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**The assessment of dietary iron bioavailability
using the piglet as an animal model for the
human infant**

A thesis presented in partial fulfilment of the requirements for
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ABSTRACT

The bioavailability of five different iron sources added to a bovine milk based formula was investigated in the anaemic, suckled piglet. These iron sources were ferrous pyrophosphate (FP), ferrous sulphate (FS), milk protein-iron complex (MPIC), ferrous lactate (FL), and haemin (Hm). Forty eight male piglets were removed from the sow at five days of age and randomly assigned to one of six treatment groups (n=8). Iron depletion was achieved in two ways: firstly, by withholding the iron injection usually given to these piglets at birth, and secondly, by feeding the piglets an iron-deficient formula throughout an eleven day adjustment/iron-depletion period. The five formulas containing the supplementary iron and one formula containing no iron (control diet) were bottle-fed to the piglets (336g of liquid formula/kg bodyweight/day) seven times daily for a 25 day repletion period. Analysis of the formulas revealed that iron levels in each of the five iron treated formulas varied over a range of 6.0-8.3 mg/100g of powdered formula.

Iron bioavailability was assessed in the piglets by a haemoglobin repletion assay. Blood was collected from the anterior vena cava of the piglets on days 0, 11, 24, and 36 of the trial, and the piglets were weighed every three to four days throughout the 25 day repletion period. Blood haemoglobin concentration, haematocrit, unsaturated iron binding capacity, and piglet liveweights were used as indicators of iron status in the piglets. Haemoglobin Repletion Efficiency (HRE%), a measure of the proportion of ingested iron that is incorporated into haemoglobin in the body, was calculated to correct for differences in the iron content of the formula and differences in iron intake by the piglets. The HRE% for the FP, FS, MPIC, FL, and Hm fortified formulas were 23.5, 35.8, 38.0, 32.9, and 3.2%, respectively. There were no significant differences, however, in the mean blood haemoglobin concentrations, haematocrits, HRE% and UIBC for piglets fed the FS, MPIC, or the FL fortified formulas half way through, and at the end, of the repletion. This implied that there were no differences in the bioavailability of these iron sources. In contrast, these parameters were all significantly lower ($P>0.05$), for the piglets

fed either the FP or the Hm formulas. Based on relative biological value, the bioavailability of each iron source, when ranked in order from highest to lowest, was MPIC<FS<FL<FP<Hm. These findings have important implications for the development of iron-enriched milk products destined for human consumption.

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GENERAL INTRODUCTION

Today, iron deficiency is one of the most prevalent nutritional deficiencies among infants and young children in both industrialised and developing countries (Kuvibidila *et al.*, 1984; FAO/WHO, 1988; Zeigler and Fomon, 1996; Looker *et al.*, 1997). The effects of iron deficiency on erythropoiesis, work capacity, cognitive performance (Pollitt, 1997), behaviour (Andraca *et al.*, 1997), and more recently cell mediated immunity (Kuvibidila *et al.*, 1984; Walter *et al.*, 1997) are well documented. These effects highlight the need for early detection and treatment of iron deficiency and its associated anaemia during this age of increased risk.

Infants and young children have an increased risk of deficiency because of the demands imposed in times of increased growth. The growth of the body necessitates an increase in the total volume of blood to keep pace with its size. Moreover, there is a steady increase in the haemoglobin content per unit blood volume from age six months. A greater demand for iron comes also, from the expanding muscle mass during growth. At birth, the full-term human infant is endowed with iron stores sufficient to satisfy the body's demand for iron for the first four to six months of life. The infant can be fed a low iron diet during this time without demonstrating overt signs of iron deficiency. After the first four to six months, however, the depletion of iron stores that results from increased growth rates and a diet low in iron, introduces a high risk of iron deficiency to the infant. While the high bioavailability of breast milk iron confers a partial protection against deficiency, the quantity of iron absorbed from an unsupplemented diet, from about four months of age onwards, is no longer sufficient to meet the needs of infants (Saarinen, 1978; Dallman *et al.*, 1980).

The fortification of foods with iron offers a suitable means of prevention and treatment of iron deficiency and its associated anaemia. Infant formula provides an appropriate vehicle for iron fortification for infants who are not breast-fed because it is consumed by these infants in large quantities. Iron fortification of infant formulas and cereals was introduced in the 1960's (Zeigler and Fomon,

1996). Today, the level of fortification of infant formula typically ranges from 8-12mg/L, primarily in the form ferrous sulphate or ferrous lactate. The iron sources of choice are those with acceptably high bioavailability. The absorption of dietary iron depends on the bioavailability of the iron which is, in turn, affected by various dietary factors. It is, therefore, important to get reliable estimates of the bioavailability of different iron sources under appropriate conditions.

The objective of this study was to test the bioavailability, to the human infant, of iron from different sources. Central to this objective was the use of a suitable animal model for infant iron nutrition.

CHAPTER 1

REVIEW OF LITERATURE

1.1 IRON NUTRITION AND METABOLISM IN MAMMALS

Iron is the fourth most abundant element known to man, making up approximately 5% of the Earth's crust. The importance of this malleable, tough, grey metal can be traced back to prehistoric times. Prehistoric man used iron for tools, weapons and other domestic implements. History documents its popularity with Assyrians, Egyptians and Aztecs (Everyman's Encyclopaedia, 1978) and it is fascinating to note that the Aztecs prized this metal above gold (Encyclopaedia Britannica, 1970). Today, iron is predominantly used commercially in structural steels. The importance of iron, however, is not limited to its use in metal implements. Iron also plays a major role in the biochemistry of humans and other organisms.

As the most abundant trace element in the body, iron is required in the nutrition of all mammalian species. A characteristic constituent of the porphyrin complex in blood haemoglobin (Stryer, 1988), iron is also associated with numerous specific proteins (Table 1.1) involved in many vital reactions in the body. Since the importance of this element was established in the 17th century (Encyclopaedia Britannica, 1970) scientists and nutritionists alike, have sought to characterise the nature mammalian iron metabolism.

The amount of iron required by the body is an important focus of iron nutrition research. In healthy humans the total quantity of, and hence requirement for, body iron varies according to sex, weight and age (Fairbanks *et al.*, 1994). Iron deficiency can be the result of impaired absorption, blood loss, haemolysis, repeated pregnancies, or when dietary requirements for iron are not serviced (Fairbanks (1994). Pregnancy and menstruation increase the demand for iron in young women. Times of increased growth in infancy, childhood, and adolescence also augment the bodies requirement for iron. These factors, when

coupled with a diet low in available iron, expose infants, children, and young women to an increased risk of iron deficiency.

Table 1.1

Major iron containing and iron binding proteins and their functions

Protein	Function
Haemoglobin Myoglobin	Oxygen transport
Cytochrome oxidase Cytochrome C and other cytochromes Catalase	Mitochondrial electron transport H ₂ O ₂ reduction (oxidative stress response)
Cytochromes P450's	Metabolism of fatty acids, steroids, prostaglandins, leukotrienes and xenobiotics such as drugs
Ribonucleotide reductase	DNA synthesis
Aconitase	TCA cycle enzyme
Iron responsive element binding protein	Regulator of iron binding proteins
Succinate dehydrogenase	Oxidation of fatty acids
Procollagen prolyl hydroxylase	Collagen synthesis
Transferrin Lactoferrin	Iron transport
Ferritin	Iron storage
Phenylalanine hydroxylase Tyrosine hydroxylase	Amino acid catabolism
Neutrophil phagocyte oxidase	Bacterial killing

In adults severe iron deficiency and iron deficiency anaemia (IDA) are commonly associated with disturbances in work performance and thermoregulation (Fairbanks, 1994). Iron deficiency in infancy and childhood has a negative impact on growth (Fairbanks, 1994). Recent studies have shown that IDA at an early age is also associated with permanent behavioural disturbances (Andraca *et al.*, 1997) and impaired cognitive function (Pollitt, 1997). It is, therefore,

important to ensure that dietary iron intake is sufficient to meet requirements, especially in infancy and childhood when the risk of iron deficiency is high.

Babies are born with adequate iron stores which become depleted in the first four to six months. The dietary iron intake of the infant is insufficient to maintain body iron stores after first four to six months of life (Lonnerdal, 1996). This can be attributed to the low iron bioavailability of breast milk and infant formula (Dallman *et al.*, 1980), which form the sole source of nutrition at this time. Dietary iron supplementation can protect against iron deficiency in late infancy. The iron fortification of infant cereals, infant formulas and follow-on infant formulas to increase the iron intake of infants was introduced in the 1960's (Zeigler and Fomon, 1996).

Knowledge of iron intake alone is of little value without a sound knowledge of iron bioavailability. Iron bioavailability can be defined as the proportion of total iron in a food, meal, or diet that can be utilised for normal bodily functions. In seeking to establish strategies for meeting an infant's dietary requirement for iron it is necessary to estimate the bioavailability of the iron source. The body of literature measuring the bioavailability of iron directly in the infant is limited. Routine use of humans in iron bioavailability research has its limitations both from the point of view of obtaining willing subjects, especially in infant studies, and due to the stringent ethical constraints imposed by the nature of iron bioavailability research. Although studies using infants are possible, the use of *in vitro* determinations and animal models in iron studies has long been the practice in allowing experimental protocols that are inappropriate for human subjects. Animal models offer particularly attractive means of investigating iron bioavailability under the appropriate physiological conditions.

An understanding of the mechanisms of mammalian iron metabolism is necessary in order to evaluate the bioavailability of different iron sources. The following discussion describes the fundamental aspects of mammalian iron metabolism including iron absorption, availability, transport and storage. This is followed by an outline of the current practices relating to iron nutrition especially in the human infant. The different methods used to investigate iron

bioavailability will be outlined and used to identify a suitable means for investigating the bioavailability of different iron sources to the human infant.

1.1.1 Iron Absorption and Availability

Although different mammalian species vary in their requirement for iron, absorption in most species has a number of common salient features. Apart from the negligible losses from sloughed body cells or bleeding, most mammals do not have a mechanism for the excretion of iron. Research from early this century revealed that increasing levels of oral or parenteral iron administration did not increase urinary excretion (McCance and Widdowson, 1938). Instead, what resulted was the accumulation of iron in the epithelial cells of the small intestine (McCance and Widdowson, 1938). It was concluded from this investigation that normal iron balance in the body must largely be regulated by the control of dietary iron absorption. This is now a widely accepted phenomenon of iron homeostasis (Raja *et al.*, 1987).

The role played by intestinal iron absorption in body iron homeostasis implies that tight regulation of absorption is necessary to ensure that iron in the body does not reach toxic levels, but, there is enough iron to maintain normal physiological function (Dallman *et al.*, 1980). The intestinal mucosa appears to assist in the regulation of iron content in the body by enhancing absorption in the iron deplete individual and, as body iron stores increase iron absorption decreases (Monsen *et al.*, 1978; Dallman *et al.*, 1980). Although the exact mechanisms of iron absorption at the molecular level are unknown, the following paragraphs discuss current knowledge in this area.

1.1.1.1 *Mechanisms of Intestinal Cell Iron Uptake*

The mechanisms of intestinal iron uptake are summarised in Figure 1.1. It is well documented that the absorption of iron depends not only on the amount of iron supplied by the diet but also on the form of that iron, the composition of the meal with which it is ingested, and on the physiological status of the individual (Bjorn-Rausmassen *et al.*, 1974; Monsen *et al.*, 1978). The iron content of a food is, therefore, a poor indication of the iron available from that food.

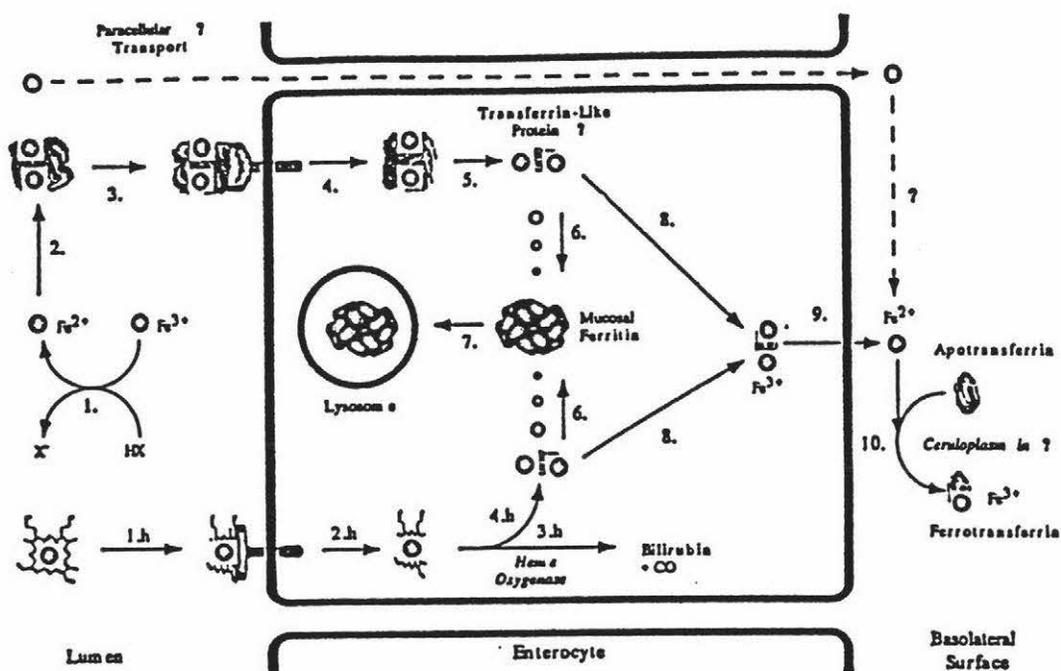


Figure 1.1

Enterocyte uptake and transfer of iron. Non-haem iron: a reductant such as ascorbic acid reduces non-haem ferric iron to ferrous iron. (1). Chelators sequester and solubilise non-haem iron. Non-haem iron is then transferred to a binding protein within the lumen. (2). The iron-binding protein binds to a specific transporter on the luminal surface of the enterocyte. (3). Non-haem iron is transported into the enterocyte. (4). This iron is either transferred to low molecular weight chelates or to a transferrin-like protein. (5). The transferrin-like protein delivers iron to either mucosal cell ferritin (6) or, to the basolateral surface of the enterocyte. (8). Absorbed iron that is not sequestered by ferritin is delivered to the basolateral surface of the enterocyte. (9). And oxidised for binding to transferrin (10). Haem-iron: Haem binds to its receptor (1h) and is internalised (2h). After entering the cell, haem is degraded to iron, carbon monoxide, and bilirubin by the enzyme haem oxygenase (3h). This iron enters the common intracellular (enterocyte) pool of iron (4h) and is processed like non-haem iron (6-10).

(Adapted from Beard *et al.*, 1996)

The primary determinant of iron absorption and availability is the form of iron in a food. Iron in the diet can exist in two forms, haem iron as part of the haemoglobin or myoglobin haem ring, and non-haem iron eg. the iron in plant foods and milk. Iron in the haem form has a higher availability than that of non-haem iron (Monsen *et al.*, 1978) and its absorption is relatively unaffected by dietary factors or gastrointestinal secretions (Jacobs *et al.*, 1963). During the digestion of haemoglobin and myoglobin haem is split from the protein and the haem is taken up by the intestinal mucosal cells (Conrad *et al.*, 1966; Sweeten *et al.*, 1986; Beard *et al.*, 1996). Following absorption, the iron is separated from the porphyrin ring by the action of haem oxygenase (Wheby *et al.*, 1970).

Most dietary iron, however, is of the non-haem type, being sourced mainly from vegetables, cereals, fruits, and eggs. Examples of non-haem dietary iron include ferritin, phosvitin-bound iron, monoferric phosphate, phytoferritin and ferrous sulphate. Due to the chemical nature of iron in the non-haem form, absorption of non-haem iron is dependent on a number of factors both dietary and physiological (Monsen *et al.*, 1978). Consequently, certain forms of this dietary or fortification iron may not be as available for absorption as other forms (Monsen *et al.*, 1978; Whittaker *et al.*, 1989; Reddy and Cook, 1991).

An understanding of the mechanisms of non-haem iron absorption is necessary in order to appreciate why some sources are more bioavailable than others. While no iron absorption occurs in the mouth, esophagus or stomach (Beard *et al.*, 1996), the luminal phase of non-haem iron absorption begins in the stomach. Secretion of hydrochloric acid and pepsin in the stomach helps to remove protein-bound iron and reduce the ferric iron to its ferrous form (Jacob *et al.*, 1963; Forth and Rummel, 1973). Ferrous salts stay in solution at a higher pH than the ferric salts. The higher solubility of ferrous iron promotes iron absorption by facilitating the traversal of iron across the mucous layer of the intestinal epithelium (Jacobs *et al.*, 1964; Beard *et al.*, 1996). This reduction process has long been considered an obligatory step in the absorption of non-haem iron. Without the reduction of ferric iron (FeIII) to its ferrous (FeII) form the ferric iron is precipitated (Forth and Rummel, 1973). Unless the ferric iron is chelated and kept in solution until reaching the absorption site the

precipitated iron is biologically unavailable for absorption. Ferrous iron crosses the mucous layer where it is oxidised to ferric iron for absorption. Absorption is believed to be via an iron dependant RNA binding protein known as the iron responsive element binding protein (IRE-BP) on the epithelial cell (Harford and Klausner, 1990). At this time, however, the description of the process of iron absorption is incomplete.

As well as the form (ie. haem or non-haem) of iron in the diet, it is necessary to consider the composition of the meal with which that iron is ingested. Many dietary components such as ascorbic acid (Monsen *et al.*, 1978; Dallman *et al.*, 1980; Fairbanks, 1994), histidine (Kapsokefalou and Miller, 1991) and citrate (Nadeau and Clydesdale, 1987) chelate non-haem iron and thereby increase its availability. Ascorbic acid, in particular, is included in infant formulas at a level that promotes iron absorption (Stack *et al.*, 1990). In addition to its role in iron reduction, the enhancing effect ascorbic acid has on iron absorption is due to the fact that it forms a soluble ligand with iron. This in effect, enhances the absorption of both ferric and ferrous compounds (Brise and Hallberg, 1962). However, this may not be the case with mammals like the pig (Perks and Miller, 1996), rat and mouse (Pla *et al.*, 1973) who do not have a dietary requirement for ascorbic acid (Whittmore and Elsey, 1977). Other substances that form low molecular weight chelates with iron include succinic acid (Brise and Hallberg, 1962; Nadeau and Clydesdale, 1987), cysteine, and some sugars (Fairbanks, 1994). Iron ingested in the haem form has also been shown to enhance the absorption of non-haem dietary iron, possibly by promoting ferric iron reduction in the lumen (Kapsokefalou and Miller, 1991) and reducing the iron chelating effect of some inhibitory factors (Fairbanks, 1994; Rossander-Hulthen and Hallberg, 1996).

Another dietary component pertinent to the iron nutrition of the infant and other young milk-fed mammals in particular, is lactoferrin. Lactoferrin is the major iron binding protein in the milk of many mammals (Lynch and Hurrell, 1990; Fransson and Lonnerdal, 1980; Pabon and Lonnerdal, 1992). Lactoferrin is thought to be responsible for the high bioavailability of breast milk iron (Lonnerdal, 1996). Although specific receptors for human lactoferrin have been

found in the infant (Kawakami and Lonnerdal, 1991), the role of lactoferrin in iron absorption is still a matter of controversy. There is very little evidence *in vivo* for a direct role of lactoferrin in the iron absorption in infants (Lonnerdal, 1996).

A host of substances have also been identified as inhibitors of non-haem iron absorption. The phosphates and phytates of fibrous dietary components form insoluble iron compounds decreasing the availability of iron (Rossander-Hulthen and Hallberg, 1996). Other substances that suppress absorption are tannins in tea, phosvitin of egg yolk, calcium, and antacids (Monsen *et al.*, 1978). The inhibitory effect calcium is believed to have on iron absorption is especially important with respect to iron availability from milk products and formulas which are characteristically high in calcium. Calcium in iron nutrition is a matter of concern because milk formulas are consumed in large quantities by many infants.

In addition to the form of iron present in the diet and the effect of dietary components, iron status of an individual can influence iron absorption. Iron absorption is enhanced in times when body iron stores are low (Monsen *et al.*, 1978). The proportion of iron absorbed from a meal, however, is negatively correlated with the level of iron in that meal (Bothwell *et al.*, 1958). As mentioned earlier, it is generally accepted that regulation of iron homeostasis is controlled at the absorptive level. Intestinal absorptive cells respond to the state of iron repletion in the body in terms of differential absorption of iron. In an attempt to clarify the mechanisms operative in the regulation of absorption, a number of theories have been proposed and tested (Hahn, *et al.*, 1943; Heblock and Saltman, 1967).

Intestinal mucosal iron levels may be a major regulating factor on iron absorption and these levels may be mediated in some way. It is interesting to note that an initial lag period exists between the onset of iron depletion in the body and the resultant increase in intestinal iron absorption (Monsen *et al.*, 1978). This period might be required for the intestinal cells to 'unsaturate' (Heblock and Saltman, 1967), or for the deactivation or reactivation of intestinal

carriers via iron responsive transcriptional and translational regulation (Harford and Klausner, 1990; Klausner *et al.*, 1993). It may also be the time required for new, reprogrammed absorptive cells to travel and develop their new functional capacity from villi crypts to the site of absorption.

1.1.3 Iron Transport and Storage

Once absorbed the iron enters a common cellular pool (Beard *et al.*, 1996). The iron is then taken up by a carrier protein, called transferrin, for transport around the body. Having entered circulation, iron must then be transported around the body to sites that require it. Transferrin keeps circulating iron soluble, bioavailable and benign so as to avoid the catalysis of toxic side reactions and to allow cellular metabolism to exploit the chemical properties of iron (Huebers, 1990).

Iron in excess of requirements is stored in the body bound to the proteins ferritin and haemosiderin. Storage is necessary because of the body's inability to excrete iron. Ferritin bound iron can be rapidly released to transferrin in order to meet the body's demands (Siimes, 1990; Beard, 1996).

1.1.4 Disorders of Iron Nutrition and Metabolism

Iron deficiency occurs when the body's iron content is diminished and is the end result of a defined sequence of changes that usually accompany iron depletion. These changes are outlined in Table 1.2 (Roper *et al.*, 1995). The most common causes of iron depletion and deficiency are:

- i. a diet inadequate in iron
 - ii. impaired iron absorption (eg. malabsorption syndromes, high levels of dietary inhibitors)
 - iii. blood loss (eg. menstruation, gastrointestinal blood loss, wounding)
 - iv. periods of rapid growth (eg' infancy and childhood)
- (Roper *et al.*, 1995; Fairbanks, 1994).

Deficiency of iron in the body is a systemic disease which can lead to abnormalities in physical mobility, gastrointestinal function (Rector, 1982) and

immunity (Kuvibidila *et al.*, 1984; Walter *et al.*, 1997). Iron deficiency anaemia (IDA) is a haematological state resulting from severe iron deficiency. The presence of IDA implies the exhaustion of iron stores in the body in the last stages of serious iron depletion. The characteristic symptoms of iron deficiency noted above (Table 1.2) are followed by depression of haemoglobin levels in the blood. This represents the initial stage of IDA. If left untreated, iron deficiency, culminates in microcytic divisions of the red cells leading to hypochromia.

With over 80% of iron in the body present in blood haemoglobin (Wintrobe, 1967a; Beard *et al.*, 1996), recognition of the latter stages of iron deficiency and ensuing iron deficiency anaemia (IDA) is possible by a simply determining blood haemoglobin concentrations (Wintrobe, 1967; Roper *et al.*, 1995). More sensitive haematological indicators are used in the early detection of iron deficiency (Roper *et al.*, 1995; Wintrobe, 1967b). These include transferrin saturation, as an indicator of availability of iron to tissues; serum ferritin concentration which reflects tissue iron stores; and red cell protoporphryn levels as a marker for haemoglobin synthesis.

Table 1.2

The development of iron deficiency anaemia

Stage I: Iron Depletion	Stage II: Iron Deficient Erythropoiesis	Stage III: Iron Deficiency Anaemia
serum ferritin ↓ bone marrow iron ↓	serum ferritin ↓ bone marrow iron ↓ serum iron ↓ TIBC ↑	serum ferritin ↓ bone marrow iron ↓ serum iron ↓ TIBC ↑ haemoglobin ↓ haematocrit ↓

Abbreviations: TIBC, total iron binding capacity; ↑, increased; ↓, decreased.

(adapted from Roper *et al.*, 1995)

Iron deficiency anaemia manifests itself in a number of non-haematological conditions including trophic changes such as brittle hair, atrophic rhinitis, generalised pruritus (Lonnerdal, 1990b), and koilonychia (spooned shaped nails) (Roper *et al.*, 1995). Anaemic infants and children are often under weight for

age and height (Fairbanks, 1994). Gastrointestinal symptoms such as achlorhydria (Bothwell, 1979) and histological changes in the duodenal mucosa (Lonnerdal, 1990b) have also been linked with IDA. Iron deficiency anaemia has been shown to impart negative effects on behaviour and permanent effects on cognitive function in young individuals (Andraca, 1997; Pollitt, 1997). These consequences of iron deficiency highlight the importance of meeting iron requirements, especially in infancy and childhood when the risk of iron deficiency is high. Dietary iron supplementation is an appropriate means to ensure iron intake is sufficient to meet requirements at any age.

1.2 SUPPLEMENTATION OF IRON IN THE DIET

In seeking to establish strategies for meeting an individual's needs for absorbed iron, knowledge of iron intake alone is of little value without a sound knowledge of iron bioavailability. The concept of iron bioavailability can be explained as the proportion of total iron in a food, meal, or diet that can be utilised for normal bodily functions. Numerous iron compounds have been tested for their bioavailability and in a variety of food stuffs in a quest to identify the most suitable compound for food fortification. These are primarily either iron salts or electrolytic iron.

A suitable iron source must have adequate bioavailability and should not affect the functional and sensory properties of the food. The recognition that iron compounds that are more soluble, for example ferrous sulphate, and compounds of smaller particle size, such as elemental iron (reduced elemental or carbonyl iron), have a greater degree of bioavailability (Mitchell *et al.*, 1985) brought about a number of changes in food iron fortification in the 1970's. Ferrous sulphate has long been accepted as a fortificant with high bioavailability due to its high solubility in the intestinal environment (Amine and Hegsted, 1974). Ferrous sulphate is generally recognised as the standard against which all other compounds are evaluated (Fairbanks, 1994; Fritz and Pla, 1972; Fritz *et al.*, 1970; Amine and Hegsted, 1974). This allows for a meaningful comparison of availabilities of different iron sources. Several compounds have

been found to be absorbed as well as Ferrous sulphate (Brise and Hallberg, 1962). These include, ferrous succinate, ferrous lactate, ferrous fumarate, and ferrous glutamate.

Unfortunately some of the iron sources with higher availability do not have a good compatibility with foods (Huebers *et al.*, 1986; Hegenauer *et al.*, 1979; Peres *et al.*, 1997). Depending of the type of food to be fortified and the form of the iron, there is a potential for iron fortificants to impart unfavourable oxidised flavours and colour changes in certain foods (Hegenauer, 1979; Zubigalla, 1996). This is particularly the case for milk-based products including infant formulas because of their high fat content. The addition of bioavailable, but very reactive ferrous sulphate and ferrous lactate to milk-based infant formula is currently the practice.

Technology has seen the development of techniques to overcome the influence of highly bioavailable and highly reactive iron compounds on the organoleptic properties of iron fortified milk products, especially infant formulas (Hegenauer *et al.*, 1979; Zubigalla *et al.*, 1996; Peres *et al.*, 1997). Hegenauer *et al.*, (1979) found that microencapsulated iron, bound to casein phosphoproteins, removes the iron from the lipid phase of milk thus preventing lipid oxidation. Zubigalla *et al.*, (1996) also found microencapsulation of an iron source in fluid milk to retain a high degree of bioavailability and appropriate physiological behaviour of the element. Microencapsulation may also provide opportunities in the fortification of other food products with high lipid content.

Further development of iron fortification sources for use in infant formulas dictates the need to develop a rapid, inexpensive, and reliable method for routine testing of bioavailability.

1.3 METHODS USED TO INVESTIGATE IRON BIOAVAILABILITY

The bioavailability of iron from various sources can be investigated by using either *in vivo* or *in vitro* techniques.

1.3.1 *In vitro* Measures of Bioavailability

In vitro measures of iron bioavailability offer a relatively inexpensive, simple and fast means of assessing the availability of iron in different foods. *In vitro* analyses do not, however, allow for the physiological factors involved in the control of iron absorption. While the methods may vary, most *in vitro* techniques do attempt to simulate gastrointestinal conditions (British Nutrition Foundation, 1995). Using simulated digestion the amount of dialysable, soluble or ionisable iron are the indices used to estimate the available iron content of the test food.

The determination of ionisable iron by extracting test foods with a solution of α,α' -dipyridyl was an early method used to quantify available iron in foods (Shacklton and McCance, 1936). Since then *in vitro* chemical analyses have advanced in an attempt to account for physiological conditions. These advanced analyses measure the iron released from food treated in an enzyme solution, usually pepsin and hydrochloric acid (Jacobs and Greenman, 1969). Narasinga Rao and Prabhavathi (1978) further modified the technique to account for alkalinity of the small intestine at which solubility of iron decreases considerably (Jacobs *et al.*, 1963). The result was an *in vitro* technique to successfully estimate iron availability at the site of absorption. Availability measurements such as equilibrium dialysis (Miller *et al.*, 1981) or continuous flow dialysis (Minihane *et al.*, 1993) involve the use of a test meal prepared with dialysable or ionisable iron. The iron passes from *in vitro* enzymatic digestion to the interior of a dialysis bag (Miller *et al.*, 1981). The extent of iron dialysis into the bag is taken as a measure of bioavailability of iron from that source. Intestinal vesicle preparations, and everted small intestinal rings or sacs are also used in *in vitro* estimation of iron bioavailability by measuring iron uptake into the biopsies (Forth and Rummel, 1973; Forbes *et al.*, 1989; Raja *et al.*, 1992).

With *in vitro* techniques it is not possible to simulate physiological factors that account for variations in iron absorption. The use of *in vitro* techniques, therefore, can only serve to rank foods in order of available iron and for the prediction of the relative effects of enhancers and inhibitors (Fairweather-Tait, 1992).

1.3.2 *In vivo* Measures of Iron Bioavailability

1.3.2.1 *Haemoglobin repletion*

Haemoglobin repletion is the most widely used *in vivo* methods for estimating bioavailability of iron sources (Cowan *et al.*, 1966; Fritz *et al.*, 1970; Amine *et al.*, 1972; Fritz *et al.*, 1973; Pla *et al.*, 1973; Forbes *et al.*, 1989; Zhang *et al.*, 1992; Howard *et al.*, 1993; Weink and Beynen, 1996; Perks and Miller, 1996), it is a direct measure of dietary iron utilisation. The haemoglobin repletion method relies on iron deficient subjects exhibiting enhanced iron absorption. This absorbed iron is assumed to be preferentially used in haemoglobin synthesis in the body (Dallman *et al.*, 1980).

A low iron status in the individual is a prerequisite to the accurate use of a haemoglobin repletion assay. The technique involves the repletion of iron status of an iron deplete individual, as measured by blood haemoglobin concentration, in response to dietary iron treatment. This repletion period is usually 12-30 days (Anderson *et al.*, 1974; Forbes *et al.*, 1989; Howard *et al.*, 1993; Weink and Beynen, 1996). The degree of recovery of haemoglobin levels in an iron deplete individual serves as a biological indicator of iron repletion and therefore bioavailability.

1.3.2.2 *Isotopic Methods*

Advent of the extrinsic tag method for labelling iron in foods represented a significant development in iron absorption methodology. Labelling foods extrinsically with a radioisotope of iron, that is ^{59}Fe or ^{55}Fe , made it possible to follow the fate of the labelled iron and thereby determine the bioavailability of iron from that food (Cook *et al.*, 1972). By labelling extrinsic iron and intrinsic food iron with two different isotopes Hallberg and Rasmaussen (1972) found the

fraction of extrinsic iron absorbed to be identical to the fraction of intrinsic iron absorbed from a meal. Both haem and non-haem iron can be tagged extrinsically because the labelled element is assumed to mix completely with the intrinsic iron (Fairweather-Tait, 1992). The techniques used to measure absorption of the isotope include metabolic balance, whole body counting (Fairweather-Tait, 1981) and haemoglobin repletion (Fomon *et al.*, 1989).

Isotopic metabolic balance studies involve the quantification of iron intake and excretion over a fixed period. These studies provide a crude estimate of iron retention and therefore bioavailability. Balance studies are cumbersome by nature and are limited by their potential for error. Incomplete excreta collection, unaccounted-for endogenous losses, and imprecise measurements lead to gross inaccuracies in absorption estimates (Fairweather-Tait, 1992). Balance studies are best applied to whole diet studies rather than the testing of iron bioavailability in individual meals or foods.

Whole body counting offers a more precise method of iron bioavailability determination (Miller *et al.*, 1981; Ziegler, 1989). This technique requires oral administration of labelled iron followed by a whole body iron count to determine retention levels (Fairweather-Tait, 1992). The fraction of iron absorbed is then estimated from total retention and losses after the oral dose. The main limitation of such a technique is requirement for special scintillation counting equipment (Fairweather-Tait, 1992).

The use of isotopic methods is common in haemoglobin repletion. Radioisotopes, ^{59}Fe and ^{55}Fe , are employed to label foods and other iron sources. The labelled iron in blood is measured after a set period (often around 14 days) and be used to determine the degree of incorporation of a given dose of iron into haemoglobin. This degree of incorporation into haemoglobin is used to estimate the extent of absorption from iron sources. In using isotopes in the haemoglobin repletion method the experimenter must also make an assumption as to the magnitude of iron incorporation into haemoglobin when using isotopic labelling (usually 80-90%) (Fairweather-Tait, 1989).

Although the use of isotopically labelled iron poses negligible risk to the subject, a reluctance to use isotopes in infant studies has imposed a requirement for new safer techniques in bioavailability studies. Recently, the feasibility of using a stable isotope (^{58}Fe) of iron has been ascertained with the development of suitable mass spectrophotometers (Zeigler, 1989; Fomon *et al.*, 1989). While a technique equivalent to whole body counting does not exist with this label, erythrocyte incorporation offers suitable precision for estimating iron absorption using a stable isotope.

1.3.2.3 *Other Parameters of Iron Status*

In addition to the major haemoglobin repletion and isotopic methods used to investigate iron metabolism there are other parameters of iron status can be measured *in vivo*. Body weight can be used as an indication of the later stages of iron deficiency anaemia. More sensitive indicators of iron status can be measured in the blood of subjects as a part of the course of haemoglobin repletion or isotopic studies. These include serum ferritin, total iron binding capacity (TIBC), serum iron, unsaturated iron binding capacity (UIBC) and haematocrit.

1.3.3 The Need for an Animal Model

The use of human subjects for experimental purposes remains the ultimate means of obtaining reliable and applicable results in iron nutrition research. Forbes *et al.*, (1989) favoured human experimentation suggesting that a varied diet with a host of enhancers and inhibitors of absorption is difficult to replicate with the laboratory animal which has a more homogenous diet. This, however, is not the case for the human infant whose diet consisting of breast milk or formula, and is homogenous in the first four to six months of life. However, from four to six months onwards the infants diet becomes increasingly complex as it is weaned onto solid food and milk is no longer the sole source of nutrition. This makes research in infant iron nutrition very difficult.

The absorption of iron from human milk (MacMillan *et al.*, 1977) and infant formula (Hurrell *et al.*, 1998) has been studied in adults. The study by MacMillan *et al.*, (1977) confirmed the capacity for human milk to promote iron

absorption. However, the relevance of iron nutriture in the adult to that of the growing infant needs to be considered. Making the assumption that iron absorption in infants is twice that of adults, MacMillan and associates' iron absorption values from human milk in adults were in agreement with estimates from similar studies in infants (Saarinen *et al.*, 1977). Making the similar assumption that iron absorption is of a higher magnitude in infancy, Hurrell *et al.*, (1998) concluded that results from iron absorption studies in adults can be used to assess the influences of enhancers and inhibitors in infant formulas fed to infants.

There are logistical, safety and ethical constraints to using humans for experimental purposes in research. This is especially so where human infants are concerned. The development of stable isotopic methods to avert from the use of radioisotopes in iron research methodology has made human experimentation a more viable option than had previously been the case (Zeigler, 1989).

A possible alternative to using humans for experimental purposes would be an animal model for infant iron nutrition. The use of an animal model in iron nutrition research would allow for rapid, inexpensive and routine testing of iron sources before they are taken to the next level of clinical testing. This would also circumvent problems associated with refining and developing new assays.

1.3.4 Animal Models used to Study Iron Bioavailability

A reliable animal model for studying iron nutrition in humans has yet to be identified despite the abundance of literature spanning the fields of mammalian iron nutrition. The majority of studies looking at human iron nutrition have employed the rat or mouse breeds as a model animal (Weintraub *et al.*, 1965; Pla and Fritz, 1971; Amine and Hegsted; 1972; Schricker *et al.*, 1981; Whittaker and Ologunde, 1990; Weink and Beynen, 1996). Also documented is the use of pigs (Kuznestove and Kal'nitskii, 1987; Harada *et al.*, 1988, guinea pigs (Narasinga Rao *et al.*, 1977) and certain breeds of monkey (Narasinga Rao *et al.*, 1977; Davidson and Lonnerdal., 1988; Davidson *et al.*, 1990). Remarkably, studies

using cockerels, chicks (Amine *et al.*, 1972, Pla, 1973) or calves (Kuvibidila *et al.*, 1984) have also been used to draw conclusions about human iron nutrition.

The use of animal models in iron studies can offer the researcher control over environmental, social and behavioural factors that often complicate human studies. Only if the model animal is suitable though, can any reliable interpretations be made from experimental data using that animal. A suitable animal model for the human infant should be physiologically comparable with respect to iron nutriture at a parallel stage of development or developmental age. The paragraphs that follow detail the literature concerning animal models currently accepted for use in iron availability studies.

There is little doubt as to the position held by the rat in the history of iron nutrition research. The rat has been used as an animal model in iron research for many decades (Amine *et al.*, 1972; Fritz *et al.*, 1970; Ranger and Neale, 1984; Huebers *et al.*, 1986; Adams *et al.*, 1991; Galves-Morros *et al.*, 1992; Zubigalla *et al.*, 1996). Recently, however, the validity of the rat model in human nutrition has been under scrutiny (Lonnerdal, 1990; Kawakami *et al.*, 1990; Reddy and Cook, 1991; Zhang *et al.*, 1992). The suitability of the rat has been questioned with regard to the physiological similarities in iron assimilation in the rat and the human infant. Further refinement in the understanding of mammalian iron nutrition has facilitated the demise of the rat model and in the identification of more suitable (Davidson and Lonnerdal, 1986; Davidson and Lonnerdal, 1989; Forbes *et al.*, 1989; Davidson and Lonnerdal, 1990; Kawakami *et al.*, 1990; Moughan *et al.*, 1992; species; Perks and Miller, 1996).

When deciding which animal model to use in evaluating iron sources for food fortification it is necessary to consider the distribution of iron in milk from the animal model species. The milk from nearly all other species has a higher casein concentration than that found in human milk (Lonnerdal, 1990a), and casein is the major iron binding protein for most species (Table 1.2). Hegenauer *et al.*, (1979) found that iron was associated with negatively charged phosphoserine groups on the β -subunit of bovine casein. In contrast the major portion of iron in human milk is bound in the whey fraction attached to

lactoferrin with a considerable amount also bound in the fat fraction (Fransson and Lonnerdal, 1980; Pabon and Lonnerdal, 1992). The major iron-binding protein in the whey fraction of milk, however, varies among species (Table 1.3) (Lonnerdal, 1990a). Whey-bound iron of the pig, cow, guinea pig and the rhesus monkey share the characteristic iron distribution of human milk where lactoferrin is the major binding protein. In contrast to humans, iron is primarily bound to transferrin of the whey milk fraction in rodents like the rat and the rabbit (Kawakami *et al.*, 1990). It has been postulated that specific receptors for lactoferrin are responsible for the high bioavailability of iron from human milk (Fransson *et al.*, 1983). Exclusively breast fed infants maintain adequate iron status in the first six months of life (Lonnerdal, 1990). This, and considering the low iron content of human milk, is an indication of the importance of lactoferrin in iron bioavailability in early life.

Table 1.3

Concentration and Distribution of Iron in Milk of Various Species

Species	Fe Content	Fe Distribution (% total Fe)		
	Mature milk µg/ml	Casein %	Whey %	Fat %
Human	0.2 - 4.0	2 - 14	65 - 81	19 - 26
Cow	0.1 - 0.2	61 - 73	9 - 15	13 - 18
Goat	0.3 - 0.5	33 - 49	28 - 51	12 - 20
Sheep	0.2 - 0.5	37 - 49	25 - 36	20 - 32
Horse	0.3 - 1.0	30 - 80	5 - 33	10 - 43
Pig	1 - 2	32 - 52	35 - 50	18 - 43
Rat	5 - 10	34 - 42	17 - 65	3 - 25
Dog	2 - 8	63 - 79	6 - 26	5 - 19
Cat	1 - 4	4 - 68	40 - 85	2 - 18

Adapted from Lonnerdal (1990a)

Iron-binding proteins in milk have been proposed to influence the ability of iron to penetrate the mucosal barrier via a facilitated uptake mechanism (Cox *et al.*, 1979; Fransson *et al.*, 1983; Kawakami *et al.*, 1990). Davidson and Lonnerdal (1988) demonstrated specific and saturable ⁵⁹Fe-lactoferrin binding to the intestinal brush border membrane in rhesus monkeys at different ages (foetal, suckling infant, weaned infant, juvenile and adult) as an indication that a receptor for human lactoferrin on the intestinal brush border membrane of the

infant may function in the absorption of iron. The concentration and structural properties of lactoferrin from the milk of rhesus monkeys are similar to that of human milk (Davidson and Lonnerdal, 1986) and numbers of intestinal receptors for lactoferrin are highest in infancy in both species (Lonnerdal, 1990). In parallel to these studies looking at lactoferrin receptors in rhesus monkeys Gislason *et al.*, (1993), attempted to demonstrate intestinal binding of human lactoferrin to receptors in suckled piglets. While specific binding of porcine lactoferrin was found, neither human nor bovine lactoferrin displayed evidence of binding to the lactoferrin receptors in the piglet's intestine (Gislason *et al.*, 1993). This highlights species differences with respect to the role of lactoferrin in iron absorption. There may be similar differences between the human and a model animal, depending on the animal chosen.

In an attempt to clarify the issue of iron retention from milk and infant formula and the influence of lactoferrin on this, Davidson *et al.*, (1990) intubated infant rhesus monkeys with either FeSO₄ supplemented infant formulas or human milk with and without human or bovine lactoferrin. No significant difference in iron retention from any of the diets was found and so it was concluded that the monkeys retain iron equally as well from the formula as from human milk. This contrasts indices in human infants which suggest anaemia is lower in breast fed infants than formula fed infants (Lonnerdal, 1990). These differences may be because the presence of lactoferrin in addition to natural levels may exceed requirements for maximal receptor binding. Another reason for the discrepancy could be that species-specific differences in lactoferrin facilitated iron absorption, despite the similarities in lactoferrin characteristics. In studies of infant iron nutrition using an animal model species-specific differences with respect to lactoferrin facilitated iron absorption need to be taken into account. A role lactoferrin in iron absorption, however, has yet to be confirmed (Lonnerdal, 1996).

Kawakami *et al.*, (1990) investigated, using the neonatal rat, iron uptake in the presence of bovine transferrin and lactoferrin, and human lactoferrin. In this study no binding of human lactoferrin in the rat occurred. Deglycosylation of the bovine lactoferrin had no influence on intestinal binding of the lactoferrin,

instead results suggested that the lactoferrin bound via a polypeptide interaction to the rat transferrin receptor (Kawakami *et al.*, 1990). This in contrast to lactoferrin binding in the rhesus monkey where fucosylated glycans on the human lactoferrin are necessary for receptor recognition on the intestinal brush border of the monkey (Davidson and Lonnerdal, 1988). As bovine milk is a common base for infant formulas, however, these species differences in binding profiles may not be relevant in choosing an animal model due to a low lactoferrin content in bovine milk.

Further discrepancies between rat and human iron absorption mechanisms were highlighted by Reddy and Cook (1991). Reddy and Cook (1991) eliminated any methodological factors, insofar as possible, that might account for differences in absorbance of iron humans and rats due to dietary factors. In humans vitamin C, meat, soy protein and phytates are all dietary factors known to significantly influence the absorption of non-haem iron (Monsen *et al.*, 1978; Rossander-Hulthen and Hallberg, 1996). In the study by Reddy and Cook (1991) species differences in iron absorption were measured as the ratio of ^{59}Fe to ^{55}Fe incorporation into circulating whole blood of iron replete rats. The rats clearly demonstrated a relative insensitivity to the factors that affect non-haem iron absorption in humans, absorbing an immense ten-fold more iron in some cases. This was not improved by reducing iron status prior to iron administration, implying no effect of iron status on the effect of dietary factors in rats.

Frolich and Lyso (1983) found a similar situation in anaemic piglets offered a bran-based ferrous sulphate fortified diet. There were no significant differences in bioavailability when iron or bran levels were altered. This indicates an insensitivity of the pig to a dietary factor which influences iron bioavailabilities in humans. These differences could be attributed to differences in the luminal environment of the gastrointestinal tract or a differences in mucosal cell function between species, however this factor has yet to be ascertained. Bran, however is rarely a component of the infants diet nor that of the suckled piglet, hence this difference are not considered relevant to either infant iron nutrition or nutrition of the baby pig.

The enhancing effect of ascorbic acid on iron absorption from the human digestive tract is well accepted. This vitamin has been successfully exploited in many product formulations to enhance the absorption of iron. Unlike the human, pigs, rats and mouse all synthesise this vitamin and this may be an intrinsic mechanism to increase iron absorption in these species (Perks and Miller, 1996). This offers another complication when considering the pig as an animal model for iron absorption in the human. Species such as the guinea pig, who share a dietary requirement for vitamin C with humans, would be the preferable model animal when altering dietary vitamin C levels for iron research. In studies where the vitamin C requirement in the human is not compromised, however, it is feasible to use the pig, rat or mouse as a model animal.

Narasinga Rao *et al.*, (1977) looked at distinguishing differences in iron absorption of ferrous and ferric iron sources between monkeys, guinea pigs and humans. The study by Narasinga Rao *et al.*, (1977) raised the question of how the absorption of both ferric and ferrous iron compound compare between the different species. In man ferric iron is less well absorbed than ferrous iron (Forth and Rummel, 1973). Rats, guinea pigs and chicks, however, do not distinguish between ferric and ferrous iron sources in the way that humans do. This is very important in terms of using the rat as a model for iron research. The ionisation state of iron is one of the most important factors determining the extent of iron absorption from the human intestine. Any species which does not distinguish between the two ionisation states should not be considered a suitable animal model.

The practice of coprophagy by several species of animal serves to complicate iron research methodologies further (Ranger and Neale, 1984; Zhang *et al.*, 1992). While it is well established that coprophagy can lead to overestimates in macronutrient bioavailability studies, there is considerable controversy over the magnitude to which coprophagy effects estimates of iron availability (Zhang *et al.*, 1992). Prevention of coprophagy ensures only one passage of food through the gastrointestinal tract, a situation parallel to the human digestive process.

Although studies in the past have found no advantage in preventing coprophagy (Geever *et al.*, 1969), more recent studies have suggested the effect of coprophagy may depend on the dietary source of iron (Zhang *et al.*, 1992; Ranger and Neale, 1984). Zhang *et al.*, (1992) reported that errors of up to of 8% for the bioavailability of FeSO₄ and up to 25% for the bioavailability of the iron in plant foods could be attributed to coprophagy in anaemic rats. This lower error in estimating the bioavailability of inorganic iron (8%) may be due to the high bioavailability of FeSO₄, which would imply a greater degree of absorption on the first passage through the digestive tract. As non-haem food iron may be absorbed via a carrier system requiring a longer absorption period, the extent of absorption of this type of iron would clearly increase given the opportunity of a second passage through the digestive tract. While a psychological effect arising from the discomfort associated with preventing coprophagy can not be overlooked as having a possible effect on iron absorption, experimental design may overcome this by introducing an adjustment period. This would allow the animals a time in which to adjust to their coprophagy prevention device before the experimental treatment begins.

Zhang *et al.*, (1992) investigated the efficiency of tail cups and collars for preventing coprophagy and concluded that the use of tail cups for coprophagy prevention was not as convenient or effective as the use of collars. A separate group of rats were treated with sham collars and tail cups so as to control for the stress effects of preventing coprophagy. Again a difference was observed between ionic and food-iron sources, indicating that the stress associated with the prevention of coprophagy appeared to significantly effect the absorption of ionic but not food iron sources. The degree to which this stress affected iron absorption was smaller than that expected had coprophagy not been prevented. By including a group of sham collared or cupped animals to control for stress effects it was possible to clearly identify the significant benefits of coprophagy prevention in terms of assessing iron bioavailability. Both the pig and rat offers relative simplicity in this respect as coprophagy can be prevented when faeces are solid by using metabolic crates or cages with slatted or meshed floors which enable faeces to fall quickly out of reach. The diarrhoea associated with

anaemia, however, necessitates for total collection and removal of faecal matter. This can be achieved in the piglet using a human ostomy apparatus adapted for attachment to the piglet's anus (Darragh and Moughan, 1995).

Moreover, the pig is particularly suitable model for iron bioavailability studies for several reasons. The infant human and pig gastrointestinal tracts are remarkably similar (Miller and Ullrey, 1987; Moughan *et al.*, 1992). In the book *The Biology of the Pig*, Pond and Houpt (1978) state "In its nutrient requirements, the pig resembles the human in more ways than any other nonprimate mammalian species. This is due to the physiological and anatomical similarity of the digestive tract of the pig and man." There is an uncanny parallel between the digestive physiology of the piglet and the human at the comparative stage of peak lactation (three-weeks-old in the piglet and the three-months-old in the human infant) (Moughan *et al.*, 1992; Darragh and Moughan, 1995). Although no direct comparison of iron nutrition has been made between the piglet and the human infant, similarities in the digestive physiology and in the nutrient requirements of the two species would appear to suggest that the three-week-old piglet would make a suitable animal model for the three-month-old human.

In addition to the physiological similarities between the 3-week-old piglet and the three-month-old infant the piglet has a uniquely adaptable iron status. The use of haemoglobin regeneration measure in rats requires modification of the rat diet which may not be applicable to human nutrition (Reddy and Cook, 1991). In contrast, to the rat the neonatal piglet has an iron status that can be readily manipulated to suit experimental requirements. This is because the neonatal pig is prone to iron deficiency due to poor endowment (Brownlie 1955; Pearson *et al.*, 1983; Whalley, 1983). The piglet is born with around 50mg of iron as a reserve (Whalley, 1983), with a requirement of around 7mg/day (Pearson *et al.*, 1983) necessary to prevent anaemia. By withholding the iron dextran injection that is standard practice in intensive pig rearing units, anaemia will ensue (Brownlie 1955; Pearson *et al.*, 1983; Whalley, 1983). Withdrawal of the iron dextran injection in neonatal piglets may be more physiological for the purpose

of iron absorption studies than altering a nutritional regimen as would be the case with the rat model.

In addition to the adaptable nature of neonatal piglet iron status, piglets are logistically suitable animal models for infant iron nutrition. Following investigations into the physiological and biochemical aspects of digestion in both humans and pigs with regard to age and stage of development, Moughan *et al.*, (1989) compiled a list of logistic advantages to using the pig as a model in human nutrition research (Table 1.4). Their findings established a strong argument for the suitability of growing and neonatal pigs for estimating the bioavailability of nutrients in food destined for human consumption. Another logistical advantage to using the piglet as a model animal in human research is that the pig can be trained to bottle-feed from a young age. This facilitates the transfer of nutrients in a way that can be accurately quantified without the need for the more stressing techniques such as intubation. Bottle-feeding is also an important factor when the experimenter wishes to mimic feeding behaviour in infant nutrition studies. Where gastric emptying or transition times may be important, it ensures delivery of a diet in a manner which satisfactorily parallels the human condition. It is difficult, if not impossible, to establish a suckled rat model at a parallel stage of development to the human infant for obvious logistical reasons.

Table 1.4

Logistical Advantages To Using The Pig as a Model for Human Nutrition

1. Ready adaptation to the metabolic crate
 2. The omnivorous eating habits of the pig
 3. The pig will eat most foods that humans eat
 4. Pigs display rapid growth conducive to experimental constraints
 5. Coprophagy can be prevented
 6. Pigs can be weaned from birth
 7. Large litter size
 8. Large sample collection facilitates chemical analysis
 9. Pigs are generally readily available at relatively low cost
-

(modified from Moughan *et al.*, 1989)

4.3 OVERALL CONCLUSION AND INFERENCES FROM THE REVIEW OF LITERATURE

Having reviewed the literature concerning mammalian iron nutrition the following pertinent facts have been identified:

- Iron is an essential component of haemoglobin and a number of other proteins and enzymes of the body.
- The demands imposed at times of increased growth coupled with a diet low in iron place the infant at risk of iron deficiency.
- Dietary supplementation from four months of age onwards is necessary to reduce the risk of iron deficiency. This can be achieved by fortifying infant cereals and formulas with iron.
- There is a need for further development with respect to bioavailability and organoleptic compatibility of the current iron sources used to fortify infant foods.
- It is important to get reliable estimates of bioavailability of different iron sources under appropriate conditions.
- Routine use of humans in iron bioavailability research is limited by safety, ethical and logistical constraints.
- Animal models offer a suitable means for routine testing of iron bioavailability.
- The suckled piglet has a well defined nutrient requirement; an anatomical make up very similar to that of the infant human; an iron status that can be readily manipulated to suit experimental requirements; and apparent logistical advantages which make this animal a suitable animal model in infant iron nutrition

Therefore the purpose of this research was to use the piglet as an animal model to test the bioavailability of iron from different sources to the human infant.

CHAPTER 2

MATERIALS AND METHODS

All aspect of this study were approved by the Massey University Animal Ethics Committee.

2.1 ANIMALS AND HOUSING

Forty eight entire male Large White X Duroc piglets were selected at random, at birth, from eight litters of pigs at a commercial piggery (Wairaka Farms Ltd, Foxton). Although remaining with their dams, the piglets did not receive the iron-dextran injection routinely given, at birth, to intensively reared piglets. When the piglets were five days of age (mean body weight \pm SE, 2.4 ± 0.06 kg) they were removed from their dams. The piglets were initially housed in pairs in moulded plastic metabolism cages designed to minimise contact with iron and iron-containing materials. The cages were in temperature controlled rooms maintained at $30 \pm 1^\circ\text{C}$ with a constant 15 ½ hour light: 8 ½ hour dark cycle. After six days the piglets were separated into individual cages for remainder of the experiment.

2.2 FORMULAS AND FEEDING

The six formulas used in the trial included a low-iron preliminary formula (control formula) and five test formulas fortified with different iron sources. The ingredients list for all the formulas included in the study is given in Table 2.1. The control formula consisted of a bovine milk-based formula that had not been fortified with iron from an extrinsic source. Each treatment was formulated by fortifying the control formula with one of five different iron sources (ferric pyrophosphate (FP formula); ferrous sulphate heptahydrate (FS formula); milk protein-iron complex (MPIC formula); ferrous lactate (FL formula); and Haemin (HM formula)). The supplementary iron sources were added at a level which was assumed to deliver a set amount of iron corresponding to current regulations concerning iron fortification of infant formulas (Codex Alimentarius Commission, 1995). However, subsequent analysis of the formulas revealed iron

levels in the each formula varied. This was due to errors in initial estimation of the iron content of each iron source. The nutrient contents relating to all of the formulas used in this study are listed in Table 2.2.

Table 2.1
Ingredient composition of six bovine milk-based formulas with or without iron supplementation*

Ingredient	Experimental Formulas					
	Control	FP	FS	MPIC	FL	Hm
Demineralised whey powder (Alawhey 652)	2.6	2.6	2.6	2.6	2.6	2.6
Skim milk powder	48.9	48.9	48.9	48.9	48.8	48.8
Milk fat	31.3	31.3	31.3	31.3	31.3	31.3
Oil Blend ^c	10.0	10.0	10.0	10.0	10.0	10.0
Water	2.6	2.6	2.6	2.6	2.6	2.6
Calcium Hydroxide	0.1	0.1	0.1	0.1	0.1	0.1
Mineral/Vitamin Premix [#]	4.5	4.5	4.5	4.5	4.5	4.5
Fe sources: (mg/100g)						
Ferric Pyrophosphate	-	35.8	-	-	-	-
Ferrous Sulphate Heptahydrate	-	-	93.0	-	-	-
Milk Protein-Iron Complex	-	-	-	15.2	-	-
Ferrous Lactate	-	-	-	-	110.9	-
Haemin	-	-	-	-	-	103.0

* g of ingredient/100 g powdered formula except for the iron sources which are mg/100g powdered formula

[∞] 30% sunflower oil, 70% soybean oil.

[#] Minerals and vitamins added to supply: calcium, 680; magnesium, 6.8; zinc, 4.2; sodium, 250; potassium, 860; retinol, 12; vitamin C, 100; panthenoic acid, 5.5; vitamin B₃, 7.25; vitamin B₂, 1.9 (mg/100g powdered formula); copper, 360; manganese, 60; tocopherol, 730; vitamin D₃, 11, vitamin K₁, 75; vitamin B₁, 675; vitamin B₆, 620; biotin, 45; and vitamin B₁₂, 6.1 (µg/100g powdered formula).

The dry milk formulas were mixed (14g dry matter (DM)/100ml water) with ultrapure water (Milli-Q® Plus, Millipore Corporation, Massachusetts, USA) daily in 25L plastic containers and refrigerated (4°C). Reconstituted formulas were stored for twelve hours before use to allow maximum hydration, thus ensuring that all of the formulas were completely dissolved. All formulas were stirred every two and a half hours to prevent sedimentation of any of the iron compounds.

Table 2.2*Nutrient composition* of six bovine milk-based formulas with or with iron supplementation*

Nutrient	Experimental Formulas [†]					
	Control	FP	FS	MPIC	FL	Hm
Total fat	20.1	18.8	18.5	18.6	18.7	19.1
Protein [‡]	16.9	15.8	15.2	15.8	15.8	15.3
Lactose	55.2	51.7	50.9	51.3	51.6	52.5
Minerals/Vitamins	4.7	4.4	4.4	4.4	4.4	4.5
Moisture)	2.8	2.6	3.7	2.6	2.6	2.7
Fe (µg/100g)	0.4	6.6	8.3	7.3	6.6	6.0

* g of nutrient/100 g powdered formula except for the iron sources which are µg/g powdered formula

‡ The protein and iron contents were determined for each formula; all other values were taken from the manufacturer's specifications.

† all treatments were formulated from an bovine milk-based formula fortified with different sources of iron. The names of each formula refer to the iron source added to the base formula: control = no iron fortification; FP = Ferric pyrophosphate; FS = Ferrous sulphate heptahydrate; MPIC = Milk protein-iron complex; FL = Ferric lactate; Hm = Haemin.

The piglets' individual daily allowances were set at 336g of liquid formula/kg body weight/day. These daily allowances were calculated on a gut capacity basis with 7.6g liquid formula/cm³ of estimated stomach volume/day. Gut capacity was estimated from individual liveweights (Darragh and Moughan, 1995). Given that the piglet was being used as a model animal for the human infant, the intakes described above were calculated to correspond to a level similar to that of the three-month-old formula fed infant, assuming gut capacity in the piglet, on a body weight basis, to be twice that of the human infant (Moughan *et al.*, 1992).

2.3 EXPERIMENTAL PROCEDURE

The duration of the study was 36 days including an 11 day preliminary period followed by a 25 day experimental period. During the preliminary period the piglets adapted to the feeding method and regimen. In addition, provision of the control formula, for the duration of the preliminary period, resulted in a depletion of the piglets' body iron stores, as confirmed by low blood haemoglobin concentrations and haematocrit. Depletion of the piglets' iron status was necessary to induce an anaemic state in the animals in order to maximise the effects of treatment with dietary iron.

Following removal from the sow each piglet had *ad libitum* access, via plastic troughs, to ultra pure water (Milli-Q® Plus, Millipore Corporation, Massachusetts, USA) only, for an initial period of 24 hours. After the first day, the piglets were trained to drink the control formula from bottles with soft rubber teats attached. Half concentration formula (7g DM/100ml water) reconstituted using ultra pure water (Milli-Q® Plus, Millipore Corporation, Massachusetts, USA) was offered on the first two trial days to allow the piglets a nutritional adjustment period. The liquid formula was warmed (35°C) prior to feeding. Piglets were assigned to six treatment groups on the basis of liveweight, litter and blood haemoglobin concentration (see section 2.41) which was assessed using whole blood taken from the anterior vena cava on day two of the trial. Treatment groups were then randomly assigned to experimental formulas. The piglets were exclusively fed their designated test formulas for the remainder of the experimental period

The formula was adjusted to full strength once the piglets had adapted to the control formula (day 3) and acclimatised to their new environment. The formula concentration remained at full strength for the remainder of the trial. The piglets were offered their daily formula allowances in seven equal portions spread at two and a half hourly intervals throughout the day; at 0630h, 0900h, 1130h, 1400h, 1630h, 1900h and 2100h. Refusals were weighed after each feed and total refusals were recorded after the final feed each day. The piglets were weighed every three to four days, and their individual daily intakes adjusted accordingly to maintain their designated rate of intake on a gut capacity basis.

On day five the piglets were each fitted with an apparatus to prevent coprophagy. A 10cm radius was shaved around the anal region of each piglet. A plastic flange on a pectin, gelatin and cellulose based wafer (Sur-fit® Stomahesive®, Bristol Myers Squibb, Auckland, New Zealand), designed for the attachment of human ostomy bags, was adhered to the shaved region of each piglet so that the 32mm opening on each flange was directly over the anus. Adhesive sports strapping tape (Elastoplast, Crown Dental Medical, Christchurch, New Zealand) was used to secure the edges of the wafer to the

piglet's tail region. A closed-end pouch (Sur-fit® Plus, Bristol Myers Squibb, Auckland, New Zealand) was attached to each flange which facilitated faecal collection. The bags were changed each day when full. Any faeces collected were discarded.

To assess the degree of iron repletion in the anaemic piglets following iron supplementation, samples of the piglets' blood were collected for analysis on days 2, 10, 24 and 36 of the trial. Individual blood samples were collected from the anterior vena cava of each piglet at 1100h in the morning of each collection day. Whole blood, collected from the piglets into evacuated blood collection tubes (2ml Vacutainer® Brand Blood Collection Tubes, Becton Dickinson, Franklin Lakes) containing EDTA, was analysed for haemoglobin concentration. To minimise haemolysis, these whole blood samples were collected and cooled to room temperature (20°C), with analyses being carried out within three hours of collection. In addition, sub-samples of this whole blood were transferred into 0.2mm x 75mm heparinized capillary tubes (Chase Instruments Corporation, Norcross, USA). These capillary tube samples were used for measuring the haematocrit values for each piglet.

Another vial of blood was collected from the piglets into evacuated blood collection tubes (2ml Vacutainer® Brand Blood Collection Tubes, Beckton Dickinson, Franklin Lakes). These blood samples were left at room temperature for six hours to allow blood clots to form. The serum from each clotted sample was decanted from these samples and subsequently analysed for unsaturated iron binding capacity.

2.4 CHEMICAL ANALYSES

2.4.1 Haemoglobin Determination

The haemoglobin concentrations of the blood samples were determined by the cyanmethaemoglobin method (Crosby and Munn, 1954). Whole blood (0.02ml) was diluted using 4ml of a solution of potassium cyanide and potassium ferricyanide (Drabkins Reagent) (Roche Cobas Method, 1995). This induced the conversion of haemoglobin in the blood to cyanmethaemoglobin. After a ten

minute delay to ensure the complete conversion of haemoglobin, the absorbance of the solution was measured colorimetrically at wavelength 530-550nm on a Roche Cobas Minos Vet Autoanalyser (ABX International, France) against a reagent blank. Haemoglobin concentrations (Hb, g/dL) for the individual piglets were calculated as in equation A.

Equation A:

$$\text{Hb} = \frac{\text{Blood sample absorbance} \times \text{conc. Standard (mg/100ml)} \times \text{dilution factor}}{\text{standard absorbance} \times 1000}$$

A control standard (Bio-rad Liquicheck™, Bio-rad Laboratories, Auckland, New Zealand) was analysed with the samples to check that the assay remained within accepted specifications (ie. 14.3 g/dL ± 0.6 g/dL).

2.4.2 Haematocrit

Haematocrit was measured as packed cell volume in the whole blood samples and expressed as a percentage of blood volume (PCV%). The capillary tubes containing whole blood sub-samples were centrifuged in a microcentrifuge (Jouan Hema-C Centrifuge, Saint Herblain, France) at 5000rpm, for five minutes. Packed cell volumes were then read from the centrifuged tubes using a haematocrit card (Mikro-Haematokrit, Heraeus-Christ GMBH, Osterode, Germany).

2.4.3 Unsaturated Iron Binding Capacity

At alkaline pH, ferrous ions added to serum bind specifically with transferrin at unsaturated iron binding sites (Persijn *et al.*, 1971). A standard amount of ferrous iron (500µg/dL) was added to the serum at alkaline pH and assumed to bind to un-filled transferrin binding sites. The remaining unbound ferrous iron that was not sequestered by transferrin sites was measured colourimetrically by the use of ferrozine at wavelength 560nm (Hitachi U-2001 UV/Vis Spectrophotometer, Japan). Ferrozine is a sulphonated derivative of diphenyltriazine that forms a soluble magenta complex with iron. The unsaturated iron binding capacity (UIBC) was determined as the difference between the amount of unbound iron and the total amount of iron added, as shown in equation B.

Equation B:

$$\text{UIBC } (\mu\text{g/dL}) = 500 - \frac{(\text{sample absorbance} - \text{sample blank absorbance} \times 500)}{\text{standard absorbance}}$$

2.4.4 Protein Determination

Nitrogen content of the formulas were determined using the Leco® autoanalysis technique (Anon, 1994). Duplicate samples of each formula were weighed out and placed into a sample boat for analysis in the Leco® autoanalyzer (Leco® CNS-2000, Leco Corporation). Samples were combusted in a resistance furnace (600°C) and the nitrogen content was determined by a Thermal Conductivity cell. Results were corrected for moisture content after drying at 105°C.

2.4.5 Energy Determination

The gross energy of each formula was determined using bomb calorimetry (Gallentramp Autobomb, Watson Victor Ltd, Wellington, New Zealand). The formulas were freeze-dried (Cuddon Model 0610 Freeze Drier, W.G.G. Cuddon Ltd, Blenheim, New Zealand) and ground through a sieve (1mm diameter). Duplicate sample pellets containing 0.05-1g of each sample were prepared by placing the sample in the cylinder with a metal bung in the bottom. A second metal plug was then used to compress the sample to give a solid pellet. The weight of each pellet was recorded.

A calorimeter vessel was filled with water until the total weight was $3000 \pm 0.01\text{g}$ and the water temperature adjusted to approximately 24.5°C. The vessel was placed in the water jacket of the calorimeter and the bomb positioned in the apparatus and the temperature allowed to stabilised (5-10minutes). An initial temperature was recorded to 3 decimal places before combustion of the sample and then a final temperature was measured and recorded after ten minutes. The bomb was removed from the calorimeter and the vessel reweighed to 3000g. A standard of benzoic acid was analysed with the samples to check it was within specification (ie. $26435.1 \text{ J/g} \pm 3.5 \text{ J/g}$).

The initial temperature was subtracted from the final temperature to obtain the temperature rise. Correction factors for all temperatures were added to the temperature rise. The temperature rise was then multiplied by the water equivalent (10.873 kJ/°C) to give a calorific value for the sample. The calorific value was divided by the sample weight to give a gross calorific value (kJ/g). Finally, the gross calorific value was adjusted for dry matter content.

2.4.6 Iron Determination

The iron content of each formula was determined colorimetrically (NZDB, 1994), with a method modified from the AOAC (1993). Quadruplicate samples (1g) of each powdered formula were dissolved in 1ml nitric acid (7.5N), and subsequently dried at 100°C for 4 hours in a furnace. The temperature in the furnace was increased at a rate of 100°C per hour until the temperature was 600°C. Samples were ashed at this temperature for a further 10 hours.

Each dry ashed sample was transferred into a 50ml volumetric flask with 1ml concentrated HCL (10N) and made to volume with ultra pure water (Milli-Q® Plus, Millipore Corporation, Massachusetts, USA), to a final HCL concentration of 0.2M. A 10ml aliquot of the 0.2M solution was transferred into a 25ml volumetric flask. The iron content of each sample was determined colorimetrically (UV/VIS Spectrophotometer Lambda 11, Bodenseewerk Perkin Elmer GmbH, Uberlingen, Germany) using orthophenathroline, at wavelength 510nm.

2.5 DATA ANALYSIS

Haemoglobin repletion efficiencies (HRE%) for each treatment group were calculated based on the difference between initial and final whole body haemoglobin iron levels for each piglet expressed as a proportion of total iron intake as shown in equation C. This also corrected for the differences in iron concentration in the formula. The whole body haemoglobin iron levels were determined using blood haemoglobin concentrations and piglet liveweights at

the beginning and end of the repletion period, assuming a blood volume of 7% body weight and a haemoglobin iron content of 0.335% (Thoren-Tolling and Lindberg, 1975).

Equation C:

$$\text{HRE}\% = \frac{\text{whole body Hb-Fe(mg)}_{\text{FINAL}} - \text{whole body Hb-Fe(mg)}_{\text{INITIAL}}}{\text{Fe intake(mg) over repletion period (25d)}}$$

Average daily weight gains (ADG) were calculated for each treatment from changes in individual piglet live weights over three separate time intervals during the trial as shown in equation D. The three separate time intervals were: days 0 to 10, 11-24 and 25-35.

Equation D:

$$\text{ADG} = \frac{\text{liveweight(g)}_{\text{final}} - \text{liveweight (g) initial}}{\text{Number of days in time interval}}$$

The level of statistical significance was set at $P < 0.05$ for tests on homogeneity of variance and ANOVA. Values for group data on haemoglobin, unsaturated iron-binding capacity and nutrient intakes (appendix 1) were converted to natural logarithms for statistical analysis. The results were reconverted as antilogarithms to recover the original units. Data for haemoglobin, unsaturated iron-binding capacity and haematocrit were compared using a within-time split plot statistical model using the general linear models procedure (SAS, 1989) to compare differences in treatment effects. Average daily liveweight gains, HRE% and nutrient intake data were analysed using a one way ANOVA (Minitab, 1989).

CHAPTER 3

RESULTS

3.1 PIGLET GROWTH AND FEEDING

All piglets adjusted to the 2½ hourly feeding regimen by day five of the trial and readily consumed their set intakes of formula throughout the study. Two piglets were excluded from the study: one piglet (from the Hm formula fed group) died during the preliminary (depletion) period from a gastrointestinal infection unrelated to the experimental treatment, and the second piglet (from the MPIC formula fed group) gained access to iron by chewing through to the rust on the plastic-coated metal bars on its metabolism cage. Seven piglets fed the control formula and one piglet in the Hm treatment group were removed from the trial after the blood collection on day 24 revealed severe anaemia, as determined by a haematocrit level less than 15% packed cell volume.

Average piglet liveweights and blood haemoglobin levels at the beginning (day 1) of the trial are presented in Tables 3.1 and 3.2, respectively. There were no significant differences in piglet liveweights or the piglets' blood haemoglobin concentrations in any of the treatment groups on day one of the trial.

Average daily growth rates for each treatment group are presented in Figure 3.1. At the beginning of the repletion period there were no significant differences in average daily growth rates between treatment groups. The rate of growth in the control group of piglets progressively declined from day 0 to day 24 of the trial (Fig. 3.1). At the end of the repletion period (day 35) the differences in average daily growth rates between treatment groups (Fig. 3.1) were not significant ($p>0.05$).

Table 3.1

Initial liveweights (mean \pm SE) of piglets*, assigned to either a control formula containing no iron or one of five bovine milk-based formulas fortified with different iron sources*

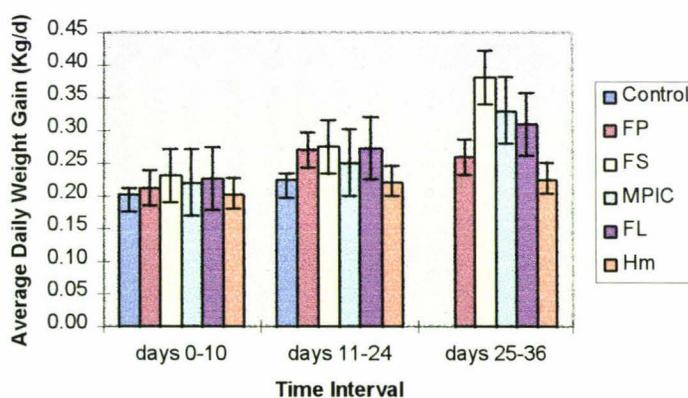
Piglet Group[⊗]	Liveweight (kg)
Control (n= 8)	2.3 \pm 0.14
FP (n=8)	2.3 \pm 0.19
FS (n=8)	2.3 \pm 0.15
MPIC (n=8)	2.4 \pm 0.10
FL (n=8)	2.4 \pm 0.12
Hm (n=8)	2.5 \pm 0.20
Statistical Significance [#]	NS

* day 1 of the 36 day trial period.

* 5-6 days old.

⊗ the treatments were formulated from a bovine milk-based formula fortified with different sources of iron. The names of each treatment refer to the iron source added to the base formula: control = no iron fortification; FP = Ferric pyrophosphate; FS = Ferrous sulphate heptahydrate; MPIC = Milk protein-iron complex; FL = Ferric lactate; Hm = Haemin.

NS = (non-significant), $P > 0.05$.

**Figure 3.1**

Average (\pm SE) daily weight gains (kg/d) for three time periods (days 0-10, 11-24 and 25-36 of the trial) in piglets fed either a control formula containing no iron and five iron fortified bovine milk-based formulas (control, FS = Ferrous sulphate heptahydrate, MPIC = Milk protein-iron complex, FL = Ferric lactate, and Hm = Haemin) over a treatment period of 25 days.

The average total intakes of formula (ml formula/feed/day) by the piglets, for each treatment, over the repletion period (days 11-36 of the trial) are given in Table 3.3. There was no significant difference ($p>0.05$) in formula intakes between treatment groups at the beginning (day 11) or middle (day 24) of the repletion period. By day 25 of the trial only one piglet remained in the control group, the rest having being removed because of severe anaemia. Consequently, this group was excluded from statistical analyses of the final period. By the end of the repletion period (day 36) the piglets fed the Hm formula had significantly lower ($P>0.05$) formula intakes than the MPIC group (Table 3.3). The formula intakes for the Hm piglets on day 36 of the trial were also lower than those of the FS, FP and FL piglets, although this difference was not significant. Protein and energy intakes for the repletion period of the trial are given in appendix 1.

Four replicate samples were analysed for determination of the iron content in each formula, and the overall mean coefficients of variation (between the quadruplicates for each formula) was 2%. Formulas were analysed in duplicate for gross energy and protein determination, the average difference between duplicates expressed as a proportion of the mean for these were 4.3% and 2.7%, respectively. The mean inter-assay coefficient of variation for the haemoglobin, haematocrit, UIBC, gross energy, and protein determination assays were 1.17%, 1.64%, 1.86%, 1.59% and 1.14%, respectively. Serum sub-samples were analysed in triplicate for UIBC, and the mean coefficients of variation between replicates for UIBC was 8.5%.

Table 3.2

Average Formula Intakes (mean ml/feed/day ± SE) in piglets fed bovine milk-based formulas fortified with different sources of iron over a 25 day experimental period*

Day of Trial	Iron Source in Formula					Statistical Significance ^{#v}
	<i>Ferric Pyrophosphate</i>	<i>Ferrous Sulphate Heptahydrate</i>	<i>Milk-Protein-Iron Complex</i>	<i>Ferrous Lactate</i>	<i>Haemin</i>	
	(n ⁺ =8)	(n=8)	(n=7)	(n=8)	(n=7)	
10	114.1 ± 0.12 _w ^⓪	126.8 ± 0.11 _w ^a	112.8 ± 0.12 _w ^a	118.8 ± 0.12 _w ^a	114.2 ± 0.11 _w ^a	NS
24	267.4 ± 0.09 _x ^a	268.8 ± 0.08 _x ^a	295.4 ± 0.10 _x ^a	263.1 ± 0.09 _x ^a	274.0 ± 0.09 _x ^a	NS
35	411.9 ± 0.05 _y ^a	411.9 ± 0.05 _y ^a	446.7 ± 0.06 _y ^a	429.3 ± 0.05 _y ^a	333.8 ± 0.08 _{wx} ^{**}	**

- * SE of the log transformed data.
- + number of piglets in treatment group.
- v statistical significance between treatment groups within time.
- # NS = non-significant, P> 0.05; ** = P<0.01; *** = P<0.001.
- ⓪ means within a row with different superscripts (a, b, c) are significantly different (P<0.05), (LSD).
- means within a column with different subscripts (w, x, y) are significantly different (P<0.05), (LSD).
- n=6, only six piglets were left in the Haemin group by day 36 of the trial, one was removed due to severe anaemia.

3.2 EFFECTS OF IRON TREATMENT ACROSS TIME

Normal values for suckled piglets for haemoglobin and haematocrit are 11.0-13.0 (g/dL) and 38-44 (PCV%), respectively (Jain, 1985; Thoren-Tolling, 1975). Haemoglobin levels from 9.0-11.0 (g/dL) are also considered adequate (Thoren-Tolling, 1975). All piglets on the control formula experienced a significant decline in blood haemoglobin and haematocrit (Table 3.4) throughout the treatment period. Only one piglet remained on the control formula after day 24 of the trial, the rest being removed and treated with iron due to severe anaemia, as previously mentioned. Difficulties in obtaining serum samples from the piglets fed the control formula meant that there was insufficient serum available to carry out UIBC analyses on this treatment group.

Table 3.3

Haemoglobin concentration (mean \pm SE[♣]; g/dL) and Haematocrit (mean \pm SE; %) in the blood collected from[⊗] piglets fed a bovine milk-based formula (containing no additional iron), for a 13 day experimental period[Ⓝ]

Day of Trial	Haemoglobin (g/dL) (n ⁺ =8)	Haematocrit (PCV%) (n=8)
normal range [∞]	11.0-13.0	38-44
0	7.9 \pm 0.02	27 \pm 2.0
11	7.0 \pm 0.02	29 \pm 2.0
24	4.4 \pm 0.02	17 \pm 2.0
Statistical Significance [#]	***	***

♣ SE of the log transformed data.

⊗ collected at the beginning of the repletion period (day 11), and half way through the repletion period (day 24).

Ⓝ following an eleven day iron-depletion period.

+ number of piglets in treatment group.

∞ normal reference range for blood haemoglobin concentration (Thoren-Tolling, 1975) and haematocrit (Jain, 1995) in suckled piglets.

*** = P < 0.001.

Average haemoglobin, haematocrit and UIBC levels in the blood (collected on days 0, 11, 24, and 36) of piglets fed the iron fortified formulas of the trial, are given in Tables 3.4, 3.5, and 3.6, respectively. The addition of dietary iron leads to highly significant ($P < 0.001$) increases in blood haemoglobin levels except for the piglets fed the FP or the Hm formulas. Adequate blood haemoglobin concentrations were reached in piglets fed the FS, FL and MPIC formulas by day 24 of the trial. The increase in haemoglobin levels during the first half of the repletion period (day 11-24) accounted for 85, 80, 75, and 65% of the total rise in blood haemoglobin over the entire repletion period for the FP, FS, MPIC, and FL treatment groups, respectively. Significant ($P < 0.01$) increases in haemoglobin were also apparent for these groups between days 24 and 36 of the trial which resulted in the mean haemoglobin level of piglets on the FS formula increasing to what is considered a normal level. While results indicate an average increase in haemoglobin concentration of 1.5 g/dL for the FP group from the beginning to the end of the treatment period, this increase was not statistically significant. The blood haemoglobin levels of the Hm treatment group showed a significant ($P < 0.001$) decline during the trial decreasing by a total of 3.0 g/dL over the entire 25 day repletion period. Any further changes in the haemoglobin levels of the Hm piglets from day 24 of the trial (half way through the repletion period) until the end of the trial (day 36), however, were not significant.

For all treatment groups, the haematocrit was below normal (Table 3.5) throughout the trial. No recovery of haematocrits were observed in the piglets fed the FP formula in either stage of the repletion period and the haematocrit for the Hm group declined significantly ($P < 0.001$) once treatment had begun. Significant increases in haematocrit were observed, however, for the FS, MPIC, and FL groups from day 11 to day 36 of the trial. As with the haemoglobin results, these increases appeared to be primarily in the first half of the treatment period with the FL and MPIC treatments showing 67 and 86% improvement in this period, respectively. The FS formula group tended toward a more steady

increase across the entire period with 45% of the total increase in PCV occurring after day 24 of the trial.

The unsaturated iron-binding capacity is a measure of the degree of saturation of the two binding sites of the iron-binding protein, transferrin, in serum. In the anaemic animal UIBC is increased. No significant change in UIBC was apparent for piglets fed either the Hm or FP formulas (Table 3.7). A significant ($P < 0.05$) decrease was observed, however, for piglets fed the FS, FL and MPIC formulas. These results support those from the haemoglobin and haematocrit data.

3.3 COMPARATIVE EFFECT OF DIFFERENT IRON SOURCES

The haemoglobin (Table 3.4) and haematocrit (Table 3.5) results for piglets fed either the FP or Hm formulas were significantly ($P > 0.05$) lower than those for FS, FL, and MPIC formulas on both days 24 and 36 of the trial. A comparison of the UIBC data (Table 3.6) between treatment groups showed a similar trend to those demonstrated for haemoglobin and haematocrit, although, the FL treatment group had significantly lower UIBC than the FS group on day 24 of the trial. Results from blood collected on days 24 and 36 of the trial show that piglets on the Hm formula had the lowest blood haemoglobin concentration and packed cell volumes of all the treatment groups, falling significantly lower ($P > 0.05$) than FP. The UIBC for piglets fed the Hm and FP formulas were also significantly lower than those determined for FS, FL and MPIC on days 24 and 36 of the trial.

Haemoglobin repletion efficiency (HRE%) is a measure of the efficiency with which the iron in the formulas is used to replace the haemoglobin-iron in the piglets. This can be interpreted as the percentage of ingested iron used for haemoglobin synthesis by the piglets. A HRE% could not be calculated for the control piglets due to insufficient piglets remaining in the trial at the end of the repletion period. Haemoglobin repletion efficiencies were, however, calculated for all the other formulas tested.

No significant differences in the HRE% were observed between the FS, MPIC, and FL treatment groups (Table 3.7) with 35.8, 38.0 and 32.9% of the ingested iron being utilised for haemoglobin synthesis, respectively. Differences in the HRE% were observed, however, between piglets fed the FS, MPIC, or FL formulas and those fed the FP and Hm formulas. Piglets fed the MPIC formula was used 14.5% less of the iron ingested for haemoglobin synthesis than piglets on the FL formula. Only 3.2% of ingested iron was utilised for haemoglobin synthesis by those piglets given the Hm formula. This was significantly lower than all the HRE% for the other iron-fortified formulas. The differences in HRE%, including the range and upper and lower quartiles for each treatment are represented pictorially in figure 3.2.

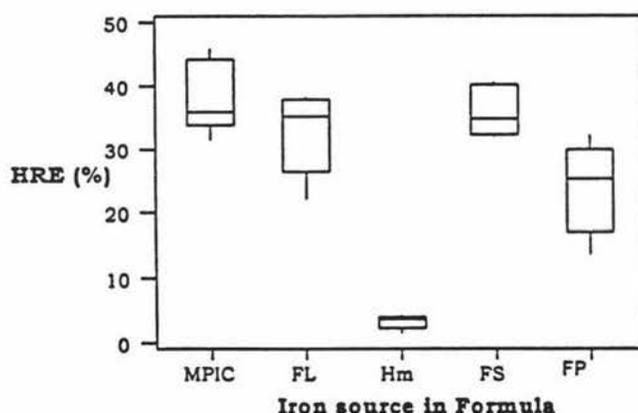


Figure 3.2

Boxplots representing the range, mean and upper and lower quartiles for the haemoglobin repletion efficiencies (HRE%) of five groups of piglets fed a bovine milk-based formula fortified with different sources of iron (FS = Ferrous sulphate heptahydrate, MPIC = Milk protein-iron complex, FL = Ferrous lactate, and Hm = Haemin).

Table 3.4

Haemoglobin levels (mean ± SE[♠]; g/dL) in blood collected[⊗] from piglets immediately after being removed from the sow and during a 25 day repletion period where the piglets were fed a bovine milk-based formula, fortified with one of five different sources of iron

Day of Trial	Iron Source in Formula					Statistical Significance [♥]
	Ferric Pyrophosphate (n ⁺ =8)	Ferrous Sulphate Heptahydrate (n=8)	Milk protein-Iron Complex (n=7)	Ferrous Lactate (n=8)	Haemin (n=8)	
0 [•]	7.7 ± 0.06 _w [Ⓢ]	7.6 ± 0.07 _w ^a	8.0 ± 0.07 _w ^a	7.7 ± 0.06 _w ^a	6.7 ± 0.07 _w ^a	NS
11	6.4 ± 0.05 _w ^a	6.5 ± 0.06 _w ^a	7.1 ± 0.06 _w ^a	6.7 ± 0.06 _w ^a	7.1 ± 0.06 _w ^a	NS
24	7.1 ± 0.04 _w ^a	10.2 ± 0.05 _x ^b	9.9 ± 0.04 _x ^b	9.2 ± 0.04 _x ^b	4.9 ± 0.04 _x ^c	***
36	7.3 ± 0.05 _w ^a	11.1 ± 0.06 _y ^b	10.8 ± 0.05 _y ^b	10.5 ± 0.05 _y ^b	4.1 ± 0.07 _x ^{c[∞]}	***
Statistical Significance [♥]	NS	***	***	***	***	

♠ SE of the log transformed data.

⊗ collected at the beginning of the repletion period (day 11), half way through the repletion period (day 24), and at the end of the repletion period (day 36).

+ number of piglets in treatment group.

• NS (non-significant) = P > 0.05; *** = P < 0.001.

♥ statistical significance between treatment groups within time, based on ANOVA.

• on day 0 the piglets were removed from the sow and blood samples taken. Between days 0-11 the piglets were fed a no-iron diet.

Ⓢ means within a row with different superscripts (a, b, c) are significantly different (P < 0.05), (LSD).

• means within a column with different subscripts (w, x, y) are significantly different (P < 0.05), (LSD).

∞ n=6, only six piglets were left in the Haemin group by day 36 of the trial, as one was removed due to severe anaemia.

• statistical significance within treatment groups across time, based on ANOVA.

Table 3.5

Haematocrit levels (mean ± SE, PCV %) in blood collected[⊗] from piglets immediately after being removed from the sow and during a 25 day repletion period when the piglets were fed a bovine milk-based formula fortified with one of five different sources of iron

Day of Trial	Iron Source in Formula					Statistical Significance ^{†‡}
	Ferric Pyrophosphate (n ⁺ =8)	Ferrous Sulphate Heptahydrate (n=8)	Milk-Protein-Iron Complex (n=7)	Ferrous Lactate (n=8)	Haemin (n=7)	
0 [*]	28 ± 2.0 _w [Ⓢ]	27 ± 2.0 _w [Ⓢ]	28 ± 2.0 _w [Ⓢ]	27 ± 2.0 _w [Ⓢ]	27 ± 2.0 _w [Ⓢ]	NS
11	25 ± 2.0 _w [Ⓢ]	27 ± 2.0 _w [Ⓢ]	27 ± 2.0 _w [Ⓢ]	25 ± 2.0 _w [Ⓢ]	26 ± 2.0 _w [Ⓢ]	NS
24	27 ± 2.0 _w [Ⓢ]	32 ± 2.0 _x ^b	33 ± 2.0 _x ^{cb}	31 ± 2.0 _x ^{db}	19 ± 2.0 _x ^c	***
36	28 ± 1.0 _w [Ⓢ]	36 ± 1.0 _x ^b	34 ± 1.0 _x ^{cb}	34 ± 1.0 _x ^{db}	17 ± 2.0 _x ^{c∞}	***
Statistical Significance ^{†‡}	NS	***	**	***	***	

⊗ collected at the beginning of the repletion period (day 11), half way through the repletion period (day 24), and at the end of the repletion period (day 36).

+ number of piglets in treatment group.

Ⓢ NS = non-significant; P > 0.05; ** = P < 0.01; *** = P < 0.001.

♥ statistical significance between treatment groups within time, based on ANOVA.

* on day 0 the piglets were removed from the sow and blood samples taken. Between days 0-11 the piglets were fed a no-iron formula.

Ⓢ means within a row with different superscripts (a, b, c, d, e) are significantly different (P < 0.05), (LSD).

∞ means within a column with different subscripts (w, x, y) are significantly different (P < 0.05), (LSD).

∞ n=6, only six piglets were left in the Haemin group by day 36 of the trial, as one was removed due to severe anaemia.

• statistical significance within treatment groups across time, based on ANOVA.

Table 3.6

Unsaturated iron-binding capacity (mean UIBC ± SE^v; µg/dL) in blood collected[⊗] during a 25 day repletion period when the piglets were fed a bovine milk-based formula fortified with one of five different sources of iron

Day of Trial	Iron Source in Formula					Statistical Significance ^{#v}
	Ferric Pyrophosphate (n ⁺ =8)	Ferrous Sulphate Heptahydrate (n=8)	Milk protein-Iron Complex (n=7)	Ferrous Lactate (n=7)	Haemin (n=6)	
11	243.6 ± 0.10 _w [⊕]	241.6 ± 0.09 _w ^a	208.7 ± 0.10 _w ^u	202.7 ± 0.10 _w ^a	236.7 ± 0.11 _w ^a	NS
24	230.2 ± 0.29 _w ^a	33.7 ± 0.31 _x ^b [⊙]	77.1 ± 0.70 _x ^{bc} [⊙]	137.2 ± 0.29 _w ^c [♣]	238.5 ± 0.29 _w ^a	***
36	243.0 ± 0.22 _w ^a	65.44 ± 0.22 _x ^b [♣]	95.5 ± 0.26 _x ^b [♣]	126.6 ± 0.22 _x ^b	263.4 ± 0.22 _w ^a [∞]	***
Statistical Significance ^{#v}	NS	***	***	***	***	

- ^v SE of the log transformed data.
- [⊗] collected at the beginning of the repletion period (day 11), half way through the repletion period (day 24), and at the end of the repletion period (day 36). Serum was decanted off these blood samples and used for analyses of UIBC.
- ⁺ number of piglets in treatment.
- [#] NS = non-significant, P > 0.05; *** = P < 0.001.
- [⊕] means within a row with different superscripts (a, b, c) are significantly different (P < 0.05), (LSD).
- means within a column with different subscripts (w, x) are significantly different (P < 0.05), (LSD).
- [♣] n=6, insufficient sample volume for analyses of all animals in this treatment group.
- [♥] statistical significance between treatment groups within time, based on ANOVA.
- [⊙] n=1, insufficient sample volume for analysis of all animals in this treatment group.
- [♣] n=5, insufficient sample volume for analyses of all animals in this treatment group.
- [∞] n=5, only six piglets were left in the Haemin group by day 36 of the trial, as one was removed due to severe anaemia.
- [•] statistical significance within treatment groups across time, based on ANOVA.

Table 3.7

Haemoglobin repletion efficiency (%) (mean HRE ± SE) of different iron sources used to fortify a bovine milk-based formula as determined in anaemic piglets over a 25 day haemoglobin repletion period

	Iron Source					Statistical Significance
	<i>Ferric Pyrophosphate</i> (n ⁺ =8)	<i>Ferrous Sulphate Heptahydrate</i> (n=8)	<i>Milk-Protein-Iron Complex</i> (n=7)	<i>Ferrous Lactate</i> (n=8)	<i>Haemin</i> (n=7)	
HRE% ^Φ	23.5 ±2.44 ^{a♦}	35.8 ±1.32 ^b	38.0 ±14.39 ^b	32.9 ±2.22 ^b	3.2 ±0.48 ^c	***

⁺ number of piglets in treatment.

^Φ Haemoglobin Repletion Efficiency = $\frac{\text{whole body Hb-Fe(mg)}_{\text{final}} - \text{whole body Hb-Fe(mg)}_{\text{initial}}}{\text{Fe intake(mg) over repletion period (25d)}} \times \frac{100}{1}$

where:

initial = day 0 of the repletion period (11 of the trial).

final = day 25 of the repletion period (day 36 of the trial).

[♦] means with different superscripts are significantly different (P<0.05).

CHAPTER 4

DISCUSSION

The aim of the present study was to evaluate the hypothesis that there are differences in the bioavailability of different iron sources in a bovine milk-based formula. Accordingly, repletion tests (based on the repletion of blood haemoglobin and haematocrit levels) were conducted, using the iron-deplete piglet as a model animal for the human infant, with five different iron sources that had been used to fortify a bovine milk-based formula.

Pigs have been routinely used as animal models to study various aspects of human nutrition (Waddel and Desai, 1981; Low, 1992; Darragh and Moughan, 1995). In the present study, the suckled piglet was chosen as an animal model for the human infant. The post-natal piglet can be regarded as a foreshortened and accelerated model of growth and development for the post-natal human infant (Moughan *et al.*, 1992). Moughan *et al.*, (1992) concluded that based on the comparison of anatomy, physiology, histology, and microbiology of the digestive tract, the three-week-old piglet appears to be an appropriate model for studying aspects of digestion and absorption in the three month old infant. Furthermore, the use of the suckled piglet as an animal model for studying protein digestion in the human infant has been validated (Darragh and Moughan, 1995). A reliable animal model for iron nutrition in humans, however, has yet to be identified. The majority of studies investigating human iron nutrition have adopted rat breeds as an animal model. Apparent differences in iron absorption between man and the rat (Forth and Rummel, 1973; Reddy and Cook, 1991), and other models previously used in human iron nutrition studies such as the mouse, chick, or guinea pig, (Forth and Rummel, 1973) have prompted questions as to whether the rat and other animals are acceptable models for studying iron nutrition in the infant. The piglet has also been used to evaluate iron bioavailability in the human infant (Harada *et al.*, 1988; Howard *et al.*, 1990), and is thought to be a more acceptable animal.

In the present study piglets were taken from the sow at one week of age and used for a total of five weeks. In doing so, the assumption was made that the similarity between the digestive system of the piglet and human infant still existed from the earlier age of 1 week, until the piglets were six weeks old. Furthermore, when using the suckled piglet as an animal model it is important to consider their physiological difference from humans in vitamin C metabolism. It is well recognised that the piglet, rat, and mouse, unlike the human infant, do not have a dietary requirement for vitamin C (Perks and Miller, 1996). Vitamin C is a known enhancer of iron absorption in humans but not pigs or rats, and this may have precluded the use of the piglet as an animal model. In this study, however, the formula contained sufficient vitamin C so that the piglets did not need to metabolise their own. Thus, the piglet has been assumed to remain a feasible model animal for studying iron bioavailability under the circumstances of the present study.

In studies of dietary bioavailability the potential for over or under-estimation of availability can arise from errors in the estimation of intake of the compound being tested. This problem is often augmented in species such as the pig, rabbit or rat who practice coprophagy (Ranger and Neale, 1984; Zhang *et al*, 1992). In the present study the potential for overestimation of iron-bioavailability was avoided in two ways. The first was in the prevention of coprophagy using a human ostomy apparatus attached to the piglets' anal region. The ostomy apparatus was used to collect and contain each piglet's faeces. It is important to note that few iron bioavailability studies have employed techniques for coprophagy prevention. The second was to limit access to alternative sources of iron by housing the piglets in a totally plastic environment.

Formula intakes were not influenced by dietary treatment. Formula intakes were used, therefore, as an indication of the piglets' overall well being. Average protein and energy intakes (appendix 1) for each treatment group were considered sufficient to maintain satisfactory growth rates in normal, iron-replete piglets (Agricultural Research Council, 1981a; Agricultural Research Council, 1981b). Therefore, any differences in average daily growth rates observed between experimental groups should have been attributable to

the treatment with iron, alone. However, no significant differences in average daily growth rates between the treatment groups were observed during the repletion period (days 11-36). This would imply that iron intake had no effect on the growth rate of the piglets. These results from the present study support evidence from the literature which indicate that growth rate is an insensitive indicator of anaemia status (Cowan *et al.*, 1966; Kuznestov and Kal'nitskii, 1987; Harada *et al.*, 1988). The insensitivity of growth rates and intake data make it necessary to concentrate on biological indices of anaemia status that can be measured in the blood of the piglets.

The neonatal piglet has an iron status that can be readily manipulated to induce an anaemic state, which makes it useful for the purpose of investigating iron bioavailability. Iron is a rate limiting factor in erythropoiesis and neonatal piglets not treated with supplemental iron develop haemoglobin values as low as 3.0g/dL within two weeks of birth (Sjaastad *et al.*, 1996). In the present study, all piglets entered the trial and, therefore, the repletion period, in an anaemic state as determined by blood haemoglobin concentration and haematocrit. The continued decline in blood haemoglobin concentrations and haematocrit in the control piglets demonstrated an ongoing negative effect on iron status in anaemic piglets who did not receive dietary iron supplementation following a period of iron depletion. This confirms the sensitivity of the piglet model to the chosen haemoglobin repletion bioassay.

Despite differences in the actual concentrations of iron in each iron fortified formula, there were no significant differences in the bioavailability in the iron sources FS, MPIC, and FL, used to fortify the bovine milk-based formula based on blood haemoglobin concentrations, haematocrit and unsaturated iron-binding capacity (UIBC) levels. Therefore, it would appear that MPIC and FL are as effective as FS in facilitating the recovery of haemoglobin, haematocrit and UIBC levels in anaemic piglets. Supplementation with FP, although not sufficient to induce any recovery in the piglets' anaemic state, did effectively maintain each piglet's iron status at initial levels, as determined by blood haemoglobin concentrations, haematocrit and UIBC levels. In contrast, supplementation of the formula with Hm iron source, at

the same level of iron content as was used in the FP formula, did not maintain haemoglobin or haematocrit at initial levels and the piglets' iron status declined, accordingly.

Supplementation of the piglets' formulas with either FS, MPIC, or FL induced a faster rate of repletion, based on haemoglobin and haematocrit data, in the first half of the repletion period than in the second. This may be the result of a physiological increase in iron absorption in response to low iron levels in the body, indicating that the efficiency of iron repletion is dependant on iron status. This was recently demonstrated by Weink and Beynen (1996) who reported a decline in the efficiency of iron absorption in anaemic rats as the iron status of the rats approached normal levels.

While differences in bioavailability of the FS, MPIC, and FL iron sources were not significant, supplementation of the bovine milk-based formula with iron induced a total recovery of haemoglobin and haematocrit to initial levels in the FS, but not the MPIC or FL treatment groups. While there were no significant differences in bioavailability between FS, MPIC, and FL based on blood parameters, this may have been due to the higher concentration of iron present in the FS formula (11.2mgFe/L) compared to the MPIC (8.1mgFe/L) and FL (9.9mgFe/L) supplemented formulas. Therefore the efficiency with which ingested iron was incorporated into blood haemoglobin in each piglet was determined to allow a more effective comparison between the iron sources. The HRE% calculated for each iron source (FS = 35.8%; MPIC = 38.0%; FL = 32.9%; FP = 23.5%; and Hm = 3.2%) support evidence from the haemoglobin and haematocrit results that FS, MPIC and FL are more available iron sources than FP or Hm, and that FP is more available than Hm.

Iron status and food composition can affect the bioavailability of a single iron source. When comparing different compounds, therefore, the bioavailability is measured relative to a standard compound. Ferrous sulphate is the standard against which the availability of most experimental iron compounds are evaluated (Fritz *et al.*, 1970; Fritz and Pla, 1972; Amine and Hegsted, 1974; Fairbanks, 1994). To facilitate this comparison, relative biological values (RBV) are calculated by comparing the HRE% of the test iron relative

to the HRE% for FS ($RBV = \text{HRE\%}(\text{test}) / \text{HRE\%}(\text{FS}) * 100 / 1$) The RBVs for of each of the iron sources investigated in the present study are given in Table 4.1.

Table 4.1

The relative biological value[∞] (RBV) of five different iron sources used to fortify a bovine-milk based formula

Iron source used in Formula					
	<i>FP</i>	<i>FS</i>	<i>MPIC</i>	<i>FL</i>	<i>Hm</i>
RBV	0.66	1.00	1.06	0.92	0.09

[∞] RBV= [HRE%(iron source)] / HRE% (Ferrous sulphate heptahydrate)

The present study confirmed the relatively high bioavailability of FS with respect to other iron sources, especially FP. Based on relative biological value, the bioavailability of each iron source when ranked in order from highest to lowest was $MPIC < FS < FL < FP < Hm$, and these results indicate that the MPIC iron source, with an RBV 6% greater than that of the FS iron source, has the greatest bioavailability of the iron fortification sources tested.

Comparison of the present findings with those of other studies is complex because many of the studies reported in the literature concerning the repletion efficiency of FS and other iron sources in the haemoglobin repletion model are inadequate with respect to experimental design. The diversity of methods employed in different studies, including differences in animal species, levels of iron supplementation, and the extent of depletion and repletion in test animals, all serve to confound study comparisons.

Weink and Beynen (1996) found that anaemic rats incorporated 41% of ingested iron, in the form of FS, into haemoglobin after 7 days of repletion. This utilisation of FS was close to the 35% found for anaemic piglets supplemented with FS in the present study where the repletion period was 25 days. Weink and Beynen (1996) also noted, however, that the HRE% in the rats decreased to 22% after 15 days. Forbes *et al.*, (1989) reported a 75% haemoglobin repletion efficiency in anaemic rats. This, however, was at a level of supplementation nearly three times that used by Weink and Beynen

(1996). No difference in initial anaemia status of the rats between the studies of Forbes *et al.*, (1989) and Weink and Beynen (1996) was apparent, as determined by blood haemoglobin levels, and although the rats from the study by Forbes *et al.*, (1989) had slightly lower initial haematocrit levels, this does not explain the scale of difference observed between these trials. Differences between the Forbes *et al.*, (1989) and Weink and Beynen (1996) would most likely be explained by differences in dietary levels of iron. Howard *et al.*, (1993) reported that 19.8% of ingested FS was incorporated into the haemoglobin of anaemic, 21-day-old, weaned piglets, fed a FS fortified infant cereal, after a 10 day repletion period. A number of factors may have contributed to the lower HRE% observed by Howard *et al.*, (1993), compared with those determined in the present study. Differences in the composition of a milk formula and cereal could have influenced iron absorption because each formulation may have contained a different complement of inhibitors and enhancers of iron absorption. There was also the potential that the shorter repletion period in the study by Howard *et al.*, (1993) (10 compared to 25 days in the present study) resulted in a lower repletion efficiency as suggested by Weink and Beynen (1996). In addition, the piglets used by Howard *et al.*, (1993), had initial haemoglobin concentrations higher than those piglets used in the present study, and this may have resulted in the lower efficiency of repletion.

Anderson *et al.*, (1974) investigated the bioavailability of different sources of iron in five-day-old piglets fed an iron-fortified cereal-milk diet for a repletion period of 28 days. Although the findings from Anderson *et al.*, (1974) indicated that the repletion efficiency for FS was 30%, very close to the 32% HRE% found in the present study, the piglets in their study were not within the haematological parameters indicative of anaemia (Thoren-Tolling, 1975) when the repletion period began. This highlights how methodological differences between studies can confound comparisons between iron absorption studies.

The low bioavailability of FP from infant formula has been well established (Rios *et al.*, 1975). Although there is evidence to suggest that the

bioavailability increases following heat treatment of FP fortified formula, the FP used in the present study was dry blended with the preliminary formula. Therefore, heat treatment could not have influenced the bioavailability (RBV = 0.66, see Table 4.1) of this iron source. A similar study of iron bioavailability in the suckled piglet haemoglobin repletion model (Harada *et al.*, 1988) also demonstrated a low bioavailability (RBV = 0.15) for FP. This was considerably lower than the RBV of FP in the present study. It was evident, however, from haemoglobin levels, total iron-binding capacity (TIBC) and haematocrit, that the FS group in the study by Harada *et al.*, (1988) began the repletion period in a more anaemic state, compared to those fed the Ferric pyrophosphate supplemented formula in the same study. The recovery of the piglets on the FS supplemented formula would, therefore, have been expected to be enhanced by the severity of their anaemia. This could explain the magnitude of difference in RBV of the FP supplement between the study by Harada *et al.*, (1988) and the present study.

The MPIC supplement used in the present study appears to be more readily available for absorption than the recognised standard FS supplement. This high bioavailability of the milk protein-iron complex (RBV = 1.06) in the present study is consistent with results from Kuznetsov and Kal'nitskii (1987) who reported an RBV of 1.08 for their casein-iron-complex using a suckled piglet-haemoglobin repletion model. High bioavailability of an iron-casein complex was also confirmed by Zhang and Mahoney (1989) in anaemic weanling rats. The positive effect of the milk protein, casein, on iron absorption is not clear. Casein derived phosphopeptides exhibit a potent ability to form soluble complexes with ferrous iron (Meisel and Frister, 1989) and this may in effect enhance the absorption of this iron. Hurrell *et al.*, (1989), however, demonstrated an inhibitory effect of intact bovine casein on the absorption of iron from an infant formula *in vitro* under simulated gastrointestinal conditions. This inhibition was decreased by hydrolysis of the protein, and probably reflected the less extensive digestion required to free iron for absorption in the upper intestine. *In vitro* techniques, however, do not simulate physiological factors that account for variations in iron absorption, so the use of *in vitro* techniques are inappropriate in situations

where a more physiological response to different iron sources and dietary factors is sought (Fairweather-Tait, 1992).

Haemin is an iron-porphyrin complex free from the globin protein ligand of the haemoglobin molecule from which it is derived. The extremely low bioavailability of the Hm supplement found in the present study was consistent with the findings of Weintraub *et al.*, (1965). Weintraub *et al.*, (1965) compared the absorption of Haemoglobin and Hm with a comparable dose of FS in non-anaemic rats, and found that absorption of Hm was nearly half that of haemoglobin and that both sources were less well absorbed compared to FS. A possible explanation for the low bioavailability of Hm in the present study could be the low solubility of the Hm product in the aqueous formula solution. Haemin, due to its chemical structure, is characteristically insoluble in aqueous solution. This was apparent, in the present study, from the black crystalline particles of Hm which settled in the bottom of the 25L formula containers, and adhered to the sides of the plastic feeding bottles at the end of each meal. Despite physical efforts to keep the compound in suspension it became apparent that the piglets were not ingesting all of the 8.9mg Fe/L contained in the formula. Colorimetric analysis of the liquid formula revealed that the iron content of the formula the piglets were ingesting was, in fact, closer to 0.88mgFe/L, considerably less than the formulated concentration. This would have had a significant impact on subsequent measures of iron repletion and could explain the poor iron status of the piglets throughout the repletion study.

Serum ferritins were measured, in the present study using an immunoturbidimetric assay (Appendix 3a & 3b) for the purpose of assessing body iron stores in the piglets. Investigation of this assay, however, revealed that the human anti-ferritin antibody used in the analyses was insensitive to piglet serum ferritin. Consequently, these results were not used in the assessment of iron bioavailability. Also, due to the insufficient volume of serum collected from the piglets it was not possible to measure total iron binding capacity or serum iron concentrations. Although results from studies in anaemic chicks and rats indicate that that both blood haemoglobin concentration and haematocrit are suitable measures of iron repletion (Pla

and Fritz, 1971), it would be preferable in future, to measure serum-ferritin levels (using an anti-ferritin antibody sensitive to piglet serum ferritin), total iron binding capacity, and serum iron determinations, for completeness of the piglet haemoglobin repletion model.

The findings from the present study were that there are differences in the bioavailability of the five iron sources tested, as determined by repletion tests using the iron-deplete piglet as a model animal for the human infant. A negative effect on the iron status in anaemic piglets who did not receive dietary iron supplementation following a period of iron depletion was demonstrated, thus confirming the sensitivity of the repletion assay used. Of the iron sources investigated, MPIC had the highest bioavailability based on RBV. This was probably due to the higher solubility of this compound preventing the precipitation of iron in the alkalinity of the duodenum which would have reduced availability. Likewise, the FP and Hm formulas had a much lower bioavailability relative to the other iron sources most likely due to the increased potential for precipitation in the intestinal environment which would render them unavailable.

CHAPTER 5

GENERAL SUMMARY AND DISCUSSION

The demands imposed in times of increased growth augment the risk of iron deficiency and its associated anaemia (IDA) in infants and young children (Fairbanks, 1994). Prevention of IDA is important, as this disorder, in the second sixth months of life and beyond infancy, has been associated with impaired work capacity, reduced cognitive performance (Pollitt, 1997), altered behaviour (Andraca *et al.*, 1997), and lowered cell mediated immunity (Kuvibidila *et al.*, 1984; Walter *et al.*, 1997).

It is generally recognised that breast milk, although characteristically low in iron (Hernell and Lonnerdal, 1996), can deliver an adequate supply of iron to the human infant in the first 4-6 months of life. The iron content of breast-milk, however, declines during the course of lactation (Hashcke and Male, 1996). As a consequence of this, the iron stores of exclusively breast-fed, full-term infants become depleted by the age of six months (Dallman *et al.*, 1980). The iron absorbed from an unsupplemented diet is often insufficient to meet the infant's needs in the second half of the first year of life (Saarinen, 1978; Fairweather-Tait, 1989). Dietary iron fortification in late infancy can, therefore, prevent and cure iron deficiency and IDA.

Solid foods are introduced to the dietary regimen of the infant usually in the second six months of life. It is at this time that the premature introduction of unfortified cows milk as the liquid part of the diet, and an intake of food with a low iron content both contribute to the high prevalence of iron deficiency in infants (Haschke and Male, 1996). Cows milk, in addition to being low in iron, contains high levels of calcium and protein both of which interact negatively with iron to limit absorption (Hurrell *et al.*, 1989). Follow-on infant formulas are an alternative to cows milk in providing the liquid component of the infant's diet and are preferable to cows milk because they are not only 'humanised' to meet the infant's nutritional requirements in late infancy but are often fortified with iron.

Infant formulas and also infant cereals offer an appropriate vehicle for the delivery of dietary iron supplementation because they are regularly consumed in adequate quantities by the infant (Zeigler and Fomon, 1996). The effectiveness of infant cereals in preventing iron deficiency, however, is still under discussion (Hashcke and Male, 1996; Zeigler and Fomon, 1996). Poor bioavailability of the iron sources currently used limits the effectiveness of fortified infant cereals. Although there have been several studies documenting success with iron fortification of formula (MacMillan *et al*, 1977; Stekel *et al.*, 1986; Zeigler and Fomon, 1996), there still appears to be some differences in the iron sources available in terms of their nutritional value to the infant. It is important, therefore, to test the bioavailability of both current and new iron sources that may be used in infant nutrition. It is also important to consider whether the iron source will induce unfavourable changes in functional or organoleptic characteristics.

Those iron sources that are the most bioavailable (eg. ferrous sulphate and ferrous lactate), usually due to their increased solubility in gastric acid, will often cause unacceptable colour and flavour changes, particularly relating to fat oxidation, when they are added to infant formulas (Hegenauer, 1979). Despite this, the current fortification of milk products is predominantly by the use of ferrous sulphate or ferrous lactate (both very reactive and highly bioavailable) for infant products, and ferric pyrophosphate (which has no-off flavour but low bioavailability) for adult products. The quest for novel iron sources continues to challenge the manufacturers of infant formula and infant foods. The ideal iron source would be organoleptically compatible with infant formulas and foods, and have an at least comparable, if not improved, bioavailability compared to that of FS or FL.

The MPIC used in the present study was, essentially, a caseinate that has been manufactured using ferrous cations instead of calcium, potassium or sodium cations. MPIC appeared to have a higher bioavailability relative to FS when added to the bovine milk-based formula. This finding is of considerable consequence with respect to iron fortification of foods and formulas. Complexing ferrous sulphate with a milk-protein not only reduces the

reactivity of the iron with the other components of the formula, it also limits the formation of the 'beany' flavour characteristic of ferrous sulphate fortified formula. The unique advantage evident when fortifying a milk-based formula with MPIC in place of ferrous sulphate, ferric pyrophosphate or ferrous lactate, is that the iron is provided in an apparently "more natural" yet highly bioavailable form.

The advantage of adding iron in a more natural form may also be of consequence to the manufacturer of bovine milk-based products other than infant formula. The use of MPIC could be expanded beyond the infant market to products that target other specific populations at risk for iron deficiency. Young women and children are two populations that could be targeted. It has been shown that iron-milk protein may reduce the inhibitory effect of calcium on iron absorption (Peres *et al.*, 1997). MPIC may, therefore, be appropriate to fortify high calcium low-fat liquid milk or milk powders, for example, targeted towards the young health conscious women. Other possible avenues for the use of MPIC are flavoured milks and milkshake powders for children. Further studies are required, however, to investigate of the use of MPIC in other foods such as soy-based infant formula or infant cereal products.

Two further considerations governing the choice of iron compound, after organoleptic compatibility and bioavailability, are cost and safety. The addition MPIC at the level used in the present study provided iron in the range conforming to current regulations concerning iron fortification of infant formulas (Codex Alimentarius Commission, 1995). This did not alter the macro-nutrient profile of the formula, however, indicating that MPIC can, therefore, be added to infant formulas at a safe and effective level without the need to adjust nutrient levels in the formula.

The production of MPIC from an initial iron compound (eg. ferric pyrophosphate or ferrous sulphate) and sodium caseinate is more costly due to the extra level of processing involved. The cost effectiveness of using MPIC above other accepted and common iron sources must be weighed against the advantages mentioned in the above paragraphs. In infant formula, for example, flavour is probably not as great a concern as it would be in fortified

fluid milk destined for the adult market. The idea of a iron in a 'natural' form, however, may offer an attractive and healthy alternative that justifies the extra expense. These marketing issues should be considered with regard to the target market of the iron fortified product.

The FP used in the present study had a low bioavailability relative to FS, MPIC, and FL. This was probably due to the fact that it was dry-blended into the bovine milk-based formula. Studies investigating the bioavailability of ferric pyrophosphate in milk products have revealed that the bioavailability of this iron source may in fact be related to the protocols followed in the manufacturing process pyrophosphate (Theuer *et al.*, 1973; Tschuta *et al.*, 1991). Heat treatment of fortified milk products appears to improve the bioavailability of ferric pyrophosphate (Theuer *et al.*, 1973; Tschuta *et al.*, 1991). It is possible that the formation of iron protein complexes, similar to the MPIC, may occur during the heating process. These complexes could be responsible for the observed increase in bioavailability of ferric pyrophosphate (Peres *et al.*, 1997). This could also provide the foundation for identification of new iron fortification compounds.

Haemoglobin-iron is believed to be well absorbed by humans (Monsen *et al.*, 1978) and this absorption is relatively unaffected by dietary factors or gastrointestinal secretions (Jacobs *et al.*, 1963). Haemin is an iron-porphyrin complex and is free from the globulin protein ligand of the haemoglobin molecule from which it is derived. Although the low bioavailability of the haemin found in the present study was consistent with the findings of Weintraub *et al.*, (1965), the problems experienced with the solubility of haemin in the present study meant it was not possible to draw conclusions as to the bioavailability of the haemin iron fed to piglets. Due to the incompatibility of haemin with the bovine milk-based formula it appears that this iron source is not suitable for iron fortification in such a formula.

The routine use of humans in iron bioavailability research has limitations. This is especially so in infant studies because of the difficulties encountered in obtaining willing subjects and because of stringent ethical constraints. Although studies using infants and adults are possible (Hurrell *et al.*, 1998),

the use of *in vitro* techniques and animal models in bioavailability studies, enables the experimenter to implement protocols that could not justifiably be conducted with human subjects. In the present study, the piglet was used as an animal model to test the bioavailability of iron to the human infant.

Numerous techniques have been employed to estimate the bioavailability of different iron sources in foods using animal models. Methodological differences, however, make it difficult to draw conclusions from inter-study comparisons. For example, inter-study comparisons are not possible where different species are used as animal models and assays are used to determine the absorption of iron. Dietary influences of dietary components and the physiological state of experimental subjects also confound inter-study comparisons.

In fulfilling the need for a logistically appropriate animal model it appears that the piglet (Moughan *et al.*, 1992; Moughan *et al.*, 1989) is superior to the commonly accepted rat (Reddy and Cook, 1991). As well as having a physiology and metabolism that is considered an acceptable model of the human infant's gastrointestinal tract (Moughan *et al.*, 1992), the suckled piglet offers a number of unique logistical advantages as an animal model in infant iron nutrition. These include an ability of the piglet to be weaned at birth and trained to feed from a bottle, the access to large sample sizes which facilitates chemical analyses, and, the ease with which coprophagy can be prevented in the piglet. The present study was unique in that it relied on the bottle feeding of piglets, seven times a day. The bottle-feeding of piglets facilitates transfer of nutrients in a manner and frequency which satisfactorily parallels the human condition. Few other studies of iron bioavailability have done this. It is important when using an animal model for nutritional research to reproduce the conditions of the population under study as closely as possible.

Human infants, under normal circumstances, do not ingest their faeces. It follows that coprophagy by any animal model species used in human bioavailability trials should be prevented, thus allowing only one passage of nutrients for digestion and absorption. It is likely that methodological errors

in the estimation of bioavailability of iron are increased in studies where coprophagy is not prevented. This is evident from findings such as those of Zhang *et al.*, (1992) who reported errors of up to 8% for the bioavailability of FeSO₄ and 25% for the bioavailability the iron in plant foods. These errors could be attributed to coprophagy by the anaemic rats used in their study. While in most animal studies every care and precaution is taken to ensure animals are housed in an iron-free environment, overestimation of iron intake, which can result from the recycling of nutrients through coprophagy, is often disregarded. Most studies incorporated slated cage-floors to facilitate the removal of faeces. This is unacceptable, particularly in haemoglobin repletion studies where there is a propensity for animals to develop diarrhoea as a symptom of severe anaemia. In the present study, the complete collection and removal of faeces using a human ostomy apparatus ensured that no coprophagy, and hence recycling of nutrients, occurred. Consequently, it was assumed that the measurements of iron intake accurate.

In addition to the logistical advantages conveyed in the prevention of coprophagy and the capacity to bottle-feed, the piglet's iron metabolism is also uniquely suitable to iron absorption studies. Unlike the animal models previously used for iron absorption, the neonatal piglet has an iron status that can be readily manipulated to suit experimental requirements. It is because of this that the piglet lends itself to the study of haemoglobin repletion as a biological marker for iron status. Unfortunately, due to ethical and methodological constraints, no haemoglobin repletion studies in human are available for comparison. This makes it difficult to discern whether haemoglobin repletion values in the different species are relevant to human iron nutrition. However, normal levels for haemoglobin and haematocrit (Table 5.1), in the healthy, three-week-old suckled piglet (Thoren-Tolling, 1975; Jain, 1985) at a comparable developmental age (ie. peak lactation in the dam) appear to be similar to those for the healthy, full-term, three-month-old, human infant (Wintrobe 1967a; Siimes, 1990). The depletion and subsequent repletion of iron stores in the suckled piglet in the present study prompted a similar pattern of physiological response, ie. an increase in blood haemoglobin concentrations and haematocrit to that of the human infant

recovering from iron deficiency anaemia (Siimes, 1990). Although no reference values are available for comparison of UIBC between the infant and the piglet, the decrease of serum UIBC in response to addition of iron to the diet of the iron-deplete piglet also parallels the human situation in recovery from iron deficiency anaemia (Wintrobe, 1967b).

Table 5.1

The normal range for blood haemoglobin concentration and haematocrit in the three-week-old piglet and the three-month-old human infant

	Human Infant	Piglet
Blood haemoglobin (g/dL)	11.0-14.0 [∞]	11.0-13.0 [*]
Haematocrit (PCV%)	30-45 [∞]	38-44 [*]

* From: Thoren-Tolling (1975)

* From: Jain, (1985)

∞ From: Wintrobe (1967a)

Haemoglobin repletion alone is not sufficient to provide a complete picture of the dynamics of iron status. Validation of the suckled piglet as a model animal for iron nutrition in the human infant would require a comprehensive assessment of the dynamics of iron status in the piglet from age one to six weeks, under the appropriate experimental conditions. This would include the assessment of storage iron levels using serum ferritin and liver haemosiderin levels, liver non-haem iron levels, serum iron and total iron-binding capacity (TIBC), in addition to the parameters measured in the present study. Validation of the piglet as an animal model would require a direct comparison of these parameters in the piglet and the human infant, under comparable experimental conditions.

Given the physiological and anatomical similarities noted between the digestive systems of the piglet and human infant, together with the obvious logistical advantages, and the observations from the present study, the suckled piglet appears to be a suitable animal model for studying iron absorption in the human infant. Although the validity of using the piglet as an animal model for studying certain aspects of digestion and absorption of

protein in the human infant has been established (Darragh and Moughan, 1995), validation of the piglet as a model for iron absorption in the human is necessary before sound conclusions can be drawn as to how relevant studies using the piglet model are to the human situation.

The work described in the present study, which used the piglet to investigate the bioavailability of various iron sources, suggests that the milk protein-iron complex offers a suitable alternative, with respect to bioavailability and functionality, to the standard iron compounds used in the fortification of bovine milk-based formulations destined for the infant/toddler market. A possible role for this novel iron source in foods other than bovine milk-based formula warrants further investigation.

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APPENDIX 1

Daily intakes of energy and protein (mean \pm SE^{}) on three days' of a 25 day repletion period, by piglets fed a bovine milk-based formula fortified with different sources of iron*

Diet	Energy Intakes (GE kJ/d)			Protein Intakes (g/d)		
	11	Day of Trial 24	33	11	Day of Trial 24	33
<i>Ferric Pyrophosphate</i> (n=8)	2352.4 \pm 0.11	5514.1 \pm 0.09	8494.0 \pm 0.05	19.2 \pm 0.11	45.0 \pm 0.08	69.3 \pm 0.05
<i>Ferrous Sulphate Heptahydrate</i> (n=8)	2726.7 \pm 0.10	5778.4 \pm 0.08	8856.2 \pm 0.05	21.0 \pm 0.10	44.4 \pm 0.08	68.1 \pm 0.05
<i>Milk-Protein-Iron Complex</i> (n=7)	2328.5 \pm 0.12	6100.3 \pm 0.10	9224.4 \pm 0.06	19.0 \pm 0.12	49.8 \pm 0.10	75.3 \pm 0.06
<i>Ferric Lactate</i> (n=8)	2512.3 \pm 0.11	5561.3 \pm 0.09	9075.6 \pm 0.05	20.1 \pm 0.11	44.4 \pm 0.09	72.5 \pm 0.05
<i>Haemin</i> (n=7)	2393.6 \pm 0.11	5778.4 \pm 0.09	8856.2 \pm 0.08 ^Σ	18.3 \pm 0.11	43.9 \pm 0.09	53.5 \pm 0.08 ^Σ
<i>Control</i> (n=8)	2313.2 \pm 0.11	4789.1 \pm 0.09	-Φ	18.8 \pm 0.11	39.0 \pm 0.09	-Φ
Statistical significance[†]	NS	NS	**	NS	NS	**

[▲] SE of the log transformed data.

⁺ day 11, 24 and day 33 of the trial.

[∞] number of piglets in treatment group.

^Φ insufficient piglets remaining in study to provide accurate data.

^Σ n=6.

[†] NS (non-significant) = P > 0.05; ** = P < 0.01.

APPENDIX 2

Average daily growth rates (kg/d \pm SE*) over a period of 25 days, for piglets fed bovine milk based formula fortified with different sources of iron

	Iron Source in formula					Statistical Significance
	<i>Ferric Pyrophosphate</i>	<i>Ferrous Sulphate Heptahydrate</i>	<i>Milk-Protein-Iron Complex</i>	<i>Ferric Lactate</i>	<i>Haemin</i>	
	(n=8)	(n=8)	(n=7)	(n=8)	(n=7)	
Average Daily Gain (kg/d) ^Φ	0.23 \pm 0.016	0.26 \pm 0.015	0.24 \pm 0.018	0.25 \pm 0.016	0.21 \pm 0.017	NS

* SE of the log transformed data

Φ ADG = $\frac{\text{weight (final)} - \text{weight (initial)}}{\text{repletion time (days)}}$

APPENDIX 3a

Serum ferritin levels in the piglets blood were determined in an immunoturbidimetric assay (Boehringer Mannheim Systems). Antiferritin antibodies bound to latex (Tina-quant® Ferritin, BM/Hitachi reagent kit) were reacted with the serum ferritin to form a antigen-antibody complex. The resultant agglutination was measured turbidimetrically at wavelength 700nm (Boehringer Mannheim/Hitachi 917 Keysystems Autoanalyser).

Serum ferritin results are reported in Appendix 3b. Investigation of this assay, however, revealed that the human anti-ferritin antibody used in the analyses was insensitive to piglet serum ferritin. Consequently, these results were not used in the assessment of iron bioavailability.

APPENDIX 3b

Serum ferritin levels (mean \pm SE⁺; μ g/ml) in blood collected[⊗] from piglets during a 25 day repletion period where the piglets were fed a bovine milk-based formula, fortified with one of six different sources of iron

Day of Trial	Iron Source in Formula					Statistical Significance ^{††}
	<i>Ferric Pyrophosphate</i> (n ⁺ =8)	<i>Ferrous Sulphate Heptahydrate</i> (n=8)	<i>Milk protein-Iron Complex</i> (n=7)	<i>Ferrous Lactate</i> (n=8)	<i>Haemin</i> (n=8)	
11 [*]	4.44 \pm 0.04	3.86 \pm 0.05	3.99 \pm 0.04	3.91 \pm 0.04	3.94 \pm 0.05	NS
24	4.10 \pm 0.10	4.80 \pm 0.10	6.35 \pm 0.11	3.98 \pm 0.10	4.07 \pm 0.11	NS
36	3.13 \pm 0.12	4.62 \pm 0.13	5.26 \pm 0.12	4.9 \pm 0.12	4.02 \pm 0.13 [∞]	* * *

⁺ SE of the log transformed data.

[⊗] collected at the beginning of the repletion period (day 11), half way through the repletion period (day 24), and at the end of the repletion period (day 36).

⁺ number of piglets in treatment group.

^{*} NS (non-significant) = P > 0.05; *** = P < 0.001.

^{††} statistical significance between treatment groups within time, based on ANOVA.

^{*} on day 0 the piglets were removed from the sow and blood samples taken.

[∞] n=6, only six piglets were left in the Haemin group by day 36 of the trial, as one was removed due to severe anacmia.