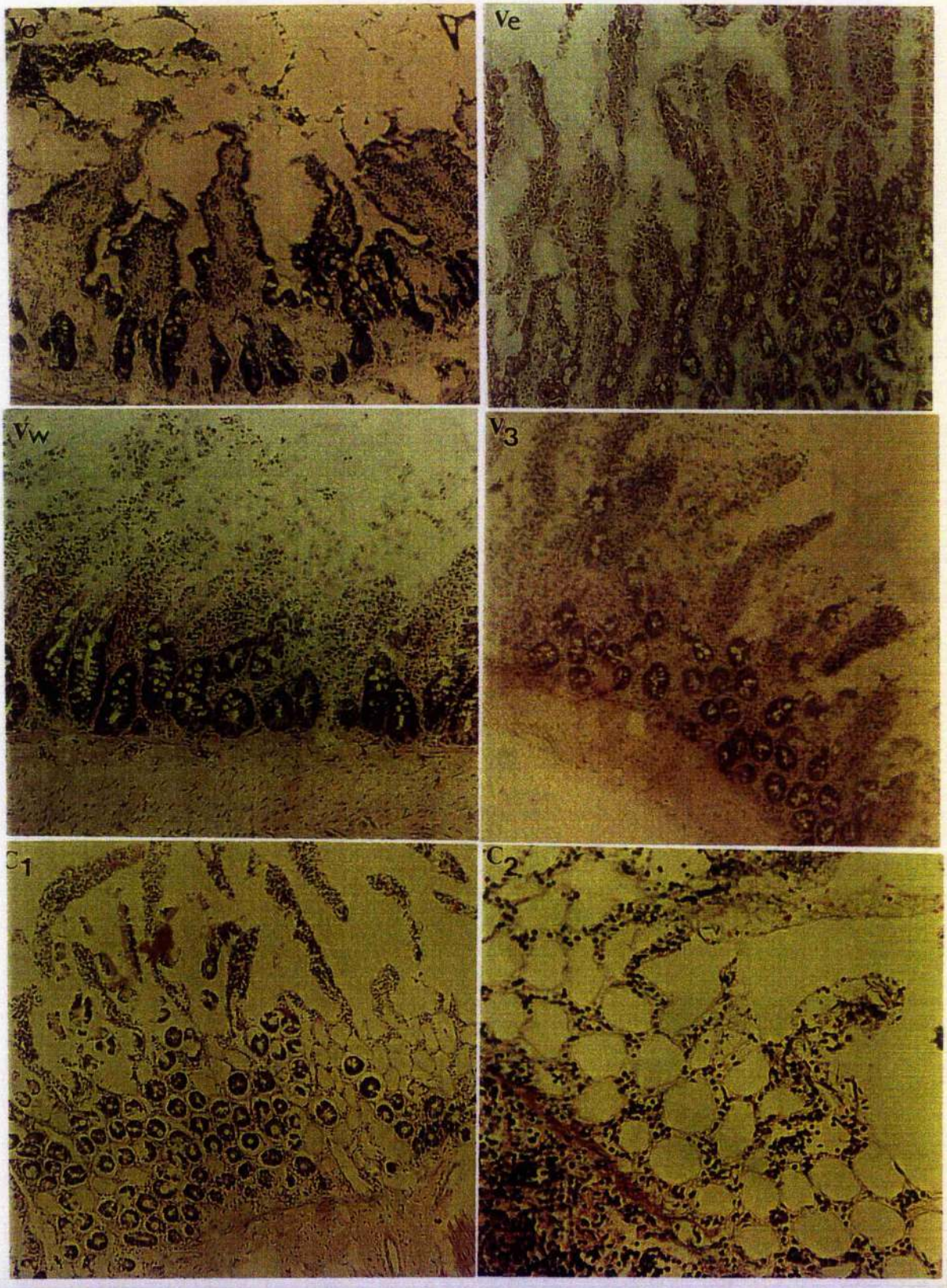


Figure 2-4

Histological verification of separation technique for crypt and villus cells

Key: Vo: longitudinal section of rat small bowel, Ve: everted small intestine, Vw: after 25 minutes in chelation buffer, V_3: after 90 inversions, C_1: after further incubation and 30 inversions and C_2: after 30 inversions (magnification X 200).



2.2 Cloning and development of DNA probes

2.2.1 Introduction

The techniques available for examining molecular expression evolved rapidly during the period of the thesis. Accordingly, in the course of these studies, I utilised a variety of techniques in my attempt to find suitable probes. These are described here both as evidence of my efforts and also as a testament to the rapid progress in development of techniques during the relatively short course of these studies.

To investigate the hypothesis, I wished to examine ferritin transcripts (L and H ferritin) in crypt and villus cells of the proximal rat intestinal mucosa over a 3-5 day period after manipulation of body iron stores with diet. I will require L and H-ferritin probes. Initially I had planned to use oligonucleotide ferritin probes and selected sequences (30 mers) for rat L and H-ferritin verified using Genbank database. These sequences (shown in the appendix at the end of this chapter) were synthesised by Dr. Barraclough in the Department of Biochemistry, University of Liverpool. When these probes were tested on rat tissues, the hybridisation signal for L-ferritin (Figure 2-5a) was located in the predicted position below the 18S rRNA but the hybridisation signals for H-ferritin (3 different sequences selected) were on 28S rRNA (Figure 2-5b) where the predicted hybridisation signal is below the 18S rRNA. This observation could be explained by complementarity between ferritin and 28S rRNA which has been reported previously (Swatantra *et al* 1985). This is thought to affect the stability and/or the translatability of ferritin mRNA. My search for a rat ferritin cDNA probe was unrewarding and mouse ferritin cDNA probes were examined in rats tissues.

Mouse L (578 bp) and H-ferritin (347 bp) were a generous gift from Dr. Andrew McKie, in the Department of Molecular Medicine, Kings College, University of London. These probes were generated by the polymerase chain reaction (PCR). When I tested these probes on rat tissues (liver and small intestinal RNA), the hybridisation signal was in the predicted position below the 18S rRNA (Figure 2-6a & b). Furthermore, sequence

verification between mouse and rat ferritin was confirmed using Genbank database. There was sufficient mouse ferritin cDNA for subsequent experiments and further manipulation was not required.

Preliminary experiments also reveal that mouse ferritin probes were not compatible with human tissues which I also wanted to examine (Chapter 5). Human ferritin probes (cDNA) probes were a kind gift from Dr. Antonello Pietrangelo, Department of Internal Medicine, Modena, Italy. The pFr and Pr3 clones containing the L-ferritin gene (approximately 850 bp) and the H-ferritin gene (approximately 900 bp) respectively had been incorporated into the plasmid PBR 322. To generate sufficient cDNA for subsequent experiments, further manipulation (gene cloning) was required.

The gene cloning experiments were undertaken by me in the School of Tropical Medicine, Liverpool, by which the plasmid PBR 322 containing the ferritin gene sequence were transformed to DH5 1-α competent cells (Promega, Biotech, Liverpool, U.K.). Furthermore, since PBR 322 is a relatively 'primitive' plasmid and as such the yield of plasmid DNA is low, amplification using chloramphenicol (an inhibitor of protein synthesis and chromosomal replication but not plasmid replication) was used to increase its copy number. Plasmid DNA was obtained using the Large-Prep alkaline lysis method and fragment DNA of interest obtained using restriction enzymes as described below.

TfR protein and mRNA expression was examined (Chapter 3), in crypt and villus cells of the rat intestinal mucosa. The latter was achieved using an oligonucleotide probe. This is a 30 base oligonucleotide antisense to $TfR^{301-331}$ (Kuhn *et al* 1984) with the following sequence (5'-GCT-CAT-CTG-GGG-ACA-GGT-GAC-CCT-TAC-ACA-3'). Sequence verification to rat TfR was again confirmed using Genbank database and preliminary experiments have shown this oligonucleotide to hybridise liver and small bowel TfR mRNA in both human and rat tissues (Figure 2-7).

Figure 2-5 (a)

Autoradiogram for L-ferritin (oligonucleotide probe)

Key: lane 1: rat liver RNA (20 μg), lane 2: rat intestinal RNA (20 μg)

Note that the hybridisation signal is below 18RS rRNA

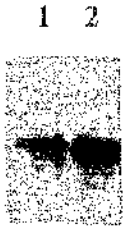


Figure 2-5 (b)

Autoradiogram for H-ferritin (oligonucleotide probe)

Key: lane 1: rat liver RNA (20 μg), lane 2: rat intestinal RNA (20 μg)

Note that the hybridisation signal is on 28S rRNA

Figure 2-6 (a)

Mouse L-ferritin (cDNA probe)

Key: lane 1: rat liver RNA (20 μg), lane 2: rat intestinal RNA (20 μg)

Note that hybridisation pattern is below 18S rRNA

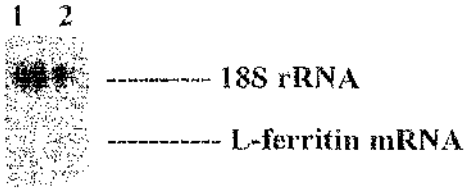


Figure 2-6 (b)

Mouse H-ferritin (cDNA probe)

Key: lane 1: rat liver RNA (20 μg), lane 2: rat intestinal RNA (20 μg)

Note hybridisation pattern is below 18S rRNA

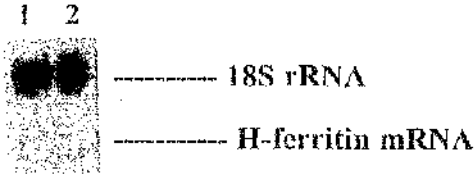


Figure 2-7

Comparison of rat and human TfR mRNA in small bowel and liver

Key: lane 1: liver RNA (20 μg), lane 2: mucosal RNA (20 μg), lane 3: human liver RNA (20 μg) and lane 4: mucosal RNA from one endoscopic biopsy

2.2.2 Methods

(i) Preparation of nutrient broth medium

Nutrient broth No.2 (containing beef extract powder, peptone and sodium chloride) obtained from Difco, East Molesey, U.K. was rehydrated by suspending 25 g of medium to 1 litre of deionised water and heated to boiling to dissolve completely. Media was autoclaved at 15 lb/sq. in. in a liquid cycle for 20 minutes and stored at 4 °C until required.

Agar base No.2 dehydrated (containing protease peptone, liver digest, yeast extract, sodium chloride and agar) obtained from Difco, East Molesey, U.K. was rehydrated by suspending 39.5 g of medium in 1 litre of deionised water and heated to boiling to dissolve completely. This was autoclaved under the same conditions and allowed to cool in a fume cupboard. Ampicillin (50 µl/ml medium) was introduced to the medium and then transferred to petri dishes. Agar plates with and without ampicillin were labelled and stored inverted at 4 °C until required.

(ii) Transformation and plasmid preparation

Competent cells DH5 1-α (500 µl) were thawed, stored in ice (10 minutes) where 10 µl of plasmid DNA was introduced (the volume of plasmid DNA solution should not be more than 5 % of the volume of the competent cells). In general, approximately 50 µl of competent cells are usually sufficient to be saturated by 1 ng of plasmid DNA. The mixture was left in a water bath (42 °C) for 2 minutes with care not to shake the eppendorf tube followed by ice (30 minutes) and 800 µl of nutrient broth. Reaction mixture was then placed in a shaking water-bath (37 °C) for 60 minutes and plated onto agar plates containing ampicillin (50 µl/ml) to select out transformed cells. Agar plates were incubated at 37 °C for 12-16 hours to obtain bacterial colonies. For the pFr and Pr3 clones (L and H-ferritin) in plasmid PBR 322, after transformation onto bacterial DH5 1-α competent cells, amplification was undertaken to increase its copy number.

(iii) Amplification

Ten mls of bacterial culture carrying the plasmid pBR322 was grown to the late log phase (optical density of 0.6 at 600 nm). Five hundred mls of nutrient broth medium (prewarmed to 37 °C) containing ampicillin (50 µl/ml) was introduced in a 2-litre flask to 2 mls of the late-log phase culture. Incubation was undertaken for approximately 3 hours at 37 °C with vigorous shaking on a rotary shaker or until the optical density of the culture was approximately 0.4. Chloramphenicol (2.5 mls of 34 mg/ml in ethanol) was introduced (final concentration in the culture is 170 µg/ml). The culture was incubated for a further 12-16 hours at 37 °C with vigorous shaking in a rotary shaker.

(iv) Harvesting and recovery of plasmid DNA

Bacterial cells were harvested by centrifugation (400 rpm for 15 minutes at 4 °C) in a Sorval GS3 rotor centrifuge. The supernatant was discarded. Bacterial pellet was resuspended in ice-cold STE buffer* and bacterial cells obtained by centrifugation as before. Alkaline lysis and ethidium bromide-caesium chloride gradient centrifugation was utilised to separate plasmid from chromosomal DNA following the method described by Ish-Horowica & Burke (1981).

Bacterial pellet was resuspended in 10 mls of Solution 1*. The suspension was incubated for 10 minutes (room temperature) followed by 20 mls of Solution 2* (freshly prepared). Contents were thoroughly mixed by inverting the bottle several times and left in ice (5 minutes) followed by 10 mls of ice-cold Solution 3* and stored in ice for at least 20 minutes. A flocculent should form consisting of chromosomal DNA, high molecular weight RNA and potassium/SDS/membrane complexes. Bacterial lysate was centrifuged (7000 rpm for 10 minutes at 4 °C) and supernatant divided into 35 mls centrifuge tubes with 12 mls isopropanol and left for 10 minutes at room temperature. Plasmid DNA was recovered by centrifugation (10,000 rpm for 10 minutes at 20 °C). Pellet was rinsed in

70 % ethanol at room temperature and excess ethanol removed by a vacuum deciccator. Plasmid DNA was obtained by centrifugation in a caesium chloride-ethidium bromide gradient.

(v) Centrifugation in a caesium chloride-ethidium bromide gradient

Plasmid DNA (pellet) was dissolved in 13 mls of TE buffer* with 13.9 g of caesium chloride and 1.3 mls (5 mg/ml) ethidiun bromide. The tube was inverted to dissolve caesium chloride and centrifuged (10,000 rpm for 10 minutes at 20 °C) to pellet proteins and to remove the pellicle layer on the surface. The density of the mixture was adjusted if necessary to 1.56 g/ml by adding caesium chloride or TE buffer. The solution was transferred to an ultracentrifuge tube, filled with paraffin and centrifuged at 42,000 rpm (T865 fixed angle rotor) for 20 hours at 18 °C. Two bands of DNA, located in the centre of the gradient will be visible (the upper band consisting of contaminating bacterial chromosomal DNA and linear open-nicked plasmid DNA [discarded] and the lower band consisting of supercolied plasmid DNA collected with a hypodermic needle).

(vi) Removal of ethidium bromide from purified plasmid DNA

An equal volume of saturated isoamyl alcohol was introduced to the DNA solution and the two phases mixed by vortexing. A Pasteur pipette was used to transfer the lower aqueous phase to another tube. The above procedure was repeated (6 times) or until all the pink colour had disappeared from the aqueous and organic phase.

**Details of buffe or solution can be obtained in the appendix at the end of this chapter*

(vii) Removal of caesium chloride from purified plasmid DNA

This was undertaken by dialysis for 24-48 hours against TE buffer* and buffer was changed several times during the dialysis period. The expected yield is 2-10 mg of plasmid DNA per 500 mls culture. DNA concentration was determined spectrophometrically at 260 nm and estimates of purity obtained by the ratio of absorption at 260 and 280 nm as described in section 2.3.2.

(viii) Restriction enzyme digest

Restriction endonucleases were used to cleave double-stranded DNA. By convention, one unit of restriction enzyme is defined as the amount of enzyme needed to cut 1 μg of DNA in 1 hour. Restriction digest was performed in the following way:
DNA solution (in a sterile eppendorf tube) was mixed with sufficient water to give a volume of 18 mls with 2 mls of 10 X restriction buffer and 1-2 units of restriction enzyme. Incubation at 37 °C was undertaken for the required period and the reaction terminated by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM.

(ix) Analysing the products of a restriction digest

Restriction digest will result in a number of DNA fragments of varying sizes where these fragments are separated by agarose gel electrophoresis. DNA fragments can be visualised by staining with ethidium bromide under ultraviolet irradiation provided sufficient DNA (not less than 25 ng) is present. A representative restriction digest is illustrated in Figure 2-8.

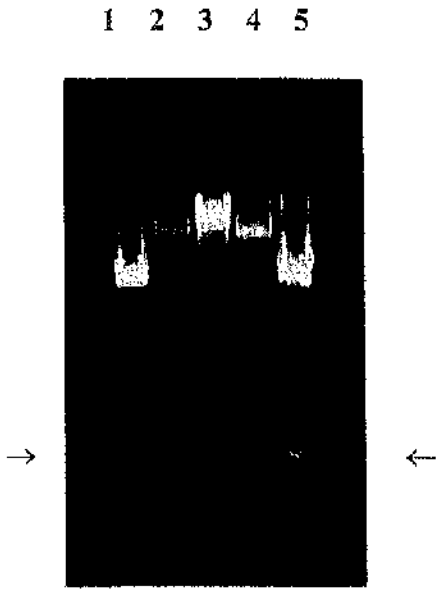
2.2.3 Comment

Modern biological techniques such as gene cloning have only been recently introduced and many of the techniques which I have used have already been superseded by more efficient ways of making DNA probes which were unavailable to me at the time

of study. Furthermore, molecular signal amplification techniques have evolved such that quantitative mRNA can now be undertaken more easily than heretofore. However, I believe that some of the techniques I have adapted such as the separation of human crypt and villus cells (Chapter 5) could be much more informative using the more sensititive assays.

Figure 2-8

Agarose gel electrophoresis for a restriction digest for human L and H- ferritin with PsT1. (DNA stained with ethidium bromide, lane 1: digested L-ferritin, lane 2: uncut L-ferritin, lane 3: markers, lane 4: uncut H-ferritin and lane 5: digested H-ferritin). The cDNA fragments (arrows) were collected, concentrated and used for hybridisation (Chapter 5).



2.3 Molecular biochemistry

The molecular biological techniques employed in the current thesis are now described.

2.3.1 Single step RNA extraction

RNA separation was undertaken as described by Chomyczynski & Sacchi (1987), a robust method which enables completion of RNA extraction within 4 hours. All chemicals used for RNA extraction were obtained from Sigma unless stated otherwise and solutions made up with 0.1 % diethylpyrocarbonate (DEPC) water and autoclaved.

Tissue or cells were homogenised in 0.5 mls of solution D (0.36 mls of 2-mercaptoethanol/50 mls solution C) which is stable for at least 1-3 months at room temperature. Solution C consists of 250 g guanidinium thiocynate in 293 mls water, 7.6 mls 0.75M sodium citrate and 26.4 mls 10 % sarcosyl. This was followed by the addition of 50 µl 2M sodium acetate (pH 4), 500 µl phenol and 100 µl chloroform-isoamyl alcohol (49:1). The mixture was vortexed for 10 seconds and cooled in ice for 15 minutes and centrifuged (12000 g for 20 minutes at 4 °C). The supernatant was obtained and introduced to 0.5 mls of isopropanol. Great care was taken to avoid transferring contaminating material (proteins and phenol) located in the lower region of the supernatant. Samples were left overnight at -20 °C or 1 hour at -80 °C to precipitate the RNA which was recovered by centrifugation (12000 g for 20 minutes at 4 °C). RNA was dissolved in 0.3 mls of solution D with an equal volume of isopropanol and left -20 °C for another hour or overnight. (From experience, RNA yield was greater if precipitation was carried out overnight). RNA was recovered by centrifugation (12000 g for 10 minutes at 4 °C), washed in 1 ml 75 % ethanol, dried at room temperature for 5 minutes, (vacuum drying of the RNA pellet from experience was not necessary) and finally dissolved in 50 µl 0.5% sodium dodecyl sulphate (SDS) at 65 °C for 10 minutes. Samples were stored at -80 °C until required.

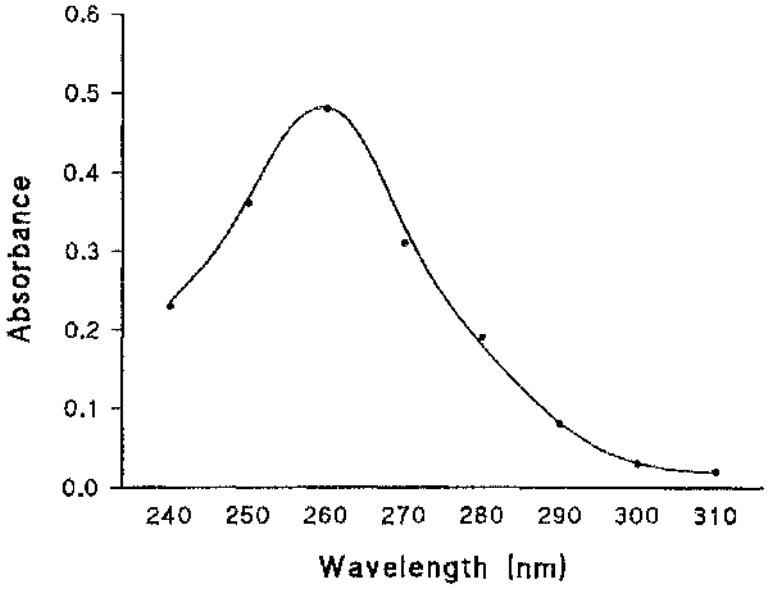
2.3.2 Spectrophometric determination of RNA and DNA

Determination of the absorption spectrum at 260 nm allows quantification of the amount of nucleic acid in the sample. By definition, an optical density of 1 is equivalent to 50 μg/μl for double stranded DNA, 40 μg/μl of single stranded DNA or RNA and 20 μg/μl of oligonucleotides. In addition, the ratio of the absorption at 260 nm to 280 nm allows an estimate of the purity of the nucleic acid. Pure DNA preparations have values of greater than 1.8 and pure RNA preparations have values greater than of 2.0. Contamination by phenol or proteins will lead to lower ratios. Oligonucleotides, however, do not observe this rule. The ratio of absorption at 235 nm to 280 nm is used, with a ratio of 1 regarded as being free from contaminants. A representative absorption spectrum of rat liver RNA is shown in Figure 2-9.

Figure 2-9

Absorption spectrum of rat small intestinal RNA.

Ratio of optical density at 260 nm to 280 nm can be used to estimate the purity of RNA



2.3.3 RNA separation by formaldehyde 5X MOPS-agarose gel electrophoresis

RNA molecules are biological compounds that carry a negative electrical charge. Therefore, when subjected to an electrical field, they will migrate towards the positive pole. Agarose gel electrophoresis can therefore be used to separate RNA molecules according to their size. Composition of formaldehyde gels and conditions for electrophoresis have been summarised in the appendix. RNA can be visualised with certain stains such as ethidium bromide which intercalates with these molecules and fluoresces under ultraviolet irradiation. Figure 2-10 illustrates the presence of the 28S and 18S ribosomal RNA (rRNA) stained with ethidium bromide. Messenger RNA (mRNA) cannot be visualised by this technique as they contribute less than 1 % of the total RNA. A more sensitive method of detection is autoradiography. In this, a complementary DNA sequence (double stranded) or an oligonucleotide sequence (single stranded) is labelled with a radioactive marker such as ^{32}dCTP. This will hybridise to complementary sequences of specific mRNA's of interest that have been separated by agarose gel electrophoresis, transferred to nylon membranes by capillary blotting (Northern blotting) and fixed to these membranes by ultraviolet radiation. The mRNA of interest can be visualised by placing an X-ray sensitive photograph over the nylon membranes (autoradiography); the intensity of the signals reflecting the population of the transcripts and are quantified by desitometery.

2.3.4 Northern blot transfer

The technique for RNA transfer to nylon membranes by capillary blotting is as described in Sambrook *et al* Molecular Cloning manual (1989). Briefly, the filter paper (Whatman chromatography paper, Maidsione, England) and membranes (Amersham UK) were cut to the same size as the gel, in 0.1% DEPC water and capillary transfer was set up as shown in Figure 2-11. The sides of the gel were sealed with parafilm (American

National CanTM) to ensure efficient transfer and capillary blotting was carried out in 10 × SSC for 16-18 hours.

Following transfer, membranes were dried at room temperature sandwiched between 2 filter papers for 5 minutes and covered in saran wrap (Dow chemical company). RNA was fixed to the membranes by exposing to UV light for 3 minutes where membranes were kept at 4 °C until required or stored at -80 °C if not to be used immediately.

Figure 2-10

Agarose gel electrophoresis showing rat liver 28S and 18S ribosomal RNA stained with ethidium bromide. Note that mRNA cannot be visualised by this technique

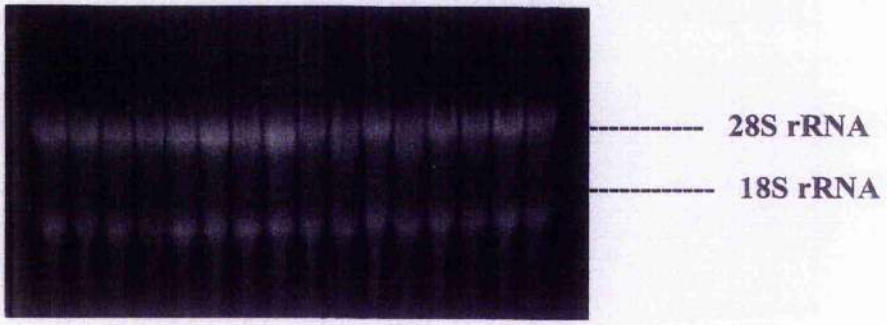
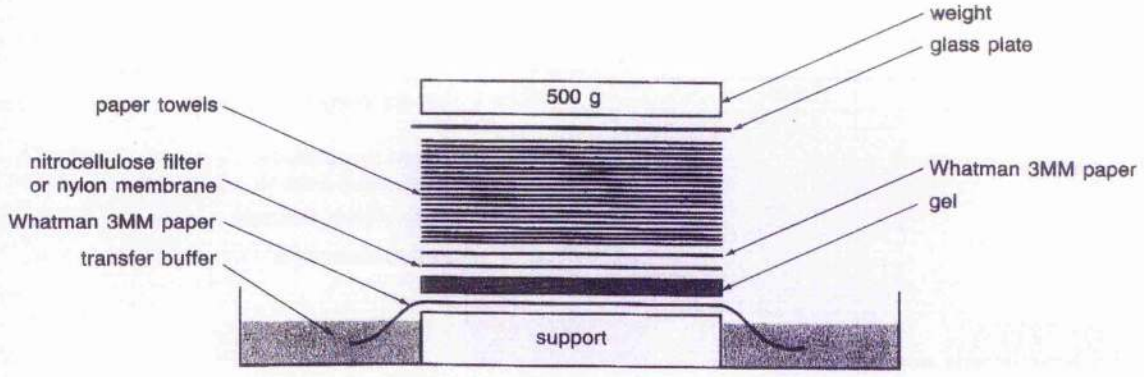


Figure 2-11

Northern transfer of RNA by capillary blotting



2.3.5 Preparation and use of DNA probes

Both cDNA (mouse and human ferritin) and oligonucleotide probes (TfR) were utilised in the experiments. However, in order to normalise for RNA loading, two additional oligonucleotide ribosomal probes (oligonucleotide sequences corresponding to rat 18S rRNA (Torczynski *et al* 1983) and human 28S rRNA (Barbu and Dautry 1990) were used. The oligonucleotide sequences for TfR, 18S and 28S rRNA were synthesised by Dr. Barraclough, in the Department of Biochemistry at the University of Liverpool.

Probes were labelled by different enzyme systems, cDNA with a random priming kit utilising Klenow enzyme (Promega, Biotech) and the oligonucleotides with a terminal transferase kit (Boehringer, Manheim, Biochemica, East Sussex, England).

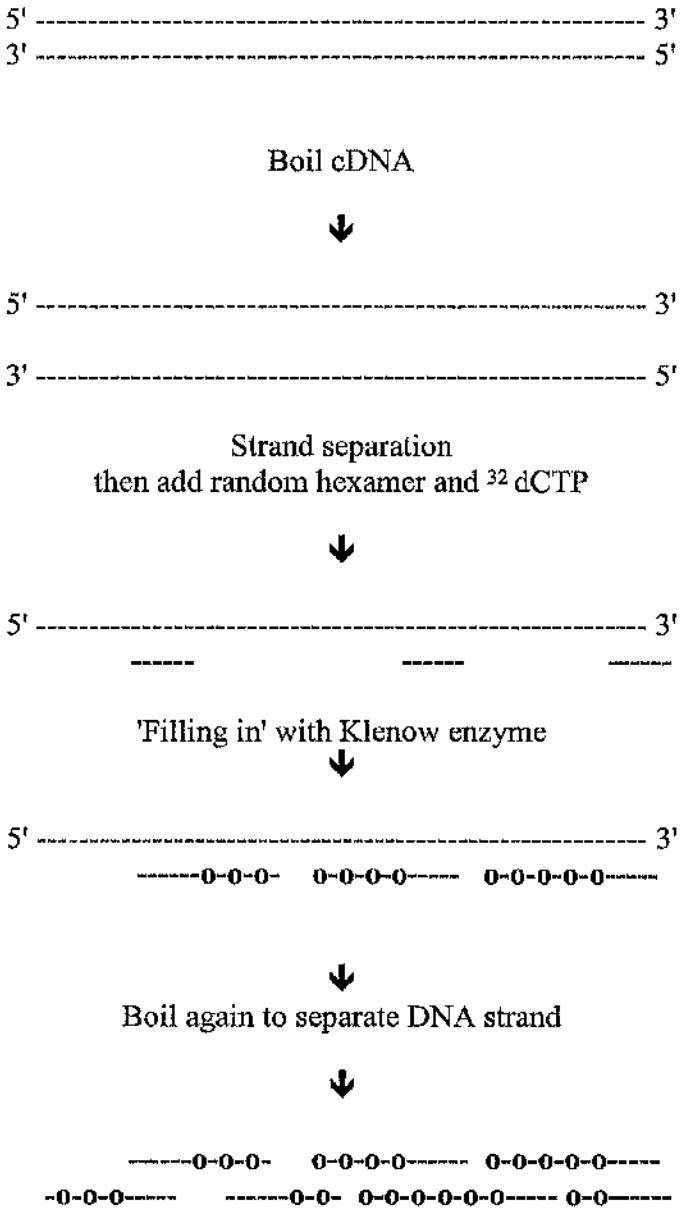
(i) Labelling of cDNA sequences by random priming

Complementary DNA was labelled with the prime-a-gene kit based as described by Finneberg and Vogestian (1983). Table 2-2 summarises labelling reactions by random priming. Six µl of cDNA (approximately 25 ng) in 6 µl TE buffer was boiled and cooled in ice (5 minutes) followed by 2 µl of dNTP (obtained from mixing 2 µl of dATP, dTTP and dGTP), 2 µl bovine serum albumin (BSA), 2 µl Klenow enzyme, 19 µl sterile water, 10 µl 5 X buffer and 3 µl ^{32}dCTP (Amersham) introduced sequentially. The mixture was incubated for 3 hours at room temperature, boiled for 5 minutes to separate double stranded DNA and cooled in ice (5 minutes) before hybridisation.

Table 2-2

Random Priming with hexamer cDNA

Key: ------ random hexamer, -o-^{32}dCTP



(ii) Prehybridisation and hybridisation conditions for cDNA probes

Quickhyad® approximately 6 mls (Stratagene, Cambridge, U.K.) was activated by incubating at 37 °C for 10 minutes in a hybridisation oven. Prehybridisation was carried out at 68 °C for 20 minutes. The probe was introduced (gently to avoid splashing) and hybridisation carried out at 68 °C for 1 hour.

(iii) Washing conditions for cDNA probes and autoradiography

Membrane was washed in the following solutions with increasing stringency : 2 x SSC/0.1% SDS (15 minutes at room temperature), 2 x SSC / 0.1% SDS (15 minutes at 60 °C) and finally twice in 0.1 x SSC / 0.1% SDS (15 minutes at 60 °C). In between washings, the membrane was checked with a Geiger counter for intensity of background activity before proceeding to a higher stringency wash. Membrane was placed in X-ray film (Kodak) and left -80 °C for varying periods to generate an autoradiogram. Autoradiograms were developed and the intensity of the signals quantified using a Schimadzu C5 9000 dual chamber flying spot scanning densitometer.

(iv) End labelling of the oligonucleotide sequences by terminal transferase

Labelling conditions were carried out in accordance to the manufactures instructions (Boehringer). The mixture, 5 µl 5x buffer, 4.5 µl $CoCl_2$, 1.5 µl oligonucleotide (approximately 10 ng), 1 µl terminal transferase enzyme and 3 µl ^{32}dCTP was left to incubate at 37 °C for 1 hour and the reaction terminated with 5 µl 2M EDTA, pH 8. Excess radioactive material was removed with a Sephadex G-50 column prepared as follows:

Two grams of Sephadex G50 (Pharmacia, Fine Chemicals, Milton Kynes, U.K.) was dissolved in 100 mls of 6 x SSPE at room temperature and left for at least 24 hours before use. A plastic Pasteur pipette (top end removed and the bottom secured with glass wool, autoclaved previously) was filled with Sephadex G50. Labelling reaction mixture

was introduced with 200 µl aliquots of 6 x SSPE collected onto eppendorf tubes (16 altogether). In this way, preliminary experiments reveal that the first 6-12 fractions contained the labelled probe with later fractions containing excess radioactive material. The eppendorf tubes were placed in scintillation vials and radioactivity determined on a LKB 1219 Rackbeta counter (counted for 1 minute).

(v) Prehybridisation and hybridisation of oligonucletide sequences

Prehybridisation was carried for 12 hours at room temperature (ribosomal probes) and at 42 °C (transferrin receptor probe) in 20 mls prehybridisation *buffer. A higher temperature for TfR was utilised to reduce non-specific binding where preliminary experiments reveal this to be the optimal temperature for hybridisation. Labelled oligonucleotide sequence was introduced and hybridisation carried out for a further 16-18 hours in 10 mls hybridisation *buffer.

(*details of buffers can be obtained in the appendix at the end of this section) .

(vi) Washing conditions and autoradiography of oligonucleotide sequences

The following conditions were used with increasing stringency:

TfR probe

1 x SSC/0.1 % SDS, 0.1x SSC/0.1 % SDS (both for 15 minutes at room temperature), 0.1 x SSC/0.1 % SDS (15 minutes at 30 °C).

Ribosomal probes(18S and 28S)

1 x SSC/0.1 % SDS (15 minutes at room temperature), 1 x SSC /0.1 % SDS (15 minutes at 60 °C) and finally twice in 0.1 x SSC/0.1 %SDS (15 minutes at 60 °C).

(vii) Determination of specific activity of probe

This is essential to find the minimum volume of the labelled probe required for hybridisation and was determined as follows. One µl of the labelled cDNA or oligonucleotide was mixed to 9 µl of water by gentle shaking. One µl was placed onto 6 Whatman DE 81 filter paper of which 3 of 6 filter papers were washed in 20 mls of 0.5 M $NaP0_4$ for 5 minutes. This represents the labelled counts and the other 3 unwashed filter paper represents the total counts. Filter papers were allowed to dry at room temperature and with 5 mls scintillant in each vial, radioactivity was recorded (5 mls of scintillant was used as a blank for background). Percentage labelling was determined by taking the ratio of the means of the labelled samples (washed filter papers) to the means of the total counts (unwashed filter paper).

The volume of the probe required for hybridisation was calculated as follows :

$$\text{Percentage labelling (x)} = \frac{\text{mean counts of the washed samples}}{\text{mean counts of the unwashed samples}} \times 100$$

If the mean counts for the unwashed samples is Y cpm

The activity of the probe is Y x 10 = Z cpm / µl

For 1 ml of hybridisation buffer, the required counts would be 1.25×10^6 cpm /ml

Hence the volume of the probe required in 6 mls hybridisation buffer is:

$$\frac{1.25 \times 6 \times 10^6}{Z \text{ cpm}} \text{ per ml}$$

(viii) Expression of results

A representative autoradiogram is shown (Figure 2-12) for rat H-ferritin mRNA and 18S rRNA at different RNA concentrations. The intensity of these signals were quantified by densitometery (Figures 2-13a & 2-13b) and results were expressed as a ratio of H-ferritin mRNA to 18S rRNA densitometer readings (Table 2-3)

(ix) Histology and immunohistochemical illustration

All the illustrations (haematoxylin, neutral red and immunohistochemistry) in this thesis have a magnification of X100 unless stated otherwise.

(x) Statistics

All data are shown as mean ± SEM unless stated otherwise. Food intake, body weight were analysed using the nonparametric Mann Whitney test. A significance level of $p<0.05$ was chosen for any differences revealed. All statistical procedures were carried out using the Arcus-Pro II™ statistical package (Medical Computing Ltd.; Aughton, Lancs, U.K.)

Figure 2-12

Autoradiogram of H-ferritin mRNA and 18S rRNA in rat liver

Rat liver RNA at 2.5 μg, 15 μg, 30 μg with the H-ferritin mRNA normalised for RNA loading with 18S rRNA

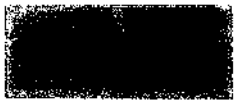
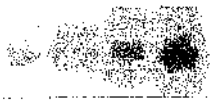
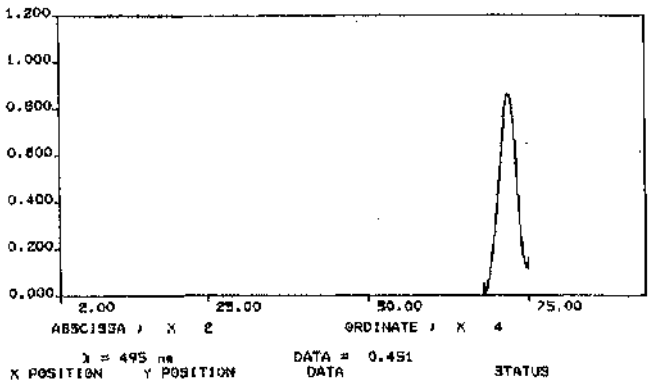
Amount of RNA (μg)	2.5 5.0 15 30
H-ferritin mRNA	
18 S rRNA	
H-ferritin mRNA : 18S rRNA:	0.77 0.75 0.72 0.68

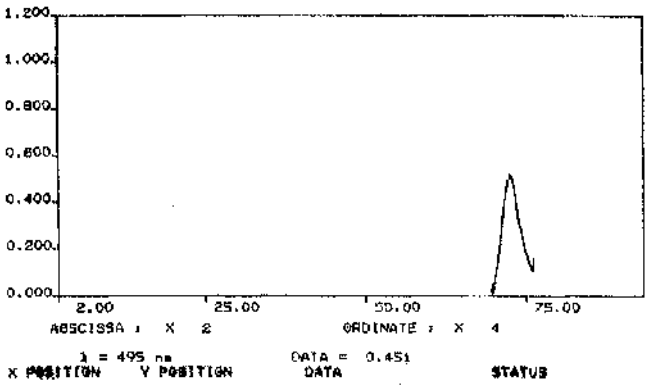
Figure 2-13a

Densitometery tracings of H-ferritin autoradiogram in rat liver at different RNA concentrations



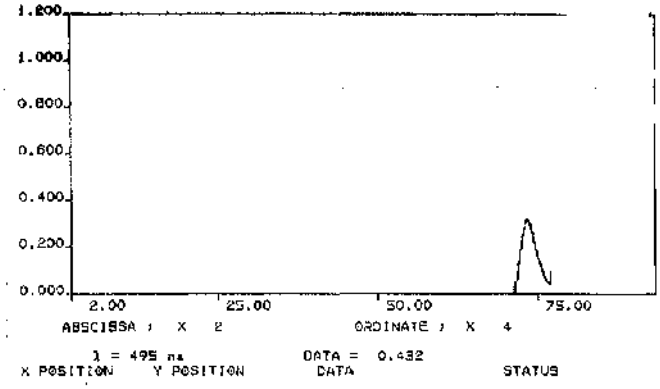
LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
72.0	71542.630			100.000
TOTAL	71542.630			



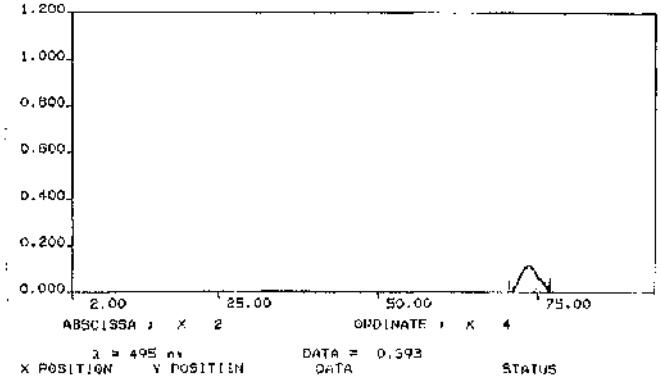
LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
72.6	44462.460			100.000
TOTAL	44462.460			



LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
73.4	23604.780			100.000
TOTAL	23604.780			

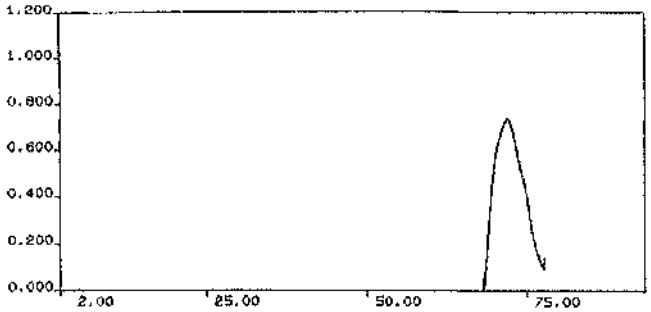


LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
73.6	9658.644			100.000
TOTAL	9658.644			

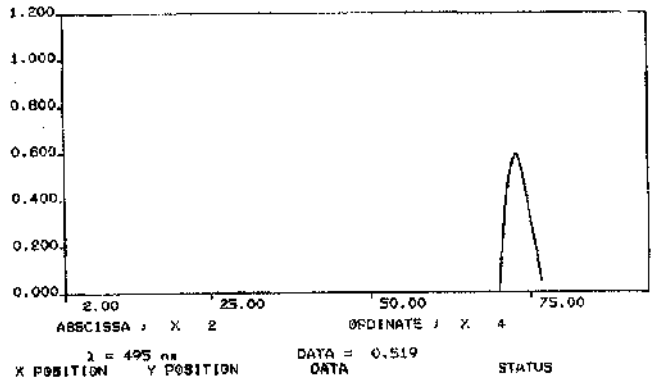
Figure 2-13b

Densitometery tracings of 18S rRNA autoradiogram in rat liver at different RNA concentrations



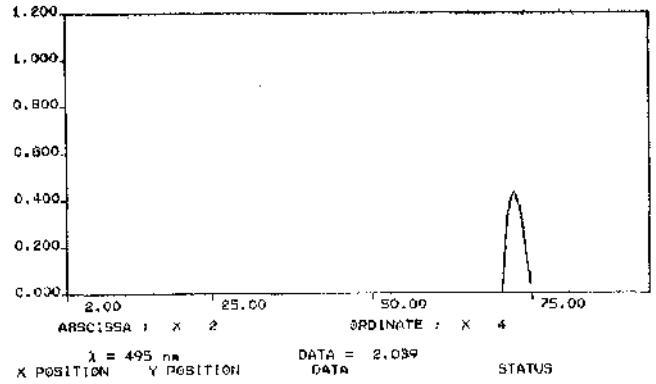
LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
72.0	105824.700			100.000
TOTAL	105824.700			



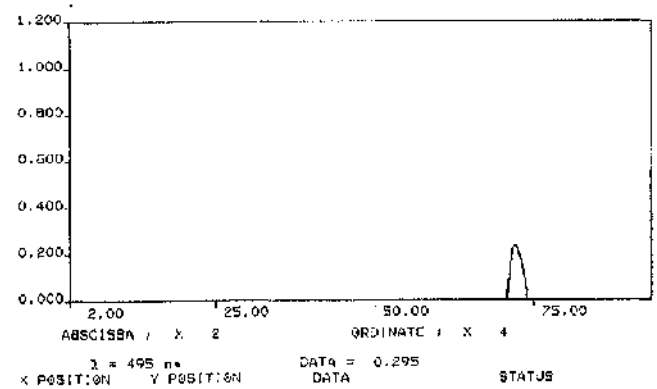
LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
72.8	61774.830			100.000
TOTAL	61774.830			



LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
72.2	31417.300			100.000
TOTAL	31417.300			



LANE NO. 1

Y POS.	AREA	CONC.	MARK	%
72.0	12478.140			100.000
TOTAL	12478.140			

Table 2.3

Summary of densitometer readings for ferritin and 18S rRNA in rat liver

Ferritin mRNA : 18S rRNA densiometer readings (au:arbitary units)

Amount of RNA(μg)	H-ferritin densitometer readings (au)	18S rRNA densitometer readings (au)	H-ferritin:18S
2.5	9658	12478	0.77
5.0	23604	31417	0.75
15	44462	61774	0.72
30	71542	105624	0.68

2.4 Histology

2.4.1 Immunohistochemistry for TfR

Immunohistochemical studies were undertaken using specific monoclonal antibodies to TfR in rat OX 26 [Serotec, Oxford, England; 1:10 dilution in phosphate buffer solution (PBS)] and human OK T9, (Ortho Pharmaceuticals, Raritan, New Jersey; 1:10 dilution in PBS) in small intestinal tissues. The second (labelling) antibody was peroxidase conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark; 1:50 dilution in PBS).

Tissues were fixed in OCT (Miles Scientific, Elkhart, Ind.) and sectioned to 6 μm thickness using a microtome cryostat (Cryotome 620, Anglia Scientific, U.K.). Sectioned tissues were dried in air (20 minutes), fixed in acetone (15 minutes) and further dried in air (5 minutes). Rabbit serum, 50 μl of a 1:20 dilution (50 μl of rabbit serum to 950 μl

PBS, pH 7.6) was placed onto each tissue section in a humid chamber for either 30 minutes at room temperature or 10 minutes at 37 °C. This is to minimise non-specific binding. Rabbit serum was then carefully removed but ensuring that tissues remain well hydrated. The primary antibody was next placed onto tissue sections and incubated either for 1 hour at room temperature or 37 °C for 30 minutes or overnight at 4 °C in a humid chamber. As a control, incubations were undertaken without the primary antibody. Tissue sections were washed 3 times in PBS, 5 minutes for each wash. The second antibody was introduced and incubation undertaken at either 1 hour at room temperature or at 37 °C for 30 minutes. Tissue sections were washed as before. Approximately 5 mg of diaminobenzidine [DAB] (freshly prepared by dissolving in 9.97 mls PBS and 33 µl hydrogen peroxide) and kept away from light until used. Tissue sections were covered with DAB for approximately 2-5 minutes until a brown colour develops (Weir *et al* 1974), washed in distilled water for 5 minutes, counterstained with haematoxylin (BDH Chemicals, England) for 10 seconds, soaked in water bath for a further 5 minutes and finally immersed in the following: 70% ethanol (2 minutes), 90% ethanol (2 minutes), 100% ethanol (5 minutes) and xylene (twice for 5 minutes). The final immersion is crucial to obtain a clear slide. A small drop of distrene plasticizerxylene (BDH Chemicals) was placed onto each tissue section and the slide mounted with a cover slip with care so as not to introduce any air bubbles. The slide was allowed to dry before viewing under light microscopy.

2.4.2 Perls prussian blue staining for haemosiderin

This stains haemosiderin deposits in tissues blue. Perls potassium ferricyanide (BDH Chemicals, Poole, U.K.) approximately 4 g was dissolved in 25 mls of distilled water until a pale yellow coloured solution was obtained followed by 25 mls of 2 % concentrated hydrochloric acid in a Caplan jar (Solution A). Then tissue sections were taken from 100 % ethanol (in a series of decreasing concentrations of ethanol) to water. Tissue sections were incubated in solution A in a Caplan jar for 10 minutes. Solution A

was removed by washing in distilled water and tissue sections were counterstained with 1 % neutral red. Tissue sections were quickly dehydrated in increasing concentrations of ethanol (70 %, 90 %, 100 %) and the slide mounted in DPX as before.

2.5 *Appendix*

Section 1: Crypt-villus separation

Chelation buffer for crypt/tip separation (pH 7.3)

27mM trisodium citrate; 5mM Na_2HPO_4; 96mM NaCl; 8mM KH_2PO_4; 1.5mM KCl; 0.5 mM dithiothreitol (DTT); 55 mM D-sorbitol and 44mM sucrose.

Section 2: Ferritin sequences

L-ferritin sequence

5'- GCT-GAA-GAT-GAG-TGG-GGT-AAA-ACC-CCA-GAC-3'

This sequence produced a hybridisation pattern in the predicted position in human and rat tissues

H-ferritin sequence

Sequence 1: 5'-GAC-TGT-GAT-GAC-TGG-GAG-AGC-GGG-CTG-3'

No hybridisation signal was obtained from this sequence

Sequence 2: 5'-AAG-AAC-TTT-GCC-AAA-TAC-TTT-CTT-CAC-CAA-3'

Sequence 3: 5'-GCA-TGT-TGG-GGT-TTC-CTT-3'

Both sequences produced hybridisation signals on the 28S rRNA

Solution 1

50 mM glucose, 25 mM Tris.Cl (pH 8.0), 1% SDS, 10 mM EDTA (pH 8.0)

Solution 1 was made in batches of 100 mls and autoclaved.

Solution 2

0.2N NaOH (diluted from 10N NaOH)

Solution 2 was prepared fresh each time before use.

Solution 3

5M Potassium Acetate	*60 mls*
Glacial Acetic acid	*11.5 mls*
Water	*28.5 mls*

The resultant is 3 M with respect to potassium and 5 M with respect to acetate. Solution 3 was not autoclaved

TE Buffer

10 mM Tris Cl (pH 8.0), 1 mM EDTA (pH 8.0)

TE Buffer was also autoclaved.

STE buffer

0.1 M NaCl, 10 mM Tris.Cl (pH 8.0), 1 mM EDTA (pH 8.0)

This buffer was also autoclaved.

DNA minigel

For a 1.2 % agarose gel, 0.5 g of agarose in 5 ml 5x TBE buffer and 35 mls of DEPC water was brought to boil and then cooled for 1 hour to allow the gel to set.

5 x TBE buffer

The running buffer is 5 x TBE buffer prepared by dissolving 54 g of Tris base, 27.5 g of boric acid and 20 ml of 0.5M ETD (pH 8.0) in 1 litre of DEPC water. This buffer was autoclaved.The working solution is 0.5 x TBE.

Sample buffer

The sample buffer consists of equal volumes of 0.25 % bromoblue, 0.25 % xylene cyanol, 30 % glycerol and ethidium bromide.

Agarose gel electrophoresis

Gel electrophoresis was under taken at 100 volts in 0.5 x TBE buffer for 1-2 hours or until the markers have migrated to three-quarters the distance down the gel.

Section 3: Molecular biochemistry

RNA Agarose gel

Large 1 % agarose gel	*Small 1 % agarose gel*
1.5 g agarose	*0.25 g agarose*
13.5 mls 5 x MOPS	*2.5 mls 5 x MOPS*
110 mls 0.1 % DEPC water	*20 mls DEPC water*
21.5 mls formaldehyde	*4 mls formaldehyde*

Protocol for making gel

Agarose was dissolved in 0.1 % DEPC water and 5 x MOPS in a water-bath and the mixture cooled to 50 °C followed by the addition of formaldehyde in a fume cupboard. The gel was then allowed to set. This takes approximately 45 minutes for a mini gel and 2 hours for a large gel.

RNA Running buffer (5 X MOPS) pH 7.0

(3-N-morpholino propanesulfonic acid)

20.6 g of 3-N-morpholinopropanesulfonic acid was dissolved in 800 mls 0.1 % DEPC water containing 3.28 g of sodium acetate (40 mM). The pH was adjusted to 7.0 with 2M sodium hydroxide. Then, 1.86 g of EDTA in 10 mls of DEPC water pH 8.0 (0.5M) was

added and the final volume adjusted to 1 litre. This buffer should not be autoclaved and has to be protected from light. The working solution is 1 x MOPS.

Denaturing solution (DS)	***Loading Buffer***
0.5 mls deionised formamide	*1 ml 50 % glycerol*
162 µl formaldehyde	*12 µl 5 % bromophenol 0.05 g/ml*
100 µl 5 X MOPS	*2 µl (10 µg/µl) ethidium bromide*
	7 µl 1M NaOH

Blotting Buffer (20 x SSC)

175.3 g of sodium chloride and 76.2 g sodium citrate was dissolved in 1 litre of 0.1 % DEPC water and then autoclaved. Working solution is 10 x SSC.

Prehybridisation and hybridisation buffers for oligonucleotide probes

Pre-hybridisation buffer	***Hybridisation buffer***
6 mls 20 x SSPE	*6 mls 20 x SSPE*
0.2 mls 10 % SDS	*0.2 mls 10 % SDS*
0.2 mls tRNA (10 mg/ml)	*0.2 mls tRNA (10 mg/ml)*
1 ml 100 x Denhardts	*1ml 100 x Denhardts*
12.6 mls 0.1 % DEPC water	*2.6 mls 0.1 % DEPC water*

100 x Denhardts solution	***10 x SSPE***
2 g Bovine serum albumin	*175.5 g NaCl*
2 g Ficoll	*27.6 g $NaH_2PO_4.2H_2O$*
2 g Polyvinylpyrrolidone	*7.4 g EDTA*

Denhardts solution was filter sterilised using a size 0.4 mm pore filter and then stored in aliquots of 1 ml at -20 °C.

3 Examination of transferrin receptor expression in rat

3.1 Introduction

Three proteins, namely TfN the major iron transport protein in plasma, TfR on cell surfaces and ferritin, the main intracellular iron storage protein are essential in regulating the availability of iron for cellular requirements (Bomford & Munro 1985). These proteins are expressed by most cells in the body and are co-ordinately regulated with a view to ensure sufficient iron delivery and availability for cellular requirements whilst at the same time avoiding delivery of excessive iron and preventing toxicity (section 1.3.7). Previous studies have reported and confirm a relationship between body iron stores and the regulation of iron absorption in the proximal intestine (section 1.3.2 iv). However, it is not yet clear whether these three proteins play a role in regulating iron supply to the body in response to demand.

Human TfN is a glycoprotein (molecular weight 80 KD) with two iron binding sites (Fletcher & Huehns 1968). The liver is the major site of TfN synthesis (Aisen 1984) but is not the exclusive site because other cells such as lymphocytes (Morgan & Peters 1971), Sertoli, (Skinner *et al* 1984) muscle and brain cells (Levine & Seligman 1984) are also known to synthesise transferrin. However TfN is not synthesised in the intestinal cell (Pietrangelo *et al* 1992). A role for TfN in iron absorption involving secretion of apotransferrin into the gut lumen to bind luminal iron has been postulated (Huebers *et al* 1983) with TfN iron absorbed onto mucosal cell via TfR. To avoid depletion, excess apotransferrin was thought to recycle via the enterohepatic circulation. However, the inability to demonstrate TfR on the luminal brush border discounted this hypothesis.

Living cells require iron for growth and iron is delivered to cells by TfN interacting with specific receptors (TfR) that reside on cell surfaces (Aisen 1980). Like TfN, the TfR gene ia located on chromosome 3. To utilise TfN iron, cells must express this receptor. TfR is found on most cells in the body especially on cells that have a high requirement for iron such as immature erythroid cells and some malignant cells. It is a

glycoprotein with two identical subunits, 95 KD each (Schneider *et al* 1982). Besides being embedded on cell surface membranes, TfR are also found in endocytic vesicles or endosomes (Dautry-Varsat *et al* 1983). Within intestinal epithelial cells, these receptors are most prominent on the basal, lateral and intercellular membranes (Banerjee *et al* 1986, Anderson *et al* 1990) where the most likely function is to supply iron to developing cells. Cell cultures have demonstrated a direct relationship between iron requirement and cellular TfR expression (Rouault *et al* 1985) and immunohistochemical studies have revealed greater receptors density in proliferating crypts cells compared to villus cells in the small intestine (Anderson *et al* 1990, Levine & Woods 1990). In addition, there appears to be a close relationship between TfR expression and cell division/migration in the intestine of the neonatal rat (Anderson *et al* 1994). Consistent with this observation is the inverse relationship between TfR receptor expression in the small intestine in relation to body iron stores (Anderson *et al* 1990).

Despite this growing knowledge on receptor distribution and regulation, there is relatively little quantitative data on receptor expression *invivo* especially on the response to changes in iron stores on regulatory adaptation at the mucosal level.

Physiological studies on iron absorption in animals revealed that iron absorption does not increase or decrease for some 3-5 days following artificially induced acute anaemia or iron overload (Bannerman *et al* 1962, Crosby *et al* 1963, Wheby *et al* 1964a). It has been suggested that cells in the crypts have been "conditioned" in accordance to body iron stores where they may later influence iron uptake as they migrate the villus architecture some 3-5 days later (Bannerman *et al* 1962, Conrad & Crosby 1963). An obvious candidate to mediate this mechanism was ferritin (Hahn *et al* 1943, Granick 1946) but subsequent demonstration of ferritin synthesis in the presence of iron deficiency (Britten & Raval 1980) discounted this hypothesis. However, recent insights into the molecular regulation of ferritin allow for this observation and ferritin as a mediator merits further consideration. It has also been hypothesised that TfR at the

basolateral surface of enterocytes may also serve to 'inform' enterocytes regarding body iron stores (Fairweather-Tait & Wright 1984, Lombard *et al* 1997).

3.2 Plan of investigations

In the present study, I have adapted and utilised a method of segregating intestinal epithelial cells to provide quantitative data on some characteristics of TfR under different dietary iron conditions. By this, I hoped to determine whether TfR behaves in a way which would allow it to influence the control of mucosal iron uptake by informing the enterocytes in the crypts and villus regarding body iron stores. To determine whether TfR influenced the ability of both crypt and villus cells to respond to changes in iron store, it was important to separate the cell types.

TfR protein and TfR mRNA expression in the proximal small intestine was examined in the four groups of animals; iron replete, iron deficient, anaemic and iron loaded animals. The iron replete group served as the control, carbonyl iron group as iron overload, iron deficient and anaemic groups served as depleted iron stores. Two additional studies were undertaken. After the iron loaded animals had received the carbonyl iron supplemented diet for 5 weeks (steady state conditioning), these animals were switched to an iron deficient diet and immunohistochemical studies of mucosal TfR receptors examined at day 0, 1, 3 and 5 after the switch. Similarly, in the iron deficient group which had received the iron deficient diet for 5 weeks, these animals were switched to the carbonyl iron diet and studied at day 0, 1, 3 and 5 after the switch. The 'switch diet' model/conditioning was adopted to see if the change in TfR expression occurred differentially in a 'crypt-villus' cell fashion.

This chapter has two experimental sections. Section 3-1 examined TfR mRNA expression in crypt and villus cells of the rat intestinal mucosa after iron stores were manipulated by dietary means. Crypt-villus cell separation was undertaken by adapting a

previously described method (section 2.1.2). Section 3-2 examined TfR protein expression in the proximal intestine using a monoclonal antibody to rat TfR.

3.3 Quantitative distribution of transferrin receptor mRNA along the crypt-villus axis of the rat small intestine: Relationship to body iron stores and response to dietary alterations

3.3.1 Materials and methods

(i) Animals and diets

Male Wistar rats (Charles Rivers, Ramsgate) were used for all experiments. The experiments were conducted in accordance with Animals Scientific Procedures Act 1986 code of practice for the care and use of animals at the University of Liverpool. Animals received diets from the time of weaning and were housed individually in plastic bottom cages and kept at 22 ± 2 ^{0}C in a room with a 12 hour light-dark cycle (0900-2100 hrs). Diet was provided in excess of normal intake (50 g) and deionised water was provided ad libitum with iron removed by dialysis with conalbumin. Food intake and general condition were checked daily. Animals were studied in four groups, six animals per group.

(a) Control group: (n=6, mean weight 338 ± 9.2 g) receiving a standard rodent pellet diet (Special diet services, Essex) for 5 weeks. The determined iron content of this diet was 110 mg/kg diet.

(b) Iron deficient group: (n=6, mean weight 267 ± 9.5 g) consisting of animals maintained on an iron deficient diet (iron content of diet was less than 10 mg/kg diet) for 5 weeks from the time of weaning.

(c) Anaemic group: (n=4, mean weight of 44.2 ± 1.6 g) These were rats that had just been weaned and the study undertaken immediately where the maternal animal had received the standard rodent diet.

(d) Iron-supplemented group: (n=6, mean weight 265 ± 12.6 g) received the standard rodent diet supplemented with 2.5 % wt/wt (dry) carbonyl iron for 5 weeks. This has been previously shown to produce hepatic parenchyma cell iron loading (Bacon *et al* 1983).

(ii) Intestinal crypt and villus separation

Intestinal crypt-tip separation were undertaken by the chelation/mechanical method detailed in section 2.1.2.(iii). Briefly, at the end of the dietary manipulation, animals were sacrificed by carbon dioxide inhalation and the proximal small intestine (25 cm) from the pyloric antrum was removed and flushed with iced HBSS containing 0.5mM DTT. Tissues were everted using a blunt-ended spinal needle by a 'sleeve-pull through' technique. In a series of incubations and inversions at 4 °C, various sediment were removed to obtain cell fractions designated Vw,V_1,V_2,V_3,C_1 and C_2 (mixture of villus and crypt cells). After separation, cells were obtained by centrifugation (Mistral 6L, Fission instruments) at 2000 rpm for 10 minutes at 4 °C. The pellet was resuspended in 0.5 mls chelation buffer and stored at -80 °C until RNA extraction or biochemical analysis were undertaken.

(iii) Characterisation of cell subpopulation

The purity and character of the cell fractions obtained by this method was assessed histologically, enzymatically for alkaline phophatase (villus cell) and by 3H-thymidine incorporation (crypt cell) detailed in section 2.1.2 iv).

(iv) RNA extraction and Northern hybridisation

- Northern hybridisation for TfR mRNA were undertaken from the segregated crypt and villus cells in the above four groups of animals. In addition, TfR was also studied in RNA extracted from cells segregated sequentially (Vw, V_1, V_2,V_3, C_1 and C_2) from the proximal small intestine.

- Total RNA was extracted from cells using acid guanidinium thiocynate-chloroform technique (Chomcynski & Saachi 1987) as detailed in section 2.3.1.

- A 30 base oligonucleotide sequence, antisense to $TfR^{301-331}$ (Kuhn *et al* 1984) was utilised where sequence verification to rat TfR was confirmed using Genbank database. Preliminary experiments have reveal this oligonucleotide sequence to hybridise to both human and rat TfR transcripts (Figure 2-9). A 32-base oligonucleotide sequence (5'-TTT-CTC-AGG-CTC-CCT-CTC-CGG-AAT-CGA-ACC-CT-3') previously shown to bind to rat 18S rRNA (Torczynski 1983) was used as a RNA control. The latter was chosen as a control because as far as it is known, 18S rRNA expression is unchanged between crypt and villus cells compared to 'standard' probes such as α-tubulin, actin or histone. Oligonucleotide sequences were labelled with ^{32}dCTP using a terminal transferase enzyme kit (Boheringer) with excess radioactivity removed using a Sephadex G-50 column as described in section 2.3.5 (iv).

- Prehybridisation, hybridisation and washing conditions were as detailed in section 2.3.5 (iv). After washing, membranes were exposed to Kodak autoradiographic film (Amersham) at -80 °C to generate an autoradiogram. Autoradiographs were quantified using densiometery. Results are expressed as a ratio of TfR mRNA to 18S rRNA.

- Perls potassiun ferricyanide and counter stain 1% neutral red (Williams *et al* 1962) to stain haemosiderin deposits in the small intestine and liver to access iron storage contend. This has been described in section 2.4.2.

- Haemoglobin determination was undertaken in 5 mls of blood with an Automated STKS Coulter Counter (Miami,Florida).

3.3.2 Results and commentary

(i) Body weight and food intake

Compared to control animals of similar age, body weight was significantly reduced in animals receiving the carbonyl iron (338 $\pm$ 9.2 g vs 265 $\pm$ 12.6 g $p<0.001$; Figure 3-1) but no significant difference in food intake (Figure 3.2). The effect of chronic iron treatment on body weight is variable. Some studies (Pietrangelo *et al* 1990) reported no change while others (Bacon *et al* 1983) reported a significant increase. This discrepancy may be ascribed to differences in experimental protocol, different strains of animals used, source of carbonyl iron and the age of animals used (Pietrangelo *et al* 1990).

The iron deficient group had significantly greater food intake compared to controls ($p<0.01$) but body weight was significantly reduced (338 $\pm$ 9.2 g vs 267 $\pm$ 9.5 g; $p<0.001$). This may be the effect of iron deficiency stimulating food intake. There was no difference in body weight between the iron deficient and chronic iron treatment group although food intake in the iron deficient group was significantly greater than the chronic iron treatment group ($p< 0.01$).

(ii) Iron stores in animals

Perls potassium ferricyanide staining was used to access iron loading. Rats receiving the carbonyl iron diet for 5 weeks had substantial haemosiderin deposits in the mucosa of the small intestine and liver (Figure 4-10a & 3-6a). However, no haemosiderin was observed in either the mucosa of the small intestine or liver in the iron deficient, anaemic and control animals.

The iron deficient group had significantly greater haemoglobin compared to the anaemic group (11.9 ± 0.3 g/dl vs 5.5 ± 0.02 g/dl, $p<0.001$) but significantly lower when compared to controls (11.9 ± 0.3 g/dl vs 13.0 ± 0.2 g/dl, $p<0.05$). Iron loaded animals had significantly greater haemoglobin compared to controls (15.0 ± 0.7 g/dl vs 13.0 ± 0.2 g/dl, $p<0.01$). Body weights and haemoglobin concentration in the different groups have been summarised in Table 3-1.

Table 3-1

Body weights and haemoglobin concentration of rats under different iron regimes (data n=6 mean ± SEM)

Group	Control	Carbonyl	Anaemic	Deficient
Haemoglobin (g/dl)	13.0 ± 9.2	**15.0 ± 0.7	***5.5 ± 0.2	*11.9 ± 0.2
Body weight (g)	338 ± 9.2	***265 ± 12.6	+ 44.2 ± 1.7	*** 267 ± 9.5

*p<0.05, **p<0.01, ***p<0.001 when compared to control

+: body weights were not compared as animals were of different age

Figure 3-1

Body weights of rats under different iron regimes (n=6 mean ± SEM)

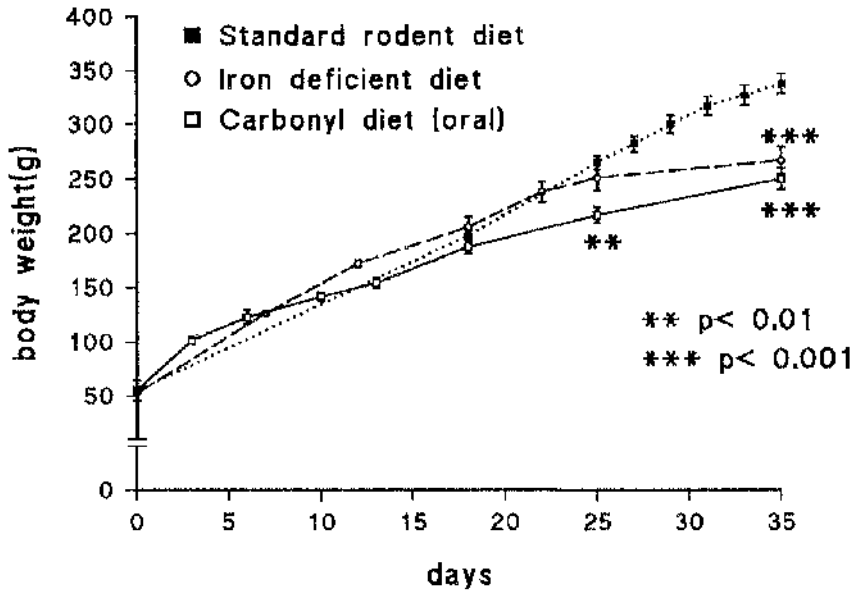
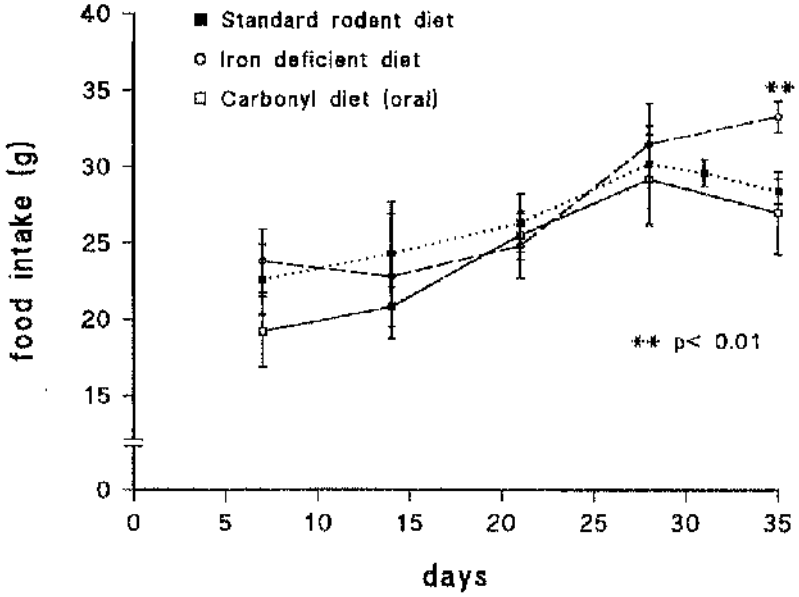


Figure 3-2

Food intake in rats under different iron regimes (n=6 mean ± SEM)



(iii) Northern hybridisation

RNA was extracted from the segregated cell populations and TfR mRNA expression examined using a 30 mer-oligonucleotide probe. Two studies were undertaken. TfR expression in various cell fractions (Vw, V_1, V_2,V_3, C_1 and C_2) were determined in animals maintained on a standard rodent diet. This revealed a progressive increase (three fold) in expression from villus to crypt cells (Figure 3a & b).

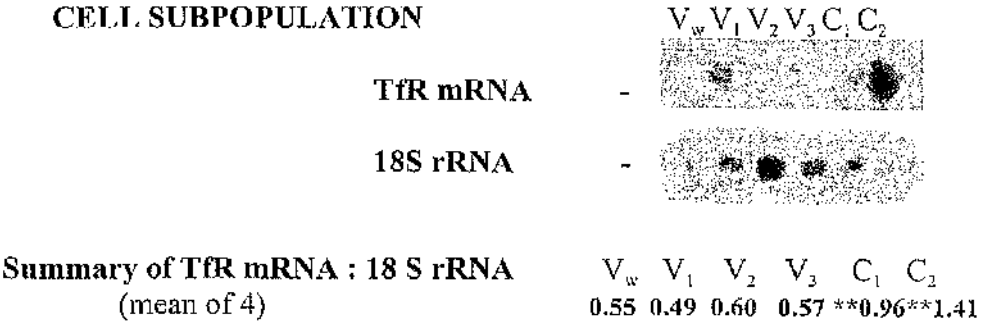
Secondly, TfR was also determined at different iron states in the intestinal mucosa. Autoradiograms for TfR mRNA in rats fed on a standard rodent diet, an anaemic group, an iron deficient diet and an iron loaded group are shown in Figure 3-4.

In anaemic animals a significant increase in TfR mRNA expression was observed in both crypt ($p<0.01$) and villus ($p<0.05$) cells when compared to control animals (Figure 3-5). However, this increase ($p<0.01$) was only observed in the villus cells in the iron deficient group. By contrast, there was a significant reduction in TfR mRNA expression in the iron loaded animals but this difference was confined to crypt cells ($p<0.01$).

(iv) Comment

It was not possible to study TfR mRNA expression in the 'switch diet' model as I had encountered difficulties in obtaining autoradiograms of reasonable quality for quantification by densitometery.

Figure 3.3(a) Representative autoradiogram for TfR



Summary of TfR mRNA : 18 S rRNA (mean of 4)	V_w	V_1	V_2	V_3	C_1	C_2
	0.55	**0.49**	**0.60**	**0.57**	****0.96**	****1.41**

Figure 3-3(b)

Sequential TfR mRNA expression in crypt and villus cells

Note the sequential increase in mRNA expression from villus to crypt cell

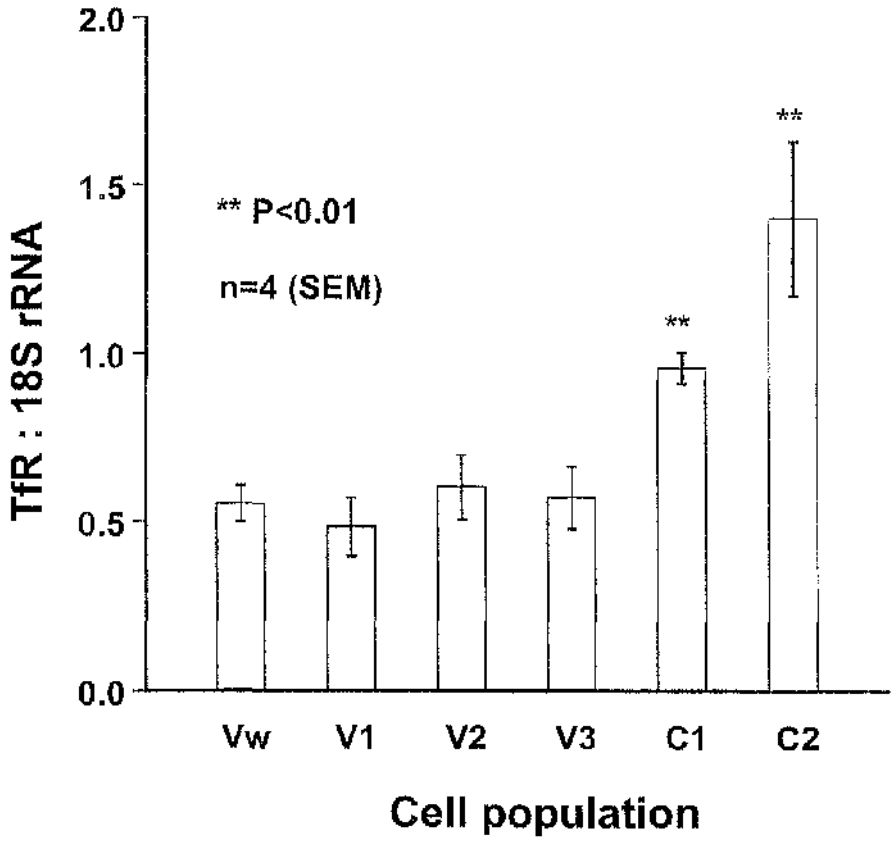
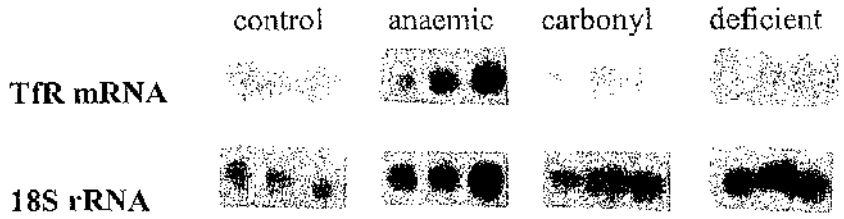


Figure 3-4

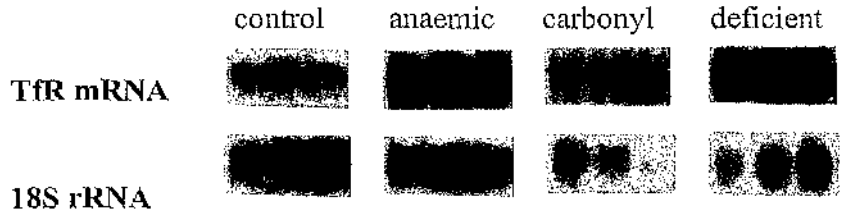
Autoradiogram of TfR mRNA in crypt and villus cells under different iron regimes

(data n= 3 ± SEM)

(a) Villus cell



(b) Crypt cell



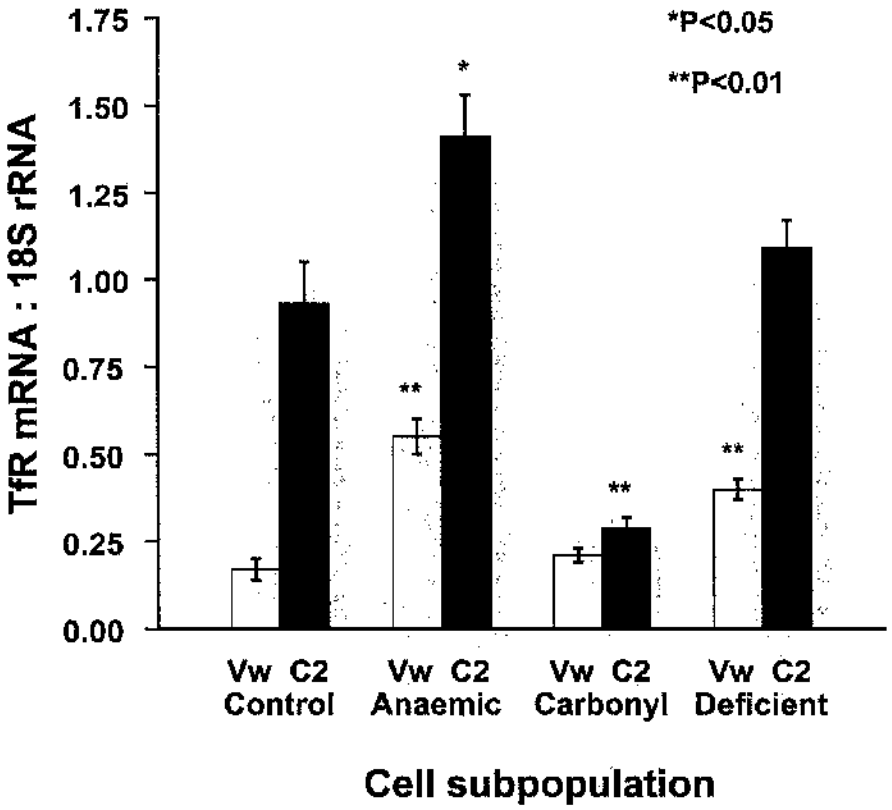
Summary of results

		Villus cell	Crypt cell
TfR mRNA: 18S rRNA (Mean of 6 ± SEM)	$V_{w\ control}$	**0.17 ± 0.03	**0.93 ± 0.12
	$V_{w\ anaemic}$	****0.55 ± 0.03**	***1.41 ± 0.12**
	$V_{w\ carbonyl}$	**0.21 ± 0.01	****0.29 ± 0.03**
	$V_{w\ deficient}$	***0.40 ± 0.03**	**1.09 ± 0.08**

*p<0.05, **p<0.01

Figure 3-5

TfR mRNA expression under different iron regimes (data n=6 ± SEM and statistical significance was as compared to control. Receptors were up-regulated in iron deficient state and down-regulated in iron replete state. Note that the gradient of expression between crypt and villus (crypt greater than villus) was retained throughout but was least prominent in the iron loaded state.



3.4 Immunohistochemical study of TfR along the crypt-villus axis of the rat small intestine: Relationship to alterations and response to body iron stores.

3.4.1 Methods

A murine monoclonal antibody to rat TfR (MRC OX-26) obtained from Serotec, Oxford was used in the following experiments. Immunohistochemical procedures were undertaken as described in section 2.4.1. Rat small intestinal tissues were mounted in OCT (BDH chemicals, Poole) and isopentane in liquid nitrogen. Isopentane was essential to preserve cell morphology during mounting. To ensure uniform and comparable conditions of staining, several cryostat tissue sections (6 μm) from all animals in each group were mounted in a grid fashion on glass slides. A subjective assessment of staining intensity for the receptor was made by two independent observers and graded from 0 (none), +1 (mild), +2 (moderate) to +3 (intense) staining.

3.4.2 Results

(i) Perls stain

(a) Carbonyl iron (5 weeks) to iron deficient diet and studied at day 0, 1, 3 and 5

In liver tissue, no difference in haemosiderin staining was observed until day 5 from the switch in iron deficient diet (Figure 3-6 a & b). In the small intestine however, haemosiderin deposits were absent by day 3 from the switch to the iron deficient diet (Table 3-2).

(b) Iron deficient diet (5 weeks) to carbonyl iron and studied at day 0, 1 ,3 and 5

In liver tissue, haemosiderin deposits were only present in liver tissues at day 5 on the carbonyl iron diet (Figure 3-6c). However, in the small intestine, haemosiderin remained absent at day 5 on the carbonyl iron diet. These observations have been summarised in Table 3-2.

Table 3-2

Perls staining in rat small intestine and liver under different iron regimes and changes in diet at day 0, 1, 3 and 5 (See text for discussion)

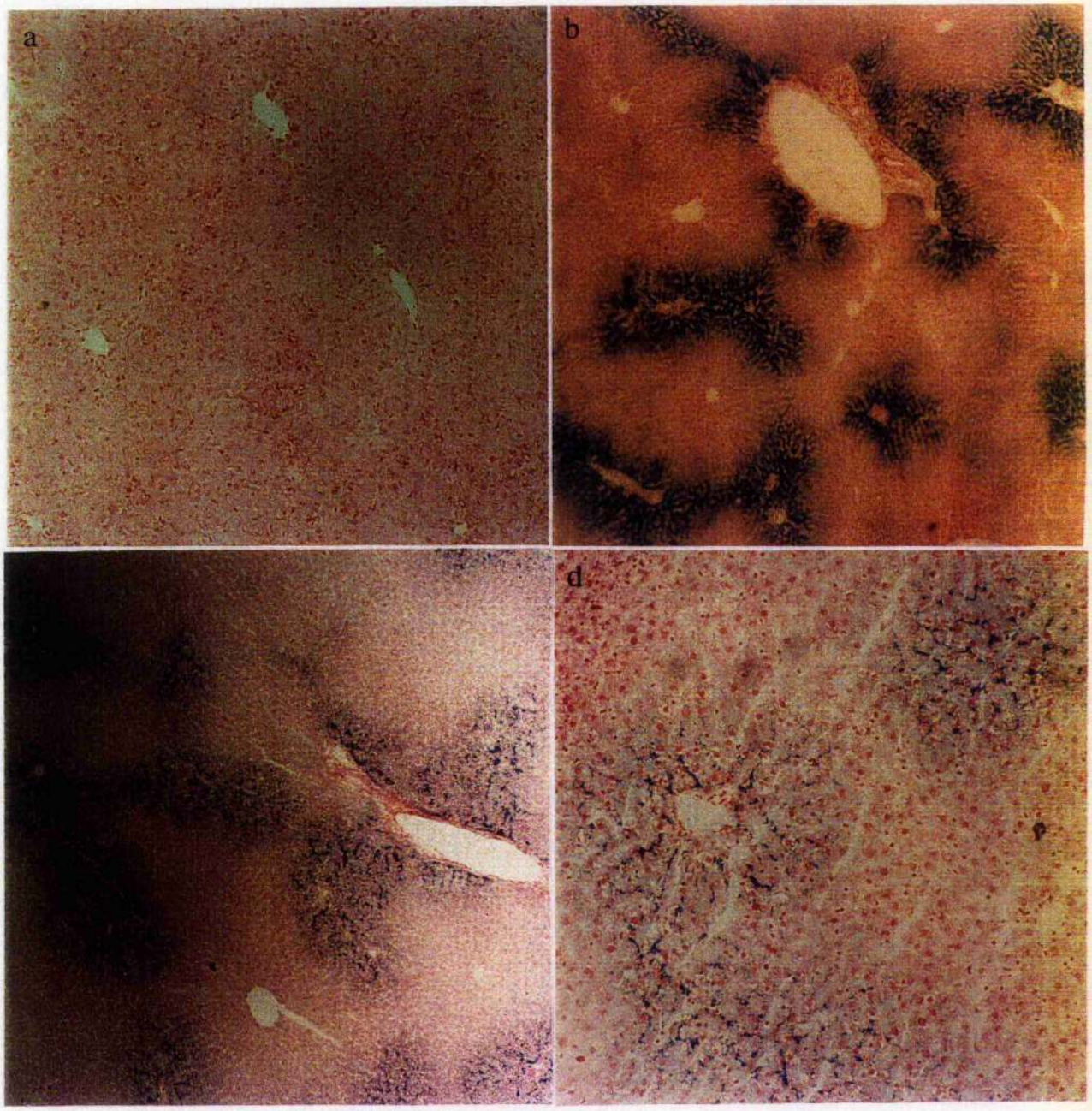
Days after switch	Control		Carbonyl		Anaemic		Deficient	
	liver	intestine	liver	intestine	liver	intestine	liver	intestine
0	-	-	+++	+	-	-	-	-
1	not assessed		+++	+	not assessed		-	-
3	not assessed		+++	-	not assessed		-	-
5	not assessed		++	-	not assessed		+	-

(+) degree of staining
(-) negative staining

Figure 3-6

Liver stained for haemosiderin

(a) On standard rodent diet (top left); (b) Carbonyl iron for 5 weeks (top right); (c) b followed by an iron deficient diet at day 5 (note reduced haemosiderin deposits; bottom left); (d) Iron deficient diet for 5 weeks followed by carbonyl iron at day 5 (bottom right)



(ii) Immunohistochemistry for TfR

TfR expression in crypt and villus cells after dietary iron manipulation have been summarised in Table 3-3.

Table 3-3

TfR expression in rat crypt and villus cells under different iron regimes and changes in diet at day 0, 1, 3 and 5 (see text for discussion)

Days after switch	Control		Carbonyl		Anaemic		Deficient	
	crypt	tip	crypt	tip	crypt	tip	crypt	tip
0	++	+	+	-	+++	++	+++	++
1	not assessed		+	-	not assessed		+++	++
3	not assessed		++	+	not assessed		++	+
5	not assessed		++	+	not assessed		++	-

(+) degree of staining

(-) negative staining

(a) Control, iron deficient, anaemic and iron loaded animals

Within the villus epithelium of control animals, TfR are only confined to the basolateral surface of enterocytes, principally in the crypts (+2) with reduced receptor expression (+1) up the villus architecture (Figure 3-7a). However, this crypt-villus gradient of receptor expression is no longer apparent in both iron deficient and anaemic group, with more intense staining observed in crypt (+3) and villus cells (+2) (Figure 3-7b & 3-7c). In the iron loaded animals, TfR were only confined to crypt cells (+1) and were virtually absent in villus cells (0) (Figure 3-7d)

(b) Iron loaded animals switched to an iron deficient diet

Receptor staining was only confined to the crypt cells (+1) but when these animals were switched to an iron deficient diet, TfR staining observed in the basal villus architecture by 24 hours (+1) and by day 3 on the iron deficient diet, TfR staining was observed up to the villus tip (+1) with decreasing receptor gradient from crypt to villus still apparent (Figure 3-8a-c).

(c) Iron deficient animals switched to an iron loaded diet

In iron deficient animals, TfR staining was observed to the villus tip (+2) but, by day 3, down regulation to mid to basal villus could be observed (Figure 3.9b). By day 5 from the switch to an iron loaded diet, receptor staining was mainly confined to crypt cells (+1) and absent in villus cells (0) (Figure 3.9c).

Figure 3-7

(a) TfR expression in control rats (top left; **(b) TfR expression in anaemic rats** (top right); **(c) TfR expression in rats on an iron deficient diet** (bottom left) **and (d) TfR expression in rats on a carbonyl iron diet** (bottom right). See text for discussion.

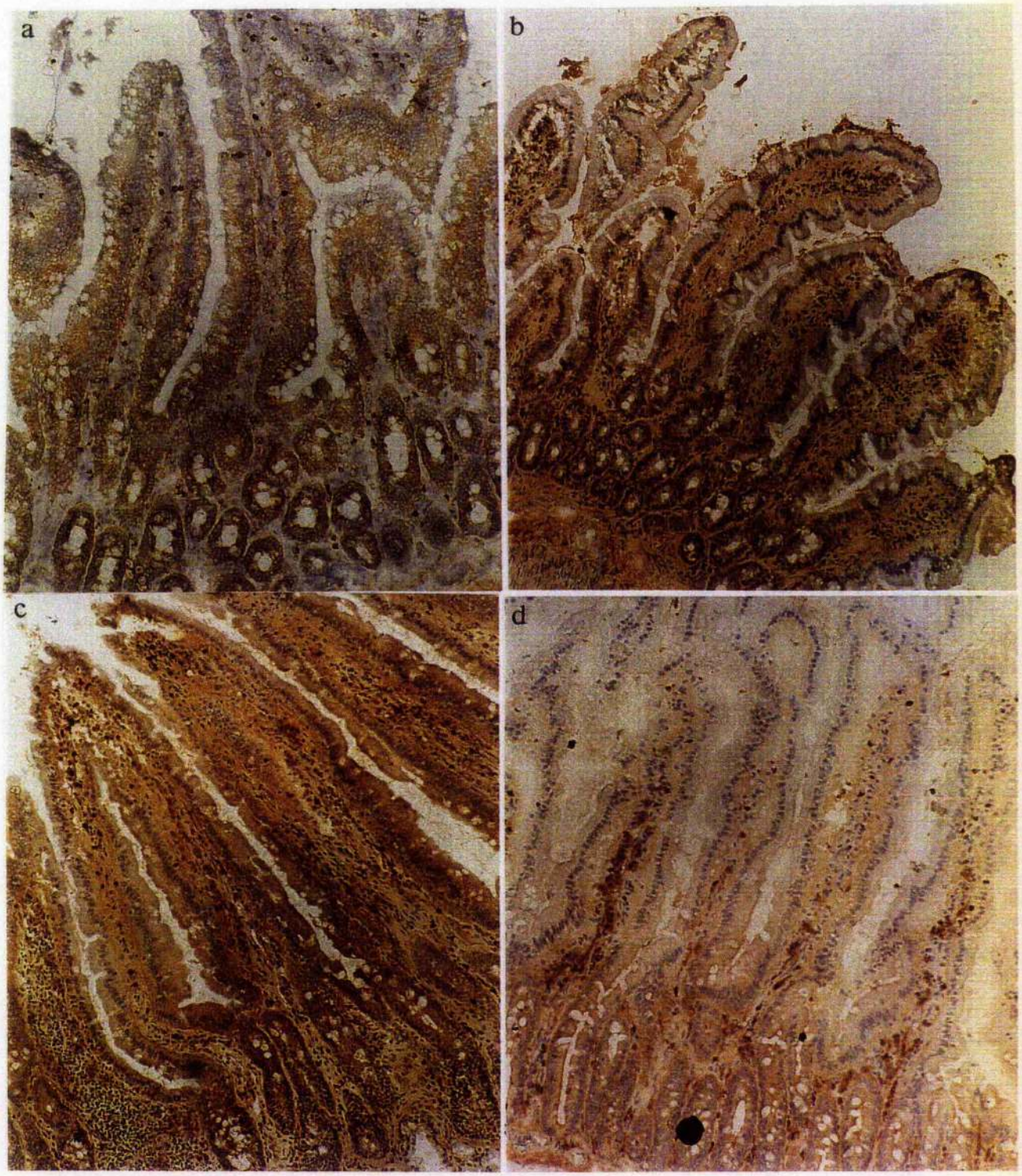


Figure 3-8 (a-c)

TfR expression from a carbonyl iron to an iron deficient diet at day 0 (left), **1** (middle) **and 3** (right). See text for discussion.

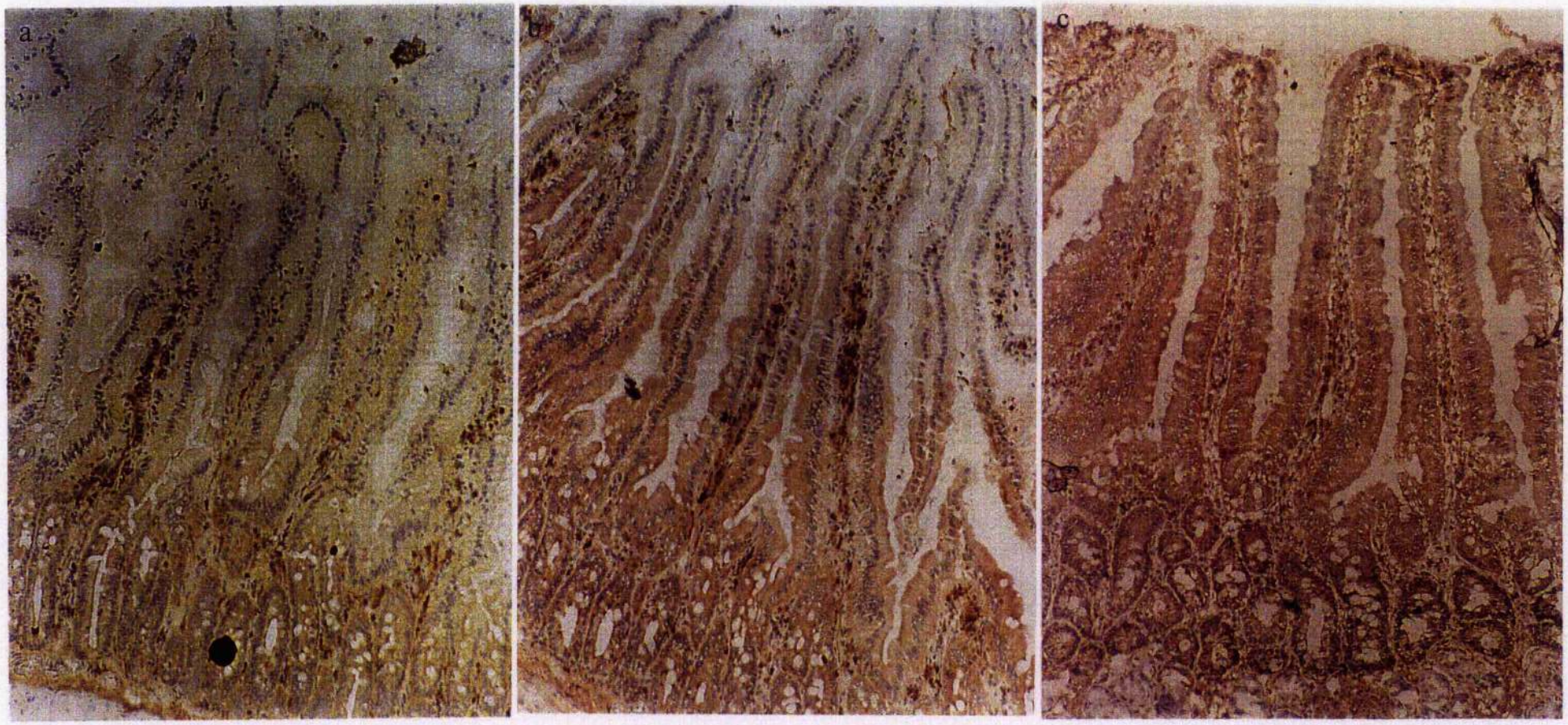
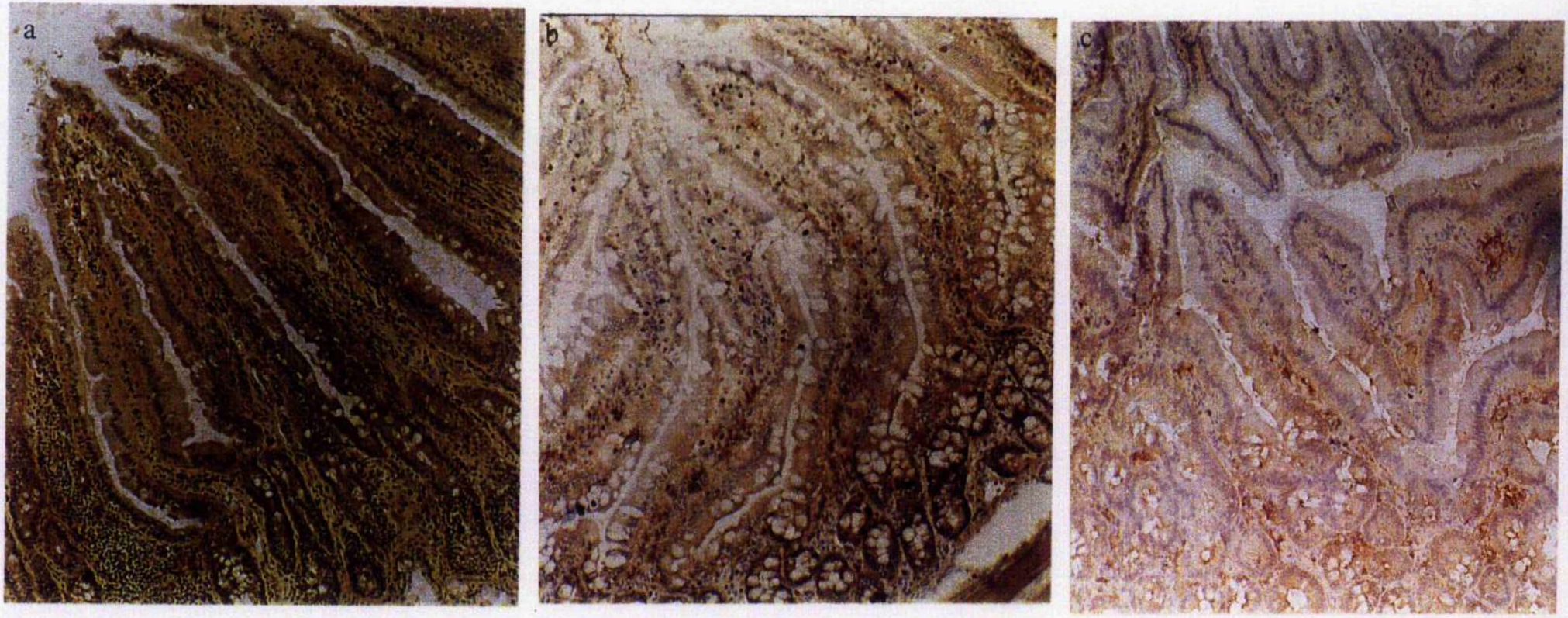


Figure 3-9 (a - c)

TfR expression from an iron deficient diet to a carbonyl iron diet and studied at day 0 (left), **3** (middle) **and 5** (right). See text for discussion.



3.4.3 Discussion

Polypeptide receptors can be classified into two categories on the basis of function (Kaplan 1981). Class I receptors, e.g. β-agonists function mainly to transmit information whereas class II receptors, e.g. TfR requires internalisation of ligand to provide the cell with a certain required factor. TfR is regulated by a complex mechanism involving several factors such as haem, protoporphyrin and certain growth factors which modulate receptor synthesis (Testa 1985). However, it is generally believed that expression of TfR is directly correlated with the rate of cell growth (Trowbridge & Omary 1981, Pelosi-Testa *et al* 1986) and inversely related to the amount of iron accumulated in cells (Ward *et al* 1982, Louache *et al* 1984, Pelosi *et al* 1986, Testa *et al* 1985). The correlation with cell growth may be partly explained by the absolute requirement for iron by ribonucleotide reductase; a key enzyme in DNA synthesis (Thelander & Graslund 1983, Kucera *et al* 1983).

The amount of iron entering the cell is dependant on receptor population which can be modified by several mechanisms; a short-term but rapid change via translocation of receptors between the cell surface and the endocytic pool; and secondly, a longer but slower change which involves changes in receptor synthesis and breakdown (Bomford & Munro 1985). This has been reported in Hela cells (Ward *et al* 1982), K 562 cells (Mattia *et al* 1984) and haematopoietic cell lines (Louache *et al* 1984).

Within the small intestine, due to its localisation on the basolateral surface (Banerjee *et al* 1986), TfR seemed to be the ideal candidate for the final pathway by which iron was transferred to the portal circulation. However, current evidence do not support this role. It is well recognised that in the iron deficient state, both mucosal uptake of iron and TfR expression are increased whereas in the iron replete state, mucosal uptake and TfR expression are reduced. However, when TfR density was measured in rats from the neonatal period and up to 50 days old, receptor density was found to be greatest in crypt cells at all ages, whereas TfR at the villus tip declined after birth in conjunction

with increased iron absorption but increased again at the time of weaning (Anderson *et al* 1991). Furthermore, administration of phenylhydrazine to rats (induces an acute haemolysis) resulted in enhanced mucosal iron absorption but not TfR expression (Anderson *et al* 1990). Expression of TfR was interpreted in these studies as signifying increased cell proliferation and therefore increased iron requirement rather than TfR having a role in iron absorption. Perhaps more importantly, for iron transfer to take place from enterocyte to portal blood via TfR, this would require a "reverse endocytosis" mechanism which is not feasible on kinetic grounds (Baker & Morgan 1994). The most likely role for TfR is to facilitate iron entry to mucosal cells from plasma by internalising plasma-derived diferric TfN (Pietrangelo *et al* 1992). To support this, TfN mRNA could not been detected in rat nor in human intestinal mucosa by Northern hybridisation (Idzerda *et al* 1986, Pietrangelo *et al* 1992) and mucosal TfN endocytosis has also been described in rats (Anderson *et al* 1994).

The present immunohistochemical data supports previous immunohistochemical studies (Banerjee *et al 1986* , Anderson *et al* 1990) on the inverse relationship between body iron stores and TfR expression. TfR were up regulated in anaemic and iron-deficient rats and down regulated during iron supplementation. Furthermore, by changing iron loaded animals to an iron deficient diet, increased TfR density in villus cells (initially confined to the basal portion of the villus architecture) was observed by day 1 from the switch in diet implying that mucosal TfR was also sensitive to changes in luminal iron as body iron stores were unlikely to be depleted in this time period. Conversely, TfR density was reduced (observed on day 5 from the change in diet), with expression virtually confined to crypt cells during the change from an iron deficient to an iron loaded diet. It would seem that TfR expression can be influenced by body iron status as well as changes in environmental (luminal) iron. The above system illustrates a fundamental physiological concept in that when cells become iron replete, TfR are down regulated to prevent further accumulation of iron but when cellular iron is low and the cells need more

iron, TfR are upregulated to permit accumulation of cellular iron. This system is vital to cells as iron is required for cell growth and the synthesis of haem containing compounds.

Using Northern hybridisation technique, I was able to demonstrate an increasing gradient of TfR mRNA expression in a 'villus to crypt cell manner' and confirmed the reciprocal relationship between TfR mRNA expression and body iron stores. Mucosal TfR mRNA were significantly lower in iron loaded animals compared to control animals but significantly higher when compared to the anaemic and iron deficient animals. TfR mRNA expression was also consistently higher in crypt than villus cells in the four groups of animals studied. This may be reflected in crypt cells having a higher requirement for iron due to their rapid cell turnover.

TfR mRNA expression has also been examined in the human intestinal mucosa and in mice, although crypt and villus cells were not segregated (Pietrangelo *et al* 1992, McKie *et al* 1996). These studies have also supported the inverse relationship between TfR mRNA and body iron stores. However, the ability of crypt and villus cells to respond by altering TfR transcripts to environmental iron remains to be determined.

The present study, together with previous *invivo* (Pietrangelo *et al* 1992, McKie *et al* 1996) and *invitro* studies on a variety of cell types such as fibroblasts (Ward *et al* 1982), leukaemic cell lines (Louache *et al* 1984), lymphocytes (Pelosi *et al* 1986) and hepatoma cells (Zucherman *et al* 1979) indicate that TfR expression is mediated by iron through a negative feedback mechanism. There is reduced receptor expression by the addition of iron and enhanced receptor synthesis by treatment with iron chelators. This negative feedback system is mediated by the regulation of the post-transcriptional mechanisms through IRP and ferritin (section 1.3.7). However, in conjunction with previous *invitro* studies (Mattia *et al* 1984, Louache *et al* 1984, Rao *et al* 1986), the present study also suggest that transcriptional control of TfR by iron may be important. Transcription appears to be maintained in crypt cells as well as the more mature villus cells coming towards the end of their life span.

Other mechanisms of TfR regulation may be present since in cultured human monocytes-macrophages (Testa *et al* 1989) and more recently, *insitu* hybridisation analysis using iron loaded rat small intestinal tissues (Jeffrey *et al* 1996), have revealed persistent TfR and TfR mRNA expression. This may be a protective mechanism against iron toxicity. Increased TfR may allow macrophages and mucosal intestinal cells to store large amounts of iron whose toxic effects is neutralised by simultaneous synthesis of ferritin such that iron retained in cells are lost when cells are shed from the intestinal epithelium.

Although TfR does not appear to have a direct role in mucosal iron uptake, the ability of both crypt and villus cells to respond to changes in body iron stores support the notion that TfR may subserve an indirect role by conveying information regarding iron stores to the enterocytes in the depths of the crypt such that when these cells mature and migrate to their functional positions at the villus tip (some 3 days later) mucosal cells may influence the handling of dietary iron. However, to translate this information into 'action' requires a second mediator of which the most promising is ferritin. Using the present techniques, it is now possible to explore this in detail (Chapter 4).

4. Examination of ferritin expression in rat liver and small intestine

4.1 Introduction

Iron in free solution is toxic to biological systems due to its ability to generate free radicals resulting in destruction of lipid membranes by oxidation (Bacon & Britton 1990). Chronic iron overload is also associated with organ injury. In liver, it leads to fibrosis, cirrhosis and hepatomas (McLaren *et al* 1983). The mechanism of liver injury also involves the generation of reactive hydroxyl radicals (Freeman 1982), perferryl and ferryl ions (Tien 1981) from the interaction of iron with oxygen. To limit iron toxicity, it is essential that the absorbed iron is complexed to a protein which is easily available in times of need and this role is fulfil by the iron storage protein ferritin.

Ferritin is the major iron storage protein in eukaryotic cells. The ferritin apoprotein shell (molecular weight approximately 480 kDa) is a heteropolymer with a 24 subunit structure which has a vast storage capacity for iron with more than 4000 atoms of iron per molecule (Harrison 1986, Harrison *et al* 1987). Two immunologically distinct sized subunits of ferritin have been described (Arosio *et al* 1978) which differ in molecular weight, share 55 % peptide sequence homology and appear to have similar folding (Jain *et al* 1985). These have been characterised as heavy (H) and light (L) chain ferritin. In cells, these are expressed in different proportions to generate families of isoferritins or heteropolymers which are L-rich (basic) or H-rich (acidic) ferritin (Harrison *et al* 1987). L rich-subunit ferritin (molecular weight 19 kDa) predominates in liver, spleen and placenta (Bomford *et al* 1981). H-rich subunits (molecular weight 21 kDa) predominate in tissues such as heart (Powell *et al* 1974 & 1975a & 1975b), pancreas, cancers and red blood cells (Arosio *et al* 1976 , Kohgo *et al* 1980, Peters *et al* 1983).

In principal, 25 ferritin isomers could be expected by the different assembly of the two chains: $L_{24}H_0$, $L_{23}H_1$, ..., L_0H_{24} and possibly many more if they occupy

non-equivalent positions on the molecule. However, it is unknown whether the distribution of H and L chains in the ferritin molecule is random or follows a particular pattern. There are metabolic differences in these isoferritins. This will be discussed in the next section as they may have important consequences on mucosal iron absorption.

The chromosomal location of ferritin genes has been examined in humans using cell fusion and *insitu* hybridisation. Cell fusion studies reveal ferritin genes at 2 chromosomal sites, L-ferritin subunit on chromosome 19 (Caskey *et al* 1983) and H-ferritin subunit on chromosome 11 (Cragg *et al* 1985). However, *insitu* hybridisation techniques have demonstrated the existence of H-type sequences on at least seven chromosomes (1,2,3,6,11,13, and X) and L-ferritin genes and related sequences on at least 3 chromosomes (19,20 and X) (McGill 1987).

On reflection of the hypothesis, I postulated that body iron stores (of which liver is the main storage organ) relays information regarding iron status through serum TfN and TfR located on the basolateral surface of enterocytes to 'condition' crypt cells such that it will set up a constitutive ratio that will be primarily L-rich subunit ferritin or H-rich subunit ferritin which will impede or facilitate the transfer of iron from mucosal cells to the portal circulation. I therefore set out (i) to examine whether ferritin transcripts in liver could be altered by diet and (ii) if so, whether dietary manipulation affected predominantly a specific ferritin subunit transcript.

4.2 Plan of investigations

Body iron stores were manipulated by dietary and parenteral iron. At the end of 5 weeks, the carbonyl group was switched to an iron deficient diet for 1, 3 and 5 days. The iron deficient group was switched to the carbonyl iron supplemented diet for similar periods. After four IP injections of iron sorbitol, the parenteral group, (initially maintained on a standard rodent diet) was switched to an iron deficient diet for 6 hours, 1

and 3 days. Animals were sacrificed and liver tissues obtained for RNA extraction and Perls staining. It was necessary to use the 'switch model' to determine if changes in luminal iron (intestinal iron) could affect ferritin expression in liver.

This chapter has been divided into 2 experimental sections. The first section (section 4.3) examined L and H-ferritin gene expression in liver after iron stores have been manipulated by oral and parenteral iron. The second section (section 4.4) examines ferritin expression in the small intestine along the crypt-villus axis when iron stores have been manipulated by similar means.

4.3 Effect of chronic dietary iron overload, iron deficiency and parenteral iron overload on ferritin mRNA expression in the rat liver

4.3.1 Methods and materials

(a) Animals and diets

Male Wistar rats (Charles Rivers, Ramsgate) were used for all experiments. Housing conditions were as previously described in section 3.3.1. Animals were divided into control, iron deficient and iron loaded groups with six animals per group as previously described and the data has been summarised in Table 3-1.

An additional group for these experiments were rats who were loaded with iron parenterally using ip injections of iron sorbitol (Astra Pharmaceuticals Limited, Langley, U.K), 25 mg per week for 4 weeks (mean weight 359 $\pm$ 11.9 g). This has been shown to produce higher mucosal ferritin associated with reduced mucosal iron absorption (Savin & Cook 1980). Animals could only tolerate injections when their body weight exceeded 200 g and were maintained on a standard rodent diet (Special diet services) while receiving weekly iron injections. On the fourth injection, these animals were switched to an iron deficient diet and studied at 6 hours day 1 and 3.

(b) RNA extraction and Northern hybridisation

Total RNA was extracted from liver tissues using the acid guanidinium thiocynate-chloroform protocol described in section 2.3 as was RNA transfer by capillary blotting and fixation to membranes.

(c) Probes and hybridisation conditions

Mouse L (578 bp) and H-ferritin cDNA (347 bp) were a generous gift from Dr. Andrew McKie, Kings College, University of London. Preliminary experiments on rat tissues (liver and small intestinal RNA) revealed the hybridisation pattern was in the predicted position below the signal of 18S RNA. (Figure 2-8a & 2-8b). In addition, sequence verification between mouse and rat ferritin was confirmed using Genbank database. An oligonucleotide sequence (30 mer) for rat 18S RNA (Torczynski *et al* 1983) was used an RNA control to normalise for RNA loading. Labelling (random priming for ferritin, end labelling for oligonucleotide), hybridisation and washing conditions were undertaken as described in section 2.3.5.

(d) Other methods

Blocks of rat liver were mounted in OCT in isopentane and 6 μm cryostat sections were prepared and stained with Perls Prussian blue stain for haemosiderin to assess storage iron content as described in section 2.4.

4.3.2 Results

(i) Assessment of hepatic iron loading

After 5 weeks on oral carbonyl iron, macroscopic inspection of livers from these animals appeared darker compared to control animals (Figure 4-1a). By contrast, livers of animals on the iron deficient diet appeared lighter (Figure 4-1b). There was no difference to controls in the animal liver receiving parenteral iron (Figure 4-1c)

Parenteral iron sorbitol resulted in hepatic iron overload with iron deposition evenly distributed throughout the hepatic lobule (both parenchyma and RE cells). By contrast, dietary iron overload produced iron deposition in parenchyma cells in a periportal distribution (Figure 4-2a and 4-2b).

There were no detectable differences in haemosiderin staining when diet of the parenteral group was changed to an iron deficient diet after 3 days. However, on changing from a carbonyl iron supplemented diet to an iron deficient diet, diminished haemosiderin staining was observed at day 5. Similarly, haemosidern deposits could be observed on changing animals initially on the iron deficient diet to the carbonyl iron diet at day 5. These findings have been summarised in Table 3-3 , section 3.4.2 (i).

Figure 4-1

Macroscopic appearance of livers under different iron regimes

(a) Carbonyl iron. Note that liver is much darker than control animals (top)

(b) Iron deficient diet. Note that liver is much lighter than control animals but after day 5 on the carbonyl diet, liver was darker than control animals (middle)

(c) Intraperitonal iron sorbitol. Note that there is no difference compared to control animals (bottom)

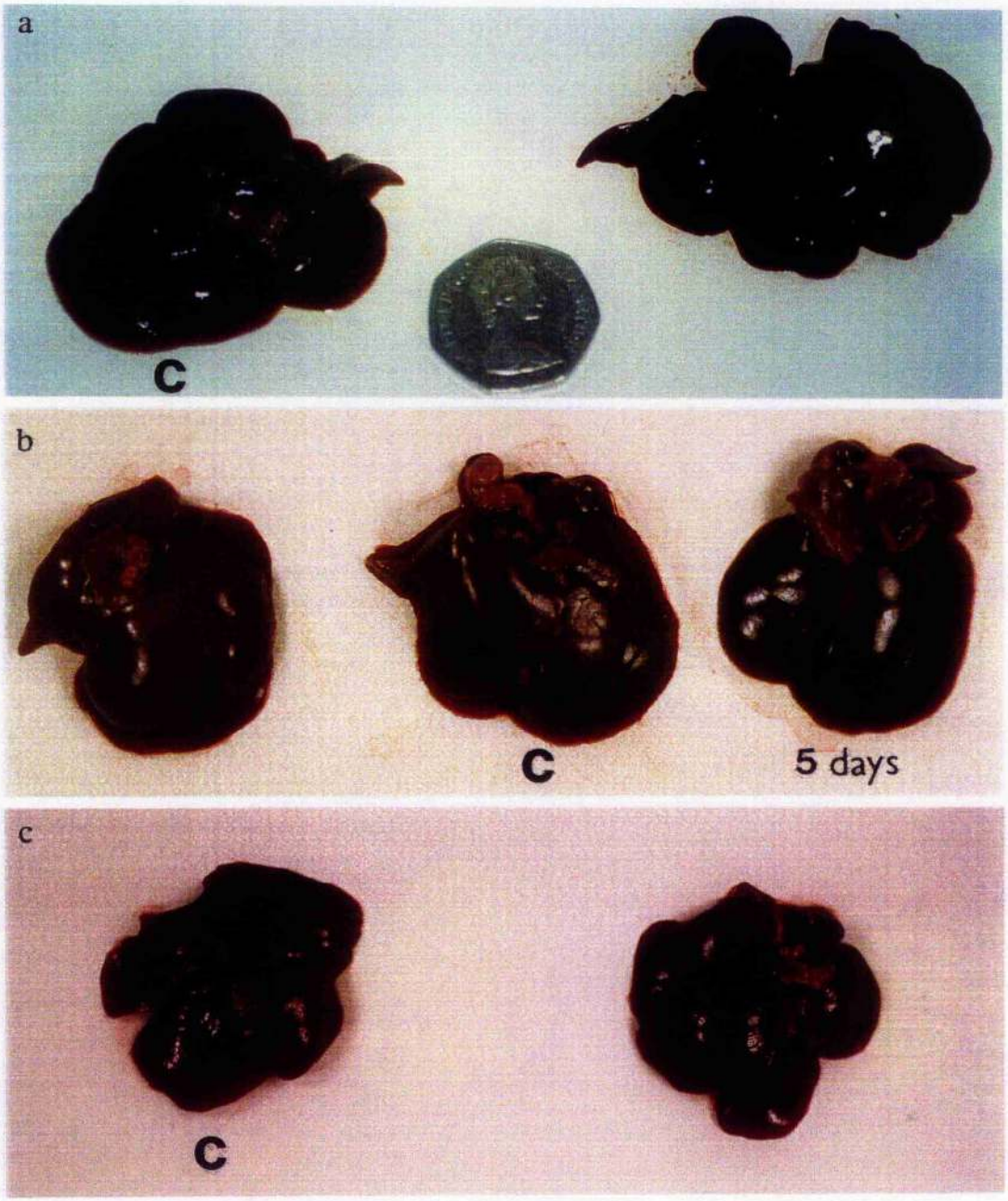
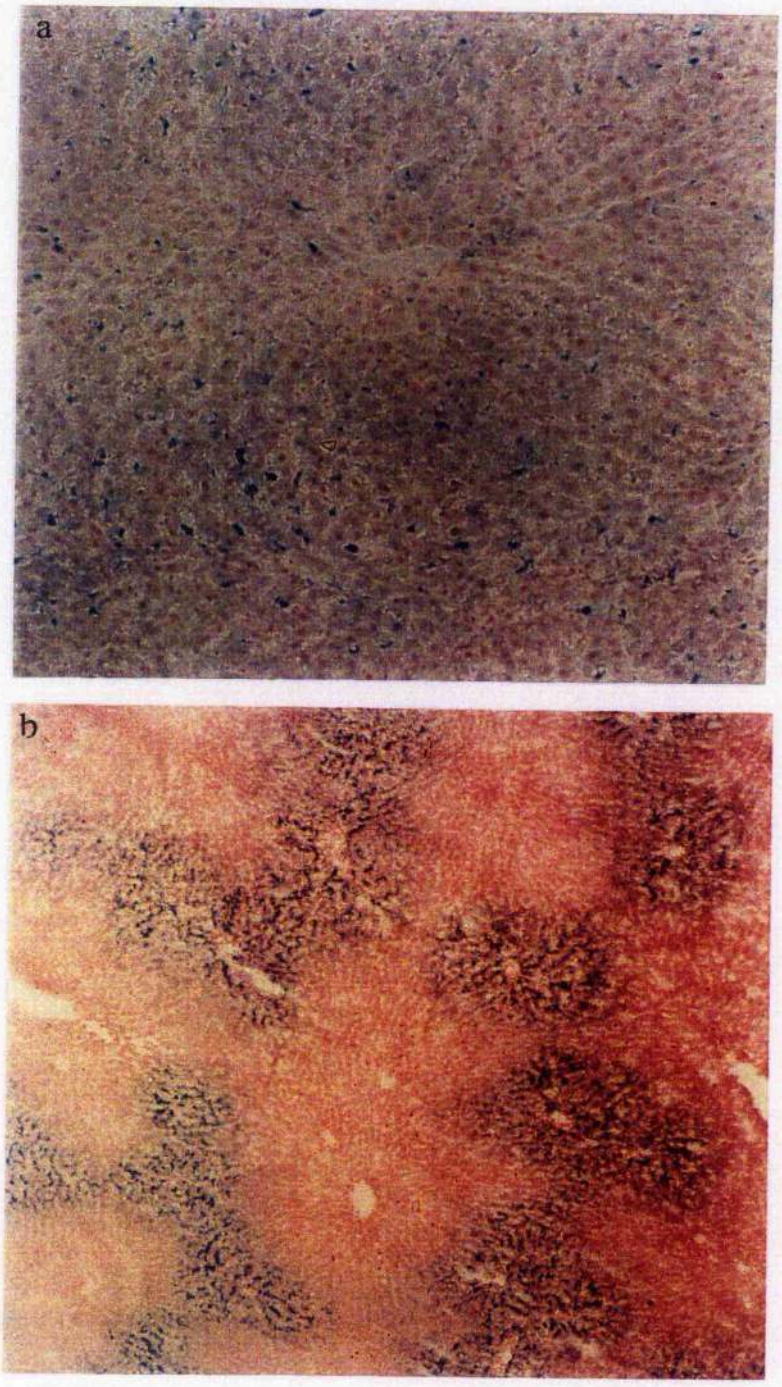


Figure 4-2

Perls stain in liver under different iron regimes

(a) In parenteral iron loading haemosiderin staining is situated throughout the hepatic lobule (top)

(b) Oral iron (carbonyl) loading result in staining in a periportal distribution (bottom)



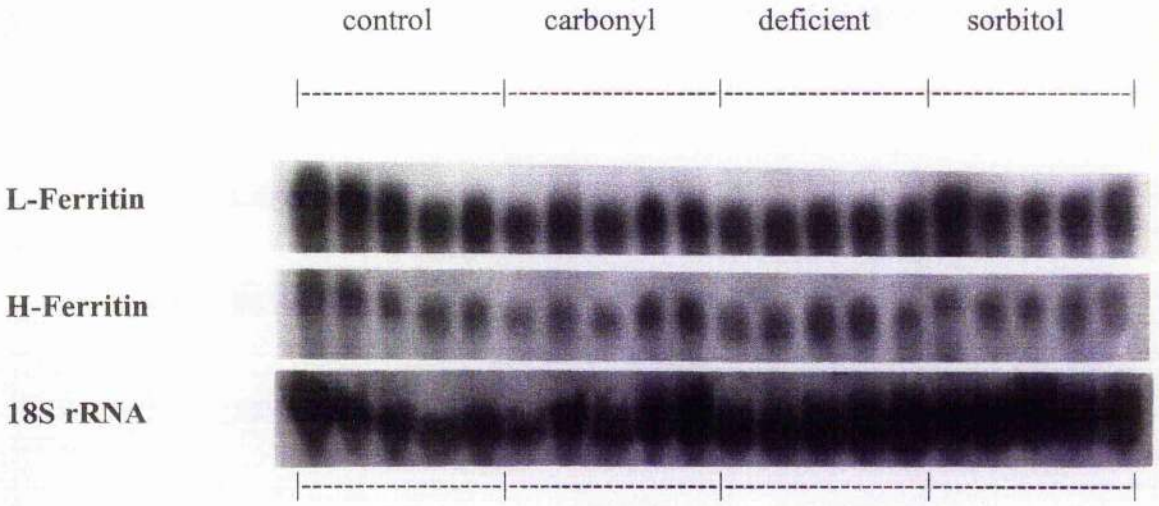
Steady state conditioning

(ii) Effect of iron loading and iron deficiency on hepatic ferritin expression

Five animals from each group were analysed. In both iron loaded groups (oral carbonyl and IP sorbitol), L and H-ferritin subunit mRNA tended to be greater than control animals although this was not statistically significant. Similarly, in the iron deficient group, both L and H-ferritin subunit mRNA tended to be lower compared to controls although this was not statistically significant (Figure 4-4 & Figure 4-5).

Figure 4-4

Autoradiogram of ferritin mRNA expression in liver under different iron regimes

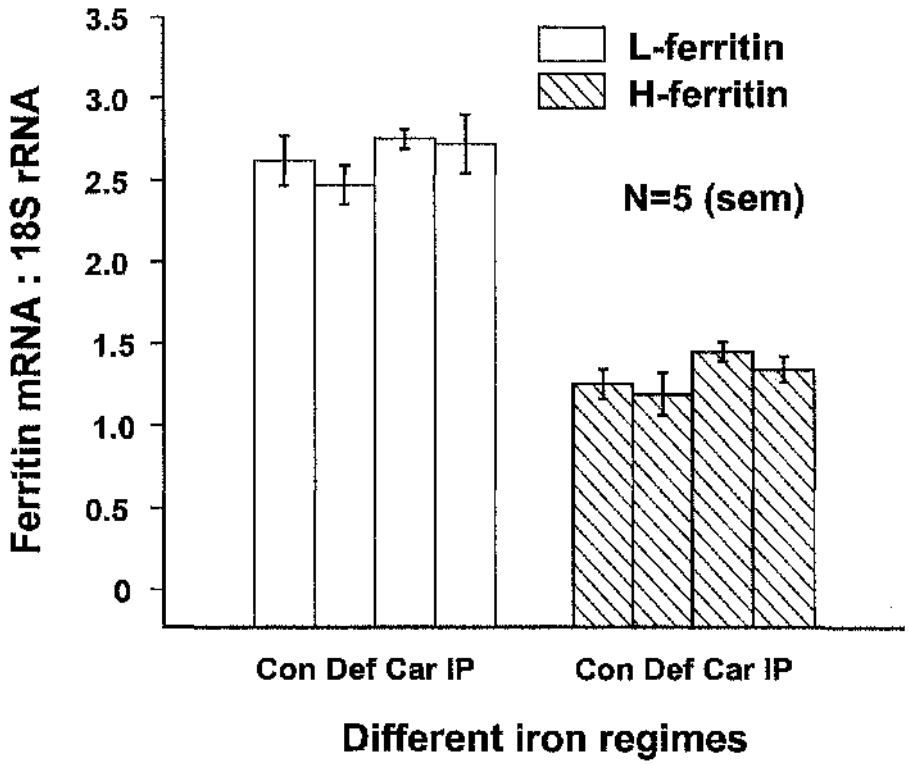


		L-ferritin	H-ferritin
Ferritin mRNA: 18S rRNA	**Control**	**2.62 ± 0.15**	**1.22 ± 0.09**
(Mean of 5 ± SEM)	**Carbonyl**	**2.75 ± 0.06**	**1.45 ± 0.06**
	Deficient	**2.47 ± 0.12**	**1.19 ± 0.13**
	Sorbitol	**2.72 ± 0.18**	**1.34 ± 0.08**

Figure 4-5

Ferritin mRNA expression in liver under different iron regimes

(data n=5 ± SEM) Con=Control, Car=Carbonyl iron, Def=Iron deficient diet, and IP=Intraperitoneal iron sorbitol.



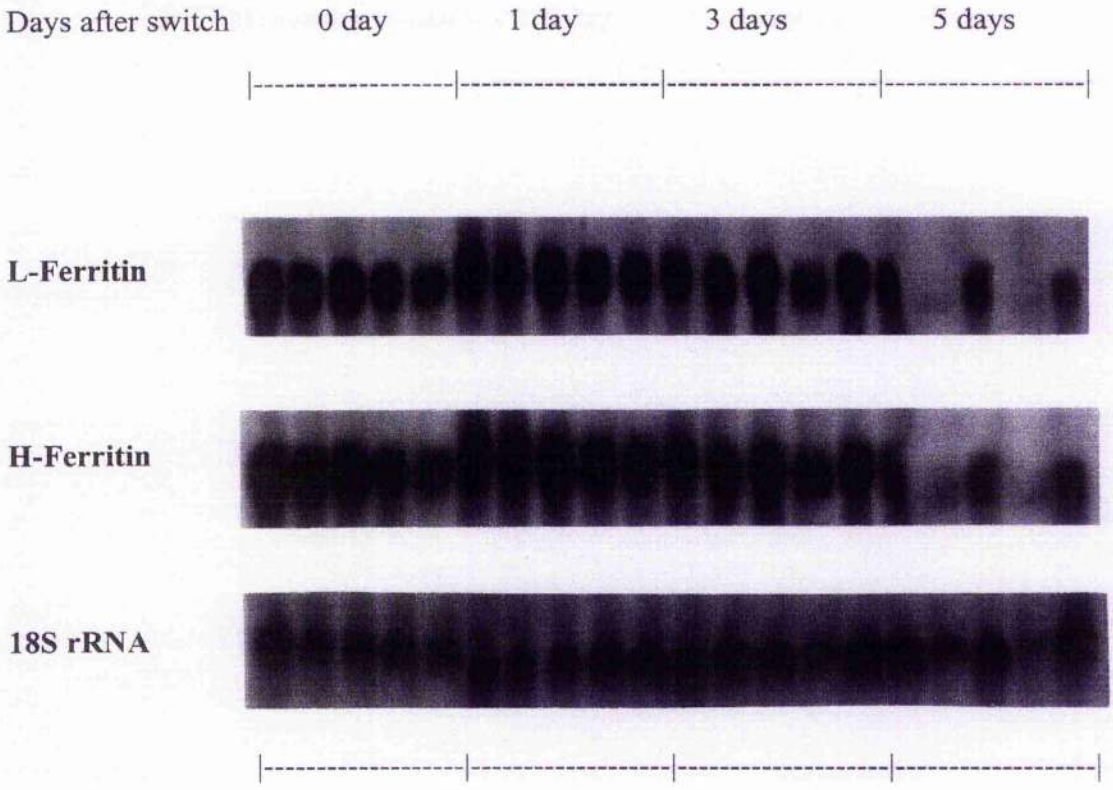
'Switch diet' conditioning

(iii) Effect of an iron loaded diet (carbonyl iron) for 5 weeks followed by an iron deficient diet at day 0, 1, 3 and 5 on hepatic ferritin expression

Within 24 hours of changing to an iron deficient diet, there was a significant reduction in L-ferritin subunit mRNA expression ($p<0.01$) maintained up to day 5 on this diet. This was also observed in H-ferritin subunit mRNA ($p<0.01$) (Figure 4-6 & Figure 4-7).

Figure 4-6

Autoradiogram showing effect of a carbonyl diet to an iron deficient diet at day 0, 1, 3 and 5 on hepatic ferritin expression



L-ferritin mRNA: 18S rRNA 2.63 ± 0.13 **1.20 ± 0.03 **1.26 ± 0.10 **0.68 ± 0.05

(mean of 5 ± SEM)

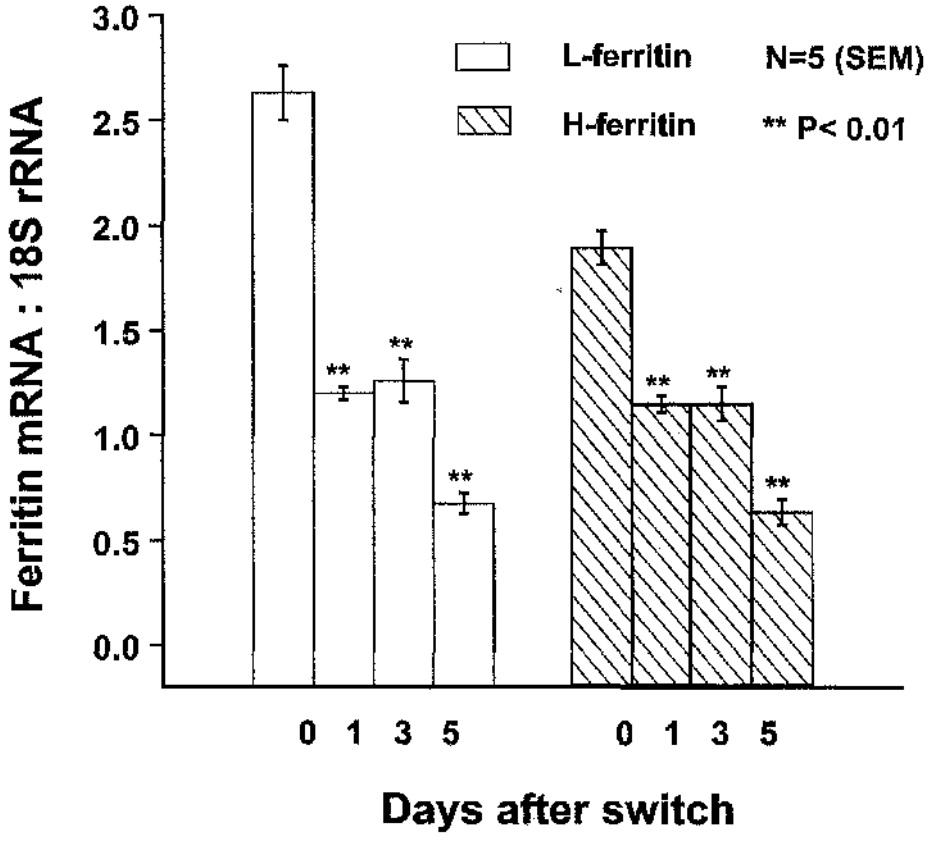
H-ferritin mRNA: 18S rRNA 1.89 ± 0.08 **1.15 ± 0.13 **1.15 ± 0.08 ** 0.64 ± 0.06

(mean of 5 ± SEM)

** $p<0.01$

Figure 4-7

Effect of a carbonyl diet to an iron deficient diet at day 0, 1, 3 and 5 on hepatic ferritin expression (data n=5 ± SEM and statistical significance as compared to day 0)



(iv) Effect of an iron deficient diet for 5 weeks followed by an iron loaded (carbonyl) diet at day 0, 1, 3 and 5 on hepatic ferritin expression

On changing the diet from a iron deficient to a loaded diet, there was no change in L and H-ferritin until day 5 where a substantial and significant rise was observed for L-ferritin ($p<0.01$). There was also a tendency for H-ferritin to rise by the day 5 on the iron loaded diet although this did not reach statistical significance (Table 4-1 & Figure 4-8).

Table 4-1

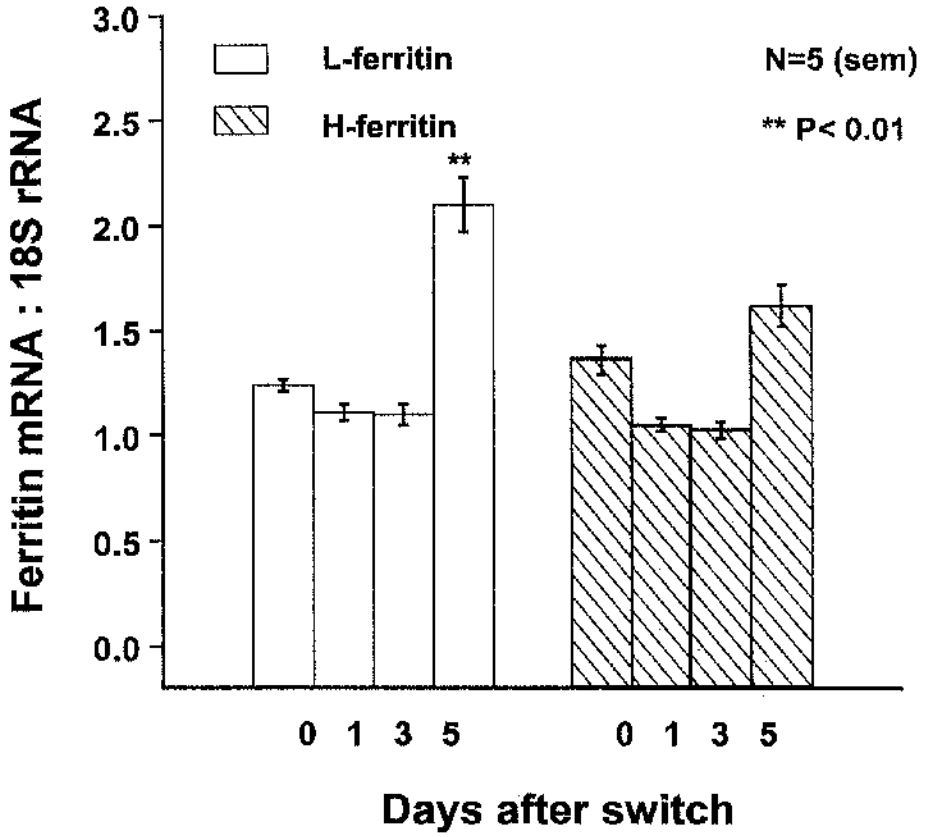
Densitometer readings (a.u.) on the effect of iron deficient diet to a carbonyl diet at day 0, 1, 3 and 5 on hepatic ferritin expression(data n=5 ± SEM)

Key: a.u.(arbitrary units for densitometery readings), **$p<0.01$ when compared to day 0

Days after switch	H-ferritin (a.u. x10^5)	L-ferritin (a.u. x10^5)	18S rRNA (a.u. x10^5)	H-ferritin : 18S rRNA	L-ferritin: 18S r RNA
0	1.85	1.92	1.60	1.16	1.20
	1.62	1.73	1.37	1.19	1.26
	1.74	1.89	1.44	1.21	1.32
	1.80	1.90	1.52	1.18	1.25
	1.81	1.89	1.63	1.11	1.16
mean ± sem				1.17 ± 0.03	1.20 ± 0.03
1	1.33	1.38	1.15	1.16	1.20
	1.80	1.92	1.59	1.13	1.21
	1.74	1.81	1.69	1.03	1.07
	1.60	1.75	1.67	0.96	1.05
	1.73	1.82	1.75	0.99	1.04
mean ± sem				1.05 ± 0.04	1.11± 0.05
3	1.68	1.84	1.70	0.99	1.08
	1.49	1.59	1.69	0.88	0.94
	1.80	1.77	1.70	1.06	1.04
	1.60	1.76	1.45	1.10	1.21
	1.75	1.88	1.62	1.08	1.16
mean ± sem				1.02 ± 0.04	1.10 ± 0.05
5	2.01	2.82	1.47	1.37	1.92
	2.48	2.96	1.46	1.70	2.03
	2.66	3.29	1.77	1.50	1.86
	2.03	2.65	1.03	1.97	2.57
	2.50	3.39	1.60	1.56	2.12
mean ± sem				1.62 ± 0.10	**2.10 ± 0.13

Figure 4-8

Effect of an iron deficient diet to a carbonyl diet at day 0, 1, 3 and 5 on hepatic ferritin mRNA expression (data n=5 ± SEM and statistical significance as compared to day 0)



(v) Effect of IP iron sorbitol followed by an iron deficient diet at 68 hours, day 1 and 3 hepatic ferritin expression

Within 6 hours of changing the diet, there was initially a significant rise in L-ferritin subunit mRNA ($p<0.05$) which then decreased. This was also observed for H-ferritin although the initial rise did not reach statistical significance. (Table 4-2 & Figure 4-9).

Table 4-2

Densitometer readings (a.u.) on the effect of IP iron sorbitol to an deficient diet at 6 hours and at day 1 and 3 on hepatic ferritin expression(data n=5 ± SEM)

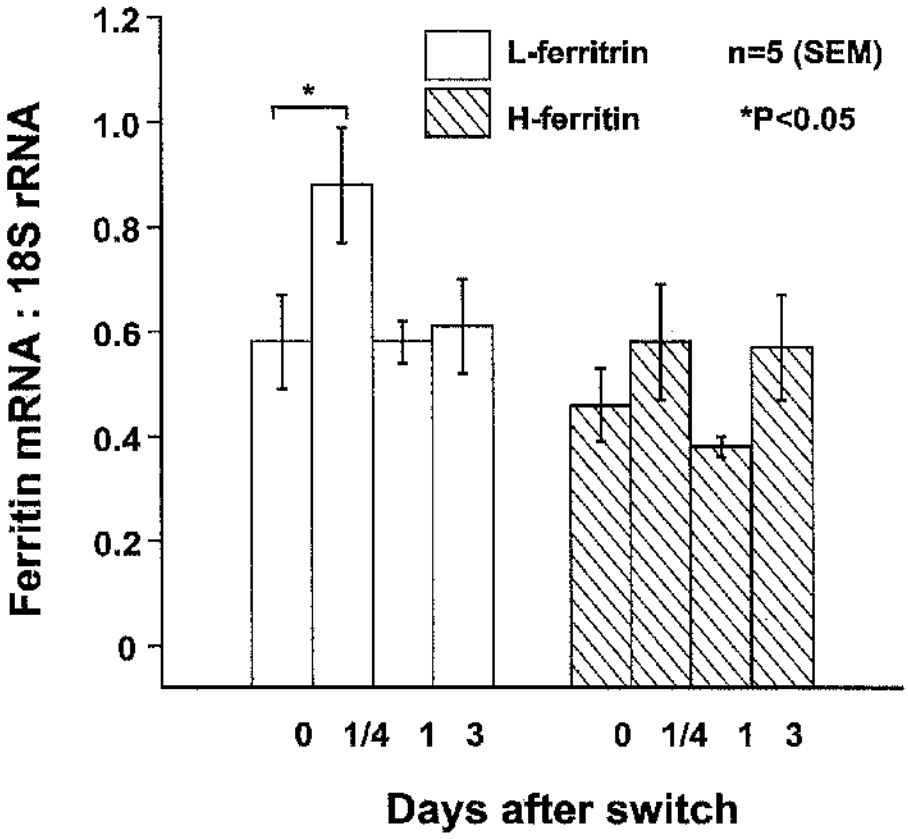
Key: a.u.(arbitrary units for densitometery readings)*$p<0.05$ when compared to day 0

Days after switch	H-ferritin (a.u. x10^5)	L-ferritin (a.u. x10^5)	18S rRNA (a.u. x10^5)	H-ferritin : 18S rRNA	L-ferritin: 18S r RNA
0	3.42	4.36	4.95	0.69	0.88
	1.90	2.31	5.14	0.37	0.45
	1.91	2.15	5.80	0.33	0.37
	1.79	3.28	4.97	0.36	0.66
	4.34	4.26	7.75	0.56	0.55
mean ± sem				0.46 ± 0.07	0.58 ± 0.09
1/4	2.74	6.10	8.84	0.31	0.69
	4.22	6.48	7.53	0.56	0.86
	2.99	4.41	6.49	0.46	0.68
	3.25	4.22	3.35	0.97	1.26
	4.50	6.46	7.26	0.62	0.89
mean ± sem				0.58 ± 0.11	*0.88± 0.11
1	3.62	6.53	10.05	0.36	0.65
	3.78	5.95	9.44	0.40	0.63
	3.55	5.27	9.09	0.39	0.58
	2.70	3.69	8.99	0.30	0.41
	4.90	7.01	11.13	0.44	0.63
mean ± sem				0.38 ± 0.02	0.58±0.04
5	5.04	5.17	12.3	0.41	0.42
	2.72	2.91	4.77	0.57	0.61
	4.80	4.98	5.86	0.82	0.85
	3.53	4.76	8.21	0.43	0.58
	2.80	2.62	4.59	0.61	0.57
mean ± sem				*0.57 ± 0.09	0.61 ± 0.09

Figure 4-9

Effect of IP iron sorbitol to an iron deficient diet at 6 hours, day 1 and 3 on hepatic ferritin expression (data n=5 ± SEM and statistical significance as compared to day 0)

Rats were given IP iron sorbitol (25 mg weekly for 4 weeks) and maintained on a standard rodent diet. On the 4th IP injection, diet was switched to an iron deficient diet for 6 hours, day 1 and 3



4.3.3 Discussion

Dietary iron overload was achieved by supplementing carbonyl iron which is an extremely pure form of elemental iron (greater than 98 %), prepared by reacting iron at high temperatures with carbon monoxide. This forms gaseous iron pentacarbonyl, where, after further heating is deposited as metallic iron submicroscopic crystals less than 5 μm in size (Carbonyl iron powders, GAF Corporation). Carbonyl iron was supplemented to the standard rodent diet (2.5 % wt/wt) rather than iron deficient diet as the latter produced intense diarrhoea when introduced to animals. As shown, supplementation of the diets with carbonyl iron produced predominantly hepatocellular iron deposition which is very similar to that observed in patients with GH. Prolonged administration of greater than 6 weeks will cause staining in Kupffer cells, endothelial cells and macrophages but the periportal to centrilobular gradient is still maintained (Bacon *et al* 1983). However, parenteral administration of iron sorbitol produced a more homogenous deposition of iron throughout the hepatic lobule in both hepatocytes and Kupffer cells.

A similar study on ferritin expression in hepatocytes had been undertaken by Pietrangelo *et al.*, (1990). However, the present study differs from Pietrangelo's study in several ways. Firstly, in animals on chronic carbonyl iron and parenteral iron sorbitol, L-ferritin subunit mRNA tended to rise although this did not reach statistical significance whereas Pietrangelo demonstrated enhanced L-ferritin subunit mRNA expression in chronic iron overload (parenteral iron was not examined). Secondly, H-ferritin subunit in the present study also tended to change whereas H-ferritin subunit mRNA was unaffected by iron treatment in Pietrangelo studies. There are several reasons for this discrepancy. The animals in the present study were maintained on the diet for 5 weeks by contrast to Pietrangelo's animals for 10 and 30 weeks. It seems unlikely (although cannot be entirely excluded) that the mouse ferritin probes used may account for some of the discrepancy even though verification of mouse L and H-ferritin sequences were shown to be

compatible to rat L and H-ferritin using Genbank Database and by preliminary Northern hybridisation experiments.

On switching diets (carbonyl iron to iron deficient and iron deficient to carbonyl iron for specific time periods) a corresponding fall and rise (respectively) in ferritin mRNA expression (L and H) was observed. In the parenteral group, there was an initial rise in ferritin mRNA expression (L greater than H) within 6 hours following ip injection of iron sorbitol, (due to elevated plasma iron sorbitol by declining by 24 hours; Charlton *et al* 1965) followed by a decline in ferritin mRNA expression as the animals were subjected to and ate the iron deficient diet. The present study suggests that ferritin mRNA in liver is predominantly L-subunit ferritin mRNA; the resulting isoferritins more suitable for long-term iron storage.

Ferritin gene expression can be regulated by iron at both transcription and translation (White & Munro 1988). In addition, iron may also be involved in other steps of ferritin gene regulation including the assembly and maintaining the stability of the ferritin shell (Drysdale 1983). The ability of iron to stimulate ferritin synthesis was first describe some 30 years ago (Drysdale and Munro 1965) suggesting that there is usually a surplus of ferritin mRNA within cells where translation is repressed until iron is present. However, in the present study, the changes observed in both L and H-ferritin mRNA in animals with chronic hepatic iron overload and switching to an iron deficient diet or vice versa suggest that (i) control of ferritin gene expression at the level of transcription may be also important (ii) luminal iron may also influence the synthesis of ferritin. However, it is difficult to reconcile the fact that the steady state (after 5 weeks) there is little difference in ferritin expression between iron deficiency and overload.

4.4 Effect of manipulating body iron stores on L and H-ferritin mRNA expression on crypt and villus cells of the rat intestinal mucosa

4.4.1 Introduction

As mentioned previously (Chapter 3) the major proteins implicated in the cellular control of iron metabolism are TfN, TfR and ferritin. Ferritin, in particular has been viewed as the key protein in controlling duodenal iron absorption. Body iron stores are controlled by a feedback mechanism which limits its absorption at the proximal intestine. However, the precise mechanism(s) remain a mystery. Over half a century ago, Hahn *et al* (1943) and later Granick (1946) proposed that ferritin may have a role in controlling body iron balance by acting as a mucosal "acceptor" for iron which can impede absorption of unwanted mucosal iron by retaining iron within the mucosal cells. Iron was lost when mucosal cells were shed onto the gut lumen. In an iron deficient state, this mucosal acceptor disappeared to permit the absorption of luminal iron.

As described in Section 4-3, two immunologically distinct ferritin subunits have been described designated L and H- subunit ferritin (Arosio *et al* 1978). Both L and H-ferritin are also found in the mucosal cells (Halliday *et al* 1978, Whittaker 1989). L-rich subunit ferritin in mucosal cells correlate with storage iron status, with raised duodenal concentration after oral iron administration thus reflecting increased amount of iron entering these cells (Halliday *et al* 1978). L-rich isoferritins function mainly as an iron storage protein as it is preferentially synthesised with iron loading (Bomford *et al* 1981). However, the role of H-rich ferritin is less well understood. It may be involved in immunosupression (Matzner *et al* 1979), regulating myelopoeitic activity (Dezza *et al* 1986) and the synthesis of haem (Blight & Morgan 1983). Perhaps more importantly, H-rich ferritin subunit appear to incorporate and release iron more readily than L-rich ferritin (Wagstaff *et al* 1978, Jones *et al* 1978, Levi *et al* 1988) i.e it serves a short-term storage for iron (a cellular house-keeping function) and is the ideal candidate protein for the intracellular shuttling of iron to the portal circulation. Structural and functional

relationships of these ferritins (deduced from cDNA clones) suggest that hecteropolymers containing H and L subunits have essentially the same three dimensional structure. However, several amino-acid substitutions at certain sites on the H-chain especially the amino-acids that form the channel through which iron gain access to the interior of the ferritin shell may be responsible for the metabolic and functional differences between H-rich and L-rich ferritin (Boyd *et al 1985).*

Previous physiological studies on iron absorption in rodents demonstrated that iron absorption did not increase or decrease for 3-5 days following artificially induced acute anaemia or iron overload respectively (Crosby *et al* 1963, Pollack 1964, Wheby 1964a). This interval corresponds to the time taken for proliferating crypt cells to migrate to the villus tip (Messier & Leblond 1958). Conrad and Crosby (1963) proposed that information regarding body iron stores may have conditioned crypt cells whereby these cells could later modulate the absorption of dietary iron following migration to the villus tip. Fairweather-Tait (1984) further postulated that TfR at the basolateral surface of enterocytes may perform this function of relaying information on body iron stores to the enterocyte. This may be achieved via a mucosal endocytic mechanism based on plasma transferrin saturation (Anderson *et al* 1994).

When body iron stores are replete and plasma transferrin is saturated, TfR is down regulated at an early stage and conversely whereas ferritin synthesis is un regulated. In crypt cells, this would set up a constitutive transcript ratio which is predominantly L-rich ferritin which would retain iron and impede iron transfer to plasma. Retained iron is lost when mucosal cells exfoliate into the gut lumen. Similarly, in an iron deficient state, TfR are persistently expressed and the information 'imparted' to enterocytes will favour a constitutive ratio which is predominately lower in L-rich ferritin transcripts, whereby, a predominantly H-rich ferritin protein is translated. This protein will retain iron less avidly thus allowing iron transfer to plasma when mucosal cells are presented with luminal iron. Hence, within the enterocyte, changing the proportions of L and H-rich ferritin subunits

could be exploited respectively to reduce or increase transfer of dietary iron to mucosal cells/plasma depending on whether iron stores were high or low.

I therefore set out to determine if alterations in body iron stores will influence ferritin expression in crypt and villus cells that is consistent with the 'lag period' of some 3 days, the time for crypt-villus migration that correspond to the previously noted time interval of alteration in iron stores and the response of mucosal cell to dietary iron. To explore this hypothesis, a 'switch diet model' and segregation of crypt and villus cells would need to be undertaken.

4.4.2 Methods and materials

(i) Animals and diet

Male Wistar rats were used for all experiments. Animals were divided into 4 groups, 6 animals per group. These have been described previously in Chapter 4 consisting of (a) Rat pellet fed animals, (b) Carbonyl iron-supplemented animals (c) An iron deficient group and (d) An iron loaded group given IP iron sorbitol (25 mg/week for 4 weeks).

(ii) Molecular biochemistry

RNA was extracted from segregated cells, Northern capillary transfer, labelling of cDNA and oligonucleotide ribosomal sequences by random priming and terminal transferase respectively, hybridisation conditions, washing conditions, generation of autoradiograms and quantification by densitometery were carried out as described in section 2. 3.

(iii) Plan of investigations

Body iron stores were manipulated by diet and by parenteral iron as described in the previous section. After 5 weeks, the oral carbonyl group was switched to an iron deficient diet and studied at day 1, 3 and 5, the iron deficient group was switched to the carbonyl diet and studied at day 1, 3 and 5. On the last week of the injection of iron

sorbitol, the parenteral group (maintained on a standard rodent diet) was switched to an iron deficient diet and studied at 6 hours, 1 and 3 days. Animals were sacrificed and crypt and villus cells segregation undertaken as described in section 2.1.

4.4.3 Results

(i) Perls potassium ferricyanide staining in intestinal mucosa

Haemosiderin deposits in intestinal cells were observed with Perls potassium ferricyanide after 5 weeks on oral iron and only 6 hours following iron sorbitol injection (Figure 4-10a and 4-10b). No haemosiderin deposits were observed in the intestinal mucosa of control animals and animals on the iron deficient diet nor at day 5 after the switch from the iron deficient diet to the carbonyl iron diet (Table 3-3, section 3.4.2). Haemosiderin deposits were also absent in the small intestine by day 3 from switching carbonyl iron loaded animals to an iron deficient diet.

Steady state conditioning

(ii) Effect of iron loading (oral carbonyl and IP iron sorbitol) and iron deficiency on ferritin expression in the rat intestine

(a) Villus cells

In both the iron loaded groups (oral and IP), no significant increase in either L or H-ferritin subunit mRNA was observed. However, in the iron deficient group, both L-ferritin ($p<0.01$) and H-ferritin ($p<0.05$) subunit mRNA were reduced compared to control animals (Figure 4-11a and Figure 4-12a).

(b) Crypt cells

No significant change in either L or H-ferritin subunit mRNA was observed between the 3 treatment groups was observed compared to control animals (Figure 4-11b & Figure 4-12b).

Figure 4-10

Perls staining for haemosiderin in the rat small intestinal mucosa

(a) Oral carbonyl iron for 5 weeks (left) and (b) 6 hours following IP iron sorbitol (bottom)

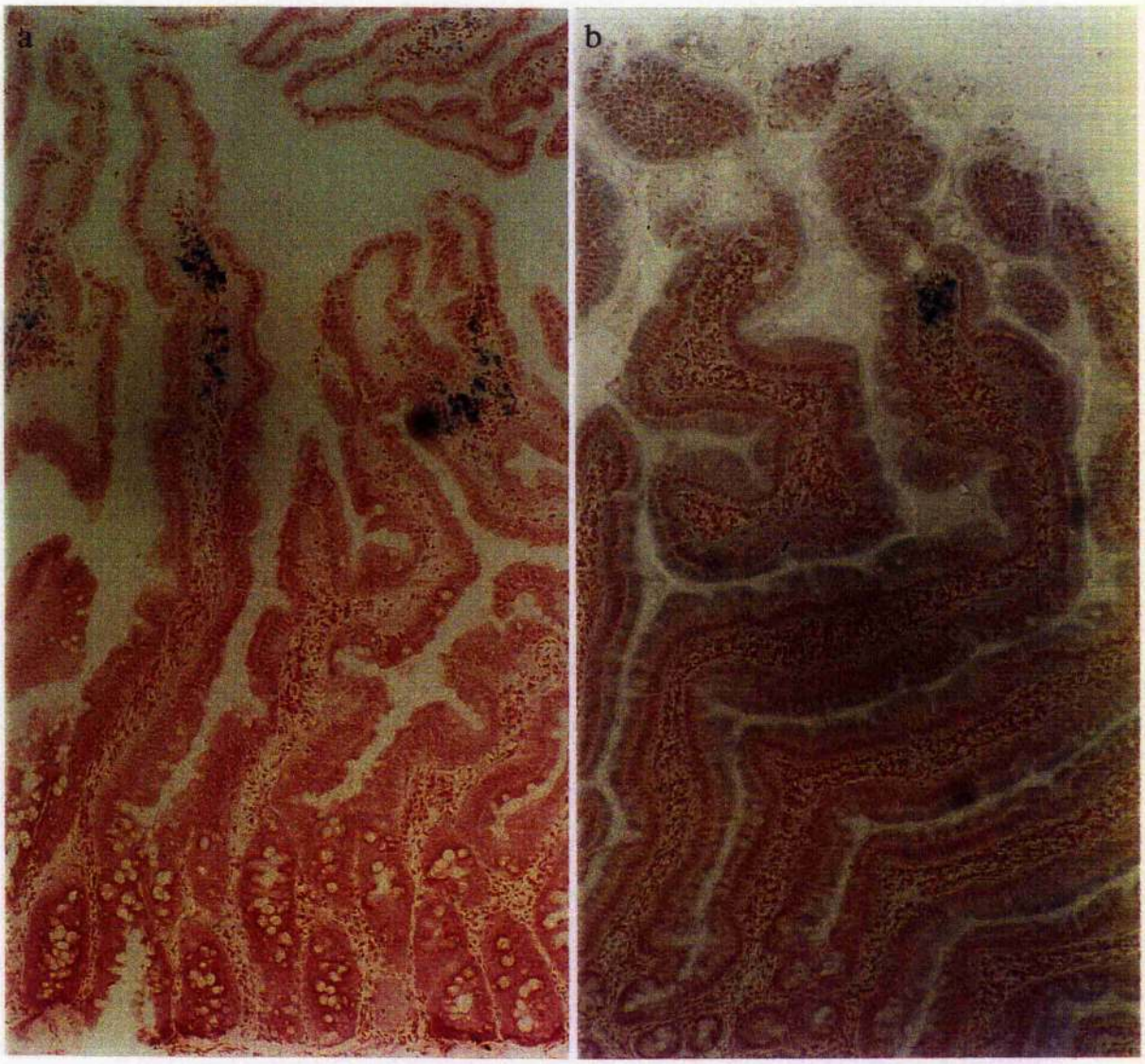
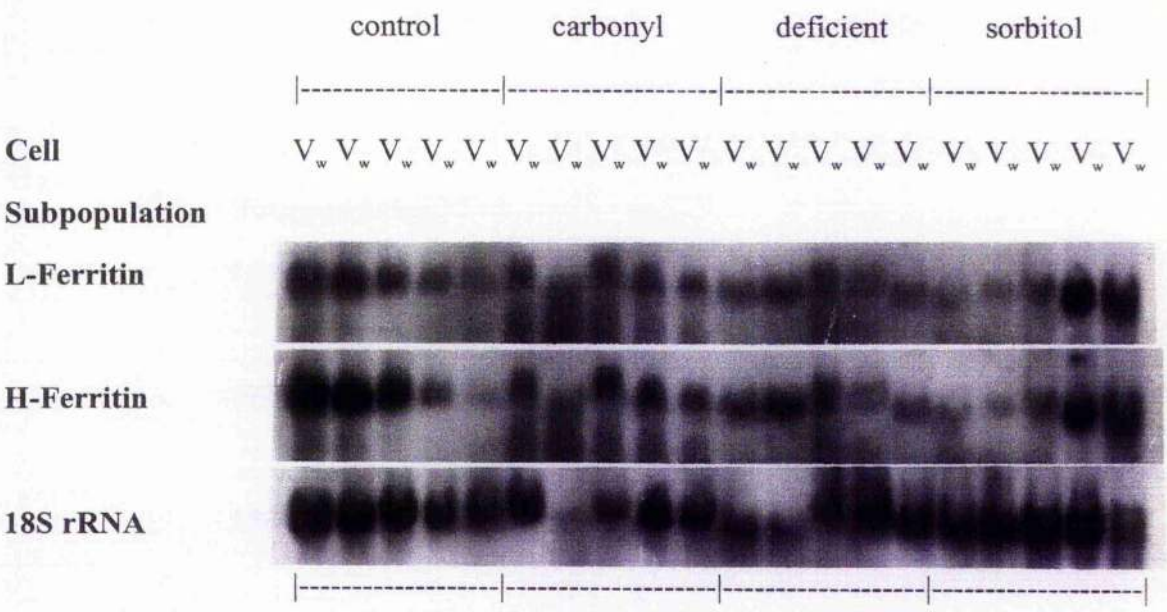


Figure 4-11

Autoradiograms showing the effect of oral carbonyl iron, IP iron sorbitol and iron deficient diet on ferritin expression in the rat intestine

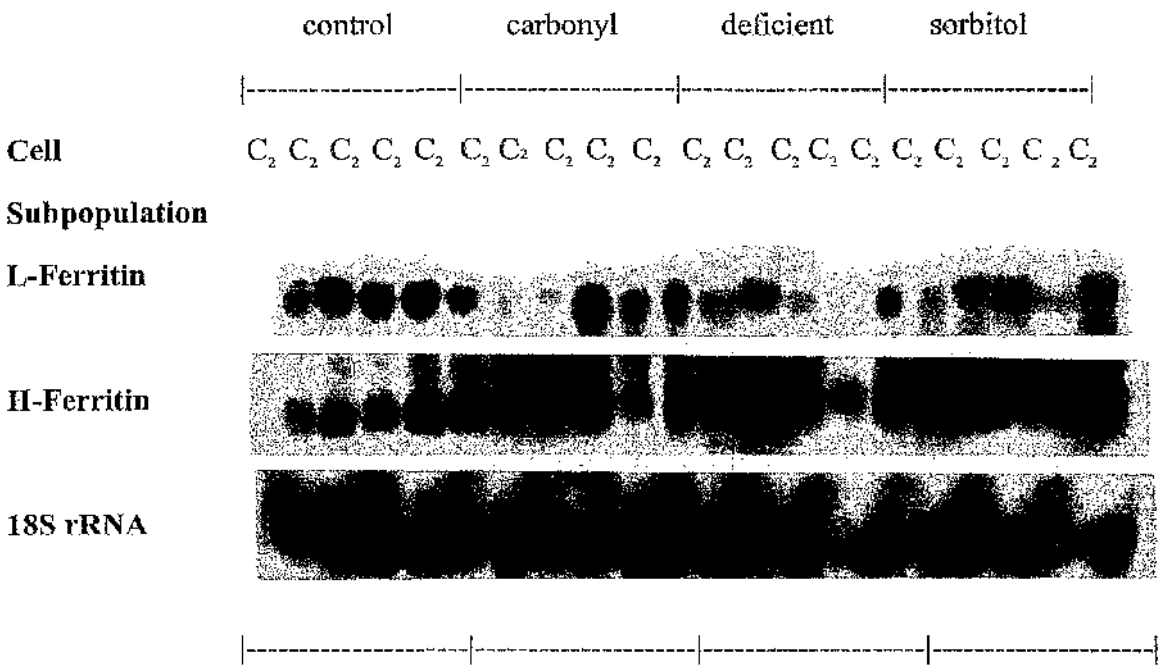
(a) Villus cell



		L-feritin	H-ferritin
Ferritin mRNA: 18S rRNA	$V_{w\ control}$	**0.50 ± 0.05**	**0.80 ± 0.13**
(Mean of 5 ± SEM)	$V_{w\ carbonyl}$	**0.49 ± 0.09**	**0.55 ± 0.06**
	$V_{w\ deficient}$	****0.20 ± 0.01**	***0.47 ± 0.06**
	$V_{w\ sorbitol}$	**0.46 ± 0.07**	**0.53 ± 0.08**

* $p<0.05$, ** $p<0.01$

(b) Crypt cell



		L-ferritin	H-ferritin
Ferritin mRNA: 18S rRNA	$C_{2\,control}$	**0.54 ± 0.03**	**0.72 ± 0.09**
(Mean of 5 ± SEM)	$C_{2\,carbonyl}$	**0.52 ± 0.21**	**0.58 ± 0.19**
	$C_{2\,deficient}$	**0.52 ± 0.11**	**0.72 ± 0.18**
	$C_{2\,sorbitol}$	**0.69 ± 0.14**	**0.48 ± 0.09**

Figure 4-12

L and H-ferritin mRNA expression in villus cell under different iron regimes

(data n=5 ± SEM and statistical significance as compared to controls)

Key: Con=Control, Def=Iron deficiency, Car=Carbonyl iron, IP=Intraperitoneal iron sorbitol

(a) Villus cell

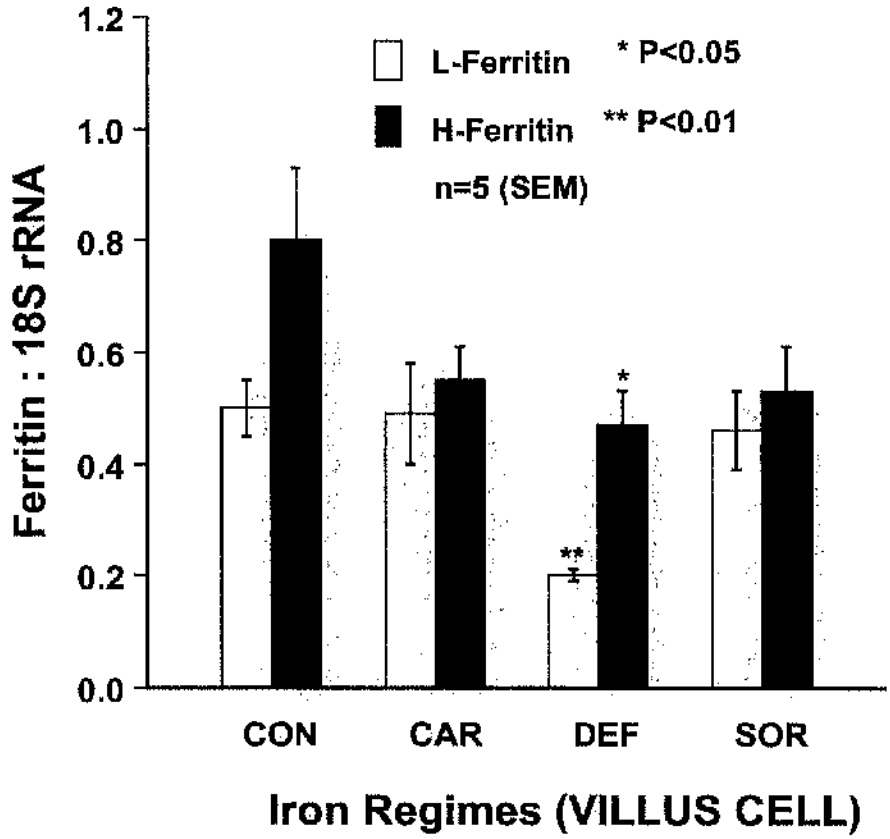


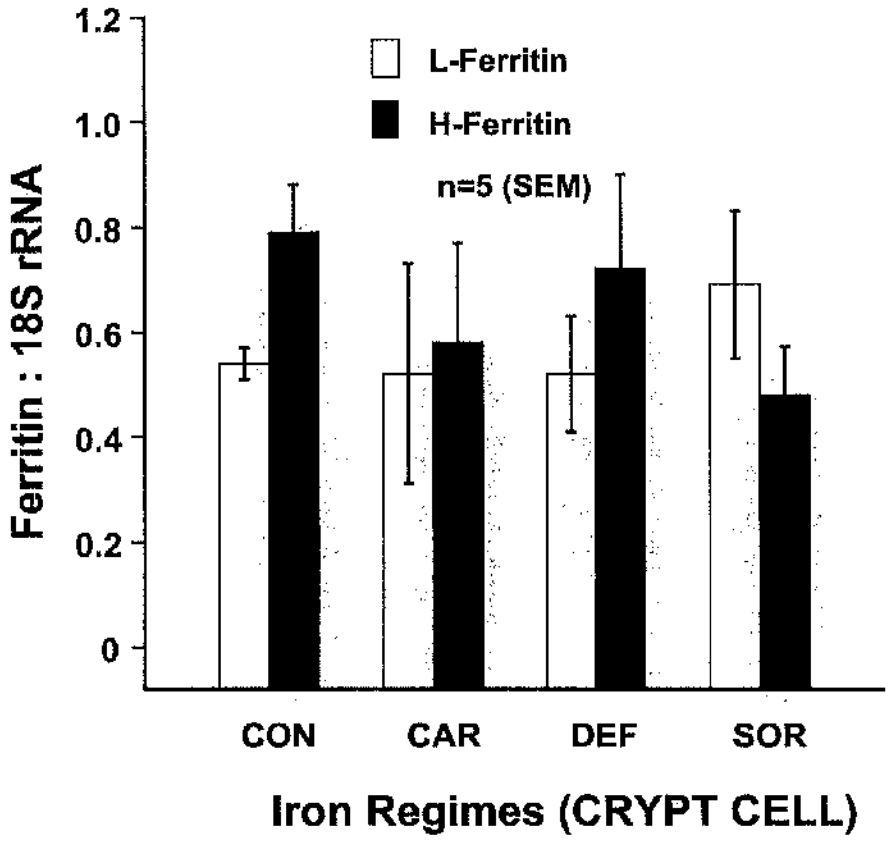
Figure 4-12

L and H-ferritin mRNA expression in crypt cells under different iron regimes

(data n=5 ± SEM and statistical significance as compared to controls)

Key: Con=Control, Def=Iron deficiency, Car=Carbonyl iron, IP=Intraperitoneal iron sorbitol

(b) Crypt cell



Switch diet conditioning

(iii) Effect of oral carbonyl iron for 5 weeks followed by an iron deficient diet at day 0, 1, 3 and 5 days on ferritin expression in the rat intestinal mucosa

Six animals were used for each group (4 groups altogether). On each autoradiogram, there were 2 animals from each group at day 0, 1, 3 and 5 after changes in diet.

(a) Villus cell

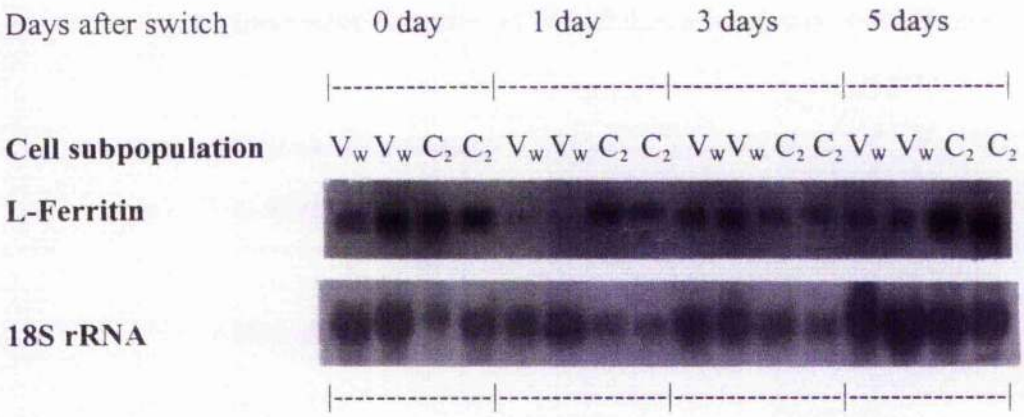
Within 24 hours on switching to an iron deficient diet, a significant reduction in L-ferritin subunit mRNA expression ($p<0.01$) was observed and maintain to day 5 while on the iron deficient diet ($p<0.01$). However, this was not observed in H-ferritin subunit mRNA which intended to rise although not statistically significant. (Figure 4-13 and Figure 4-14a & b)

(b) Crypt cell

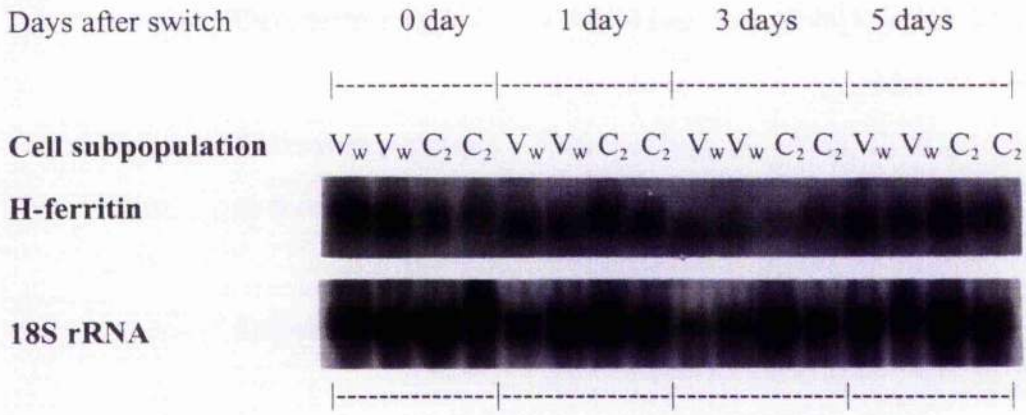
Similarly, L-ferritin subunit mRNA expression in crypt cells was reduced within 24 hours on changing to the iron deficient diet ($p<0.05$) and up to day 5 ($p<0.05$). There was no significant change in H-ferritin subunit mRNA expression (Figure 4-13 and Figure 4-14 a & b).

Figure 4-13

Autoradiogram on the effect of changing from a carbonyl iron diet to an iron deficient diet at day 0, 1, 3 and 5 on ferritin expression in the rat intestinal mucosa



L-ferritin mRNA: 18S rRNA	V_w	**0.34 ± 0.05**	****0.11 ± 0.03**	****0.16 ± 0.02**	****0.14 ± 0.02**
(mean of 6 ± SEM)	C_2	**0.33± 0.10**	***0.08 ± 0.02**	***0.07 ± 0.02**	***0.09 ± 0.03**



H-ferritin mRNA: 18S rRNA	V_w	**1.41 ± 0.19**	**1.54 ± 0.23**	**1.66 ± 0.33**	**1.96 ± 0.42**
(mean of 6 ± SEM)	C_2	**1.94 ± 031**	**1.39 ± 0.14**	**1.27 ± 0.24**	**1.72 ± 0.51**

* $p<0.05$, ** $p<0.01$

Figure 4-14 (a)

L-ferritin expression in crypt and villus cell from a carbonyl iron diet to an iron deficient diet at day 0, 1, 3 and 5 (data n=6 ± SEM and statistical significance as compared to day 0 before the change in diet)

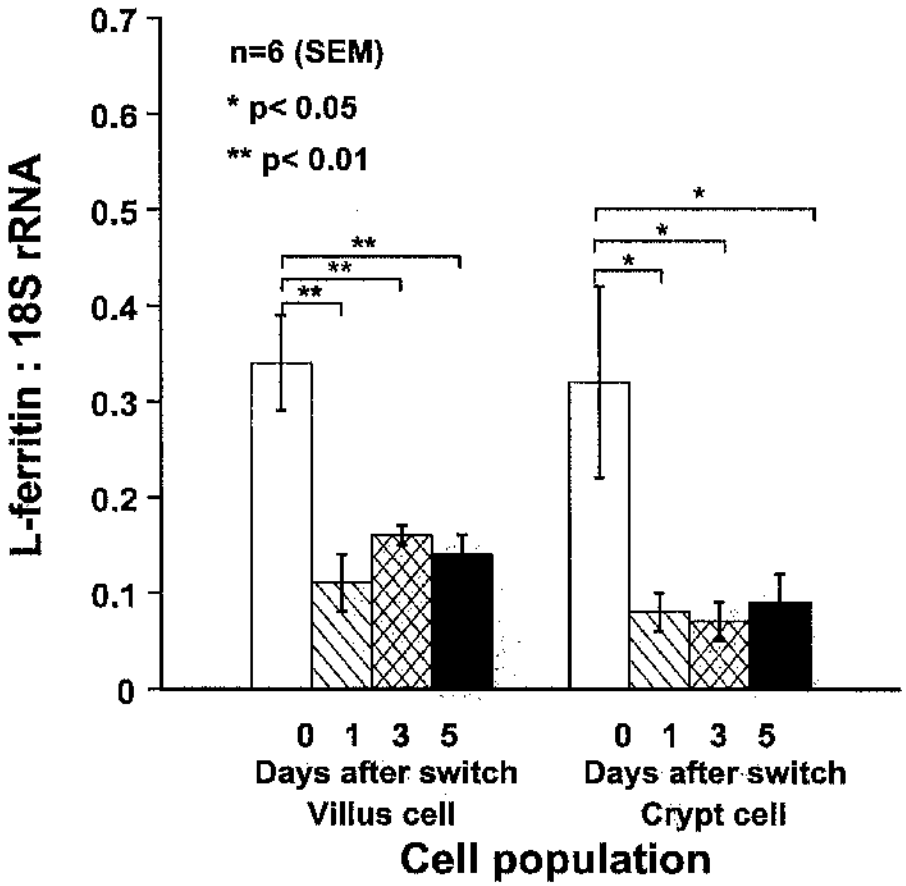
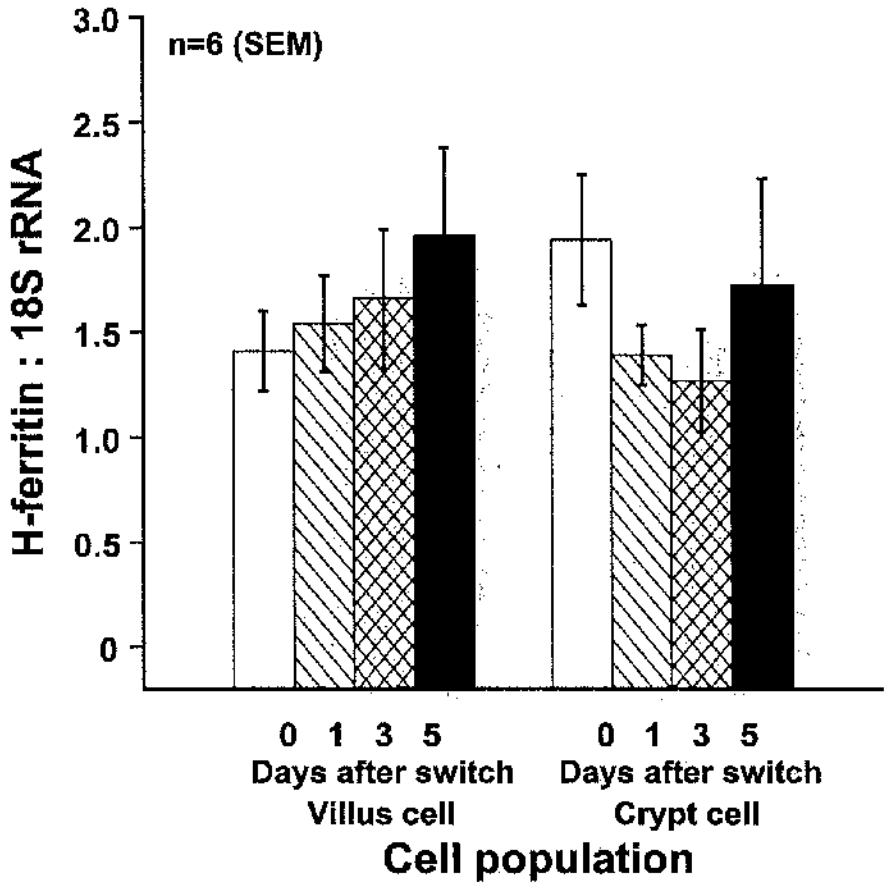


Figure 4-14(b)

H-ferritin expression in crypt and villus cell from a carbonyl iron diet to an iron deficient diet at day 0, 1, 3 and 5 (data n=6 ± SEM and statistical significance as compared to day 0 before the change in diet)



(iv) Effect of an iron deficient diet for 5 weeks followed by an iron loaded (carbonyl) diet at day 0, 1, 3 and 5 on ferritin expression in rat intestinal mucosa

(a) Villus cells

There was no difference between L and H-ferritin subunit mRNA expression in villus cells during dietary manipulation from an iron deficient diet to an iron loaded diet up to day 5 (Table 4-3 and Figure 4-15 a & b).

(b) Crypt cells

There was also no difference in L-ferritin subunit mRNA expression in crypt cells during dietary manipulation although there was a tendency for L-ferritin to rise by day 5 but this was not statistically significant (Figure 4-15a). Similarly, there was no significant change in H-ferritin subunit mRNA expression during dietary manipulation (Figure 4-15b).

Table 4-3 Densitometer readings (a.u.) on the effect of an iron deficient diet to a carbonyl iron diet at day 0, 1, 3 and 5 on ferritin expression in crypt and villus cells

Days after switch	H-ferritin villus cell (a.u. x10^5)	L-ferritin villus cell (a.u. x10^5)	18S rRNA villus cell (a.u. x10^5)	H-ferritin : 18S rRNA (villus cell)	L-ferritin: 18S rRNA (villus cell)	H-ferritin crypt cell (a.u. x10^5)	L-ferritin crypt cell (a.u. x10^5)	18S rRNA crypt cell (a.u. x10^5)	H-ferritin : 18S rRNA (crypt cell)	L-ferritin: 18S rRNA (crypt cell)
0	1.11	0.63	1.08	1.03	0.58	1.61	0.70	1.11	1.45	0.63
	1.23	0..63	1.22	1.01	0.52	1.48	0.66	1.46	1.01	0.45
	2.59	1.67	2.29	1.13	0.73	1 21	0.77	1.32	0.92.	0.58
	2.22	1.32	1.79	1.24	0.74	1.71	0.91	2.03	0.84	0.45
	0.68	0.35	0.54	1.25	0.64	0.46	0.20	0.32	1.45	0.63
	0.39	0.19	0.35	1.11	0.53	0.22	0.18	0.31	0.71	0.57
mean ± sem				1.13 ± 0.04	0.62 ± 0.04				1.06 ± 0.13	0.55 ± 0.03
1	0.17	0.19	0.22	0.77	0.86	1.38	1.11	1.48	0.93	0.75
	1.16	0.66	1.38	0.84	0.48	0.91	0.72	1.72	0.53	0.42
	2.41	1.22	1.96	1.23	0.62	4.08	1.66	4.25	0.96	0.39
	2.24	1.39	2.02	1.11	0.69	3.63	1.25	2.98	1.22	0.42
	0.19	0.14	0.18	1.07	0.77	0.67	0.29	0.51	1.31	0.56
	0.35	0.20	0.29	1.21.	0.68	0.61	0.29	0.67	0.91	0.44
mean ± sem				1.04 ± 0.08	0.68 ± 0.05				0.98 ± 0.11	0.50 ± 0.06
3	1.12	0.47	1.18	1.02	0.40	0.95	0.27	2.10	0.45	0.13
	1.15	0.77	1.32	0.87	0.58	1.08	0.39	2.03	0.53	0.19
	0.51	0.46	0.81	0.63	0.57	1.47	0.99	1.91	0.77	0.52
	0.58	0.30	0.66	0.80	0.46	2.96	1.48	3.08	0.96	0.48
	0.69	0.30	0.56	1.24	0.54	0.31	0.42	0.73	0.42	0.57
	0.79	0.41	0.69	1.15	0.59	1.46	0.86	1.57	0.93	0.55
mean ± sem				0.95 ± 0.09	0.52 ± 0.03				0.68 ± 0.09	0.41 ± 0.08
5	1.18	0.54	1.15	1.03	0.47	0.20	0.10	0.14	1.46	0.72
	0.44	0.46	0.61	0.72	0.75	0.92	0.53	0.81	1.13	0.65
	1.45	0.38	1.25	1.16	0.30	0.66	0.29	0.99	0.67	0.29
	1.82	0.58	2.02	0.91	0.29	1.61	1.30	1.71	0.94	0.76
	1.72	0.90	1.92	0.90	0.47	0.24	0.41	0.54	0.44	0.76
	0.27	0.20	0.33	0.82	0.61	0.61	0.58	1.06	0.60	0.55
mean ± sem				0.92 ± 0.07	0.48 ± 0.07				0.87 ± 0.16	0.62 ± 0.07

Figure 4-15 (a)

L-Ferritin expression in rat intestinal cells from an iron deficient diet to a carbonyl iron diet at day 0, 1, 3 and 5 (data n=6 ± SEM and statistical significance as compared to day 0 before the change in diet)

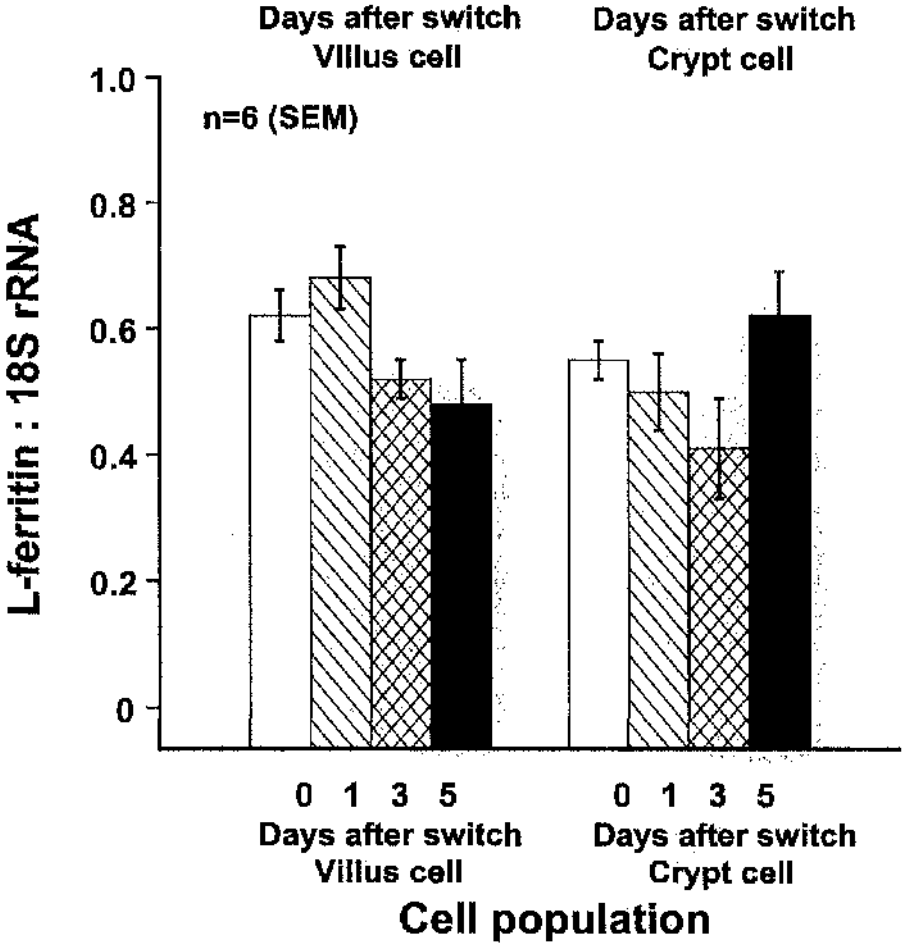
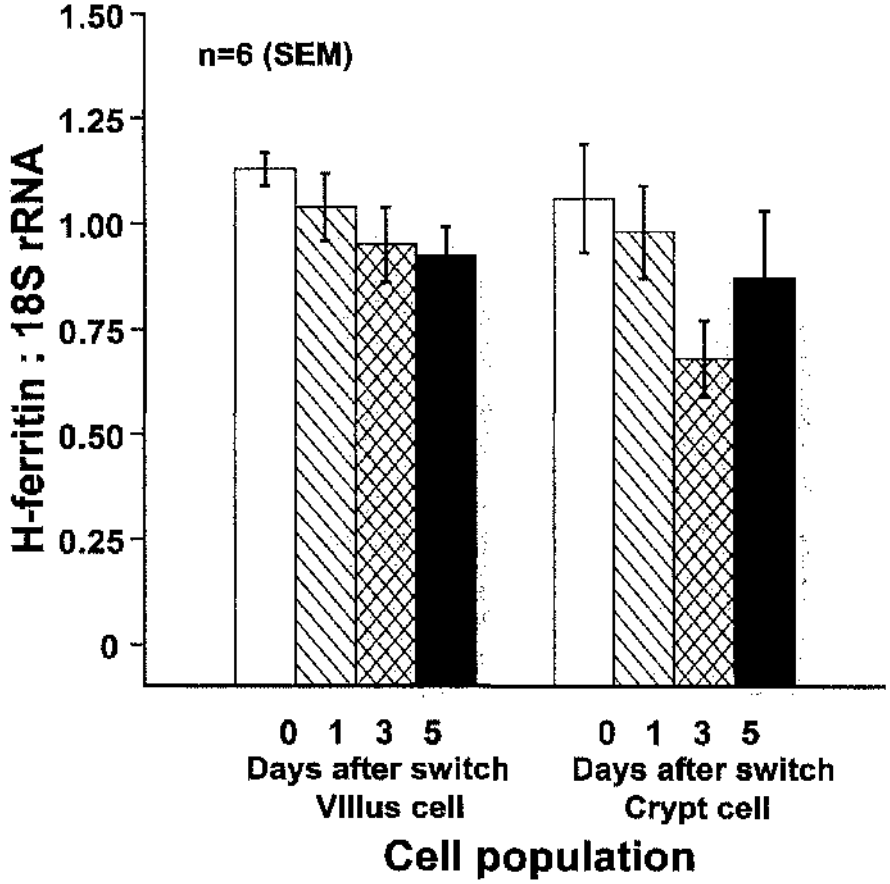


Figure 4-15 (b)

H-ferritin expression in rat intestinal cells from an iron deficient diet to a carbonyl iron diet at day 0, 1, 3 and 5 (data n=6 ± SEM and statistical significance as compared to day 0 before the change in diet)



(v) Effect of parenteral iron sorbitol followed by an iron deficient diet for 6 hours, 1 and 3 days on ferritin expression in rat intestinal mucosa

(a) Villus cells

Reduced expression of L-ferritin subunit mRNA ($p<0.01$) was observed within 6 hours of changing the diet and is maintained up to day 3 ($p<0.01$). H-ferritin subunit mRNA expression also tended to fall but only significant at day 3 on the iron deficient diet ($p<0.01$) (Table 4-4 and Figure 4.16 a and b).

(b) Crypt cell

L-ferritin subunit mRNA in crypt cells also tended to fall but is only significant at day 1 ($p<0.05$) from changing to the iron deficient diet. Similarly, H-ferritin subunit mRNA expression also tended to fall and only significant at day 1 ($p<0.05$) and maintained to day 3 ($p<0.01$) from the change in diet (Table 4-4 and Figure 4-16a and b).

Table 4-4 Densitometer readings (a.u.) on the effect of IP iron sorbitol to an iron diet at day 0, 1, 3 and 5 on ferritin expression in crypt and villus cells

Days after switch	H-ferritin villus cell (a.u. x10^5)	L-ferritin villus cell (a.u. x10^5)	18S rRNA villus cell (a.u. x10^5)	H-ferritin : 18S rRNA (villus cell) (a.u. x10^5)	L-ferritin: 18S rRNA (villus cell) (a.u. x10^5)	H-ferritin crypt cell (a.u. x10^5)	L-ferritin crypt cell (a.u. x10^5)	18S rRNA crypt cell (a.u. x10^5)	H-ferritin : 18S rRNA (crypt cell) (a.u. x10^5)	L-ferritin: 18S rRNA (crypt cell) (a.u. x10^5)
0	0.50	0.25	0.67	0.74	0.37	0.72	0.43	0.89	0.81	0.50
	0.77	0.38	1.03	0.75	0.37	0.47	0.22	0.90	0.52	0.24
	1.14	0.97	1.03	1.11	0.94	0.61	0.13	0.88	0.69	0.15
	0.94	0.48	0.59	1.6	0.82	0.58	0.09	0.56	1.03	0.17
	0.90	0.85	1.02	0.88	0.83	1.25	0.45	1.74	0.72	0.26
	0.85	1.05	0.99	0.80	1.06	1.25	0.52	2.05	0.61	0.25
mean ± sem				0.98 ± 0.14	0.73 ± 0.12				0.73 ± 0.09	0.26 ± 0.06
1/4	0.63	0.48	1.66	0.38	0.29	0.75	0.26	1.66	0.45	0.16
	0.21	0.20	1.05	0.20	0.19	0.29	0.20	1.05	0.29	0.19
	0.38	0.18	0.51	0.75	0.36	0.42	0.07	0.51	0.83	0.14
	0.41	0.18	0.38	1.07	0.48	0.49	0.05	0.38	1.28	0.14
	1.18	0.25	1.27	0.93	0.20	0.14	0.02	0.12	1.14	0.16
	1.25	0.11	1.13	1.11	0.10	0.11	0.02	0.11	1.00	0.19
mean ± sem				0.74 ± 0.15	0.27±0.05**				0.83 ± 0.16	0.16 ± 0.01
1	0.45	0.13	1.11	0.41	0.12	0.32	0.26	1.61	0.2	0.16
	0.44	0.19	1.23	0.36	0.15	0.38	0.31	1.72	0.22	0.18
	0.25	0.06	0.28	0.90	0.22	0.39	0.04	1.05	0.37	0.04
	0.25	0.06	0.33	0.77	0.18	0.43	0.08	1.79	0.24	0.05
	0.16	0.03	0.16	0.97	0.19	0.79	0.14	0.92	0.86	0.15
	0.21	0.03	0.22	0.93	0.23	0.49	0.11	0.93	0.53	0.12
mean ± sem				0.72 ± 0.11	0.18±0.02**				0.40±0.01*	0.12±0.02*
3	0.68	0.37	1.78	0.38	0.21	0.30	0.23	1.93	0.16	0.12
	0.77	0.41	2.58	0.30	0.16	0.33	0.20	1.96	0.17	0.10
	0.61	0.17	1.07	0.57	0.16	0.80	0.49	2.35	0.34	0.21
	0.55	0.18	0.12	0.49	0.16	0.55	0.27	1.68	0.33	0.16
	0.91	0.17	1.34	0.68	0.13	0.75	0.20	2.50	0.30	0.08
	0.89	0.25	1.79	0.50	0.14	0.98	0.47	3.64	0.27	0.13
mean ± sem				0.49± 0.07*	0.16±0.01**				0.26±0.03**	0.12 ± 0.02

Key: *p,0.05,**p<0.01 compared to day 0

Figure 4-16 (a)

L-ferritin expression in crypt and villus cell on the effect of IP iron sorbitol to an iron deficient diet at 6 hours, 1 and 3 days (data n=6 ± SEM and statistical significance as compared to day 0 before the change in diet)

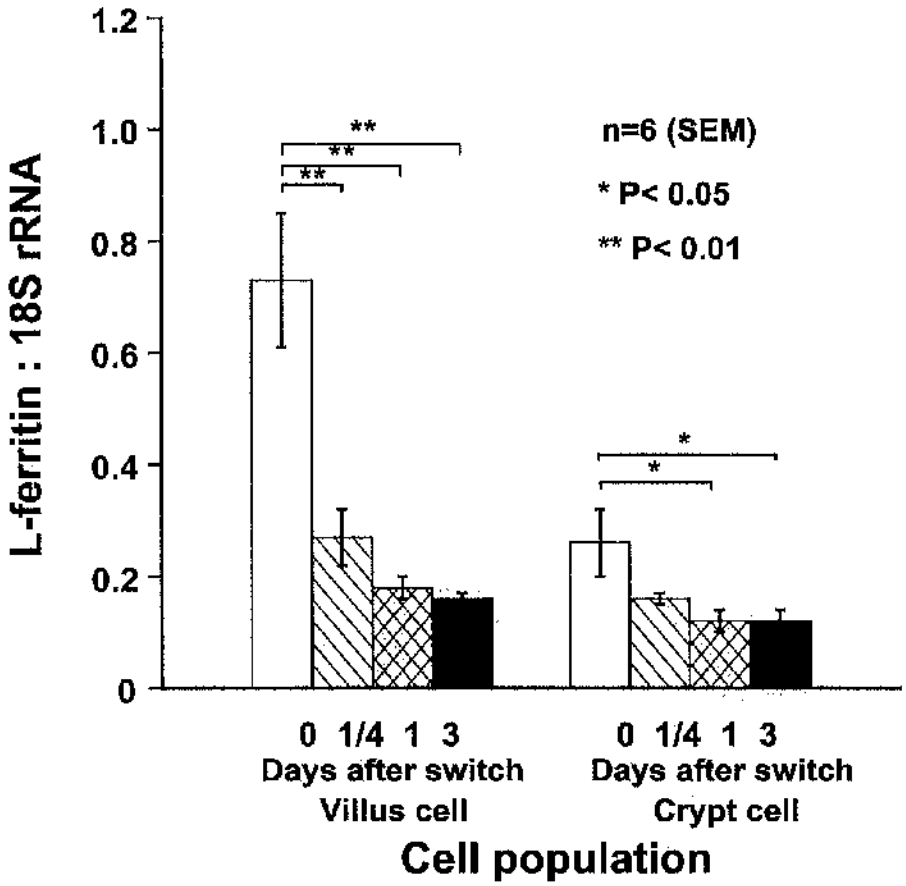
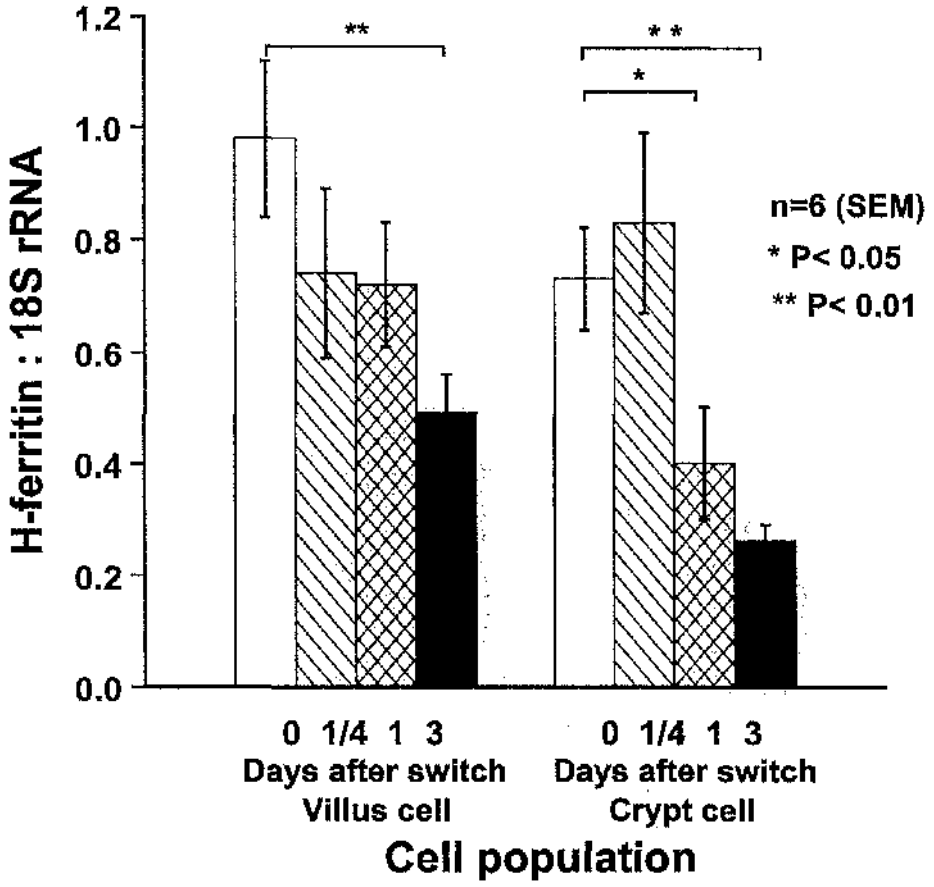


Figure 4-16 (b)

H-ferritin expression in crypt and villus cell on the effect of IP iron sorbitol to an iron deficient diet at 6 hours, 1 and 3 days (data n=6 ± SEM and statistical significance as compared to day 0 before the change in diet)



4.4.4 L to H subunit transcript ratio or constitutive ratio

On reflection of the hypothesis (section 1.4), it has been postulated that body iron stores conditioned the crypt cells in the intestinal mucosa. This 'conditioning' sets up a specific constitutive transcript ratio which may influence the handling of luminal iron. The L:H transcript ratios in the various regimes have been summarised in Tables 4-5a & 4-5b.

4.4.5 Summary of results

(i) Steady state conditioning

Regime 1 (iron loading in animals)

In animals loaded with iron (oral carbonyl and IP iron sorbitol), there is a tendency to transcribe more of the L-rich ferritin in both villus and crypt cells compared to controls, although this was only statistically significant in crypt cells in the IP iron sorbitol group.

Carbonyl iron:

(villus; 0.91 ± 0.07 vs 0.67 ± 0.09; $p>0.05$; crypt; 0.86 ± 0.07 vs 0.79 ± 0.09; $p>0.05$)

IP iron sorbitol:

(villus; 0.88 ± 0.06 vs 0.67 ± 0.09; $p>0.05$; crypt ; 1.45 ± 0.09 vs 0.79 ± 0.09; $p<0.05$)

In the iron deficient group, there was also a tendency to transcribe a lower L-rich ferritin in both crypt and villus cell although this did not reach statistical significance.

Iron deficient:

(villus ; 0.46 ± 0.04 vs 0.67 ± 0.09; $p>0.05$; crypt ; 0.74 ± 0.05 vs 0.79 ± 0.09; $p>0.05$)

(ii) Switch diet model

(a) Regime 2 (carbonyl iron to iron deficient diet at day 0, 1 ,3 and 5)

In the switch diet model from an iron loaded diet to an iron deficient diet, there was a tendency to transcribe less L-rich feritin in both villus and crypt cell by day 1 and

maintained to day 5 although this was only statistically significant in villus cells (Table 4-5a).

(b) Regime 3 (iron deficient diet to a carbonyl diet at day 0, 1 ,3 and 5)

In this regime, there was a tendency to transcribe predominately L-rich ferritin (only observed in crypt cells) but this did not reach statistical significance (Table 4-5b).

(c) Regime 4 (IP iron sorbitol to an iron deficient diet at day 0, 1/4, 1 and 3)

This produced observations very similar to regime 2, with predominately lower L-rich transcripts although this phenomena appears to be confined to the villus cell (Table 4-5b).

Table 4-5 (a)

Densitometer ratios of L and H-ferritin in the rat intestinal mucosa

Regime 1: Animals maintained on different iron regimes for 5 weeks (data n=5 ± SEM)

Regime 2: Changing from a carbonyl iron diet to an iron deficient diet at day 0, 1, 3 & 5 (data n=6 ± SEM) *p<0.05, **p<0.01 (statistical significance as compared to day 0 from the change in diet)

Regime (1) (n=5)	Villus L:H ratio	Crypt L:H ratio	Regime (2) and days after switch (n=6)	Villus L:H ratio	Crypt L:H ratio
Control	0.55	1.15	0	0.26	0.09
	0.51	0.75		0.18	0.09
	0.51	0.73		0.38	0.48
	0.91	0.66		0.18	0.26
	0.89	0.65		0.18	0.10
				0.32	0.09
mean ± SEM	0.67 ± 0.09	0.79 ± 0.09	mean ± SEM	0.25 ± 0.03	0.19 ± 0.07
Carbonyl	1.10	1.00	1	0.02	0.05
	0.91	0.74		0.03	0.05
	0.83	1.02		0.07	0.09
	0.78	0.88		0.18	0.15
	0.94	0.67		0.09	0.13
				0.08	0.09
mean ± SEM	0.91 ± 0.07	0.86 ± 0.07	mean ± SEM	**0.08 ± 0.03	0.09 ± 0.02
Deficient	0.39	0.91	3	0.12	0.01
	0.37	0.70		0.11	0.02
	0.34	0.67		0.08	0.35
	0.68	0.83		0.05	0.10
	0.54	0.61		0.19	0.03
				0.14	0.04
mean ± SEM	0.46 ± 0.06	0.74 ± 0.05	mean ± SEM	*0.12 ± 0.02	0.09 ± 0.05
IP sorbitol	0.86	1.15	5	0.05	0.11
	0.65	1.08		0.13	0.07
	1.06	2.18		0.10	0.19
	0.91	1.42		0.07	0.13
	0.90	1.41		0.04	0.03
				0.04	0.12
mean ± SEM	0.88 ± 0.06	*1.45 ± 0.09	mean ± SEM	**0.09 ± 0.02	0.11 ± 0.02

Table 4-5b

Densitometer ratios of L and H-ferritin in the rat intestinal mucosa

(data n=6 ± SEM)

Regime 3: Changing from an iron deficient diet to a carbonyl iron diet at day 0, 1, 3 & 5

Regime 4: Changing from IP iron sorbitol to an iron deficient diet at day 1/4, 1 and 3

*p<0.05, **p<0.01(statistical significance as compared to day 0 from the change in diet)

Regime (3) and days after switch (n=6)	Villus L:H ratio	Crypt L:H ratio	Regime (4) and days after switch (n=6)	Villus L:H ratio	Crypt L:H ratio
0	0.56	0.43	0	0.50	0.62
	0.52	0.45		0.49	0.46
	0.64	0.92		0.85	0.21
	0.60	0.54		0.51	0.16
	0.51	0.43		0.94	0.36
	0.47	0.81		1.31	0.41
mean ± SEM	0.55 ± 0.02	0.60 ± 0.09	mean ± SEM	0.77 ± 0.13	0.37 ± 0.08
1	1.12	0.81	1/4	0.76	0.05
	0.57	0.79		0.95	0.05
	0.50	0.41		0.48	0.09
	0.62	0.34		0.45	0.15
	0.72	0.43		0.22	0.13
	0.56	0.48		0.10	0.09
mean ± SEM	0.68 ± 0.10	0.54 ± 0.08	mean ± SEM	0.49 ± 0.13	0.09 ± 0.02
3	0.63	1.61	1	0.29	0.80
	0.73	0.91		0.42	0.82
	0.56	0.17		0.24	0.11
	0.53	0.20		0.23	0.21
	0.44	1.36		0.20	0.17
	0.51	0.59		0.25	0.23
mean ± SEM	0.56 ± 0.04	0.73 ± 0.20	mean ± SEM	**0.27 ± 0.03	0.39 ± 0.13
5	0.46	0.49	3	0.55	0.75
	1.04	0.58		0.53	0.59
	0.26	0.43		0.28	0.62
	0.32	0.81		0.37	0.48
	0.52	1.72		0.19	0.27
	0.74	0.92		0.28	0.50
mean ± SEM	0.56 ± 0.06	0.82 ± 0.19	mean ± SEM	**0.37 ± 0.07	0.54 ± 0.06

4.4.6 Discussion

Ferritin synthesis is regulated mainly by a post-transcriptional mechanism (Harford & Klausner 1990) and to a lesser extent by transcription (White & Munro 1984). Little is known of transcriptional control other than that it occurs in response to increasing iron availability (Bomford *et al* 1981). Post transcriptional control of ferritin is mediated by the action of IRP with surplus ferritin mRNA in cells being repressed until iron is available such that when cellular iron is increased, ferritin synthesis also increases.

The concept of body iron stores influencing iron absorption by conditioning proliferating crypt cells in the intestinal mucosa is intriguing for a number of reasons. There is no other micronutrient known to have a similar mechanism. There is a delay in the effect of some 3 days before these cells are able to 'respond' and alter luminal iron absorption. Thirdly, this 'delay' corresponds to the time for crypt to villus migration. Using the current separation and molecular biological techniques it was possible to explore some of these mechanisms.

Contrary to expectations, steady state conditioning in the present study, revealed that rats supplemented with oral (chronic loading) or parenteral iron (acute iron loading) did not significantly alter either L or H-subunit ferritin mRNA in both villus and crypt cells. This was also reported recently by McKie *et al;* (1996) in the intestinal mucosa of mice although crypt and villus cells were not segregated (published while the current studies were undertaken). However, the L:H transcript ratios in the present study, do indicate a tendency towards an L-rich ferritin, which was observed in both crypt and villus cell (but was only significant in crypt cells in the IP iron sorbitol group). Upon translation (not studied here), this will produce isoferritins more suited to retain iron within the enterocytes and reduce iron influx to the portal circulation. Conversely, in the iron deficient group, a significant reduction in both L and H subunit ferritin mRNA was observed in only villus cells. This was also reflected in the L:H transcript ratio of villus cells although not statistically significant. It is difficult to explain the why this

phenomena was only observed in the villus cells. It may be an adaptive response following iron deficiency to conserve iron for proliferative crypt cells rather than the 'mature' villus cells coming towards the end of their life-span. Following on to this, it would appear that body iron stores do not have the tendency to stimulate the production of specific ferritin-subunit ferritin transcripts in mucosal cells by contrast to the well recognised association between body iron stores and serum ferritin (Addison *et al* 1972, Jacobs *et al* 1972, Lipschitz *et al* 1974, Powell *et al* 1975a).

The purpose of the 'switch model conditioning' (from an iron loaded diet to an iron deficient diet and from an iron deficient diet to an iron loaded diet) was to explore whether ferritin could be the mediator in the 'mucosal block' responding to information imparted to the enterocyte regarding body iron stores.

In the oral carbonyl group, a significant reduction in L-ferritin subunit mRNA expression was observed in both crypt and villus cells within 24 hours of changing to the iron deficient diet and maintained to day 5. This was also reflected in the L:H transcript ratio (a reduction) although this was only statistically significant in the villus cell. A similar observation has been reported (Topham *et al* 1992) on iron uptake by brush-border membrane vesicles whereby transfer of control rats to a low-iron diet resulted in a 2-fold increase in iron uptake after only 24 hours.

A number of inferences can be drawn from the current study: firstly, ferritin is unlikely to be the mediator since villus cells were able to 'respond' within 24 hours by altering (decreasing) L-ferritin subunit mRNA expression. If ferritin had been the mediator, one would predict an initial response in crypt cells followed by a response (reduction in L-ferritin subunit expression) in the cells along the crypt-villus axis over next 3-5 days with villus cells at the tip, perhaps demonstrating the most delayed response following from a change in diet. Secondly, this response in L-ferritin expression observed in both villus and crypt cells was more likely due to changes in luminal derived

iron rather than alterations in body iron stores as it is unlikely for body iron stores to be depleted within 24 hours.

Interestingly, the above findings were not observed for H-ferritin subunit mRNA. This may reflect that the two subunits of ferritin protein are co-ordinately regulated at the translational level but differentially regulated at the transcriptional level (Munro & Linder 1978). A third possibility would be that crypt cells are 'programmed' not by body iron stores but by a microcirculation governed by dietary iron supply i.e. the amount of iron absorbed in the villi will have an immediate effect on villus cells, such that when iron is scarce, the animal need not wait for iron stores to be reduced in order to 'relax' the control on the regulation of iron absorption. This is supported by a previous study (Topham *et al* 1991) using brush-border membrane vesicles demonstrating an increase in plasma iron and iron incorporation into mucosal ferritin (following intragastric dose of ^{59} Fe) in rats subjected to a short-term and long-term iron depletion suggesting that mucosal cells were capable of processing of iron prior to major changes in body iron status.

However, there were no demonstrable differences in ferritin mRNA expression in the iron deficient group switched to an iron loaded diet although, the ability of inorganic iron to stimulate synthesis of basic isoferritin protein in the intestinal mucosa (after 5 days on iron) has been described in patients with iron deficiency anaemia given oral iron (Halliday *et al* 1978). Several explanations are possible for the current observation. Northern hybridisation technique may not be sensitive enough in detecting mRNA changes compared to solution hybridisation (White & Munro 1988). This may be reflected in the L:H subunit transcript ratio especially in crypt cells which tended to rise by day 3 and 5 after the introduction of the carbonyl iron diet although this did not reach statistical significance. Furthermore, the duration of iron treatment was 5 days whereas Pietrangelo *et al.*, (1990) was able to demonstrate a preferential change in hepatic L ferritin subunit mRNA expression in rats maintained on an iron loaded diet for at least 10

weeks. Furthermore, in studies on iron uptake using brush-border membrane vesicles (Topham *et al* 1992), the transfer of iron-deficient rats to a control diet resulted in a progressive decrease in iron uptake which only reached that of control rats in 2 weeks.

Parenteral iron produces an acute iron overload. Rather than synthesising basic isoferritin transcripts (L-rich ferritin) which will retain iron in mucosal cells, both crypt and villus cells responded by reducing ferritin expression which was observed within 6 on introduction to the iron deficient diet. This was also reflected in the reduced L:H transcript ratio especially in the villus cell. This is perhaps a further illustration that mucosal cells were responding to changes in luminal iron rather than body stores. Reduced ferritin expression was observed for both L and H ferritin subunits by contrast to the oral iron loaded group where H-ferritin subunit mRNA expression was relatively unaffected by the subsequent dietary change. This difference could be reflected in the different iron preparations used to load animals (carbonyl iron vs iron sorbitol) and iron administration (oral vs ip injection).

In conclusion, the present study has revealed that body stores do not have the tendency to stimulate the production of specific subunit ferritin transcripts in mucosal cells. However, mucosal cells themselves were capable of responding to changes in luminal iron by synthesising specific ferritin transcripts independent of body iron status. Furthermore, this response in mucosal cell did not follow the lag-period that will account for crypt-villus migration. Nevertheless, the concept of the body iron stores conditioning the crypt cells which can subsequently influence mucosal handling of dietary iron as they migrate to functional cells at the terminal villus remains attractive although the present data does not support that this is mediated by ferritin.

5. Iron protein expression in human intestine in relation to body iron stores

This chapter has been divided into three sections. Section 5.1 describes the development of a method for isolating subpopulations of crypt and villus cells from human intestinal mucosa obtained endoscopically. Section 5.2 utilises this separation technique to examine L and H-ferritin mRNA expression in crypt and villus cell from patients with disordered iron metabolism. Section 5.3 examined expression of TfR using a monoclonal antibody to human TfR in subjects with disordered iron metabolism.

5.1 Development of a technique for separating crypt and villus cell in the human intestinal mucosa

5.1.1 Introduction

The absorptive surface of the small intestine is lined by epithelial cells which have their origin as stem cells in the crypts of Lieberkuhn. As mentioned in section 2.1, when these cells migrate out of the crypts onto the architectural villus support structure in the small intestine, they undergo differentiation to give rise to a variety of metabolically active functional cells specialised for absorption. However, factors controlling proliferation, cell migration and terminal differentiation of these cells are incompletely understood. In addition, in terminally differentiated cells, the homeostatic mechanisms influencing absorption of particular substrates have been difficult to study because of the intricate relationship between different epithelial cell subtypes lining the villus structure.

The initial development of methods to isolate relatively pure cell subpopulations along the crypt-villus axis reviewed by Remke *et al.*, (1988), have made it possible to study aspects of the life cycle and differentiation of these cells. However, in most of these reports, little histological or morphological evidence were provided about the segregated cell subpopulations. The most successful of these methods have been described by

Weiser (1973) more recently, Flint *et al.*, (1991) used a low temperature at 4 °C to demonstrate that intact RNA could be isolated from the relatively pure subpopulations of cells obtained. These methods were developed using rat and mouse small intestine where sample size is not a problem. The utilisation of methods for isolating RNA from human small intestine from endoscopic biopsies as a routine presents a challenge because of the limited amount of tissue that would normally be available.

5.1.2 Materials and methods

Hanks balanced salt solution (HBSS: low calcium and magnesium) was obtained from Hyclone, U.K. Methyl(3H)-thymidine (specific radioactivity 79 Ci/mmol) was obtained from Amersham, and media for incubating endoscopic biopsies from Gibco-Betsada Research Laboratories, U.K. which consisted of Gibco-Trowells T8 (65% vol), Gibco-NTC-135 (20%vol), Gibco-foetal calf serum (15% vol), Gibco-L-Glutamine 200mM, benzylpenicillin (600 mg/ml) and streptomycin sulphate (167 mg/ml). Incubating conditions for biopsies are in section 5.1.2 (ii).

(i) Isolation of crypt and villus cells from human duodenal mucosa

Human small bowel tissues were obtained from patients who were undergoing routine endoscopy for dyspepsia at Aintree Hospitals Liverpool. Approval for this study has been granted by South Sefton Ethical Committee. Endoscopic duodenal biopsies (n = 4 to 6), each weighing between 15-20 mg were obtained using an Olympus IT 20 endoscope and large FB 13K biopsy forceps. These biopsies were amalgamated in HBSS with 0.5mM DTT on ice and epithelium was isolated as described in Table 5-1.

Essentially, the separation technique entails serial processing of tissue samples for progressively longer duration in chelation buffer and with progressively more vigorous mechanical inversions. Cell fractions thus obtained were frozen in liquid nitrogen and

stored at -80 °C until required for extraction of RNA or biochemical analysis. RNA can be reliably isolated from such material stored for up to two weeks.

(ii) Validation of segregated cell population subtypes

The purity and character of the cell fractions obtained were then determined. Serial removal of different cell types was assessed histologically by staining the biopsy remnant at each step with haematoxylin and eosin, assessing enzyme activity (villus cells) and by 3H-thymidine incorporation (crypt cells).

Enzyme activity (alkaline phophatase) was determined as described in section 2.1.2 (iv) using absorption spectrophotometry to detect the release of p-nitrophenol at 420 nm. Results (Figure 5-1[top]) are expressed as standardised units per mg protein assayed by the modified Lowry technique (1951) as described in section 2.1.2 (iv).

Crypt cell enrichment of all cell fractions were identified by 3H-thymidine uptake. Endoscopic biopsies were incubated in Falcon culture dish (Figure 5-3) for 6 hours at 37 °C in 95 % oxygen, 5 % carbon dioxide containing 0.9 mls of media (section 5.1.2) and 2 µl of 3H-thymidine (specific activity 79 Ci/mmol). Under these culture conditions, preliminary experiments have shown these endoscopic small bowel biopsies to be viable for up to 48 hours. Following incubation, biopsies were washed six times in 10 mls chelation buffer. Separation of cell subpopulations was then undertaken. Segregated cells were recovered as described in section 2.1.2 (iv) and results, (Figure 5-1[bottom]) expressed as dpm per mg protein also assayed by the Lowry method (1951).

At each sequential stage of the isolation procedure, a biopsy remnant was removed and processed for histological examination after staining with haematoxylin and eosin (Figure 5-2).

Table 5-1

Isolation of human duodenal mucosa along the crypt-villus axis

Incubate endoscopic biopsies (four to six) in 5 mls Hanks buffer

in 0.5mM DTT at 4 °C for 1 minute

Discard supernatant

Transfer biopsies to 10 mls of *chelation buffer

Incubate at 4 °C with constant stirring for 25 minutes. Remove biopsies

Pellet cells at 10,000g for 10 minutes

This cell fraction is **Vw**

Transfer biopsies to 2 mls *chelation buffer

Shake by hand, approximately 30 inversions

Collect supernatant

Repeat twice in 2 mls of cold *chelation buffer

Pellet cells as before. This gives cell fractions V_1, V_2 and V_3

Transfer biopsies to 10 mls *chelation buffer

Incubate at 4 °C for 10 minutes with constant stirring

Discard supernatant

Transfer biopsies to 4 mls of cold *chelation buffer

Shake by hand (approximately 60-90 inversions) or until tissues float

to the top on standing. Remove biopsy remnant. Pellet cells as before

This cell fraction is C_2

* Details of chelation buffer can be obtained in the appendix in Chapter 2

(iii) RNA extraction, separation and Northern hybridisation

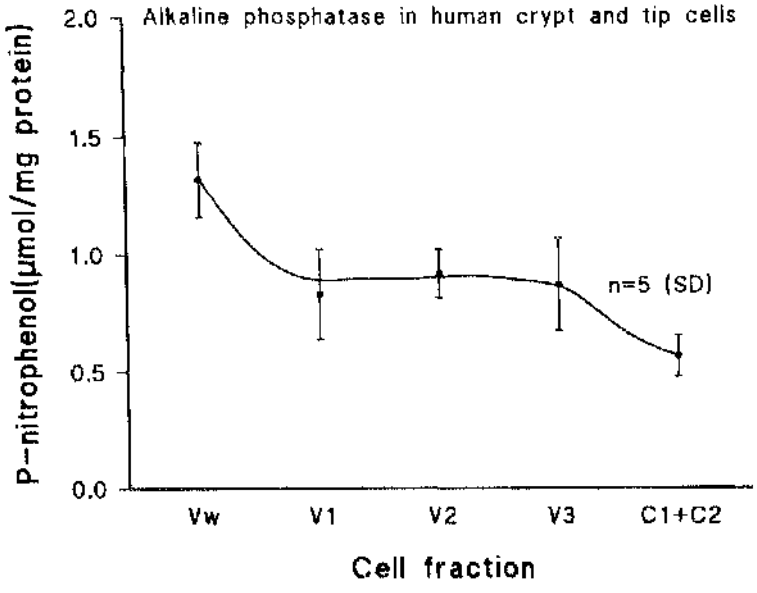
RNA was extracted from endoscopic duodenal cells using acid guanidinium thiocynate-phenol-chloroform method (section 2.3.1). RNA separation and capillary transfer of RNA were undertaken as described in section 2.3.3 and 2.3.4.

Two probes were available to examine mRNA expression whose proteins have a predilection for either proliferating cells in the crypts i.e. TfR (Anderson *et al* 1990, Pietrangelo *et al* 1992) and sucrase-isomaltase for absorptive functional cells in the villus architecture (Green *et al* 1987). Sucrase-isomaltase cDNA (300 bp) was a generous gift from Drs. X. Wang and D. Swallow, University College, London. This was labelled using a random primer kit as described in section 2.3.5 (i). TfR probe is a 30 base single stranded oligonucleotide, labelled using a terminal transferase enzyme kit as described in section 2.3.5 (iv). As a control to normalise for RNA loading., a 26-base oligonucleotide the sequence (5'-AAC GAT CAG AGT AGT GGT ATT TCA CC-3') corresponding to human 28S rRNA (Barbu & Daultry 1987) was used. This ribosomal sequence was labelled using terminal transferase as for TfR. For both oligonucleotide probes (TfR and 28S rRNA), excess label was removed using a Sephadex G-50 column which was prepared as described in section 2.3.5 (iv). Removal of excess label was not necessary in random priming.

Figure 5-1

Alkaline phophatase activity and 3H-thymidine incorporation in segregated cells on the human intestinall mucosa

Note that enzyme activity is highest in villus cell (Vw) and lowest in crypt cell (C2) and thymidine incorporation is highest in crypt cells (C2) and least in villus cell (Vw)



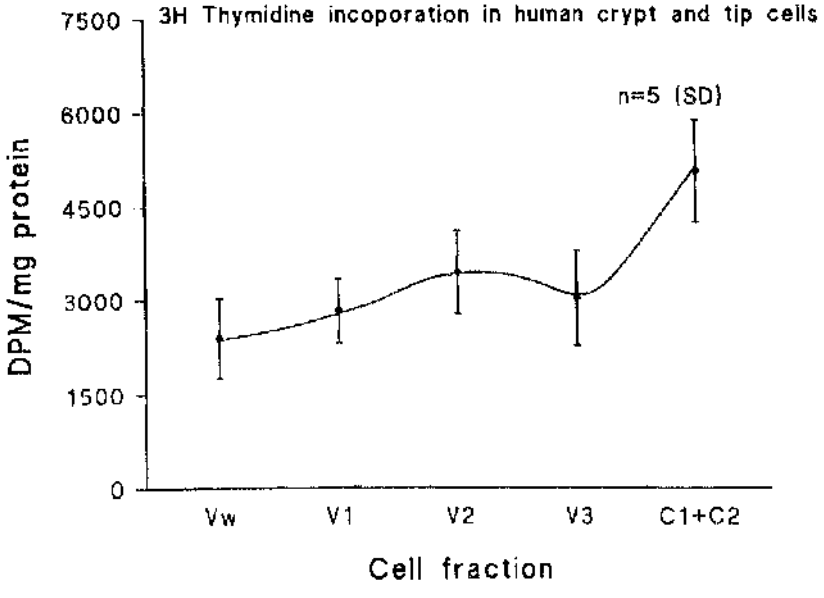


Figure 5-2

Histological verification of separation technique

Key: Vo: longitudinal section of rat small bowel, Vw: after 25 minutes in chelation buffer, V1: after 30 inversions, V3: after 90 inversions, C1: after further incubation and 30 inversions and C2: after 30 inversions (X 200)

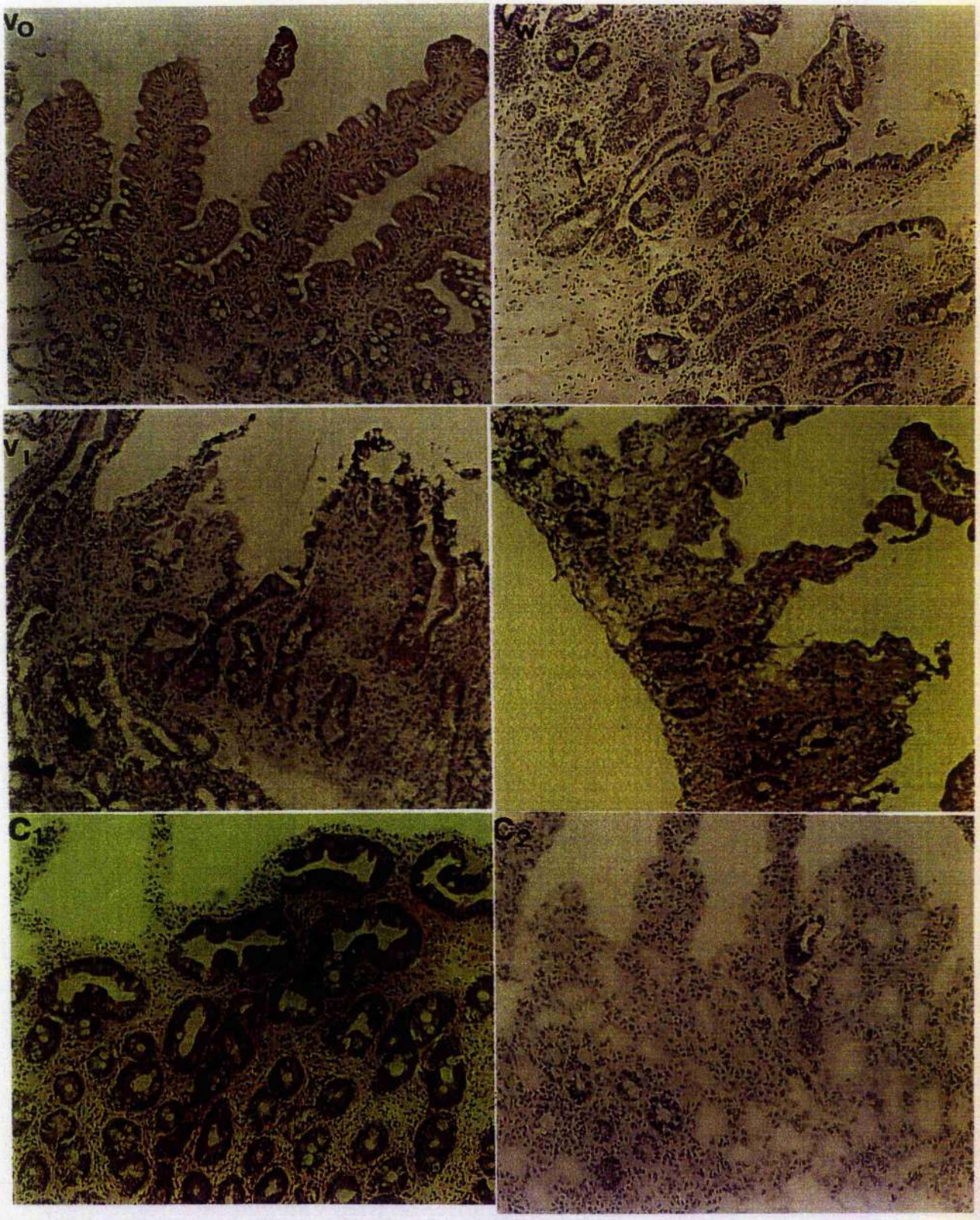
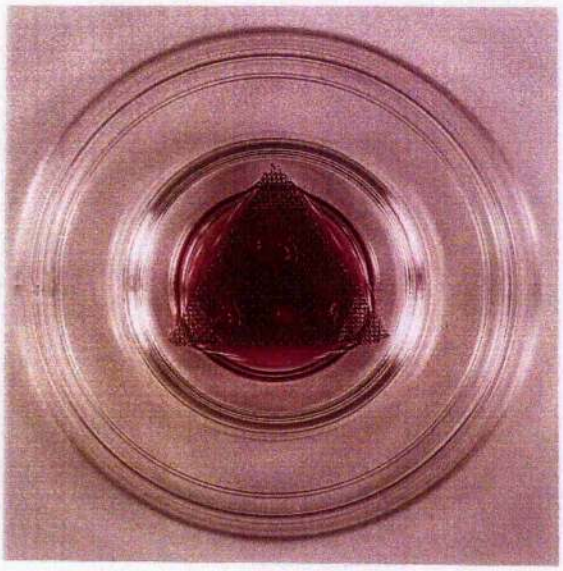


Figure 5-3

Falcon culture dish for incubating intestinal biopsies

Note the larger duodenal biopsies (top) obtained from FB 13 biopsy forceps compared to smaller duodenal biopsies (bottom) from conventional biopsy forceps



(iv) Prehybridisation, hybridisation and washing conditions

For sucrase-isomaltase, membranes were prehybridised at 68 °C for 20 minutes in 6 mls of Quickhyb^{R} (Stratagene, Cambridge). Hybridisations were then carried at 68 °C for 1 hour. After hybridisation, membranes were washed in the following solutions with increasing stringency: 2x SSC / 0.1 % SDS (15 minutes at room temperature), the same solution (another 15 minutes at 60 °C) and finally twice in 1x SSC/0.1 %SDS (15 minutes at 60 °C). For TfR, optimal conditions for pre-hybridisation, hybridisation and washing conditions were carried out at 42 °C as described in section 2.3.5 (iv). For 28S rRNA control, prehybridisation and hybridisation reagents were similar to TfR but hybridisation was carried at room temperature. Washing conditions were undertaken as for sucrase-isomaltase. Autoradiographs were quantified by scanning densitometery and results expressed as a ratio of mRNA to 28S rRNA.

5.1.3 Results

Biopsies were obtained using an Olympus IT20 endoscope which can accommodate the larger FB 13K biopsy forceps. Each endoscopic biopsy obtained weight approximately 15-20 mg compared to 5 mg biopsies obtained using conventional endoscopic biopsy forceps. I require between 4 to 6 such biopsies to obtain sufficient RNA for mRNA detection by Northern blotting. To reduce RNA degradation, all equipment were autoclaved and washed in 0.1 % DEPC water and RNA extraction were undertaken within 2 weeks of cell separation after being stored at -80 °C.

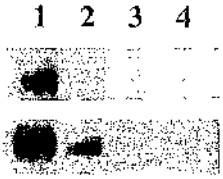
The study of alkaline phosphatase expression and 3H-thymidine incorporation (Figure 5-1) in segregated cells reveal a 3 fold gradient of activity/incorporation between crypt and villus cells. This compares favourably to previous reports (Weiser 1973, Flint *et al* 1991) on segregation in the intestinal mucosa of rats and mouse.

Study of mRNA expression revealed that sucrase-isomaltase mRNA expression is high at the villus and low in the crypt cells, a 4 fold difference (Figure 5-4a). This is consistent with the enzymatic location of sucrase-isomaltase, a family of disaccharides, which occurs in the brush-border membrane of enterocytes catalysing the breakdown of dietary di and oligosacharides. A cDNA probe for human jejunal brush border sucrase-isomaltase has been isolated (Green *et al* 1987) where mRNA expression has been demonstrated in the human jejunum and in the enterocyte cell-line Caco-2. By contrast, utilisation of the TfR oligonucleotide probe reveal TfR mRNA expression to be greater (a six fold difference) in crypt than villus cell (Figure 5-4 b). This is consistent with previous immunohistochemical studies on TfR expression in mucosal cells of rodents and humans (Banergee *et al* 1986, Lombard *et al* 1990, Anderson *et al* 1990) with greater receptor expression in crypt to villus cells and is also consistent with proliferative crypt cells having a higher requirement for iron. Relative densitometer ratios for sucrase-isomaltase and TfR mRNA illustrating higher mRNA expression of

sucrase-isomaltase mRNA in the villus and TfR mRNA in the crypt have been summarised in Figure 5-5.

Figure 5-4
(a) Sucrase-isomaltase

Sucrase-isomaltase mRNA normalised for RNA loading with 28S rRNA. Lane 1, Vw from 6 amalgamated biopsies (0.5 µg RNA/mg tissue); lane 2, C2 (0.25µg RNA/mg tissue) ; lane 3, Vw from 6 biopsies from conventional forceps (0.5µg/mg tissue) lane 4, C2 from 6 biopsies from conventional biopsies (0.25µg/mg tissue). Note the higher expression of sucrase-isomaltase mRNA in Vw (lane 1) compared to C2 (lane 2). Note also sucrase isomaltase mRNA was not detected in biopsies obtained from conventional small forceps in both Vw and C2 (lane 3 and 4)



---------- Sucrase-isomaltase mRNA

---------- 28S rRNA

mRNA : rRNA ratio Vw 1.41

C2 0.34

(b) TfR

TfR mRNA normalised for RNA loading with 28S rRNA in 6 amalgamated biopsies obtained from large FB 13K biopsy forceps. Note the higher expression of TfR mRNA in C2 [0.25 µg RNA/mg tissue] (lane 2) compared to Vw [0.5 µgRNA/mg tissue] (lane 1)

---------- TfR mRNA

---------- 28S rRNA

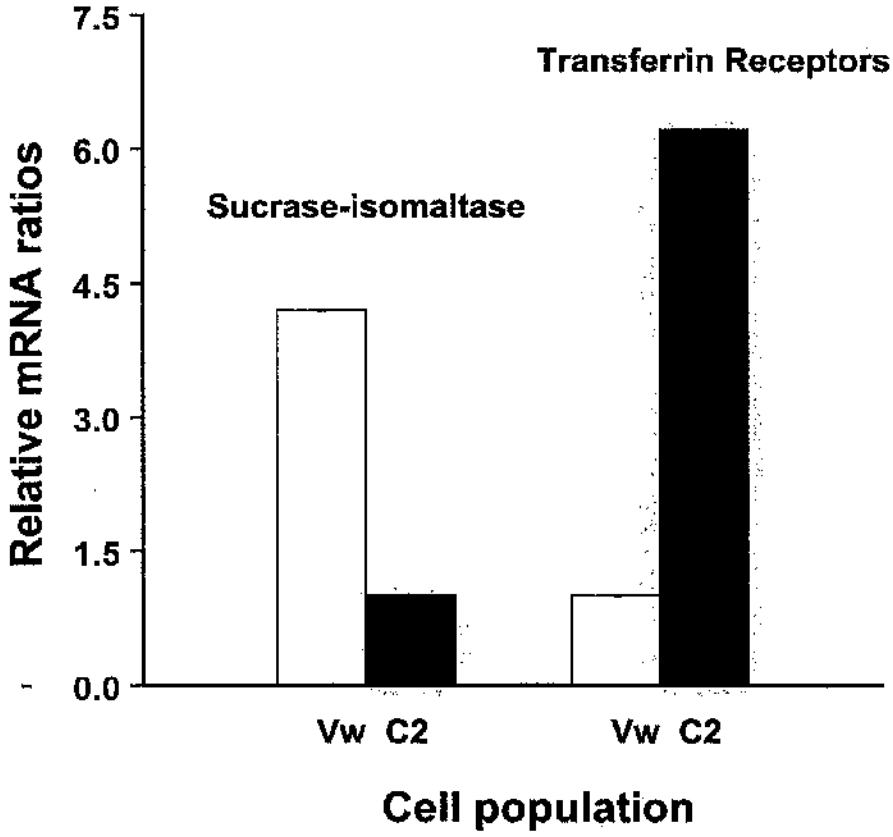
mRNA : rRNA ratio Vw 0.08

C2 0.52

Figure 5-5

Sucrase-isomaltase and TfR relative ratios

Note sucrase-isomaltase is mainly expressed in (Vw) villus and TfR mainly expressed in (C2) crypt cells



5.1.4 Discussion

The base of the intestinal mucosa contains crypt cells which are undifferentiated, mitotically active cells. Differentiation occurs as the cell moves up the villus architecture. The initial methods developed to segregate these cells provided little histological and morphological evidence for the different cell subpopulations. Weiser (1973), however, isolated crypt and villus fractions from adult rat small intestine using a bivalent cation-chelating agent at 37 °C and provided biochemical evidence for the different cell subpopulations. Fractions were analysed for certain brush-border enzymes (e.g. sucrase and alkaline phophatase activity in villus cells, thymidine kinase in crypt cells) and incorporation of 3H-thymidine into the primitive undifferentiated crypt cells. Weiser was able to demonstrate an apparent gradient of functionally villus cells (Vw) from primitive undifferentiated crypt cells (C_2).

Isolation of epithelial cells at 37 °C, however, did not preserve cell integrity as internalisation of certain surface receptors e.g. insulin and epidermal growth factor receptors to intestinal cells can occur within an hour at body temperature (Gallo-Payet & Hugon 1984,1985). In addition, isolation at 37 °C can result in a loss of mRNA transcripts and their protein products (Weiser *et al* 1987). These deleterious effect could be overcome to some extent if isolation was carried out at 4 °C and this has been achieved in the rat and mouse intestinal mucosa where tissue availability is not a problem (Flint *et al* 1991).

I have developed a technique by which a purified population of proliferative and terminally differentiated epithelia of the human intestinal mucosa could be obtained at 4 °C. This method produced initial fractions (Vw) of relatively pure villus cells which express characteristics of terminally differentiated enterocytes found on the villus but not in the crypt. Similarly, fractions obtained at the end of the separation (C_2) consisted of an enriched suspension of crypt cells.

Relatively pure subpopulations of crypt and villus cells can be obtained by this low temperature method and the yields are suitable for mRNA studies. Morphological integrity is maintained in these cells. This separation technique will also be potentially useful to study changes in the control of proliferation, cell migration, terminal differentiation in the human intestinal mucosa.

5.2 Ferritin expression in crypt and villus cells in subjects with iron deficiency anaemia and genetic haemochromatosis

5.2.1 Introduction

Having adapted and verified the separation technique for crypt and villus cells in the human intestinal mucosa, I then proceeded to examine ferritin mRNA expression in these cell subpopulation in patients with iron deficiency anaemia (IDA) and GH. Iron absorption is a carefully regulated biological process which is able to respond by increasing iron absorption in the proximal small intestine in iron deficiency or blood loss but to decrease uptake from the intestinal mucosa when iron stores become replete. In GH, an autosomal recessive inherited defect, the regulating mechanism is no longer intact resulting in excessive iron absorption from the diet despite raised body iron stores. Unrecognised and untreated, GH leads to a lethal accumulation of body iron. The pathogenesis of GH remain unclear. It is thought to involve defect(s) in the control of duodenal iron absorption either on the luminal site, which would result in raised iron deposition in the enterocytes; and/or defects on the serosal iron transfer which will result in enhanced release of adsorbed iron to the portal circulation.

Several proteins have been implicated in the control of cellular iron metabolism. Three of these TfN, TfR and ferritin have been discussed in Chapter 3 and 4. Ferritin, the major iron storage protein has been viewed as the key factor in controlling mucosal iron uptake, 'the mucosal block' which has been discussed in Chapter 4 with two

immunologically distinct ferritin chain, with different affinity to accept and release iron (Arosio *et al* 1978). Recent molecular biological techniques have provided some insights on iron metabolism specifically in the regulation of expression of TfR and ferritin by the interaction of mRNA elements like the IRE and the cytoplasmic IRE-BP or IRP as discussed in section 1.3.7. Potentially, these may provide new insights into the metabolic abnormalities in GH.

Using the technique for separating crypt and villus cells in the human intestinal mucosa, I undertook a study to examine the relationship between body iron stores and ferritin mRNA expression in mucosal cells of patients with IDA and GH.

5.2.2 Methods and Materials

(i) Subjects

This study was undertaken in accordance with the ethical committee at Aintree Hospital, Liverpool and had been approved by South Sefton ethical committee. Informed consent was obtained from all subjects who underwent peroral endoscopy after a 10 hour fast to obtain duodenal biopsies. The study was carried in 3 control volunteers, 3 patients with GH and 4 patients with IDA. Endoscopic small bowel tissues were obtained from patients who were undergoing routine endoscopy for dyspepsia at Aintree Hospital Liverpool. Control subjects (1 male and 2 female; age 23-43 years) had no history of disordered iron absorption. There were three male patients with GH aged between 54-69 years. The diagnosis of GH was based on a liver biopsy showing raised parenchymal iron, a transferrin saturation of more than 62% and a raised serum ferritin greater than 600 mg/litre. All three patients had not been venesected prior to the study. There were 4 patients with IDA (2 male, 2 female; age 42-58 years). The diagnosis of IDA was based on a haemoglobin concentration of less than 10 g/dl, a mean corpuscular volume of less than 76 f/l and a serum ferritin of less than 10 mg/litre.

(ii) Probes and gene cloning

The pFr and Pr3 clones for L (approximately 900 bp) and H subunits (approximately 850 bp) of human ferritin (a generous gift from Dr. Antonello Pietrangelo) in the vector PBR 322 was transformed into DH5 1-α competent cells (Promega, Biotech). This was undertaken as described in section 2.2.2 (ii). Plasmid DNA was extracted using the large scale preparation method in caesium chloride-ethidium bromide in section 2.2.2 (iv-vii).

Human L and H-ferritin sequences are almost full-length complementary DNAs that have been shown to recognise specifically ferritin mRNA (Santoro *et al* 1986, Costanzo *et al* 1984) were removed from the plasmid using the restriction enzyme Pst1 (Figure 2-8 and Figure 5-6a). A 28S rRNA probe containing a 26-base oligonucleotide which recognises human 28S rRNA (Barbu & Daultry 1989) was used as control. Labelling of cDNA's and oligonucleotide sequence with ^{32}dCTP, hybridisation/washing conditions for human ferritin probes and 28 S rRNA were as described in section 2.3.5.

5.2.3 Results

Iron status of the subjects in the present study have been summarised in Table 5-2. Densitometery readings of all the subjects for each group are shown in Table 5-3 and summarised in Figure 5-6b. In controls, IDA and GH, ferritin transcripts (both L and H) did not differ significantly in their respective crypt and villus cells (Figure 5-6b). However, ferritin L mRNA was significantly lower in both crypt ($p<0.01$) and villus cells ($p<0.01$) in patients with IDA compared to control subjects (Figure 5-6b). This was also observed for ferritin H mRNA being significantly lower in crypt ($p<0.01$) and villus cell ($p<0.001$). Contrary to expectations, patients with GH also had significantly lower L and H ferritin subunit transcripts in both crypt ($p<0.01$ for L-ferritin, $p<0.001$ for H-ferritin) and villus cells ($p<0.05$ for L-ferritin and $p<0.01$ for H-ferritin) compared to control subjects (Figure 5-6b).

Figure 5-6a

Autoradiogram for L and H-ferritin (human cDNA probe)

1= villus cells from 6 (15-20 mg) endoscopic biopsies; 2= crypt cells from 6 (15-20 mg) endoscopic biopsies

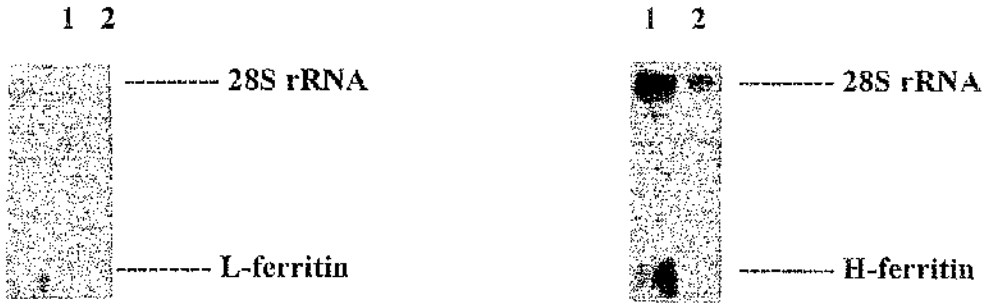


Table 5-2

Iron status of subjects studied

Subjects	Age (yrs/sex)	Haemoglobin (g/dl)	Transferrin Saturation (%)	Serum ferritin (μ/L)
GH 1	57/M	14.3	97	2400
GH 2	54/M	14.8	93	2510
GH 3	69/M	13.7	90	1730
IDA 1	53/M	9.2	10	7
IDA 2	45/M	8.4	6	9
IDA 3	42/F	7.8	3	3
IDA 4	55/F	7.6	7	11
Control 1	43/F	13.2	39	36
Control 2	33/F	13.8	29	28
Control 3	40/M	14.6	42	42

GH: Genetic haechromatosis, IDA: Iron deficiency anaemia

Table 5-3 Densitometer readings (a.u.) in patients with diiferent iron states on ferritin expression in crypt and villus cells

Data n=3 ± sem for controls & GH and n=4 ± sem for IDA. Key: *p<0.05, **p<0.01, ***p<0.001 compared to controls, a.u: arbitary units, IDA:iron deficiency anaemia, GH:genetic haemochromatosis

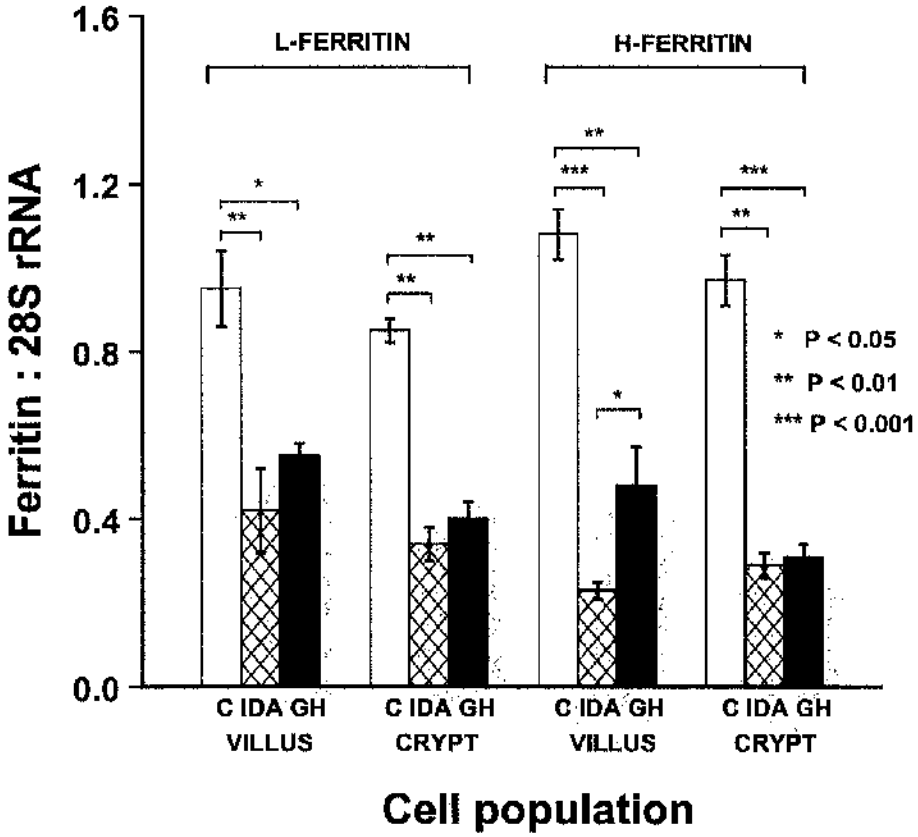
Iron state	H-ferritin villus cell (a.u. x10^5)	L-ferritin villus cell (a.u. x10^5)	28S rRNA villus cell (a.u. x10^5)	H-ferritin : 28S rRNA (villus cell)	L-ferritin: 28S rRNA (villus cell)	H-ferritin crypt cell (a.u. x10^5)	L-ferritin crypt cell (a.u. x10^5)	28S rRNA crypt cell (a.u. x10^5)	H-ferritin : 28S rRNA (crypt cell)	L-ferritin: 28S rRNA (crypt cell)
Control 1	0.461	0.422	0.391	1.18	1.08	0.163	0.154	0.175	0.93	0.88
Control 2	0.326	0.276	0.413	0.79	0.67	0.143	0.100	0.132	1.08	0.76
Control 3	0.600	0.556	0.551	1.09	1.01	0.076	0.081	0.091	0.83	0.89
mean ± sem				1.08 ± 0.06	0.95 ± 0.09				0.97 ± 0.06	0.85 ± 0.03
IDA 1	0.082	0.253	0.409	0.20	0.62	0.033	0.037	0.124	0.27	0.30
IDA 2	0.109	0.148	0.496	0.22	0.30	0.050	0.047	0.138	0.36	0.34
IDA 3	0.106	0.159	0.469	0.23	0.34	0.042	0.069	0.169	0.25	0.41
IDA 4	0.144	0.233	0.555	0.26	0.42	0.037	0.044	0.148	0.25	0.30
mean ± sem				***0.23 ± 0.02	**0.42 ± 0.01				**0.25 ± 0.03	**0.34 ± 0.04
GH 1	0.049	0.083	0.159	0.31	0.52	0.066	0.056	0.174	0.38	0.32
GH 2	0.064	0.067	0.108	0.59	0.62	0.083	0.116	0.297	0.28	0.39
GH 3	0.105	0.092	0.184	0.57	0.50	0.029	0.052	0.107	0.27	0.49
mean ± sem				**0.48 ± 0.09	*0.55 ± 0.03				**0.31± 0.03	**0.40± 0.04

Figure 5-6b

Ferritin expression in crypt and villus cell in subjects with disordered iron metabolism (n=3 ± SEM for Con & IDA and n=4 ± SEM for IDA

(statistical significance as compared to control subjects)

Key: Con :Control, IDA: Iron deficiency and GH: Genetic haemochromatosis



5.3 Immunohistochemical study of TfR expression in the intestinal mucosa of patients with iron deficiency anaemia and genetic haemochromatosis

5.3.1 Methods

(i) Subjects investigated

Duodenal biopsies were obtained from 3 subjects with GH, 4 subjects with IDA and 3 controls who underwent peroral endoscopy with dyspepsia. In addition there was a 32 year of female subject who was previously noted to have an IDA . She was prescribed ferrous sulphate (200 mg three times a day for 3 months). At the time of endoscopy, her serum ferritin was similar to control subjects.

(ii) Immunohistochemistry

These were undertaken using the monoclonal antibody OKT9 for detection of TfR (Sutherland *et al* 1981), obtained from Ortho Pharmaceuticals, Raritan, on duodenal biopsies in subjects with IDA, on iron supplements, control subjects and in GH as described in section 2.4.1. To ensure uniform and comparable conditions, cryostat sections (6 μm) from all patients were mounted in grid fashion on glass slides in duplicate for the antibody. As a negative control, incubations were undertaken without the first antibody. A subjective assessment of the staining intensity for each group was made by 2 independent observers and graded from 0 (none) , 1 (mild), 2 (moderate) and 3 (intense) staining.

5.3.2 Results

(i) Distribution and localisation of TfR staining in duodenal mucosal cells

There were differences in the TfR staining in the of the duodenal mucosa between the groups studied (Figure 5-7 a-d). Receptor staining in the columnar villus epithelial cells was confined to the subnuclear and basolateral regions in control subjects, iron deficient patients and in patients with primary overload. However, staining was more

intense in the iron deficient subjects which was observed on the entire length of the villus architecture (Figure 5-7c). In a patient receiving iron supplements, staining was less intense than control and was mainly confined to cells in the basal villus architecture with decreasing receptor expression from crypt (+2) to villus tip (0) (Figure 5-7 d).

By contrast, in subjects with GH, intense staining for TfR (Figure 5-8) was observed in both crypt (3) and almost the entire length of the villus architecture (2). Perls potassium ferricyanide staining in GH subjects reveal haemosiderin deposits in liver but not in duodenal tissues (Figure 5-9 a- b).

Figure 5-7

TfR expression in duodenal mucosa in subjects with disordered iron metabolism

(a) Control subjects (note reduced expression from crypt to villus, top left); **(b) Negative control** (top right); **(c) Iron deficiency anaemia** (note enhanced receptor expression up to the terminal villi, bottom left); **(d) Effect of iron supplementation** (note receptors are down-regulated and are mainly confined to crypt cells, bottom right)

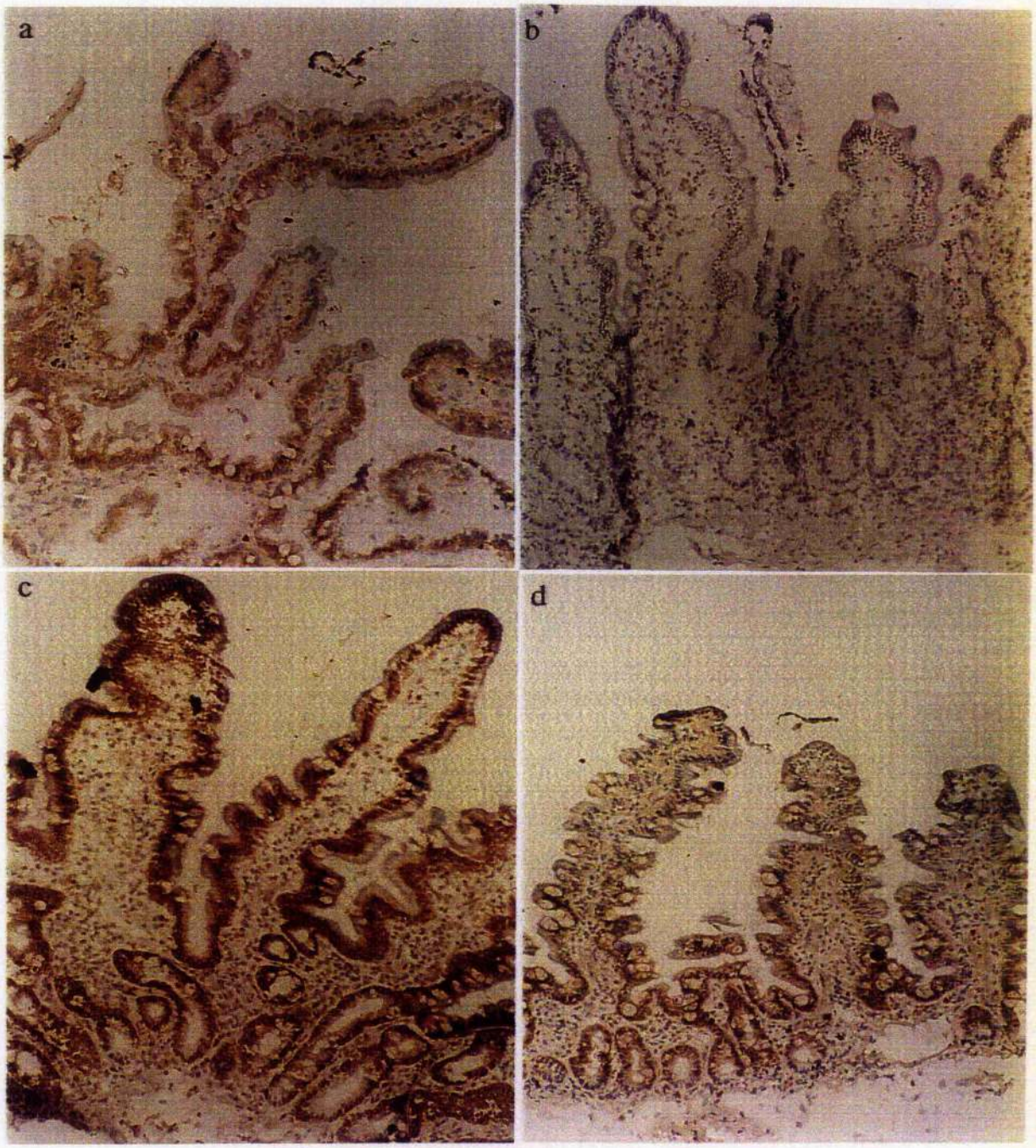


Figure 5-8

TfR expression in duodenal mucosa in GH

Immuohistochemistry from 2 patients GH1 and GH2 (note enhanced receptor expression from crypt and almost the entire length of the villus architecture)

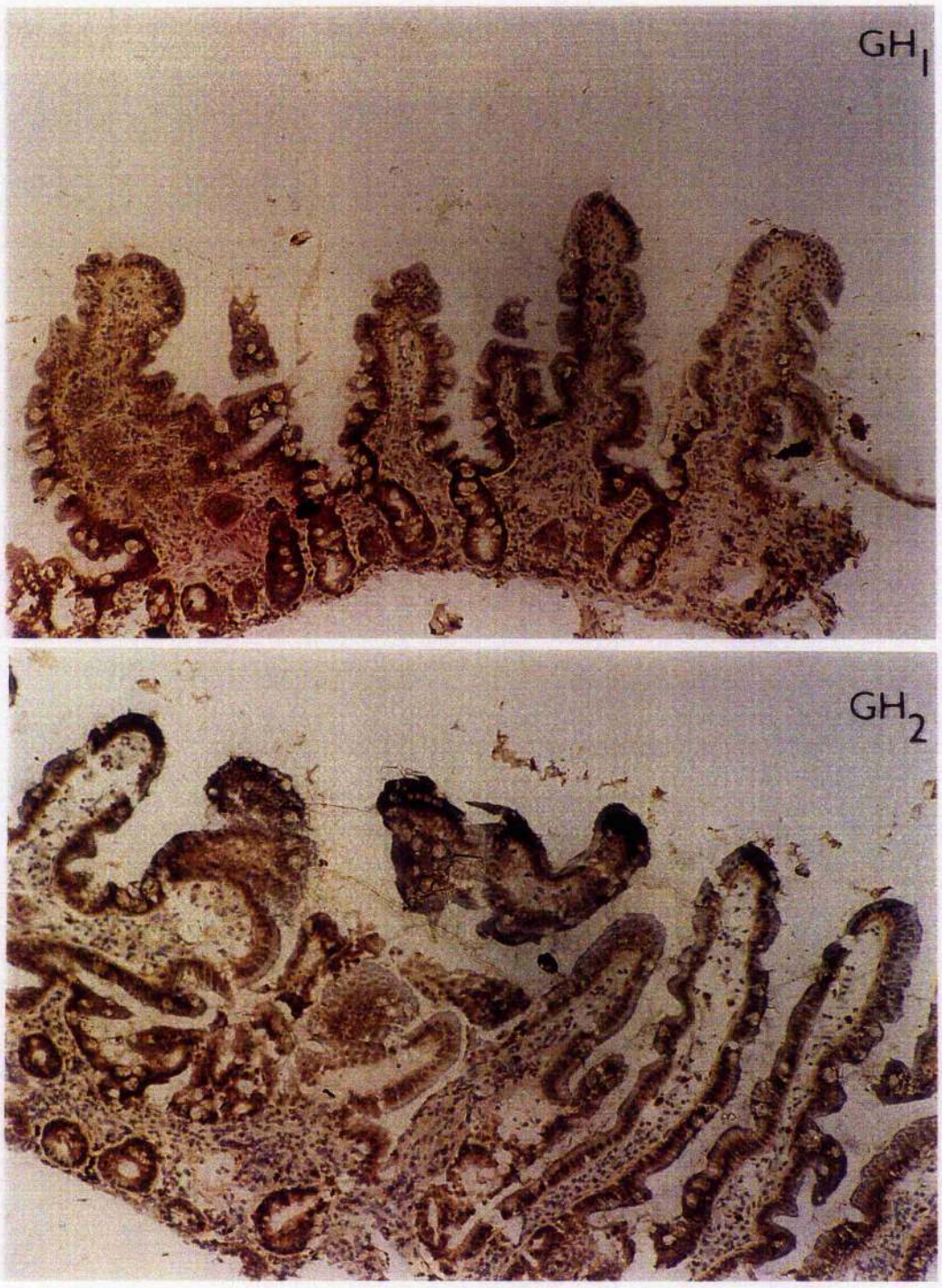
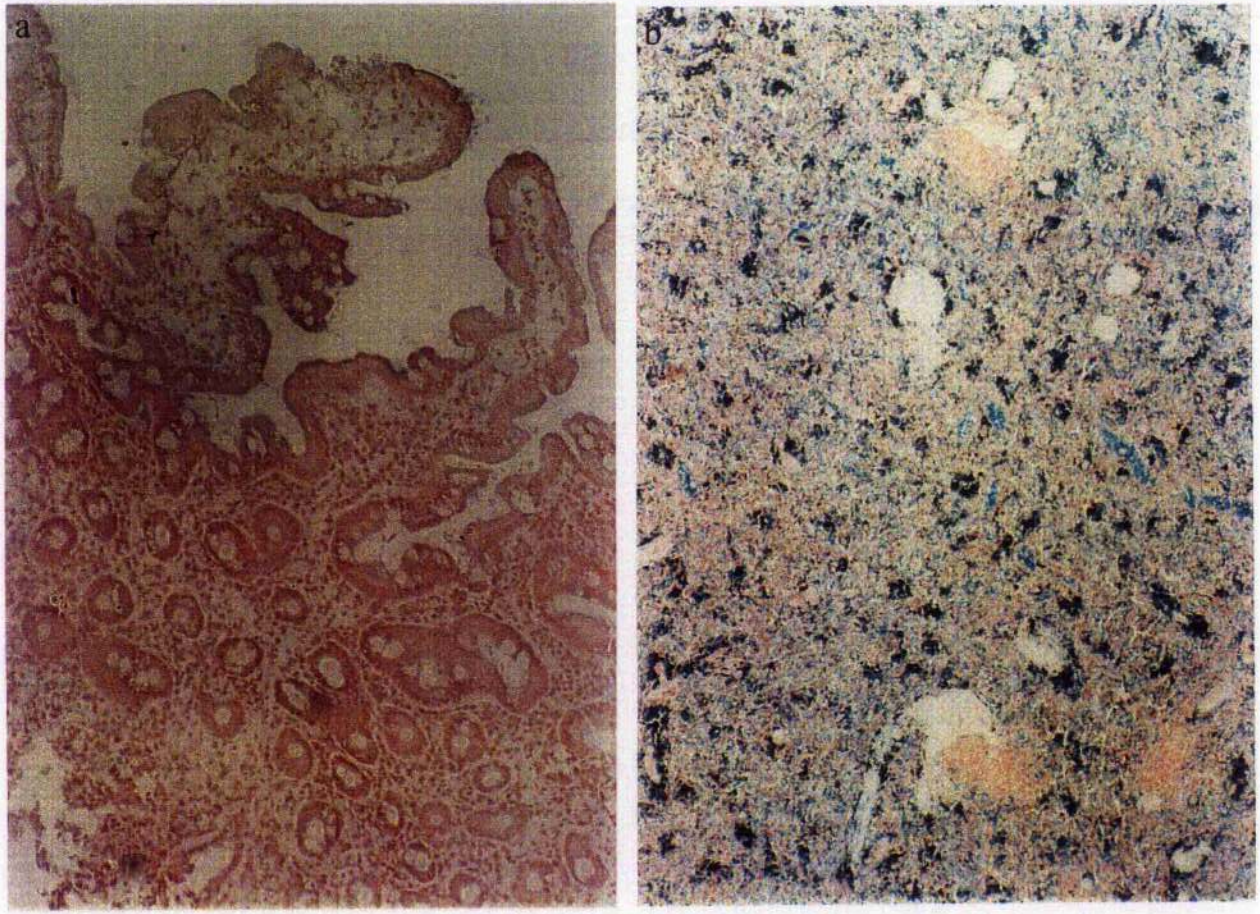


Figure 5-9

Perls staining in genetic haemochromatosis

(a) Small intestine (left). Note absence of haemosiderin **(b) Liver** (right). Note the different intensity of haemosiderin staining.



5.3.3 Discussion

Immunohistochemical studies on TfR expression confirmed the inverse relationship between receptor expression and body iron stores. However, in GH, TfR were not downregulated. Initially it was thought to represent a site specific defect (Lombard *et al* 1990) but this may just reflect enhanced enterocyte iron transfer to plasma.

In the present study a semi-quantitative analysis of duodenal crypt and villus cells was undertaken to study ferritin mRNA expression in subjects with disordered iron metabolism. In IDA subjects, both L and H ferritin transcripts were reduced in the absorptive and the non absorptive cells. As cellular iron is diminished in IDA, ferritin expression may be expected to be is reduced as less protein will be required for iron storage and detoxification.

Trousseau (Trousseau 1865) was the first to describe iron overload in a diabetic patient who had bronze skin and a granular liver on post mortum in the later half of the nineteenth century. Von Recklinghausen assigned the term 'haemochromatosis' to this condition in 1889. The hereditary nature of the disease was only recognised by Seldon in 1927 and it took half a century before the inheritance of this condition was recognised to be autosomal recessive (Simon *et al* 1977). Recently, a Cys-282-Tyr mutation in the HLA-H region of chromosome 6 was found to be associated with GH (Feder *et al* 1996). However to date, the precise role of HLA H in iron metabolism is currently not known.

Previous immunohistochemical and electron microscopy studies on ferritin expression in the duodenum of patients with GH have attempted to clarify the role of ferritin but with conflicting results. Two separate groups using similar techniques (Whittaker *et al* 1989, Francanzani *et al* 1989) failed to detect ferritin in the absorptive cells of the duodenum although ferritin was observed in cells of the lamina propria. Sibille *et al;* however (1988), could not identify ferritin in the macrophages of the lamina propria.

The site of the metabolic defect in GH remains unknown. In theory, this defect could be on the enterocyte (on the luminal surface, mucosal or both) or a more widespread disorder affecting the liver and the RE system. Since iron absorption is primarily controlled at the site of absorption in the proximal intestine, a generalised membrane defect in the cells of the RE and liver seems less likely. Previous physiological studies on iron metabolism seem to suggest that the defect may lie in both mucosal uptake and the release of iron to the portal circulation (Boender & Verloop 1969, Marx 1979). To date, the most promising candidate protein to mediate enhanced mucosal iron uptake in GH is the 160 kDa iron binding protein (Teichman & Stremmel 1990) especially when it was revealed that the protein was inappropriately upregulated in GH (Stremmel 1991). Further support for a mucosal defect is seen in a copper loading disorder (Menke disease), a condition not too dissimilar from GH where the abnormality has been localised to a defective membrane copper transporting ATPase (Davies 1993). On the other hand, iron kinetic studies in enterocytes of GH subjects (McLaren *et al* 1991) tended to suggest that mucosal transfer of iron to the portal circulation may be the major determining factor. The reality is that neither has been definitely identified as the rate limiting step.

The present data revealing that both L and H-ferritin transcripts in GH subjects were significantly lower compared to control subjects is in agreement with a recent observation (Pietrangelo *et al* 1995) utilising *insitu* hybridisation techniques. In a previous study by the same group (Pietrangelo *et al* 1992), on whole small bowel endoscopic biopsies (containing both villus and crypt cells) ferritin transcripts were also inappropriately low in these subjects. Furthermore, ferritin excretion studies in GH also reveal lower faecal ferritin (both L and H) than expected for their body iron status (Skikne *et al* 1995). Therefore could the defect in GH be due to a failure of the molecular mechanisms governing ferritin expression?. The molecular regulation of ferritin expression is linked to the molecular regulation of TfR which controls iron entry from

plasma to target cells (section1.3.7). This mechanism for controlling the availability of iron to target cells probably operates in the enterocyte since an inverse expression of these proteins in relation to body iron stores has also been demonstrated (Pietrangelo *et al* 1992, Anderson *et al* 1990). However, this mechanism in the bowel is only likely to govern iron entry from plasma since TfR are only found on the basolateral side.

IRP activity (a reflector of duodenal iron pool) and TfR expression has been studied by several investigators in the intestinal mucosa of GH. TfR 'fail' to downregulate (Banerjee *et al* 1986, Lombard *et al* 1990) and IRP activity (Pietrangelo *et al* 1995) is increased in patients with GH both reflecting increase iron transfer from mucosa to plasma. Increased mucosal iron transfer to the portal circulation will render mucosal cells in GH relatively 'iron deficient' and may explain ferritin expression comparable or less than control subjects. This observation would tend to imply that enhanced iron-uptake by iron transport proteins at the luminal surface of the enterocyte e.g. Stremmel's iron binding protein (Stremmel & Teichman 1990) or Conrad's mobilferrin (Conrad *et al* 1990, 1993) being less likely candidates as these proteins will expand the iron storage compartment within the enterocyte unless it can be shown that these proteins are also capable of mediating enhanced iron transfer to plasma.

In conclusion the results of the present study indicate that ferritin mRNA expression in IDA and GH were both reduced in the absorptive and the non absorptive cells of the duodenum. It appears that reduction in mucosal ferritin GH is not due to a failure of the molecular mechanisms that control ferritin synthesis but rather, mucosal cells have enhanced iron transfer to plasma leading to raised body iron stores but themselves becoming relatively 'iron deficient'.

6. Synopsis of thesis

6.1 General discussion

The control of iron absorption is a carefully regulated biological process. It is able to respond by increasing iron absorption in the iron deficient state and to decrease absorption in the iron replete state. Iron absorption can be viewed to occur in several stages: (i) uptake of luminal iron onto mucosal cells (ii) iron transport across the enterocyte and (iii) the release of iron to the portal circulation. It is not always possible to separate these stages in absorption studies leading to conflicting results. Intestinal iron absorption needs to be a finely tuned process, because, unlike many other trace elements, there is no regulatory excretory mechanism for iron and iron homeostasis largely controlled by regulating absorption of dietary iron (McCane & Widdowson 1937). Iron excretion in human is limited to approximately 1 mg/day (Bothwell 1979) but excretion can be increased up to 4 mg/day with the principal loss in the gastrointestinal tract (Crosby *et al* 1963). This loss is balanced by a daily intake of approximately equal amount in adults except in GH where iron absorption is inappropriately high but the mechanism for the apparent lack of regulation remains a mystery.

6.1.1 Candidate proteins in iron metabolism

Studies on iron absorption have concentrated on mucosal proteins known to be involved in iron metabolism namely TfN, TfR and ferritin. These are not the only proteins implicated in mucosal iron metabolism. Others include the mucosal iron transport protein of Teichmann & Stremmel (1990) and the transmembrane protein, mobliferrin working in conjunction with integrin (Conrad 1990, 1992). However, their precise role(s) in mucosal iron homeostasis remains unclear.

This thesis is primarily concerned with the effect of body iron stores on mucosal iron proteins (namely ferritin and TfR) that are implicated in mucosal iron uptake. However, it is perhaps important to reiterate that there are other factors known to affect

mucosal iron uptake. These factors have been discussed in Chapter 1 and are now summarised below.

i. *Non-haem iron* vs *haem iron* with greater mucosal uptake of *haem iron* (30 % vs 10 %)

ii. Interactions between iron constituents in foods and the secretions of the gut such as gastric HCl which enhance, while bile and pancreatic secretions impede mucosal iron uptake.

iii. Nature of the iron constituents in foods e.g. dietary *non-haem iron* are more affected by luminal secretions than *haem iron*. Animal tissues (proteins) also promote mucosal uptake of *non-haem iron*.

iv. The interplay of promoting ligands like ascorbic acid and inhibitory ligands like phytates and calcium have a major influence on *non-haem iron* uptake in mucosal cells.

v. The proportion of *haem iron* and *non-haem iron* in a mixed meal since *non-haem* iron uptake is influenced by the total iron content in a meal whereas *haem iron* is relatively unaffected.

vi Mucins (high molecular glycoproteins) may have a role in promoting mucosal iron uptake. Iron bound to gastric mucins enhances solubility where on approaching the duodenum, iron is released (due to the alkaline conditions), then passes to a transmemenbrane protein integrin, and eventually to mobileferrin.

vii. The mucosal cell itself may influence iron uptake since transplantation of small intestine from iron deficient rats and iron loaded rats to isogenic iron deficient rats revealed significant changes in mucosal iron uptake.

viii. Internal factors such as body iron status, erythropoeitic activity and hypoxia also influence iron processing at the mucosal surface.

ix Iron uptake by mucosal cells from plasma involves a receptor-ligand endocytic mechanism via an ATP-ase where iron is released to the cell and apotransferrin is recycled to the plasma surface. This delivery of iron is dependant of receptor expression and iron saturation of plasma TfN.

x. The mechanism(s) governing iron transfer to the portal circulation, although poorly understood, is an additional factor. In this respect, although TfN may normally play some role, it appears not to be essential. IRP may be important in performing this function.

xi. Iron supplied to cells control expression of ferritin and TfR. These mRNA's contain a sequence of nucleotides forming a stem-loop structure, such that when bound by an iron binding protein, a cytoplasmic aconitase, (dependant on body iron stores) mediates translation of these mRNA's.

xii. The ferritin heteropolymer is composed of subunits (L and H-rich) which have different iron uptake and iron retaining properties such that the isoferritin produced could have a role in influencing iron uptake onto the mucosal cell and iron export to plasma.

xiii. Migration of proliferating crypt to functional villus cells is another aspect that is conceivable important with a constitutive ratio of ferritin transcripts being pre-determined in accordance to body iron status such that mucosal uptake of dietary iron is determined by time for cells to migrate to the villus tip and iron delivery to plasma dependant on the initial pre-determined constitutive ratio in the crypt cells

(i) Transferrin

Within mucosal cells in the gastrointestinal tract, most of the iron is bound to one or two proteins, mucosal ferritin and mucosal TfN, a modified form of plasma TfN (Johnson *et al* 1983, Purves *et al* 1988). Initial studies with ^{59}Fe and following the

labelling pattern of these proteins in iron replete and iron deficient animals, suggested mucosal TfN to be the iron transport protein from mucosal cell to plasma. Mucosal ferritin was postulated to bind iron that was not transported to plasma which served as a reservoir of iron for these cells (Savin & Cook 1980, Topham *et al* 1985). However, the transport role of mucosal TfN has been contested because radioimmunoassay techniques have demonstrated TfN throughout the small intestine (Osterloh *et al* 1985, Johnson *et al* 1983) whereas iron absorption in man occurs mainly in the duodenum and jejunum. Furthermore, studies utilising Northern hybridisation techniques (Pietrangelo *et al* 1992) have failed to demonstrate TfN mRNA in mucosal cells implying that TfN was not synthesised within the enterocytes and is probably derived elsewhere, most likely, from plasma via a mucosal endocytic mechanism (Anderson *et al* 1994).

(ii) Transferrin receptors

The role of TfR has also been the subject of some controversy. Based on immunological and immunohistochemical studies (Parmely *et al* 1985, Banergee *et al* 1986, Anderson *et al* 1990) these receptors are embedded within cell membranes on the serosal surface of enterocytes, more specifically on the mucosal basolateral surface. These receptors are abundantly expressed in the proliferating crypt cells due their higher requirement for iron (Meissner & Leblond 1960) and there appears to be a gradient of receptor expression which diminishes with increasing distance up the villus architecture (Anderson *et al* 1990, Lombard *et al* 1990). TfR are unregulated in iron deficiency and downregulated in the iron replete states (Anderson *et al* 1990). It's role on the basolateral surface of enterocytes remains unclear with postulated functions that include (i) governing the release of mucosal iron to the portal circulation (ii) regulation of cell growth (iii) a role in "informing" the enterocytes regarding body iron stores and (iv) facilitating the entry of iron from plasma to enterocytes for cellular functions and growth.

However, maintaining cellular functions and growth is the general accepted view for the role of TfR.

(iii) Ferritin

The concept of ferritin as regulator of iron entering the body was proposed some 50 years ago, initially by Hahn *et al.*, (1943) and later modified by Granick (1946) who proposed the "mucosal block" with mucosal cells having the ability to store iron which could be transferred to the body according to need. Mucosal ferritin is low in iron deficiency and is raised in secondary iron overload (Charlton *et al* 1963, 1965, Smith *et al* 1966). The "mucosal block" was challenged with the demonstration of ferritin synthesis in iron deficient animals supplemented with small doses of iron (Brittin & Raval 1970, Richmond *et al* 1972) and the prospect of ferritin having a regulatory effect was largely discontinued. However, the "mucosal block" hypothesis was rekindled by the elucidation isoferritin profiles with different iron retention properties (Ariso *et al* 1978, Wagstaff *et al* 1978, Jones *et al* 1978) with L-rich ferritin subunits mainly for iron storage and H-rich ferritin subunits subserving a 'housekeeping' function.

The "mucosal block" is not the only hypothesis to account for the relationship between iron absorption and body requirements. Others include plasma TfN iron turnover concept by Cavill (1975) and the Fletcher-Huehns concept (1968). The former proposes that the rate of tissue iron uptake from plasma and the labile iron pool in the various body compartments influenced mucosal iron absorption with enhanced mucosal absorption when uptake of plasma TfN iron was increased in the same way that the liver maintains blood glucose if peripheral utilisation was high. Several studies have supported this hypothesis. Increased iron absorption occurs following exchange transfusion of reticulocytes in rodents (Finch *et al* 1982 , Raja *et al* 1988) but was not accompanied by a reduction in plasma iron nor an increase in iron binding capacity suggesting that iron delivered to the reticulocytes was derived from the labile iron pool in the various body

compartments. However, several other studies have disputed this view (Levine *et al* 1972, Cox & Peters 1980). The Fletcher-Huehns hypothesis (1968) was introduced after *invitro* experiments revealed functional differences between the two binding sites of TfN. It assumed the amount of circulating TfN with both binding sites occupied reflected body iron stores and this in turn, determined mucosal iron concentration in the small intestine. One site was suggested to give up it's iron preferentially to red cell precursors and the other from the intestinal epithelium. In this way, the demands of the bone-marrow can affect intestinal iron uptake by regulating transfer of iron from the intestinal epithelium to plasma. However, with the passage of time, this concept has been disputed as no difference in the ability of either site of TfN to donate or receive iron has been demonstrated in *invitro* or *invivo* studies in man (Aisen & Brown 1975).

6.1.2 Mucosal block, lag period and 3-dimensional villus architecture

The concept of pre-conditioning of crypt cells stems from previous physiological studies on iron absorption revealing a 'lag period' between changes in body iron stores and alterations in mucosal iron uptake. This 'lag period' (approximately 3 days in rats) correlated with the migration of cells in the crypts of Lieberkuhn to the villus tip architecture. It was suggested, that, the behaviour of the mucosal cells may have been "conditioned" during their developmental period in the crypts, and, any cellular manipulation of the environment must be followed by a 'lag' period during which the "conditioned" cell must first move out to the their functional position on the villi. Upon migration to the villus tip, these cells determined the capacity of iron transfer to the body according to need. The mechanism(s) by which crypt cells are conditioned by body iron stores are poorly understood, but, I postulated that TfR at the basolateral surface of enterocytes may subserve this role (in accordance to the plasma transferrin saturation) via mucosal endocytosis.

Using an adapted method of separating crypt and villus in the rat model and Northern hybridisation techniques to examine L and H-rich ferritin subunit mRNA, I set out to examine this concept of the 'lag period' and pre-conditioning of the crypt cells by body iron stores with ferritin as the candidate protein for the mucosal block.

6.2 Summary of presented results

6.2.1 TfR

Immunohistochemical studies using OX 26, a monoclonal antibody to rat TfR, confirmed the established inverse relationship between TfR expression and body iron stores with enhanced receptor expression when body stores were low and diminished receptor expression when body iron stores became replete. It also confirmed previous studies (Banergee *et al* 1986, Anderson *et al* 1990) on greater TfR expression in proliferating crypt cells compared to villus cells.

This inverse relationship between body iron stores and receptor expression was again demonstrated on further manipulation of the diet of these animals. In rats given carbonyl iron orally for 5 weeks, TfR expression was down-regulated and was virtually confined to the cells. On introducing an iron deficient diet to these animals, enhanced receptor expression was observed, initially in crypt cells and later in villus cells. Interestingly, this response was observed by day 1 from the change in diet where body stores were unlikely to change significantly suggesting that the mucosal cell itself may be responsive to changes in environmental iron. Conversely, in rats maintained on an iron deficient for 5 weeks, receptor expression was enhanced and extended right up to the terminal villus architecture. On introducing a carbonyl iron supplemented diet to these animals, TfR were down regulated, with diminished receptor expression, initially, in villus cells followed by crypt cells.

TfR expression was also examined in human duodenal mucosa in subjects with disordered iron metabolism. Using OKT9, a monoclonal antibody to human TfR, the reciprocal relationship between iron stores and TfR expression was also confirmed in human duodenal mucosal cells.

6.2.2 TfR mRNA

A 30-mer oligonucleotide probe was used to study TfR mRNA expression in animals with different iron stores. TfR transcripts also demonstrated the inverse relationship between body iron stores and receptor expression. The sequential studies on TfR expression in cell subpopulations have provided a semi-quantitative analysis which had not been previously reported. Furthermore, there was greater receptor mRNA expression in crypt cells than villus cells reflecting proliferating cells to have a higher requirement for iron. Similar changes have been observed for TfR mRNA expression in human but using whole endoscopic biopsies containing both crypt and villus cells (Pietrangelo *et al* 1992) and in mice (McKie *et al* 1996) with increase expression in iron deficiency and reduced expression in secondary iron overload

6.2.3 Liver ferritin

Ferritin mRNA expression in liver were also examined. The hybridisation signals for L-rich ferritin subunits tended to be stronger than H-rich ferritin subunit, implying that the predominant isoferritin in liver was L-ferritin.

In animals loaded with oral and parenteral iron, ferritin (L and H) expression tended to rise, although did not reach statistical significance when compared to controls. This observation was different from a previous study revealing a substantial increase in L-rich ferritin subunit but not H-rich ferritin subunit expression (Pietrangelo *et al* 1990). The reasons for these discrepancies were discussed in section 4.3.3.

However, when animals maintained on a carbonyl iron supplemented diet and in animals receiving parenteral iron were switched to an iron deficient diet, there was a significant decrease in both L and H-rich subunit ferritin mRNA within 24 hours. Similarly, on changing animals from an iron deficient diet to a diet enriched with carbonyl iron, a significant increase in liver ferritin transcription was observed for both L and H- rich ferritin subunits which was only observed at day 5 after the change in diet. These observations suggest that hepatocytes are also capable of 'responding' to changes dietary iron.

6.2.4 Mucosal ferritin

Ferritin mRNA expression were also examined in crypt and villus cells of the intestinal mucosa. Hybridisation signals for H-rich ferritin tended to be stronger than L-rich ferritin, implying that the predominant isoferritin within the enterocytes was H-rich ferritin subunits.

In animals loaded with carbonyl iron and parenteral iron, there was a tendency to transcribe an L-rich ferritin (although not statistically significant) in both villus and crypt cells compared to their respective controls. This was also observed in a recent similar study in mice, although intestinal cells were not segregated (McKie *et al* 1996). However, the L:H transcript ratios of these two groups in the present study (only significant in the parenteral group) do reflect that mucosal cells were capable of responding to changes body iron.

In animals maintained on the iron deficient diet, there was a significant reduction in both L and H-subunit ferritin expression but confined to villus cells. A possible explanation for this is that in the iron deficient state, steady state ferritin transcripts may be lower (presumably due to diminished iron stores), perhaps an adaptive process to conserve iron for proliferating crypt cells. Villus cells, coming to the end of their life

span, have lower ferritin transcripts such that 'less iron' will be lost when these cells are shed into the gut lumen.

In animals maintained on a carbonyl iron supplemented diet and then switched to an iron deficient diet, a significant reduction in L-subunit ferritin mRNA expression was observed in both crypt and villus cells within 24 hours from the change in diet. However, H-subunit ferritin mRNA did not change significantly. Similar changes (observed within 6 hours from the change in diet) were also observed in both crypt and villus cells in the parenteral iron group but both L and H-subunit mRNA were significantly reduced. These differences may be related to the mode of administration of iron (oral vs IP) and iron preparation (carbonyl iron vs iron sorbitol). The converse, however, was not observed, where both ferritin subunit mRNA remain unaltered (although there was a tendency towards a raised L:H transcript ratio) on changing animals maintained on an iron deficient diet to a carbonyl iron supplemented diet The reasons for these discrepancies have been discussed in section 4.4.6.

A number of inferences can be drawn from these observations. (i) if there was conditioning of crypt cells by body iron stores, this was not mediated through ferritin mRNA levels since a response in villus cell was observed within 24 hours from the change in diet. (ii) both absorptive and non-absorptive cells were capable of responding to changes in luminal iron. (iii) environmental iron scarcity may have an effect on the controlling mechanisms by affecting ferritin in the villus via a microcirculation effect. (iv) although body iron stores demonstrated a tendency to influence the production of specific ferritin transcripts in the mucosal cell, the mucosal cell itself may be more important in processing iron prior to major changes in iron stores. There is supportive evidence for this from several previous studies (Fairweather-Tait & Wright 1984, Fairweather-Tailt *et al* 1985, Adams *et al* 1991, Topham *et al* 1991).

6.2.5 Genetic haemochromatosis

Several reports have suggested that TfR (Banerjee *et al* 1986, Lombard *et al* 1990) and TfR mRNA (Pietrangelo *et al* 1992) are inappropriately expressed (failure to down-regulate) in mucosal cells of patients with GH. Similarly, mucosal ferritin in subjects with GH have also been examined using radioimmunological and immunohistochemical techniques (Halliday *et al* 1978, Whittaker *et al* 1989, Fracanzani *et al* 1989). These studies demonstrated lower mucosal ferritin (both subunits) when compared to patients with secondary iron overload but comparable mucosal ferritin levels when compared to control subjects. Similar results have been obtained using Northern and *insitu* hybridisation techniques (Pietrangelo *et al* 1992, Pietrangelo *et al* 1995).

The present immunohistchemical study confirmed previous studies on the "inability" to down regulate of TfR in GH (Banerjee *et al* 1986, Lombard *et al* 1990). This is now thought to reflect enhanced iron transfer to the portal circulation rather than a site specific defect.

Examination of subpopulation of crypt and villus cells in the human small intestinal mucosa have not been described to date. I have addressed this by developing a method for separating subpopulations of these cells from endoscopic biopsies. The purity these cell subpopulations were confirmed by measuring 3H-thymidine incorporation for crypt cells and assaying alkaline phosphatase activity for villus cells. In addition, I have also demonstrated that sufficient RNA could be obtained to analyse specific mRNA transcripts utilising specific probes e.g. TfR (expression mainly in crypt cells) and sucrase-isomaltase (expression mainly in villus cells).

Ferritin expression in crypt and villus cells were also examined using this separation technique. Ferritin transcripts, demonstrated by Northern hybridisation were consistent to a recent study (published while my studies was undertaken) using *insitu* hybridisation (Pietrangelo *et al* 1995) with mucosal cells (both crypt and villus cells) in GH being relatively 'iron deficient'. By demonstrating raised IRP activity (a refection of

increase iron transport to plasma), it was concluded that ferritin expression was preserved in GH and also accounted for mucosal cells of GH being relatively 'iron deficient' (Pietrangelo *et al* 1995).

6.3 Potential for future work and the future

Although there have been major advances in our understanding of mucosal iron absorption since the days of Hahn, there are still major gaps in our understanding in iron metabolism. There is little data especially on the mechanism(s) on mucosal iron transfer to plasma. TfR seems to be the ideal candidate due to its location but is not technically feasible on kinetic considerations (Baker & Morgan 1994).

Clues in our understanding of mucosal iron uptake may lie in the elucidation of the defect in GH. There is much debate on the location of the defect. Is the defect on the mucosal side, the serosal site or both? At the mucosal surface of the enterocyte, the most promising candidate is the 160 KD iron transport protein. However, to date, the gene for this mucosal iron transport protein has yet to be localised. Its concentration in the duodenum appears to be increased in iron deficiency and reduced in iron overload. Potentially, this protein could be responsible for increasing uptake of iron at the luminal membrane of the enterocyte which would also result in a higher influx of iron into the cell. It remains to be shown if this protein is also capable of rapid removal of iron from the mucosal cell.

On the serosal side, the 90 KD iron regulating factor protein appears promising. It is conceivable that the regulatory mechanism involving the IRE-IRP1 is abnormal in GH such that IRP's role as the physiological iron sensor is impaired. This will result in excessive transfer/release of iron to plasma. However, this has yet to be confirmed. Against this is the recent localisation of the gene for IRP1 to chromosome 9 (Hentze 1989a). Nevertheless, IRP may constitute a family of regulatory proteins with the recent

elucidation of a second IRP (named IRP2), molecular weight of 105 KD (Kim *et al* 1994). The role of this and possibly other IRP need to be addressed.

It has been suggested that Northern hybridisation may not be the most sensitive method of demonstrating changes in ferritin/TfrR mRNA expression who advocate the technique of solution hybridisation (White & Munro 1988). It may be worthwhile re-examining the hypothesis using this technique or more recent and sensitive techniques such as reverse transcriptase polymerase chain reaction (PT-PCR) and RNA protection assays. The alternative explanation from the current findings that environmental availability of iron may determine regulatory response in the mucosa rather than body iron stores warrants further investigation.

Ultimately, a key factor lies on chromosome 6, the site of the haemochromatosis gene. The elucidation of the haemochromatosis gene product will lead to a better understanding of the pathogenesis of GH and therefore mucosal iron processing. The defect in GH may not be any of the proteins described so far especially with the recent identification of a Cys-282-Tyr mutation in the HLA H region of chromosome 6 (Feder *et al* 1996). The precise role of HLA H in iron metabolism is not known. However, some clues may be inferred by the fact that it codes for a membrane protein for a binding site for β_2- microglobulin. Interestingly, a deficiency in β_2- microglobulin has been shown to be associated with iron overload in mice. Endocytosed vesicles (containing TfN and TfR) are also known to interact with HLA H proteins (Cresswell 1985). It is conceivable that a 'faulty' or absent β_2- microglobulin results in inappropriate mucosal iron uptake such that information regarding body iron stores have not been conveyed to the mucosal mechanism(s) controlling iron uptake. These are exciting and challenging avenues for future studies. It is perhaps pertinent to conclude that, the more we know about mucosal iron uptake, iron transport and storage, the more there is the need to know!

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