EFFECTS OF DIETARY CALCIUM ON INTESTINAL NON-HAEM IRON ABSORPTION DURING WEANING

by

Peggy Efua Oti-Boateng, B Sc Biochem (Hons.)

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Corrigenda

Page xvi	Line 2	"beast" should read "best"
Page xxvii	Line 6	"Malao" should read "Malo"
Page xxvii	Line 12	"Hapatol" should read "Hepatol"

Chapter 1:

Page 4,	Line 14	replace "other nutrients" with "Calcium".
Page 5	Line 5	", an increase vascular volume" should read "an increase in vascular volume"
Page 10	Line 26	"by four folds" should read "by four fold"
Page 11	Line 7	"oxynenase" should be "oxygenase"
Page 11	Line 11	"folds" should be "fold"
Page 12,	Line 8	"hydroxyl ions" should read " hydroxide ions"
Page 13	Line 20	"dependant" should read "dependent"
Page 15	Line 16	"diary" should read "dairy"
Page 17	Line 17	Should read "The solubility of ferric pyrophosphate may be improved...."
Page 19	Line 20	"bioavilability" should read "bioavailability"
Page 19	Line 25	"a marked increase on" should read "a marked increase in"
Page 24	Line 13	"homologous" should read "homologs"
Page 24	Line 21	"folds" should read "fold"

Chapter 2:

Page 61	Line 11	reference to Fairweather - Tait *et al* (1992) is inappropriate and has been deleted.
Page 62	Line 1	Sentence "The method has been automated...." Deleted.
Page 66	Line 19	"period, and" should read "period."
Page 69	Fig 2.1	Control group where "Iron sufficient and Calcium sufficient (IS + Ca, Control).
Page 71	Table 2.1	% saturation refers to transferrin saturation.

Chapter 4:

Page 151	Line 18	Statement "Serum ferritin abnormalities also decrease with age" has been deleted.
Page 157	Line 6	"difference" should read "differences"
Page 157	Line 18	"differences" should read "difference"
Page 167	Line 22	Riboflavin should have been classified as a water - soluble not a fat - soluble vitamin.
Page 170	Line 5	replace "overt" by "allow"

Chapter 5:

Page 186	Line 26	The statement "These interactions of calcium...." Should read "These potential interactions of calcium....".
Page 198	fig 5.2	Title should read " A cross over growth profile of 35 day old weanling rats assigned to iron deficient diets with varying calcium contents".

Page 199 Line 15	The statement "The removal of calcium……but marginally improved iron stores…" should read "The removal of calcium……but marginally (although not significant) improved iron stores…"
Page 199 Line 21	"no significant" should read "no significant difference"
Page 202 Line 6	The statement "Overall, there was a 30% negative association …" has been deleted.
Page 203 Fig 5.4	Error bars. Error bars are omitted for clarity, however standard errors where within 5% of the mean.
Page 205 Fig 5.5	Figure legend. The text "or other treatment groups" should read "A similar effect of calcium was observed in the Iron deficient conditions."
Page 209 Line 9	The statement "This is the first time a negative…" should read "This is the first time that an apparent negative…".
Page 214 Line 6	"However, considered work is required to test this hypothesis."
Page 214 Line 7	"Nranp2" should read "N ramp2"
Page 215 Line 3	"human" should read "humans"
Page 215 Line 7	"condition" should read "conditions"
Page 217 Line 1	delete "which are commercially available at the molecular level"

Chapter 6:

Plate 6.3 A	Magnification of plate 6.3A is 40 and not 25.
Page 219 Line 5	delete "at all"
Page 240 Line 6	"negative iron status" should read "negative iron balance"
Page 244 Line 6	"dilema" should read "dilemma"

Chapter 7:

Page 247 Line 23	replace "most" with "more"
Page 253 Line 25	"inhibiting duodenal mucosal iron transport" should read "inhibiting duodenal iron transport"

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ABSTRACT

Iron absorption during weaning is regulated by complex interactions between the rate of erythropoiesis, body iron stores, dietary factors and gastrointestinal tract adaptation. Chronic iron deficiency anaemia in the first two years of life is associated with long term deleterious effects on behaviour and learning capacity. The aim of this study was to investigate the iron status and dietary intakes in 6-24 month old children in Australia and Ghana and to assess the effects of dietary calcium on intestinal iron absorption. The true prevalence of non-anaemic iron deficiency (NAID) and iron deficiency anaemia (IDA) and dietary intakes in infants and toddlers from a broad socio-economic background were assessed by haematological and biochemical parameters semi-quantitative diet recall and anthropometric measurements. The rat was used as an experimental model to investigate the effects of dietary calcium on duodenal iron uptake by employing haematological, biochemical, morphometric, immuno-histochemical and *in situ* hybridisation techniques.

Using strict laboratory diagnostic criteria, 25% of Caucasian children (6-24 months and full-term at birth) living in Adelaide were NAID and 6% had iron IDA. In a targeted group of 6-24 month old Asian children, there was a 14 % prevalence of both NAID and IDA (Oti-Boateng *et al.*, 1994). A recent study in Ghana showed a 55% prevalence of IDA in apparently healthy 6-24 month old children in metropolitan Accra and Kumasi (Oti-Boateng *et al.*, 1997). The high prevalence of iron deficiency and anaemia in Australian and Ghanaian children can be attributed to the low intake of bioavailable iron in weaning diets which are often ingested with large amounts of calcium. Calcium has been shown to inhibit the absorption of iron, but its mechanism of interaction with iron absorption at the intestinal level is not known, hence the use of the rat as a model to investigate this interaction during the weaning period.

Four semi-purified rat diets with varying iron and calcium contents were compared: Iron sufficient with high or low calcium (diets 1 and 2), and iron deficient

with high or low calcium (diets 3 and 4). These diets were fed to weanling rats (21 days old) for 14 days. Body weight gain, dietary intake and food conversion efficiency was measured every second day. Haemoglobin (Hb), haematocrit (Hct), serum ferritin (SF), transferrin (Tf), iron (SI) and serum calcium concentrations were determined. The duodenum mucosal iron uptake was measured in intact tissue and brush-border membrane vesicles using radio-labelled iron (^{59}Fe). Morphometrical changes were measured using image analysis and changes in gastrointestinal digestive enzymes were also analysed. The duodenal localisation of L- and H-ferritin and transferrin receptor messenger RNA expression were determined using radio-labelled *in situ* hybridisation technique. Immuno-cytochemistry was employed to assess the expression of TfR binding proteins in the duodenum.

There was no difference in the body weight gain per dietary intake in three of the groups but animals in group 3 (iron deficient high calcium diet) had arrested growth up to six days. There were significant differences ($p < 0.05$) in haemoglobin (Hb), haematocrit (Hct) serum ferritin (SF) and transferrin concentrations between iron sufficient and deficient treatment groups. Reduction of dietary calcium improved Hb and Hct values by 7% and significantly increased body iron stores and mucosal iron uptake under iron deficiency conditions. Villus height and crypt depth in control animals were significantly lower than those of animals in the treated groups. The protein content and sucrase activity of both mucosa homogenate and vesicle fraction of animals on iron deficient calcium sufficient diet ID + Ca was significantly lower than iron deficient calcium deficient animals. There was, however, an increase in the intensity of ferritin mRNA expression in villus enterocytes from tissues of rats fed iron deficient calcium sufficient diet.

These results indicate that there is a critical period during weaning when the consumption of high dietary calcium with low iron can retard growth potential. Dietary calcium significantly inhibits intestinal non-haem iron absorption at the intracellular level

by up-regulating villus enterocyte ferritin concentrations under iron deficiency conditions. The results also confirm earlier clinical findings in Australia and Ghana that marginal iron diets with a high calcium content increase the risk of iron deficiency and this occurs at a period when iron deficiency anaemia can have long-term deleterious effects on behaviour and school performance. An appreciation of this interaction is essential when formulating weaning diets for optimal iron bioavailability.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any University or institution and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis when deposited in the University Library, being available for loan and photocopying

3/3/1998

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ABBREVIATIONS

Abbreviations not explained in the text:

Ab	Absorbance
ad libitum	Without restraint
AR or AnalaR	Analytical reagent
ATP	Adenosine triphosphate
bp	base pair
cDNA	complementary DNA
Ci	Curie
cm	centimetre
CO2	Carbon dioxide
cpm	counts per minute
RNA	Ribonucleic acid
cRNA	complementary to DNA (synthesised in vitro)
DEPC water	Diethyl pyrocarbonate-treated water
DNA	deoxyribose nucleic acid
dNTP	deoxynucleoside triphosphate
dpm	disintergrations per minute
eg.	for example
et al.	*et alia* (and others)
etc.	*et cetera* (and so on)
G	gauge
g	gram
g	unit of gravitational field (acceleration of gravity)
HCL	Hydrochloric acid
hr	hour
Hz	Hertz
i.d.	internal diameter

i.m.	intramuscular
in situ	in a natural or original position
in utero	within the womb
in vitro	outside the organism in an artificial environment
in vivo	occurring in the living organism
i.v.	intravenous
kb	kilobase pair
kDa	kilodaltons
kg	kilogram
L	litre
M	molar
mA	milliamperes
MBq	MegaBequerel
mg	milligram
MJ	Megajoules
mL	millilitre
mm	millimeter
mM	millimolar
mmol	millimole
MQ water	Milli-Q-filtered water
MW	Molecular weight
n	number of samples
ng	nanogram
nm	nanometer
nmol	nanomole
Nramp	Natural resistance-associated macrophages protein
ns	not significant
o.d.	outer diameter
OD	Optical density

P	probability
per se	as such
poly (A)	polyadenylic acid
RNA	Ribonucleic acid (mRNA messenger RNA, rRNA ribosomal RNA, tRNA transfer RNA)
RO water	Reverse osmosis water
rpm	revolutions per minute
sec	seconds
SE	standard error of the mean
UV	Ultraviolet
V	Volts
v/v	volume:volume
W	Watts
w/v	weight:volume
^0C	Degrees Celcius
µCi	microCurie
µg	microgram
µL	microlitre
µm	micrometer
µM	micromolar
%	percent
/	per
<	less than
≤	less or equal to
>	greater than
≥	greater or equal to

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LIST OF PUBLICATIONS, CONFERENCE PAPERS AND SUBMISSIONS ARISING OUT OF THIS THESIS

Oti-Boateng P, Seshadri R, Petrick S, Gibson RA. Simmer, K. (1998) Iron status and dietary iron intake of 6-24-month-old children in Adelaide. *J. Peadiatr. Child Health* **30**; (in press)

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Oti-Boateng PE, Butler R, Tivey, DR. (1996) Dietary calcium and intestinal non-haeme iron absorption during weaning period: implications for infant iron nutrition. *J. Peadiatr. Child Health* **32**:A8

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CHAPTER 1

THE IMPORTANCE OF DIETARY IRON IN INFANT NUTRITION

Chapter 1

GENERAL INTRODUCTION

Iron is essential for all living cells. The ease with which iron can be reversibly oxidised or reduced makes it an essential component of, or a cofactor, for many cellular processes some of which involve energy metabolism, neurotransmitter systems, fatty acid biosynthesis, deoxyribonucleic acid (DNA) synthesis or detoxification (Koziol *et al.*, 1978; Yehuda 1992; Bruce *et al.*, 1993; Hunt *et al.*, 1994 (b), Klein and Fulco, 1994). Two-thirds of the body iron is in haemoglobin which is vital for the transport of oxygen, carbon dioxide and hydrogen in blood. Iron also participates in the synthesis of myoglobin, the pigment in muscle which intervenes in the storage of oxygen for release during muscle contraction. Iron is also an important component or essential cofactor of several enzymes involved in mitochondrial electron transport for cellular respiration which is linked to oxidative phosphorylation for the production of energy in the form of adenosine triphospate (ATP) (Vyas & Chanders 1984).

The iron-dependent tissue enzymes belong to three broad groups:

1. Enzymes with iron haem centres similar in structure to haemoglobin and including myoglobin, the cytochromes, catalase and the peroxidases. The main subcellular location of cytochromes a, b and c is within mitochondria. They are responsible for the oxidative production of cellular energy in the form of ATP. Other cytochromes are located in the membranous structure of the endoplasmic reticulum: cytochrome P 450 plays a role in the oxidation of xenobiotics and endogenous substances, and cytochrome b5 appears to be involved in protein synthesis (Holloway and Katz, 1975).

2. Enzymes that contain iron in a non-haem iron form. These compounds include the iron-sulphur proteins and metalloflavo proteins like xanthine oxidase, NADH dehydrogenases, succinate dehydrogenases and aconitase .

3 Enzymes that do not contain iron *per se* but require iron or haem as a cofactor such as tryptophan pyrolase (Hercberg & Galan 1989).

Iron deficiency anaemia has been association with lethargy (as a result of decreased physiological energy production), impaired fatty acid synthesis (which is essential for myelination and dendrification of the neuronal network of the brain particularly in children under two years) an increased risk of infection (as a consequence of reduced immune response) and decreased intestinal nutrient absorption (resulting from alteration of intestinal enterocyte morphology) (Walter *et al.*, 1989; Sayer and Long, 1993; Berant *et al.*, 1992; Benkovic and Connor 1993; Smuts *et al.*, 1995).

Although an essential nutrient, iron is also a potent toxin. Ferrous iron (Fe^{2+}) has a high affinity for oxygen and thus catalyses the reduction of oxygen *in vivo*, resulting in the formation of harmful superoxide and hydroxyl radicals at physiological pH's of 7.4 and 5.6 respectively (Carthew *et al.*, 1993; Vatassery *et al.*, 1997). The hazardous effects of iron are more apparent in diseases of iron overload such as haemochromatosis (Leggett *et al.*, 1990; Olsson *et al.*, 1995;). To prevent iron toxicity, a refined system made up of haemoglobin, myoglobin, cytochromes, transferrin-transferrin receptors and ferritin have evolved to either contain or regulate the availability and timely delivery of iron to cells (Salonen *et al.*, 1991; Kivivouri *et al.*, 1993). As indicated previously, haemoglobin contains 60 - 70% of the total body iron, with myoglobin, the cytochromes and other iron-containing enzymes accounting for further 10%. The iron in haemoglobin is constantly being recycled into circulation as red cells are destroyed at the end of their lifespan. The catabolism of haemoglobin by macrophages in organs such as the liver and spleen maintains iron homeostasis by keeping the iron derived from spent haemoglobin in circulation (Brock *et al.*, 1995). The remaining 20-30% of body iron is distributed in the storage protein ferritin and haemosiderin while the extracellular transport protein transferrin accounts for only 0.1-0.2% (Sassa 1992). Adequate iron status is only achieved when all the iron-containing compartments/components are in balance. The severity of depletion or plethoric of an iron-containing compartment is used as an indication of the degree of iron deficiency or iron overload.

Iron requirements and intake are governed by the need to maintain iron balance relative to loss and growth. The need for iron is most pronounced during infancy and early childhood when there is a three fold increase in blood volume and body weight (Smith and Rio, 1974; Dallman 1992). At the onset of weaning, the neonatal iron reserve is reduced and exogenous dietary iron intake becomes important for maintaining balanced iron status. Homeostatis of body iron is maintained by regulation of iron absorption across the proximal small intestine, whereby a signalling mechanism seems to exist to modulate iron uptake by mucosal body cells in response to body iron stores which play a major role in the supply of iron for metabolic needs and the duodenal intestinal (Komza *et al.*, 1994; Oates and Morgan, 1997). Although iron absorption has been the topic of many investigations, the mechanism by which other nutrients moderate intestinal mucosal iron uptake from the lumen and transport into the body is not known. The complexities of iron metabolism and its regulation are beyond the scope of this thesis. The aim of this thesis is to elucidate the effect of other nutrients on iron status and intestinal iron absorption in young children, employing clinical studies and animal models.

The first part of this review will concentrate on our understanding of iron metabolism in young children, nutritional factors that mitigate against iron absorption and its regulation and the effects of imbalance in iron homeostasis. Prevalence studies of inadequate iron status in children and measures that have or are being put in place to avert these adverse consequences of iron deficiency in infants and young children will also be discussed. The second part of the review will examine briefly the general aspects of 1) the development and maturation of the gastrointestinal tract during perinatal and weaning periods; 2) postnatal maturation of the intestinal epithelium and patterns of enzyme activity; and 3) factors that control intestinal proliferation and maturation and prime the epithelium into adulthood. This will help us to appreciate the physiological changes and adjustments that occur in the gastrointestinal tract during the weaning period and the need to critically evaluate iron nutrition during this vulnerable period in life.

LITERATURE REVIEW

THE IMPORTANCE OF DIETARY IRON IN INFANT NUTRITION

1.1 Iron status in infants and children

Due to the inevitable role iron plays in all living cells, full term infants are born with sufficient iron to supply physiological needs for the first two months of extrauterine life. Seventy five percent of neonatal iron is in circulating haemoglobin, 9% in lean tissue and 14% in the liver. During this time, decreased erythropoiesis, the termination of extra-medullary erythropoiesis, an increase vascular volume and haemolysis cause an approximate 30% physiological fall in haemoglobin concentration from an average of 16.5g/dL to 11.0g/dL, (Dallman and Yip, 1989; Dallman 1992). Iron is redistributed to storage sites and this is reflected by a sharp rise in the level of serum ferritin over the first month (Smith and Rios, 1974). Since erythropoiesis is not usually resumed until 2-3 months the young infant's requirement for exogenous iron is relatively small until that time. At about six months when the trans-placental iron endowment is exhausted, the infant becomes dependent on continuous supply of exogenous iron for maintenance of normal erythropoiesis (Dallman and Siimes, 1979; Yip 1995). This occurs earlier in preterm and low birth weight infants. Depletion occurs if there is an imbalance between the amount of iron present at birth and the rate at which the element is needed for anabolic processes or is lost in blood or desquamated gut and skin cells (Elian *et al.* 1966).

Nutrition is, therefore, the major determinant of iron status in infancy, more so than at any other time in life. Sketel (1984), using estimates established by Smith and Rios (1974) calculated an average daily body requirement of 0.49 mg for the first six months and 0.90 mg between the ages of six and twelve months in non-breast fed infants. At one year of age 30% of the haemoglobin iron was derived from the diets in comparison with only 5% of absorbable iron needed in adult haemoglobin (Dallman *et al.* 1980). These differences in percent incorporated of absorbable iron into haemoglobin in

infancy and adulthood further support the importance of exogenous dietary iron in children.

Infants fed exclusively on human milk are estimated to have an average daily iron intake of only 0.29 mg at six months and the majority of exclusively breast fed term infants are able to maintain an iron status equal to that of the infants receiving iron supplemented formula for six months. This apparent anomaly is largely attributed to the relatively high bioavailability of the iron in human milk, 45-75% being absorbed (Saarinen & Siimes 1977). The high enhancing capacity of breast milk has been proposed to be to associated with the high levels of lactoferrin in breast milk (Ismail and Brock, 1995), although a study by Davidsson and coworkers (1994) failed to show any association. The pattern of milk iron concentration during lactation is very similar to that for lactoferrin which is synthesised in the mammary gland, but whether lactoferrin acts to sequester iron entering the mammary epithelial cells has not been conclusive. However, the iron content of human breast milk falls during lactation with levels of 0.5-0.6 mg/L in the first 2 weeks, 0.4 to 0.5 mg/L up to 3 months of lactation and 0.2 to 0.4 mg/L thereafter (Siimes *et al.* 1979).

Siimes *et al.* (1984) studied 36 infants who were exclusively breast fed and 32 infants who were completely weaned off the breast before three and a half months and who received iron supplementation in the form of formula (6 mg/L) and wheat cereal (40 mg/kg). They found that the concentration of haemoglobin remained higher in the breast fed infants (122 and 124 g/L) than in the controls (114 and 118 g/L) at 4 and 6 months respectively. However, after 6 months, 8% of the exclusively breast-fed infants had biochemical evidence of iron deficiency and by nine months all the exclusively breastfed infants without iron supplementation had high total iron binding capacity, low serum ferritin level and low serum iron, all of which are indications of iron depletion. The iron supplemented infants maintained an adequate iron status. The differences in the mean values of the biochemical parameters considered were significantly different from those

of the exclusively breast-fed infants. The authors also demonstrated that maternal iron supplementation during breast feeding did not affect the iron status of infants nor prevent iron deficiency. This study, and a previous one by Fomon and Strauss (1978), indicate the need to introduce iron supplementation in weaning diets of exclusively breast-fed infants from six months to maintain an adequate iron balance.

Iron balance is achieved in the body by the regulation of iron absorption and the recycling of the majority of total body iron stores. The average adult has 3-5 gm of body iron distributed into functional iron and storage iron (1.5 to 3g in haemoglobin and myoglobin, 0.5g in other proteins and 0.3 to 1.5g stored). Ideally the amount of iron absorbed should be equal to iron lost and in normal adults this amount is approximately 1mg per day (Conrad & Barton 1981). Infants are more susceptible to iron balance is more vulnerable to depletion between six to eight months as indicated above. Iron stores are utilised during infancy as reflected in a drop in serum ferritin from approximately 250 µg/L to 40 µg/L at nine months (Worwood, 1982). The full-term infant can maintain positive iron balance for up to 4 months, however, due to rapid growth the tripling of blood volume there is a more than two fold increase in daily iron requirement (3 mg Fe/day at six months to 9mg/day at nine months) in both breast and formula-fed infants (NHMRC, 1991). Iron losses in infants can also affect the balance as demonstrated by Ehrenkranz *et al.* (1992). They calculated a 50% loss of iron from erythrocyte incorporation studies in low birth weight infants. However, in term infants, iron loss is minimal and the main determinant of iron balance in infancy is absorption .

1.2 Iron Absorption

The processes involved in iron absorption are complex and multifaceted. The efficiency of the absorption of iron from the diet is significantly influenced by its chemical form, gut transient time and the presence of other factors that can either enhance or inhibit its absorption and utilisation (Huebers *et al.*, 1983; Monsen 1984; Dreosti 1993; Davidson *et al.*, 1994; Reddy *et al.*, 1996). The presence of favourable agents such as ascorbate or

citrate in the diet leads to the chemical reduction of dietary iron from the Fe^{3+} form to the more absorbable Fe^{2+} form. Ionic competition for other divalent nutrients like calcium (which is often taken in high quantities relative to iron), magnesium and zinc have also been shown to influence iron absorption (Fairweather-Tait *et al* ., 1989; Hallberg *et al.*, 1992 (a); Oti-Boateng *et al.*, 1994). Known inhibitors like phytate, phosphorous and oxalate reduce the bioavailability of iron by virtue of their ability to chelate the metal iron in the gut lumen (Monsen and Cook, 1984).

Another important factor in evaluating the capacity of a diet to provide bioavailable iron is the fraction of iron bound to haem. Haem is more readily absorbed than "ionic" iron. The oxidation state of haem, (Fe^{2+}) does not affect its absorption because the entire haem complex is taken up into the mucosal cells (Rucker *et al.*, 1994). Total body iron stores, haematopoietic activity also influence iron absorption and other nutrients may enhance or inhibit its absorption (Massey 1992). For example calcium concentration in the diet has been shown to inhibit haem iron absorption although the degree of inhibition was less than observed for non-haem iron (Gleerup *et al.*, 1995). The exact mechanism of haem iron absorption is not well understood, however, it is suggested that cysteine and methionine (thiolsulphide amino acids) in meat may be the enhancing factors

1.2.1 Dietary iron

Dietary iron exists in either the haem (organic) or non-haem (inorganic) forms. In a typical Western diet about 10% of food iron is in the haem iron form which mainly comes from haemoglobin and myoglobin in meat, fish or poultry. This iron is relatively well absorbed and is less influenced by iron stores and the presence of other dietary constituents (Hallberg 1981; Bothwell *et al.* 1989). The other 90% of food iron is in the form of iron salts which are derived mainly from plant foods and is referred to as non-haem iron. Non-haem iron absorption is considerably lower than that from haem sources. Studies by Hallberg (1981) and Bothwell (1989) showed that between 10 and

25% of iron was absorbed from meat and fish with only 1-7% from cereal and legume staples. Subsequent studies demonstrated that almost all forms of non-haem iron in a meal behave as a single pool in the small intestine, the major site of iron absorption (Bjorn-Rasmussen *et al.* 1972; Bothwell *et al.* 1979).

The pool of non-haem iron is more subject to variations in the diet than haem iron. Many dietary constituents affect the availability of non-haem iron from foods. Unfortunately, non-haem iron is the most readily accessible form of iron in most developing and underprivileged societies. The majority of the food iron available for infants and young children is also in this form. The enhancers of iron absorption in infants are ascorbic acid and haem iron (Cook & Monsen 1976; Hallberg 1981; Haschke *et al.* 1988). The presence of phytic acid, fibre, tannins and oxalic acid, which are derived from plant, have been shown be responsible for the poor availability of dietary iron (Simpson *et al.* 1981; Cook *et al.* 1983; Gillooly *et al.* 1983; Hallberg *et al.*, 1989; Hurrell *et al.*, 1992 (c)).

Since most of the foods introduced early to infants are predominantly, cereals vegetables and cow's milk with relatively little meat or fish (Mills and Tyler 1992; Retallack *et al.* 1994), almost all of their dietary iron is in the non-haem form and is highly subject to modulating influences. Food manufacturers have attempted to remedy this problem by fortifying cereal-based infant foods and milk formulae. Unfortunately, because the added iron enters the 'common pool' of non-haem food iron, it is absorbed as poorly as the native iron in the diet (Bjorn-Rasmussen *et al.* 1972; Hurrell 1984) and is influenced by the same inhibiting and enhancing factors.

Vegetable and cereal based diets are not only low in iron but also contain plant phosphates such as phytate and inositols which are strong inhibitors of iron absorption (D'Souza *et al.*, 1987; Hurrell *et al.*, 1992; Lynch *et al.*, 1994). In spite of the attempts made to solve this problem through food processing and fortifying cereals and legumes

with different forms of inorganic iron (such as electrolytic iron and ferrous sulphate which have 5 and 50% relative bioavailability respectively (Monsen 1984; Fomon 1989), iron deficiency remains one of the most prevalent nutrient deficiencies in both privileged and under-privileged societies (Kuvibilida *et al.*, 1993; Karr *et al.*, 1996). This is probably due to the adverse interaction of iron with other nutrients. Iron-fortified cereal (IFC) when mixed with iron-fortified milks (formula) have been shown to reduce the risk of iron deficiency in 6-12 month old children ($p < 0.05$) relative to those who consumed IFC with cows' milk (Fuchs *et al.* 1993). Formula-fed infants also have better iron stores compared to their breast-fed counterparts at 12 months of age due to the relatively high content of bioavailable iron in formulae which contains high levels of ascorbic acid (Saarinen and Siimes 1979; Oti-Boateng *et al*. 1994). After 12 months when either IFC or formula with supplemental iron ceases and cows' milk becomes the main drink, toddlers are at risk of developing iron deficiency iron at a period when their iron stores are depleted. At the same period the diet becomes varied with an increased consumption of vegetables but there is still very little fish, meat or seafood.

1.2.2 Enhancers of iron absorption

There are three dietary factors that can enhance iron absorption. These are i) the amounts of non-haem iron reducers such as ascorbic acids as described in section 1.2.1, ii) haem iron and iii) 'meat factor' which will enhance both non-haem and haem iron absorption.

Haem iron

The major source of haem protein is meat which is not only a source of highly bioavailable haem iron but it also enhances the absorption of the non-haem iron in a meal (Cook & Monsen 1976; Bjorn-Rasmussen & Hallberg 1979). No significant differences in the enhancing effects of beef, pork lamb, liver chicken or meat has been observed (Cook and Monsen, 1976). Absorption of haem iron was increased by four folds in patients with iron deficiency compared with normal subjects (Lynch *et al.*, 1989). The

absolute iron absorption from standard meal is increased four-folds higher than non-haem iron absorption at serum ferrtin levels of 100 μg/L in humans (Hallberg *et al.*, 1979; Lynch *et al.*, 1989;), while veal muscle induces a ten-fold increase in non-haem iron absorption from a maize meal (Layrisse *et al.*, 1976). The physiology of intestinal uptake of haem iron has important differences from the uptake of non-haem iron (Roberts *et al.*, 1993). Iron ingested as haemoglobin is split from haem (ferroprotoporphyrin IX, 650 mol wt) in the intestinal mucosa by oxynenase-like enzyme and joins a soluble pool of absorbed non-haem iron. Haem is then taken up by the mucosal cell via a pathway different from non-haem iron which involves binding to specific haem receptors (Roberts *et al.*, 1993). The specific binding of ^{14}C-labelled haem to brush border membrane was 3.3 folds more than non-haem iron under iron deficiency.

Meat factor

The presence of meat in the stomach promotes the absorption of both haem and non-haem iron. The release of amino acid and polypeptides in the upper small bowel may be the explanation for the enhancing effect of meat or fish on the absorption of non haem iron although the mechanism is poorly understood (Charlton & Bothwell 1983). Alternatively, the specific binding of haem to the brush border membrane that facilitates the increased absorption of haem iron in iron deficient compared to iron sufficient may explain the meat factor.

Nutritional implications

Therefore, the addition of meat to the diets of infants of populations subsisting on cereal diets would be expected to have a major positive impact on iron absorption, but it is financially impractical and unrealistic since meat is not a childhood favourite and is often not introduced into the diets before the age of 18 months.

Of more practical value are the observations of Sayer and co-workers (1986) who demonstrated the marked enhancing effect of ascorbic acid on the absorption of dietary

non-haem iron making the consumption of fruit or juice a desirable alternative in infancy. The effect of ascorbic acid is dose-related and is quite marked in all kinds of diets. Hallberg and co-workers (1989) have shown that a 50% reduction of the natural content of ascorbic acid in a meal reduces the absorption of non-haem iron by 75%. Ascorbic acid plays a normal physiological role in the conversion of the ferric iron in the average diet to the ferrous form that is necessary for absorption. It is involved in reducing the formation of poorly soluble and poorly available ferric complexes including those with hydroxyl ions, certain phosphate ions, phytates and iron polyphenol complexes in the intestinal lumen (Wollenbert & Rummel 1987).

1.2.3 Inhibitors of iron absorption

The two most important dietary inhibitors of iron absorption are phytates and polyphenols. High levels of calcium, phosphate, bran or egg yolk also have inhibitory effects on iron absorption (Cook & Monsen 1976; Hallberg 1981; Hallberg *et al.* 1991; Hurell *et al.* 1992).

1.2.3.1 Phytates

Phytates are widely dispersed in foods and are found in particularly high concentrations in bran (Bjorn-Rasmussen *et al.*, 1974; Hallberg *et al.*, 1987). Phytates are strong inhibitors of iron absorption, and the balance of evidence today indicates that the content of phytates in the diet is a major determinant of iron bioavailability (Brune *et al.*, 1989; Fairweather-Tait *et al.*, 1989). *In vitro,* phytates bind several irons irrespective of its valency state while they can interfere also with the absorption of other dietary minerals such as calcium, zinc and magnesium *in vivo.* Hallberg and coworkers (1989) showed there is a dose-dependent inhibitory effect of sodium phytate on iron absorption (18% when there is 2 mg/g of phytate in the diet, 64% with 25 mg/g phytate and 82% with 250 mg/g phytate). The addition of ascorbic acid to these three doses of phytate consistently counteracted the inhibition whereas the corresponding effect of meat only occurred with 25 mg/g phytate. There is probably the same dose-effect relationship

between inositol hexaphosphate (the main form of phytate in seeds, tubers and roots) and the inhibition of non-haem iron absorption. Fermentation and baking cause a partial degradation of phytates to simple phosphates and inositol phosphate, it is possible that the phosphates in the bread may have other iron binding properties than those of hexaphosphates. Since there are high amounts of phytate in many diets, phytate is a major determinant of iron nutrition. Even processed foods are never phytate-free and there is the need to reduce the content of inosotol hexa and penta-phosphates by suitable food processing techniques to improve the bioavailability of iron (Hallberg *et al..*, 1986).

The balance between phytate and ascorbic acid has nutritional implications especially for iron availability in weaning diets which are predominantly cereal-based. The addition of ascorbic acid or fruit juice may be beneficial, but this is not a common practice.

1.2.3.2 Polyphenols

Another tenacious set of inhibitors of iron absorption are polyphenols. These are responsible for the marked inhibitory effect of tea on iron absorption and are present in a number of vegetables used as weaning foods, including sorghum, legume, spinach, fruits and seeds (Gillooly *et al.* 1984; Brown *et al.* 1990). Some natural phenolic compounds also occur as tannins and caffeine. These form a dark brown colour with iron and are commonly found in tea and coffee. Polyphenols have been found to be responsible, in a dose-dependant manner, for the poor bioavailability of the non-haem iron present in these foods. Brown and co-workers (1990) investigated the influence polyphenol-containing beverages on iron absorption in rats. They demonstrated that black tea, green tea, coffee and cocoa have total polyphenols ranging from 80 -136 mg/100 mL. They found a marked inhibitory effect of cocoa, coffee and tea on the absorption of iron by as much as 50-62% when taken with a non-haem iron diet. This has enormous implications especially for infants and young children in cocoa and tea growing areas of the world where these beverages are drunk at the same time with meals.

It can be presumed that this practice will have an effect on their iron nutritional status although the interaction between iron and various dietary phenolic compounds is poorly understood.

1.2.3.3 Plant Proteins

The inhibitory effect of proteins in a number of plant foods deserves mention. Soybeans, nuts and lupins, which are suitable as protein supplements, are known to be strong inhibitors of dietary iron absorption (Hallberg *et al.* 1982; Macfarlene *et al.* 1988a,b). The exact nature of the inhibition has not been elucidated, but it has been proposed that lectins in soya beans and particularly in soya bean flour inhibit iron uptake by the intestinal mucosal membrane (Loser 1987; Erdman and Fordyce, 1989; Hisayasu *et al.*, 1992b). The effect of soya protein has nutritional implications in infant feeding as it is a major ingredient in some infant formulas especially in the United States where soya formulas account for one-quarter of infant formula sales (Witherly 1990). The dietary use of soya protein is also increasing in Australia both in dairy-type foods and oils. Soya protein has a good protein quality, low cost, plentiful supply and excellent functional properties which makes it an attractive material for developing new product lines (Erdman *et al.* 1989) However, soya products markedly reduce non-haem iron absorption with the isolated protein having the greatest inhibitory effect (Cook *et al.* 1981).

Hurrell *et al.* (1992) conducted 4 iron absorption studies on the role of phytate in modifying non-haem iron absorption from 11 different soy protein isolates in four groups of 7-9 human subjects. They showed that 8.4mg/g of phytic acid in the soya meal resulted in a mean iron absorption of 1.5% which increased to 3.15% when the phytic acid was reduced to 0.2 mg/g. From this study they showed that phytic acid was the major inhibitory factor in soya-protein and the removal of phytic acid to < 0.01 mg/g of isolate increased iron absorption by four to five folds. Even in a mixed diet, soya protein itself also has a relatively strong inhibitory effect on iron absorption.

1.2.3.4 Calcium

Recent studies have suggested that the high calcium content of cow's milk may be responsible for the low bioavailability of iron in cow's milk compared with human milk (Monsen and Cook, 1976; Cook *et al.*, 1991; Hallberg *et al.*, 1991). Hallberg and coworkers (1992b) found that the addition of calcium to breast milk to levels found in cow's milk caused a 42% reduction in iron absorption of from breast milk. The amount of iron absorbed from the reconstituted breast milk was still more than that from cow's milk. They showed that the calcium content in breast and cow's milk was responsible for the 70% differences in the iron bioavailability from these milk types. They also showed a dose-dependent inhibitory relationship between calcium intake and iron bioavailability. Furthermore, calcium has been found to reduce the absorption of iron from both haem and non-haem iron in meals (Hallberg *et al.* 1991; Cook *et al.* 1991b, 1992; Gleerup *et al.*, 1995). When pharmaceutical doses of calcium carbonate are taken without food, no reduction of iron occurs, however, reduced iron absorption occurs when calcium carbonate is taken with food. The exact mechanism of calcium on iron absorption is not known.

These finding have nutritional implications for the consumption of diary products as these are a primary source of calcium and protein in infants and young children. Since iron and calcium are both essential nutrients in infancy, it is important to find feasible and practical solutions that satisfy the requirements of both nutrients. Consequently, the marked inhibitory effect of calcium on iron absorption ought to be considered in the design of infant formulas and diets. It is also important to limit the amount of cow's milk or dairy products consumed in childhood to moderate amounts to achieve an optimal balance between calcium and iron. However, an optimal balance between calcium and iron uptake cannot be achieved without understanding the mechanism of interaction of dietary calcium on intestinal iron absorption. It will be important to elucidate the mechanism of dietary calcium on intestinal non-haem iron absorption during the weaning period to optimise as a means of optimising intestinal iron absorption.

1.3 Iron Fortification of Infant Cereal

The approach to increasing the amount of iron in the weaning diet by fortifying basic foods with iron is likely to be the easiest long term approach for preventing iron deficiency (Rees *et al.* 1985), although the selection of an appropriate form of bioavailable iron for supplementing weaning diets is far from being resolved. An overview of iron compounds which have been used in diets is given below.

Ferrous sulphate

Ferrous sulphate is used as an adult dietary supplement and has been shown to be an effect therapy for deficiency in adults in infants and adults (Cook *et al.*, 1992). Iron sources that are freely water soluble and have high bioavailability, notably ferrous sulphate, are used to fortify formulae. However, organoleptic properties such as the effect on taste and flavour and the catalytic effect of iron on fat oxidation during storage, make such compounds unsuitable for fortifying dry infant cereals (Sketel *et al.* 1986). Ferrous sulphate is used in the United States by the Gerber Product Company who are producing wet cereal for infants 6 months and over but its efficacy has not been widely tested clinically (Gerber, personal communication, 1993).

Elemental iron

Elemental iron can be reduced, electrolytic or carbonyl depending on the method of manufacture and is the only iron source used in infant cereals in Australia (Personal communication, Nestle', 1997). Due to variations in production, each type may itself vary in shape, size and surface area. Its bioavailability depends on solubility in gastric juice and is governed as much by differences in particle size and surface area as the type of powder (for example, the bioavailability can be increased by ensuring small particle size). Therefore, variation in bioavailability may be as great using the same type of product as when different products are compared (McPhail *et al.* 1985; Fomon 1987; Hurrell 1984; Lynch & Hurrell 1990).

Ferric pyrophosphate

Iron sources that are poorly soluble in water such as ferric pyrophosphate and sodium iron pyrophosphate have been added to dry infant cereals. Their bioavailability is usually low and such compounds, have been replaced in Australia, Europe and America by elemental iron. The solubility of ferric pyrophosphate can be improved by forming a chelate with sodium citrate or ammonium citrate relatively to sodium iron pyrophosphate. Organoleptic problems however makes the chelated form of ferric pyrophosphate unattractive (Hurrell 1985).

Ferric orthophosphate and ferric saccharate

Ferric orthophosphate and ferric saccharate are occasionally added to infant cereals in European countries. Ferric orthophosphate has a low and variable bioavailability while ferric saccharate is well utilised. However, brown colour of the latter compound may cause discolouration of food when higher levels are added.

Ferrous succinate and fumarate

Ferrous succinate and ferrous fumarate are the best alternatives to elemental iron and ferric pyrophosphate for infant cereal fortification. Organoleptic problems have not been encountered at fortification levels up to 60 mg iron in 100g food. Although both are insoluble in water, they dissolve in dilute acids. Ferrous fumarate is already in use in America where about 3% of infant cereals are fortified with this form of iron (Rees *et al.* 1985; Hurrell *et al.* 1991).

Bovine blood

There have been a few studies conducted to incorporate bovine blood into infant cereals as a means of increasing the iron availability, however, there are concerns with these. Calvo *et al.* (1989) in their search for adequate foods to supplement breast feeding infants in Chile, conducted a study by fortifying extruded rice flour with 5% bovine haemoglobin concentrate (BHC) to raise the amino acid content by 59%. Ten 8-10

month old infants were fed with 20g of the bovine fortified rice cereal on one occasion. Iron absorption was measured by a double isotope technique. They concluded that the use of a haem-iron fortified cereal as a weaning food was feasible, as it provided 37% bioavailable iron, energy (kilojoules) and protein. Subsequently, Hertrampf *et al.* (1990) carried out a field trial to assess the effectiveness of this extruded rice-BHC weaning cereal in infants 4-12 months old with a control group of the same age who ate the regular weaning foods. They found a significant difference in the serum iron concentration, serum ferritin, and free erythrocyte protoporphyrin at 9 months between the intervention group and the control and by 12 months, iron deficiency was found in 17% in the control subjects and 10% of the intervention group.

Although the consumption of 60g of the Rice-BHC cereal weaning diet daily can sustain adequate iron status, the use of cow's milk in the control could have also contributed to the stronger evidence of anaemia in the infants in the control group. In future studies, it will be prudent to control for foods containing strong iron inhibitors. Despite the relevance of the above study in showing the relative iron availability of rice-BHC weaning foods and its relative effectiveness in preventing iron deficiency, the use of bovine haemoglobin as iron fortifer in commercial weaning cereals still needs further investigation. The effect of temperature on the shelf life of such a high protein weaning cereal and its microbial quality on packaging and storage are still to be assessed, as is the organoleptic acceptance of this kind of a weaning diet in infants. Unless the shelf life and organoleptic properties of bovine fortification can be extended, food manufacturers will need to improve the absorption of other dietary iron supplements.

1.4 Improving the bioavailability of fortification iron

The bioavailability of fortification iron depends on the effects of ligands in the meals that act on the common non-haem iron pool as well as on the nature of the iron source itself. Studies have indicated that about 4% of extrinsically labelled iron is absorbed from food, about the same percentage as is absorbed from iron-fortified cow's

milk based formula (Morck *et al.* 1981). Since the absolute absorption of iron source is difficult to predict, food manufacturers use its relative bioavailability value (RBV) as a guide to its potential absorption. The RBV is obtained by measuring the absorption and utilisation of a given iron source relative to ferrous sulphate (RBV for ferrous sulphate is set at 100). Iron sources with an RBV similar to ferrous sulphate are expected to be equally well utilised. The anticipated absorption of an equivalent amount of iron source with a lower RBV would be correspondingly less; however, poorly soluble iron sources enter the common pool to a variable extent, and their relative bioavailability may not be predictable with certainty.

As mentioned above, the bioavailability of fortification iron can be improved by the addition of enhancers of iron absorption to food such as ascorbic acid. Once the fortification iron is dissolved in gastric juice, it can be reduced by ascorbic acid to its more soluble ferrous state, and then be combined in soluble chelates that are available for absorption. Ascorbic acid is sensitive to food processing and storage loss, although over addition and careful storage can ensure sufficient ascorbic acid remains for the shelf life of the product.

Food processing such as vacuum drying or heat sterilisation can also improve the bioavailability of some fortifcation iron compounds. Hurrell *et al.* (1991) investigated the fortification of cocoa drink with ferrous fumarate, ferrous sulphate and ferric pyrophosphate. From organoleptic and bioavilability point of view, ferrous fumarate was judged to be an acceptable iron fortificant for a chocolate drink powder when the cocoa powder was mixed with water or milk and heated at up to 80^{o}C. In contrast, there was no change in the relative bioavailability of iron in chocolate drink when ferrous sulphate and ferric pyrophosphate fortification iron forms were used. There was, however, a marked increase on the relative availability of the different iron compounds when the products were subjected to vacuum drying, such that the RBV of ferric pyrophosphate and the other insoluble iron compounds was significantly increased.

Ferrous fumarate was ten times better absorbed than ferric pyrophosphate and absorbed twice as well as ferrous sulphate (the bioavailability changed significantly on processing).

The RBV of ferrous fumerate fortified in a glass of cocoa drink was shown to increase by 40% in children with borderline (ferritin below 15 μg/L) iron deficiency (Hurrell et al., 1991). They suggested that in the absence of food, one glass of chocolate milk drink would provide approximately 0.5 mg of absorbable Fe/serve and, two glasses a day will be more than adequate for the daily recommended intake for a one year old. This is encouraging since cocoa and milk separately have been shown to inhibit iron absorption substantially. Such an iron fortified chocolate and milk drink would have a significant impact on iron nutrition in children.

Wood and coworkers (1978) found that heat treatment of a chicken diet as a liquid slurry increased the RBV of ferric pyrophosphate to chicken from 7 to 90. On the other hand, the baking of bread seems to have little effect on the RBV of ferric orthophosphate (Cook *et al.* 1973). It thus appears that different methods of heat processing have different effects on the bioavailability of different iron compounds used as fortificant. This must be due to the influences of the processing methods on the solubility of the food iron in the gastrointestinal tract.

1.4.1 Methods for measuring dietary iron absorption

Several methods can be used to estimate the availability of iron from foods. These are chemical balance, haemoglobin repletion, plasma iron tolerance, radio-iron balance, whole body counting, stable isotope balance and red cell incorporation of iron.

Iron absorption from many foods has been determined from studies using radioactive isotopes of iron (Hallberg *et al.*, 1991). Since virtually all the iron absorbed from the diet is utilised for haemoglobin formation, with negligible excretion of endogenous iron, absorption can be used to accurately predict availability. In adults,

balance studies have been largely abandoned in favour of more precise methods using the radioisotopes ^{55}Fe and ^{59}Fe, either by means of radioiron balance or red blood cell incorporation. For example a study by Hallberg *et al.* (1991) assessed the effect of calcium on iron absorption from different types of food labelled with the radioisotopes ^{55}Fe and ^{59}Fe. A blood sample was drawn after two weeks to determine the content of ^{55}Fe and ^{59}Fe in red blood cells. In addition the total retention of ^{59}Fe was measured by whole body counting and at the same time the total retention of ^{55}Fe was calculated from the ratio ^{55}Fe and ^{59}Fe in red cells. From this study they were able to conclude that iron absorption is inhibited by as much as 50-69% by calcium in a dose-related manner.

There are ethical problems associated with the administration of radioisotope to infants. Although a few studies have been permitted in the past, the growing concern over the hazards of ionising radiation will limit the future use of radioisotopes. Stable isotopes (^{58}Fe and ^{57}Fe) offer a safer alternative means of following the fate of iron in infants. Janghorbani *et al.* (1986) applied the method of inductively coupled plasma mass spectrometry to determine the ratio of ^{58}Fe and ^{57}Fe in the erythrocytes of infants before and after oral administration of the least abundant isotope ^{58}Fe. Each child was given ^{58}Fe as a single dose (which provided 2 mg iron and 1.4 mg ^{58}Fe) or as one dose on each of two consecutive days. From the increase in erythrocyte enrichment with ^{58}Fe, they calculated the percentage of iron absorption. They showed that this method of erythrocyte incorporation of ^{58}Fe has a measurement precision of 0.1%. From this they were able to establish the relationship between the amount of iron absorbed and the amount appearing in the circulating erythrocytes. Ehrenkranz and co-workers (1992) used the stable isotope ratio (^{58}Fe / ^{57}Fe) methodology developed by Janghorbani for measuring iron absorption and incorporation into the red blood cells in very low birth weight infants. They calculated a 28.7% incorporating of absorbed ^{58} Fe into the red blood cells (RBCs) on day 15, which correlated with the haemoglobin concentration and reticulocyte count on day 1. Fomon and coworkers (1993) also used non radioactive iron

to assess erythrocyte incorporation of ingested ^{58}Fe-enriched ferrous sulphate by 56 day old breast and formula fed infants. They showed a negative correlation ($r = -0.61$, $p < 0.001$) between formula feeding and erythrocyte incorporation of ^{58}Fe. Breast fed infants incorporated 15% more of their absorbable iron than formula fed infants at 70 days old, and suggested that the calcium content of the formula may have inhibited erythrocyte incorporation of labelled iron 58Fe in formula-fed infants.

This non radioactive methodology has a significant potential for future assessment of the bioavailability of various sources of iron in infants and young. It however, has a limitation in that the initial cost of investment in the equipment required to measure these isotope is well beyond the budgets of many research laboratories. Since the models used to predict dietary iron bioavailability have been from single meal data, further work to determine more clearly the relationship between single meal and dietary bioavailability estimates are needed.

1.5 Intestinal absorption of iron

Although there have been several studies on the erythrocyte iron incorporation of dietary iron, there are fewer studies on the mechanism of intestinal iron absorption during infancy and childhood, particularly from composite diets. The upper intestine is the most active site for iron absorption where the acidic pH from gastric juice maintains iron in its soluble ferric iron form (Jacobs *et al.*, 1966; Chowrimootoo *et al.*, 1992; Oates and Morgan, 1997). Chelates are formed to maintain iron in a soluble state, and pancreatic juices inhibit protein binding to iron which may inhibit its absorption. A small quantity of iron may enter by simple diffusion as proposed by Charlton and Bothwell (1983). However recent genetic and molecular studies have led to unexpected advances in our understanding of iron transporters associated with iron metabolism.

Iron transporter proteins

The first step in intestinal iron uptake is the mucosal uptake of iron whereby ferrous iron from the lumen is bound to specific sites of iron mediated-carriers that transport iron across the enterocytes, which involves events at the brush-border and basolateral membranes (Chowrimootoo et al, 1992). The iron is then transferred from the basolateral surface of the enterocytes into the blood (Chowrimootoo *et al.*, 1992; Oates and Morgan, 1995; 1997). Several intracellular iron-binding proteins have been identified. Three molecules of particular interest, differric transferrin-transferrin receptor, mobilferrin and DCT1 which have high iron affinity and can actively traverse the intestinal membrane have been proposed (Egan *et al.*, 1993; Conrad *et al* , 1994; Gunshin *et al.*, 1997; Fleming *et al.*, 1997). Ferritin iron binding protein has also been shown to be associated with intestinal iron homeostasis.

The transferrin receptor plays a major role in cellular iron acquisition by facilitating the uptake of iron-laden transferrin from the extracellular medium (Huebers and Finch, 1983; Bali and Aisen, 1991; Egan *et al.*, 1993). The receptor protein has also been shown to be prominent on the basal, lateral and intracellular membrane in the intestinal epithelial cells (Anderson *et al.*, 1990, 1994; Pietrangelo *et al.*, 1992). However recent studies have shown that diferric transferrin, transferrin receptors have no direct role in the uptake of iron from the luminal membrane to the basal lateral membrane in enterocytes (Pietranagelo *et al.*, 1992; Anderson *et al.*, 1994; Oates and Morgan 1997). The available apo-transferrin in the intestinal lumen is insufficient to fully account for dietary iron absorption. The evidence that the localisation of TfR in the epithelia is more intense in the crypt than in the villuos cells further suggests that transferrin may not be directly involved in intestinal iron absorption as initially proposed (Anderson *et al.*, 1994; Oates *et al.*, 1997).

Using ultra centrifugation, immunological, immunoprecipitation and western blot techniques, Conrad and co-workers (1994, 1996) have speculated that an alternative Tf-

independent pathway of integrins-mobilferrin may be involved in iron transport across the mucosal epithelium. Consequently it has been suggested that the intergrins on the cell surface facilitate transfer of iron through the cell membrane prior to iron binding to mobilferrin within the cell. Mobilferrin may then transport iron into the villous epithelia cells by endocytosis (Conrad *et al.*, 1993; Egan *et al.,* 1993). The hypothesis by Conrad *et al.*, (1994, 1996) is, however, not widely accepted (Jeffries *et al.*, 1996). In view of the inconclusive evidence in mechanism of intestinal iron uptake, the hypothesis by Fleming's group (1997) and Gunshin *et al.*. (1997) that another non-receptor mediated uptake system existed was convincing.

Flemming and colleagues (1997) in their search to identify the gene responsible for microcytic anaemia (*mk*) in mice, as a result of severe defects in intestinal iron absorption, have identified a single protein (*Nramp2*) that plays a central role in mammalian iron uptake. They have shown that the *mk* gene was positioned on chromosome 15 and homologuos to Nramp1, an earlier protein they had identified. In the analysis of *Nramp2* cDNA clones by reverse transcription-polymerase chain reaction (RT-PCR) amplification of RNA from Mk and wild type alleles, they detected a single missense mutation. This missense differed only in substituting Arginine for Glycine in the predicted transmembrane domain of the original *mk* alleles.

Gunshin and coworkers (1997) further confimed that the *Nramp2* was indeed an iron transporter. By employing *Xenopus* oocytes expression and distribution assay to screen for iron uptake mediated by duodenal mRNA from rats fed low iron diets, they characterised a single cDNA divalent-cation transporter called DCT1. The DCT1 encoding the rat isoform of Nramp2, was found to stimulate iron uptake by 200 folds in the duodenum. A voltage clamped analysis of DCT1 mediated iron transport indicated a pH mediated electrogenic process similar to that of other transmembrane transporters. Gunshin *et al.* (1997) also detected intense DCT1 mRNA hybridisation signals in the enterocytes lining the basal villus, crypts but not at the villus tips.

Detailed expression studies by both groups have been consistent with the fundamental role of Nramp2 in iron uptake (Fleming *et al.*, 1997; Gunshin *et al.*, 1997). A proximal to distal localisation of Nramps 2 expression were observed in the small intestine and these results were consistent with studies of the intestinal sites of iron uptake (Chowrimootoo *et al.*, 1992; Vulpe and Gitschier, 1997; Fleming *et al.*, 1997; Gunshin *et al.*, 1997). An iron response element (IRP) consensus sequence within the putative stem-loop in the 3' untranslated region detected in DCT1 cDNA, suggests regulation by an iron binding proteins (IRP) of transferrin receptor and ferritin mRNAs (Thiel, 1993; Gunshin *et al.*, 1997).

These two elegant and comprehensive studies by Fleming *et al.* (1997) and Gunshin *et al.* (1997) have provided an alternative to mobilferrin, integrin and the transferrin-transferin receptor as iron mediated transporters. Further application of genetic and molecular approaches to identifying possible iron transporters in the intestine will advance our understanding of the intricate mechanism of intestinal iron absorption and regulation both in health and disease.

1.5.1 Regulation of intestinal iron uptake

The cellular mechanism and control of duodenal iron absorption has not been fully elucidated despite the fact that enterocyte handling of iron is crucial in maintaining body iron status. Body iron status provides a stimulus for iron absorption such that there is an increased intestinal iron uptake when the body iron stores are low and a decrease when the body iron is replete. Humans have a limited capacity to excrete iron, so the amount of iron in the body is controlled at the point of absorption at duodenal mucosa. Studies have implicated the intestinal mucosal cell in the regulation of iron absorption by a transferrin-transferrin receptor pathway in the crypt cells, or passive sequestering of iron by ferritin that is not transferred to the plasma (Anderson *et al.*, 1990; Kivivouri *et al.*, 1993). It has been suggested that when the level of body iron is low, the iron concentration in the intestinal crypt cells modulate the expression of genes encoding

portions for transferrin receptors (TfR) (Pietrangelo *et al.*, 1992; Anderson *et al.*, 1994) . These TfRs are able to bind, internalise and recycle transferrin to the cell surface for vectorial iron absorption across the matured enterocytes (Anderson, 1996).

The gene expression of both ferritin and transferrin-transferrin is highly regulated by the amount of intracellular iron (Kuhn 1991; Pietrangelo et al., 1992; Baynes *et al.*, 1994). There is evidence to suggest that the regulation of ferritin translation in response to changes in iron availability occurs at the level of translation initiation (Klausner *et al.*, 1993; Kuhn *et al.*, 1994; Hentze and Kuhn 1996). It has been demonstrated that while the IREs in ferritin and transferrin receptors are identical, IRE localised in 5' untranslated regions of ferritin H and L mRNA controls the translation of iron storage while their localisation in the 3' UTR of transferrin receptors mRNA participate in the regulation of TfR mRNA stability (Heintze *et al.*, 1991; Kuhn *et al.*, 1994). DCT1 has also been shown to be upregulated in response to iron deficiency in most cells. DCT1 also contains in its 3' and 5' untranslated region, a putative iron response element which forms a stem loop similar to those found in the 3' and 5' IRE found in TfR and ferritin (Gushin *et al.*, 1997). By analogy with TfR mRNA, the iron response element may regulate DCT mRNA levels by RNA degradation mediated by a protein that binds that element (Gunshin *et al.*, 1997). This phenomenon of analogy will need to be investigated by immunocytochemistry. Although these hypotheses could explain the down regulation of transferrin in the presence of ferritin at the translational levels, the regulation of intestinal TfR mRNA and ferrtin mRNA in the presence of other divalent cations such as calcium is not known.

1.5.2 External factors

Some of the factors operating outside the alimentary canal to increase iron absorption includes low iron stores, erythropoiesis and hypoxia (Oates and Morgan, 1996; O'Riodan*et al.*, 1997;). Each of these factors have been shown to exert an

independent effect on the mucosal iron absorption, but the mechanism for their collective effect has not been fully elucidated (Kivivouri *et al.*, 1993; Baynes *et al.*, 1994).

Low iron status and rate of erythropoiesis

As discussed previously, the amount of iron in the body stores is one of the factors controlling the absorption of iron from the gastrointestinal tract. Even a small change in iron stores as reflected by serum ferritin values is accompanied by a reciprocal alteration in iron absorption (Cook *et al.*, 1974, 1990; Bezwoda *et al.*, 1978). In the event of a positive iron balance apoferritin is synthesised to soak up the excess iron for storage in the mucosal cell (Oates and Morgan, 1996). The rate of erythrpoiesis is also believed to influence iron absorption. Skikne and Cook (1992) assessed the effect of enhanced erythropoiesis, recombinant human erythropoietin (rHEPO), on food iron absorption of radio-labelled (^{55}Fe and ^{59}Fe- enriched ferrous sulphate) in 13 women subjects (between the ages of 22-43 years) with normal haemoglobin and serum ferritn > 12µg/L. They showed that the administration of rHEPO resulted in a significant increase (mean absorption ratio 8.8, $p < 0.001$) in both haem and non-haem iron absorption when differences in iron status were corrected for. They supported the hypothesis that the iron absorption was influenced by the rate of tissue iron uptake and the size of the labile iron pool in various body tissues. They showed that the administration of rHEPO induced labile iron compartments and increased tissue iron uptake as indicated by an increased reticulocyte counts, haematocrit and serum transferrin receptors (Skikne and Cook, 1992).

Hypoxia

Hypoxia has been shown to promote duodenal iron uptake in both rats and human (O'Riordan *et al.*, 1997). It has been shown that at normal mucosal concentration of sodium and potassium, duodenal hypoxia increases duodenal accumulation of iron by altering the potential difference across the apical brush border membrane (BBM) (O'Riordan *et al.*, 1997). By using autoradiograph to detect the distribution of iron in the

villus brush border membrane in hypoxia and control rats. They also suggested that the mechanism by which hypoxia influences intestinal iron uptake involved an enhanced BBM electrical gradient resulting from Na+ permeability. The alteration in the expression of 59Fe complexed with nitrilotriacetate (NTA) in response to hypoxia may suggest an upregulation of increased membrane iron uptake. The study by O'Riordan *et al.* (1997) has helped in evaluate the effect of hypoxia on iron uptake, however more studies at the molecular level are needed to further clarify the effects on hypoxia on the expression of iron binding proteins eg DCT1 in intestinal iron transport.

1.6 Iron storage and transport

1.6.1 Transferrin and transferrin receptors

The major iron transport protein transferrin, is a b-1-globulin. It is a single polypeptide with two binding sites for ferric iron. In an individual with normal iron stores, approximately one-third of plasma transferrin is saturated with ferric iron at any one time. (Huebers & Finch 1984; Wollenberg and Rummel 1987). In iron deficiency the degree of saturation is reduced and hence transferrin saturation is a useful indicator of iron status (Skikne and Cook, 1992). Iron is transported between the site of absorption and the sites of, storage and utilisation by transferrin. The delivery of iron into cells require the transferrin to transferrin receptors (TfRs) on the cell surface . The resulting complex of transferrin and receptor is then internalised by endocytosis (Aisen 1994).

In the event of a negative iron balance, *eg* nutritional anaemia, there is an increase in the synthesis of Tf-TfR in all tissues which increases the capacity of cells to acquire more transferrin bound iron (Hubert *et al.*, 1993; 1992; Kivivuori *et al.*, 1993). Although the exact mechanism of iron passage from the endosome is not fully understood, it is suggested that the translocation of iron from Tf is facilitated by acidic pH of 5.6 of the transferrin-receptor binding sites in the membrane. These attributes modulate the kinetics of iron release from transferrin (Aisen 1994; Egan *et al.*, 1993). For example, it has been demonstrated *in vivo* that for the release of iron from Tf-TfR

complex in the endosome to occur, the kinetic sites must be occupied by a simple non-chelating anion such as chloride or perchlorate on the transferrin molecule at pH 7 (Cook *et al.*, 1990; Aisen 1994). This process is enhanced as the ionic strength (anion concentration) increases (Egan 1993) suggesting that the release of iron from transferrin is pH dependent.

Transferrin receptors have also been suggested to be involved in iron release, but in a more complex way (Bali *et al.*, 1991). Bali *et al.*, (1991) showed that at a pH of 7.4 (such as that found on the cell surface where the transferrin receptor first comes in contact with iron-laden transferrin), the receptor delays the release of iron from both sites of transferrin. But in the endosomes at a pH of 5.6 the engagement of Tf by the receptor accelerates iron release from transferrin. This has been proposed by Egan *et al.*, (1993) to explain the speed with which transferrin relinquishes iron to iron-dependent cells. The apo-transferrin (transferrin devoid of iron) that remains in the endocytic vesicle has a high affinity for its receptor but on return to physiological pH (pH 7.4) at the cell surface, the strength of the binding decreases and apo-transferrin is released back into circulation to bind more iron (Baynes *et al.*, 1994; Oates and Morgan 1997).

1.6.2 Iron storage (ferritin)

Ferritin is a ubiquitous iron storage protein found in all cells (Munro and Linder, 1978; Ford *et al.*, 1984). It consists of a protein shell penetrated by channels through which ferrous iron (Fe^{2+}) enters the 3-fold inter-subunit channels of apoferritin (a 24-subunit protein). Ferrous sulphate is catalytically oxidised by ferroxidase centres in the apoferritin shells to the ferric form prior to storage. Apoferritin sequests iron (Fe^{3+}) as an organic complex of ferrihydrite and protein. First ,small clusters of ferrihydrite are formed and these continue to grow by accretion of further iron to give crystalline ferrihydrite particles containing up to a maximum of 4500 Fe^{3+} atoms (Baumingeret *al.*, 1991 (ab)). Ferrihydrites nucleation into the cavity forms a Light (L) or Heavy (H) chain ferritin (Wade *et al.*, 1991; Baumingeret *al.*, 1991 (ab)). L-rich ferritin chains are

physically stable and are associated with longer term storage. Arosio *et al.*, (1994) showed that L-ferritin has an in-built functional specificity for efficient iron mineralisation and plays little or no role in the rate of iron oxidation. The accretion of iron in the small intestine in the form of L-ferritin is consistent with this. H-rich ferritin molecules (eg those in the heart and brain) contain Fe^{3+} atoms which are relatively mobile (ferroxidase activity), and hence these ferritins play a role in rapid iron sequestration and detoxification (Xu and Chasteen 1991; Benkovic and Connor, 1993). H-rich ferritin binding has also been shown to be increased in actively proliferating cells and decreased in quiescent cells, thus supporting the putative regulatory role of H-ferrtin to be mediated by specific binding to H-ferrtin receptor on the cell surface (Moss *et al.*, 1993).

These differences in the mechanism of iron incorporation between H- and L-rich heteropolymers have been suggested to explain their functional differences: H- rich ferritins chain promote iron oxidation via the ferroxidase centres while L-chain rich ferritins promote iron hydrolysis mineralisation inside the protein core. Whether H-chain rich ferritin present in the intestinal plays a role in mucosa is yet to be fully elucidated.

1.6.3 The use of transferrin, transferrin receptors and ferritin in clinical diagnosis

The use of serum transferrin and transferrin receptors as indices of tissue iron deficiency and serum ferritin as a measure of iron stores is being encouraged because by using these parameters it is possible to evaluate the entire spectrum of body iron status (Skikne and Cook, 1992; Sikstrom *et al.*, 1993; Hallberg *et al.*, 1993a, b). The "gold standard" for the diagnosis of iron deficiency is confirmation of absence of iron stores in a bone marrow aspirate. Bone marrow aspirate, however, is invasive and time consuming. Serum ferritin is the best single blood test for the diagnosis of iron deficiency, but its interpretation especially in infants and elderly patients, is complicated by a tendency for it to rise with infection, chronic disease and aging (Holyoake *et al.*, 1993; Hallberg *et al.*, 1993a).

Clinical and biochemical studies have demonstrated that serum transferrin and transferrin-receptor levels correlate with the number of red cell precursors in the bone marrow in various haematological disorders and with deficits in functional iron in patients with iron deficiency (Huebers *et al.*, 1983; Cook *et al.*, 1992; Singhal *et al.*, 1993). Serum TfR level was markedly elevated in 8 year old sickle cell anaemic children compared with normal haemoglobin control group. Even within the sickle cells anaemic children, the heterogenous trait (SC) had a better sTfR levels compared to their SS counterparts (23.4 ± 9.7 mg/L vs 38.3 ± 12.7) (Singhal *et al.*, 1993). A raised serum Tf or a low TfR is the earliest biochemical indicator of depletion of the functional iron pool (Skikne *et al.*, 1990; 1992; Thorstensen and Romslo, 1993; Baynes et al., 1994), and the most reliable indicator of nutritional iron deficiency in children where other measures such as haemoglobin concentration, serum iron and serum ferritin are often not very helpful. Serum iron and ferritin concentrations tend to be influenced (elevated) by viral and bacterial infections such as the common cold and urinary tract infections (UTI) respectively while haemoglobin counts are reduced by diseases such as hookworm infection and malaria.

1.7 Assessing iron status

Multiple biochemical and haematological tests provide a framework for characterising the spectrum of iron nutritional status. Red blood cells indices such as haemoglobin (Hb), haematocrit, free mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), red cell count (RCC) and mean cell volume (MCV) are measures of severe iron deficiency. Serum iron concentration, transferrin and transferrin saturation measurements as described in section 1.2.6, historically have been the preferred technique for determining depletion of iron and are sensitive to the body supply of mobilizable iron. The relative patterns of change of Hb and serum iron reflect two functionally different body iron pools (the erythropoietic pool and total body iron stores). As mentioned earlier, serum ferritin is directly related to the level of iron store

and is currently the preferred index of iron status. It is sensitive to tissue iron levels across a substantially greater range than any of the other indices, and it is a useful index of iron nutrition before impairment of haematopoiesis (Worwood 1982). Serum transferrin and sTfR also reflect tissue ion mobilisation.

Each of these parameters reflects different aspects of iron metabolism, and a number of conditions other than iron deficiency can alter the test values. This potential limitation of altered accuracy due to factors other than iron deficiency, one strategy to improve accuracy of diagnosing iron deficiency is to employ them all and regard the individual with multiple test results in the abnormal range as having iron deficiency, in addition to a complete assessment of hematologic as well as iron nutritional status.

One consistent aspect of two epidemiological studies carried out by Lozoff *et al.* (1987) in Costa Rica and that of Walter *et al.* (1989) in Chile, is the effect of iron nutrition on cognitive function and behaviour. In these concurrent studies, the authors used multiple criteria based on red cell indices and body iron stores to determine the effects of nutritional iron deficiency on mental and psychomotor development of infants. Subjects were divided into iron sufficient, intermediate iron deficient anaemia and iron deficient anaemia groups as follows;

1. Iron sufficient

Haemoglobin > 110 g/L
Mean corpuscular volume (MCV) > 70 fl
Transferrin saturation (Fe/TIBC) >10%
and serum ferritin > 10 ng/mL.

2. Iron deficiency anaemia

Haemoglobin < 110 g/L and one or more abnormal biochemical measures (see 1)

3. Non anaemic iron deficiency (NAID)

Serum ferritin < 10 ng/mL and haemoglobin levels of >110 g/L in addition to one or more abnormalities in the haemoglobin such as Fe/TIBC.

Walter's (1989) iron status assessment at 9 and 12 months enabled a preliminary classification based on haemoglobin (Hb) concentration, mean corpuscular volume (MCV) and serum ferritin. The NAID infants were further reclassified into grades of severity depending on response to therapeutic trials and measures of iron status. Seven to ten days after the initial iron status assessment, the first Bayley scale infant development (BVID) mental and psychomotor development test was conducted. When a complete haematologic assessment of 189 infants at 9 months and 196 infants at 12 and 15 months was done for a final classification of the infants, the mean values for biochemical measurements showed that because such a stringent criteria for classification was used, only 30 controls and 39 anaemic subjects were identified. The study of Lozoff *et al.* (1987), which was very similar to that of Walter *et al.* (1989), further demonstrated that NAID infants will become anaemic unless supplemented with iron.

These studies also demonstrated an association between iron deficient anaemia and impaired psychomotor development. Infants with NAID had poorer mental and psychomotor development than non-anaemic controls. If the refined classification deficiency had not been employed, 70% of the NAID infants in Walter's studies would passed for being iron-sufficient. Oski *et al.* (1983) also showed that iron deficiency in non-anaemic infants had a significant response to treatment with a 22 point increase in Mental Developmental Index (MDI). The response to iron therapy suggests that infants with even mild iron deficiency will benefit from supplementation and therefore many more children may need to be identified than those who are anaemic. It will be therefore important for health workers to classify iron status using a spectrum of biochemical

parameters rather than just haemoglobin. The classification mentioned would be appropriate for programmes aiming to identify children who would benefit from iron supplementation including iron fortified foods.

1.7.1. Prevalence of iron deficiency

Despite an increased knowledge of iron nutrition, iron deficiency remains one of the most prevalent nutritional problems, particularly in infants and young children, in both privileged and underprivileged societies (Dallman 1990). In the Carribean, 70% of pre-school children have iron deficiency (Simmons & Gurney 1980). In India nearly 50% of children are anaemic while in Chile, 28% of infants under two years of age are anaemic and 65% have biochemical evidence of iron deficiency (Rios *et al.* 1983). Iron deficiency is also a problem in more developed countries. In 1992 Kristy and co-workers in their study of five disadvantaged communities in Montreal found anaemia, mainly due to iron deficiency, in 25% of infants between 10-14 months of age. There is little data available in Australia at the commencement of this research in 1994, although dietary surveys have shown that 65% of infants aged 6-12 months fail to consume even 50% of the recommended dietary intake of iron, putting them at risk of iron depletion (Gibson and Kneebone, personal communication).

The high prevalence of iron deficiency in infancy can be largely attributed to the low intake and low bioavailability of iron in the diet at a time when there is a high demand for exogenous iron. In the developing countries where most of the populations subsist largely on cereal-based and vegetable diets with very little fish or meat, infants are most vulnerable to iron deficiency. In the industrialised world the introduction of cows milk before the age of one has been shown to be a major contributing factor to iron deficiency. Consequently, in South Australia, dietary guidelines consistently recommend that infants receive breast milk or iron fortified formula until twelve months of age and that iron fortified infant cereals are introduced at 4-6 months. Retallack *et al.* (1994) conducted a dietary survey of 258 infants in Adelaide to document the types of foods being consumed

and how these compare with current guidelines. Consumption of cow's milk after 6 months was common, with 10% of 5-8 month old infants, 44% of 8-10 month old infants and 61% of 10-12 month old infants receiving cows milk as their sole source of milk. After 6 months, there was a sharp decline in the use of iron fortified infant cereal and an increase in the consumption of adult cereals. These trends, together with the early introduction of vegetables that contain polyphenols, known inhibitors of iron absorption, and the very late introduction of meat to infant diets, suggest that the diet of Adelaide infants > 6 months may have both a low iron content and poor availability.

Although iron fortification of infant cereals is practised widely in the developed world as a means of preventing iron deficiency (section 1.3), the absorption of iron from weaning cereals is not clear and fortification policies are largely *ad hoc* and not completely based on experimental evidence of availability and efficacy (Wharton 1989). In addition, pertinent studies of cereals and vegetables have also been done primarily in rats and adults human. Studies in infants have concentrated on formula with little information available on the effectiveness of iron fortified cereal in preventing iron deficiency (Hurrell 1984; Rees *et al.* 1985; Lynch *et al.* 1990). There is the need to evaluate the effectiveness of food iron supplementation if iron the prevalence of iron deficiency has to reduced in young children because of the adverse effects of iron deficiency.

1.8 Functional and biochemical effects of iron deficiency anaemia

When iron requirements exceed iron absorption a negative iron balance results and the iron stores are used to supply the needs of functional compartments. Haemoglobin synthesis is compromised only after all storage iron has been utilised. Similarly, the levels of functional tissue iron compounds are also affected once iron stores have been exhausted. The effects of a negative iron balance on body iron stores and haemoglobin production have been studied extensively, with less attention on functional effects on other organs.

Iron deficiency has been associated with lethargy, irritability, loss of concentration, gastrointestinal absorption and cardiac failure. Recently, a further justification for the prevention of iron deficiency emerged from increased evidence that psychomotor deficits accompany even mild iron deficiency anaemia at about 12 months of age (Lozoff *et al.*, 1987; Walter *et al.* 1989). Since infants rarely develop iron deficiency before six to eight months of age, it appears that only a brief period of relatively mild deficiency between 6 to 12 months of age may result in long-term impairment of behaviour and school performance. The age and duration at which iron deficiency occurs appear to be vital with regard to the effects on brain metabolism and physical growth (Dobbing 1990; Walter 1993; Mokni *et al.*, 1993).

1.8.1 Effect of iron deficiency on the cardiac muscle

Blayney (1976) showed that iron deficiency produced a significant reduction in the activity of several respiratory enzymes in the mitochondrial fractions isolated from rat hearts: NADH cytochrome c reductase, succinic cytochrome c reductase, succinic dehydrogenase, and NADH ferricyanide oxidoreductase. The decrease in the activity of these enzymes appeared at the 7th week of the iron deficiency, and cytochrome c oxidase activity was decreased at the 14th week of the deficit, as were the concentrations of cytochrome a_3 and cytochrome b. Most of these enzymes contain non-haem iron and the depression in the activity of these enzymes could be related to a critical shortage of non-haem iron. The exception is cytochrome c oxidase activity, which is not dependent on non-haem iron and was decreased only at 14 weeks in association with a reduction in cytochrome a_3 concentration (Blayney 1976). Many studies have demonstrated the absence of changes in the concentration of cytochrome c in cardiac muscle during iron deficiency whereas it is clearly decreased in skeletal muscle. This difference could be due to a biochemical specificity inherent to these two types of muscle.

1.8.2 Effect of iron deficiency on skeletal muscle

Several physiological studies have shown that moderate iron deficiency anaemia may affect effort tolerance with a reduction in maximum work capacity. Many alterations of the enzymatic activity of skeletal muscle, similar to that of cardiac muscle, in iron deficiency have been described. Age, severity and duration of iron deficiency are known to affect the activity of tissue enzymes (Edgerton *et al.* 1980). Finch *et al.* (1976) demonstrated a decrease in the levels of a-glycerophosphate oxidase in the mitochondria of skeletal muscle in iron-deficient rats. After iron administration, they observed a rapid increase in the activity of this enzyme in parallel with the recovery of physical ability, which occurred before the return to normal haemoglobin concentration. Myoglobin and cytochromes did not return to their normal concentrations until long after the return to a normal haemoglobin level.

In a later study, Finch *et al.* (1979) confirmed these results and observed in a group of iron-deficient rats reduced levels of a-glycerophosphate oxidase in the animals with the lowest physical capacity. Iron deficiency is associated with a decrease in the activity of the a-glycerophosphate oxidase, thus modifying glycolysis which results in an excess lactate formation: at high levels this leads to cessation of physical activity. The changes in the levels of *a*-glycerophosphate oxidase appear to be the essential factor explaining muscle disorders, i.e. the decrease in physical performance observed in iron deficiency. Other reports have described a decrease of about 20-50 % in myoglobin concentration in iron-deficient rats during the growth period (Wheby 1987). By contrast, no decrease was apparent when the iron deficiency occurred in adult rats. The age at onset of the iron deficiency appears to play a particularly important role with regard to the effects of the deficit (Koziol *et al.* 1978; Weinberg *et al.* 1980).

1.8.3 Effects of iron deficiency on the gastrointestinal tract

Functional gastrointestinal abnormalities associated with iron deficiency, including a reduction of acid secretion by the stomach and impairment in intestinal absorption, have been described in man and animals (Jacobs *et al.* 1966; Ghosh *et al.* 1972; de Vizia *et al.*, 1992). In 1965, Dallman & Schwartz showed a 50% decrease in the concentration of cytochrome c in the intestinal cells of iron-deficient rats. These results were confirmed by Hoensch *et al.* (1976) who also reported a decrease in both cytochrome oxidase and cytochrome P 450. Cytochrome P 450 was especially dependent on iron as its enzymatic activity was modified after only two days of iron deficiency. This phenomenon was reversible with 24-hour oral iron supplementation. Dhur *et al.*, (1989) also observed a decrease in cytochrome P450 concentration in the intestine microsomes of iron-deficient rats even in moderate iron deficiency. Using mitochondrial preparations of the intestinal mucosa of rats, the effects of different types of diets (iron-restricted, normal and iron-enriched diets) on the activities of succinic dehydrogenase and cytochrome oxidase were studied. The activity of these enzymes was significantly decreased in the groups given an iron restricted diet, while no difference was observed in the two other groups. This may explain why overt anaemia can result in diarrhoea and loss of blood in the stool.

1.8.4 Iron deficiency in relation to lipid metabolism and the brain

The effect of iron deficiency on lipid metabolism in tissues such as liver or plasma may be reflected in the brain, thereby altering the normal functions and development in the central nervous system (Vyas and Chandras 1984). In two separate animal studies, the importance of iron in lipid metabolism was demonstrated. The multi-enzyme complex which catalyses the desaturation of stearoyl-CoA to yield the monounsaturated oleoyl-CoA (^ 9-desaturation) contains cytochrome b_5 and a terminal desaturase enzyme which is a non-haem iron protein (Rao *et al.* 1983). Iron is also required for the production of polyunsaturated fatty acids which are necessary for the transport of long-chain fatty acids into the mitochondria for beta-oxidation. Carnitine is synthesised from trimethyl lysine which involves two hydrolyases that require ferrous iron (Holloway *et al.* 1975).

Bartholomew and Sherman (1985) in a rat experiment reported that the hepatic level of carnitine in iron-deficient rats was reduced compared to iron-supplemented controls.

Rao *et al.* (1983) in their study of the fatty acid composition of the liver in adult rats showed that since iron is required for fatty acid desaturase activity, the ratio of monoenoic to saturated acids (16:1/16:0 and 18:1/18:0) in tissue lipids is reduced by iron deficiency. Such effects of iron deficiency can also occur in pups. Analysis of the fatty acid composition of liver lipids shows that iron deficiency induces many changes in lipid metabolism occur in the liver during the initial growth period of pups. They concluded that this could be reflected in the circulating lipids and may influence the lipid composition and development of the brain (Rao *et al.* 1980). These studies show the importance of iron in various metabolic events related to lipids, such as oxidative degradation of fatty acids in the synthesis of mono- and polyunsaturated fatty acids, plasmalogens and prostaglandins (Rao *et al.* 1983).

Although the human infant is more protected from iron deficiency than the rat, extrapolations to man are useful in recognising that iron deficiency can affect the brain in ways other than by altered fat metabolism (Rao and Larkin 1984). Due to the obvious limitations in the use of human subjects, animal models for nutritionally-induced iron deficiency have played an important role in our understanding of the numerous behavioural manifestations associated with the deficiency, such as lethargy, irritability, apathy, fatigue, lack of concentration, hypoactivity and reduced IQ performance (Walter 1989; Lozoff *et al.* 1987).

There are at least a dozen studies in which the role of iron deficiency in behaviour alterations in infancy has been established. Since lack of iron ranges from iron depletion to iron deficiency anaemia, the degree of iron deficiency has become an important dimension in studies on human infants. In a study of 6 - 24 month old Costa Rican infants, Lozoff *et al.* (1987) found that the mean mental development test score of 28

infants in an iron-deficiency anaemia group (Hb < 105 g/L) was 87, compared to the mean score of 100 among the 40 non-anaemic infants (Hb > 120 g/L). A 9-point pretreatment difference in motor scores was also observed. Mental test score deficits were especially marked in older anaemic infants (19-24 months old) (Lozoff *et al.* 1987). The developmental assessments at 12 months indicated that 39 infants with iron deficiency anaemia (Hb 84-104g/L) had significantly lower mental and psychomotor scores than the 127 iron-deficient non-anaemic infants. The infants with mild anaemia showed no graded improvement at higher Hb level.

1.8.5 Iron deficiency and behaviour

The effects of ID on behaviour are mediated by metabolic processes which are dependent on the presence of iron, as demonstrated by Siimes *et al* (1980) using an animal model. They offered graded amounts of iron to iron-deficient rats and demonstrated that tissue iron proteins were not affected until the saturation of transferrin increased significantly. The haemoglobin concentration as well as tissue cytochrome and myoglobin decreased steadily, showing the limited availability of iron to both the erythroid marrow and other tissues. When iron availability becomes a limiting factor for Hb synthesis in humans, a stage known as iron-deficient erythropoiesis develops and Hb concentration begins to decrease, presumably with other tissue iron proteins. Walter, using the findings of Siimes, concluded that, since overt anaemic conditions can be attained with prolonged Hb values under 110g/L for the infants in his study, as in the case of long-term non-anaemic iron deficient subjects, these metabolic processes may explain the decrease in tissue iron. This is reflected in the absence of deleterious cognitive effects in the NAID population (Walter 1989).

There is enough evidence to suggest that a degree of iron deficiency sufficient to cause anaemia is associated with lower developmental scores. Deficits in cognitive function may have their origin in alterations of non-cognitive behaviour patterns. Anaemic infants are said to be irritable and to demonstrate lack of interest in their

surroundings (Oski 1979). Walter *et al.* (1983) found that iron deficient anaemic infants were unhappier than control infants but all other measures of behaviour were within the normal range. Deinard *et al.* (1981) found infants with low ferritin to be more fearful, less visually and auditorily attentive, more vocal and less likely to mouth toys.

The question of whether iron therapy can completely correct any abnormality, regardless of how soon changes might be detected, was examined by Lozoff *et al.* (1982 a,b). Three months of oral iron therapy was carried out on the anaemic children, and some of those who responded to treatment and became non-anaemic still showed chemical evidence of ID. Even those who became iron sufficient failed to improve their mental or motor test scores after three months. These results indicate that iron deficiency anaemia even when mild can have long term deleterious consequences on mental and cognitive development and the data emphasise the importance of iron in the metabolism of the central nervous system, neurotransmitters that influence emotional responses, and alertness and arousal status (Lozoff *et al.,* 1982a, b). These studies emphasise the need to maintain an adequate iron status in infancy, at the time when the brain is most vulnerable. This period of vulnerability also coincides with the optimal period of gut growth and development.

1.9 Gastrointestinal development and adaptation

Weaning is a vulnerable period in childhood which coincides with a period of rapid growth, increased erythropoiesis and gut development and maturation (Henning, 1987; Dallman, 1990; Aurrichio 1991; Edward, 1993). This is also a period when the infant's food becomes varied with the introduction of weaning foods. The onset of weaning is a time when breast milk volumes becomes inadequate (700 - 970 mL) to meet the energy requirements of a normal infant at 3 - 6 months and there is a parental concern to offer weaning foods other than the breast milk or milk formula (Jason *et al.,* 1984, Whitehead, 1985, WHO, 1985). Nutrients of public health concern and emphasis are carbohydrate, fat, protein, minerals and vitamins. The change in dietary components also

involves physiological maturation in terms of 1) renal function and the capacity to cope with increased concentrating and excretory requirements, 2) gastrointestinal function, to handle the digestion of the complex diet and 3) the development of the mucosal barrier against the penetration of harmful substances.

1.9.1 Renal system

At birth, the renal system performs all its normal functions but has a limited concentrating ability due to the infant's relatively low urinary urea excretion and high electrolyte retention as a consequence of the infant's high anabolic state. During the neonatal period, however, the functional capacity of the kidney increases rapidly, and by four months their ability to handle the increased solute load is adequate for the gradual introduction of weaning foods (Ziegler and Fomon, 1989). By 12 months the kidney size has doubled to tolerate the significant dietary variation. Theoretical calculations of renal concentrations in five months old children who weigh an average of 7 .0 kg indicate that breast fed infants ingesting 110 kcal /kg/per day of breast milk would produce 756 mL of urine with an osmolarity of 130 mmol, well below the infant's renal concentration of about 600 mmol/kg (Ziegler and Fomon, 1989). Therefore healthy exclusively breast fed infants at five months would have adequate (78%) renal capacity to cope with the introduction of weaning diets with higher solute loads.

1.9.2 Gastrointestinal development

Gastrointestinal development is achieved through increased gut length and matured microvillus membrane structure with a concomitant increase in gut surface area to cope with increased absorption of nutrients (Grand *et al.*, 1976; Akre 1989; Hendrick, 1992). It has been demonstrated that the surface area of the intestine increases by 100,000 fold between six weeks post conception and time of birth, and the length of the small intestine increases in proportion to the body length of the infant two and a half times (Grand 1976). Gastric motility also increases during the fetal stage and intestinal transit time is in the range of 4.5 - 7 hours at term (Grand *et al.*, 1976). Gastric capacity

also increases 10 times during the first postnatal two weeks. The increased gastrointestinal length, motility and gastric capacity are necessary physiological changes at the time of weaning.

1.9.3 Gastrointestinal enzymes

During the period of weaning enzyme activity and the surface area of the intestine must be adequate for the digestion of carbohydrate, fat and protein and absorption must be efficient. Several studies have shown that there are functional inverse changes in lactase and amylase activity in humans and animals (Sevenhuyen *et al.*, 1984; Auricchio 1991). In term infants lactase activity is two to four fold more than in 2 - 12 month olds with a dramatic postnatal decline in lactase activity usually occurring in the fifth year. This decline is, however not the same in all races (Brown *et al.*, 1979). There is ethnic variation in the timing of lactase activity decline with Asian children unable to digest lactose as early as two months of age. As the weaning diet contains more carbohydrate there is a three fold increase in pancreatic amylase activity from birth to three months and the amylase activity reaches adult levels by six months (Sevenhuyen *et al.*, 1984). The is also a significant increase in the bile acid pool for micellar formation in fat digestion during the first month of life with a corresponding improvement in fat absorption. Although pepsin secretion for protein digestion is present at birth, it reaches adult levels by two years. Brush border peptidase activity and amino acid transport are adequate at birth, however, the mechanism for protein absorption is not fully developed at this time.

1.9.4 Adaptation of mucosal barrier

Both breastfeeding and weaning require significant changes in the adaptation of the gastrointestinal tract to cope with the penetration of harmful substances (Yoshioka *et al.*, 1983). These include the development of a mucosal barrier against pathogens and foreign proteins. At birth the intestinal defense mechanism is passive or is not well developed. Breastfeeding has been shown to provide the stimulus necessary for the development of an active defense mechanism or passive protection. Immunoglobulin

A, the predominant Immunoglobulin in breast milk may transfer the mother's immunity to specific pathogens to the neonate through plasma cells to the intestine. Human milk also contains growth hormones, growth factors (epidermal growth factor, EGF, insulin-like growth factor, IGF and nerve growth factor, NGF) which may play a role in the maturation of the small intestine (Elian *et al.*, 1966). However, during weaning there is the introduction of an additional foreign pathogens and proteins. The type of food and animal protein exposure during the weaning period may either enhance or retard growth with a possible increase in the risk of hypersensitivity (Erdman *et al.*, 1989). For example, the exposure of infants to cow's milk protein before the age of twelve months has been shown to be associated with occult intestinal bleeding and diarrhoea which can lead to iron deficiency in young children (Ziegler *et al.*, 1990).

1.10. CONCLUSION

Nutrition is the major determinant of iron status in infancy. Dietary trends the world over suggest that iron deficiency will become an increasing problem in infants. Improving the bioavailability of iron in the weaning diets at a time when the infant is growing rapidly and is most vulnerable to iron deficiency should be addressed with a multi-disciplinary approach to include paediatrics, haematology, nutritionists and food technology.

Although attempts have been made to resolve this through food iron fortification and strategic national nutrition programmes, iron deficiency anaemia in 6-24 months old children is estimated as 3-7% and 10-20% in western industrialised and developing countries respectively. Iron deficiency without anaemia ranges from 30-50% in the same populations (Rios *et al.*, 1983; Dallman *et al* ., 1984; Wood *et al.*, 1993; Fuchs *et al.*, 1993). The high incidence of iron deficiency in this age group may probably be due to a low intake of iron and the antagonistic interaction between iron and other nutrients, such as calcium, in the developing proximal intestinal mucosa which is undergoing rapid physiological adjustments from "simple" breast-milk to more complex weaning diets.

Future studies to assess bioavailability of iron with regard to infant nutrition will need to investigate the following issues:

(a) the impact of changes currently taking place in the diet of many adults on foods offered to infants and toddlers

b) the effects of these diets on the iron status of these young children

(b) the increasing popularity of cow's milk and soya bean products before the age of one

(c) the relative effectiveness of iron-fortification of infant weaning foods by measuring the bioavailability of iron in the diet in relation to other nutrients

(d) the mechanism of interaction of other dietary nutrients, such as calcium, on intestinal iron absorption during the weaning period, a period of significant changes in intestinal development and adaptation.

1.11 Specific aims of this thesis

1 To conduct clinical studies to assess the iron status and dietary intakes of 6 - 24 month old children from broad socio-economic backgrounds in metropolitan cities in Australia and Ghana,

2 To develop an animal model of iron sufficiency and iron deficiency during the weaning period.

3 To use this animal model to investigate the mechanism of intestinal non-haem iron absorption in both membrane vesicles and intact tissue using radio-labelled iron

4 To investigate the effects of dietary calcium on intestinal iron absorption using haematological, biochemical indicators of iron status, morphometric, biochemical enzyme activity measurements and

5 To investigate the mechanism of the effects of dietary calcium on intestinal (i) ferritin and transferrin expression using immunocytochemistry and (ii) L- and H-ferritin and transferrin genes expression using *in situ* hybridisation techniques.

CHAPTER 2

DEVELOPMENT OF RESEARCH PLAN AND METHODOLOGIES

INTRODUCTION

Iron metabolism can be summarised as the maintenance of iron-related physiological functions through the balance of intake, transport, storage and loss of iron (Sarineen and Siimes, 1977). The maintenance of this balance is affected by a number of factors which may vary at different stages of life. Positive iron status can only be achieved when there is an adequate bioavailable dietary iron intake to balance the requirements of erythropoiesis, growth and development. Iron deficiency occurs when the utilisation of storage iron exceeds intestinal iron absorption (Bothwell, 1979).

Low iron status, if not diagnosed early, can develop into iron deficiency anaemia with sequelae which include adverse neurological and developmental consequences (Walter *et al.*, 1992; Beard, 1994). Despite the need for early diagnosis of iron deficiency, its detection in young children is difficult. Currently available biochemical tests, particularly for serum ferritin, can be affected by common childhood infections or inflammation leading to falsely elevated ferritin levels. These factors decrease the specificity and sensitivity of this test in diagnosing iron deficiency. (Lipschitz *et al.*, 1974; Yip, 1995). Accurate diagnosis of nutritionally adequate iron status can only be achieved through the use of several haematological and biochemical assays.

Different laboratory cut off ranges for serum ferritin and haemoglobin levels have also contributed to the variation of the reported prevalence of iron deficiency in young children (Yip 1990; Hallberg *et al.*, 1993, Karr *et al.*, 1996). In addition, there are also significant developmental variations in serum ferritin and haematological indices such as Hb and mean corpuscular volume which need to be taken into consideration when these tests are being used for characterising iron status (Saarinen and Siimes, 1977; Yip *et al.*, 1984; Baynes *et al.*, 1994; Yip 1995), but these precautions are often ignored. The use of improved and appropriate diagnostic tools to increase the specificity of diagnosing iron deficiency and strict cut off criteria to assess iron status in 6-24 month old children were an essential component of this research.

Changes in dietary habits the world over suggest that iron deficiency will become an increasing problem in infants and young children (Retallack *et al.*, 1994; Preziosi *et al.*, 1994; Olsen *et al.*, 1995). These changes also impact on weaning practices. For example, there is a trend in western countries towards reduced consumption of meat due to its fat content and price. Also the pressure of combining motherhood and working life of carers these days has affected the practice of preparing fresh home made weaning diets in industrialised countries. Food insecurity in the less privileged developing countries has also limited the choice of foods used during the weaning period (Berg, 1992; Hendricks *et al.*, 1992). Generally education in nutrition has increased mothers awareness in maintaining nutritionally balanced diets in industrialised countries, but they are confused when it comes to making choices about weaning foods for maximum iron content and availability. The low economic status of some parents has also influenced the choice of family diets and restricted them to diets of low iron bioavailability, thereby contributing to the limited variety of foods ingested during the weaning period. In this study, methods for assessing the dietary intake of children were developed to estimate nutrient intakes and to ascertain the effects of nutritional practices and socio-economic factors on the iron status of children from two geographical locations.

The interaction of other nutrients, particularly calcium, on iron absorption has received considerable attention (Cook *et al.*, 1991; Hallberg *et al.*, 1992a; Gleerup et al., 1995). Both calcium and iron are important nutrients required for optimal physical growth during infancy and childhood. However, the ingestion of these two nutrients at the same time has been shown to inhibit iron absorption by 30-60 % (Hallberg *et al.*, 1992; Gleerup *et al.*, 1995b). Improving the bioavailability of iron in weaning diets at a time when the infant is growing rapidly and is most vulnerable to iron deficiency and anaemia cannot be achieved without understanding how intestinal iron absorption is influenced by other dietary factors. A detailed study of the effect of the bioavailability of dietary iron, particularly non-haem iron, on infant iron nutrition was carried out in this

study using morphometric methods, uptake studies and biochemical, haematological and molecular techniques.

This chapter discusses the methodologies employed to investigate the issues raised. The methods used were carefully selected to increase specificity and sensitivity in the assessment of iron status and to determine the effects of dietary intakes on iron metabolism. Clinical studies were conducted to assess iron status in populations from two different geographical locations and animal models were used to investigate the effects of other dietary components on intestinal absorption of other nutrients during the weaning period. Brush-border membrane vesicles and intact duodenal tissues were also used to measure duodenal mucosal iron absorption under iron-deficient and sufficient conditions using radiolabelled iron (^{59}Fe). Changes in intestinal digestive enzymes were also measured to ascertain changes that may occur under iron deficiency conditions. Morphometric, histological, immuno-cytochemical and *in situ* hybridisation techniques also were used to further elucidate the mechanism of these interactions. The general principles underlining these methodologies will be outlined in this chapter with relevant details in subsequent chapters where a particular methodology is used.

2. 1 CLINICAL STUDIES

Two clinical epidemiological studies were designed to assess the nutritional iron status and intake of full term 6-24 month old children from heterogenous socio-economic backgrounds representing populations in metropolitan Australia and Ghana. Haematological, biochemical and anthropometric measurements and semi-quantitative dietary assessments were used in these clinical investigations.

2.1.1 Anthropometric measurements

Anthropometrics can be used as indicators of nutritional status in children (Haschke *et al.*, 1988; 1993; Heinig *et al.*, 1993a). Measurements of weight, length (height) and head circumference were assessed in both clinical studies as a means of evaluating growth trends and the ability of the children to thrive. These anthropometric measurements were either corrected to Z-scores or reported as quadratic multiple regression measurements which depict the sampled population quartiles and compared to the reference data developed by the country under investigation (Heinig*et al.*, 1993; WHO, 1985). In calculating Z-scores for weight, height and head circumference, individual measurements were compared to the mean for that age group. The deviations from the mean were then assessed relative to the deviation from that population 3rd to 97th percentile for that particular age. Cut off points of ± 3 standard deviations were used in the two clinical studies to reflect biological outcomes (Mokni *et al.*, 1993). These anthropometrics were supported by the biochemical parameters listed in this chapter.

2.1.2 Full blood examination

Haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell count (RCC) and red cell distribution width (RDW) are good haematological indicators of iron deficiency anaemia. A low Hb, MCV, MCH and high RDW are associated with iron deficiency anaemia. They can be used alone without additional biochemical assays in areas where

iron deficiency anaemia is believed to be endemic (Cook and Skikne 1989). A full blood count or examination was carried out to determine the degree and type of anaemia present using an auto-analyser.

Two millilitres of blood was collected by venipuncture, by a registered nurse phlebotomist to ensure limited discomfort to children and also to reduce haemolysis of blood samples during blood collection, using mL sterile and disposable syringes with 22G needles. The size of the needles and the time taken to draw the blood were critical in order to avoid haemolysis which can influence both blood count and biochemical assays. One mL of this blood was collected in an electrolytically coated EDTA paediatric tube for platelet stability and for full blood count, while the other mL was used for biochemical analyses. These blood samples were transported to the laboratory at a temperature between 4-8 ^{o}C in an electric cooler because temperatures outside this range were found to induce haemolysis. Full blood count was carried out using an auto-analyser within 3 hours of sample collection, but when the samples had to be transported over 200 km (as was the case in Ghana), this was done within 24 hours to avoid changes in red and white cell sizes. Risk factors such as contamination were guarded against by using one off disposable anti-septic swabs, needles and syringes.

For the determination of haemoglobin the auto-analyser systems used in the present studies measured haemoglobin colorimetrically as cyanomethaemoglobin (International Committee for Standardisation in Haematology, 1977; 1978; Dacie and Lewis, 1991). In these autoanalysers the erythrocyte membranes are disintegrated by lysing agents which release the haemoglobin (Technicon Corporation, New york USA; Serono Diagnostics, England. UK). The haemoglobin iron is then converted from the ferrous to ferric state to form methaemoglobin, which then combines with potassium cyanide to form stable cyanmethaemoglobin. A rapid reaction and complete transformation of haemoglobin to a cyanated product ferrihaem $(CN)_2$ is achieved with the autoanalysers (Dacie and Lewis 1991). The rapidity of the reaction is very important

because it increases the accuracy of measuring the absorbance of soluble cyanide forming micellized ferrihaem $(CN)_2$ at approximately 546 nm within 12 seconds. This eliminates turbidity of the sample which may result from emulsification of cellular debris and plasma lipids.

The proportion of blood occupied by erythrocytes (Haematocrit, Hct) is indirectly estimated by the correlated measurement of whole blood electrical conductivity on the auto-analyser. Counting of red cells and white cells is based on an optical method where the passage of the blood cells through a narrow channel interrupts a light or a beam and the electrical impedence is measured (Technicon T2M Manual, 1990; Serono Diagnostic, 1994). Internal standards used for the two machines showed them to be comparable.

These machines provided measurements of red cell volume and haemoglobin content and by counting large numbers of cells provided much greater accuracy than manual techniques. The auto analysers had the additional advantage of being able to use small blood samples (0.5-1.0 mL), which makes it useful for analysing paediatric samples.

2.1.2.1 Red cell indices

In the investigation of anaemia, analysis of red blood cell numbers and sizes in addition to the amount of and concentration of Hb in each erythrocyte, gives important diagnostic clues to the aetiology of the anaemia. For example, microcytic and hypochromic red cells are associated with impaired haemoglobin synthesis (inadequate iron supply) and can be distinguished from macrocytes associated with impaired nuclear maturation (due to Vit B_{12} or folate deficiency) (Yip, 1990; Worwood, 1995). Red cell counts can also reveal clues to other causes of anaemia such as hypoproliferative disorders, haemolytic processes or the presence of haemoglobinopathy (eg. sickle cell anaemia). A high red cell distribution width (RDW) is also associated with anaemia. The RDW is a coefficient of variation (CV) of the RBC distribution and is expressed as a

percentage (Massey, 1992) . This expression of the red cell distribution of the red cell population quantifies the degree of anisocytosis (heterogeneity) of the red cells size (Ansley and Orntein, 1971).

Red cell indices like HCT, MCH and MCHC were calculated automatically using the following unbuilt formulae

1. Haematocrit (Hct)

$$= \frac{\text{mean corpuscular volume (MVC) x Red blood cell count (RBC)}}{100}$$

2. Mean corpuscular haemoglobin (MCH)

$$= \frac{\text{haemoglobin (Hb) per 1000 mL blood}}{\text{red blood cell count (RBC)}}$$

3. Mean corpuscular haemoglobin (MCH)

$$= \frac{\text{haemoglobin (Hb) in grams/100 mL of blood}}{\text{RBC}}$$

4. Mean corpuscular haemoglobin concentration (MCHC)

$$= \frac{\text{haemoglobin (Hb) in grams/100 mL x 100}}{\text{Haematocrit (Hct).}}$$

2.1.2.2 White blood cells

White blood cell, lymphocyte, neutrophils and eosinophil counts were also estimated to exclude infants with intercurrent infections or inflammation. These values were analysed on the same analyser systems used for haemoglobin and utilise the principles of enzyme peroxidation and differential cellular staining (Ansley and Ornstein, 1971). According to these principles, morphometry of leucocytes can be characterised using cytochemical staining. A photo-detector monitors the light absorbance created by the presence of hydrogen peroxide which is linked to a photo-detector that calculates the cell types. Blood films were screened by qualified haematology technicians for any

abnormalities. In the case of abnormal white cell distributions all print out with "review differentials" were repeated by manual peripheral blood smear differentials. Manual blood films were also prepared to confirm mycrototic and hypochromic anaemia if samples were identified as anaemia or out of the normal range. Blood films were also prepared to screen for malaria plasmodium, sickle cell traits in Ghanaian children, and α– and ß- thalessaemia in both populations.

2.1.3 Biochemical measurements

Although the changes in blood cell parameters associated with iron deficiency anaemia such as a low mean cell volume (MCV), a decreased mean cell haemoglobin (MCH) and a high red cell distribution width (RDW) are good indications of reduced supply of iron to the erythroblasts in the bone marrow (Williams *et al.*, 1977), they do not necessarily indicate depletion or absence of body iron stores. Additional measurements using biochemical assays are therefore required to increase specificity in the diagnosis of iron deficiency. These measurements include, serum ferritin (SF), serum iron (SI), total iron binding capacity (TIBC) and transferrin (Tf).

2.1.3.1 *Serum ferritin (SF)*

Serum ferritin is specific for assessing tissue iron and has been shown to correlate with body iron stores although SF can be influenced by infection (Lipschitz *et al.*, 1974; and Siimes, 1978 (a); Cook *et al.*, 1990; Hallberg *et al.*, 1993; Worwood, 1995). The availability of sensitive methods for measuring serum ferritin has significantly advanced the ability to detect iron deficiency and overload (Addison *et al.*, 1972; Jacob *et al.*, 1972; Miles *et al.*, 1974; Cook *et al.*, 1980) These earlier studies used radio-labelled immunoassays for ferritin determination.

Serum ferritin was estimated in the present clinical studies using an enzyme-linked immunosorbent assay (ELIZA) which uses a two step sandwich technique calibrated against human liver ferritin (Boehringer-Mannheim Immunodiagnostics kit,

Cat no 1135317, 1990). The principle involved the attachment of the ferritin in the sample to latex-bound antibodies to form an antigen-antibody complex which is measured colorimetrically on a Cobas Bio analyser. In the first incubation step, ferritin (antigen) from the sample is bound to antibodies (sheep's antibody) coated onto the inside wall of the test tube. Since ferritin possesses several antigenic determinants, sandwich ferritin-antibody complexes are formed in a subsequent second incubation with POD-labelled ferritin antibodies. The quantity of sandwich-ferritin-POD complex formed is a measure of the ferritin content of the sample. The POD conjugate not bound by the antibody is removed in a bound/free separation step. Hydrogen peroxide and the chromogen ABTS (2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonate) are then added and the POD-activity bound to the tube wall is measured photometrically. The developing colour intensity is measured against a blank which consists of substrate-chromogen solution. Results of the amount of ferritin present are obtained from a calibration curve prepared using known standards provided in the kit.

The accuracy of the measurement was found to be affected by a red coloration (bilirubin) which is a consequence of haemolysis, and anticoagulants like EDTA. Serum separation from whole blood was carried out in a refrigerator at 4^{o}C to avoid haemolysis. Normal ferritin values are dependent on the distribution of SF from a healthy population available to the laboratory, therefore the Flinders Medical Diagnostic laboratory has determined its own normal values for serum ferritin as follows; females 15 - 250 µg/L and males 20 - 300 µg/L. No reference values for children had been established before this study was undertaken so values of 10-150 µg/L for children 2 - 12 years of age from the Adelaide Women's and Children's Hospital were used as a guide, and compared to reference ranges from Germany (wiedermann and Jonett-Mentzel, 1993).

2.1.3.2 Serum iron (SI)

The measurement of serum iron concentration alone provides little useful information about iron status due its considerable physiological fluctuation on an hourly

to daily basis. It is, however, a complimentary assay for evaluating ferritin levels since a false positive result for ferritin can result from inflammation or infection. The iron concentration is normally within a 10 - 30 μmol/L but changes in supply or demand by renal infection or iron deficiency can cause rapid changes in iron concentration (Bothwell *et al.*, 1979; Cemeroglu and Ozsoylu 1994; Worwood, 1995). Serum iron estimation was carried out using a colorimetric technique on a Cobas auto-analyser. In this method, iron bound to transferrin in blood is split off by guanidine hydrochloride and reduced to ferrous iron (Fe^{2+}) by hydroxylamine. A red chelate is produced with ferroxamine and the intensity of this chromogen which is proportional to the iron concentration is measured colorimetrically.

2.1.3.3 *Transferrin (Tf)*

As indicated (section 1.5), transferrin (Tf) is the major transporting iron binding protein in plasma. Transferrin transports iron from sites of absorption and red cell destruction (sequescent or haemolysed red cells) to developing red cells in the bone marrow. Transferrin levels can be raised due to iron deficiency or reduced due to iron overload, hepatic cirrhosis, chronic infection, malignancy and other pathological conditions associated with low serum iron other than iron deficiency. Transferrin assays on their own cannot be used as conclusive diagnosis of iron deficiency but they were employed as a complimentary assay to give further clues to the aetiology of iron deficiency. Transferrin iron is only 0.1% of the body iron and the transferrin pool turns over 10-30 times each day (Huebers & Finch 1984; Wollenbert and Rummel 1987; Sikstrom *et al.*, 1993). Transferrin concentrations were estimated using an immunological reaction between transferrin in the plasma and a specific anti-human transferrin antibody which is measured turbidometrically. Transferrin estimation was performed on a Behring Nephelometer (BNS) using a Boehringer Mannheim Tinaquant Transferrin kit with a rabbit anti-human transferrin antibody (of approx 3g/L) and two different control protein sera (Behring N/T control serum and Ciba abnormal) for quality control purposes. The accuracy of the assay was checked weekly and between and

within assay quality checks were performed using quality control standards. The Flinders Medical Centre Diagnostic laboratory accepted a coefficient of variation (CV) of not more than 2.8 % or standard deviation of (SD) of ± 2 for assays. Diluted antisera remained stable for 2 weeks at 2 to 8°C.

2.1.3.4 *Serum transferrin receptor (sTfR)*

To increase the probability of diagnosing iron deficiency, a new biochemical protocol that estimates transferrin receptors (TfR) was employed. Measurement of serum transferrin receptors (sTfR) provides a sensitive clinical index of an early impairment of iron delivery to the erythron in subjects with absent iron stores but who have not developed anaemia (Skikne *et al.*, 1992; 1995). Transferrin receptor estimations have also been shown to be less influenced by infection than ferritin levels (Hueber *et al.*, 1990; Cook *et al.*, 1990; 1992; Singhal *et al.*, 1993; Baynes *et al.*, 1994;). This new laboratory tool represents a significant advance in the assessment of nutritional iron deficiency and the evaluation of anaemic patients. A recent study in Zaire has shown that sTfR levels are more sensitive indicators of iron deficiency in children with malaria (Kuvibidila *et al.*, 1995). As serum TfR is a sensitive indicator of tissue iron deficiency and less influenced by infection, the combination of sTfR and SF provides a quantitative measure of iron status over a broad range of body iron levels. It is particularly useful in areas like Ghana where infections from hookworm and *Plasmodium falciparum* malaria and sickle cell anaemia are endemic. This was the basis upon which the additional assay of sTfR was employed in the laboratory diagnosis of iron status in Ghanaian children.

The serum TfR test is an immunoassay based on the double antibody sandwich method and is measured colorimetrically using an enzyme substrate chromogen on a plate reader. This is a new and expensive diagnostic test and is not readily available in either Ghana or Australia. Serum samples were therefore frozen at -20°C and sent to the Queensland Institute of Medical Research (the centre with the most experience in

carrying out this test) where the tests were carried out by Ms Kym Doyle in the laboratory of Dr Greg Anderson.

Methodology of sTfR assay

The sTfR test is an immunoassay based on a double antibody sandwich method which is measured colorimetrically using an enzyme substrate chromogen. There are several diagnostic kits available but Ramco sTfR test kit was used in this study. Fifty µl of subject's serum and two control samples, each diluted to 1:100 with phosphate buffered saline were pipetted into microwells pre-coated with poly colonal antibody to TfR. One hundred and fifty µl horseradish peroxide (HRP) conjugated murine monoclonal antibody specific for TfR was added to each of the wells and incubated for two hours at room temperature. During the incubation period the TfR binds to the polycolonal anti-TfR antibodies adsorbed to the wells and the second HRP-conjugated antibody binds to the captured TfR. The wells were washed with deionised water to remove any unbound TfR and excess HRP-conjugate and 200 µL composed of a combination of Enzyme substrate chromogen (tetramethylbenzide, TBM) and TBM in citric acid buffer containing hydrogen peroxide was added to each well and incubated for 30 minutes. A colour reaction produced through the action of HRP and TBM was stopped with 50 µl of acid stop solution (2.5M Sulphuric acid). The absorbance (Ab) of the wells (controls, standards and subject samples) were read on a microplate reader at a wavelength of 450 nm. The reader was zeroed using blanks prepared with 200 µL substrate solution and 50 µL of Acid stop solution. Results of samples assayed were calculated by plotting a standard curve of Ab versus concentration of pre-diluted TfR standards provided in the kit. Standard curves constructed by connecting the points with the best fit lines were used to calculate the concentration of transferrin receptor in the unknown samples (Ramco Laboratories Inc., 1996). The normal range determined by RAMCO TfR assay was 2.9 - 8.3 µg/mL with no sex or age differences. Variation in sTfR values in healthy subjects has been shown to reflect differences in the rate of red cell production (Cook *et al.*, 1990).

2.1.4 Diagnostic classification of iron status

There is a wide variation in cut off ranges for haematological and biochemical diagnosis of iron deficiency in different laboratories (Lovric 1970, Dallman 1984; Yip, 1990, Hallberg *et al.*, 1993). These cut off ranges have influenced the reported prevalence of iron deficiency and anaemia in children. For example, Lovric in 1970 used a cut off value of < 100g/L for Hb; thus diagnosing chronic anaemia but missing out on children with mild anaemia. Yip and coworkers (1990) on the other hand used the cut off value for Hb as < 110g/L and ferritin as 12 µg/L to diagnose anaemia. The cut off value used for anaemia in Ghana at the time of this study was Hb < 100g/L. Hallberg and coworkers (1992) have reported in a Swedish study that a cut off of 12 µg/L for ferritin falsely diagnoses iron deficiency as iron sufficiency.

In view of these discrepancies in reported cut off values used in the studies reviewed, the pre-determined haematological and biochemical cut off ranges used in this present study were based on international cut off values of Hb < 110g/L (Yip, 1990; ICHS, 1986) and a proposed serum ferritin level of < 15 µg/L (Hallberg, 1993a), in addition to standard values established by the Flinders Medical centre in Adelaide and Nyaho Diagnostic Laboratory in Ghana, to define anaemia in 6-24 months old infants and toddlers. By combining Hb and other red cell parameters such as MCV, MCH and RDW in screening for iron deficiency, the diagnostic accuracy of IDA was increased in the current studies. A cut-off value of sTfR < 8.5 µmol/L was also used in the Ghanaian study based on values established by Ramco Industries, USA.

Children were classified into three categories of iron status using the following criteria;

1). Iron sufficient (IS):

Hb concentration ≥ 110 g/L , MCV > 72 μm^3, MCH > 23.0 pg, RDW < 15 %, SF ≥ 15 µg/L, and/or Tf ≤ 3.0g/L, SI > 8.0 µmol/L, Sat >12%, TfR ≤ 8.5 µmol/L;

2). Non anaemic iron deficient (NAID):

Hb concentration ≥ 110g/L MCV > 72 μm^3, MCH > 23.0 pg, RDW < 15 %, and SF < 15 µg/L, and /or Tf > 3.0 g/L, Sat < 12% and SI < 8.0 µmol/L, TfR > 8.5 µmol/L,

3). Iron deficiency anaemia (IDA):

as for 2 with Hb concentration < 110g/L, and /or MCV < 70, RDW > 15 %.

2.1.5 Measurement of riboflavin status.

An association between microcytic anaemia and iron deficiency in children has been suggested because the relative effectiveness of iron and riboflavin in correcting iron deficiency is better than the administration of iron alone(Powers *et al.*, 1981; Fairweather-Tait *et al.*, 1992). A nutrition study in the Gambia by Powers *et al.* 1981 showed an indirect association between iron and riboflavin deficiency.

Riboflavin status was assessed in 120 children by measuring the activation coefficient for the red blood cell enzyme, glutathione reductase (ERGAC). Haemolysate was prepared by washing red cells with 1% saline solution and centrifuged at 4°C in a microcentrifuge (Mikro Zentrifuge, Germany). The plasma was removed and the blood cell frozen at -20°C until needed for analysis at the Department of Paediatrics, University of Sheffield laboratory in England.

The method for measuring red cell riboflavin relies on monitoring the oxidation of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) during the enzyme catalysed conversion of oxidised glutathione to the reduced form. The activity of the enzyme is measured in the presence of (stimulated activity) and absence of (basal activity) of added flavin adenine dinucleotide (FAD) which is a cofactor for glutathione reductase and the results expressed as a ratio of stimulated to basal activity. Values of 1.4 or greater are conventionally used as a threshold for deficiency when EGRAC is

measured by this method. The method has been automated for the Cobas Bio autoanalyser (Powers *et al.*, 1993).

2.1.6. Dietary assessment

Dietary iron intake is the major determinant of iron status as reviewed in chapter 1. Assessment of nutrients from individual foods and the total nutrients derived from these foods are important in estimating the prevalence of dietary intakes above or below fixed cut points, referred to as recommended daily allowance (RDA) in America, Ghana and the United Kingdom or recommended daily intake (RDI) in Australia. Through out the study however, RDA will be used for consistency. In studies of iron nutrition, dietary assessment is essential in evaluating the blood measurements, the effectiveness of food iron fortification, weaning practices and factors that may inhibit or enhance iron absorption (Fomon *et al.*, 1981; Penrod *et al.*, 1990; Mira *et a.l,* 1996). There are three dietary survey methodologies namely, Diet Recall, Diet History and Food Frequency Questionnaire. The choice of the dietary survey methodology used in this study was of fundamental importance. It was based on the anticipated use of the information to investigate the effectiveness of weaning diets in the prevention of iron deficiency and other issues raised earlier. A 24-hour diet recall was used in the present clinical studies for the following reasons:

i) it provides a more accurate estimate of the previous day's intake than a food frequency questionnaire,

ii) it can be used to estimate absolute nutrient intakes or acute single doses,

iii) it is capable of monitoring changes in intakes,

iv) it has inherent flexibility in handling diverse recipes, mixed dishes and food preparation methods, and

v) it offers the precision necessary for dietary surveys (Sempos *et al.*, 1992).

These recommendations were made based on findings from the National Health and Nutrition Examination Survey (NHANES III) and extensive review of the literature by Ernst (1990) and Sempos *et al.* (1992).

The 24-hour semi-quantitative diet recall questionnaire was designed specifically to assess iron and other nutrient intakes in young children. Specific questions included duration of breast and formula feeding, volume of formula or cow's milk (CM) intake per day and frequency of consumption of iron fortified baby or adult cereal. Details of the frequency of meat, chicken, pork or fish consumption were also requested in the dietary questionnaire (Zulkifli and Yu, 1992). This latter question was particularly important because these animal proteins contain the haem form of iron which has been shown to be 10-25% more bioavailable than non haem forms of iron in plant protein. The consumption of vegetables, fruits and fruit drinks was also recorded. Recipes of family foods offered to children, the method of cooking and the brand names of foods were asked also. The nutrient content of the foods consumed were calculated using values from the literature and the Australian Food Composition Nutrient Table (1990). The nutrient data was entered into a nutrient analysis package designed with the assistance of a registered dietitian at the Flinders Nutrition Unit because there was no analytical dietary programme available at the time. Dietary iron was classified as low, medium or high (as described in detail in chapter 3, Table 3.1). Due to the diverse nature of foods consumed by Ghanaian children, the dietary analysis table designed for the Australian children was not used. Dietary data from Ghana were analysed using the Ghanaian, Australian and American Food Composition tables.

A comprehensive nutrient analysis of the different foods consumed by each child in the Ghana study was carried out employing a nutrient analysis programme developed by the Centre for Scientific and Industrial Research Organisation (CSIRO) Human Nutrition Division in Adelaide, Australia with the assistance of Mrs Sally Record. In this method, precoded single core foods and codes for foods from mixed dishes were entered into the CSIRO dietary analysis programme. Nutrients in these foods were grouped together based on similar nutrient composition for selected key nutrients and the total of all nutrient intakes like energy, macro and micro nutrients was calculated for each child. Internal checks were done by a registered Dietitian.

2.1. 7. Ethics approval

Ethics approvals were obtained from the appropriate clinical investigations committees in Australia and Ghana after extensive scrutiny of clinical methodologies and written information for parents. Parental consent was also obtained before children were recruited into the various clinical studies. The information for parents and parental consent forms are shown in appendix 1

2.1.8. Exclusion criteria

Infants were excluded if they were born pre-term, were younger than 6 months or older than 24 months, had acute or chronic gastrointestinal, respiratory, or neurological disease, had any infection identified from clinical history or from a full blood examination, or if they had received iron supplements. Children identified to have thalassaemia or sickle cell anaemia or malaria were also excluded. Children who had been immunised two weeks prior to recruitment into the study were also excluded because anecdotal evidence from my experience with previous blood sample analyses indicated that immunisation could raise serum ferritin levels.

2.1.9. Statistical analysis

Statistical analyses were performed using the *Statistical Package for Social Science* (SPSS, 1989) for Windows and GENSTAT programmes. The frequency of Hb, weight, height and head circumference of children in age groups 6-12, >12-18, >18-24 months were plotted and a normal distribution confirmed. Logarithmic transformation for SF, Tf and TfR were plotted to assess whether it was necessary use log transformed values to ensure normal distribution before analysis. The data were normally distributed so there was no need to use log transformed data for the analysis of variance. Analysis of variance (ANOVA) was used to compare iron status between races, age groups, breast and formula fed infants, cow's milk, total dietary iron and other nutrients intakes. Correlations were carried out to determine associations between iron status,

anthropometrics, dietary iron and other nutrient intakes. Simple and multiple regression analyses were also done to predict confounders of body iron sufficiency or depletion.

2.2 ANIMAL STUDIES

Although blood collection in children is ethically acceptable, it is difficult to obtain samples of human gut tissues for clinical research unless these can be donated by patients during gut resections or when being assessed by biopsy for gastrointestinal disease. Secondly the gut biopsies are often from patients with underlying gastroenterological problems which make them inappropriate for studying iron absorption under normal healthy conditions. It was also difficult to obtain parental consent to obtain 50-100 samples in a year at the Adelaide Women's and Children's hospital. As a result of these problems, an animal model was chosen for intestinal iron uptake studies.

2.2.1 Species and strain

The rat is the most commonly used animal model in the study of gastrointestinal function. Several authors have shown that the Wistar rat is a good model for studying iron metabolism in man (Huebers *et al.*, 1975; Koziol *et al.*, 1978; Weinberg *et al.*, 1980; Conrad *et al.*, 1994; Oates and Morgan, 1997). In this study the Wistar rat was chosen as an appropriate animal model for the following reasons:

i) Results obtained with weanling Wistar rats aged 21 to 35 days can be extrapolated to 6-24 months old humans (Rao *et al.*, 1983; Larkin and Rao, 1990),

ii) they are inexpensive laboratory animals when bred by the user,

iii) the litters can be culled at birth to maintain desired weights during the weaning period, and

2.2.2. Animal ethics and care

Adult male and female rats for breeding were kindly donated by the Reproductive Biology Group at the Department of Animal Science, University of Adelaide. The experimental protocol for all the animal studies in chapters 6 and 7 was approved by the Animal Care and Ethics Committee of the University of Adelaide. All adult and suckling rats used in these studies were maintained at the Department of Animal Science,

University of Adelaide's Animal Biotechnology holding facility. Operative care was provided with occasional veterinary advice from Professor Brian Setchel and Mr Jim Zupp of the Department of Animal Science, University of Adelaide. All experimental protocols were designed with due care to minimise the number of animals used and followed the Australian Code of Practice for the care and use of animals for scientific purposes. To maintain constant post weaning weights of rats, litter sizes of more than 16 pups were culled to a maximum of 10 pups per dame on day two after birth.

2.2.3. Diets and Dietary Intervention

Four semi-purified rat diets with varying iron and calcium contents were prepared at the CSIRO Human Nutrition Division at Glenthorne in accordance with the recommendations of the American Institute of Nutrition (AIN- 89) (Bieri, 1980) as follows:

Semi-purified Rat diet

Composition		
Casein	20 %	
Sucrose	10 %	
Corn Starch	50.15%	
Sunflower seed oil	5 %	
Bakery fat	5 %	
Cellulose (Sulkaflock)	5 %	
Choline Chloride	0.2 %	
Methionine	0.15%	
Mineral mix	3.5%	(see appendix for detail composition)
Vitamin Mix	1.0%	(see appendix for detail composition)

The diet had a calculated digestible energy levels of 14.5 MJ/kg. The amount of iron (ferric chloride) and calcium (calcium carbonate) added to the vitamin mix were 420 mg and 12.5 g per kilogram of diet; (a calcium/iron ratio of 30). The exact formulation of the diet was intended for growth and maintenance during the first year of life. The iron and calcium deficient diets contained no added iron or calcium supplements but were found to contain residual iron and calcium nutrients when the diets were analysed

using an Inductively Coupled Plasma (ICP) spectrometer at the Plant Nutrition Analytical laboratory of the University of Adelaide. The iron deficient diets contained 4% residual iron (16.8mg Fe/kg) while the calcium deficient diets had 2% residual calcium (250 mg Ca/kg) relative to 420 mg Fe/kg diet and 12.5g Ca/kg diet respectively. The following is the actual composition of the varied AIN-89 diet:

- Iron and calcium sufficient (Control); IS + Ca (420 mg Fe and 12.5g Ca /kg diet)
- Iron sufficient calcium deficient; IS-Ca (420 mg Fe and 0.25 g Ca/kg diet)
- Iron deficient calcium sufficient; ID + Ca (16.8 mg Fe and 12.5 g Ca/kg diet)
- Iron and calcium deficient; ID-Ca (16.8 mg Fe and 0.25 g Ca/kg diet)

Complete mineral analysis was conducted (in triplicate) on the four experimental diets on the ICP and nitrogen content of the same diets were determined using the Kjeldahl method. The composition of the four diets is shown in appendix. All diets were prepared in 10 kg batches and stored at -20 ^{o}C to prolong shelf life. Diets were thawed overnight at 4 ^{o}C before being fed *ad libitum* to the rats. Weanling Wistar rats (21 days old) of mean weight 50 ± 5 g were marked by ear clipping and kept in plastic cages. Rats were assigned to one of the above four diets for 14 days and body weight gain and food intake was measured every other day over the 14 day period. The welfare of animals was observed daily.

2.2.4 Growth assessment

The effectiveness of the diet in inducing iron deficiency in 14 days was tested by running four experiments with two diets, iron sufficient (IS + Ca) as the control and iron deficient (ID + Ca). Each treatment had 15 animals. The growth profile and food intake were assessed as shown in Figure 2.1. Results of the preliminary study indicated that the varied AIN-89 diet formulation can be used to maintain adequate growth in weaning rats for 14 day. This dietary composition was used with various modification in subsequent animal trials.

Figure 2.1. Weight gain in 35 day old Wistar rats (a) and feed intake (b) of animals on Iron sufficient calcium deficient (IS + Ca; Control) (n = 15) and Iron deficient calcium sufficient (ID + Ca) diets for 14 days post weaning.

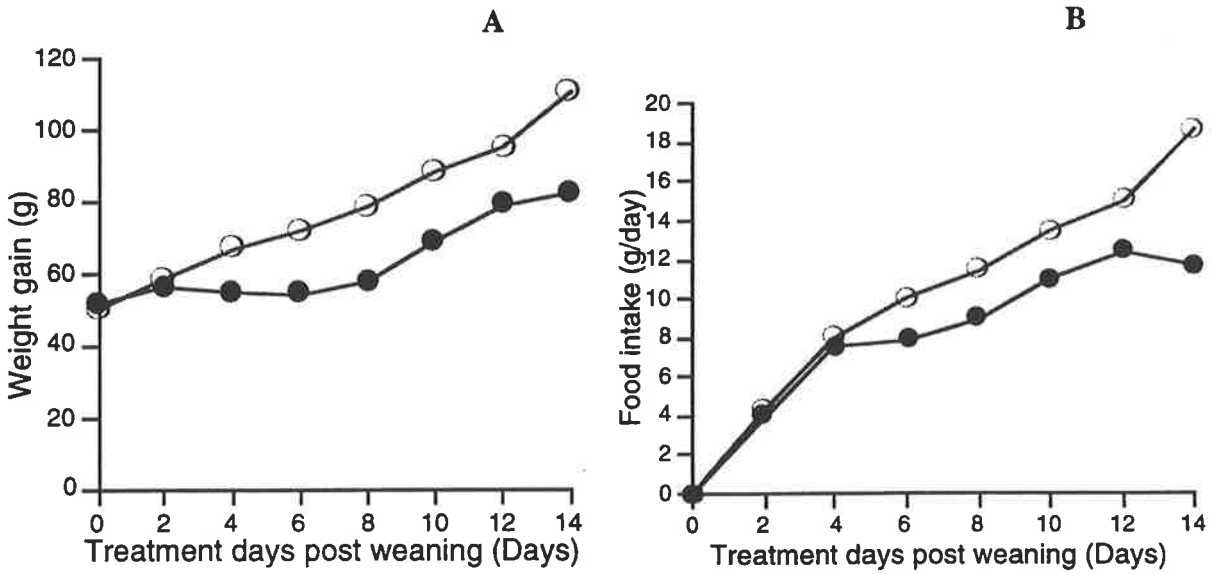


Figure 2.1 represents the mean weight of 15 animals (g) and food intake mean of weaning rats assigned to one of two diets Iron sufficient calcium deficient (IS + Ca) (solid circle) or Iron deficient calcium sufficient (ID + Ca) (Empty circle) for 14. * Standard deviations (± SD) are not shown for clarity but are within 5% of the means

days.

2.2.5 Assessment of iron status

Iron status has been shown to correlate with dietary iron intake and body weight in animals (Barton *et al.*, 1983; Oates and Morgan, 1996). For these reasons, animals were fasted overnight on day 13 for 16 hours prior to blood collection. On day 14 animals were weighed and anaesthetised intraperitoneally with 0.2 mL of 60 mg/mL pentobarbitone sodium per 150 g body weight. The abdomen and thorax were opened and a 2.5 mL blood sample was collected via the vena cava using a sterile 3 mL disposal syringes with 22 G needles. One mL of the blood was collected into heparinised micro tubes (AdeLAB, Australia) for platelet stability and subsequent haemoglobin and

haematocrit measurements while the other 1.5 mL of blood was put into a 2 mL micro-tube for serum collection and subsequent serum ferritin, serum iron and transferrin total iron binding capacity analyses.

2.2.5.1 Haemoglobin concentration and % Haematocrit determination

Haemoglobin concentration was measured using the cyanmethaemoglobin method as described under section 2.1.3. Haemoglobin determination was performed manually in the animal studies because the auto analyser had not been calibrated for animal work.

One hundred millilitres of reagent 1 (containing 0.6 mM KFeCN, 0.75 mM KCN, 3.00 mM PBS, pH 7.2 and 10 mg/100 mL Triton x 100) was prepared in a fume cupboard and stored in a brown glass bottle for two months. The reagent was discarded if a brown coloration developed before that time. ´On the day of analysis, 80 µL of heparinised blood and 1 mL of reagent -1 were pipetted into three 2 mL test tubes. The pipette was flushed out thoroughly with the mixture. One millilitre of the resulting mixture (blood + reagent) was pipetted into a 1 mL plastic cuvette and the absorbance read at 546 nm against redistilled water on a Shimazu spectrophotometer 120. Haemoglobin concentration was determined using a Boehringer Mannheim GmbH Diagnostica table for values for measurements of Hg 546 nm (1984). Haematocrit was measured manually using microhaematocrit tubes which were filled with heparinised blood by capillary action. These tubes were spun for 5 minutes on a Cellokrit. The packed cell volume was measured using haematocrit plates.

2.2.5.2 Biochemical analyses

Whole blood was collected into plain microtubes tubes and centrifuged in a micro centrifuge (Mikro 12 - 24, Hettich Zentrigugen, Germany) within two hours of collection for 5 minutes for serum collection. The sera were used for estimating SF, Tf, SI and TIBC similar to indicators assayed in the human samples using a Hitachi 717

autoanalyser instead of the Cobas autoanalyser used in the human study. The Hitachi uses the same ELIZA principle for ferritin determination but instead of measuring the complex colorimetrically as in the Cobas auto analyser, the Hitachi uses a turbidometric method. The Hitachi auto analyser has the advantage of being able to measure SF, Tf and SI at the same time. TIBC can also be calculated. The coefficient of variation of the determination within and between runs for rat SF, Tf and SI was less than 10%. Serum iron levels were often much higher than those in human samples for the same iron status conditions.

Table 2.1 Haematological and biochemical iron status measurements on iron sufficient and iron deficiency weaning rats (35 day olds)

Blood parameters	**Dietary Treatments**	
	Iron and calcium sufficient (Control; IS + Ca)	Iron deficient calcium sufficient (ID + Ca)
Haemoglobin (g/dL)	14.5 ± 1.5	*8.0 ± 2.0
Haematocrit (%)	39.2 ± 3.0	*26.5 ± 2.0
Serum ferritin (μg/L)	199.0 ± 95.0	**74.0 ± 20.0
Transferrin (μmol/L)	1.24 ± 0.1	1.20 ± 0.1
Serum iron (μmol/L)	38.0 ± 14.0	*16.0 ± 7.0
% Saturation	135.0 ± 11.0	*98.0 ± 7..0
Coefficient of variation	2.0 -10%	2.0 - 8.0 %

Table represent result in mean (± SD) haematology and biochemical indicators of iron status in post weaning rats (35 day olds). IS + Ca is control diet which is iron and calcium sufficient, ID + Ca is iron deficient with sufficient calcium. *Significant values ($p < 0.01$) and ** ($p < 0.001$) from control (IS + Ca) are as marked

2.2.5.2 Effect of dietary treatment on iron status in experimental rats model

Haematological and biochemical results (Table 2.1) indicate that the rat can be used as an experimental model for investigating iron status. A 96 % reduction in dietary iron was associated with 45 % reduction in haemoglobin, a 32 % reduction in Hct , 62% reduction in SF, 58% decline in serum iron and a 27% reduction in transferrin saturation in iron deficient weaning rats within 14 days.

2.2.6 Assessment of gut growth

The most commonly used parameter for assessing gut growth are wet weight and length of the gastrointestinal tract. Although these measurements can be done quickly without the use of sophisticated equipment, they provide no information on the adaptive changes that occur at the cellular level during growth and dietary manipulations. A better understanding of tissue growth and adaptation in response to dietary manipulation can be gained by assessing the biochemical and morphological status of the gastrointestinal tract, hence the use of these measurements in this study.

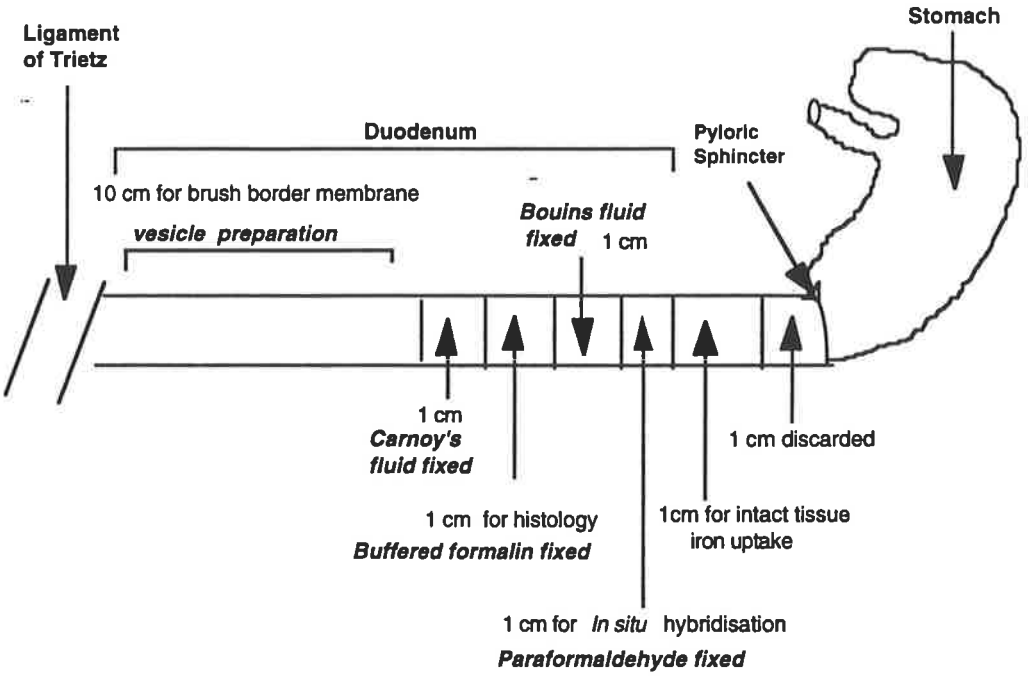
The proximal duodenum, cut 1 cm away from the junction with the pyloric sphincter (Figure 2.2), were collected and placed on ice. These duodenal segments were flushed three times with 10 mL ice cold phosphate buffer saline solution (PBS; pH 7.4) for morphological and biochemical studies. Ten centimetres of the tissue was wrapped in aluminium foil and stored at -40 ^{o}C for vesicle preparation when required. One centimetre was cut for intact iron uptake. The rest was cut as illustrated (Figure 2.2).

2.2.6.1 Morphometric measurements in histological sections

In addition to biochemical assessment of gut growth, changes in the mucosa and functional cells of the mucosa were assessed. Quantitative evaluation of the small intestine, particularly the small intestinal mucosa was carried out because of the importance of this compartment in tissue proliferation and absorptive function. This

evaluation was done by measuring villus height and crypt depth of the duodenal mucosa of rats on the four experimental diet regimes.

Figure 2.2 Schematic diagram of tissue collection after preliminary assessment of optimal fixatives and fixation times



2.2.6.2 Histological preparation and selection criteria

Before experimental work commenced, a preliminary study was conducted to establish a fixation protocol that would achieve good preservation of cytological detail for routine quantitative histological assessment and also be suitable for studying the expression of receptor ferritin and transferrin mRNA using *in situ* hybridisation. Strict selection criteria for good and complete spherical sections and for the measurement of morphometric structures were observed to reduce variability in histological measurements. The procedure followed involved the washing of 4 cm of gut tissue on the day of cull. This was cut into four 1 cm sections, 1cm was fixed in 4 % buffered formalin, 1cm in Bouin's fluid, a third cm in Carnoy's fixative and the fourth was put in 4 % buffered paraformaldehyde (section 3..2.5) .

To evaluate the different fixation protocols, multiple duodenal tissue samples were collected from iron and calcium sufficient (IS + Ca) and iron deficient calcium sufficient (ID + Ca) weanling rats and fixed for 6, 12 or 24 hours in each of the 4 fixatives. After the specified times, tissues were processed for routine paraffin embedding using an automated system (Shandon Citadel, 2000, Tissue Tex, England). Two transversely oriented segments were embedded in the same mould. Wax blocks were sectioned at 8 or 10 µm. From each block, 4 serial sections were collected onto either TESPA (3-aminopropyltriethoxysilane)or poly-l-lysine coated slides. Sections were dewaxed with histolene, hydrated serially in ethanol 100, 80, 70 and 30% ethanol, stained with haematoxylin and washed under a gentle stream of running RO water. The sections were counterstained with eosin and dehydrated serially in 30%, 70%, 80% and 100% ethanol and 2x histolene. Five slides each of iron sufficient and iron deficient of the first of the serially cut formalin fixed sections were stained with haematoxylin and eosin and mounted with DePex mounting medium (Gurr, BDH Chemicals , Kilsyth, Australia). The second series of sections were collected on a 0.2 % TESPA coated slides and stained in the same way. The remaining sections were stored for subsequent *in situ* hybridisation studies. Tissue sections were examined under a light microscope at 40X magnification and histological measurements done on images captured with a colour video camera. Captured images were digitised by an Image Analysis Program linked to an IBM computer (Video Pro 32, Adelaide, Australia). From each microscopic field, crypt depth and villus height measurements were taken. Means and analysis of variance were calculated for the two variables for each of the 6, 12 and 24 hour treatments.

2.2.6.3 Selection criteria

The presence of a single epithelial layer and clear visibility of the base and neck of the crypt in each microscopic field was the basis for the selection of a particular crypt. In the case of the villus, it was essential that the villi were cut in a perfect transverse plane with the tip of the villus and the crypt/villus junction clearly visible in each

microscopic field. A minimum of 5 perfectly oriented crypt and villus structures were selected and measured in each of the 3 tissue segments present on a microscopic slide .

Good cytological preservation was achieved in duodenal segments for the two dietary treatment groups with all four fixatives. Shrinkage of tissue was minimal with formalin and paraformaldehyde fixed tissue at 6 and 12 hours but good preservation was only possible in Bouin's fluid at 6 hours and in Carnoy's at 8 hours. The Bouin's and Carnoy's fixed tissues were very brittle after 6 hours and sections were fragmented. In later experiments in which ID - Ca tissues were used, segments were found to be very delicate and had to be handled with extreme care. On the basis of these observations, optimal histology and morphometry conditions were found to be 12 hours in formalin or paraformaldehyde followed by storage in 70% alcohol for 24 hours. In all, 15 measurements each of crypt depth and villus height were carried out on each slide after carefully scanning the histological samples that met the selection criteria above.

2.2.7 Preparation of Brush-border membrane vesicles.

Brush border membrane vesicles were prepared using a modified protocol from Shirazi-Beechey and coworkers (1991). One hundred Wistar weanling rats (35 days old) on the four dietary treatments (25 per treatment) were anaethesised with pentobarbitone injection as indicated in section 2.2.4. Ten centimetres of proximal duodenal tissue was immediately removed and washed with ice-cold PBS (pH 7.2) as described in section 2.2.6. The washed duodenal tissue segments were either used immediately or were stored at - 80 ^{o}C until needed for vesicle preparation.

All further procedures were performed at 0 - 4 ^{o}C. Frozen gut segments were weighed, cut into small pieces and defrosted in buffer 1 [100 mM mannitol, 2 mM HEPES/Tris, pH 7.1] in a 25 mL centrifuge tube using approximately 10 volumes buffer (1g tissue/10 mL buffer) and vibromixed on a Vortex-genie (Scientific Industries, Massachusetts, USA) for 2 x 30 seconds. The resulting homogenate was filtered through

a buchner funnel. The homogenate was treated in a homogeniser (PCU- 2 Polytron Kinectica GmbH, Lauzern, Switzerland) on speed 6 for 2 x 30 seconds and 2 aliquots of it (500 µL) each were taken and stored in liquid nitrogen until needed. To the remaining homogenate, magnesium chloride (2.5M stock) was added to a final concentration of 10 mM (40 µl of 2.5M $MgCl_2$/10 mL of homogenate), mixed and allowed to stand on ice for 20 minutes. The homogenate was then centrifuged for 15 minutes using a JA 20 rotor in a Beckman centrifuge at 3,000 g at 4 ^oC. The resulting pellet was discarded and the supernatant collected into a clean tube. This was centrifuged again at 30,000g for 30 minutes. The resulting pellet was resuspended in 10 mL buffer 2 (100 mM mannitol, 2 mM HEPES/Tris, pH 7.4, 0.1mM $MgSO_4$) by passing it through a 21G needle 3 - 5 times. The sample was again centrifuged for 45 minutes at 30,000 g and the final pellet containing the vesicle membrane was resuspended in approximately 500 µL buffer 3 (100 mM mannitol, 2 mM HEPES/Tris, pH 7.4, 0.1mM $MgSO_4$, 0.02 % sodium azide (Na_2N_3)) The membranes were resuspended in buffer 3 by passing them through 25G and 27G needles and were stored in liquid nitrogen in three aliquots in screw capped mL microcontainers until needed. Protein concentration and alkaline phosphate activities in the mucosal homogenate and final vesicle suspensions were determined in order to calculate an enrichment value for enzymes in the membrane preparation.

2.2.7.1 Protein determination

The Bradford method (1976) for protein determination, which involves the binding of Coomassie brilliant blue G-250 dye to protein with the formation of a protein-dye complex which can be measured colorimetrically was used. The method is rapid and sensitive for the quantitation of macro-and micro protein levels compared to other commonly used ones developed by Lowry (1951) and Schaffner and Weissman, (1973). A manual version of the Bradford method was used in this study but was adapted for use on the plate reader (Dynatech 700, USA) to measure the protein concentrations in vesicles and homogenate samples. Bovine serum albumin (BSA) was used as a standard.

The purity of the vesicle samples was checked by assaying protein and alkaline phosphatase concentrations in both the homogenate and final vesicle preparations. Alkaline phosphate activity was assayed by a modified Holdsworth (1970) method using p-nitrophenol (Sigma, ,Australia) standard and an enzyme phosphatase substrate (Sigma, Australia). The ratio of alkaline phosphatase activity in the final vesicle preparation to that in the homogenate was indicative of percentage recovery. The alkaline phosphate activity in the vesicle samples (n=152) was 6 - 10 times more than in their respective homogenates (n = 152).

2.2.7.2 Brush border membrane (BBM) enzymes.

At the point of weaning, changes in the function of digestive enzymes include a decrease in lactase-phlorizin activity, the appearance of sucrase-isomaltase and a rapid increase in maltase, glucoamylase and alkaline phosphatase activities (Henning, 1985 and Menard and Calvert, 1991). Many brush-border membrane enzymes like glutamyltranspeptidase, tripeptidase and enteropeptidase which are the key enzymes in the activation of proteolytic enzymes, increase rapidly to adult levels (Menard and Calvert, 1991). Although the intrinsic timing of the mechanisms involved in the onset of intestinal maturation at the weaning period has been shown to be important, it is also possible that the transition to an adult-like diet is equally essential for normal gastrointestinal development. If this logic is true, extreme weaning practices which may impair this process will probably have long term effects on gastrointestinal development well beyond the period of weaning.

The brush border of the small intestine contains four glycosidases which split dietary disaccharides and oligosaccharides (Aurichio and Sebastio, 1989), but only two of these enzymes, lactase-phlorizin hydrolase (LPH), and sucrase-isomaltase (SI), were evaluated in this study because of their distinct variation in the small intestines of young children and adults which is associated with the developmental profile in mammals (Raul *et al.*, 1986). Lactase and sucrase activities in the mucosal homogenate and brush

border membrane vesicle were measured using the method of Dahlqvist (1968) and measured colorimetrically using a plate reader (Dynatech 700, USA).

2.2.7.3 Radio-labelled iron uptake in Brush border membrane vesicles.

Iron labelled non-haem iron (59 Fe) uptake into vesicle was measured in vesicle samples from the four treatment group to assess the effects of diet on iron transport into duodenal brush border membrane. This is discussed in detail in chapter 5.

2.2.8 Intact tissue iron uptake

This investigation was carried out to elucidate the effects of dietary calcium manipulations on mucosal iron uptake. A one centimetre piece of the duodenum (1 cm away from the pyloric sphincter) was everted longitudinally over a perspex rod and flushed with a 2 x 2 mL ice cold PBS; pH 7.4. The mucosal uptake of radiolabelled iron ($^{59}Fe^{2+}$) is described in detail in chapter 5.

2.3 Development of molecular techniques to assess the effect of calcium on intestinal Ferritin (L and H) mRNA expression and Transferrin Receptor (TfR) mRNA expression.

2.3.1. Materials and Methods

Materials

2.3.1.1. Tissues

Wistar rat (35 day old) tissues collected from IS + Ca, IS - Ca, ID + Ca and ID - Ca animals that were housed in special facilities at the University of Adelaide.

2.3.1.2 Bacteria strains

The host Escherichia coli (*E.coli*) strain used to propagate plasmid clones were XL1-Blue, and ED 8799. Stocks were obtained as gifts from Dr Simon Bawden.

2.3.1.3 Plasmid strains and phagemids

The detail of the Recombinant clones and base vectors and their sources are as follows;

pGEM-3Zf (+), pGEM-1 were obtained from Promega Corporation.

pUC-19-rTfR: a pUC-19 clone containing 3.413 kb DNA fragment encoding the rat TfR sequence (subcloned from pTZ19U-rTfR Griswold *et al.*, 1990) was a gift from Dr Greg Anderson, Queensland Institute of Medical Research, Australia.

pGEM-1-L-Ferritin: a 532 bp partial cDNA encoding the rat ferritin light chain subunit (see Leibold *et al.*, 1984 for complete sequence) inserted into the XbaI site of pGEM-1. The clone (KB 003) was a gift from Kerrie Basclain, Department of Medicine, University of Western Australia.

pGEM-3Zf (+)-H-Ferritin: a 900 bp cDNA encoding the rat ferritin heavy chain subunit (see Murray *et al.*, 1987) for sequence inserted into the EcoRI site of pGEM-3Zf(+) pUC 19-rTfR. The clone, KB 016, was a gift from Kerrie Basclain, Department of Medicine, University of Western Australia

2.3.1.4 Enzymes

Restriction endonucleases were purchased from either Boehringer Mannheim (Australia) or Pharmacia.

Ribonuclease T1 (RNase T1) and Ribonuclease A (RNase A) were purchased from Boehringer Mannheim (Australia)

E.Coli, DNA polymerase I (Klenow fragment), was purchased from Bresatec, Pty Ltd, Australia.

T7 and SP6 RNA polymerases and T4 DNA ligase were purchased from Promega Corporation.

Calf intestinal phosphatase (CIP) and Proteinase K were supplied by Boehringer Mannheim .

2.3.1.5 Radiochemicals

[α-^{32}P]rUTP and [α-^{33}P]rUTP (specific activity, 3000 Ci/mmole), were purchased from Bresatec Ltd and Amrad respectively.

2.3.1.6 Molecular Biology Kits

Molecular biology kits were used according to manufacturers' instruction. Qiagen columns were purchased from Qiagen Inc., and Promega Riboprobe System were purchased from Promega Corporation.

2.3.1.7 General chemicals and reagents

The following chemicals were purchased from the Sigma Chemical Co.: acrylamide and bisacrylamide, agarose, ampicillin, rATP, chloramphenicol, EDTA

(ethylenediaminetetraacetic acid), 2-mercaptoethanol, mineral oil, salmon sperm DNA, SDS (sodium dodecyl sulphate), TEMED (N,'N,'N,' N'-tetramethylethylenediamine), TESPA (3-aminopropyltriethoxysilane) and tetracycline.

CsCl (technical grade) was purchased from Metallgesellschaft.

Dextran sulphate and Ficoll (type 400) were purchased from Phamarcia.

Low melting point agarose (ultra pure) was obtained from Bethesda Research Laboratories, MD.

Agarose (type 1: Low EEO) was purchased from Sigma.

Magnesium chloride, polyethylene glycol (6000), potassium ferricyanide and potassium ferrocyanide were purchased from BDH Laboratories.

Urea (ultra pure) was obtained from Merck.

General chemicals not listed were obtained from one of the following suppliers; Ajax Chemical Pty Ltd, BDH Chemicals Pty Ltd., May and Baker Pty Ltd., Merck, Pharmacia or Sigma Co. Chemicals were of the highest purity available.

Trypsin (Difco) was made as a1% solution x Versene (EDTA: CSL)

2.3.1.8 Bacterial culture reagents

LB Medium was used for the growth of *E.Coli,* XL1-Blue and ED 8799.

Agar plates were made using LB medium as described by Sambrook *et al.* (1989).

2.3.1.9 Miscellaneous

DNA Markers

DMW-S1 : EcoRI digested SPPI phage (360bp - 8.51 kb) : Bresatec

DMW-P1 : HpaI digested pUC19 (26-501 bp) : Bresatec

X-ray film was obtained from Konica Corporation or Fuji Photo Film Corporation. Hyperfilm was purchased from Amersham.

Ektachrome film and D19 Developer (used for *in situ* hybridisations) were purchased from Kodak, Ltd.

L4 emulsion (used for *in situ* hybridisations), Hypam Rapid Fixer and Film were purchased from Ilford Ltd.

2.3.2 DNA Methods

Molecular Biology protocols

The following protocols were carried out essentially using the methods described by Sambrook *et al.*, (1989).

2.3.2. 1 Ethanol precipitation

Ethanol precipitation was conducted by modified method of Sambrook *et al.* (1989). In situations of low concentration of DNA, 1-2 µL of glycogen carrier was added to facilitate precipitation of DNA.

2.3.2.2 Phenol extraction

Before use, phenol (Wako, Chemical. Co) was equilibrated four times with buffer; one time with 1M Tris-HCl pH 8.5 followed by three equilibrations with 100mM Tris-HCl pH 8.5. 8-hydroxyquinoline (0.1 w/v) was added prior to the last equilibration step. The extraction was carried out by mixing the aqueous sample with an equal volume of phenol/chloroform (1:1). After centrifuging, the top aqueous phase was transferred into new tubes.

2.3.2.3 Isolation of plasmid DNA

Isolation of bacteria containing recombinant plasmid/phagemids using selection media containing ampicillin was prepared by modified procedure of Birnboim and Doly (1980) described by Sambrook *et al.*, 1989).

Large scale preparation of plasmid DNA was made using Qiagen columns, the following manufacture's protocol.

Small scale preparation of plasmid DNA (analytical scale minipreps) was conducted essentially by the method described by Sambrook *et al.*, (1989)

2.3.2.4 Restriction enzyme digestion and analysis of DNA

Restriction endonulease digestions were performed using the conditions recommended by the supplier for each enzyme. Usually 2-5 units of enzyme were used per μg of DNA and reactions were performed for approximately 2 hours.

2.3..2.5 Agarose gel electrophoresis.

DNA fragments were size fractionated by agarose gel electrophoresis which was performed in a horizontal apparatus (Sambrook *et al.*, 1989). Analytical electrophoresis was performed on gels of 75 mm x 55 mm whilst larger gels were used for DNA preparation.

All gels were run in TAE buffer and samples were loaded in 2.5% Ficoll 400, 0.1% lauryl sarkosyl, 0.025% bromophenol blue, 0.025% xylene cyanol. Electrophoresis was performed at 110 mA (Pharmacia Electrophoresis Power supply EPS 500/400). After electrophoresis, the DNA was detected by staining with 0.1% ethidium bromide for 10 -15 minutes and viewed under short wave UV light. Gels were photographed using a Polaroid camera with 667 (ASA 3000) Polariod film.

2.3.2.6 Polyacrylamide gel electrophoresis

Radio-labelled electrophoresis of RNA fragments was carried out on a vertical 14 cm x 14 cm slab gel containing 6% (w/v) acrylamide/bisacrylamide gel (19:1) and 8M Urea. The acrylamide gel was polymerised in 1xTBE buffer by addition of 0.1% (w/v) ammonium persulphate (APS) 0.1% (w/v) TEMED. The polyacrylamide slab gel was pre-electrophoresed at 15-20mA for 20 minutes before loading. Gels were

electrophoresed at 15-20 mA for 40 minutes or until the loading dyes had migrated the desired distance. RNA was detected by autoradiography (section 2.3.5)

2.3.2.7 Isolation of DNA from gel

Restriction fragments ranging in size from 1kb to 3.5Kb were isolated from 1.0 % low melting point agarose gel (type 1) by phenol extraction (Sambrook *et al.*, 1989).

Restriction fragments ranging in size from 80 to 1kb were extracted from agarose or acrylamide gels by elution from gel slice in 0.5 ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% SDS at 37C for 16hours. The DNA was then ethanol precipitated. A check gel was run to characterise size and purity of cDNA fragment.

2.3.3 DNA cloning

(i) *Vector preparation*

Phagemid cloning vectors were prepared by linearising with appropriate endonuclease(s) followed by removal of the 5' terminal phosphate groups as described by Sambrook *et al.* (1989). Calf intestinal alkaline phosphatase (CIP) was the enzyme of choice for the removal of 5' -phosphate groups from linear DNAs to suppress self ligation and circularisation of the plasmid DNA. The vectors were then subjected to agarose gel electrophoresis, the linear vector band excised and the DNA prepared by phenol extraction (2.3.2.5).

ii) *Preparation of insert DNA fragments*

DNA fragments with 5' or 3' protruding termini were "blunted" with Klenow fragments. Fragments were fractionated in agarose gels and isolated by elution (2.3.2.7).

(iii) *Ligation*

Approximately 40 ng of vector DNA was combined with the desired DNA fragment in a 1:3 molar ratio. The ligation was performed in 50 mM Tris-HCl pH 7.4, 10 mM magnesium chloride, 10 mM dithiothreitol, 1 mM rATP, 100 µg/mL BSA. Approximately 1unit of T4 DNA ligase (Promega) was added and ligation was allowed to proceed at room temperature overnight.

(iv) *Plasmid and phagemid transformation*

E.coli , XL1-Blue cells and ED 8799 cells were made competent and transformed by the modification of the calcium chloride method described in Sambrook *et al.* (1989).

2.3.4 RNA method

2.3.4.2 Tissue *in situ* hybridisation

In situ hybridisation is a powerful molecular technique which allows the identification of mRNA expression within intact tissues at both the regional and cellular levels. Synthetic oligonucleotide probes of approximately 20-50 bases in length or cDNAs are ideal because they can penetrate tissues easily and are sufficiently specific to recognise a single mRNA species. Probes of this length can be designed to be complementary and specific to unique segments of mRNA and may be designed to distinguish between mRNAs differing by only a single base. This method has been shown to be useful in the study of tissues containing heterogenous cell types such as brain, ovary, testis and more recently, in intestines and mRNA regulation in specific tissues or cells (Davis *et al.*, 1986; Oates and Morgan *et al.*, 1996).

2.3.4.3 *In vitro* transcription

Inserts in plasmids pGEMTfR-685, pGEMHFer-418 and pGEMLFer 532 were transcribed either with SP6 or T7 RNA polymerase according to the methods of Kreig and Melton (1987) using a Promega kit. The RNA was labelled to high specificity by

the incorporation of α–[^{33}P]-rUTP or α–[^{32}P]-rUTP. Transcripts were purified by phenol extraction and ethanol precipitation.

The tissue *in situ* hybridisation technique which will be described in detail in chapter 6 was based on the method of Cox *et al.*, (1984) with modification by Powell and Rogers (1990).

2.3.4.4 Size reduction of RNA probes

To increase the hybridisation signal, large sense and antisense RNA probes were reduced in size by alkaline hydrolysis with 0.2M sodium carbonate (AnalaR, BDH Chemicals, Port Fairy, Vic) at pH 10.2 and 60 ^{o}C for 10 minutes. The solution was neutralised by adding 3 µL 3M sodium acetate and 5µL 10% glacial acetic acid and incubated at -20^{o}C for 20 minutes. Transcripts were then isolated by phenol:chloroform extraction and ethanol precipitation with the addition of l0 µg tRNA (Cox *et al.*, 1984). The probes were resuspended in 46.5 µL DEPC water, 2.5µL 200 mM vanadyl RNase inhibitor (New England Biolabs Inc, Beverley, MA), 1µL 0.5M β-mercaptoethanol (Sigma, St Loius, USA) and 1 µL RNasin (20 units/µL; Promega Co.) and stored at 4^{o}C.

2.3.4.5 Determination of the specific activity of cRNA probe

Prior to use, an aliquot (1µL) was taken from each RNA solution and scintillation counted in 3 mL Biodegradable Counting Scintillant (BCS; Amersham, Riverton, USA) to determine the level of α-[^{33}-P]-rUTP incorporation. Another aliquot was size fractionated in a 6% polyacrylamide denaturing gel to assess success of alkaline hydrolysis. For the latter procedure, a 1µL cRNA aliquot was combined with 2 µL DEPC water and 3 µL Ficoll load buffer (Appendix 3.2), and heated at 90^{o}C for three minutes before gel loading and electrophoresis in 1X TBE buffer (Appendix 3.1). Radiolabelled RNA was visualised by autoradiography using X-ray film (Fuji RX, Fuji Corp) in an Ilford autoradiograph cassette.

2.3.5 Autoradiography

Radiolabelled RNA was visualised by autoradiography using X-ray film in an Ilford autoradiograph cassette. X-ray film (Agfa) exposures were carried out at room temperature or at -80^{o}C using tungsten intensifying screen.

2.3.6 Containment facilities

All work involving recombinant DNA was carried out under C1 containment conditions required for work with viable organisms as defined by the Genetic Manipulation Advisory Committee and the Biohazards Committee of the University of Adelaide.

2.4 Immunocytochemical Localisation of TfR

2.4.1 Antibodies

A mouse monoclonal antibody against rat TfR were used. The antibody used in this study was donated by Dr Philip Oates, Department of Physiology University of Western Australia (Oates and Morgan, 1997).

Preliminary studies showed that formalin fixed duodenal tissues gave better results with minimum background compared with those fixed in paraformaldehyde. Formalin fixed tissues were therefore processed as descried in section 2.2.6.1 and sectioned to 3 μm thickness.

Liver sections (3μm thick) from rats fed IS+Ca and ID-Ca were used as positive controls. Duplicate slides with sections were deparaffinated in histoclear for ten minutes, rehydrated in serial ethanol concentrations of 100% ethanol for 2 minutes, 100% ethanol and 0.3% hydrogen peroxide solution for 30 minutes and 80%, 50% and 30% serial ethanol in Tris buffer, each for two minutes. To eliminate endogenous peroxidase activity, rehydrated sections were treated with 2.5% aqueous periodic acid for 5 minutes and 0.02% sodium borohydride for 2 minutes. Prior to incubating sections

with primary antibodies, they were treated with a blocking solution comprising 10% foetal calf serum in TBS buffer (20 mM Trizma base, 500mM NaCl, pH7.5) containing 0.2% saponin for 20 minutes. Sections were incubated with monoclonal antibody directed against rat TfR (Chemicon International, Temecula, CA) for 1 hour at ^{o}C . The immuno reaction was stopped by rinsing the sections three times with TBS-saponin followed by incubation with a secondary antibody. Peroxidase-coupled mouse immunoglobulin G (IgG) directed against rabbit IgG (Silenus, Laboratories Pty Ltd, Melbourne, Australia) was diluted 10 fold for rat TfR. After incubation for 1 hour at room temperature with the secondary antibody, the sections were washed three times in TBS buffer and biotinylated with kit (Amersham) prior to the detection reaction. Detection was carried out using section of 3 mM 3, 3' diaminobenzidine (DAB) (Sigma Chemical Co., St Louis, USA) and 0.02% hydrogen peroxide in phosphate-buffered isotonic saline.

The reaction was stopped after three minutes by washing the sections in double distilled water. Sections were counterstained with Mayer's Haematoxylin (Sigma Diagnostics, St Loius, USA) for five minutes and washing in distilled water for 3 minutes (2x). The sections were dehydrated in serial ethanol concentrations of 30%, 50% (2x), 100% (2x) for two minutes each, histolene for five minutes and mounted in Depex mounting medium (BDH, Gurr). Sections used to determine non-specific binding were treated identically, except that non immune rabbit serum was substituted for ferritin or TfR antibodies.

2.4.2 Assessment of Immunoperoxidase Staining.

A semi-quantitative grading of intensity of expression was performed by scoring each section on a four point scale of 0 (no expression) - 3 (high expression). For each treatment, TfR localisation was assessed along the length of 6 perfectly oriented villi, crpts and muscularis using an image analysis software program (Video Pro, Leading Edge, Adelaide, Australia).

CHAPTER 3

IRON STATUS AND DIETARY INTAKES OF 6-24 MONTHS OLD CAUCASIAN AND ASIAN CHILDREN IN LIVING IN ADELAIDE

IRON STATUS AND DIETARY INTAKES OF 6-24 MONTHS OLD CAUCASIAN AND ASIAN CHILDREN IN LIVING IN ADELAIDE

3.1 INTRODUCTION

Young children, whether in developed or developing countries are at risk of iron deficiency because rapid growth imposes high iron needs which is coupled with the low bioavailability of iron in the diet of infants. Although formula fed infants are able to maintain adequate iron status at six months compared to breast fed infants, both breast fed and formula fed infants are at risk of iron deficiency during weaning. This can be attributed to increased erythropoiesis as a consequence of a two fold increase in physical growth (Siimes and Saarinen, 1978; Messer *et al.*, 1980, Worwood, 1982; Dallman 1992; Yip, 1995). A concomitant chronic iron deficiency anaemia will develop, if a weaning diet adequate in iron to meet the physiological daily requirements is not given (Siimes *et al.*, 1984; Penfold, 1989; Penrod et al., 1990; Haschke *et al.*, 1993). A negative iron balance then occurs when the utilisation of body iron stores exceeds absorbable dietary iron needed to replenish these iron stores. Prolonged depletion of body iron stores results in iron deficiency anaemia.

Chronic iron deficiency is associated with anaemia, lethargy, and alteration in small intestinal mucosa predisposing the gut to nutrient malabsorption (Berant *et al.*, 1992; Trowbridge *et al.*, 1993). Evidence also suggests that iron deficiency anaemia during infancy and early childhood can have lasting and perhaps irreversible effects on cognitive functions and behaviour (Oski *et al.*, 1983, Walter *et al.*, 1989, Lozoff *et al.*, 1991; Beard *et al.* 1993). The duration of iron deficiency beyond three months and its severity in the first two years of life has been associated with impaired behaviour and school performance at five years of age, even when, data is corrected for socio-economic status and educational background of parents (Lozoff *et al.*, 1987; Walter, 1993). Walter and coworkers (1989) showed that an oral iron supplementation three months can raise the Hb levels of iron deficiency anaemic toddlers from Hb < 105g/L to

normal levels of Hb ≥ 110g/L. The mental developmental index (MDI) and psychomotor development index (PDI) scores of these children were however, 10 MDI and 15 PDI lower than their iron sufficient counterparts (Walter *et al.*, 1989). These studies clearly indicate that the first two years in a child's development offers a window of opportunity to achieve his maximum intellectual potential. This genetic endowment can however, be missed all together if iron deficiency anaemia persists even for a short time between the ages of 6 - 24 months. This vulnerable period of brain growth and development in childhood also coincides with a period of transitional food, motor development and gut maturation (Henning 1987, Dallman, 1990; Clark *et al.*, 1990; Menard and Calvert 1992; Heinig *et al.*, 1993).

At the onset of transitional or weaning foods, when breast milk volumes become inadequate to meet the energy and micronutrient requirements of a normal infant at 3- 6 months (Jason *et al.* 1984, Whitehead, 1985, WHO, 1985; Horowitz, 1989), children must rely on additional foods to meet their daily nutrient needs. During the first year, infants rapidly develop rhythm in feeding pattern and as gastric capacity and motility increase, infants progress from feeding small amounts frequently to ingesting higher volumes less frequently (Clark *et al.*, 1990; Pridham, 1990). In spite of the increase in the volume of food ingested, these serving portions are often too small to provide the needed amounts of nutrients, particularly, iron (Horowitz, 1989). The introduction of weaning foods requires an adaptation of the gastrointestinal tract to cope with the presention of potential harmful substances including both animal and plant proteins (Walker, 1985). These have been speculated to either enhance or retard growth with a possible increase in the risk of hypersensitivity (Ziegler *et al.*, 1992). For example, the exposure of infants to cows' milk protein before the age of twelve months has been associated with occult intestinal bleeding and diarrhoea which can lead to iron deficiency in young children (Ziegler *et al.*, 1992).

In spite of these findings, there is a paucity of data in Australia on the iron status and dietary intake by 6 to 24 months old children. Although dietary surveys have indicated that infants aged 6-12 months consume less than 50% of the recommended dietary intake of iron (Hitchcock and Coy, 1988). Dietary guidelines in Australia consistently recommend that infants receive breast milk or iron fortified formula until the age of 12 months and that iron fortified cereals be introduced by 4 - 6 months. Despite these guidelines Rettalack *et al.* (1994), have shown in a shopping centre survey, that cow's milk consumption after six months was common in 5-12 months old, with 44% of children receiving cow's milk as the sole source of milk by 8 months. There was also a sharp decline in the use of iron fortified cereals and an increase in the consumption of adult cereals at 6 months.

It is hypothesised that these dietary trends together with the limited knowledge on the efficacy of absorption of iron from iron fortified cereals and composite diets in infants, put children in Australia at risk of iron deficiency. Consequently, the aim of this clinical study was to investigate the iron status and dietary intake of 6 -24 months old Caucasian and Asian children from a broad socio-economic background in Adelaide using haematological, biochemical, dietary and anthropometric measurements.

3.2 SUBJECTS AND METHODS

3.2.1 Subjects

Healthy full-term (6-24 months old) children living in Adelaide were recruited from immunisation clinics, Child and Adolescent Family Health Service (CAFHS) centres and the Flinders Medical Centre (FMC). The centres were spread over Adelaide and were chosen to include the different socio-economic groups. Cambodian and Vietnamese population were studied specifically because subjective analysis by CAFHS nurses working in that area, indicated that Asian children were at risk of iron deficiency anaemia. The Cambodian and Vietnamese children resided in north-western Adelaide and home visits were arranged with an interpreter for 20 Cambodian families. The study protocol included parental consent and written information which was reviewed and approved by the FMC and CAFHS Ethic committees.

3.2.2 Exclusion criteria

Children were excluded if they had less than 39 or more 42 weeks gestation period, were younger than 6 months or older than 24 months or had any acute or chronic infections as specified under section 2.1.8. Children who had been immunised less than two weeks prior to being recruited into the study were also excluded. Children residing outside the metropolitan Adelaide or without parental consents were also excluded. Children who were identified to have α or β thalessaemia were excluded.

3.2.3 Anthropometric measurements.

Weight, height and head circumference which are measures of the infants ability to thrive were taken on the day of assessment. Children were weighed bare ($\pm$ 0.10 kg) using a Paediatric digital scale. Paediatric measuring mats were used to measure the lengths of 6-20 month olds. Where children were older, a vertical rule was used instead. Due to the population variation in anthropometric measurements these values were corrected to Z-scores as described in chapter 2 using values supplied by the Adelaide Women's and Children's Hospital, South Australia (Penfold, 1989).

3.2.4 Dietary intake assessment

A 24-hour semi-quantitative diet recall questionnaire designed specifically to assess iron intake in young children was filled in by parents with the assistance of the investigator. Specific questions were asked as indicated under section 2.1.6. Parents were also asked about children's meat, chicken, pork or fish consumption. The consumption of vegetables, fruits and fruit drinks were also recorded. The dietary questionnaire for Asian children was modified to include the frequency of rice porridge and any additive used such as fish, milk, or meat stock.

Nutrient intake of children was calculated using estimates from a nutrient table designed (Table 3.1) which was based on nutrient values obtained from the Australian Food composition Table (Greenfield and Wills 1979). Three main categories of foods commonly consumed by 6-24 months old children were selected; (1) breast milk, formula and cow's milk, (2) cereals and (3) meats. The iron contents of the specified amounts depicted by the serving portions of the selected foods were calculated. Based on theoretical relative bioavailability of the different forms of iron in these foods compared to ferrous sulphate, the estimated absorbable iron from these diets were calculated. The Australian recommended daily allowance (RDA) for iron intake for a particular age group, as well as information in published reports, were used to assess the RDA of iron for each child (Hendrick *et al.*, 1992, Saarinen *et al.*, 1979, NHMRC, 1991, 1995; Hurrell 1984; Sketel *et al.*, 1986; Fomon 1987; Fairtherweight *et al.*, 1989).

The dietary iron intake of each child was rated 0 to 3. Scores of 3 was ranked as optimal, 2 medium, 1 as poor and 0 as negligible. Scores were added with the total possible ranging from 0-6 for an individual child. These semi-quantitative dietary intake scores were compared between groups. Using the estimated Australian RDA for children at 6-9; 9-12 and 13-24 months, the percent RDA contributed by the different diets were calculated. For example, the RDA for iron for 6-9 months is 9 mg, so if the calculated daily iron intake for an infant comprises 500 mL of fortified formula (4.4 mg

of Fe), 15 g of weetbix mixed with 150 mL of cows' milk (1.36 mg Fe), cooked potatoes, carrots and peas (0.24 mg Fe), then total daily iron intake of the infant is calculated as 6 mg Fe/day scored 2 and ranked medium RDA (66%).

Table 3.1. Ranking of dietary iron contents of common weaning foods in comparison with % Australian recommended daily allowance (RDA) and relative bioavailability of iron in these foods.

Food	Fe content (mg)	Bioavailable iron relative to Ferrous sulphate (mg)	Iron content in relation to % RDA*	Ranking
Milk				
Formula (500)mL	4.43	1.8 (a)	44	3
Breast milk (500 mL)	0.15-0.35	0.25 (b)	2.5	2
Cow's milk (500 mL)	0.3	0.06 (c)	0.3	1
Cereal				
10g IFC+ 100 mL J (h)	2.5	0.37 (d)	25	2
10 IFC + 100 mL F	3.7	0.56	37	2
10 IFC + 100 mL CM	2.6	0.13 (e)	26	1
15g IAC +Fr	1.65	0.12	13	1
or 15g IAC + 100 mL F	2.5	0.13	25	1
15g IAC + 100 mL CM	1.36	0.05	14	1
15g UFC + 100g CM	0.13	0.003 (f)	1.3	0
Meat				
Chicken (30g)	0.4	0.1 (g)	4	1
Beef (20g)	0.6	0.15	6	1

IFC = Iron fortified infant cereal; J = fruit juice; CM = cow's milk ; IAC = iron fortified adult cereal; Fr fruits; F = formula; UFC = unfortified adult cereal*

Recommended dietary intake of iron for children 6-24 months in Australia is as follows: 6 months, breastfed is 0.5 mg and formula fed is 3.0 mg; 7-12 months, 9mg and 12-24 months is 6-8 mg (NHMRC, 1982-89; 1995).

a) Sketel *et al.* (1986); (b and c) Saarinen *et al.*, 1979; (d) Fomon (1987); (e and f) Hendrick *et al.*, 1992; (g) Layrisse *et al..*, 1969.; Wills 1980; (h) Hunt *et al.*, 1994; Heinz, 1990. The amount of absorbable iron intake was ranked optimal (3) medium (2) and low (1) or nil (0) taking into account the quantity and the relative bioavailability of iron in the specified food in relation to ferrous sulphate and how they compare with the recommended iron intake for 6-12, and >12 - 24 month olds

3.2.5 Laboratory testing for iron status

A two mL sample of peripheral blood was obtained by venipuncture, by a registered nurse as described in section 2.1.2, for full blood examination, serum iron (SI), transferrin (TF) and serum ferritin (SF) estimation, Eighty Caucasian infants recruited from immunisation clinics had blood taken between three to four weeks after immunisation because anecdotal evidence suggests that vaccines may elevate serum ferritin measurements.

3.2.5.1 Full blood examination

Haemoglobin (Hb), Haematocrit (Hct), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin concentration (MCHC), red cell count, (RCC) and red cell distribution width (RDW) were measured using Technicon H. 2™ System auto-analyser (Technicon Corporation, USA) to determine the degree and type of anaemia if present as described in chapter 2. Blood films were prepared by staining for any abnormalities and to classify the type of anaemia in children who had any abnormal red blood cell indices. Blood films were screened microscopically by a qualified senior Haematologist, Professor Ram Shesadri, at the Flinders Medical Centre.

White blood cell, lymphocyte, neutrophils and eosinophil counts were also estimated, using Technicon H. 2™ System which uses the principle of enzyme peroxidation and differential cellular staining to exclude infants with infections or inflammation (Ansley and Ornstein, 1971).

3.2.5.2 Biochemical measurements

A one mL blood serum was taken into plain top 2 mL eppendorfs for biochemical analyses. These include, Serum ferritin (SF), Serum iron (SI), Total iron binding capacity (TIBC), and Transferrin (Tf). Serum ferritin was estimated using an enzyme immunoassay on Cobas Auto analyser at the Flinders Medical Diagnostic laboratory as described in section 2.1.4.

3.2.5.2.2 Serum iron (SI)

Blood serum (65µL) was taken for SI estimation using a Cobas auto-analyser. In this method, iron bound to transferrin in blood is split off by guanidine hydrochloride and reduced to ferrous iron (Fe2+) by hydroxylamine. A red chelate is produced with ferroxamine and the intensity of this chromogen, which is proportional to the iron concentration, is measured colomimetrically as described in section 2.1.3.2.

3.2.5.2.3 Transferrin (Tf)

Transferrin estimation assay was employed to give further clues to the aetiology of iron deficiency as indicated earlier on in chapters 1 and 2. Transferrin concentration (Tf) was estimated using a standard anti-human antibody (from rabbit), transferrin antibody (of approx 3g/L) and two different control protein sera (Behring N/T control serum and Ciba abnormal) for quality control purpose, on a Behring Nephelometer (BNS, USA), as described in section 2.1.3.3 More information was obtained by calculating TIBC from which the percentage of transferrin saturation with iron was calculated.

3.2.6 Laboratory diagnosis of iron deficiency

Normal haematological and biochemical values for 6-24 months old infants and toddlers were obtained from published studies and standard values established by the FMC Diagnostic Laboratory Service. A cut-off value of < 15µg/L SF was chosen based on a study by Hallberg *et al.*, (1993a) which showed that SF <15 µg/L was associated with the absence of stainable bone marrow iron (2.1.3.1). The infants were classified into three categories of iron status using the following criteria; 1). Iron sufficient (IS): Hb concentration ≥ 110 g/L, MCV > 72 μm^3, MCH > 23.0 pg, SF ≥ 15 µg/L, and/ or Tf ≤ 3.0g/L, SI > 8.0 µmol/L, and Sat ≥ 12%; 2). Non anaemic iron deficient (NAID): Hb concentration ≥ 110g/L and SF < 15 µg/L and/ or Tf >3.0 g/L, Sat < 12% and SI < 8.0 µmol/L and 3). Iron deficiency anaemia (IDA): as for 2 with Hb concentration < 110g/L.

3.2.7 Statistical analysis

Statistical analyses were done using SPSS for Window programme (Chicago, USA 1986). Frequency of Hb, weight Z-scores and age groups 6-12, >12-18, >18-24 months in Caucasian and Asian children were plotted and a normal distribution confirmed. Logarithmic transformation for SF Tf and SI were necessary to ensure normal distribution before analysis. Analysis of variance (ANOVA) was used to compare iron status between races, age groups, breast and formula fed infants, cow's milk and total dietary iron intake. Comparative differences between Caucasian and Asian children were examined using Kruskal-Wallis one way Anova due to the categorical nature of the data. Correlations were done to determine associations between iron status and recommended dietary iron intake. Multivariate regression analyses was also done to predict the effects of different nutrients on iron status.

3.3 RESULTS

3.3.1 Description of survey population

Three hundred and twenty five children were approached and 245 (80%) were recruited into the study. A total of 235 young children (123 boys, 53%; 112 girls, 47%) were assessed of which 43 (18% of total children population) were of Asian origin. Thirty eight percent of the total population were in the 6-12 month age group, while 35% and 27% were in >12-18 and >18-24 months old age groups respectively .

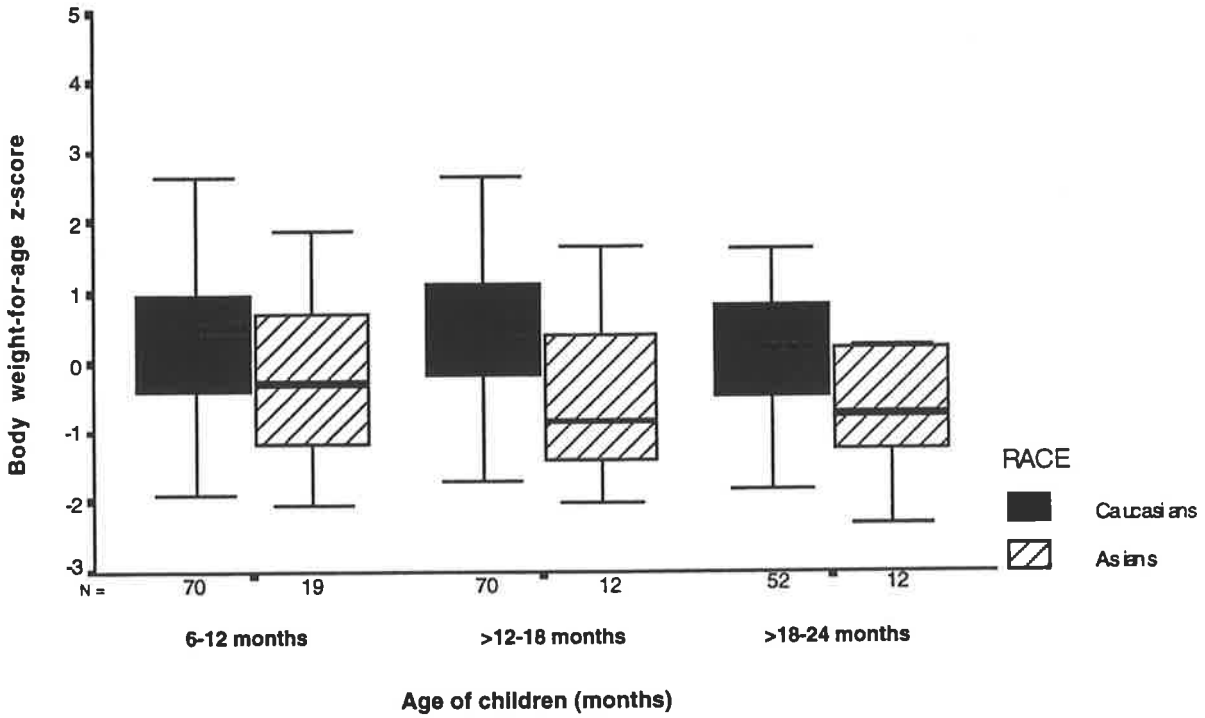
3.3.2 Anthropometric measurements

Growth indicators

All children were within the normal range for age based on the Australian growth curves for children compiled by the Adelaide Women's and Children Hospital (Penfold, 1989) and the Australian National Child Health Anthropometric charts (Kitchen *et al.*, 1983). Weight-z-scores were normally distributed with over 97% of all children within three standard deviations (Figure 3.1). Weight-for-age-z-score was significantly higher in Caucasians than Asians, $Chi^2 = 15.33$; $p < .0001$ in all three age groups (Figure 3.1). Fifty three percent of the weight gain in 6 - 24 month olds can be predicted from the age of the child, $r^2 = 0.53$, $p < 0.001$ (Figure 3.2). Quadratic regression analysis of height for all children showed that all children were also within the 95% confidence interval (CI) (Figure 3.3). A height-for-age-z-score analysis of the data also showed that all children were within the normal 3rd to 97th percentile of distribution .

Figure 3.1

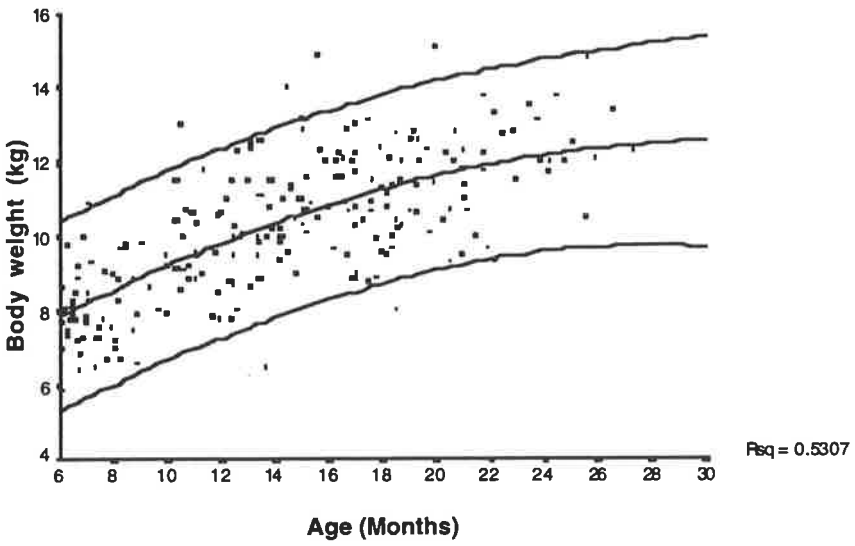
Body weights in z-scores ± SD, for 235 six to twenty four month old Caucasian and Asian children surveyed from a broad socio-economic background in metropolitan in Adelaide.



Box plot of the weight z-scores ± standard deviation from the median of 235 Caucasian and Asian children show that all children were within the normal 3 standard deviations for weight for age. The bar across each box denotes the median of the population of children in the specified age group in either Caucasian or Asian ethnic groups.

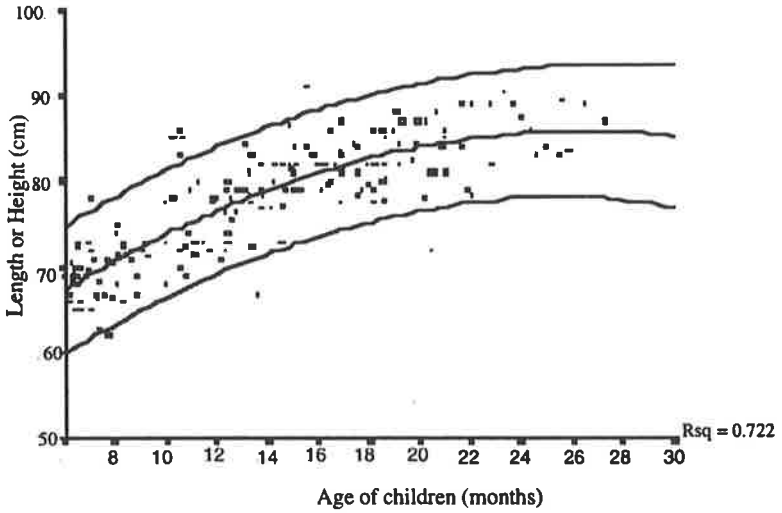
Figures 3.2 and 3.3 Relationship between age and body weights (Fig. 3.2) and length or height (Fig.3.3) for 235, six to twenty four month old Caucasian and Asian children surveyed from a broad socio-economic background Adelaide.

Figure 3.2



Scatter plot of body weight against age of 235 children. Quadratic regression equation line is fitted with 5th, individual mean and 95th percentile distribution. Plot shows that 98% of all surveyed are within the 5-95th percentile distribution. The remaining 2% were within the 3rd and 97th percentile for body weight. Plot indicates a strong positive relationship (53%) between age and length or height in the sample population of the children surveyed in Adelaide.

Figure 3.3



Scatter plot of body weight against age of 235 children . Quadratic regression analysis of individual length of children in the population showed that 98% of children were within the 95th percentile distribution. Plot indicates a strong positive relationship (72%) between age and height in 235 children surveyed in Adelaide.

3.3.3 Haematological and biochemical analyses

3.3.3.1 *Haemoglobin (Hb)*

Complete haematological results showed that the mean Hb for the children surveyed was 120.4g/L (SD ± 10.23) with a median of 120 g/L. Analysis of the 95% confidence interval showed the 5th and 95th percentiles distribution for haemoglobin in the surveyed population as 105 g/L and 137 g/L respectively. Twenty-five percent of all children had Hb < 110g/L which is the cut off point for the pre-determined criteria used in this study while 4% of the population sampled had an Hb < 100 g/L (Figure 3.4a). Values for red cell indices also indicate the range between the minimum and maximum haematological parameters for the three age groups. Haemoglobin concentrations are also presented in table 3.2 to show the mean and standard deviations for the frequency plot (Figures 3.4a and 3.4b) in the three age groups. The minimum Hb value for the 6-12 month olds was higher than those for the other two age groups (Table 3.2). A second plot of only Caucasian children to determine haemoglobin concentration showed very similar mean Hb values of 120.3g/L ± 9.6 with 1SD being equal to 110.7g/L, compared to 109.7g/L in figure 3.3a.

3.3.3.2 *Mean corpuscular volume, (MCV)*

The mean MCV in the 6-24 month old Caucasian children sampled was 78fL, ranging from 72 - 88fL. The mean MCV of the Asia children was significantly lower than those of the Caucasian children in the three age groups (Table 3.2).

Table 3.2. Haematological and biochemical iron status values for 235 children, within the ages of 6-24 months surveyed from a broad socio-economic background in metropolitan Adelaide, South Australia.

Age and race		Haemoglobin conc. (g/L)	Mean corpuscular volume (fL)	Mean corpuscular haemoglobin conc. (g/L)	Serum ferritin conc. (µg/L)	Serum iron conc. (µmol/L)	Transferrin conc. (g/L)	Saturation (%)
6-12 months								
Caucasian (n=69)	mean	119	*78.0	334	38.0[a]	9.0	3.1	13
	range	104 - 142	72 - 88	306 - 358	9 - 142	4 - 19	2.1 - 3.8	3 - 25
Asians (n=19)	mean	121	74.0	331	37.0	10.0	2.6	11
	range	96 - 138	56 - 83	291 - 355	6 - 114	5 - 17	2.2 - 2.8	10 - 12
>12-18 months								
Caucasian (n=70)	mean	122	77.0	331	26.0[a]	10.0	3.3	14.0
	range	101 - 142	27 - 90	304 - 357	6-230	5 - 17	2.6 - 4.5	4 - 30
Asians (n=12)	mean	120	71.0	326	34.0	8.0	3.2	9
	range	87 - 150	62 - 83	293 - 347	5-64	7 - 9	2.4 - 4.5	3 - 26
>18-24 months								
Caucasian (n=52)	mean	120	78.0	332	23[a]	11.5	3.2	16
	range	81 - 152	54 - 86	283 - 361	3-52	4 - 23	2.5 - 4.8	3 - 33
Asians (n=12)	mean	121	73.0	323	21.0	NA	NA	NA
	range	89 - 138	57 - 82	279 - 353	3-70			

NA Data was not collected due to insufficient serum samples

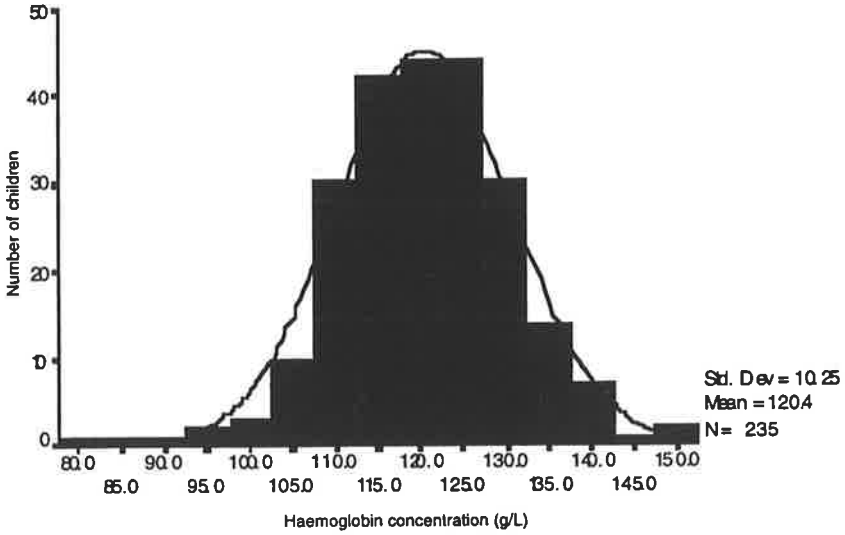
* There was a difference between the mean corpuscular volume in Asians and Caucasians at all ages ($p < 0.05$)

a: There was a significant difference between the mean serum ferritin levels of Caucasian children at all ages. ($p<0.01$)

Figure s 3.4 A and B

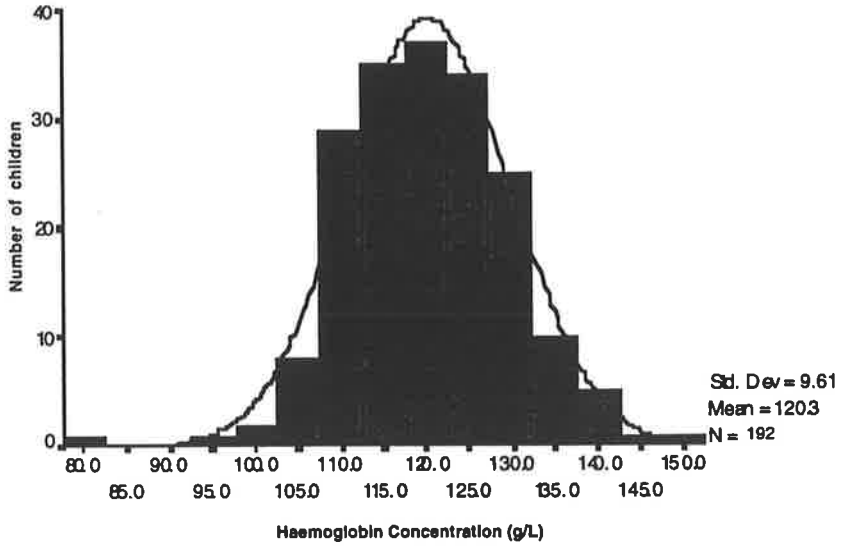
Haemoglobin distribution with normal fitted curve for 235 six to twenty four month old Caucasian and Asian children (a) and 192 Caucasian children (b) surveyed from a broad socio-economic background in metropolitan Adelaide.

A



Haemoglobin concentration of 105g/L represents 5th percentile distribution of the population while Hb level of 110 g/L is equivalent to the 10th percentile distribution of the population of 6-24 month old Caucasian and Asian children sampled. The 10th percentile levels coincides with the cut-off point for determining anaemia.

B

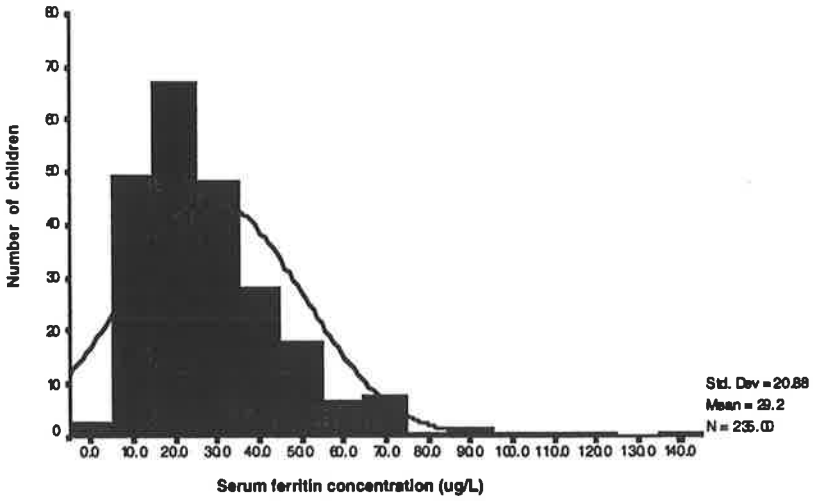


Haemoglobin concentration of 105 g/L represents 5th percentile distribution of the population, Hb level of 110 g/L is equivalent to the 10th percentile distribution and 137g/L is the 95th confidence interval of the population of 6-24 month old Caucasian children sampled. The 10th percentile levels coincides with the lower cut-off point (Hb < 110g/L) for determining anaemia.

3.3.3.3 Serum ferritin (SF)

The mean SF levels for the pooled Caucasian and Asian population sampled was 30.1 µg/L (SD± 26.9) and the median and 95th percentile values were 24 and 68 µg/L respectively. The median SF levels in Caucasian and Asian children were 24 µg/L (range, 10-68 µg/L) and 31 µg/L (range 6-69 µg/L) respectively (Table 3.2). A total of 59 Caucasian and Asian children had SF levels below 15 µg/L and 30 children (13% of both Caucasians and Asian) had < 10 µg/L (Figure 3.5). Mean serum ferritin concentration differed significantly ($p < 0.001$) between the three age groups with 18-24 month olds having the lowest SF concentration in Caucasian children (Table 3. 2). There was a negative correlation between haemoglobin and serum ferritin ($r = -0.24$, $p < 0.001$) which is indicative of reduction in iron stores to meet requirements for erythropoiesis. When serum ferritin was assessed to find if there was any association between iron stores and body weight, there was no correlation found.

Figure 3.5. Serum ferritin distribution with normal fitted curve for 235, six to twenty four month old Caucasian and Asian children surveyed from a broad socio-economic background in metropolitan Adelaide



Plot shows that the mean distribution in the population of 235 children sampled was more than the cut off value of 15 µg/L for serum ferritin. The mode distribution was 20 µg/L in 68 children. This SF distribution further supports the use of a higher SF cut-off value of 15 µg/L.

3.3.3.4 Serum iron (SI) and transferrin (Tf)

Overall the mean SI increased with age in Caucasian children, but decreased in Asian children (Table 3.2). The mean SI was higher than the lower cut off value of 8μmol/L in the three age groups. There was a wide variation in SI concentration in the population sampled, with a minimum of 4 μmol/L to a maximum of 23 μmol/L. The wide variation may be due to either individual hourly of daily fluctuation in SI, although all blood samples were taken in the morning.

The mean Tf in all age groups was higher than the lower limit of 3.0g/L, indicating increased transferrin protein in response to tissue iron need. Asian children in 6-12 months age group however, had Tf concentration (2.6 g/L) lower than the cut off limit for diagnosing NAID. This trend in tissue iron sufficiency is also reflected in the high mean Hb (120g/L) seen in this age group. The variation in the transferrin saturation was also not significant different among the age groups in both Caucasian and Asian children (Table 3.2).

3.3.4 Diagnostic classification of Iron status

Based on the classification criteria used as indicated in section 3.2.6, 31% of Caucasian children were iron deficient with a sixth of these being anaemic. Twenty eight percent of the Cambodian and Vietnamese children were iron deficient with half of them being anaemic (Table 3.3). The prevalence of NAID and IDA was more common in Caucasian infants >12 months of age compared to younger infants less than 12 months (Table 3.3). Although the cross tabulation summary of the iron status indicates a two and a half times higher incidence of IDA in the Asian population than in Caucasian children (Table 3.3), the incidence of IDA was no more common in Asian than the Caucasian children ($p < 0.08$) using Pearson's chi-squared analysis of variance between iron status and race.

TABLE 3.3 Iron status of 235, six to twenty four month old Caucasian and Asian children surveyed from a broad socio-economic background in metropolitan Adelaide in South Australia.

Age and ethnic origin	Iron Status		
	No. of Iron Sufficiency children (% ; IS)	No. of Non Anaemic Iron Deficiency children (%; NAID)	No. of Iron Deficiency Anaemia (%; IDA)
6-12 months			
Caucasians	53 (77%)	14 (20%)	2 (3%)
Asians	16 (84%)	1 (5%)	2 (11%)
>12-18 months			
Caucasians	46 (66%)	19 (27%)	5 (7%)
Asians	9 (75%)	0	3 (25%)
>18-24 months			
Caucasians	33 (63%)	14 (27%)	5 (10%)
Asians	6 (50%)	5 (42%)	1 (8%)
Sub-total			
Caucasians	**132 (69%)**	**47 (25 %)**	**12 (6%)**
Asians	**31 (72%)**	**6 (14%)**	**6 (14%)**

Children were classified into the three iron status category using the following haematological and biochemical criteria; 1). Iron sufficiency (IS): Hb concentration > 110 g/L, SF ≥ 15 µg/L, and Tf ≤ 3.0g/L, SI ≥ 8.0 µg/L and iron saturation ≥ 12%; 2). Non anaemic iron deficiency (NAID): Hb concentration > 110g/L and SF < 15 µg/L and/ or Tf >3.0 g/L, Sat < 12% and SI < 8.0 µmol/L and 3). Iron deficiency anaemia (IDA): as for 2 with Hb concentration < 110g/L. Pearson chi-quared analysis of variance between race and iron status was $X^2 = 6.2$; $p < 0.18$)

3.3.5 Dietary intake

3.3.5.1. Breast feeding

Analysis of the frequency of breast feeding showed that, 80% of all infants were exclusively breast-fed at birth, 77% for at least a month, 34% for 6 months and 11% for 12 months after birth. Caucasian infants were breast-fed longer than their Asian counterparts (6 ± 2 vs 3 ± 1 months; $p < 0.001$). Iron sufficiency was more common in children breast fed for up to 6 months of age than those for more than 12 months (Table 3.4). Non anaemic iron deficiency increased after 6 months. Twenty seven percent of children breast fed beyond 6 months of age were diagnosed as NAID (Table 3.4). The prevalence of IDA increased in children breastfed for >6-12 months (10%) compared to the other groups. Children who were breast fed for more than 12 months run the most risk of becoming IDA. A multivariate analysis in which race, age and body weight were corrected for showed that, 15 % of the variation found in SF could be explained by prolonged breastfeeding ($p < 0.001$).

3.3.5.2 Formula feeding

A small proportion of infants (11%) were formula-fed from birth but the majority of non breast fed infants were fed iron fortified infant formula at six months. Fifty-three percent of children were formula fed between 6-12 months. Formula feeding from four months of age was more common in Asian than Caucasian infants (40% of all Asian vs 18% of all Caucasian children; $p < 0.05$). Iron sufficiency was also more common (77%) in children formula fed between 6-12 months (Table 3.5). There was weak association between serum ferritin levels with the duration (months) of formula feeding in the population sampled ($r = 0.23$, $p < 0.001$). Caucasian infants who were formula fed for less than one month had a higher incidence (13%) of becoming iron deficient than those formula fed for >6-12 months (5%). Asian children formula fed 6-12 months had better iron status than those formula fed >1-6 months (Table 3.5). There was no incidence of IDA in children formula fed children for more than 12 months, with could probably due to low number of children.

Table 3.4. Relationship between length of breast feeding and iron status in 235 six to twenty four month old Caucasian and Asian children surveyed from a broad socio-economic background in metropolitan Adelaide in South Australia.

Duration of Breastfeeding (Months)	Iron Status		
	Iron Sufficiency (IS)	Non anaemic Iron Deficiency (NAID)	Iron Deficiency Anaemia (IDA)
<1 (n=55)	46 (84%)	5 (9%)	4 (7%)
1-6 (n=73)	51 (70%)	20 (27%)	2 (3%)
>6-12 (n=79)	50 (63%)	21 (27%)	8 (10%)
>12 (n=28)	16 (57%)	8 (29%)	4 (14%)

Table shows the prevalence of the three iron status in relation to the number of months of breast feeding in 6-24 month old children. The risk of developing NAID increased in children who were breast fed longer. Chi-squared analysis shows an association between low iron stores and duration of breast feeding although this was not significant ($X^2 = 13$ $p < 0.13$).

Table 3.5. Relationship between duration of formula feeding and iron status in 6 - 24 month old Caucasian and Asian children surveyed from a broad socio-economic background in metropolitan Adelaide in South Australia.

Duration of formula feeding (Months) in the two race categories	Iron Status		
	Iron Sufficiency (IS)	Non anaemic Iron Deficiency (NAID)	Iron Deficiency Anaemia (IDA)
<1 month			
Caucasian (n=85)	53 (62%)	21 (25%)	11 (13%)
Asian (n=11)	5 (45%)	2 (18%)	4 (36%)
1-6 months			
Caucasian (n=43)	31 (72%)	11 (26%)	1 (2%)
Asian (n=7)	4 (58%)	2 (28%)	1 (14%)
>6-12 months			
Caucasian(n=100)	77 (77%)	18 (18%)	5 (5%)
Asian = (24)	22 (92%)	1 (4%)	1 (4%)
>12 months			
Caucasian (n=6)	3 (50%)	3 (50%)	Nil
Asian (n=2)	1 (50%)	1 (50%)	Nil

The percentages indicate the percent of children being formula fed when the age category was assessed. It does not represent the number of children relative to the total 235 children assessed in the study. Some mothers stopped formula feeding due to financial reasons and re-introduced it again.

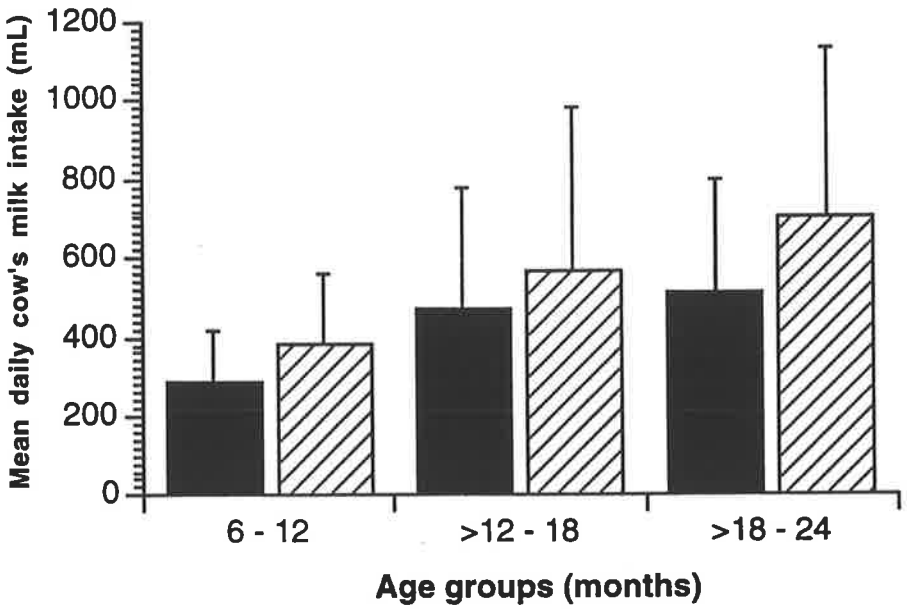
3.3.5.3 Cows' milk intake

Figure 3.6 shows that the consumption of cow's milk increased in both Caucasian and Asian infants after 6 months of age. Five percent of 6-9 months and 13% of > 9-12 months old infants consumed over a litre of cow's milk a day (Data not shown). The mean daily cow's milk intake was, 470 ± 150 mL in the combined group of NAID and IDA. Caucasian infants compared with IS infants 320 ± 130 mL (p<

0.05). Iron deficiency and IS Asian infants consumed 550 ± 170 and 395 ± 180 mL per day respectively. A multivariate analysis of variance of age and cows milk intake fitted into a model with serum ferritin as a dependent variable showed significant effects of age and cowsmilk intake on low iron stores ($r^2 = 0.22$; $p < 0.001$). By using the calcium content of South Australian cow's milk (1150 mg of calcium/of 1000 mL), the calcium content of cow's milk consumed by each child was calculated (Allen, 1993). A multivariate analysis of variance, in which age, duration of breast feeding and iron status were corrected for, showed a 12% coefficient of variation of calcium content (in cow's milk) on serum ferritin which is indicative of body iron depletion ($p < 0.01$). There was no association between calcium and haemoglobin concentration.

Figure 3.6

Mean daily cow's milk (mean ± SD) ingested by 192 Caucasian and 43 Asian 6-24 month old children surveyed from a broad socio-economic background in metropolitan Adelaide in South Australia.

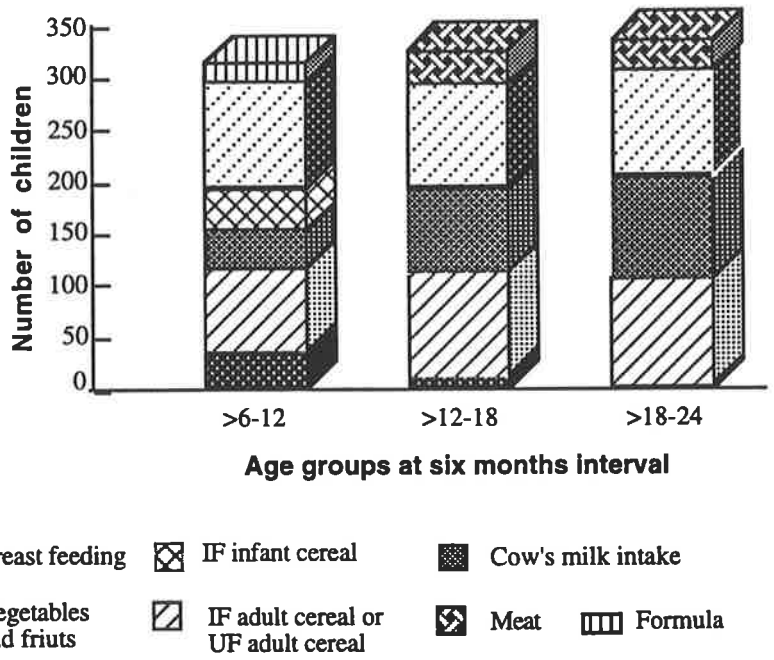


This graph shows an increase in the volume of cow's milk (±SD) with age in the 235 Caucasian(solid bar) and Asian (cross hatched bar) children surveyed. There was a significant ($p<0.01$) increase in the mean cow's milk ingested a day in children >12-18 months compared to those in 6-12 months, with a further increase in >18 -24 month old Caucasian and Asian children.

3.3.5.4 Iron fortified cereals and meat intake

Iron fortified infant cereal was offered to 58% of infants up to the age of six months. Iron fortified adult cereal consisting mainly of wheat bran and unfortified adult cereals of oats and rice were consumed for breakfast by 40% and 80% of all infants by 6 and 12 months of age respectively. Meat was sparingly consumed by 20% of all children, at most three times a week after the age of 12 months (Figure 3.7). Majority of these children (10%) who consumed haem iron ingested less than 20 g of meat or 30 g of poultry of fish a day.

Figure 3.7. Number of children consuming any given food in the 235 six to twenty four months old children surveyed from a broad socio-economic background in metropolitan Adelaide, South Australia.



The frequency graph represents the number of children consuming any given food for any give age group. Children who consumed adult cereals either ingested iron-fortified (IF) adult commercial breakfast cereals or home made unfortified cereals (UF). Majority of Caucasian children (87%) however ingested commercial adult cereals while Asian children consumed unfortified rice cereals.

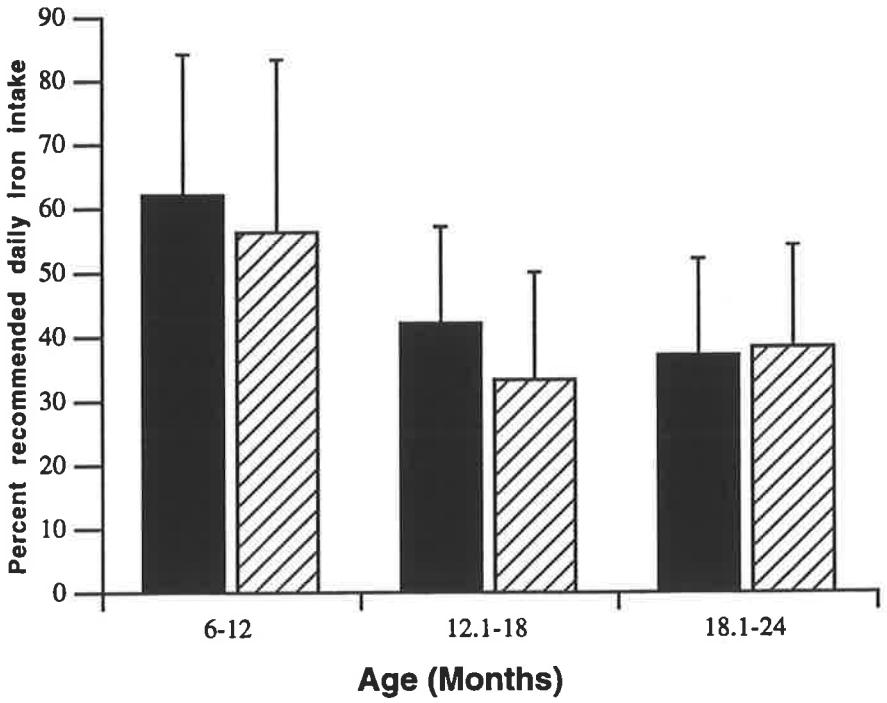
3.3.5.5 Summary of weaning practices

The frequency of foods intake of all children surveyed as described in sections 3.3.5.1 -3.3.5.4 and shown graphically in figure 3.7 clearly indicates that as breast and formula feeding decreased or ceased from 6 months, cow's milk became the main drink in 6 - 24 month old Caucasian and Asian children. Vegetables formed a major food type consumed in 6- 24 month olds with an increase in adult cereal intake with age. Although meat has high bioavailable iron both in content and availability this was consumed sparingly at all ages after 12 months (Figure 3.7). Meat was consumed approximately three times a week in both ethnic groups.

3.3.5.6 Dietary iron intake

Semi-quantitative dietary analysis of the mean daily iron intake relative to the Australian RDA for 6-24 month old Caucasian and Asian children showed that, over 70 % of all children consumed less than 50% of their RDA for iron. Only 10% of the population surveyed ingested the RDA for age. Over all children dietary iron intake decreased with age. Mean intake of children 6-12 months was 61% of RDA for iron, but that for children >12-24 months was only 41%. In Caucasian children, the mean % RDA for 6-12 months old was significantly higher (62%; $p< 0.001$) than that for >12-18 months (33%) and 18-24 months (37%). A similar trend was also seen in the Asian group 56%, 33 % and 38 % respectively, $p < 0.03$. (Figure 3.8). This decrease in iron intake with age in both Caucasian and Asian children probably reflects changes in the diets of children from the use of iron fortified formula or iron fortified cereal up to 12 months. Dietary iron intake did not appear to influence iron status in all children and did not differ significantly between Caucasians and Asians. Multivariate analysis showed that the risk factors for depletion of body iron in 6-24 months old were, the effect of age, duration of breast feeding, growth, and cow's milk intake.

Figure 3.8. Mean percent of recommended daily iron intake (RDA ± SD) of 6 - 24 month old Caucasian and Asian children in metropolitan Adelaide.



Daily iron intake calculated from a semi-quantitative dietary intake scores in relation to percent of recommended daily allowance (RDA ± SD) in Caucasian (solid bar) and Asian (cross hatched bar) children.

3.4 DISCUSSION

3.4.1 Growth profiles

Anthropometric measurements of body weight and height or length are often used to assess growth in relation to the adequacy of the diet to the biochemical requirements for tissue growth during infancy and childhood (Haschke *et al.*, 1993). The results of this current study indicate that, the growth pattern of 6-24 month old children surveyed in Adelaide were within the 3rd and 97th percentile distribution for both body weight and height. Caucasian children on average weighed more than their Asian counterparts. This difference in body weight may be due to the use of growth charts which were compiled from Caucasian children. Genetic difference between the two races may probably have contributed to the moderate difference observed in body weight and height measurements.

Results from this current study are similar to those from a study by Dewey *et al.*. (1992). They collected monthly anthropometric data from 46 breastfed and 41 formula fed infants from 0 to 18 months. They demonstrated that both breast and formula fed children were normally distributed within 3 standard deviations for weight and height, up to 12 months, although breastfed children gained significantly less weight than formula fed infants at 3-6, 6-9 and 9-12 months. The difference in growth was more pronounced in breastfed children by 13 months of age (Dewey *et al.*, 1992). The assessment of growth profile relative to human or formula milk was not assessed in the present study due to the semi-quantitative nature of the dietary assessment. Relating the observations of Dewey and coworkers (1992) to the present study may suggest that the 28 children who were breastfed longer than 12 months may have had impaired growth. Growth profiles of all children are, however, consistent with those in the Darling study (Dewey *et al.*, 1992; Heinig *et al.*, 1993) which showed a strong positive correlation between age and growth in healthy children who had ≥ 3 kg birth weight. Although children in Adelaide may have been within the 3 standard deviations for anthropometric

measurements, the effect of milk type and the nutritional quality of weaning foods on iron status were assessed.

3.4.2 Diagnosis of iron status

Results obtained from the combination of haematological and biochemical criteria in this current study, indicate that iron deficiency without anaemia was common in apparently healthy older Australian children with increasing prevalence from 17% at 12 months to 23% at 18 months and 29% by 2 years. The incidence of anaemia also increased with age with 12-18 month olds at most risk. This is the first study in Australia to use the relative patterns of change in body iron stores (SF <15 μg/L), Tf, SI and red cell indices to assess iron status in 6-24 month old children (Oti-Boateng *et al.*, 1998). Consequently, it was difficult to draw any comparison between this study and other studies which have assessed iron status in young children in Australia.

Nevertheless, a comparison of the prevalence of IDA in 6-36 month olds with an earlier study which used haematological indicators, is the survey by Lovric (1970). Comparison between the current and Lovric's study (1970) showed that iron status of Australian children has not improved since 1970 when Hb < 100g/L and microcytic and hypochromic blood films were used. Lovric (1970) diagnosed a 3% incidence of IDA in healthy 6-36 months old children compared to 20% prevalence in hospital patients in Sydney (Lovric, 1970). By using a cut-off value of 100g/L for Hb (5th percentile of distribution) compared to 110 g/L (10th percentile of distribution) used in this current study, Lovric (1970) classified iron deficiency at a stage when iron stores had declined to the point of decreased erythropoiesis. The undiagnosed anaemic children at the time could have suffered impaired growth and mental development (Oski *et al.*, 1983, Lozoff *et al*,. 1987, Walter *et al.*, 1989; Walter 1993, Beard 1993). Walter and coworkers (1992) demonstrated that children under 18 months of age whose Hb concentrations were < 105 g/L and duration of anaemia persisted for > 3 months had significantly lower motor and mental scores despite iron therapy. The lower limit of Hb < 100 g/L

for anaemia for 6-60 months infants and children, has since been changed to Hb < 110 g/L, by the World Health Organisation (1972), to avert mis-diagnosing children with nutritional anaemia, which can be easily prevented by modifying dietary intakes.

The prevalence of NAID and IDA in Adelaide infants and toddlers also differ from other studies. A recent study by Karr*et al.* (1996), in which similar cut off values for red cell indices (Hb and MCV) and a different SF value of 12 µg/L were used to diagnose prevalence of NAID and IDA in 6-23 month old children, showed an improved iron status. They diagnosed iron depletion and iron deficiency anaemia as 18.7% and 5.4% respectively, in 182 children in Sydney, relative to 25% prevalence of NAID in Caucasian children in Adelaide. By combining the two classes of iron depletion and deficiency in Sydney children, however, the prevalence of NAID (24.1%) did not differ significantly from results obtained for the Adelaide survey. The similarity in the prevalence of NAID, which was not initially detected because of different classification criteria further supports the need to establish a uniform criteria for diagnosing iron status.

In contrast to similar prevalence of NAID in Adelaide and Sydney children, there was 4.6% more IDA children in Adelaide than Sydney (Karr *et al.*, 1996). The differences in the prevalence of NAID and IDA found in these two studies can be attributed to two reasons. The first being the use of different lower limits for serum ferritin and the other is the inclusion of MCV < 70fL in the diagnosis of NAID instead of IDA as in the present survey in Adelaide. A value of SF < 15 µg/L as a laboratory indicator of iron deficiency was used in the current study based on the observation by Hallberg *et al.*, 1993. Hallberg's group (1993a) showed that at SF level of 15 µg/L, indices such as Hb, MCV, MCH and iron saturation were also lower. This lead them to suggest that SF < 15 µg/L is probably the best cut-off for diagnosing iron deficiency while SF value of 12 µg/L may under-estimate the prevalence of iron deficiency by 10%.

Reassessment of the present data using Hallberg's criteria (1993a) confirmed this. For example, by changing the cut-off value from SF < 15 to < 12μg/L, 11% of infants initially assessed as NAID in this Adelaide study may be classified as IS. If the cut-off value for anaemia was maintained at Hb < 110g/L but SF lowered to ≤ 12μg/L, 7 (39%) of IDA infants in this study would have been classified as anaemia due to "causes other than iron deficiency" and these children would have been subjected to further unnecessary investigations. It may appear that SF < 15μg/L for low iron stores in this study, compared to < 12μg/L in the Sydney study (Karr *et al.*, 1996), is over estimating iron deficiency. However, 33 out of 47 NAID Caucasian children with SF < 15 μg/L also had Tf > 3.0 g/L. At SF < 15μg/L, the probability of identifying children with impaired erythropoiesis as measured by elevation of tissue iron transport was 70%. The use of an extra diagnostic parameter (Tf) increased the probability of identifying children with low iron stores in the Adelaide study.

These prevalence data show that although biochemical and haematological parameters are more accurate in assessing iron status than dietary intake as reviewed in chapters 1 and 2, the sensitivity of haematological parameters can be affected by other confounders particularly when strict criteria are not used. The problem of sensitivity of haemoglobin parameters was resolved in this present study by using multiple assessment criteria. For example, five children could not be classified due to the presence of chronic inflammatory disease which presented as either very high SF with normal iron studies, or abnormally high iron studies with normal SF.

Another study in Spain which used an additional biochemical indicator of erythrocyte protoporphyrin, EP to those described in chapter 2, showed high prevalence of NAID and IDA in Spanish children. They used lower limits of Hb < 120 g/L, MCV < 70 fL, EP > 3 μg/g Hb, SF ≤ 12 μg/L and TIBC 16 % to classify inadequate iron status in 6 -24 month old children (Fernandez-Ballart *et al.*, 1992). They found low iron stores (SF < 12 μg/L) in 10% of the 73 children with other biochemical evidence of

iron deficiency, 10% prevalence of anaemia (Hb < 120g/L) and 75% with low TIBC. By comparison, the prevalence of IDA (10%) was higher in Spanish infants and toddlers compared to the 7% prevalence found in Adelaide children. It is however possible that if a cut off value for Hb < 110g/L had been used, fewer children could have been diagnosed as NAID. This further demonstrates the need to use the international lower limit of Hb < 110g/L to diagnose anaemia.

3.4.2 Dietary intake assessment

3.4.2.1 Breast milk and formula intake

Although dietary intakes may not be accurate in diagnosing iron status, they are useful guides in determining the pattern of food consumption which influences optimal iron nutritional status in a particular population (Fernandez-Ballart *et al.*, 1992; Mira*et al.*, 1996; Karr *et al.*, 1997a). Dietary intakes when supported by biochemical analysis can be powerful tools in assessing dietary trends that either enhance or inhibit iron bioavailability (Fuchs *et al.*, 1993; Mira *et al.*, 1996; Karr*et al.*, 1997a). Good examples of how dietary intake assessments can be used to follow nutritional changes over time, are the current Adelaide study and the epidemiological studies in United States of America (Yip, 1992) and Sydney (Karr*et al.*, 1996). In the US and Sydney studies, multiple classification criteria and dietary intakes have shown a steady decline in the prevalence of iron deficiency anaemia in infants and toddlers. In the United States over the past 20 years, the prevalence of anaemia had dropped from 10% in 1980 to 3 % in 1986 (Yip, 1990, Ernst *et al.*, 1990; Looker *et al.*, 1997) while the decline in Sydney was from 3.0% in 1970 to 1.4% in 1996 (Lovric, 1970; Karr *et al.*, 1996).

The differences in the prevalence of anaemia in America (Dallman and Johnson, 1980; Yip, 1990), Sydney (Karr *et al.*, 1996) and the current study, can be attributed to the several probable differences in weaning practice. Although the National Health and Medical Research Council (NHMRC, 1991) has provided recommendation for dietary practice in 6-24 month old children in Australia; including the introduction of solids

between four to six months (starting with iron fortified cereals), introduction of meat between the age of six months and delaying the introduction of cow's milk as predominant milk source until 12 months of age, this recommendation was not available in 1994.

Contrary to the recommendations of NHMRC, children in Adelaide were exclusively breastfed for more than six months and this was reflected in their iron status in the present study. Iron sufficiency was more common in 70% of children breastfed up to six months with a reversed trend by the seventh month. After six months there was an increase in NAID and IDA as indicated by significant decrease in serum ferritin. This is consistent with the findings of Siimes *et al.*, 1984, who showed that the full-term infant is born with adequate iron store to last the first two months of life, but by four months of age, exclusively breastfed infants require supplementary iron from weaning diets to maintain an adequate iron status.

Studies by Makrides and coworkers in Australia (Makrides *et al,* 1994, 1996) have recommended prolonged breast feeding because exclusively breast-fed infants (75% breast-fed) tend to have better maturity of the visual pathway than formula-fed infants. This current study has however demonstrated that any advice to exclusively breast feed beyond the age of six months may be at the expense of iron adequacy. When the body's endowment of iron store is exhausted, breastfeeding may induce iron deficiency anaemia thereby increasing the risk of reduced cognitive functions and motor development. This assertion has been supported in part by a study from South Africa which demonstrated that low iron status can influence the metabolism of specific n-3 fatty acids which are precursors for alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Smuts *et al.*, 1995). These polyunsaturated fatty acids, such as EPA and DHA have been associated with improved visual acuity of breastfed infants (Makrides *et al.*, 1996). Since prolonged breast feeding and iron adequacy in infancy are essential for improved cognitive functions and motor

development, there is the need to consider the effects of these two nutrients on mental and motor functions at the same time. It is possible that there is a window of opportunity during which breast feeding may offset the dangers of iron deficiency on ALA and DHA metabolism, but this has not been tested.

Although formula fed children may have impaired visual acquity (Makrides *et al,* 1996), they had better iron status compared to their breastfed counterparts in this present study. Iron sufficiency was more prevalent in children who continued to receive iron fortified formulae (IFF) after six months of age compared to those who consumed IFF for less than a month. Children who received IFF for less than a month had a six fold increase risk of IDA compared with those who were fed IFF beyond six months. This present study complements and confirms the results from a similar clinical intervention study, in healthy 3-9 months, by Haschke and coworkers (1993). They showed that breastfed infants who were supplemented with IFF (3 or 6 mg Fe/L) at 3 months up to the age of 9 months (group1), had better iron status than breastfed infants who received IFF at 6 months until the age of 9 months (group 2). Children in group 1 had significantly higher Hb and MCV levels of 126g/L and 81fL respectively compared with 124g/L and 80fL respectively in group 2. A third group of children who had formula from birth had significantly higher iron stores (102 µg/L of SF) compared to group 1 (42 µg/L) and group 2 (29 µg/L) at 9 months of age. These findings are consistent with earlier recommendation that breastfed children must receive iron supplementation from three months of age to conserve their body iron stores (Saarinen and Siimes, 1978; Siimes *et al.*, 1984).

3.4.2.2 Cow's milk intake

While the actual types of food introduced during weaning are important, the choice of milk has been shown to affect iron status (Sarinen and Siimes, 1979, Fuchs *et al.*, 1993). Infants continue to derive a significant amount of energy and other nutrient intake from their milk during weaning. This present study showed that as breast and

formula feeding ceased or decreased, cows' milk, a high source of calcium with a dose-related inhibitory effect on iron, became the main drink for over 60% of infants after six months and by 10 months 90% of all the children surveyed were ingesting over 450 mL of cow's milk a day. As expected, there was a significant increase in cow's milk intake with age, with 18-24 month olds consuming twice as much cow's milk as their 6-12 month old counterparts. This increase in diary product intake by young children can be attributed to National Health and Medical Research Council, NHMRC (1989) recommendation that 1-2 year olds should consume a mean daily intake of 700mg of calcium; equivalent of 609 mL of cow's milk. Although the Australian College of Paediatrics (1990) recommends cow's milk intake as main milk source after 12 months to safeguard against a nutritionally inadequate weaning diet, 20% of all the children in the present study ingested cow's milk in the second six months of age. The CAFHS guideline (1988) given to mothers at the time of this survey stated that cow's milk should not be introduced for the first 6-8 months, but after 6 months whole cow's milk can be introduced gradually as a supplement to infant formula or breast milk. This study indicates that majority of parents adhered to the CAFHS guidelines with detrimental consequences. Non-anaemic iron deficiency children received 45% more cow's milk than their IS counterparts (493 ± 294 mL vs 343 ± 300 mL). This is consistent with a an intervention study by Gill *et al.*, (1997) who have shown a progressive 34% fall in iron stores in children who received cow's milk from 6 to 15 months compared to 16% decrease in SF levels in children who had neither formula or cow's milk between 6-15 months of age.

3.4.2.3 Introduction of solids

The main role of weaning diets is to meet the infants nutrient requirements. This is particularly true for iron, once its stores begin to deplete at the age of 4-6 months. The progression from breast milk to solids in Adelaide infants was based not only on the infant's nutrient requirements but also on environmental influences. Although iron fortified infant cereals are recommended by the NHMRC (1989) as

preferred follow on diets, most children consumed adult fortified cereal, by six months of age. As breast and formula feeding ceased or decreased, there were only three main diets consisting of adult cereals, cow's milk and vegetables for children after the age of 12 months. The most common solid foods for 6-24 month olds were vegetables which have been shown to be high in polyphenols that can inhibit iron absorption. These vegetables consisted of carrots, pumpkins, potatoes, zucchini and pulses (predominantly baked beans). There was a significant ($p < 0.01$) increase in the number of children consuming vegetables particularly after 12 months. This results complement earlier survey in Adelaide by Retallack *et al.* 1994 who have shown that use of vegetables as weaning food was more common at 6-12 months than the recommended follow on diet of iron fortified infant cereals.

Studies in adults and children have shown that meat which is a good source of bioavailable iron because it can be absorbed several times more than non-haem iron forms similar to those found in iron fortified cereals (Bjorn-Rasmusen *et al.*, 1979; Mira *et al.*, 1996). However, meat was sparingly consumed by children at all ages in the present study, with only 10% of all children consuming 20g of beef or 30g of chicken (equivalent of 0.6 mg and 0.4 mg of Fe respectively) a day. The average daily intake of haem iron in the 10% of children who consumed beef or chicken in the present study was however more than those calculated for 12-36 month old children in Sydney (Mira *et al..*, 1996). Iron deplete children ($SF < 10$ µg/L) in Sydney consumed significantly less haem iron (0.28 mg Fe) than their iron replete counterparts (0.42 mg Fe) (Mira *et al..*, 1996). Similarly in the current study, children who consumed 0.4 - 0.6 mg of haem iron had adequate iron status, irrespective of cow's milk intake. Results from this study and those by Mira and coworkers (1996) suggests that the daily consumption of > 0.4 mg of haem iron can maintain balanced iron status in 12-24 month old children in developed countries irrespective of the amount of cow's milk intake. Whether this dietary practice can be feasible in developing countries where meat is expensive and unaffordable by most families needs to be investigated.

3.4.2.4 Dietary iron intake

Although the dietary analysis tool used in this study has not been previously validated, results indicate that it was appropriate and valid in estimating dietary iron intake of Caucasian and Asian children surveyed in Adelaide. This study showed a decreased iron intake with age, with about 70% of all children failing to consume 50 % of the Australian recommended dietary allowance (RDA) by 18 months. These children, however, did not show any signs of inability to thrive as indicated by the anthropometric measures because all children were within the 3 SD for growth. It could be argued that the dietary analysis used in this study was too crude or not specific enough to identify associations between iron intake and anthropometrics however, the results from this study are similar to those from a longitudinal study conducted by Hitchcock and Coy (1988) in which a more accurate dietary analysis package was used. They also showed that the mean iron intake at one year was less than 50 % of the RDA and even when the mean intakes increased with age there was a considerable number of children who consumed well below the RDA at two years of age (Hitchcock and Coy, 1988). A later study by Penrod *et al.* (1990) also showed similar results. In that study, nutrient intakes were assessed in infants between 8 and 13 months of age. Of the 100 infants surveyed, 52 % of these children did not meet half of the RDA (7.5mg of iron) for this age group. The decreased trend in dietary iron intake with age in this current study and that of Hitchcock and Coy (1988), Penrod *et al.* (1990) and Mills and Tyler, 1992 shows that the dietary analysis method used in the present study was specific enough to estimate daily iron intake relative to the Australian RDA.

The sharp decline in dietary iron intake of older children (>12-18 months) by 22 % and 30 % in 19 months in the present study also suggests that formula and iron-fortified cereal were the major sources of iron for 6-12 months. By 6 months of age when the consumption of iron fortified infant cereal and formula intake had decreased or ceased adult cereals which contain low bioavailable iron with a high phytate content, which inhibits iron absorption by as much as 10 % - 80% (Hallberg *et al.*, 1989a;

Hurrell *et al.*, 1992; Reddy *et al.*, 1996) was consumed by 80% of the infants. Weetbix (mainly wheat bran) which is often eaten with cow's milk was a popular cereal for children in Adelaide from as early as 4 months and this practice is similar to the findings of Retllack *et al.*, 1994. This present study also demonstrates that while most infants were taking solids at six months, majority of infants had changed from consuming high content and high bioavailable iron diets to low content and low bioavailable iron diets of cereals, legumes and cow's milk. Only 4 % of children had as little as 2 g of cooked meat, a good source of bioavailable iron, in their diet by 8 months. The low compliance in the introduction of bioavailable iron diets after 10 months, when formula or iron-fortified cereal cease despite weaning advice suggest that, parents are confused when it comes to selecting weaning diets with bioavailable iron .

Despite these dietary practice of parents not offering rich bioavailable iron weaning foods in addition to ingestion of less than 50 % of the recommended daily dietary iron intake of all the children surveyed, there was only a weak association between dietary iron intake and overall iron status, suggesting that most of the older children were relying on their reserved iron stores for physiological requirement. Alternatively, not only are the children relying on their reserved iron stores to maintain adequate iron status, but the quality of iron in the diets ingested was more important than its quantity in maintaining positive iron balance in these young children. The effects of dietary iron on iron status as assessed by serum ferritin was more frequently impaired by levels of cow's milk intake and duration of breast feeding as demonstrated in this current study. This raises the question of how accurate RDAs can be used to determine micronutrient status such as iron status which is controlled by complex interaction of other nutrients and body physiological requirement.

The discrepancy in the trend of precent RDA and iron status was evident in this current study. Only 3% of children consumed optimal RDA for iron, and it would have

been expected that 97% of children would have inadequate iron status but this was not the case. Thirty seven percent (37 %) of the surveyed population had inadequate iron status, as assessed by red cell indicators and biochemical iron studies, which was 60 % less than expected. Another way of looking at the RDA could also suggest that 50% of the Australian RDA may probably be adequate to maintain a normal iron status if iron deficiency was only present in 30 % of the surveyed population. Findings from the present study are similar to those of Wood *et al.*, (1993) in which dietary iron intake in 6-24-month olds decreased to less than 75 % of American RDA but iron deficiency (SF < 12μg/L) was not observed.

The low correlation observed between iron status and dietary iron intake in this present study and that conducted by Wood and colleagues (1993) reinforces some of the difficulties nutritionists encounter when evaluating dietary data based on RDAs. RDA is defined as the levels of intake of specific nutrient considered in the judgement of the committee on the basis of available scientific knowledge to meet the unknown nutritional need of practically every person (Truswell *et al.*, 1990; Barness and Curan, 1996). They are not the requirement of specific individuals and will indeed exceed the needs of majority of children as indicated in this present study. Iron deficient levels of 70% prevalence would have been reported in these children compared to the true biological value of 30 % if haematological and biochemical analysis had not been done. Individual variations caused by growth and development, physical activity, genetic variations, state of health, level of iron store depletion, body size and the environment in children, may contribute to differences in iron status. There is also the possibility of parents to either under or over report the dietary intake of their children to impress health workers. These within- person variability and error in food recall are major sources of error in nutrient estimation.

Sempos and coworkers (1992) have used a simple set of statistical assumptions to evaluate within-person variability in dietary intake and assessing the association

between diet and levels of the outcome variable (eg Hb and SF in the case of iron nutrition). They argue that for both daily and usual dietary surveys methods, the observed correlation is equal for the true correlation multiplied by an attenuation coefficient A: $r_{observed} = r_{true} \times A$. depending upon the accuracy of the measuring instrument. The larger the attenuation coefficient A or the closer it is to 1 the closer the observed correlation will be to the true value. This may explain the weak correlations found between the percentage recommended daily allowance (RDA) of iron intake and the true values of blood iron status in Australian children. The limitation of appropriate tools for analysis, accurate measurements and interpretation of the dietary data based on RDAs in the present study reinforces the need to quote dietary RDA data with caution when they have not been validated by biochemical analysis. Since this clinical study has assessed both dietary intake and biological outcomes it is an accurate assessment of the iron status of 6 - 24 month olds. The evaluation of individual diets in relation to composite daily diets is also useful in understanding some of these phenomena.

A third argument for the low correlation observed between dietary iron and iron status is in the bioavailability of dietary iron. There are many other dietary inhibitors like cowsmilk which are known to tenaciously bind iron thus preventing its absorption at the initial point of entry which is the intestine (Fomon *et al.*, 1981, Hurrell *et al.*, 1989). Although the relative bioavailability of iron in these diets were taken into consideration in the design of the Dietary analysis table, it appears that these measures were not adequate to explain the extent of this interaction of these diets on iron absorption, particularly in the case of calcium. There is, therefore, the need to investigate these nutrient interactions further.

Recommended Daily Allowances (RDAs) are useful guides in human nutrition but the most convincing argument from this work is to include advice on selection of foods that will enhance nutrient absorption to compliment advice on RDAs. Food types and diets vary from country to country and across cultural groups. It will be important

to replicate this study in a geographically different country to assess the accuracy of RDAs of different nutrients, particularly iron, in relation to biochemical indicators of iron status in infants and toddlers.

3.4.3 Inhibitory effects of calcium content in cow's milk

Although cow's milk in addition to weaning foods can provide the child's needs for proteins and minerals that can sometimes exceed requirements, a notable exception is iron (Penrod *et al.*, 1990). Older children who consumed large amounts of cow's milk, which contained excess amounts of calcium relative to the RDA, also had low iron intake as demonstrated in this study. Earlier studies have shown that the phosphate, calcium and casein components of cow's milk could be associated with the inhibitory capacity of cow's milk on iron absorption (Hurrell et al., 1989Fomon *et al.*, 1990; Cook *et al.*, 1991; Hallberg g*et al.*, 1992(a,b). Hallberg and coworkers (1992b) have, however, shown in adult subjects that the calcium content in cows milk is the main limiting factor for its poor iron availability, although the mechanism of inhibition at the point of absorption has not been elucidate. For example when breast milk calcium was increased to the levels found in cow's milk, the bioavailability of iron in breast milk was reduced by 70% (Hallberg *et al.*, 1992b). Cook and others (1991) have also shown that 300 mg of calcium significantly inhibits dietary non-haem iron diets containing 37 mg of iron from a simple test meal by 49 %, while non-haem iron diets containing doses of 600 mg calcium and 18 mg iron was inhibited by 62 % in adult female volunteers. Hallberg and coworkers (1994) further demonstrated in a dietary trial in adult humans and showed that 300-600 mg of calcium in a complex meal inhibited both haem and non-haem iron uptake from low-iron/high-calcium diets by 50-70%. An iron/calcium ratio dietary trial study in 21 healthy female volunteers conducted by the same group (Hallberg *et al.*, 1995) again showed that, the inhibition of iron absorption by calcium in complete daily meals containing both low and high iron content over a 10 day period was 30-35%. Although these findings demonstrate the inhibitory effects of calcium in simple tests meals (Cook *et al.*, 1991; Hallberg *et al.*,

1994; 1995), the effects of calcium on iron status during the weaning period when the diets contain 90% non-haem iron still remain to be investigated.

It can however be inferred from this current epidemiological survey that 6-24 months old children who ingested cow's milk were subjected to inhibitory effects of calcium on iron absorption. This is based on the calculation that if 263 mL of cow's milk contains the equivalent of 300 mg of calcium which has a dose dependent effect on iron absorption, then all children who consumed the mean volume of 263 mL of cow's milk with sub-optimal dietary non-haem iron contents, which is often less than 10 mg of Fe, run the risk of developing iron deficiency. The dose inhibitory effect of calcium in cow's milk was further demonstrated in this present study where Caucasian children who consumed an average of 470 mL of cow's milk a day had iron deficiency or anaemia, while their iron sufficient counterparts consumed 320 mL a day. Asian children who had a higher prevalence of anaemia consumed on average 550 mL of cow's milk a day with an equivalent of 770 mg of calcium which has been shown to inhibit iron absorption by 70 %. These results indicate that if children are to maintain adequate iron status they need to ingest not more than 400 mL of cow's milk a day. However, there is a high calcium requirement during this vulnerable period of high iron needs and NHMRC recommends a daily calcium intake of 700 mg for this age group. Out of all the weaning food intakes in Australian children, cow's milk with high calcium content was one of few foods which increased with age and was consumed in excess of RDA in 6 - 24 month olds. The reason for increase in cow's milk over other foods/drinks is because it is a cheaper milk drink than iron fortified formula.

Findings from the current study complements a clinical intervention study conducted in 6-12 months old children by Fuchs and coworkers, 1993. They have shown that consuming whole cow's milk even with iron fortified cereal (iron levels ranging from 3.5-17.5 mg/L) increased the risk of developing iron deficiency (SF < 12 µg/L) and anaemia. These results are probably due to the poor bioavailability of iron in

infant cereal coupled with the high inhibitory effect of the calcium in cow's milk. A multivariate analysis of variance of age and cows milk intake fitted into a model with serum ferritin as a dependent variable in the present study showed significant effects of age and cow's milk intake on depletion of body iron stores ($r^2 = 0.22$; $p< 0.001$). The weak correlation between calcium intake and ferritin concentration found in the current study, however suggests that the effects of inhibition of cow's milk may not have been attributed to calcium alone, hence the need to investigate the effects of calcium on iron status by varying the RDAs for iron and calcium in the normal diets of children during the weaning period.

A further multivariate analysis of variance to test the effect of calcium on iron stores, in which age and duration of breast feeding were corrected for, showed a 12 % coefficient of variation of calcium on serum ferritin which is indicative of body iron depletion. The 12 % coefficient of variation caused by calcium on inadequate iron stores in 6 - 24 month olds children in Adelaide has shown that not only does cow's milk contain a trace amount of iron, but it can also inhibit the availability of iron from other nutrients and augment the risk of iron deficiency. These findings are consistent with several other studies (Saarinen and Siimes, 1979, Fomon et al., 1981; Ziegler *et al.*, 1990; Cook *et al.*, 1991; Gleerup *et al.*, 1995; Fuchs *at al.*, 1993 (a,b). The 12% risk of the possible calcium ingestion associated with serum iron depletion in the present study compared to 30-50 % reduction in erythrocyte incorporation of radio-labelled iron in Cook's and Gleerup's groups studies (Cook *et al.*, 1991; Gleerup *et al.*, 1995) imply that, as dietary iron becomes limited for erythrocyte incorporation, there is the risk to deplete storage iron at a rate of 12 % to maintain at least 30 % rate of erythropoiesis. These findings and those from the present study indicate that calcium inhibits the transport of iron to erythropoietic sites as well as induce the depletion of body iron stores.

These findings suggest that if calcium can cause a 30 - 35 % dose inhibitory effect on iron availability, then all the children in Adelaide who drank more than 300 mL of cow's milk (equivalent to 344 mg calcium) with normal meals may derive low amounts of bioavailable iron from their diets, despite the consumption of iron fortified cereal or formula. This is especially relevant to children whose meals are often followed by some form of dairy product such as yoghurt, cheese, milk shake or milk drinks.

3.4.4 Effects of cow's milk on occult blood loss and renal solute load

Cows milk may also affect nutritional iron status adversely because of its propensity to induce occult blood loss. Two concurrent studies demonstrated in 6 - 12 month old children that infants who drank whole cow's milk in the second six months had substantial enteric blood loss with consequences of iron deficiency and anaemia (Fomon *et al.*, 1981; Ziegler *et al.*, 1990). Stool samples were not analysed in the current study, but the inadequate iron status of the children who consumed high levels of cow's milk before 12 months suggest that iron imbalance of these children could have been augmented by enteric blood loss. Tunnessen and Oski (1987) found a very low incidence of guaiac-positive stools regardless of primary beverage in the form of breast or infant formula milk, but found higher levels in cow's milk fed infants who also had lower serum ferritin levels.

Another unfavourable characteristics of cows milk is that it provides a very high potential renal solute load (PRSL) (Fomon *et al.*, 1981, Fomon 1985; Ziegler *et al.*, 1990; Ziegler and Fomon, 1993). Several studies have also found cow's milk feeding in 6-12 month olds to be associated with a marked increase in sodium, potassium chloride and protein resulting in a potential renal solute load (PRSL) that exceeds the estimated 33 mOsm / 100 kcal which can narrow the margin of safety situations that may lead to dehydration (Fomon *et al.*, 1981; Montalto *et al.*, 1985; 1986; Penrod et al., 1990; Ziegler *et al.*, 1990). In comparison with infant formula whole cow's milk

increased PRSL two folds (42 vs 21 mOsm/100kcal) in 6 month olds and nearly the same two folds in 10 month olds (39 vs 26 mOm /100kcal) (Ziegler, 1990).

Results from this present study complement and confirm earlier findings that iron status of infants fed excess amounts of cow's milk during the ages of 6-24 months. Infants and toddlers are at greater risk of being impaired than breast fed infants due to the high calcium levels and occult blood loss which impact more on iron deficiency, although anaemia may not often be precipitated in older children. There is convincing evidence from the current study and others of the dose-inhibitory effect of calcium on body iron stores and haemoglobin incorporation of dietary iron absorption, however, there is no data on the mechanism of interaction of dietary calcium on intestinal iron absorption.

There is the need to elucidate the mechanism of interaction of calcium on iron absorption at the initial point of dietary iron absorption. Anecdotal evidence indicates that children in developing countries consume less calcium but are also at risk of iron deficiency. It will therefore be important to assess the effects of different weaning practices on the iron status of children from a developing country like Ghana in order to make this PhD research more relevant to children in both developed countries such as Australia and developing country like Ghana.

3.5 CONCLUSION

In conclusion, iron deficiency and anaemia are still prevalent in western industrialised country like Australia with one out of every three 6 - 24 months old with inadequate iron status despite advancement make in food iron fortification. Iron nutrition of infants and toddlers in western industrialised countries needs careful consideration. The classification criteria for diagnosing iron status in young children needs to be evaluated because accurate diagnosis of iron deficiency is as important as the prevention of nutritional anaemia. The present study has clearly demonstrated that the disparity in reporting iron status is based on the sensitivity and specificity of assessment indicators and the cut-off criteria used. It is recommended to always quote prevalence of iron deficiency and anaemia in relation to these indicators and cut-off criteria. Accurate classification of inadequate iron status is essential to enable dietary intervention before the onset of overt anaemia which has been associated with reduced attention span, decreased immune response with an increased rate of infection, irritability and impaired mental and psychomotor development.

Breast feeding is passionately recommended in Australia largely based on findings that breast-fed infants tend to have better maturity of the visual pathway than formula-fed infants, however it is also possible that exclusively breast fed children without dietary iron supplementation at six months may prove to be counter-productive in regards to cognitive functions. This clinical study has demonstrated that one of three risk factors for body iron depletion is prolonged breast feeding beyond six months. Exclusively breast fed infants had biochemical evidence of iron deficiency at six months and by nine months all the exclusively breast fed infants without iron supplementation had lower levels of total iron binding capacity, low serum ferritin and low serum iron, all of which are indications of iron depletion. These findings are consistent with studies by Siimes *et al.* 1979, 1984 despite 13 years between these studies and the present one indicating that, the earlier advice to supplement the diets of breast-fed infants by four months has not been heeded to. Whether iron deficiency

without anaemia can have long-term effects on cognitive function is not known but we know that untreated anaemia in 6 - 24 months old is associated with impaired mental and psychomotor developmental indices. Good nutrition is crucial for optimal brain growth in the first two years of life. Without nutritionally adequate diet during the critical brain growth period, it is possible that the child may not reach his true genetic potential. Both breast feeding and iron adequacy in infancy appear to be essential for enhancing cognitive potential hence the need to investigate this interaction by establishing breast feeding as a baseline for iron absorption studies in infants and toddlers.

Cows milk intake is also promoted for strong bones and teeth in western industrialised countries, however this study has demonstrated that the calcium content in cow's milk can inhibit body iron accretion. Although several studies have shown different levels of inhibition of calcium on erythrocyte incorporation of iron, it has become more important to assess critically the effects of interaction of dietary calcium on intestinal iron bioavailability, particularly during the weaning period. Since iron and calcium are essential for physical growth, it is essential to optimise the absorption of both nutrients during the rapid growth spurt period of infancy and childhood. Elucidating the mechanism of its interaction will lay proof for understanding how other divalent cations such as zinc, which has been associated with brain growth, competes with intestinal iron absorption.

Results from this study has also shown that the majority of infants and toddlers ingest less than 50% of the Australian recommended dietary iron intake. The need for additional iron in this age group must be recognised and recommendations made for alternative weaning diets, since iron absorption is low from milk and many solids. It was assumed at the beginning of this clinical study that iron fortified cereals and other weaning diets would provide adequate intakes of iron. Values for RDAs reflected uncertainties both in estimate and in the bioavailability of iron from different diets.

While dietary estimations are based on the amount of iron required for haemoglobin synthesis and increased tissue needs in infants and young children, such estimates have been shown in this present study to be relatively imprecise compared to haemoglobin and biochemical estimates of iron status. This study has, however, showed that this is not the case. Iron in the diet is a very poor pre-indicator of iron that may actually be absorbed and therefore recommendations need to be made for the selection of weaning diets from which bioavailable iron can be optimised. The inhibitory effect of calcium on iron in doses equivalent to normal intake in toddler's meal suggests that calcium content of meals must be carefully considered in the iron nutrition of this vulnerable group who have high requirements for both nutrients. The inhibitory effect of calcium on iron status in doses equivalent to normal intake in toddlers' meal makes it imperative for critical evaluation of the two nutrients to optimise their absorption since both nutrients are essential for physical growth and mental development. Findings from this epidemiological survey has shown that RDAs for young children must be carefully evaluated particularly when they are to be used as indicators for elucidating haematological and biochemical indicators of malnutrition.

This study also suggests that recently arrived immigrants from Asia are at risk of developing iron deficiency and this group should be targeted for counselling and education about better infant nutrition practice. Since iron deficiency is prevalent in a privilege society like Australia where there is a wide selection of foods the whole year round, it will be interesting to compare the iron status of Australian children with those in a developing country such as Ghana where there is a lot of seasonal variation in the availability of foods. Such comparative studies will also increase our knowledge in understanding the effects of environment, cultural practices and socio-economic backgrounds on iron status and dietary iron intake using similar physiological cut-off points and dietary assessment tools.

CHAPTER 4

IRON STATUS AND DIETARY INTAKES IN 6 - 24 MONTHS OLD CHILDREN IN GHANA

IRON STATUS AND DIETARY INTAKES IN 6 - 24 MONTHS OLD CHILDREN IN GHANA

4.1 INTRODUCTION

The clinical study in which the iron status of 6-24 month old children were assessed in Caucasian and Asian children showed that iron deficiency and iron deficiency anaemia are still prevalent in privileged societies, in spite of technological advancement made in food iron fortification (Lynch and Hurrell, 1990; 1983; Hurrell *et al.*, 1991; Oti-Boateng *et al.*, 1994). It is estimated in industrialised and developing countries that 3-7 % and over 25-61% of children respectively, between the ages of 6-24 months have iron deficiency anaemia (Demaeyer, 1985; Taylor *et al.*, 1993; Kuvibidila *et al* ., 1993; Oti-Boateng *et al.*, 1994). For example, the prevalence of inadequate iron status as defined by serum ferritin < 15 μg/L was associated with the intakes of cow's milk (with high calcium), low iron diets and prolonged periods of exclusive breast feeding beyond six months of age (Oti-Boateng *et al.*, 1994). This study also showed that appropriate choice of foods during the weaning period was important in determining amounts of daily dietary iron ingestion. Based on 70% of the Australian children consumed less than 50 % of the recommended daily allowance (RDA) for iron for age (Oti-Boateng *et al.*, 1994).

The increased creation of low socio-economic groups, as a consequence of government cut backs in industrialised countries, and International Monitory Funds (IMF) economic restructuring policies for loan eligibility by developing countries, can be speculated to adversely affect dietary practices in these groups. These trends may particularly affect iron nutrition in infants and young children because the high bioavailable forms of iron are derived from expensive foods like meat and iron fortified food supplements, which are the first to be excluded from the diet in times of economic hardship. The problem of political instability on the African continent has given rise to variable food production, increasing the problem of iron deficiency, thus causing high

incidence of anaemia. There is very little current data on the prevalence of iron deficiency and anaemia in young children in Africa (Prual *et al.*, 1992; Kuvibidila *et al* ., 1993). A study in Niger has however shown a 47% prevalence iron deficiency anaemia in school children. Another study in 6-36 months old Zairian children showed a 61% prevalence of anaemia based on Hb < 110 g/L (Praul *et al.*,1992; Kuvibidila *et al.*, 1993). Many developing countries like Ghana have no published data on iron status in infants and toddlers as an indicator of adequate iron nutrition. Despite increased knowledge on the importance of maintaining balanced iron nutrition in the first two years, as a means of averting impaired behaviour and school performance in school age children. *Ad hoc* attempts have been made in the last five years in Ghana to improve the protein-energy status of children by promoting high protein based-weaning diets consisting of soya bean and fish meal. However, these have not been clinically assessed for their efficacy and effectiveness in improving the iron status of these children. The clinical study in chapter 3 has shown that children with high protein-energy status can still have inadequate iron status, due to inhibitory factors that can affect iron bioavailability in weaning diets.

The aim of this epidemiological study was therefore to provide additional knowledge on the nutritional adequacy of weaning diets of children in developing countries. The iron status and dietary intakes of 6-24 months old children from broad socio-economic populations in two metropolitan cities in Ghana using similar research protocols as outlined in the Australian clinical study were also investigated. Attempt was made to define the factors and weaning practices that militate against the availability of iron in weaning diets. Anthropometric measurements were assessed to investigate if there were any associations between nutrients intake and physical growth.

4.2 SUBJECTS AND METHODS

4.2.1 Subjects

Healthy 6 - 24 month old infants and children, who were full term at birth and had a birth weight not less than 3 kg, were recruited from eight well baby clinics within a 20 km radius in metropolitan Accra. Recruitment of children took place from November 1996 to January 1997. This period coincides with the dry season in Ghana when food is not abundant. Clinics were carefully selected to represent the different socio-economic and ethnic spread in Accra. In Kumasi however, children were recruited from a central Well Baby Clinic which serves half the population of metropolitan Kumasi and represents a cross section of socio-economic classes. Clinical history of children were taken and conferred with health record kept by Public Health nurses. Growth charts and vaccination records of children were checked to ascertain that they were growing well and also had been immunised against the four most common childhood diseases, these being diphtheria, whooping cough, polio and tuberculosis.

4.2.2 Anthropometric measurements.

Weights, lengths and head circumference measurements were taken employing methods used in the Australian study (section 3.2.3). Anthropometric measurements were not converted to Z-scores because raw data on the mean and standard deviation for age-in-month of these anthropometrics in 6 - 24 months old children were not available at the time of the study. Weight and height percentile values were calculated using quadratic regression equation which can give an indication of individual variation from that of the sampled population mean for the same age. The individual quadratic regression equation data of weight-for-age, length-for-age were compared with a reference chart developed by the National Co-ordinating Committee of the Ministry of Health, Ghana (1994) and percentiles of WHO/NCHS standards (Hamill *et al*, 1979). Head circumference measurements were also plotted and 3rd, 5th, 50th 95th and 97th percentiles determined.

4.2.3 Ethics approval and parental consent.

Ethics approval for the study was obtained from the Nutrition Division of the Ministry of Health in Accra and the Clinical Investigations Committee of the University of Science and Technology (UST) School of Medical Sciences in Kumasi. Parental consent was signed by parents after they had read a written explanation of the study protocol. Where parents could not read or write, the study protocol was explained to them by the investigator and a verbal consent was obtained.

4.2.4 Laboratory testing for iron status

A 2mL peripheral blood sample was obtained by venipuncture for full blood examination and biochemical iron status as described in chapter 2. Caution was taken not to recruit children who had been immunised two weeks before the assessment. Lymphocyte and eosinophil counts were also estimated to exclude infants with intercurrent infections or inflammation. Blood films were also prepared to screen for malaria plasmodium counts and sickle cell traits as described in section 2.1.2.

4.2.4.1 Biochemical assays

Biochemical assays used for iron status included SF estimation using a Boehringer ferritin kit that uses a two step enzyme-linked immunoassay principle and measured on a plate reader. To increase the probability of diagnosing iron deficiency, a new biochemical protocol that estimated transferrin receptors (TfR) was employed as described in section 2.1.3.

4.2.5 Diagnostic classification of iron status

Normal haematological and biochemical values established for 6-24 month old infants and toddlers as in chapter 3 were used in addition to standard values established by the Nyaho Diagnostic Laboratory in Ghana. The criteria described in section 2.1.4 was used to diagnose iron status. Additional parameters of RDW and TfR were used in

to increase the diagnostic accuracy of NAID and IDA in Ghanaian children because Tf, SI and %Sat were not measured.

4.2.6 Dietary assessment

A retrospective 24-hour, semi-quantitative diet recall questionnaire similar to that for Caucasian and Asian children in Australian was used as detailed in Chapter 2. A sample questionnaire is shown as appendix 1. Open or recall of all food and drinks consumed in 24 hours or where the food of the pervious day varied significantly from the usual intake because of an outing or travel, a "usual" diet recall was taken. Specific information sought in the questionnaire included the duration of breast and formula feeding, volume of formula or cow's milk intake, vegetables, fruits and fruit drinks, meat, poultry, seafood consumption per day, the frequency and the amount of food taken at breakfast, snacks, lunch and dinner in a day. Most Ghanaian diets are composite diets. Nutrient content of diets were therefore calculated from recipes, weights of specific foods using serving portions, derived from validated calculations based on household utensils such as spoons, ladles, cups and weights in grams and frequency of intake. The dietary questionnaire was validated in 30 children who were recruited into the study, by a registered Dietitian at the Nouguchi Institute for Medical Research in Ghana, using a three day weighted food record.

Total intake of nutrients was computed from all foods multiplied by the amount and frequency of intake using the Centre for Scientific and Industrial Research (CSIRO) Division of Human Nutrition's Nutrient Analysis package. Nutrients were derived from separate food items served individually, as components of mixed dishes and together with other foods were coded and entered into the Analysis Package. Food preparation methods, including type and amount of fat and salt added, whether fresh, frozen, canned or dried were taken into account to accurately calculate the amount of nutrients as a result of additives and water. Nutrients derived from usage of vitamins/mineral supplements and medicine, including brand names, were also computed. Each nutrient was a

summation and the total amount of each nutrient consumed per day was calculated. From these complex analyses, comprehensive nutrient estimations of total energy derived from sugars, protein, carbohydrate, fibre and fat were also calculated.

A comprehensive of assessment of macro- and essential micro-nutrients were also calculated from the food intakes as shown in the results section 4.3. Associations between nutrients intake of children, growth measurements and blood parameters were computed to determine the effects of diet on the iron and nutritional status of 6-24 month old children in order to make well informed nutrient intake recommendations, which are currently not available in Ghana.

4.2.7 Socio-economic status

Several epidemiological studies have shown that social factors influence well being, particularly nutrient intakes in children (Yip, 1990). Socio-economic status of parents were determined to ascertain whether there were socio-economic relationships between iron status and dietary intakes of children. The socio-economic background of children were assessed by asking specific questions about the profession of both parents. Ranking of socio-economic status was determined using the Ghana National code for educational background, income and professional classification.

4.2.8 Exclusion criteria

These were similar to those described in chapter 3. In addition to these, children identified as having impaired growth, malaria plasmodium falciparum counts in peripheral blood smears or had teenage parents were also excluded.

4.2.9 Data analysis

Statistical analyses were performed using the (SPSS) for Windows and GENSTAT programme as described in section 2.1.10. To maximise sample size, data obtained for all 345 children were analysed even if there were no corresponding blood

measurements. Correlations and regression analyses of blood and nutrient intake were only analysed for those who had both data sets.

4.3 RESULTS

4.3.1 Description of population

Four hundred and seventy eight children were approached and 378 were recruited into the study (55% boys and 45% girls). Blood samples were taken from 352 children while dietary questionnaires were completed for 345 children. Complete blood measurements were possible in 307 children due to insufficient volume of blood and/or serum samples needed for all analyses. In all, 20 children were excluded from further analysis for the following reasons; 8 children were on iron supplements of Pentavit or multivite syrup, 6 had infections or inflammations detected from white blood cells, lymphocytes and eosinophil counts or based on elevated serum ferritin measurements, a further 4 had sickle cell trait and finally 2 had malaria parasites detected on microscopic blood films. Dietary and anthropometric assessments were completed for 345 children. In summary, iron status classification was completed for 287 children (61% in the 6-12 month old age group, 27% in > 12-18 months and 12% in >18 - 24 months age groups) while dietary assessment was completed for 345 children (62% in the 6-12 month old age group, 26% in > 12-18 months and 12% in >18 - 24 months age groups).

4.3.1.2 Socio-economic background of children.

Socio-economic analysis of the 345 children included in the study showed that 28% were in the low socio-economic group (IV, LC) in which only one or both parents earned an income of less than 150,000 cedis a month (approximately US $ 100.00). This group included petty traders, hawkers and labourers. Forty-two percent of children were grouped in the second class of low-middle income (III, LMC). This second group earned between 150,000 - 500,000 cedis. This included office clerks, ancillary hospital workers, some independently owned businessmen and women. The third class which is classified as middle to upper middle class (II, MUC) earned > 500,000 - 1.5 million cedis. Twenty three percent of children were in this group. The fourth group (the upper class I or UC) earned over 1.5 million cedis a month comprised of 7 % of the population

surveyed. For simplification of analysis, children were groups into three socio-economic levels as follows (Table 4.1);

Table 4.1 Socio-economic levels of 345 children between the ages of 6-24 months surveyed in Accra and Kumasi metropolitan areas.

Age group (months)	Level 1 (UC)	Level II (LMC and UMC)	Level III (LC)
6-12 (n = 213)	18	124	71
>12-18 (n = 91)	9	67	15
>18-24 (n = 41)	4	26	11
Total	**31**	**217**	**97**
	9%	**63%**	**28 %**

Significant proportion of the children surveyed were in the Level II class, compared to nearly a tenth of the population sampled in level III class. This indicates that 63% of all the children surved were within the middles socio-economic group II.

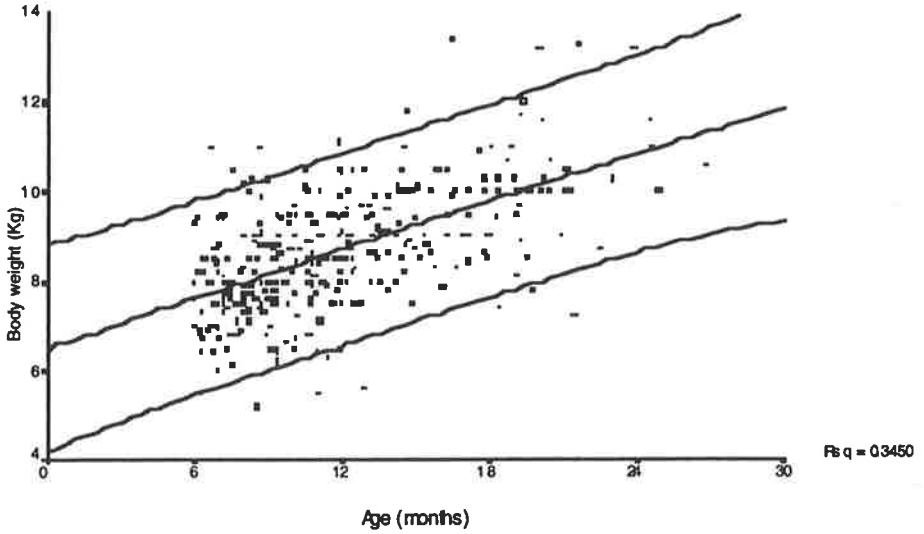
4.3.2 Growth measurements

4.3.2.1 Body weights

Figure 4.1 (a) shows the weight of all 345 children in relation to age. There was a 35% association between age and body weight ($r^2 = 0.35$, $p < 0.001$). The use of a quadratic regression equation which is appropriate for grouping the means into percentiles ranging from 5th to 95th confidence interval for the sampled population. Figure 4.1 also shows that majority of the children were within the 5th to 95th percentile range for weight. By comparison with the Ghanaian weight percentile chart compiled by the Nutrition Division of the Ministry of Health in Ghana, all children were within the Good (child is growing well) category (Appendix 4.1).

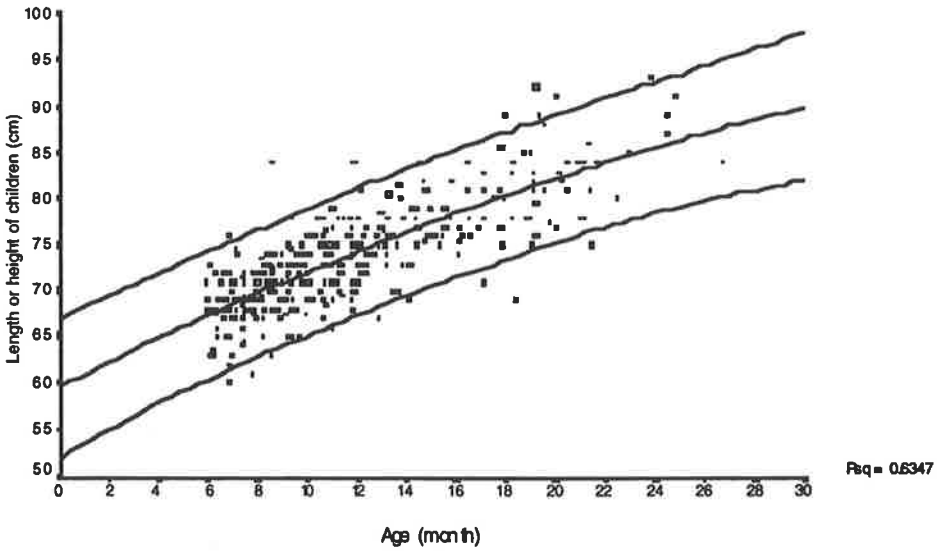
Figure 4.1
Body weight (A) and Length/height measurements (B) in relation to age in 345 children, within the ages of 6-24 month old, surveyed from a broad socio-economic background in Ghana.

(A)



This scatter plot shows the regression lines of 5th, mean and 95th percent confidence interval (CI) of distribution of the sampled population. There is a 35% relationship between age and body weight (r^2 = 0.3450). Individual Children below the 5th or 95th percent CI were, however, within the international 3 standard deviation for weight for age (Hamill *et al.*, 1979).

B



A scatter plot of the length measurements against the age in months of all children surveyed in Kumasi and Accra. Curve fits for the 5th, mean and 95th percent CI for the population sampled. Regression equation shows a 63% effect of age on length/height. Children who were out of these curves were still within the international 3 SD for weight for age (Hamill *et al.*, 1979).

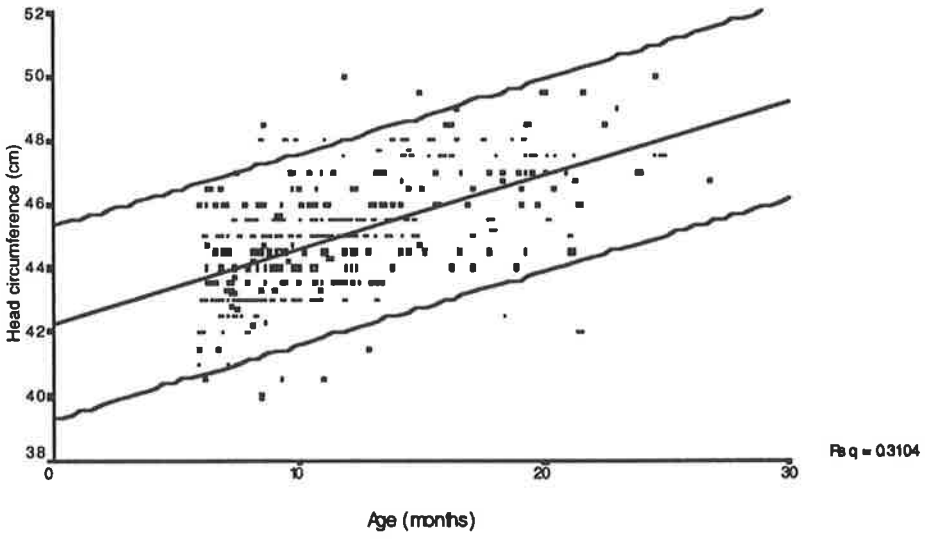
4.3.2.2 *Lengths or heights*

Using the same individual quadratic regression equation technique for data analysis, a 63% association between length /height and age ($r^2 = 0.634$; $p < 0.001$) was found (Figure 4.1b). Majority of the children were within 5th and 95th CI with only 7 children below the 5th percentile but even these were within the 3 standard deviation for height as determined by WHO/NCH (Hamill *et al.*, 1979).

4.3.2.3 Head circumference

Head circumference of children were measured and related to age (Figure 4.2). Individual quadratic regression were performed to assess the 95% CI. These values were compared with 5th, 50th and 95th percentiles of WHO/NCHS standards (Hamill *et al.*, 1979).

Figure 4.2
Head circumference measurements of 345 infants and toddlers within the ages of 6-24 months old surveyed in Ghana.



A scatter plot of the individual head circumference measurements against age of 345 children surveyed in Kumasi and Accra. Curve fits for the 5th, mean and 95th percent CI for the population sampled. Regression equation shows a 31% of age on head circumference measurements. Children who were out of these curves were still within the international 3 SD for head size for age (Hamill *et al.*, 1979).

4.3.3 Haematological indices

Full blood examination was completed in 287 children as shown in Table 4.2. The extreme percentiles of 5th, 10th and 95th distribution of all haemoglobin parameters are given to assess the degree of anaemia and iron adequacy in the population of children surveyed in Table 4.3 and supported by graphic presentation in Figure 4.3 (a). The 95% confidence interval (CI) approach in addition to the mean and standard deviation were computered because in the case of many laboratory values, particularly biochemical iron indicators, the values deviate markedly from a Gaussian distribution (Yip *et al.*, 1984). The 5th and 10th percentile values for Hb were lower than the cut off limit for defining anaemia as shown in Table 4.3. The 3rd and 97th percentiles were also 80.0 and 121.0 g/L respectively (Figure 4.3a). The frequency of distribution of Hb in all children surveyed had a mean of 103 ± 12 g/L with a skewness of - 0.76. The mean Hb in the population sampled was also below the predetermined cut off limit of 110 g/L (Table 4.2). Only 45% of all children had Hb levels more than 110 g/L, 37 % had mild anaemia with Hb levels ranging from 105 - 109 g/L and the remaining 18% had mild to chronic anaemia with Hb levels below 105 g/L (Figure 4.3a). Degree of iron sufficiency increased with age with older children (> 18-24 months) showing higher mean Hb levels (108 g/L ± 12) compared with children 6-18 months (103 ± 11 g/L) (Table 4.2). This difference tended towards significance ($p < 0.06$) (Table 4.2).

The mean for Hct in all children was 33.4 ± 0.2 %. The 5th percentile for Hct was lower than the cut off limit of 32% (Table 4.3). The differences in Hct in the three age groups was not significant ($p < 0.07$). There was a strong positive correlation between Hb and Hct as expected ($r = 0.93$, $p < 0.0001$), indicating the specificity of measuring Hct as an indication of Hb if facilities are not available for assessing Hb.

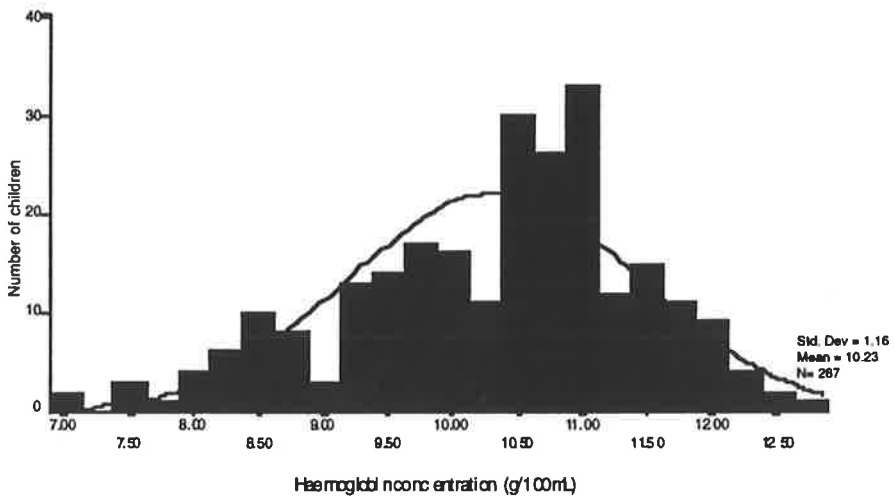
Table 4.2. Haematological and biochemical iron status values for 287 children within the ages of 6-24 months surveyed from broad socio-economic background in two metropolitan cities (Accra and Kumasi) in Ghana

Age group		Haemoglobin conc(Hb) (g/L)	Haematocrit (Hct) (%)	Mean corpuscular volume (MCV) (μm^3)	Mean corpuscular haemoglobin (MCH) (pg)	Mean corpuscular haemoglobin conc (MCHC) (g/L)	Red cell distribution width (RDW)(%)	Serum ferritin conc. (SF) (µg/L)	Transferrin receptors conc. (TfR) (µmol/L)
6-12 months (n = 174)	mean (SD)	102 ± 11	33.2 ± 3.5	72.0 ± 7.0	22.0 ± 2.5	306 ± 12.0	17.4 ± 2.7	30.0 ± 28.0	8.9 ± 4.1
	median	105	33	71.7	21.7	307	17.0	19.0	8.4
	range	52 .0 - 124.0	16.9-42.2	45.0 - 87.2	13.7 - 27.2	250 - 337	7.5 - 29.0	1 - 161	1.4 - 25
>12-18 months (n = 79)	mean (SD)	103.0 ± 12.0	33.5 ± 3.8	70.6 ± 8.1	21.5 ± 3.0	304 ± 10.0	18.1 ± 2.9*	23.6 ± 20.6*	9.9 ± 6.3*
	median	105.0	34.0	72.1	22	303	18.2	12.0	8.9
	range	71- 123.0	24 - 40.9	50.4 - 86.0	14.8 - 27.0	270 - 325	12.3 - 23.5	1.0 - 139	1.42 - 34.9
>18-24 months (n = 34)	mean (SD)	108 ± 12	34.9 ± 3.7	74.0 ± 7.0	22.9 ± 2.4*	308 ± 8.7	15.6 ± 3.0	30.6 ± 30.0	7.9 ± 3.0
	median	107	35.3	73.5	23.1	309	15.3	19.5	8.4
	range	71 - 127	24 - 41.9	58.5- 88.0	17.5 - 26.9	290 - 326	5.0 - 20. 0	1.9 - 108.0	1.3 - 12.3
*Analysis of variance and between blood values and age groups	Group total	103 ± 11.2	33.4 ± 3.6	71.6 ± 7.1	22.0± 2.6	305 ± 11	17.3 ± 2.9	28.3 ± 30	9.05 ± 4.7
	f	f = 2.7	f = 2.6	f = 2.1;	f=3.0	f = 1.2;	f = 7.9;		f = 0.7
	p<	p<0.07	p<0.07	p<0.1	p< 0.05	p<0.2	0.0004		p< 0.4)

SD is an abbreviation for ± standard deviation. * Indicates significant difference (p<0.05) between age groups.

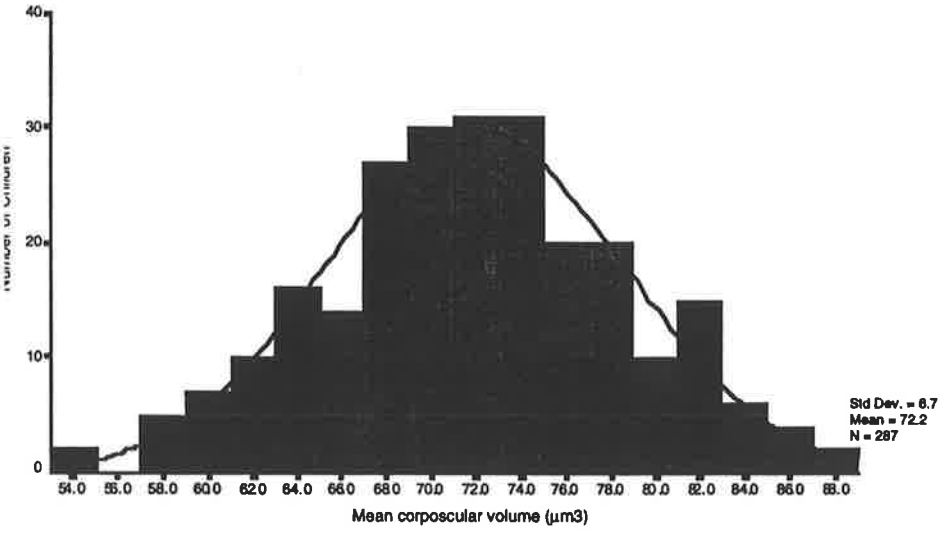
Figure 4.3. Distribution of haemoglobin (A) and Mean corpuscular volume (B) in 287 children within the ages of 6-24 months surveyed from a broad socio-economic background in Ghana.

A



This is a graphic summary of Hb values in table 4.2. The distribution curve fit shows that the mean of the population Hb is 10.3g/dL (equivalent to 103g/L), with the mode and median values of as Hb 107g/L and 105g/L respectively. There values were all lower than the cut off limit for classifying anaemia.

(B)



This plot is a graphic summary of mean corpuscular volume (MCV) values in table 4.2. The distribution curve fit shows that the mean of the population MCV (71.6 $\mu m^{3)}$ which coincides with the mode (72.5 μm^3) and median (72.1 μm^3)distribution. The mean MCV is tat the lower limit for classifying anaemia.

Mean corpuscular volume (MCV) which is a good indicator of mycrocytosis was assessed in all children. The mean MCV in the 287 children assessed was 71.6 $\mu m^3 \pm$ 0.4 and a median of 72.1 μm^3 which is at the cut off for anaemia diagnosis (Table 4.2). The 5th and 95th percentiles distributions were 62.5 and 85.8 μm^3 respectively (Figure Table 4.3). Mean corpuscular haemoglobin (MCH) for all children was 22 pg ± 0.17 pg and the median MCH value was the same (Table 4.2).

Mean red cell distribution width (RDW) in all children was 17.3 % ± 0.18. The 95% CI was 13-22 % respectively. Most of the children had higher RDW than 17% which is an indication of increased erythropoiesis (Tables 4.2 and 4.3). The RDW values were significantly different in the three age groups with older children showing better iron status ($p < 0.004$). There were strong negative correlations between RDW and MCV, and RDW with MCH but only moderate inverse correlations between RDW and Hb, RDW and Hct (Table 4.4). There was however a positive correlation between MCV and Hb ($r = 0.63$, $p < 0.001$) showing a reverse trend of association between mean corpuscular volume of red cell distribution which is the rate of erythropoiesis (Table 4.4).

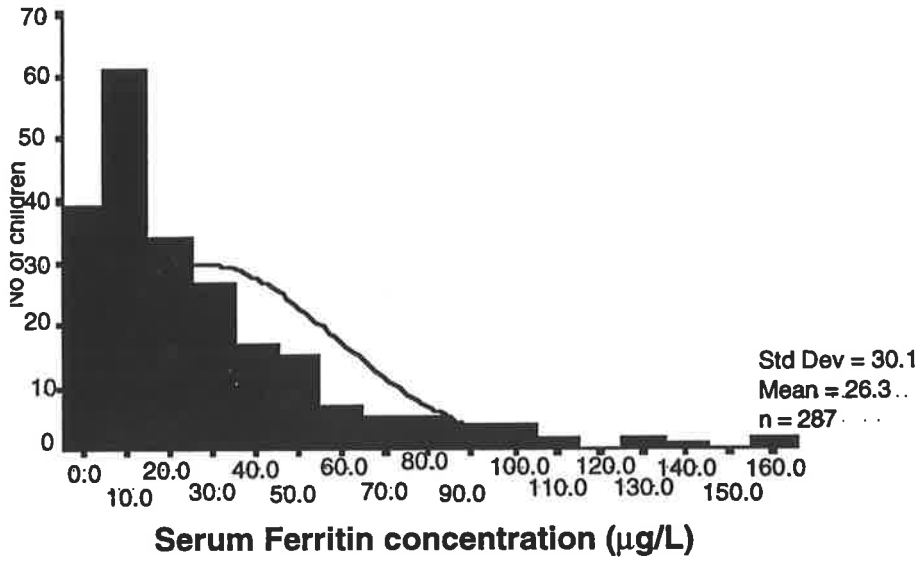
4.3.3.2 Biochemical indices

4.3.3.2.1 Ferritin

Serum ferritin abnormalities also decreased with age. The mean body iron store (SF) estimation for all children was 28.3 ± 30.1 µg/L (ranging from 2 - 161 µg/L) with a median of 18.7 µg/L (Table 4.2; Figure 4.4). The 95% CI was 1.9 - 94 µg/L respectively and a variance of 90% (Figure 4.4). These mean and median values were higher than the cut off value of 15 µg/L. Children in the >12-18 months group had lower ferritin levels than 6-12 and >18-24 month olds, although this was not significant (Table 4.2). Least square means analysis showed that IS children had the highest ferritin levels (34.0 µg/L ± 3.5 SE, $p< 0.0001$) compared to NAID (20.0 µg/L ± 7.9 SE, $p< 0.01$) and IDA children (20.0 µg/L ± 3.9 SE, $p< 0.0001$), despite a cut off value of 15µg/L for defining iron deficiency. This wide range in SF concentration in both NAID and IDA

could have accounted for a mean which was higher than the cut off limit of 15 μg/L. The use of TfR to assess iron deficiency and Hb to define anaemia increased the specificity of diagnosing iron deficiency.

Figure 4.4. Serum ferritin concentration in 287 children within the ages of 6-24 months from a broad socio-economic background surveyed in Ghana



The 95% coefficient of variation of distribution shows the 5th and 95th percentiles as 1.9 and 94.0 μg/L respectively. The mode distribution is 15 μg/L, which is the cut off limit for iron deficiency. The mean of 28 μg/L is nearly twice the cut-ff value. This indicates a non Gausian distribution of serum ferritin.

Table 4.3. Confidence interval (95th) values for haematological and biochemical iron status indicators in 287 Ghanaian children between the ages of 6-24 months.

Blood parameters	Frequency Distribution (percentiles)			
	5th	10th	50th	95th
Haemoglobin (g/L)	82	86	105	120
Haematocrit (%)	27	28	34	36
Mean corpuscular volume (µm3)	63	64	72	86
Mean corpuscular haemoglobin (pg)	18	19	22	26
Red cell distribution (%)	13	14	17	22
Serum ferritin (µg/L)	1.9	3.0	18.7	94.2
Transferrin receptor (µmol/L)	1.8	4.9	8.4	17.3

The 5th and 10th percentile distribution values for all the haematological parameters are lower than the lower limits for international classification of anaemia. For example the 10th percentile for Hb is only 86g/L which is far below the lower limit of Hb<110g/L).

Table 4.4. Pearson's correlations between age, haematological and biochemical iron status indicators of 287 children within the ages of 6-24 months, from a broad socio-economic background surveyed in Ghana.

	Transferrin receptor	Serum ferritin	RDW	MCH	MCV	Haematocrit (Hb)	Haemoglobin (Hct)
Age							
	0.02	-0.41	-0.12	0.07	0.09	0.18*	0.18*
	<0.8	< 0.5	< 0.06	< 0.27	0.20	0.004	0.02
Haemoglobin							
r	-0.563	0.04	-0.5*	0.62**	0.63**	0.93**	
p	0.0001	<0.6	<0.0001	<0.0001	<0.0001	< 0.0001	
Haematocrit							
	-0.460*	0.04	-0.41*	0.42*	0.50*		
	<0.0001	<0.61	<0.0001	0.0001	<0.0001		
MCV							
r	-0.65**	0.101	-0.63**	0.964**			
p	< 0.0001	<0.19	< 0.001	0.0001			
MCH							
r	-0.410*	0.05	-0.64**				
p	0.0001	<0.5	<0.0001				
RDW							
r	0.373*	0.036*					
p	<0.0001	< 0.63					
serum ferritin							
r	0.15						
p	<0.18						

* Values show weak to moderate associations between variables. ** Values show strong associations. There was a very weak association between age Hb and Hct. There were negative associations between Transferrin receptor (TfR), Haemoglobin (Hb), haematocrit (Hct), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) and a positive correlated with correlation between TfR and red cell distribution width (RDW). There was no association between serum ferritin and any of the blood parameters.

4.3.3.2.2 Transferrin receptor (TfR)

Transferrin receptor estimation was only possible in 180 children. The mean TfR for these children was 9.0 ± 0.5 µmol/L (1.3 - 35 µmol/L) and a median of 8.4 µmol/L indicating that majority of children were at the borderline of 8.5 µmol/L (Table 4.2). This was attributed to increased erythropoiesis. No significant differences in TfR expression was observed with age, although older children had better iron status (Table 4.2). Transferrin receptor (TfR) assay is a new diagnostic tool with few prevalence studies being compiled therefore, correlations were computed to find out if there were any associations between the rate of erythropoiesis which is indicative of transferrin receptor synthesis (TfR) and other red cell indicators such as Hb, MCV, RDW and body iron stores (SF). Rate of erythropoiesis (TfR) was inversely related to Hb ($r = -0.56$, $p < 0.0001$), MCV ($r = -0.65$, $p < 0.0001$) and body iron stores ($r = -0.31$, $p < 0.03$). Correlations between TfR and RDW were however positive ($r = 0.46$, $p < 0.0001$) (Table 4.4). These trends indicate a decline in the size of erythroid precursors and the severity of tissue iron deficiency. The correlation between TfR and RDW ($r = 0.46$, $p < 0.0001$) and RDW and Hb ($r = -0.47$, $p < 0.0001$) showed a close relationship between these three red blood cell indicators (Table 4.4).

4.3.3.2.2 Red blood cell riboflavin (riboflavin)

The mean riboflavin levels in the 170 children assayed was 1.25 ± 0.2, which is an indication of adequate body riboflavin status (Table 4.5).

Table 4.5. Riboflavin measurements in 6-24 month old children in Ghana

Age group (months)	6-12	>12 - 18	>18-24	Total mean
Riboflavin				
Mean	1.26 ± 0.22*	1.22 ± 0.11	1.23 ± 0.2	1.25 ± 0.2
Median	1.22	1.23	1.26	f = 0.14;
Range	1.0 - 1.67	1.02 - 1.34	1.02 - 1.42	p< 0.8

Riboflavin values are in mean ± SD, median, minimum and maximum values. *The mean riboflavin measurement was significantly higher in 6-12 month olds than any of the other age groups.

There were no difference in riboflavin status in the three age groups although older children had slightly better status than young ones (1.2 vs 1.3). There was no significant difference in the riboflavin levels in the three age groups ($p<0.8$) (Table 4.5

4.3.4 Laboratory diagnosis of iron status

Using pre-determined cut off limits for haematological and biochemical diagnosis of IS, NAID and IDA in 287 children for whom complete blood analysis was performed (haematological and one or two biochemical values), one-third of the population surveyed was IS with the remaining two thirds classified as having inadequate iron status (Table 4.6). Fifty five percent of all children surveyed were diagnosed as having nutritional anaemia which is consistent with microcytic and hypochromic anaemia. Iron deficiency was more common in 6-18 than >18-24 month olds. Overall the prevalence of IS, NAID and IDA was significantly different between the three age groups. The prevalence of iron sufficiency was more than doubled after 18 months (27% IS in 12 - 18 months vs 56% in > 18-24 months; $p < 0.001$) (Table 4.6). Socio-economic status as described in section 4.3.1.2 and table 4.1 had no significant effect on iron status in the age groups studied.

4.3.5 Dietary assessment

Dietary assessment analysis conducted for 345 children using the comprehensive CSIRO Dietary Analysis programme is reported under three main headings namely, energy, macro- and micro-nutrients intakes are shown in tables 4.7a - 4.7c. Nutrient intakes were compared with the international recommended daily allowance (RDA) for 6-12 and >12-24 month olds (Barness and Curran, 1996). Bivariate correlations were performed to determine associations between energy, macro- and micro- nutrients intake and iron status as well as anthropometrics and socio-economic backgrounds. Multivariate analyses were also performed to predict inhibitory or enhancing factors on haematological and biochemical indicators of iron status.

Table 4.6 Prevalence of iron sufficiency (IS), non-anaemic iron deficiency (NAID) and iron deficiency anaemia (IDA) 287 children within the ages of 6-24 months surveyed in Ghana.

AGE GROUP (Months)[a]	IRON STATUS		
	Iron Sufficiency(IS)	Non-Anaemic Iron Deficiency (NAID)	Iron Deficiency Anaemia (IDA)
6-12	54	18	102
(n=174)	31 %	10%	59%
>12-18	21	14	44
(n = 79)	27%	18%	55%
>18-24	19	2	13
(n=34)	56%	6%	38%
***Total**	**94**	**34**	**159**
	33%	**12%**	**55%**

[a]There were 61% of children were in the 6-12 month old age group, 27% were more than 12 months but less than 18 months and the remaining 12% were in the >18 - 24 months age groups.

*Pearsons correlation between iron status and age groups (degrees of freedom = 4) was Chi-squared = 12.4 ($p < 0.01$), indicating an improvement in nutritional iron status with age, in 287 infants and toddlers.

4.3.5.1 Macronutrients

4.3.5.1.1 Energy intake

The mean total energy intake derived from macro nutrients such as carbohydrates, fats and proteins was 3278 ± 1444 kjoules per day for all age groups. Mean total energy intake increased by 16% ($p < 0.04$) from 12 months and remained at similar levels up to 24 months (Table 4.7a). Overall, complex carbohydrate contributed 52% to total daily energy consumption in the 6-24 month old children surveyed. Thirty three percent of the total energy intake was derived from fats and the remaining 15% from proteins and simple sugars (Table 4.7a). The relative daily protein consumption in the children surveyed increased with age ($r = 0.26$, $p < 0.001$).

Mean daily protein intake of all these children was above the recommended daily allowance for the three age group levels, indicating that the children surveyed were not protein-energy malnourished. Both infants and toddlers consumed excess amounts of protein. Children 6-12 months exceeded the RDA for protein. Older children (18 - 24 months) exceeded their RDA for protein by 80% with more than a 100% increase by the age of 24 months. There were however, no significant difference in the energy consumption levels in the three iron status categories, although anaemic children consumed 10% more kjoules than IS and NAID children.

Mean dietary fat consumption in all children was 27 ± 15 mg per day. Older children had 36% more fat in their diets than younger 6-12 month olds ($p < 0.003$). There was an association between daily fat intake and age ($r = 0.20$; $p< 0.001$). The percent daily fat consumption was within the internationally acceptable range of 35 - 45%. Anaemic children had 14% more fat in their diets compared to NAID and IS children but this was not significant.

Fibre content in the diets of children increased with age. Toddlers (> 12 - 18 mo) eating more than twice the amount of fibre ingested by infants ($p < 0.0001$) (Table 4.7a). There was, however, no significant differences in the fibre intakes of the three iron status groups, although anaemic children consumed 22 % more dietary fibre than NAID children.

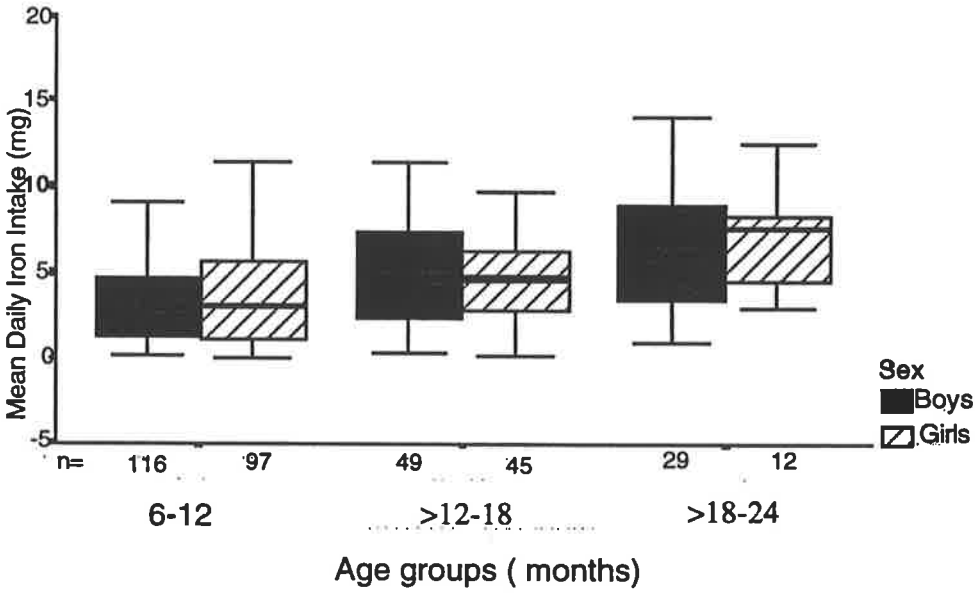
4.3.5.2 Micro nutrient intakes

The micro-nutrient intakes of children were classified into two groups. Table 4.7b shows analyses of minerals in the diets of the 345 children in the 6-24 month old age group while Table 4.7c shows vitamin content of the diets.

4.3.5.2.1 Dietary iron intake

Overall, the mean daily iron intake in the 345 Ghanaian children assessed was 4.4 ± 3.9 mg with majority (median) of children consuming only 3.6 mg Fe /day. Over 82% of all children consumed less than 50% of the recommended daily iron intake with only 24 children (6%) of all children meeting their recommended iron intake of 10 mg Fe or more a day. Although there was an increase in the mean daily iron intake of these children with age, ($r = 0.3$, $p < 0.0001$) mean daily iron intakes of infants and toddlers were significantly lower than the international RDA of 10 mg of Fe/day for children 6-24 months (Figure 4.5). Infants 6-12 months consumed on average 36% of RDA for iron while toddlers' daily iron intake reached only 60% of their RDA (Table 4.7b). There was no difference in mean daily iron consumption between boys and girls in 6-12 and >12-18. However, as children got older (>18-24 months), girls consumed on average 30% more dietary iron than boys in the same age group. (Figure 4.5)

Figure 4.5. Mean daily iron intake in 345 6-24 months old children (56% boys and 44% girls) surveyed in Accra and Kumasi metropolitan areas in Ghana.



This box plot shows the mean dietary iron intake in 6-24 month old boys and girls surveyed in Ghana. The mean RDA for infants and toddlers is 10mg Fe/day. The box indicates the mean distribution with standard deviation bars. The line across the box indicates the median of Fe intake in the various age groups in boys and girls.

TABLE 4.7a Mean daily total energy intakes derived from carbohydrates, simple sugars, protein and fat in 345 Ghanaian children between the ages of 6-24 months of age. Values are quoted in mean ± SD and compared with international RDA. Median values are also stated.

Age group		Carbohydrate (CHO) (g/day)	[a]Protein (g/day)	*Fat (g/day)	*Fibre (g/day)	*Simple Sugars (g/day)	*% Complex CHO	*% Fat	*Total energy (kjoules)
6-12 months	**RDA**		**14g**				**45 - 55%**	**35 - 45 %**	**4000**
(n = 213)	mean (SD)	101.0 ± 66.3	20.7 ± 14.5	25.3 ± 15.0	3.5 ± 4.0	27.5 ± 21.2	52.8 ± 6 10.0	33.6 ± 11.1	2975 ± 1737
	median	90.0	17.5	21.5	2.5	24.6	52.3	34.0	2683
	range	5.8 - 414.5	1.0 - 117.0	0.32 - 145.0	0.00 - 38.2	0.04 - 2712	23.8 - 81.7	3.3 - 57.7	231 - 14388
>12-18 months	**RDA**		**16g**				**45 - 55%**	**35 - 45 %**	**4800**
(n = 91)	mean (SD)	117.3 ± 52.4	26.0 ± 11.1	28.0 ± 15.0	5.3 ± 3.9	21.5 ± 14.3	54.1 ± 9.3	30.7 ± 10.5	3429 ± 1386
	median	118.5	25.9	24.2	4.8	17.6	55.0	30.2	3377
	range	22.0 - 300	4.2 - 74.2	5.4 - 108.0	0.00 - 27.7	1.9 - 66.4	23.3 - 76.2	7.8 ± 61.2	884 - 10304
>18-24 months	**RDA**		**16g**				**45 - 55%**	**35 - 45 %**	**4800**
(n = 41)	mean (SD)	100.3 ± 39.1	28.9 ± 12.7	34.2 ± 17.1	6.1 ± 3.3	15.4 - 12.7	48.0 ± 12.0	36.0 ± 12.0	3428 ± 1211
	median	92.4	25.5	35.2	5.5	11.3	47	35.5	3373
	range	28.8 - 185.7	5.5 - 66.1	3.24 - 80.2	0.00 - 15.3	2.5 - 65.7	19.0 - 77.0	6.0 - 67.0	1156 - 6519

[a]Protein is the only nutrient with RDA for energy intake. The RDAs were based on Allen (1993) and Barness and Curan (1996) . If RDAs were the same for the age groups, analysis of variance was determined. There were no RDAs in weight for the remaining nutrients in this table. Instead, the RDA is expressed as in percentages. The range indicates minimum and maximum values for each nutrient. * Shows significant differences ($p<0.01$) between age groups.

TABLE 4.7b

Mean daily micro nutrients and mineral intakes of 345 Ghanaian children between the ages of 6-24 months of age. Values are quoted in mean ± SD and median. Mean daily intakes of various nutrients are compared with international recommended daily allowances (RDA).

Age group		Dietary iron (mg/day)	Calcium (mg/day)	*Magnesium (mg/day)	*Potassium (mg/day)	*Phosphorous (mg/day)	Sodium (mg/day)	Carotene (µg/day)	Zinc (mg/day)
6-12 months	**RDA**	**10mg**	**600mg**	**60mg**	**470mg**	**500mg**	**140mg**	#	**5mg**
(n = 213)	mean (± SD)	3.6 ± 3.0*	219.0 ± 187	59.0 ± 59. 6	621 ± 438	285± 220	92.2 ± 64.0	102 ± 289	2.7 ± 1.6
	median	3.0	162.0	43.0	485 .0	239.4	72.0	24.0	2.3
	range	0.06 - 14.2	222 - 1515	2.4 - 509	46.4 - 2898	11.2 - 1553	1.1 - 405.0	0.0 - 3726	0.22 - 12.7
>12-18 months	**RDA**	**10mg**	**800mg**	**80mg**	**980mg**	**800mg**	**320mg**		**10mg**
(n = 91)	mean (± SD)	5.4 ± 5.1	255 -± 265	93.6 ± 58.7	907 ± 537	370 ± 211	147 ± 112	146 ± 187	3.5 ± 1.5 [b]
	median	4.7	159.4	86.0	797.0	316.0	107.5	67.4	3.6
	range	0.22 - 42.8	45.5 - 1538	10.2 - 257.4	193 - 2258	48.0 - 1020	29.6 - 449.3	0.00 - 956	0.9 - 8.6
>18-24 months	**RDA**	**10mg**	**800mg**	**80mg**	**1560mg**	**800mg**	**580mg**		**10mg**
(n = 41)	mean (± SD)	6.7 ± 3.8	236 ± 244	120.0 ± 70.4[a]	1002 ± 576	443 ± 221	191 ± 140	343 ± 607	4.4 ± 2.3 [b]
	median	6.5	140.1	117.0	984.0	436.3	157.2	179.4	4.7
	range	0.90 - 19.1	47.0 - 1203	10.8 - 302.0	144 - 2339	56 - 975	42.7 - 604 3	0.00 - 3792	1.1 - 11.3

RDA was not available for infants and toddlers. The RDAs were based on Allen (1993) and Barness and Curan (1996) . If RDAs were the same for the age groups, analysis of variance was determined. * There were significant differences (f=17; p<0.001) between the RDAs for iron in the three age groups. [a, b] RDA for magnesium and zinc significantly increased with age (f=23; p<0.0001 and f=20, p< 0.001). The range indicates minimum and maximum values for each nutrient. * Shows significant differences (p<0.01) between age groups even in proportion to the RDAs for the particular age group.

TABLE 4.7c. Mean daily vitamin intakes of 345 Ghanaian children between the ages of 6-24 months of age derived from simple sugars, complex carbohydrate, protein and fat. Values are quoted in mean ± SD and compared with international RDA. Median values are also stated.

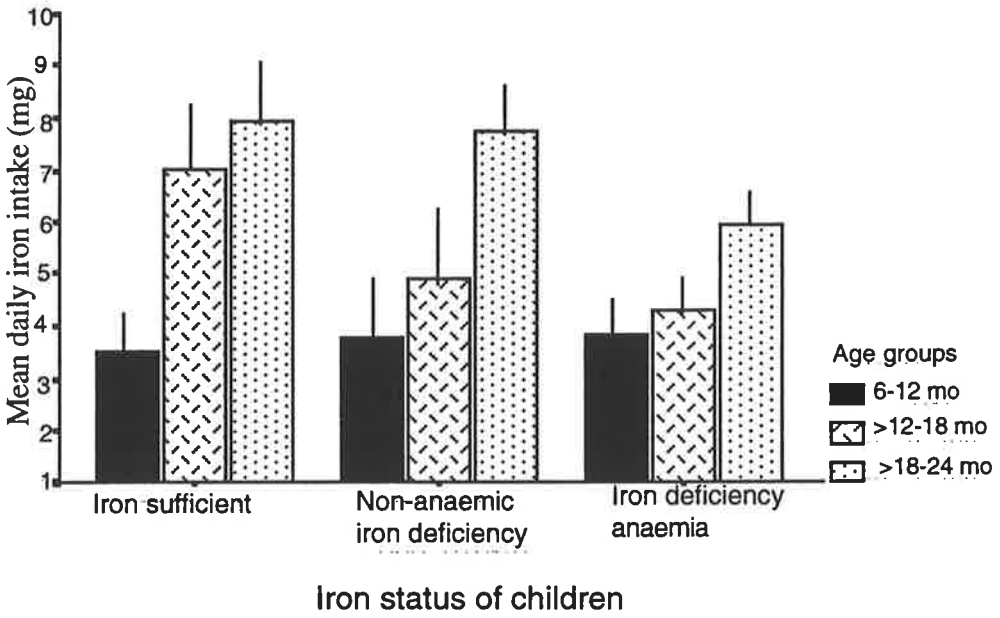
Age group		Riboflavin (mg/day)	*Nicotinic acid (mg/day)	Potassium nicotinic acid (mg/day)	Thiamine (mg/day)	Vitamin C mg/day	Vitamin D µg/day	Retinol (µg/day)
6-12 months	**RDA**	**0.5**	**6mg**	**#**	**0.4 mg**	**35mg**	**10 mg**	**375**
(n = 213)	mean (SD)	0.34 ± 0.27	4.2 ± 4.8	18.9 ± 56.2	0.48 ± 0.6	24 ± 24	3.7 ± 7.2	214.0 ± 117
	median	0.26	3.0	2.5	0.28	15.2	1.8	195.5
	range	0.02 - 2.80	0.18 - 50.5	0.0 - 478	0.02 - 4.4	1.5 - 227	0.00 - 75.6	0.0 - 1278
>12-18 months		**0.8**	**9**		**0.7**	**40**	**10**	**400**
(n = 91)	mean (SD)	0.37 ± 0.32	5.3 ± 3.7	27.5 ± 78.0	0.48 ± 0.49	34 ± 29	6.1 ± 6.7	174 ± 144.
	median	0.32	4.8	3.4	0.39	29.1	3.9	135.6
	range	0.1 - 2.8	0.71 - 26.6	1.1 - 477.0	0.1 - 4.2	2.3 - 149	0.10 - 37.0	6.2 - 725.0
>18-24 months		**0.8**	**9**		**0.7**	**40**	**10**	**400**
(n = 41)	mean (SD)	0.48 ± 0.45	6.1 ± 3.2	24 ± 72	0.63 ± 0.78	35 ± 28	8.5 ± 7.3	157± 257
	median	0.360	5.2	3.8	0.40	31.3	6.3	63.0
	range	0.10 - 2.62	1.4 - 16.0	1.4 - 319	0.11 - 4.64	0.00 - 108.0	0.00 - 32.0	0.0- 1440.0

RDAs were not available for infants and toddlers. The RDAs were based on Allen (1993) and Barness and Curan (1996) . If RDAs were the same for the age groups, analysis of variance was determined. The range indicates minimum and maximum values for each nutrient. * Shows significant differences ($p<0.01$) between age groups.

4.3.5.2.2 *Relationship between iron status and daily dietary iron intake*

The low RDA for iron in the children surveyed suggests negative iron balance in these children (Figure 4.5). Correlations were therefore done to test this hypothesis. Mean daily iron consumption varied in the three iron status groups with IS children consuming 12% more dietary iron than NAID children and 20% more than their IDA counterparts in any given age group. There was however, no significant difference between the daily iron intakes of NAID and IDA children in 6-12 months age group (Figure 4.6). Mean daily iron consumption in the IS group was only 50% of the recommended daily allowance of 10 mg Fe/day. Overall there was a 17% association between iron intake and haemoglobin concentration ($r^2 = 0.17$; $p < 0.01$). Least square means analysis of variance showed that IS children consumed more iron (5.9 mg 0.39 SE, $p<0.0001$) than children with inadequate iron status; NAID (5.3 mg 0.39 SE, $p<0.0001$) and IDA (5.1 mg 0.39 SE, $p<0.0001$) (Table 4.8). There was however, a moderate correlation between total energy and dietary iron intake ($r = 0.55$, $p < 0.001$), indicating that energy rich diets contributed at least 30 % of the dietary iron for 6 - 24 month old Ghanaian children. Dietary iron intake also increased with height ($r = 0.30$, $p< 0.001$) which implies that physical growth is associated with dietary practice. A multivariate analysis in which age and length of breast feeding were fitted into the model with ferritin as a dependent variable showed 28% of the variation in body iron deficiency to be associated with length of breast feeding ($r2 = 0.28$; $p< 0.001$).

Figure 4.6. Relationship between iron sufficiency (IS), non-anaemic iron deficiency (NAID), iron deficiency anaemia (IDA) and mean daily dietary iron intake (±SD) of 285 (6-24 month old) children surveyed in Accra and Kumasi metropolitan areas in Ghana.



The bar chart of children with both mean daily iron intake and iron status shows significant effects of age and daily iron intake on iron status. Overall iron deficiency children consumed the least RDA for iron at all age levels than iron sufficient and non-anaemic iron deficiency children. Least square means analysis of variance showed that IS children consumed more iron (5.9 mg 0.39 SE, $p<0.0001$) than children with inadequate iron status; NAID (5.3 mg 0.39 SE, $p<0.0001$) and IDA (5.1 mg 0.39 SE, $p<0.0001$)

Table 4.8. Mean daily intakes of selected nutrients and duration of breast feeding which showed significant differences (p< 0.01) with iron status in 285 children within the ages of 6-24 months, surveyed in Ghana

NUTRIENT INTAKE	IRON STATUS			CORRELATION WITH DIETARY IRON
	Iron sufficient (IS)	Non-anaemic Iron Deficiency (NAID)	Iron Deficiency Anaemia (IDA)	
Iron (mg)	5.9 (SE 0.4)	5.3 (SE 0.4)	5.1 (SE 0.4)	
Calcium (mg)	228 (SE 21)	304 (SE 50)	233 (SE 30)	r = 0.4; p< 0.001
Vitamin D	6.5 (SE 0.6)	7.6 (SE 1.4)	5.2 (SE 0.7)	r = 4; p< 0.001
Carotene	246 (SE35)	165 (SE 80)	199 (SE 37)	r = 0.35; p<0.001
Duration of BF	8 (SE 1.0)	11 (SE 4)	13 (SE 3.0)	r = 0.02; p< 0.2
Retinol	199 (SE 19)	202 (SE 20)	203 (SE 30)	r = 0.15; p < 0.0001
Vitamin C	31 (SE 8)	31 (SE 7)	25 (SE 2)	r = 0.27; p< 0.0001
Dietary Riboflavin	0.44 (SE 0.03)	0.39 (SE 0.03)	0.4 (SE 0.01)	r = 0.68; p < 0.0001
Phosphorous	331 (SE 80)	350 (SE 30)	329 (SE 70)	r = 0.69; p < 0.0001
Thiamine	0.64 (SE 0.06)	0.53 (SE 0.2)	0.52 (SE 0.07)	r = 0.70; p< 0.0001
Magnesium (mg)	99 (SE 6.4)	(88.4 (SE 15)	90 (SE 7)	r =0.76; p< 0.0001
Potassium (mg)	908 (SE 49)	878 (SE 63)	856 (SE 52)	r = 0.75; p< 0.0001
Pot-nicotinic acid	18.5 (SE 4)	58.2 (SE 12)	19 (SE 7)	r = 0.60; p < 0.0001
Energy (Kjoules)*	3029 (SE 131)	2974 (SE 136)	3324 (SE 191)	r = 0.55; p < 0.0001
Zinc	3.5 (SE 0.2)	3.2 (SE 0.1)	3.0 (SE 0.1)	r = 0.7; p < 0.0001

Table shows mean daily nutrients intake (± SD) of children in the three iron status groups. Correlations between nutrients and iron are shon in column 5. The abbreviations used in the table indicate, SE standard error of the mean. BF is duration of breast feeding, r Pearsons correlation, p level of significance.

4.3.5.2.2 Calcium, phosphorous and vitamin D

Calcium, phosphorous and vitamin D are essential for bone structure and matrix formation. The average daily intake of these three nutrients were less than one-third of the RDA for infants and toddlers (Table 4.7b). Mean daily calcium levels in the population surveyed reached only 230 mg which was only 38% of the RDA for 6 - 12 months and 29% for >12 - 24 month olds. Overall the RDA for infants and toddlers were not met compared to Australian children who consumed excess amounts of calcium a day (Oti-Boateng *et al.*, 1994). The effect of calcium on iron status was assessed in the children surveyed. Using general multiple regression model procedures to calculate least means squares for calcium consumption in the three iron status categories, results showed significant differences in the daily mean calcium intake in IS, NAID and IDA children ($p < 0.0001$) (Table 4.8). When the data was corrected for age and sex of these children, there was a significant increase in daily calcium intake with iron deficiency. Non-anaemic iron deficiency group ingested 304 ± 50 mg of calcium a day compared with IDA children who consumed 233 ± 30 mg of calcium. Iron sufficient children ingested the least amount of calcium a day (228 ± 21 mg). Iron sufficient children had 33% less calcium in their diets compared to their NAID counterparts ($p< < 0.06$). This suggest that 304 mg of calcium with low iron intake may affect iron status.

Multivariate analysis in which age, sex and iron status were fitted into the model showed that age accounted for the 34 % ($r^2 = 0.34$, $p < 0.001$) of the variation seen in the mean daily calcium intakes. There were positive correlations between calcium and other nutrients. Daily calcium intake of the children was derived mainly from fish diets that also contained high levels of phosphorus ($r = 0.90$, $p< 0.0001$) and vitamin D ($r = 0.64$, $p< 0.0001$). These are required for bone matrix and density during rapid growth years. These fish diets however contain only moderate levels of iron ($r = 0.4$, $p < 0.0001$), riboflavin ($r = 0.36$, $p < 0.0001$), protein ($r = 0.22$, $p< 0.0001$) and fat ($r = 0.22$, $p< 0.0001$) which were inadequate to supply the daily recommended amounts needed for growth and other physiological requirements.

Although mean daily vitamin D intake in all the children surveyed was only 50% of the RDA, there were significant difference in daily vitamin D consumption in the three iron status categories when data was corrected for age and sex. The least square means of daily vitamin D intake showed that IS children ingested 6.5 ± 0.6SE, NAID , 7.6 ± 1.4 SE while IDA children consumed 5.2 ± 0.7SE ($p < 0.0001$). The high vitamin D intake in NAID children may probably be due to the high calcium diet intake of that group. There were no direct relationships between vitamin D and serum ferritin but weak correlations were observed between Vit D and Hb concentration ($r = 0.20$, $p < 0.01$) and phosphorous and Hb ($r = 0.25$, $p < 0.05$). There was an inverse relationship between phosphorous and RDW ($r = -0.34$, $p<0.01$). These indicate various roles of Vit D and phosphorous in phospholipid synthesis which is required in red cell membrane formation during erythropoiesis.

4.3.5.2.3 Magnesium, zinc, potassium and sodium

Mean daily magnesium intake of the children surveyed was 75.4 ± 64.5 mg (Table 4.7b). Magnesium was the only nutrient which was consumed in excess of daily requirements. There was a significant difference in the daily magnesium intake between the three iron status groups which increased with age ($p < 0.0001$). The daily magnesium intake was close to the RDA in 6-12 month olds but was 18% higher than the RDA for >12 - 18 month olds and 50% higher than in >18 -24 months olds. There were strong associations between magnesium and dietary iron ($r = 0.76$, $p< 0.0001$) and between magnesium and zinc ($r = 0.84$, $p< 0.0001$). There was a strong positive association between magnesium and vitamin C. The strong positive relationships seen between Mg and Fe also suggest that the main dietary iron of these children was derived from plant sources. Least square means analysis of variance showed that IS children consumed an extra 10 mg per day compared to their NAID counterparts (98.8 ± 6.4 vs 88.4 ± 14.7 SE) and 18 mg per day more than IDA children (90.0 ± 6.7 mg; $p< 0.0001$) when data was corrected for age and sex of children.

Mean daily zinc intake followed a similar trend to the other nutrients with the exception of magnesium. There were significant differences in the mean daily zinc intake of the children in the three iron status groups. Although the amounts ingested increased with age ($r = 0.37$, $p < 0.001$), they were inadequate compared with the recommended daily allowances of 5mg for infants and 10 mg for toddlers. Zinc positively correlated with dietary iron ($r = 0.70$, $p < 0.0001$) which shows that the foods ingested by Ghanaian children that have high zinc also contain high dietary iron contents. Diets which have zinc were derived from high protein and high fibre sources as seen in the strong correlations between Zn and protein ($r = 0.77$, $p< 0.0001$) and zinc and fibre ($r = 75$, $p < 0.0001$).

Potassium

Mean daily potassium (K) intake followed a similar trend to magnesium with age when data was corrected for iron status and sex (Table 4.7b). Children in the >18-24 months age group and those in >12-18 months group had 66% and 46% more K^+ in their diets than those in the 6-12 month old group ($p < 0.0001$). There were strong positive relationships between K and several divalent cations indicating that diets high in K also contained several minerals. Mean daily K intake correlated with protein ($r = 0.78$, $p< 0.0001$) with Zn ($r = 0.84$, $p< 0.0001$), with Fe ($r = 0.75$, $p < 0.0001$) and with Mg ($r = 0.91$, $p < 0.0001$). There were weak relationships between K and Ca ($r = 0.30$, $p< 0.0001$). High K diets also contained high fibre ($r = 0.85$, $p < 0.0001$). This probably inhibited the availability of the minerals discussed earlier, particularly iron. Generally diets high in K were also rich in fat ($r = 0.67$, $p < 0.0001$), fat soluble vitamins such as riboflavin ($r = 0.41$, $p < 0.0001$) and vitamin D ($r = 0.40$, $p < 0.0001$) and water soluble vitamins like vitamin C ($r = 0.70$, $p < 0.0001$) and nicotinic acid ($r = 0.65$, $p < 0.0001$). They also contained high fibre which are good sources of energy for the 6-24 month old children ($r = 0.73$, $p < 0.0001$). Analysis of variance was carried out to assess whether there were any differences in the mean daily potassium consumption of the three iron status groups. Least square means showed differences in daily potassium intake between

the 3 groups. Iron sufficient children had the highest potassium intakes (908.0 mg ± 49 SE), NAID children had 878 mg ± 63SE, while IDA children had 856 ± 52 SE (Table 4.8).

Mean daily sodium intake increased with age as was the case for the other nutrients. It increased by 60% at 13 months and 107 % at 19 months ($r = 0.40$, $p < 0.001$).

4.3.5.2.4 Vitamins

Vitamins are organic compounds required in minute amounts to catalyse cellular metabolism essential for growth or maintenance. Vitamins can be categorised into two main groups based on their solubility in either water or fat. Mean daily vitamin intakes were analysed from the foods ingested by the children. Pearsons bivariate correlations were used to assess the relationship between vitamins, minerals and iron status indicators.

Retinol equivalent, RE (Vitamin A)

The mean retinol levels in the population surveyed was 197 ± 148 µg with a decline in daily mean intake as children got older. Mean daily intakes were less than the RDAs for infants and toddlers. Infants 6-12 months ingested on average 52 % of RDA while toddlers were able to meet only 42% of their daily requirement for retinol ($r = -0.30$, $p < 0.02$). As breast feeding decreased or ceased weaning diets were nutritionally inadequate to meet the retinol requirements of the children because they consumed very little of the main sources of retinol which are mainly from animal protein such as breast milk, fish, meat or egg. There was a negative association between duration of breast feeding and retinol equivalent in the diet but this was not significant. Fifty five of the children surveyed (16 % of total population) had no retinol in their diets which put them at risk for infection and growth retardation. There were weak correlations between dietary iron and retinol ($r = 0.15$, $p < 0.0001$), carotene and retinol ($r = 0.12$, $p <$

0.0001),Ca and retinol (r = 0.31, p< 0.0001), vitamin C and retinol (r = 0.35, p< 0.0001) and a moderate association between retinol and dietary riboflavin (r = 0.55, p< 0.0001). These weak associations further suggest that infants could have derived their retinol from breast milk and very little from fish and provitamin from plants. There were no significant differences in the mean daily retinol equivalent of the diets of either the IS or ID children because the small amounts of retinol in these diets were destroyed through food processing such as drying.

Carotene

The mean daily value for carotene in the diets of the children surveyed was 142.3 ± 361 µg with a significant mean daily increase in consumption with age (r = 0.32, p < 0.0001)(Table 4.7b). Carotenes (alpha, beta and gamma) are calculated as 1/6 the activity of retinol (Barness and Curran, 1996). Using this formula for calculating % of RDA for retinol, 6-12 month old children ingested 4.5% more RDA for retinol while >18-24 month olds had 14% additional RDA form carotene intake. These additional sources of retinol from carotene were still inadequate to meet daily requirements.

There were weak associations between carotene and Fe (r = 0.35, p < 0.0001), carotene and zinc (r = 0.31, p < 0.0001), Mg and carotene (r = 0.32, p < 0.0001), other vitamins such as thiamine (r = 0.31, p < 0.0001), retinol (r = 0.13, p < 0.02) and vitamin C (r = 0.24, p < 0.0001). There was also a weak association between ferritin and carotene in >18-24 month olds indicating some relationship between conservation of iron stores and carotene intake (r = 0.25, p < 0.02). A multivariate analysis in which age and sex of children were corrected for showed a 6% relationship between carotene intake and ferritin. Least square means analysis further showed that IS children ingested more carotene (246.4 µg ± 35 SE; p<0.0001) than their NAID (165 µg ± 80 SE; p < 0.04) and IDA (199 µg ± 37 SE; p< 0.0001) counterparts.

Thiamine (Vitamin B_1)

Infant consumed 20% more thiamine than the RDA for 6-12 month but this declined to 67% of the RDA in 12-18 month olds and 91% in 18 -24 month olds (Table 4.7c). Thiamine is associated with a number of oxidative decarboxylation including that of pyruvic acid and its deficiency has been linked to beriberi, irritability and fatigue in children. Whether 67% of RDA is enough to overt the clinical manifestation of these conditions is not known. There were however, strong positive correlations between thiamine and dietary iron intake ($r = 0.70$, $p < 0.001$), thiamine and riboflavin ($r = 0.70$, $p < 0.001$) and thiamine and nicotinic acid ($r = 0.65$, $p < 0.001$). Thiamine was weakly correlated with retinol ($r = 0.43$, $p < 0.001$), vitamin C ($r = 0.42$, $p < 0.001$), magnesium ($r = 0.44$, $p < 0.001$) and calcium ($r = 0.31$, $p < 0.001$), protein ($r = 0.40$, $p < 0.001$), fibre ($r = 0.53$, $p < 0.001$), vitamin D ($r = 0.12$, $p < 0.001$), and fat ($r = 0.36$, $p < 0.001$). These associations indicate that high thiamine foods ingested by the children studied also contained high iron and the B vitamins and vitamin C as well as energy sources for the children. Most of the thiamine in the diet was derived from cereals and legumes with marginal amounts from milk sources as indicated by the low correlations with calcium and vitamin D. Thiamine also correlated weakly with Hb ($r = 0.18$, $p < 0.001$). Least square means analysis showed that all IS children ingested more thiamine (0.64 mg± 0.6 SE, $p< 0.0001$) than NAID (0.53 mg ± 0.2 SE ; $p< 0.0001$) and IDA (0.52 ± 0.07).

Dietary Riboflavin (Vitamin B_2)

Mean daily dietary riboflavin in the diets of children was 0.36 mg± 0.31SD. Mean daily intake of riboflavin increased with age but were still below the RDAs of older children. Infants 6-12 months consumed 68% of RDA for riboflavin, >12-18 months ingested 46% of RDA and >18 to 24 had 60% of the RDA for riboflavin ($p<0.02$). There were significant differences in the daily intake of riboflavin between children in the three age groups. Riboflavin is an important component of flavoprotein enzymes like succinic dehydrogenases which are essential for cellular respiration. When Pearsons

correlation coefficient analyses were performed to test for associations between dietary riboflavin, iron, erythrocyte riboflavin and red cell indicators, no correlations were found between dietary riboflavin and erythrocyte riboflavin or Hb. There was, however, strong association between dietary iron and dietary riboflavin ($r = 0.68$, $p< 0.0001$). Least square means analysis of variance showed IS children to have 20% more riboflavin in their diets (0.44 ± 0.03 SE, $p< 0.01$) compared with ID children; NAID (0.39 ± 0.03 SE, $p< 0.01$) and IDA (0.40 ± 0.03 SE, $p< 0.01$). Although IS children had more riboflavin, they still consumed sub-optimal levels of the recommended daily intake for their age.

Potassium nicotinic acid (Niacin equivalent, NE)

The average daily intake of NE was 21.2 ± 4.2 mg and daily consumption increased with age. However, it did not reach the recommended daily allowance for infants and toddlers (Table 4.7c). There was a strong positive correlation between dietary iron and niacin (Table 4.8).

Vitamin C

There were significant differences in vitamin C intake of children at all ages ($r = 0.20$, $p < 0.001$), but sub-optimal levels of vitamin C was consumed at all ages (Table 4.7c). There were positive correlation between vitamin C and iron in the diet ($r = 0.54$, $p< 0.001$) which is a known enhancer of iron absorption. Weak associations were also seen between vitamin C and Ca ($r = 0.27$; $p < 0.001$), total energy intake ($r = 0.33$, $p < 0.001$) and total protein ($r = 0.32$, $p < 0.001$).

4.4 DISCUSSION

Findings from the present epidemiological study in which iron status and intake of 6-24 month old children were determined showed that iron deficiency and iron deficiency anaemia are prevalent in Ghanaian children, despite the efforts being made to improve the nutritional status in Ghana. An unacceptably high prevalence of anaemia (55%) in this vulnerable group in Ghana was observed.

Compared with the prevalence of anaemia in studies from other developing countries, the prevalence of anaemia in Ghana was 20% more than what has recently been reported in 6-24 month old Korean children, where similar cut off values for Hb < 110g/L, RDW > 15%, MCV < 70fL, but SF < 10 µg/L were used to define anaemia (Kim *et al.*, 1996). It was also 11% more than the incidence found in South African children living in informal urban settlements where anaemia was defined as Hb< 110g/L (Coutsoudis *et al.*, 1994). In politically unstable Zaire however, the incidence of anaemia in 6 - 24 as diagnosed by Hb < 110g/L, TfR > 8.5 µmol/L and SF < 12 µg/L (Kuvibidili *et al.*, 1993) was 6% more than the rate of occurrence in Ghana. Comparing this with studies from developed countries, Oti-Boateng *et al.* (1994) reported a prevalence of 7% anaemia in Australian children. Yip (1990) also reported from results of national nutrition surveys in six states in America between 1976 - 1985, that the prevalence of IDA diagnosed by Hb < 103 g/L and Hct < 3% in 6 - 23 month olds and Hb < 105 g/L and Hct <34% in 24 -60 months had declined by 54% over the 10 year period .

The initial estimated prevalence of anaemia in 1976 in America was 18-19% compared with the same prevalence (18%) in children who had Hb < 105g/L in the present when the data was reassessed. The prevalence of anaemia in America decreased to 8-9% in 1988 (Yip, 1990) with a further decline in prevalence and severity to 3% between 1988-1994 when the iron status of children was reviewed by Looker *et al.*, (1997). Prevalence studies in America have been useful in comparing trends in iron nutritional status in African-Americans, Caucasian other races and high and low income

families. Comparison with the American study would suggest that the problem of ID in Ghana is mainly a result of dietary practices and a rigorous nutritional anaemia reduction programmes through infant food iron supplementation as in America can help to reduce anaemia levels.

The effects of iron deficiency on erythropoiesis and energy metabolism are well documented. In addition the lasting effects on the mental and psychomotor development of 6-24 month old children have been observed (Oski *et al.*, 1983; Walter *et al.*, 1989). The duration and severity of iron deficiency anaemia as defined by Hb < 110 g /L and SF < 12 µg/l in 6- 15 months old, has been associated with altered attention span, lower intelligence scores, and some degree of irritability (Lozoff *et al.*, 1987, Walter *et al.*, 1989). Since infants rarely become iron deficient before six to eight months of age, it appears that only a brief period of three months of relatively mild iron deficiency (Hb < 105 g/L) during the weaning period, may result in long-lasting consequences for behaviour and school performance (Lozoff *et al.*, 1991; Walter *et al.*, 1993). The high incidence of anaemia found in 6-18 month olds in the present study, using the less severe cut off limit of Hb <110g/L, therefore suggests that a high proportion of children in Ghana may have impaired behaviour and cognitive function in later years. This has very important nutritional and physiological implications for children in Ghana and calls for programmes which can help alleviate this problem.

This current study, and perhaps other studies from African countries, also show that the magnitude of prevalence of IDA in infants and toddlers does not only depend on political stability and socio-economic status of parents, rather poor weaning practices appear to be responsible. For example, there were no significant differences in iron status between the three socio-economic groups surveyed in Ghana. The prevalence of IDA in children in the middle income families was similar to that in the low income group (Class III). The diagnosis of anaemia in class I was associated with Hb < 110 - 105 g/L and one or two biochemical indicators of SF or TfR, compared with Hb <105 g/L in

Classes II and III children, indicating a more severe anaemia in lower to middle socio-economic status. The low number of children in class I could have however biased the data. Findings from this current clinical survey also support the earlier study in chapter 3 and recent epidemiological studies from Africa, Europe, Indo-China, and America that iron deficiency and anaemia are prevalent to varying degrees (3 - 61%) in 6-24 month olds using similar haematological and biochemical indicators of iron status.

A recent study in Zambia in which iron status and hook worm infestation were assessed in school-age children showed that, iron deficiency anaemia can be attributed to intense hookworm infestation. Stolzfus and coworkers (1997) demonstrated in a multivariate model of hook worm infestation, Hb >110g/L, EP >90 nmol/mol haem, SF < 12 μg/L, malarial parasitemia and gender that, 25% of the prevalence of all anaemia, 35% of the iron deficiency anaemia and 73% of chronic anaemia were caused by hook worm infestation. The current study excluded children with malaria and sickle anaemia which can often mask the true presence of nutritional iron deficiency (Yip *et al.*, 1984; Kuvibidili *et al.*, 1993), but it is also possible that the 55% anaemia found in these Ghanaian children could also be related to hookworm infestation which was not measured. Assuming that 25% of all iron deficiency anaemia is caused by hookworm infestation, then it can be inferred that 40 of the 159 Ghanaian children diagnosed as IDA could have been caused by hook worm infestation (Table 4.6). If this assumption is true then the true prevalence of nutritional iron deficiency anaemia in 6-24 month old Ghanaian children is 41% because malaria and other forms of infections were eliminated. These findings further support the assertion that main cause of iron deficiency and anaemia in infants and toddlers is attributed to inadequate dietary iron intake at a time of high physiological needs. The weak correlation found between dietary iron intake and iron status in this study, however suggests an interaction of inhibitors of iron absorption in the diets of Ghanaian children. Other factors found to contribute to the varying degrees of global prevalence of iron deficiency and anaemia in young children can be

attributed to infection in cases of mild to chronic anaemia, prolonged breast feeding and weaning practices as reviewed in chapters 2 and shown in chapter 3.

4.4.1 Risk of iron deficiency with prolonged breastfeeding

The high prevalence of anaemia as defined by Hb < 110g/L, SF < 12 μg/L or 15 μg/L have been associated with exclusive breast feeding beyond six months of age in the previous study and by Siimes and coworkers (1984). This study also showed that prolonged breastfeeding beyond six months of age accounted for 28% of the risk factors that are associated with iron deficiency anaemia in children 6-18 months when the data was corrected for age and sex. Children in Ghana were generally breast fed longer than their Australian Caucasian counterparts (10 ± 6 months vs 6 ± 2 months) because recent nutritional advice by the Nutrition Division of the Ministry of Health in Ghana promotes exclusive breast feeding for six months with the introduction of weaning foods after six months. This was based on UNICEF's recommendation to reduce the prevalence of diarrhoea during early periods of weaning (Ministry of Health, Ghana, personal communication). The average length of breastfeeding (13 months) was more common in low to middle income families in which the severity of anaemia (Hb < 105g/L) was more prevalent. By comparison with the upper income families who breastfed on average for 8 months the severity of anaemia was Hb < 110g/L but not less than Hb < 105g/L in the present Ghana study.

Delaying the introduction of weaning foods till seven months may have decreased the frequency of diarrhoea in exclusively breast fed children (Ministry of Health, Ghana, personal communication) however, results from this study clearly show that this advice has created another nutritional problem of iron deficiency due to prolonged breast feeding. Although diarrhoea episodes were not assessed in the current Ghana study, recalls of the weaning foods suggest that the prevalence of diarrhoea in children in Ghana may be more related to the early introduction of high fibre and high solute load weaning diets. These diets consist of beans and nuts which may have high content of aflatoxin

and poor water quality than the timing of weaning food intake. These complex interaction of food and water quality on diarrhoea, timing of the introduction of weaning foods and the prevalence of anaemia will need to be investigated further.

4.4.2 Relationship between dietary intake and iron status

The high incidence of iron deficiency anaemia in 6-24 month old Ghanaian children was associated with low intakes of dietary non-haem iron. Overall, Ghanaian children consumed only 44% of the calculated daily iron requirement for erythropoiesis, physical growth and mental and psychomotor development. Over 90% of the dietary iron source was derived from plant (non-haem iron form), the rest was from mainly fish and some meat (haem iron). Younger children 9-12 months who needed to absorb 50% more than either 6-8 or 13 - 24 months as calculated by Rio and Sketel (1982) consumed the least percent of RDA for age. Six to twelve month old infants consumed an average of 3.6 mg Fe a day (36% of the RDA) with a 18% and 31% increases in >12-18 and >18-24 month olds respectively (Table 4.7b). These results are contrary to those found in Australia where % of RDA decreased with age with 6-12 month olds ingesting 61% of RDA and >18-24 had 37% of the RDA for iron. The reversed trend in the mean daily dietary iron intakes in Australia compared to Ghana clearly indicates differences in weaning practices in the two countries.

The work by Oti-Boateng *et al.* (1994) found that dietary iron intake of Australian infants (6-12 months) was mainly derived from a single food of either formula which contains 12 mg of $FeSO_4$/L and 40 mg vitamin C, iron fortified infant or adult cereal with a content of 50mg Fe /100g cereal (Gillooly *et al.*, 1984; Fomon, 1987; Hurrell *et al.*, 1989(a)), while toddlers 13-24 months diets changed to resemble those of adults which were not iron fortified and meat was sparingly ingested. By comparison, Ghanaian infants derived their exogenous iron from whole maize, soyflour and other legumes which are low in iron but high in iron inhibitors. As children got older, the consumption of soyflour porridge was excluded and the weaning diets became more varied with the

introduction of fish, green vegetables and composite foods. The quantity of iron in the diets in 12-24 month olds nearly doubled as shown by the 46% increase in mean dietary iron intake in 18-24 month old children. Marked differences in the amounts of iron absorbed from these Ghanaian weaning diets were reflected in the concentration of serum ferritin and haemoglobin. The high frequency of ingestion of soyflour porridges coincided with low Hb levels of 6-12 month olds. Overall, iron sufficiency children had 12% more iron in their diets than their NAID children and 20% more than their IDA in the present study. A multivariate model analysis of mean daily iron intake, Hb, gender and age showed a 17% increase in Hb to be attributed to daily dietary iron intake. These findings, particularly the relationship between dietary iron intake and haemoglobin, are in accord with studies of Dallman *et al.*, (1980) and Siimes *et al.*, (1984).

4.4.3 Clinical implications of soyflour as weaning food

The use of soyflour as weaning food is promoted in Ghana as a means of counteracting protein malnutrition as indicated in the excess daily protein intake in infants and toddlers in the current study. Over 70% of 6-12 month olds ingested soyflour. While soyflour may have contributed to the reduction of protein-energy malnutrition there is established evidence that soy products do not only inhibit iron absorption but also contain anti-nutritive factors regardless of food processing methods used (MacFarlane *et al.*, 1988; Lynch *et al.*, 1994). According to Cook *et al.*, (1981) non-haem iron absorption is significantly impaired in humans consuming a diet of soya products. Soyflour has also been shown to affect protein digestion (Loser *et al.*, 1987). Animal and human studies have shown that high levels of trypsin inhibitors in soyflour can induce the rapid release of the hormone cholecystokinin (CCK) from the intestine (Liener, 1988). Continuous release of CCK, as a consequence of long-term exposure to trypsin and chymotrypsin inhibitors found in soyflour diets, has been shown to cause an increase in pancreatic size and weight (hypertrophy) and proliferation of pancreatic acinar cells (hyperplasia) and a possible benign neoplastic lesions known as acinar adenoma in animals. (Gumbmann *et al.*, 1986; Calam *et al.*, 1987) The observation of Calam and

coworkers, (1987) has been confirmed by others who have demonstrated that soybean trypsin inhibitors can induce pancreatic hypertrophy in humans (Linener *et al.*, 1988; Holm *et al.*, 1992).

These deleterious effects of pancreatic hypertrophy associated with long-term soyflour trypsin inhibitor consumption observed in animals and adults may not be relevant in children. Rapid gastric emptying times (5 to 30 minutes in neonates compared to 3.5 to 4 hours in adults) at high gastric pH (3 to 5 in neonates relative to 1.5 to 2.5 in adults) (Flavin, 1984) and the limited pancreatic function in children may provide more effective protection against trypsin inhibitor activation. The growth profile of Ghanaian children within 3 ± SD suggests no relationship between growth retardation and soyflour consumption either. It is however possible that these anti-nutritive factors found in soyflour may compromise protein digestion, hence effect intestinal iron uptake especially haem iron. The regular consumption of products that induce such significant physiological changes should therefore be avoided.

High levels of lectins which are compounds that bind to simple and complex carbohydrates have also been found in soybean. Lectins are potent exogenous growth factors for the small intestine (Liener, 1986; Pusztai, 1991) and can interact with specific glycoproteins on the cell surface of red blood cells resulting in agglutination of these cells. Although there are no reported soybean lectin toxicity observed *in vivo* in humans, animal models have shown that pure soybean lectin inhibited growth of rats and caused considerable changes in morphology and functioning of both the small intestine and pancreas (Pusztai, 1991).

The severity of anaemia (Hb < 105g/L) in 29 Ghanaian children consuming large amounts of soyflour porridges suggest that these children may have intestinal mucosal damage and allergies which may predispose them to occult intestinal bleeding and severe anaemia. The use of soyflour in weaning food needs further investigation both in Ghana

and in countries that consume large amounts of soybean products. There is the need to assess the effects of these toxins, particularly, lectins on gastrointestinal permeability of these Ghanaian children.

4.4.3 Nutrient assessment of Ghanaian weaning diets.

Daily energy intakes of Ghanaian children were derived from carbohydrate, protein, fat and fibre. Fifty five percent of the total energy was obtained from carbohydrate, 33% from fat 15 % from protein as recommended (Barness and Curran, 1996). Ghanaian children consumed excess energy which shows that the weaning diets were adequate to supply energy required for growth and other metabolic processes. The daily protein intake at all ages exceeded the daily RDA but was limited in micro-nutrients such as iron, vitamin A, B complex vitamins such as thiamine, riboflavin and nicotinic acid and vitamin D which are abundant in animal protein. Daily intake of all the micro-nutrients mentioned above were well below the recommended daily required amounts. The low daily intake of essential vitamins (with the exception of Vit D due to abundant sunlight) are a concern because vitamins play a range of roles.

Effects of vitamin deficiencies include growth retardation and impaired resistance to infections. There were no negative correlations between vitamin A which is an important component of the retina responsible for vision in the dark, important in tooth and enamel development and formation and maturation of epithelia cells (Bloem *et al.*, 1989) and any of the growth parameters. This suggests that the marginal vitamin A status in the Ghanaian children surveyed was not low enough to cause any anthropometric retardation. The positive correlations seen between iron, vitamin A, vitamin C riboflavin and Hb concentration suggest, a complex interaction between these nutrients and iron homeostasis. Iron sufficient infants and toddlers had higher amounts of iron, vitamin C and riboflavin than their NAID or IDA counterparts which supports earlier findings by Bloem *et al.* (1989) in South East Asia and van Stuijvenberg *et al.* (1997) in South Africa who also found positive correlations between vitamin A and Hb,

and SI but Vit A negatively correlated with SF. They concluded from these results that iron absorption was not impaired by marginal vitamin A status but rather, the mobilisation of iron from stores was affected. Further intervention studies are needed to test the interaction between dietary non-haem iron absorption and iron store utilisation in children during the weaning period.

The mean calcium intake in 6-12 month olds was only 38% of the RDA and 29% of the RDA for >12-24 month olds compared to excess daily allowances found in Australia. Non anaemic iron deficiency children consumed more than 300 mg of calcium a day, while IS children consumed on average, 228 mg of calcium a day. Although these calcium levels were well below the RDAs for 6-24 month olds, the proportion of calcium to iron in the diets may have influenced dietary iron uptake in NAID children as previously shown in the Australian study. The ratio of mean calcium to iron in the diets of NAID children in the present study was 303 mg Ca to 5.2 mg Fe which was 19% more than that of IS children with 228 mg Ca to 5.9 mg Fe. These ratios suggest that calcium may be a risk factor for iron absorption in children with borderline iron status but has no effect under iron deficiency condition. The differences in the ratio of dietary calcium to iron in the Ghanaian weaning diets may be another limiting factor contributing to inadequate iron status. Alternatively, the difference in impart of calcium on iron status in NAID compared to IDA children could be related to the small number of children in NAID compared to 55% of children in the IDA group. Whether the association was biased on number of children or there was a true inhibitory relationship between calcium and iron status will need to be investigated further. The most important issue however, is that calcium has again emerged as one of the factors that can influence iron status in children during weaning in Ghanaian children who consumed well below the RDA for calcium at all ages. The interaction of calcium on iron absorption need to be investigated further because both nutrients are required for growth and mental development. Experiments which can control for number of subjects, length of breastfeeding, age of

children, geographical locations, socio-economic and dietary inhibitory factors that may influenced iron absorption during weaning will need to be carried out.

4.5 CONCLUSION

Iron deficiency and anaemia are prevalent in Ghanaian children during the period of weaning. The high prevalence of anaemia in this vulnerable group can be attributed to extended breast feeding beyond 6 months and the ingestion of weaning diets which are predominantly cereals, vegetables, nuts, cowpeas, soyflour and limited amounts of fish meal. These foods are not only low in iron content but also contain factors that limit iron absorption. This study has demonstrated that it is difficult to meet the iron requirements of a growing child on these diets alone. Anti-nutritive factors, such as lectin and trypsin inhibitors are consumed in very large amounts in soyflour porridge weaning diets in Ghana. The regular consumption of soyflour currently practised in Ghana may suggest that Ghanaian children are at risk of significant physiological consequences that are associated with ingestion of trypsin inhibitors, lectins and phytic acid. The poor quality of protein sources in the Ghanaian weaning diets reinforces the need to promote fish-fortified diets rather than soy-based fortification of weaning diets in Ghana. Alternatively, genetically selected maize lines with high quality protein and high iron should be tested and promoted in Ghana instead of soyflour, which is not a traditional Ghanaian food. Overall, weaning diets of Ghanaian children are limited in many minerals and micronutrients. Most were below 60 % of RDA for infants and toddlers. There is urgent need to evaluate the quality of weaning diets in meeting the nutrients requirements of young children.

Calcium is important during infancy and childhood which coincides with skeletal modelling and consolidation of bone mass. Positive balance is very necessary (Matkovic *et al.*, 1991). There is however a competitive inhibition between iron and calcium at a time when there is a high need for both nutrients. Results from this current study are in accord with earlier findings by other researchers that 300 mg of dietary calcium is a potent inhibitor of both haem and non-haem iron absorption (Hallberg *et al.*, 1992a; Cook *et al.*, 1991; Oti-Boateng *et al.*, 1994; Gleerup *et al.*, 1995). The current study has demonstrated that although the mean daily calcium intake was less than the RDA for that

age group it was sufficient to reduce the availability of iron to young children and thus increase the risk of iron inadequacy since the level of iron in their diets was low. The current study has reinforced the need to assess the mechanism of interaction of calcium on intestinal non-haem iron absorption.

CHAPTER 5

THE EFFECTS OF DIETARY CALCIUM ON INTESTINAL NON-HAEM IRON ABSORPTION DURING THE WEANING PERIOD

THE EFFECTS OF DIETARY CALCIUM ON INTESTINAL NON-HAEM IRON ABSORPTION DURING THE WEANING PERIOD.

5.1 INTRODUCTION

Results from the clinical studies reported in chapters 3 and 4 have shown that nutritional iron deficiency and anaemia continue to be a problem in infants and children in both developed and developing countries. Although overtime the prevalence has declined in developed countries. The high prevalence of iron deficiency anaemia in young children in Australia and Ghana was linked to the intake of weaning diets containing low iron with poor bioavailability at a period when a high dietary iron intake was required for erythropoiesis and growth. Weaning diets consisted mainly of cereals, vegetables and legumes in both studies, while excess cow's milk intake was only common in Australian children (Oti-Boateng *et al.*, 1994, 1998). These diets were not only low in iron (both in content and availability) but also contain large amounts of iron potential inhibitors such as calcium, which is also an essential nutrient during the period of rapid growth (Hallberg *et al* ., 1992b; Gleerup *et al.*, 1995; Oti-Boateng *et al.*, 1994; 1997).

Dose-dependent dietary calcium has been shown to reduce haemoglobin incorporation of radio-labelled iron in adults by as much as 60% (Hallberg *et al.*, 1991). The consumption of over 470 mL of cow's milk (containing 730 mg calcium) per day in children beyond 18 months of age has also been shown to be associated with a 12 % depletion in serum ferritin concentration (Oti-Boateng *et al.*, 1994). A 250 molar ratio of calcium to iron was shown to cause a 28% decrease in haemoglobin concentration in 6-24 month old children in Ghana. Whether calcium has a direct interaction with mucosal iron uptake or causes changes in intestinal enzyme activity when the gut is growing and maturing during weaning is not known. Furthermore, although the duodenum is widely regarded as the primary site of iron absorption (Chowrimootoo *et al.*, 1992; O'Riordan *et al.*, 1997), the question of whether dietary calcium interferes with iron uptake at the

brush border or basolateral membrane, particularly during weaning and associated gastrointestinal tract (GIT) development, has not been addressed.

The inhibition of iron uptake by calcium can be hypothesised to be dependent on their relative affinity for intestinal iron binding proteins such as ferritin and transferrin. This hypothesis is based on the assumption that calcium and iron are freely soluble in gastric juice but are poorly soluble at the relatively alkaline pH of the small intestine compared to that of the stomach (Bothwell *et al.*, 1979). The availability of iron for uptake at the apical brush border membrane and transport to the basal membrane of the epithelial membrane could be affected by its chemical forms and their relative affinity for mucosal binding proteins (Sanyal *et al.*, 1992; Conrad *et al.*, 1994; Gunshin *et al.*, 1997). Iron and Ca^{2+} have identical charges and hydrated atomic dimension (6A) and with similar structural binding requirements to proteins. However, Ca^{2+} seems to exhibit higher affinity for coordinating between a 7-OH and 12-OH cholanic ring group and a terminal carboxyl (COO^-) or sulphonic (SO_3^-) group of bile salts and proteins (Sanyal *et al.*, 1992). Although bile salts increase iron uptake, they are not likely to exhibit this property in children under two years, since bile salt secretion is age dependent thus giving calcium an uptake advantage (Sanyal *et al.*, 1992).

Calcium can also irreversibly interact with and modulate the structure of a class of calcium binding proteins, such as calmodulin, parvalbumin and carboxyglutamate (Gla) proteins, which facilitate the transport of calcium ions through the cell and keep the actual free intracellular calcium below toxic levels (Haiech *et al.*, 1981; Van Eldik *et al.*, 1982). It is possible that calcium may also bind with high affinity to iron binding proteins such as mobilferrin, which is associated with intestinal iron transfer (Conrad *et al.*, 1993), although this is not widely supported or with iron responsive element binding proteins (IRP) which is associated with ferritin and TfR regulation (Kuhn *et al.*, 1994; Hentze and Kuhn, 1996). These interactions of calcium on iron binding proteins will therefore limit the transport of iron from the apical membrane to the basolateral membrane of the

intestinal epithelial mucosa cells for onward delivery to peripheral blood or down regulating the translation of transferrin receptor mRNA (Parmely *et al.*, 1985; Conrad *et al.*, 1994; 1993; Kuhn *et al.*, 1994). Calcium salts may also change the physiochemical state of iron salts and render them less available for absorption.

The inhibitory effect of calcium on iron absorption has nutritional implications on the iron status of infants and young children as demonstrated in the studies in chapters 3 and 4. Improving the bioavailability of iron from weaning foods by optimising its availability in the presence of other nutrients especially calcium, would reduce the incidence of iron deficiency and related symptoms in young children. This will help to reduce the current high levels of iron-fortification which are often used as a compromise poor iron bioavailability. The analysis of the interaction of dietary calcium with iron absorption at the initial site of absorption will be valuable in assessing the modulating influence of other divalent cations such as magnesium, lead and zinc on iron-deficient or sufficient diets.

The clinical studies in chapters 3 and 4 were possible because the procedures used were not invasive with the exception of blood collection. Since it was, however, difficult to obtain human gut samples, the Wistar rat was chosen as an experimental model for assessing the effects of dietary calcium on intestinal iron absorption. Animal models which can be extrapolated to humans have been used extensively to demonstrate the importance of iron (Yehadu and Youdim 1987; Larkin and Rao 1990; Kuhn 1991; Smuts *et al.*, 1995). Notable among these have been animal studies involved in the assessment of the effects of transient nutritional iron deficiency anaemia on brain development which have been supported in recent years by longitudinal human clinical studies (Lozoff *et al.* 1987, Walter *et al.*, 1989). The Wistar rat has been used successfully as an experimental model for studying intestinal absorption of iron and other nutrients (Anderson *et al.*, 1990; Oates and Morgan; 1996; O'Riordan *et al,*. 1997). The ultimate objective of this study is to investigate the interaction between calcium and

dietary iron and provide information which will enable nutritionists to make more informed recommendations on appropriate ways of maximising the bioavailability of iron and calcium. This study aimed to use the rat as a model to investigate the effects of dietary calcium on the growth profile, haematology, biochemistry, villus morphometry and duodenal non-haem iron absorption by intact tissue and apical brush-border membrane vesicles, during the weaning period.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Dietary Intervention

One hundred weanling (21 day old) Wistar rats of mean weight 50 ± 2g were randomly assigned to one of the following four semi-purified dietary treatments for 14 days. This time period can be extrapolated to 6-24 months in human. These diets have been previously tested for their efficacy in inducing iron deficiency as shown in 2.2.3 Body weight gain and food intake was recorded every other day over the 14 day period using an electronic balance. The welfare of the animals was checked regularly as outlined in section 2.2.3. Food intake per body weight gain was calculated to determine the food conversion efficiency of each animal. Profiles of growth and food intake were plotted to assess the trends of the effectiveness of the diets in maintaining adequate growth of animals in each of these four treatment groups:

- Iron and calcium sufficient, normal rat diet (Control) ; IS + Ca (420 mg Fe and 12.5 g Ca /kg diet)
- Iron sufficient calcium deficient; IS - Ca (420 mg Fe and 0.25 g Ca/kg diet)
- Iron deficient calcium sufficient; ID + Ca (16 mg Fe and 12.5 g Ca/kg diet)
- Iron and calcium deficient - ID - Ca (16 mg Fe and 0.25 g Ca/kg diet)

5.2.2 Determination of iron status.

Blood analysis, as shown in the human study, provides an accurate indication of iron status and the extent to which the four dietary regimes can influence this was examined. On day 14, animals were fasted overnight for 16 hours and euthanased with 0.2 mL of 60 mg/mL pentobarbitone sodium per 150 g body weight. The thorax of each rat was opened carefully to avoid puncturing any vein or artery. Two and a half millilitres blood samples were collected from the vena cava into 1 mL heparin coated eppendorf tubes. These were inverted three times to ensure proper mixing and for platelet stability. After mixing, blood samples were transferred into a cooler at 4-8^{o}C . These blood samples were used for Hb and Hct measurements within two hours. The other 1.5 mL blood was collected into plain eppendorf tubes for serum collection by

immediately centrifuging the whole blood at 3,000 g for 5 minutes in an Eppendorf micro centrifuge.

5.2.2.1 Haemoglobin measurement

Haemoglobin concentration was determined colorimetically using a manual cyanomethaemoglobin method (Libansky and Cas-Lek, 1971). One mL of reagent solution (0.6 mM KFeCN, 0.75 mM KCN, 3.00 mM PBS, pH 7.2 and 10 mg/100 mL Triton X- 100) was prepared in a fume cupboard and stored in a brown glass bottle for up to two months. Solutions which developed a brown coloration within that period were discarded. Eighty µL of heparinised blood and 1 mL of the reagent were pipetted into 3 x 2 mL test tubes. To ensure thorough mixing, the pipette was flushed with the mixture and the solution was vortexed. One mL of the resulting mixture (blood + reagent) was pipetted into a 1 mL plastic cuvette. The absorbance of was read at 546 nm in triplicate against redistilled water on a Shimadzu UV 120 spectrophotometer. The haemoglobin concentration in the samples was determined using on a Boehringer Mannheim GmbH Diagnostica Table.

Haematocrit/Packed cell volume

Heparinised blood was collected into microhaematorit tubes. These tubes were centrifuged in a Cellokrit horizontal centrifuge for 5 min and packed cell volume was measured by means of a haematorule.

5.2.2.2 Biochemical analyses

The sera collected were used for SF, Tf, SI and [Ca] estimations using an immuno-turbidometric method on a Hitachi auto analyser. A Boehringer Mannhein Diagnositic kit containing a monoclonal ferritin antibody, a polyclonal transferrin antibody and enzyme standards were used. The Hitachi autoanalyser is based on the principle used in the Behring nephelometer described in 2.2.5.2.

5.2.3 Intact tissue iron uptake

Intestinal segments were removed from animals immediately after blood collection. Two centimetres of proximal duodenum cut 1 cm away from the pyloric junction. The tissue was kept on ice and the lumen washed through three times with ice cold PBS (pH 7.2) at 4°C. The 2 cm tissue segment was cut in half. The first 1 cm piece (DuoT1) was everted, stretched over and secured to the end of a 3 cm long perspex rod. The tissue was immediately immersed in oxygenated pre-incubation buffer containing (mmol/L); NaCl 125, HEPES 16, KCl 3.5, $CaCl_2$ 1 and MgSO4 10 (pH 7.4) and ferrous ascorbate complex (100 µmol/L Fe2+ : 2000 µmol/L ascorbate) to equilibrate. The tissue in the pre-incubation mixture was vortexed to ensure adequate exposure of all tissue surfaces to the pre-incubation buffer and uniform incorporation of tissue non-radioactive iron (Fe^{2+}). All incubations were carried out for 5 min at 25°C (modified method of Chowrimootoo *et al.*, 1992). One microcurrie (37,000 kBq) ^{59}Fe ($FeCl_3$ radionuclide in 0.5 M HCl; specific activity 23.17 mCi / mg) (Du Pont, Australia) was diluted to specific activity of 1 µCi/ mL and used for the uptake studies. The non-radioactive ferrous ascorbate complex buffer was replaced with fresh oxygenated radioactive ferrous ascorbate (^{59}Fe-ascorbate) complex solution and vortexed for 5 minutes at 25°C. The incubation was terminated by washing the tissue in a 10-fold excess of cold non-radioactive Fe-ascorbate at 4°C to displace surface bound iron.

The tissue was removed from the perspex rod with forceps into a previously weighed glass scintillation vial. The tissue was dissolved in 6 volumes (w/v) of NCS Solubiliser solution (Dupont, USA) at 50°C overnight. Thirty four microliters of acetic acid was added to the radio-labelled tissue solution to remove the light yellow colour formed during the tissue solubilisation. Prior to counting, 10 mL of scintillation liquid (BSC) was added and ^{59}Fe activity was measured using a Beckman Beta counter (1215 Rackbeta II, Finland). Tissue iron uptake was expressed in µmoles of ^{59}Fe/g wet weight of tissue per minute.

The second piece duodenum segment (DuoT2) was fixed in 1% buffered formalin for processing in tissuetex and embedded in paraffin. Paraffin embedded tissues were sectioned and used for morphometric, immunohistochemical and *in situ* hybridisation analyses as described in 2.3 and 2.4.

5.2.4 Brush-border membrane vesicle iron uptake

Brush border membrane vesicles were prepared by a modified protocol of Shirazi-Beechey *et al.* (1992) as described in section 2.2.7. Brush-border membrane iron uptake was assessed by the rapid filtration method of Kessler *et al.* 1978 modified Chowrimootoo *et al.* (1992). All incubations were performed in triplicate. Incubation buffer containing a radioactive ferrous ascorbate complex (1:20 molar ratio of iron to ascorbate; 100 µmol/L $^{59}Fe^{2+}$: 2,000 µmol/L ascorbate; specific activity 1µ Ci/ mL) was prepared in Buffer A (in mmol/L; NaCl 116.6, HEPES 16, KCl 3.5, $CaCl_2$ 1 and $MgSO_4$ 10 (pH 7.4) this being iso-osmotic with the final vesicle preparation. Uptake was initiated by adding 50µL of vesicle suspension to 100 µL ^{59}Fe-ascorbate uptake buffer (buffer A). After incubation for 1 min at 20^{o}C the process was terminated by filtering 100µL of the vesicle suspension and uptake buffer mixture through Millipore filters (0.22 µm) on a vacuum filtration manifold, followed by two washes with 5 mL of ice-cold NaCl (154 mmol/L) containing a 10 fold excess of cold non-radioactive iron (1000 µmol Fe). The radioactivity of the filters was measured in 10 mL BSC scintillation liquid using Beckman BetaRack Counter (1215 Rackbeta II, Finland). Vesicle iron uptake was expressed as µmol/mg of vesicle protein per minute.

Studies of Fe^{2+} uptake into BBM vesicle included the following information: time course of uptake at room temperature; temperature dependency of Fe^{2+} influx into BBM vesicles; and influx rate as a function of the Fe^{2+} concentration in the media.

5.2.5 Brush-border membrane enzymes

Brush border membrane enrichment in the vesicle preparations was assayed using alkaline phosphatase, a brush border marker (Fostner *et al.*, 1968). Alkaline phosphatase activity was measured in homogenate and vesicle samples and the activity expressed as µmol/mg protein. Lactase and sucrase activities were measured in brush border membrane vesicles to assess the effects of dietary manipulations on these digestive enzymes which are indicators of gut maturity during weaning. Lactase and sucrase activities expressed as µmol glucose/mg protein per minute were determined in both homogenate and vesicle samples by a modification of the method of Dahlqvist (1964). All enzyme activities were measured under initial rate conditions.

5.2.6 Characterisation of brush border membrane vesicles

Brush border membrane vesicles were characterised by transmission electron microscopic with the assistance of Dr Marilyn Henderson at the Centre for Electron Microscopy and Microscopic Structural Analysis (CEMMSA), University of Adelaide, South Australia. Vesicle samples were thawed on ice and centrifuged in a micro centrifuge (Mikro Zentrifuge , Germany) at 3,000 g for 5 minutes. The resulting pellet was resuspended in 200 µL of fixative consisting of 1.25 % glutaraldehyde in PBS plus 4 % sucrose at pH 7.2 and left overnight at 4^{o}C. The vesicles were then centrifuged at 3,000 g in a Hewlitt microcentrifuge (Germany) and the supernatant (fixation solution) decanted. The resulting pellet was washed by resuspending it in PBS buffer containing 4% (w/v) sucrose for 30 minutes. The pellet was washed again in sucrose for 30 minutes and post-fixed in 2% (w/v) osmium tetraoxide solution (OsO_4; Probing Structure, Queensland Australia) in PBS at room temperature for 2 hours on a rotator.

The pellet was then dehydrated through a graded series of ethanol solution (70, 90, 95 and 100%). Each dehydration process took 30 minutes and two changes of each ethanol solutions with an additional change of 1 hour in 100% ethanol. The dehydrated pellet was treated once with 50% ethanol / 50% epoxy resin for 8 hours and transferred to

100% resin for a further 3x 8 hours. The pellet was embedded in resin following the third change, polymerised in an oven at 60°C and sectioned on an ultra microtome. The sections were fixed onto aluminium transmission electromicroscopic stubs and dried in acetone and liquid CO_2. The dried vesicle sections were coated with gold-palladium-carbon and examined using a Philips XL 30 Field Emission Transmission Electron Microscope operated by 10 kV (Plate 5.1).

5.2.7 Mucosal morphometry

The proximal small intestine is one of the most responsive iron absorptive regions of the gut. The effect of treatment on mucosal iron absorption was therefore conducted using this region (Chowrimootoo *et al.*, 1992). A cm duodenal segment was collected on an ice cold glass plate and flushed gently with 2 x 2 mL ice cold phosphate buffer saline solution (PBS; pH 7.4) to avoid damaging the mucosa. The tissues were fixed for 12 hours in 10% buffered formalin and transferred into 70% ethanol until processed as described in section 2.2.6. The paraffin wax embedded tissues were sectioned at 8 micron thickness and stored on microscopic poly-l-lysine coated slides as described in 2.2.6. Sections were dewaxed with histolene, hydrated serially in ethanol, stained with haematoxylin, counterstained with eosin and mounted with DePex as described in 2.2.6 (Plate 5.2). Villus height and crypt depth were measured and quantitative morphometric analysis performed using computerised image analysis as described in section 2.2.6. Villus height and crypt depth were expressed in µm.

5.2.8 Statistical analysis

Statistical analyses were performed using the SPSS for Windows program (1995). All values in tables and figures are expressed as mean ± SD. Analysis of variance (ANOVA) was used to compare iron status, body weight gain, dietary treatments, villus height and crypt depth, intact tissue and vesicle iron uptakes. Where significance was achieved ($p<0.05$), Least significant difference (LSD) was used to

determine the differences between groups. Regression and Chi-square analyses were performed to determine associations between various measured parameters.

Transmission electron micrograph of brush border membrane vesicle prepared from duodenal tissue taken from 35 day old weaning rat.

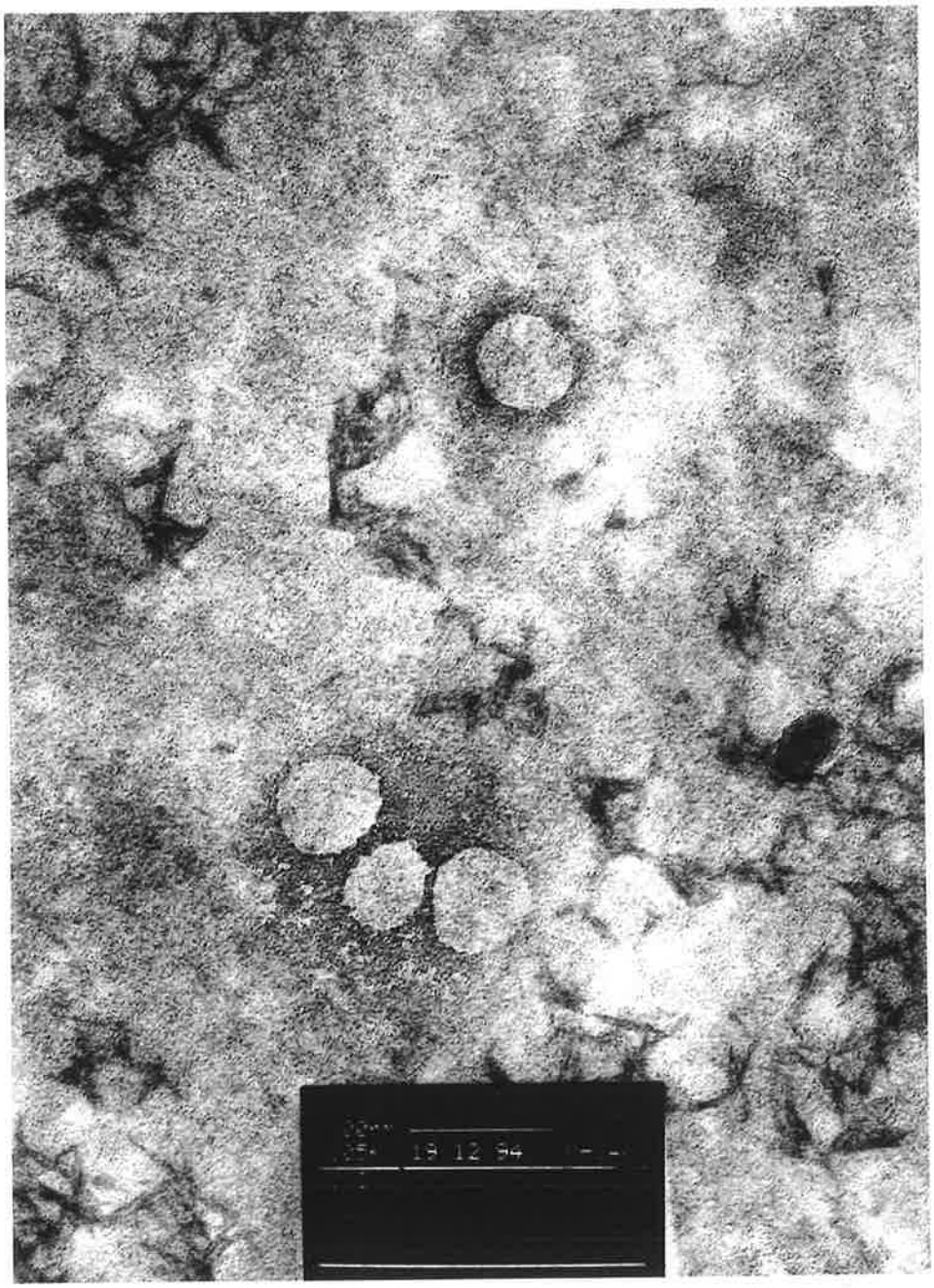


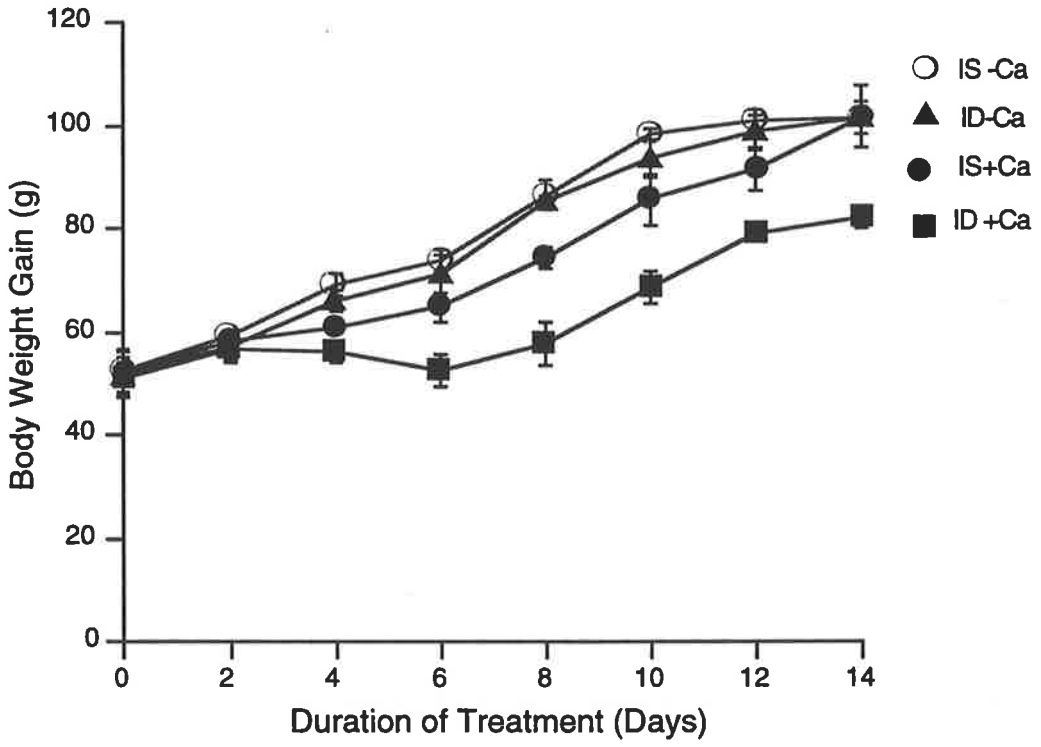
Plate 5.1 Note the presence of brush border membranes with the vesicles (105,000X magnification)

5.3 RESULTS

5.3.1 Body weight gain and dietary intake

Animals on the control (IS + Ca), IS - Ca and ID - Ca rat diets had similar growth rates during the 14 day treatment period, however, those on the ID + Ca diet had arrested growth for up to 6 days. The growth rate of ID + Ca treated animals began to increase after 6 days and they had achieved 78% of the weight of the animals on the other 3 diets at the end of the experiment (Figure 5.1). Food conversion efficiency calculated as grams of food intake per gram body weight was significantly improved in animals on the control diet compared to those on the other diets (control (IS + Ca) , 0.10 ± 0.01; IS - Ca, 0.12 ± 0.01; ID + Ca, 0.13 ± 0.03; ID - Ca 0.11 ± 0.01; $p < 0.05$) reflecting a better food conversion efficiency in the control IS + Ca group.

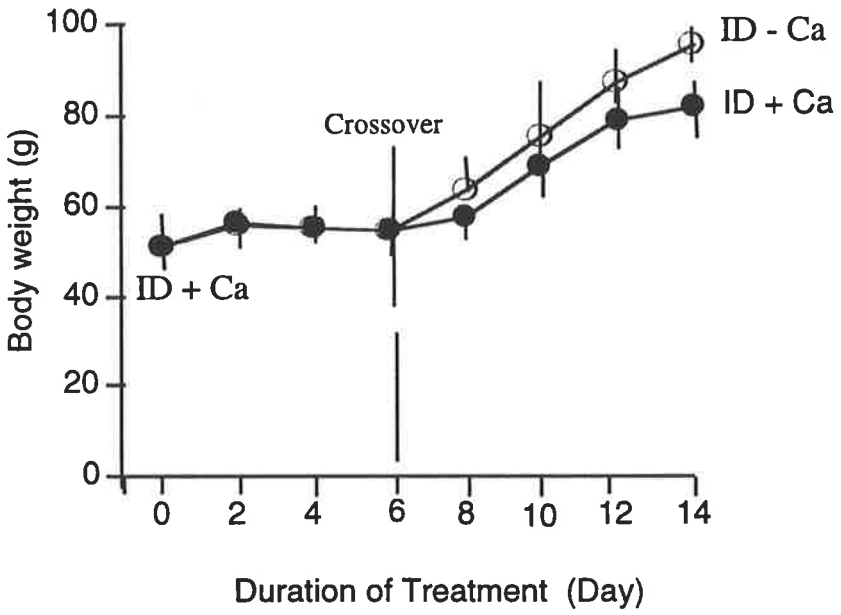
Figure 5.1 Growth profile of weanling rats assigned to either iron sufficient or deficient diets with varying calcium content.



Plot shows body weight gain (± SD) of animals 25 per treatment for 14 days. Each treatment of IS + Ca iron and calcium sufficient (control), Iron sufficient low calcium (IS-Ca), Iron deficient calcium sufficient (ID + Ca) and iron and calcium deficient diet (ID-Ca) had 25 post weaning rats per treatment group. Day 0 represents the beginning of treatment in 21 day old Wistar rats.

A positive association was found between the four dietary treatments and food conversion efficiency ($r = 0.54$. $p < 0.0001$) as defined by food intake per body weight gain of animals over the 14 day treatment period. A cross over study in which the efficacy of the two iron deficiency diets in maintaining adequate growth, was compared showed that the two groups of animals ($n = 8$) started on ID + Ca and crossed over to ID - Ca diet on day 6 had a 20% improved growth rate (Figure 5.2).

Figure 5.2 A cross over Growth profile of 35 day old weanling rats assigned to either iron sufficient or deficient diets with varying calcium contents



Plot shows mean ± SD in body weight of animals crossed over from ID + Ca diets onto ID - Ca diets on day six of treatment. Note the improved growth in animals crossed over to ID - Ca diet compared to ID + Ca group who remained on ID + Ca diet. Number of animals in each group is 8.

5.3.2 Haematological and biochemical measurements

Animals on iron deficient diets (ID + Ca and ID -Ca) had significantly lower haemoglobin, (Hb) haematocrit (Hct), serum ferritin (SF), serum iron (SI) and transferrin concentrations (Tf) compared with those on iron sufficient diets (Table 5.1). Animals on calcium sufficient diets (IS + Ca and ID + Ca) also had significantly higher ($p < 0.01$) serum calcium levels compared with the calcium deficient groups. There were strong positive associations between dietary iron intake and haemoglobin concentration ($r = 0.53$; $p< 0.001$), dietary iron and Hct ($r = 0.61$; $p<0.001$), and dietary iron and serum ferritin ($r = 0.51$; $p<0.001$), confirming an association between dietary iron consumption and both iron stores and erythropoiesis. There were also strong positive correlations between red blood cell indices and serum ferrtin; Hb and SF ($r = 0.60$, $p< 0.001$), Hct and SF ($r = 0.73$, $p < 0.001$). There was also an association between dietary calcium intake and serum calcium concentration ($r = 0.65$, $p < 0.001$) as well as serum iron and serum calcium ($r = 0.31$, $p < 0.04$).

The removal of calcium from the diet decreased SF concentration by 47 % ($p < 0.01$) under iron sufficiency conditions but marginally improved iron stores (10%) under iron deficiency conditions (Table 5.1). Addition of calcium to the diet on the other hand increased both haemoglobin and haematocrit estimations by 7% ($p<0.05$) under iron sufficiency conditions but decreased Hb and Hct under iron deficiency (Table 5.1). These results suggest a dual role for calcium as either an enhancer of storage iron protein and erythropoiesis under IS while inhibiting iron storage under ID conditions, although this was no significant (Table 5.1).

Trends similar to that of SF was found with transferrin (Tf) expression under both iron sufficiency and deficiency. The removal of calcium decreased (Tf) expression by 29% and 39% under Is and ID conditions respectively. There was no correlation between Tf and any of the red cell parameters nor SF. Although a moderate association was found between TF and serum iron, this was not significant ($r = 0.25$; $p < 0.09$).

Table 5.1. Effect of dietary calcium on haematological, biochemical iron status and serum calcium in post weaning rats (35 day old) assigned to iron sufficiency or iron deficiency diets with varying calcium contents.

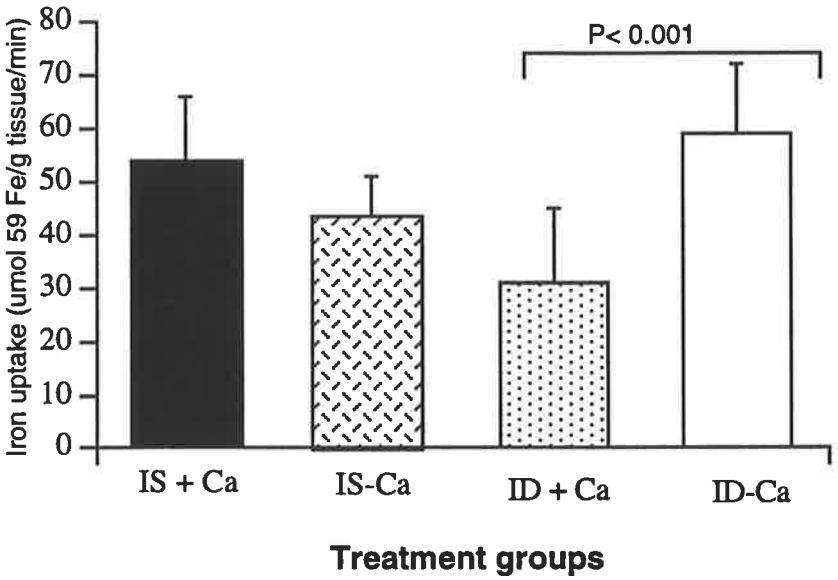
BLOOD PARAMETERS	DIETARY TREATMENT			
	Iron and calcium sufficient Control (IS + Ca)	Iron sufficient with low calcium (IS - Ca)	Low iron sufficient calcium (ID + Ca)	Low iron and low calcium (ID - Ca)
Haemoglobin (g/dL)	13.7 ± 1.4	13.4 ± 0.8	**7.8 ± 0.1	*8.5 ± 1.4
Haematocrit (%)	41.7 ± 2.4	38.8 ± 2.3	*25.6 ± 2.4	*28.2± 3.6
Serum ferritin (µg/L)	232 ± 98	*157.0 ± 82	**69.0 ± 15	**74.0 ± 17
Transferrin (µmol/L)	58.4 ± 30	41.5 ± 20	49.3 ± 14	*30.0 ± 12
Serum iron (µmol/L)	1.24 ± 0.01	1.2 ± 0.02	1.2 ± 0.01	1.1 ± 0.02
% Saturation	176 ± 69	147 ± 102	176 ± 120	*113 ± 19
Serum calcium concentration	2.64 ± 0.4	*1.6 ± 0.2	2.7 ± 0.4	*1.9 ± 0.2

Table represent result in mean (± SD) haematology and biochemical indicators of iron status in post weaning rats (35 day olds). Is-Ca is control diet which is iron and calcium sufficient; IS -Ca is iron sufficient calcium deficient; ID + Ca is iron deficient with sufficient calcium; and ID-Ca is iron and calcium deficient. *Significant values ($p < 0.01$) and ** ($p < 0.001$) from control (IS + Ca) are marked

5.3.3 Mucosal iron uptake

Intact duodenal tissue iron uptake was 17% greater in controls (IS+Ca) compared with IS-Ca animals. Under iron deficiency conditions, however, the trend was reversed, with the reduction of calcium in the diet significantly increasing duodenal iron uptake by 97 % ($p < 0.001$) (Figure 5.3). This suggests an inhibitory interaction of calcium on musosal tissue iron uptake with suboptimal iron status.

Figure 5.3 Effects of dietary calcium on intestinal non-haem radio labelled iron (^{59}Fe) absorption in 35 day old post weaning rats assigned to one of four diets of iron sufficient or iron deficient diets with varying calcium contents.



Plot shows mean ± SD of intact tissue iron (^{59}Fe) uptake in µmol ^{59} Fe /g tissue /minute. Each treatment of IS + Ca iron and calcium sufficient (control), Iron sufficient low calcium (IS-Ca), Iron deficient calcium sufficient (ID + Ca) and low Iron low calcium (ID-Ca) had 25 post weaning rats per treatment group. Uptake was carried out in triplicate. Plot shows that intact mucosal $^{59}Fe^{2+}$ uptake significantly ($p< 0.001$) decreased in post weaning rats (35 day olds) on iron deficient with calcium sufficient diet (ID + Ca) compared with those on low iron low calcium (ID - Ca). There was however a reversed trend under iron sufficiency condition (Is + Ca and IS - Ca) .

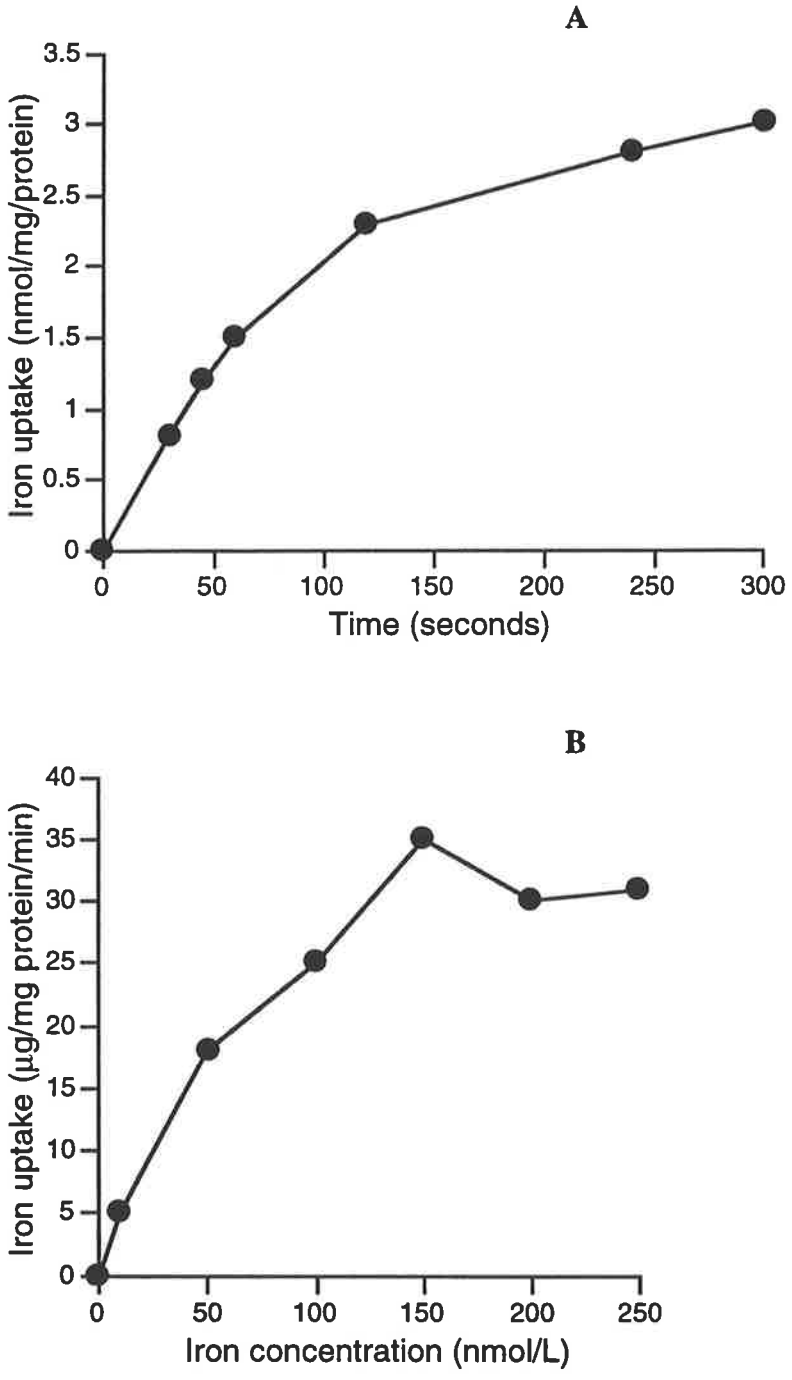
The inhibitory effect of calcium on intact duodenal tissue iron uptake under ID condition was similar to the levels of inhibition on body iron stores as measured by serum parameters of iron status, where a low-calcium diet was associated with a 10% increase in serum ferritin. There was, however, a 25% increase in duodenal iron uptake of animals on calcium and iron sufficient diets compared with iron sufficient low calcium diets (Figure 5.3). Overall, there was a 30% negative association ($p< 0.01$) between mucosal iron uptake and calcium status, although it is more pronounced under iron deficiency condition.

5.3.4 Brush border membrane vesicle iron uptake

Iron accumulation by vesicles has been shown to account almost completely for iron transport into internal space of vesicles with minimal binding to the membrane (Chowrimootoo *et al.*, 1992; O'Riordan *et al.*, 1997). Thus studies with vesicles are a useful adjunct to studies with intact mucosal enterocytes. A time course and iron concentration gradient showed iron saturation at two minutes and concentration at 100 nmol of $^{59}Fe^{2+}$ (Figure 5.4 a and b) respectively.

The enrichment for brush border membrane enzymes using alkaline phosphate activity as a marker was 6 to 10 fold relative to the original mucosal homogenate (Table 5.2). To assess the effects of diet on the apical brush border membranes, iron transport studies were carried out. A reduction in dietary calcium concentration significantly decreased vesicle iron uptake by 70 % ($p < 0.001$) under iron sufficiency and 38 % in iron deficient animals (Figure 5.5). The enhancing effect of dietary calcium on apical vesicular iron accumulation was similar to the effects of calcium on haemoglobin concentration (Table 5.1). The enhancing effect of calcium on iron accumulation was contrary to the effects on intact tissue iron uptake, where a reduction of dietary calcium significantly increased mucosal iron uptake under iron deficiency condition.

Figure 5.4 A time dependence (a) and substrate activation (b) of iron uptake by duodenal brush border membrane (BBM) vesicles of 35 day old post weaning rats on iron and calcium sufficient diet (Control; IS + Ca) at room temperature.



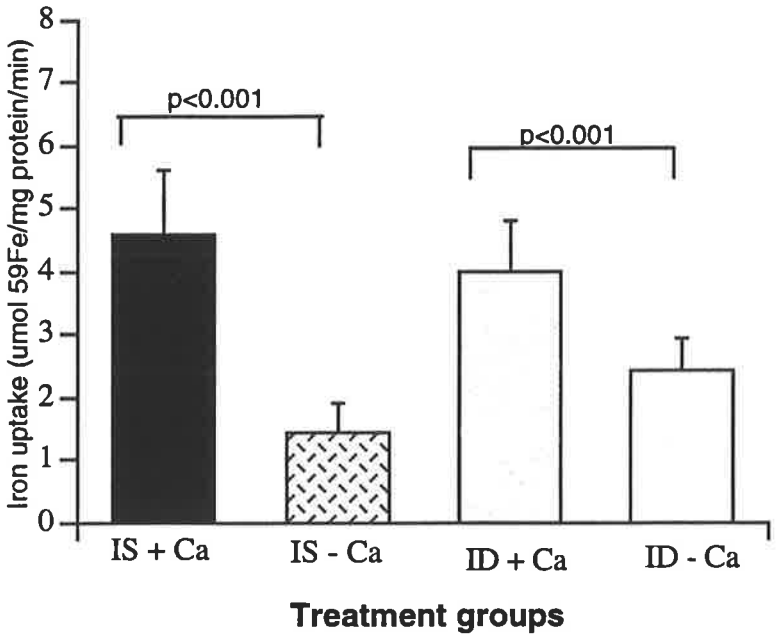
Plots are mean ± SD (n = 4-6) of vesicle preparations from post weaning rats (35 day olds) on iron and calcium sufficient diets. Uptake was carried out in triplicates at 25^0C control. The brush-border membrane vesicles showed saturation kinetics.

Table 5.2. Duodenal digestive enzyme profiles of weaning rats (35 day old) maintained on varying Iron and Calcium contents

Treatment Group	Enzyme Activity Profile							
	Protein concentration (mg protein / g tissue)		*Alkaline phosphatase (µgmole/mg protein / hour)*		*Lactase Activity (µmol glucose/mgprotein/ hour)*		*Sucrase Activity (µmol glucose / mg protein)/hour*	
	Homogenate	Vesicle	Homogenate	Vesicle	Homogenate	Vesicles	Homogenate	Vesicle
Control rats; Iron and calcium sufficient (IS + Ca)	21.4 ± 2.2	6.2 ± 1.3*	8 ± 1.0	48 ± 3.6*	26 ± 4.0	38 ± 6.0	26.5 ± 4.3	42 ± 10
Iron sufficient low calcium (IS + Ca)	21.8 ± 1.9	5.7 ± 0.8*	6± 0.8	42 ± 5.0*	19.8 ± 3.6	36 ± 10.0	28.3 ± 5.8	46 ± 5.8
Iron deficient calcium sufficient (ID + Ca)	18.3 ± 1.6	5.4 ± 0.7*	12 ± 1.0	30 ± 6.0*	27.3 ± 6.8	40 ± 7.0	24.2 ± 2.6	38 ± 7.9
Iron and calcium deficient ID - Ca	21.6 ± 2.1	6.5 ± 2.1*	10 ± 2.0	26± 7.0*	27.6 ± 7.4	38 ± 6.0	26.6 ± 7.5	45 ± 6.2

Table shows protein concentration, alkaline phosphatase and profile of digestive enzymes lactase and sucrase from homogenate and vecisle samples from IS + Ca, IS-Ca, ID + Ca and ID - Ca. * Indicates significant difference in alkaline phosphatase activity in vesicle compared with homogenate samples.

Figure 5.5 Radio-labelled iron (^{59}Fe) uptake by brush-border membrane vesicles prepared from 35 day old post weaning rats fed one of four diets of Iron sufficient or Iron deficient with varying calcium contents.



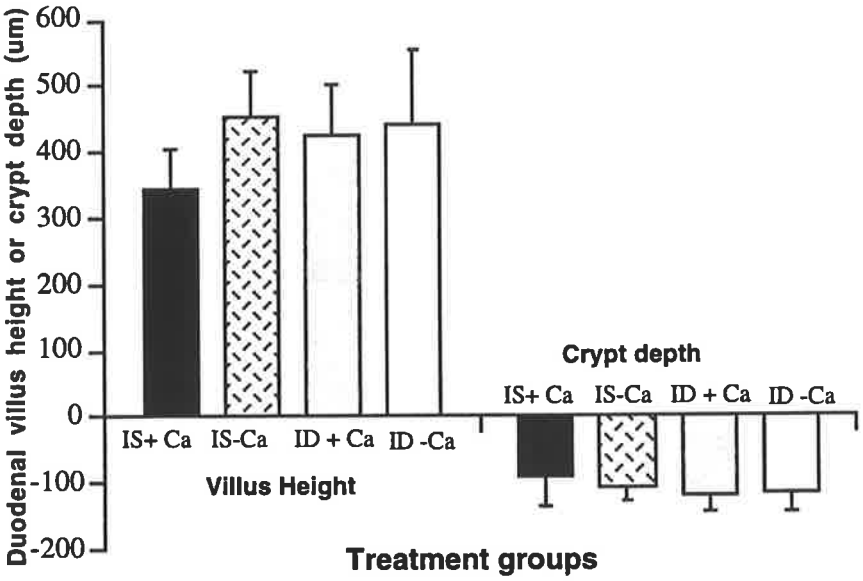
Plot shows mean ± SD. Wistar rats were fed one of the four diets for 14 days. Vesicle samples were prepared from 25 animals per each treatment group. Uptake was carried out in triplicates. Plot shows that vesicle $^{59}Fe^{2+}$ uptake significantly ($p< 0.001$) increased in iron and calcium sufficient post weaning rats (35 day olds) compared to those on iron sufficient with no calcium (IS - Ca) or the other treatment groups .

5.3.5 Duodenal morphometry

The effects of iron and calcium status on intestinal morphometry were assessed to further elucidate the effects of these dietary treatment on villus height and crypt depth. Villus height and crypt depth in control (IS + Ca) animals were significantly ($p < 0.05$) lower compared with the other treatment groups. Under iron sufficiency conditions, calcium deficiency was associated with a 32% increase in villus height and a 10% increase in crypt depth suggesting morphological changes under IS-Ca to compensate for calcium inadequacy (Figure 5.6). Under iron deficiency conditions, however, there were no significant differences in either villus height or crypt depth of animals on ID + Ca or

ID - Ca diets, suggesting that calcium had no effect on the morphometry of the enterocyte when iron status was inadequate. Villi of ID + Ca animals had leaf-shaped morphology compared with those of animals in IS+ Ca, IS - Ca and ID - Ca treated animals. The leaf shaped morphology of ID + Ca villi suggests an increase in surface area for increased absorption, although this was not measured (Plate 5.2).

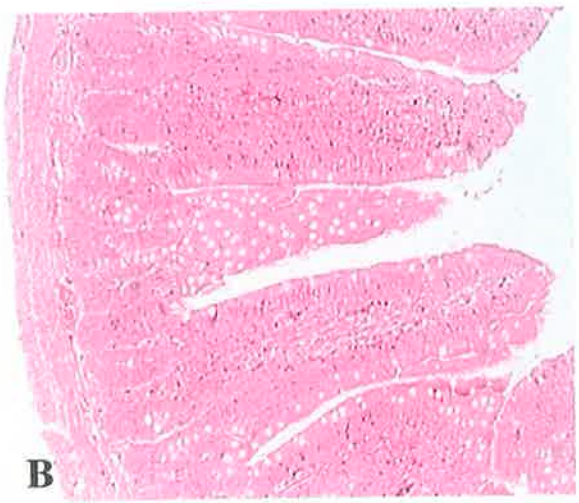
Figure 5.6 Villus heights (positive orientation) and Crypt depths (negative orientation) of duodenal mucosa of 35 day old post weaning rats assigned to one of dietary treatments of iron sufficiency or iron deficiency diets with varying calcium contents.



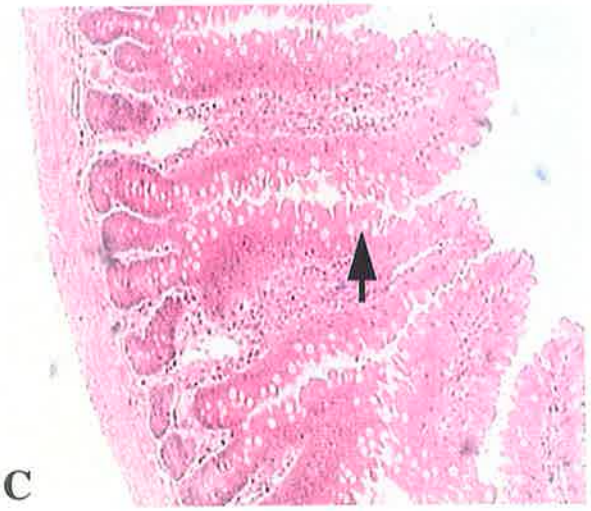
All values were calculated as mean ± S D. Each dietary treatment group of Iron and calcium sufficient (Control - IS + Ca), Iron sufficient calcium deficient (IS - Ca), Iron deficient calcium sufficient (ID + Ca) and Iron and calcium deficient (ID - Ca) diets had 25 animals per group. Note the significant differences ($p < 0.05$) in villus height and crypt depth of animals on control (IS + Ca) diet compared with those on the three treatment diets.

Plate 52. Histology of Duodenal Tissues from Weaning Rats Maintained on Varying Iron and Calcium Content Diets

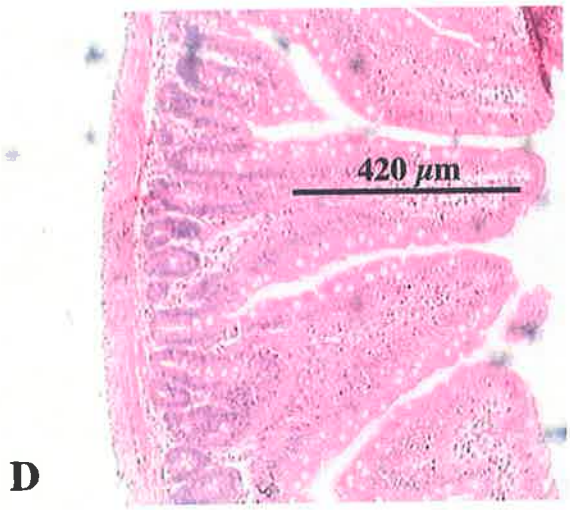
Iron and Calcium sufficient (IS + Ca; Control)



Iron sufficient, Calcium deficient (IS + Ca)



Iron deficient, Calcium, sufficient (ID + Ca)



Iron and Calcium deficient (ID - Ca)

Plate 5.1 shows histology of duodenum samples from 35 day old Wistar rats maintained on (**A**) Iron and calcium sufficient (IS + Ca; Control), (**B**) Iron sufficient calcium deficient (IS - Ca), (**C**), Iron deficient calcium sufficient (ID + Ca) and (**D**), Iron and Calcium deficient (ID - Ca) for 14 days. Note the leaf- shape morphology of the ID + Ca tissue (C). Magnification X 25.

5.4 DISCUSSION

5.4.1 Effects of calcium on haematological and biochemical indicators or iron status

Adequate iron balance is essential for erythropoiesis, growth, cognitive function and brain maturation during the weaning period in children (Cook 1990; Dobbing 1990; Walter *et al.*, 1993; Conrad *et al.*, 1994; Yip, 1995). To achieve optimal iron status during the growing period, the ingestion of daily dietary iron must be adequate to balance the amount of iron that is utilised for erythropoiesis, tissue iron storage and transport (Lipschitz *et al.*, 1974; Bothwell *et al.*, 1979; Gavin *et al.*, 1994; Sikstrom et al., 1997). When haematological and biochemical measurements were used to assess iron status and dietary iron intake of 35 day old post weaning Wistar rats, positive correlation was found between iron status and dietary iron intake. These results are similar to those in a study by Smith *et al.*, (1993), who showed that weanling rats fed low iron diets (< 2 mg Fe /kg diet) for nine days had only 45% of the Hb concentration compared to animals on iron sufficient diets (320 mg Fe/kg diet).

A consistent (43%) decline in erythropoiesis was also found in the rats as a consequence of reduced dietary iron in the current study. This was not so evident in the human studies in chapters 3 and 4 where a low association was found between dietary iron and iron status in children. The high correlations between dietary iron and haematological and biochemical indicators may be due to the weaning diets used. These were semi-purified and contained all essential micronutrients, with the exception of iron, which was manipulated. Daily food intake was also accurately measured in the animals. In contrast, the children's diets were more varied and often inadequate in most essential nutrients. Dietary recalls were unlikely to provide accurate estimations of dietary iron intake in children. There were also strong positive associations between Hb and SF ($r = 0.60$, $p < 0.0001$) and Hct and SF ($r = 0.73$, $p < 0.0001$) in this rat study, whereas only moderate correlations of these blood indicators were found in the humans studies. The low Hb concentration in ID + Ca compared to IS + Ca is likely to be due to the 70%

decline in storage iron (SF) in ID + Ca in response to a 96% decrease in dietary iron over the 14 day period.

5.4.2 Effects of calcium on iron status

This study also showed the effects of calcium on iron status. A positive relationship between calcium and serum ferritin was found under iron sufficiency conditions. A 48% reduction in body iron stores in IS - Ca rats relative to IS + Ca was found in iron-sufficient rats with low calcium diet whereas only a 7% increase in iron stores was found under ID conditions. The calcium content of the diet had no significant effect on Hb concentration in either IS or ID animals. This is the first time a negative causal effect of dietary calcium has been found on iron stores measured as serum ferritin in weanling rats using the same polyclonal ferritin antibodies used in human assays. Others have found associations between calcium and biochemical iron status parameters such as liver iron concentration, serum iron and bone marrow staining (Barton *et al.*, 1983; Hunt *et al.*, 1994b).

Barton and coworkers (1983) have shown in immature rats fed high, normal or low calcium diets containing normal or low levels of iron for 4 weeks to have evidence of iron deficiency (low serum iron and haematocrit). Evidence of ID was more prominent among rats consuming high calcium diets (Barton *et al.*, 1983). This is consistent with results in the current study which showed an improved Hb and serum ferritin in rats fed low iron and low calcium (ID - Ca) diets. Despite the differences in the age and calcium concentration used, iron deficiency was observed in the study by Barton and colleagues (1983). Barton's study used young adolescent 54 day old rats and higher calcium contents 19g Ca / kg compared to the use of 21 day olds and 12.5g Ca /kg diet in the present study. Hunt *et al.* (1994b) also found significant differences ($p< 0.05$) in liver iron stores (indicated by liver non haem iron) in iron deficient (0.79 ± 0.24 µmol/g dry wt) and iron adequate adult rats (4.58 ± 0.62 µmol/g dry wt) on high to normal calcium diets. The current study confirms those by Barton *et al.* (1983) and Hunt *et al.* (1994b)

that high dietary calcium with low or normal iron diets can induce iron deficiency irrespective of age. Evidence of the inhibitory effects of dietary calcium on the iron homeostasis of the rats also supports earlier findings by Oti-Boateng *et al.* (1994) that diets with high calcium content can induce a 12% decrease in body iron stores in children on low to moderate iron diets.

5.4.3 Effect of calcium on body weight

Calcium intake also affected body weight. Animals on optimum dietary iron with calcium maintained adequate growth whereas the ingestion of high dietary calcium with iron deficient diets significantly impeded growth ($p < 0.001$). Animals on ID + Ca diets had the lowest food conversion efficiency and this was associated with a 20 % decrease in body weight compared with animals on the ID - Ca diet on day 14. The decrease in body weight was also associated with a more severe anaemia. These results are consistent with earlier findings by Barton *et al.* (1983) and Hunt *et al.* (1994). Weaning rats fed moderately iron deficient but high calcium content foods gained less weight and had lower serum iron concentrations with diminished marrow iron stores compared with control rats (Barton *et al.*, 1983). Hunt and coworkers showed a 15% decrease in body weight over an 8 week treatment period in adult iron deficient rats (on 4.6 mg Fe /kg diet) compared with animals on marginal iron diets (19 mg Fe/kg diet) (Hunt *et al.*, 1994b). These animals were anaemic and substantially less active during both the dark and light periods.

The decreased food conversion efficiency and anaemia seen in ID + Ca rats in this current study may be associated with reduced locomotor activity which is consistent with dopaminergic function (Youdim *et al..*, 1989; Yehuda and Youdim, 1989). The low activity of the ID + Ca rats may have induced lethargy which probably affected feeding. There was however a compensatory growth in animals on ID + Ca when crossed over onto ID - Ca diets on day 6 for another 8 days. The low weight gain associated with ID + Ca treatment suggests that children who subsist on low iron with high calcium diets

may be at risk of growth impairment during the critical period of weaning. Whether these children with impaired anthropometric measurements will be able to reach the potential of their IS + Ca or IS - Ca counterparts will need to be investigated.

5.4.4 Effects of calcium on gut growth and maturation

Gut growth as assessed by morphometry showed that villus height and crypt depth of control (IS + Ca) rats were significantly lower than those from the other 3 treatment groups. This may suggest a compensatory effect where there is an elongation of villus height or crypt depth in IS - Ca, ID + Ca and ID - Ca to increase enterocyte surface area for increased absorption of nutrients. Leaf-shaped villi were found in ID + Ca rats. Although the differences between the villus height and crypt depth of ID + Ca and ID - Ca rats in the present study were not significant, morphologic abnormalities associated with nutritional iron deficiency were observed by Naiman (1987). The enterocytes of animals on ID + Ca diets in the present study contained an elaborate array of supranuclear vacuoles which may be associated with transport of and degradation of macromolecules and which are characteristic of immature enterocytes (Cornell and Padykula, 1969; Connella and Neutra, 1984). The cessation of macromolecular uptake appears to be directly linked to an increase in cellular proliferation and migration and repopulation of immature enterocytes by adult-like cells in rats (Simister and Rees, 1983).

The morphometric measurements also support an effect of calcium on gut proliferation and maturation as shown by the decrease in gut protein and an increase in lactase activity seen in rats fed ID + Ca diet respectively. At weaning an intrinsic timing mechanism triggers intestinal maturation which involves a decrease in lactase-phlorizin hydrolase activity and an up-regulation of sucrase-isomaltase and alkaline phosphatase activities (Henning, 1985; Menard and Calvert, 1991). This functional adaptation of the small intestine for the digestion of carbohydrate as part of normal gastrointestinal development requires the transition to adult-like diets in rats. Under normal conditions, there is intestinal closure to prevent the uptake of macro-molecules at weaning. Nutrients

are therefore important for the stimulation of intestinal growth in weanling and adult rats. Insulin and numerous other growth factors have been implicated in this regulation (Smith *et al.*, 1993; Steeb *et al.*, 1996). Mucosal homogenates of rats fed on ID + Ca diets had lower protein concentrations than any of the other three treatment groups. Animals on the ID + Ca diets had 17% less protein in their mucosal homogenate than of IS + Ca rats, which suggests a reduced digestive capacity of the gut of ID + Ca rats as a result of low protein synthesis.

Relating histological findings with biochemical measures of brush border sucrase and lactase activity of duodenal enterocytes, no significant differences were observed in sucrase activity in animals in the four treatments groups, although ID + Ca animals had significantly lower sucrase activity in homogenates and vesicle (17%) than ID - Ca rats. There was, however, an increase lactase activity in rats fed ID + Ca rats than ID - Ca rats. This further confirms an adverse effect of calcium on gut growth and maturation under iron deficiency conditions. Lactase activity was present in duodenal mucosal homogenates and vesicle fractions in all animals in all four treatments but the levels were only 10% of those found in mucosal homogenate of neonatal (6-12 day olds) Wistar rats (Steeb *et al.*, 1996). Lactase activity measured in duodenal enterocytes indicates a transition of the gut from weaning to adult. The morphometric characteristics and duodenal mucosal digestive enzyme activities found in this study suggest that diets containing optimal dietary calcium and a low iron content can retard gut maturation and affect the capacity of the duodenal mucosa to absorb iron.

5.4.5 Interaction of dietary calcium with duodenal iron absorption.

Results from this present study and clinical studies in chapters 3 and 4 have shown that dietary calcium can reduce iron store accretion and also retard gut maturation. The mechanism of interaction at the point of absorption is not known. Iron absorption from the intestinal lumen to the blood involves carrier-mediated steps at both the brush-border and basolateral membranes (Eastham *et al.*, 1977, Cox *et al.*, 1979 (a, b); Conrad

et al., 1993). Despite extensive studies on iron absorption, however, the cellular locus for adaptation of iron uptake in response to iron deficiency remains unclear. It is however possible that the newly discovered iron transporter which is inversely regulated by iron (Nramp2) (Fleming *et al.*, 1997; Gunshin *et al.*, 1997), is the likely brush border membrane iron transporter.

In the present study, brush border membrane vesicles and intact duodenal tissue were used to compare features of membrane iron uptake by ID and IS enterocytes with varying dietary calcium concentrations. There was a significant increase ($p < 0.001$) in brush-border membrane iron uptake of animals fed IS + Ca diets than IS - Ca, suggesting an enhancing effect of calcium on brush border iron which was also apparent in SF concentration. The increased duodenal iron absorption despite iron sufficiency in weaning rats on IS + Ca diet is consistent with the high iron requirements for increased erythropoiesis during the period of rapid growth (Kozma *et al.*, 1994). There was a similar trend under ID conditions, where dietary calcium increased apical iron uptake, suggesting that calcium may be influencing the brush-border membrane potential difference.

Transporters of iron at the brush border and basolateral membrane have been proposed and intracellular iron binding proteins which may regulate the availability of cytosolic iron for transport across the basolaeral membrane have been recently identified (Conrad *et. al.*, 1993; 1994; Fleming *et al.*, 1997; Gunshin *et al.*, 1997). Conrad and coworkers have identified alternative iron binding proteins to transferrin and ferritin, which have been associated with intestinal iron absorption and regulation although these findings are speculative and not widely supported. These proteins include, mucin, integrin, mobilferrin and paraferritin. Mobilferrin was identified in the intestinal epithelial cells by immunological methods and has been shown to competitively bind calcium but have a high affinity for iron (Conrad *et. al.*, 1994). The N-terminal sequence of mobilferrin has been shown to be homologous with calreticulin, a calcium binding

protein. Taking all these together, it can be hypothesised that mobilferrin may play a role in mediating the inhibitory effect of dietary calcium on intact tissue iron uptake under iron deficiency conditions. These findings suggest that the significant increase in the intracellular iron uptake in ID - Ca compared with ID + Ca may be associated with competition between calcium and iron for binding to intracellular mobilferrin which may in turn affect the availability of cytosolic iron for transport to peripheral blood.

Recent studies have also identified a new iron transporter protein, Nranp2, in intestinal and haematopoietic systems, which is likely to be important for normal iron transport (Fleming *et al.*, 1997). Functional expression cloning by Gunshin *et al.* (1997) has confirmed that Nramp2 is indeed an iron transporter and a single cDNA (called DCT 1) encoding the rat isoform of Nramp2 was found to stimulate iron uptake. Based on functional characterisation of DCT1, Gunshin and coworkers (1997) have also shown that DCT1 stimulates the absorption of a range of divalent cation such as Fe^{2+}, Zn^{2+} and Mn^{2+} and Cu^{2+} at physiological Ca^{2+} levels whilst excessive luminal calcium interfered with normal absorption of trace elements such as iron. Since Nramp2 is inversely regulated by iron, it is likely that dietary calcium is inhibiting Nramp2, thus decreasing intact tissue iron uptake as shown in animals fed ID + Ca diets in the present study. The marginally higher haemoglobin concentration in ID - Ca animals confirm this hypothesis. Low calcium diets under ID conditions improved haemoglobin concentration by 7% and increased intestinal iron absorption by as much as 60%. The findings of Gunshin *et al.* (1997) are consistent with an inhibitory role of calcium on iron absorption in animals on low iron diets, as shown in results from intact tissue iron uptake, SF and Hb under iron deficiency. These observations, however, differ from the observations found in BBM vesicles, where Ca^{2+} enhanced the uptake of brush-border membrane iron. The recent cumulative evidence of Nramp2 and the similarity in its characteristic iron regulating potential may suggest that, the inhibition of iron transport by calcium may be at the basolateral rather than the apical level. Alternatively, these differences seen in

the brush border membrane vesicle may be due to technical differences in the methodology.

The degree of inhibition of intestinal iron absorption by dietary calcium has also been reported by Hallberg and coworkers (1992a) in human. They showed a 30-60% inhibition of erythrocyte incorporation of radio-labelled dietary non haem iron in a dose-dependent manner but could not elucidate the exact mechanism of the interaction. These findings indicate that calcium may be exerting inhibitory effects on both intestinal iron uptake and iron transport to peripheral blood. Its antagonistic effect is particularly evident under iron deficiency condition. The increased intestinal iron absorption in control and IS - Ca animals despite their positive iron status may relate to the continuous high iron requirement for growth, mucosal epithelial cell proliferation and erythropoiesis during the weaning period.

5.4.6 Proposed mechanism of inhibition of dietary calcium on duodenal iron absorption.

A simplistic model of dietary calcium interaction with iron absorption during weaning is suggested in which calcium may not retard iron uptake at the brush border membrane during weaning as proposed by Barton *et al.* (1983) in adult rats. Instead, calcium slows down the absorption of iron into the mucosal cell and/or competes with binding proteins such as mobilferrin, Nramp2 or ferritin, common to the absorptive pathways of both iron and calcium under iron deficiency condition. It is also possible that calcium delays the movement of iron into the circulation, but this was not evident in serum Tf concentration. Calcium may therefore impede intestinal cellular iron movement, but accelerate serum iron transport for iron storage under iron sufficiency condition.

5.5 CONCLUSION

This experimental animal model confirms the findings from the two previous clinical epidemiological studies which showed that dietary iron is an important factor affecting iron repletion. A more conservative iron homeostasis was found in children compared to the rats, although animal models are useful for mimicking the effects of inadequate dietary iron intakes on red blood cell and biochemical indicators of iron status. Consumption of a diet which is consistently low in iron will produce iron deficiency. Results also indicate that consumption of low-iron weaning diets with high dietary calcium levels can reduce intestinal iron absorption . Dietary calcium did not appear to have a direct effect on villus morphology under iron deficiency conditions. However, the study has shown that under iron deficiency conditions diets low in calcium will improve initial growth rate. This suggests that high levels of calcium under ID conditions would lead to early growth retardation which may jeopardise future growth potential and which in turn can impair brain growth and behaviour (Dallman, 1990; Walter *et al*, 1993). Dietary calcium did not appear to affect intestinal maturation significantly.

An interaction of calcium with intestinal non-haem iron absorption at the cellular level through the induction of changes in the synthesis of iron binding proteins such as ferritin, mobilferrin and Nramp2 is proposed. This study gives credence to earlier clinical findings which showed that marginal iron diets with high calcium will increase the risk of iron deficiency especially during weaning. It is therefore important to carefully select weaning diets to optimise the bioavailability of both iron and calcium since both nutrients are essential for physical growth and development. The most interesting result from this current study suggests that the rate limiting process for iron transport to the blood from the mucosal cell does not involve the uptake of iron from the brush border, as suggested by Barton *et al.* (1983). The current study, showed that the site of calcium inhibition on iron is at the point where iron is transferred from the brush border to intra cellular iron transporters to peripheral blood. There is the need to further investigate the mechanism of interaction of calcium on intestinal iron binding proteins such as transferrin and ferritin

which are commercially available at the molecular level. Future studies may need to be extended to include DCT1.

CHAPTER 6

EFFECTS OF DIETARY CALCIUM ON FERRITIN AND TRANSFERRIN RECEPTOR GENES EXPRESSION IN DUODENAL ENTEROCYTES DURING WEANING.

6.1 INTRODUCTION

Although iron absorption has been the subject of many investigations, the method by which intestinal mucosal cells modulate iron uptake from the lumen of the gut and affect the transfer of iron from the cell into the body is not clearly understood. The major site of iron absorption is in the duodenum with the jejunum and ileum contributing very little, if any at all, to iron uptake (Chowrimootoo *et al.*, 1992). Homeostasis of body iron has been demonstrated in the last three chapters to be maintained by the regulation of absorption across the duodenal enterocyte. A mechanism seems to exist whereby the mucosal cells responds to information about body iron stores and regulates absorption in response to body iron requirements (Skistrom *et al.*, 1993; Gavin *et al.*, 1994; Oates *et al.*, 1997). The precise mechanism for non-haem iron absorption is still speculative (Conrad *et al.*, 1993). Recent work, however, suggests the mechanism controlling iron transport across the mucosa to be via the binding of ferrous iron to iron transporter proteins such as Nramp 2 which is regulated by DCT1, likely to have specific binding sites on the brush border membrane (Fleming *et al.*, 1997; Gunshin *et al.*, 1996). The mechanism of the interaction of calcium with iron during absorption which is in the proximal intestine is not known, although clinical studies in chapters 3 and 4 have shown iron status in infants and toddlers to be influenced by the calcium content of the diet. Animal studies in chapter 5 also confirmed the inhibitory effect of calcium on iron absorption and showed that calcium retarded movement of iron from the apical brush border membrane across the intracellular enterocytes cells to peripheral blood.

From chapter 5 it was hypothesised that there are two possible roles of calcium on mucosal iron absorption. Calcium interacts with ferritin which is thought to play major roles in iron storage and detoxification in mucosal cells. It is likely that calcium may be interacting with ferritin gene by increasing mucosal iron storage. Secondly, all cells require iron for the synthesis of iron containing enzymes and proteins and for mitosis. The high iron requirement during mitosis is evidenced by an increased expression of transferrin receptors preceding cell division and the receptor-mediated

uptake of transferrin providing the major pathway of iron uptake by cells (Oates and Morgan, 1997). It is also possible that calcium may modulate transferrin which is an iron binding protein. In the current study, changes in the levels of expression of L and H ferritin and transferrin by the duodenal enterocytes of weaning rats were investigated to determine whether there were any associations with changes in dietary calcium concentrations. *In situ* hybridisation and immunohistochemitry techniques were employed to investigate the effect of calcium on ferritin and TfR gene expression in duodenal enterocytes.

6.2 METHODS

6.2.1 *In situ* Hybridisation Technique

Localisation study was initiated to determine the distribution of ferritin and TfR mRNAs by tissue *in situ* hybridisation. Expression was evaluated in intestinal tissues obtained from rats maintained on diets varying in Iron and Calcium content as described in section 5.2.1.

Transcripts were phenol extracted and ethanol precipitated prior to use. The *in situ* hybridisation procedure was based on the method of Cox *et al.* 1988 and modified by Powell and Rogers (1990). Preliminary *in situ* work was carried out on IS and ID rat duodenal tissues to optimise the hybridisation and washing conditions and days of exposure of L-ferritin probes to these tissues fixed in either paraformaldehyde or formalin. Comparisons were made of the intensity of expression between sites and level of L-ferritin expression in the enterocytes from rats from the four treatment groups of IS + Ca, IS-Ca, ID+Ca and ID-Ca after 1, 2, 4 and 6 days exposure. Details of subcloning of original plasmids, and procedures involved in achieving successful *in situ* hybridisation are discussed in section 2.3.

6.2.2 Localisation of L- and H- ferritin and TfR in duodenal enterocytes of weaning rats by cRNA tissue *in situ* hybridisation

6.2.2.1 L-ferritn cDNA

A plasmid containing a pGEM-LFer 532 cDNA encoding for rat L-ferritin was used as a DNA template to prepare sense and antisense probes as described in section 2.3.3 and Figure 6.1). Details of these procedures are discussed later in this chapter.

6.2.2.2 Sub cloning of the original H-ferritin cDNAs

The original cDNA insert was larger than the desired molecular weight of 800 bp recommended for RNA transcription. For this reason, the original H-ferritin cDNA was subcloned are described in section 2.3.4 and Figure 6.2.

6.2.2.3 Sub cloning of the original TfR cDNA

This rat cDNA insert of 3.413kb in pUC-19 (see Figure6.1) was too large fortranscription. Consequently, a 685 bp SacI TfR fragment was excised from the pUC19 clone and inserted into SacI site of pGEM-3Zf (+) vector, such that the 5' end of the cDNA was nearest the SP6 polymerase promoter vector (section 2.3.4; Figure 6.3).

6.2.2.4 Preparation of tissue sections

Duodenal tissue sections from rats fed on IS+Ca, IS-Ca, ID+Ca and ID-Ca diets were fixed in a solution of 4% (w/v) paraformaldehyde (BDH Laboratory Supplies, Poole, England) containing 0.1M sodium phosphate buffer pH 7.2 for six hours at room temperature. Samples were later transferred into 70% ethanol at room temperature and stored until processed. Samples were processed as described in section 2.2.6.2 embedded in paraffin wax and sectioned at 7 μm. Sections were collected on TESPA coated slides.

6.2.2.5 α-[^{33}P]-UTP Labelling of cRNA Probes for *In Vitro* Transcription

Sense and antisense RNA probes were prepared by *in vitro* transcription using the Riboprobe Gemini System Transcription kit (Promega Corporation, Sydney). Prior to the *in vitro* transcription reaction, clean linearised DNA templates for RNA transcription were prepared by restriction enzyme and purified by phenol:chloroform extraction and ethanol precipitation.

A preliminary experiment was conducted to assess the purity of the DNA template for complete transcription and radiolabelling with ^{32}P prior to ^{33}-P labelling of

L and H ferritin and TfR. The procedure was identical to α-[^{33}-P]-rUTP labelling. In this case gel electrophoresis was used to determine the success of the transcription, with the exclusion of the DNA degradation step phenol extraction and ethanol precipitation.

6.2.2.6 Preparation of Tissue Sections and Prehybridisation

Tissue sections were dewaxed by passing them twice through Histolene (Histological clearing agent; National Diagnostics, Atlanta, Georgia) for five minutes and hydrated with graded ethanol solutions (two minute washes in 95% ethanol, 80% ethanol and 70% ethanol) then washed in 2 x SSPE for one minute. Sections were then acetylated in triethanolamine/acetic anhydride for ten minutes (225 mL 0.1M triethanolamine hydrochloride (AnalaR, BDH Chemicals, Kilsyth, Australia) at pH 8 and 562 µL acetic anhydride (AnalaR, BDH Laboratory Supplies, Poole, England), combined just before use, and washed in 2X SSPE and then 10 minutes. After the second SSPE wash the slides were drained and air dried for approximately 20 minutes.

When completely dried, the slides were equilibrated on a warming plate (Ratex Instruments, Australia) at 52°C for five minutes and a prehydridisation solution was applied. The prehydridisation solution (Appendix 5.2) comprised of 50% (v/v) formamide (AnalaR, BDH Laboratory Supplies, Poole, England), 10% (w/v) PEG 6000 (Polyethylene glycol; BDH Chemicals, Kilsyth , Australia), 2x SSPE (Appendix 4.6), 1% (w/v) SDS, 1 mg/mL yeast tRNA, 0.5 mg/mL autoclaved Salmon sperm DNA (Sigma, St Louis, USA). The prehybridisation solution (3.5 µL H2O in 36 µL prehyb solution), was heated at 90°C for five minutes and quick chilled on ice before the addition of BLOTTO and 0.5% (w/v) β-mercaptoethanol to 100mM final concentration. The prehydridisation solution was brought to 52°C then 36 µL, per 22 mm^2 coverslip, was slowly pipetted onto each area of tissue, avoiding formation of bubbles. Sections on slides were covered with parafilm, placed into slide holders, immersed in 2x SSPE combined in the chamber of an Omnislide *in situ* machine (Hybaid Ominislide, Hybaid Ltd, UK) and incubated at 52°C for one hour. The parafilms was carefully removed

from the sections and the slides were washed twice in a Hybaid Ominislide (Hybaid Ltd, UK) containing 2X SSPE at 37°C for two minutes, drained and air-dried.

6.2.2.7 Hybridisation of cRNA Probes to Tissue Sections

Hybridisation solution was prepared as described for the prehydridisation solution with the inclusion of an RNA probe (approx. 1,000,000 counts per 22 mm^2 area). A premix solution consisting of formamide, PEG 6000, 2X SSPE, SDS, yeast tRNA, autoclaved Salmon sperm DNA and the RNA was thoroughly mixed, denatured at 90°C for five minutes and cooled to 52°C before adding BLOTTO and β-mercaptoethanol. Hybridisation solution (36μL per area of tissue) was carefully pipetted onto each area on prehybridised slides which had previously been equilibrated at 52°C on a warming tray. Glass coverslips (22 mm^2) were placed over sections and the slides were placed into slide holders and hybridised overnight (16 hours) at 52°C in the Omnislide hybridisation machine.

6.2.2.8 RNase Treatment and Post-Hybridisation Washes

At the post-hybridisation stage, slides were placed in slide racks and washed in the Hybaid Omnislide using a solution of 50% formamide, 2X SSPE, 0.1% SDS, and 10mM β-mercaptoethanol at 52°C for five minutes. The coverslips floated off during the wash. The slides were then transferred to wash solutions without SDS (for five minutes then minute wash). Slides were then washed in 2X SSPE for five minutes, twice, to remove the formamide prior to RNase treatment. Slides were air dried, 100 μL of fresh RNase solution (1 mL RNase solution: 10 μL 1mg/mL RNase, 0.8 μL RNase T1, and 990 μL 2X SSPE) was applied to each section and the slides were covered carefully with parafilm, ensuring that no bubbles remained over the sections. After incubating in humid machine (Hybaid, UK) at 37°C for 30 minutes, the parafilm was removed, the slides transferred to slide racks and the sections washed in 2X SSPE at 52°C for five minutes. This was followed by a higher stringency washes in 0.1X SSPE at 60°C for 10 minutes and 0.1X SSPE at 52°C for 30 minutes. Slides were then

dehydrated in increasing concentrations of ethanol (75%, 80% and 90% ethanol) for two minutes each and air dried at room temperature prior to dipping in Ilford L4 emulsion as described below.

6.2.2.9 Emulsion autoradiography with Ilford L4

Preparation of emulsion

Under an Ilford Safelight, a mixture of 50% w/v Ilford L4 emulsion/2% glycerol solution was incubated at 45°C in a water bath, with occasional mixing until the L4 emulsion was fully melted.

The melted emulsion mixture was poured slowly into a dipping chamber at 45°C and allowed to stand for five minutes. Blank test slides were immersed in the emulsion then excess emulsion was removed from the back of slides with a damp wettex cloth. Slides were placed on an ice cooled tray for 10 minutes then checked for dipping artefacts. The experimental slides were then processed in the same way.

Emulsion coated slides were dried vertically in an aerated light proof box for two hours. Following drying, slides were placed in black light proof slide boxes containing granulated silica gel, the boxes sealed with tape and wrapped in two layers of foil then put in a black plastic bag. Autoradiographic exposure of the emulsion was performed for 2 days at 4°C.

Slides to be developed were equilibrate to room temperature for approximately two hours. Slides were removed from the box under safelight and placed into a Coplin jar containing Kodak D19 developer (undiluted) for 2 minutes, rinsed briefly in water and transferred into Ilford Hypam Rapid Fixer (diluted 1:4) containing Ilford hardner (diluted 1:40) for two minutes. Following fixation, slides were washed in distilled water for 30 minutes and allowed to air dry prior to staining.

6.2.2.10 Staining of *in situ* slides

The developed, fixed, washed and dried RNA slides were hydrated by immersion in 95% ethanol, 70% ethanol and 50% ethanol for one minute each, then stained in filtered haematoxylin for 3 minutes. Following staining, slides were rinsed three times in water for 15 seconds and one minute (2x), dehydrated in 70% ethanol for one minute, 95% ethanol (2X) for two minutes and Histolene (2X) for five minutes, sections then mounted under coverslips in Depex. Histological examination was carried out under lightfield and darkfield illumination (BH60 microscope fitted with dark field condenser, Olympus, Japan).

6.2.3 Immunohistochemical Technique.

A second localisation study was initiated to determine transferrin receptor (TfR) activity in duodenal enterocytes of weaning rats with varying dietary iron and calcium concentrations was determined using immunohistochemical method as described in section 2.4. This was necessary in the case of TfR since no defined signals were obtained from tissue *in situ* hybridisation. Monoclonal antibody against rat TfR, raised in the rabbit, were used.

6.2.4 Assessment of iron status

Haematological and biochemical indicators of iron status were assessed in all animals (section 2.2.5.1) from which tissues were collected for immuno-cytochemistry and *in situ* hybridisation assessments.

6.3 RESULTS

6.3.1 Cloning of rat H-ferritin and transferrin receptor recombinant DNA

The cloning of H-ferritin (Figure 6.2) and TfR (Figure 6.3) fragments with smaller molecular weights of less than 800 bp into appropriate vectors with bacteriophage promoter for RNA transcription was successful . The recombinant DNA H-ferritin 418 bp fragment cleaved from pGEM-3Zf and TfR 685 cleaved from pUC19 were successfully used for preparing cRNA probes. Sense and antisense cRNA probes generated from the restriction fragment encoding 5' terminal 685 bp rat TfR DNA, 5' terminal 418bp H-ferritin and 3' terminal L-ferrtin. The L and H ferritin and TfR probes were used to assess the effects of dietary iron on mucosal iron accretion and transport during the weaning period. The effects of calcium on ferritin and transferrin mRNA expression were also evaluated in duodenal tissue.

6.3.2 Tissue *in situ* hybridisation of L and H ferritin and transferrin receptor cRNA in rat duodenal enterocytes

Strikingly strong hybridisation signals were detected with the antisense probe for L-ferritin along the entire length of the villus and crypt epithelia of duodenal samples from rats fed on iron and calcium sufficient (IS+Ca) diets (Plate 6.1). There was negligible background when compared to the tissues hybridised with sense probe, as expected (Plate 6.1a). Stronger hybridisation signals were visualised in the enterocytes of animals fed IS+Ca and ID+Ca diets (Plates 6.1 and 6.2). L-ferritin expression showed a biphasic pattern with an intense hybridisation signal in the villus epithelium and a more diffuse signal in the differentiation zone in the crypts of duodenal samples of animals fed the ID+Ca diet (Plate 6.2d). Weak hybridisation signals were observed in the crypts of duodenal samples of IS-Ca treated animals (Plate 6.1c). No detectable hybridisation signal was noted in the entire duodenal epithelium in animals fed ID-Ca diets (Plate 6.2e).

6.3.2.1 *Effects of calcium on H-ferritin mRNA expression in the duodenal tissues of weaning Rats.*

The intensity of hybridisation and the sites of localisation of H-ferritin were assessed to ascertain if there were any differences from L-ferritin. The pattern of H-ferritin hybridisation in duodenal samples of rats fed the iron and calcium sufficient (IS+Ca) diet was essentially identical to that observed for L-ferrtin. However, signals were not as intense as those detected for L-ferritin. H-ferritin hybridisation signals varied considerably in all treatment groups with no differences observed in the level of hybridisation in response to calcium or iron status. They were also less specific localisation.

6.3.2.2 *Effects of calcium on transferrin receptor mRNA expression*

Preliminary experiments of *in situ* hybridisation for the TfR mRNA in the intestinal mucosa of rats fed the four different levels of iron and calcium have consistently showed very low hybridisation signals. Antisense cRNA probe generated from the restriction fragment encoding 5' terminal 685 bp rat transferrin receptor cDNA was shown to weakly hybridise to mRNAs in duodenal tissues of weaning rats particularly in the crypts. No TfR mRNA was detected in duodenal samples from any of the four dietary treatments when the original clone of pUC-19 with TfR fragment with 3.143 bp and the subcloned PGEM -3Zf (+) with 685 fragments were used.

Figure 6.1 Plasmid map of recombinant pGEMLFer 532

The pGEM-LFer 532 clone, (KB 006) was not subcloned. pGEM-LFer 532 was digested with SalI and transcribed with T7 RNA polymerase to make antisense transcripts, while digestion with SmaI was transcription with SP6 RNA polymerase produced sense transcripts.

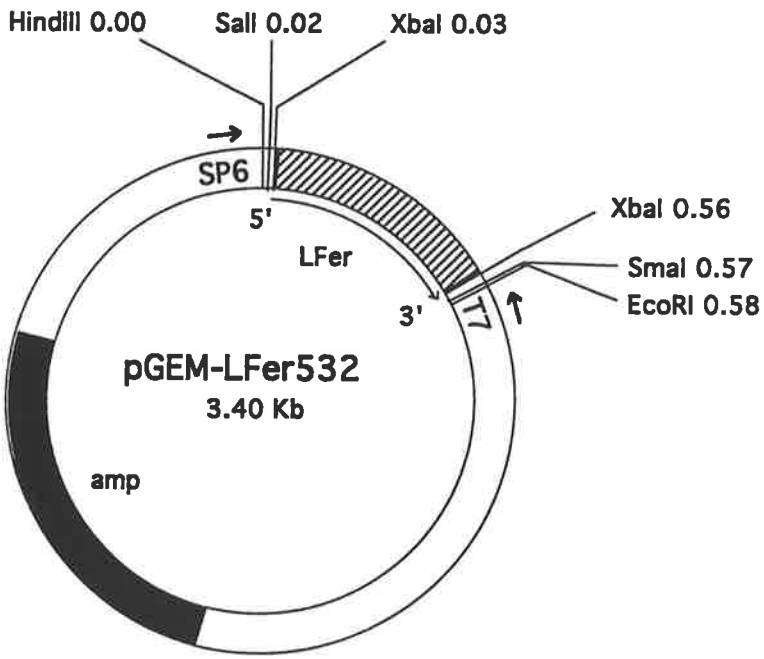


Figure 6.1 Plasmid map of recombinant pGEMLFer-532

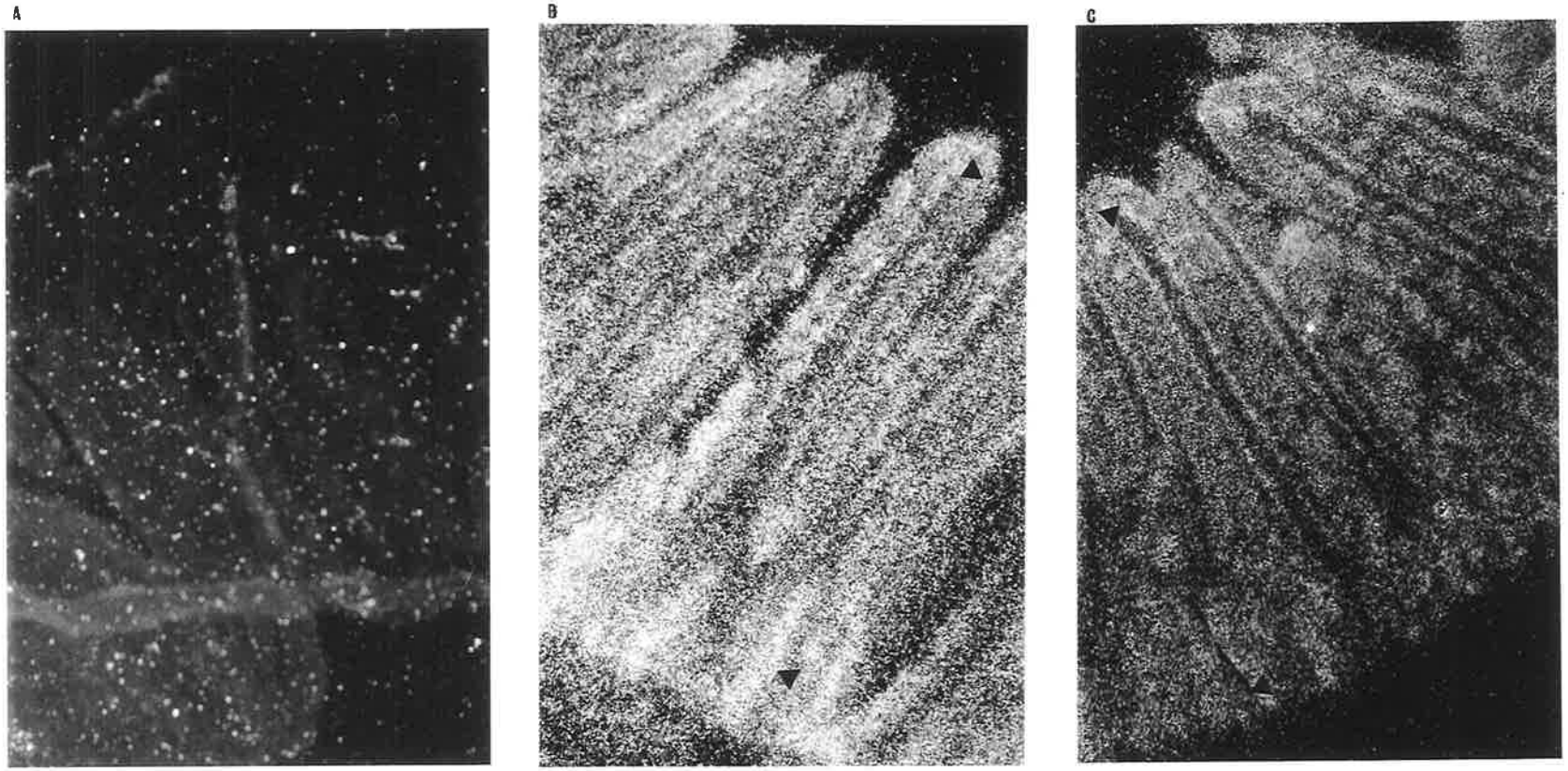


Plate 6.1. Tissue *in situ* hybridisation of L- ferritin mRNA expression in duodenal enterocyte of rats fed IS + Ca diets. (A), Sense probe for L-ferritin mRNA gave negligible background binding. In contrast, anti sense probes (B), demonstrates a strong L-ferritin mRNA expression along the entire length of the villus and crypt epithelia of IS + Ca and (C) IS -Ca shows less intesne hybridisation (Magnification 25X). Arrows show sites of intense expression.

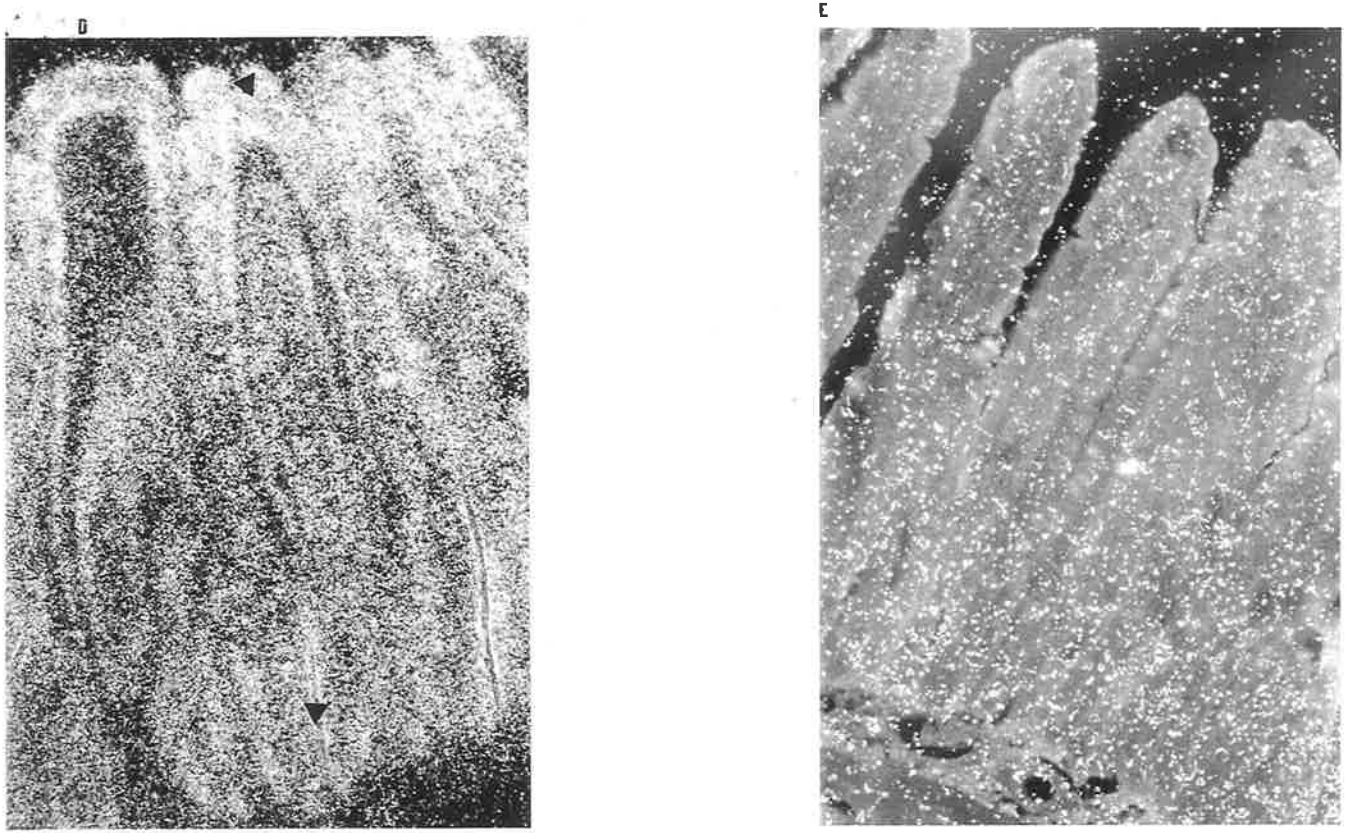


Plate 6.2. Tissue *in situ* hybridisation of L- ferritin mRNA expression in duodenal enterocyte of rats fed ID + Ca diets (D) showing L-ferritin mRNA expression along the entire length of the villus and crypt epithelia compared to (E), no expression in tissues from animals on ID - Ca diets (Magnification 25X). Arrows show sites of intense expression.

Figure 6.2 Plasmid map of recombinant pGEMHFer-418

The 900 bp H-ferritin cDNA fragment in clone KB016 was reduced in size by PstI digestion (removed approximately 450 from the 5' end of the cDNA insert) and religation. The new clone pGEM HFer-418 was digested with HindIII and transcribed with T7 RNA polymerase to make antisense transcript . EcoRI digestion and transcription with SP6 RNA polymerase produced sense transcripts.

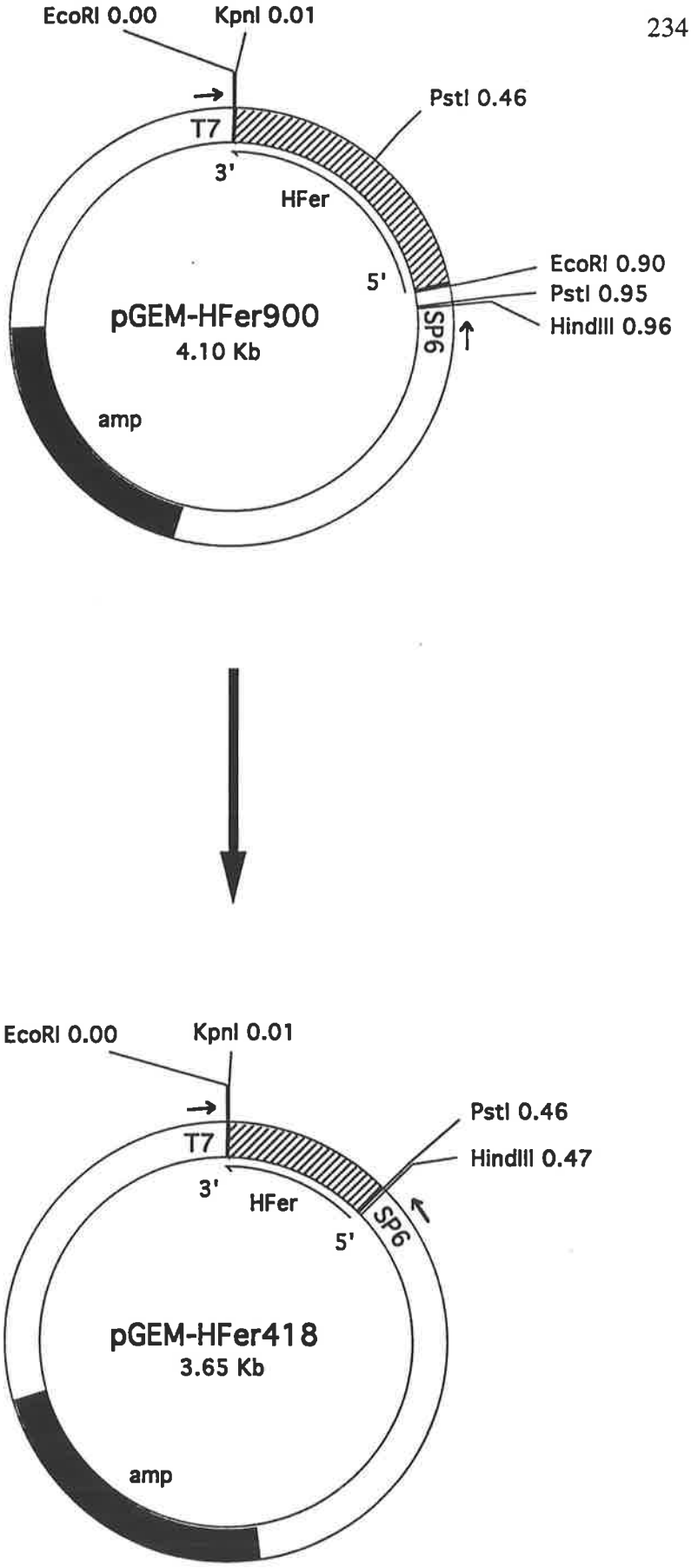


Figure 6.2 Plasmid map of recombinant pGEMHFer-418

Figure 6.3 Plasmid map of recombinant pGEMTfR-685

A 685 bp SacI fragment excised from the 3' end of the TfR cDNA (pUC-19-r-TfR; Griswold and Robert, 1990) was cloned in the SacI site of pGEM-3Zf (+), with the 5' end of the cDNA nearest the EcoRI site of the vector polylinker. The new clone was digested with HindIII and transcribed with T7 RNA polymerase to make antisense transcripts. When pGEMTfR-685 was digested with EcoRI, SP6 RNA polymerase was used.

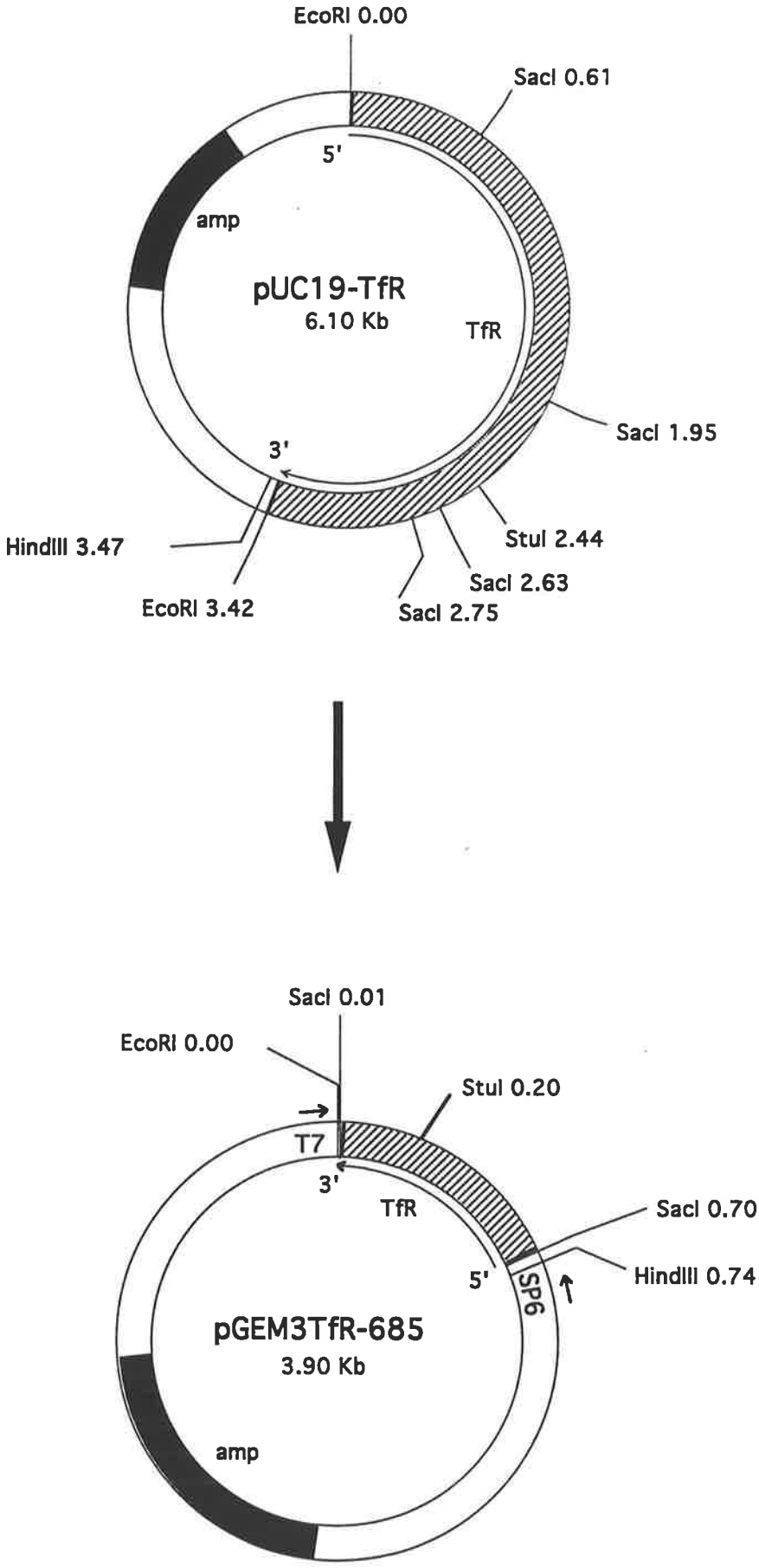


Figure 6.3 Plasmid map of recombinant pGEMTfR-685

6.3.3 Immunological assessment of the effect of dietary calcium on transferrin receptor protein in duodenal enterocytes during weaning.

The previous study in chapter 5 showed an inhibitory effect of calcium on intestinal iron uptake. Since no defined *in situ* hybridisation of neither the pUC-19-r-TfR nor the subclone pGEMTfR-685 was obtained, it was considered important to evaluate the role of calcium on TfR protein in iron metabolism. Immunohistological technique was therefore used to evaluate if there were any interactions at the translational level.

6.3.3.1 *Immunohistological assessment of TfR binding protein*

It was informative to assess the localisation and intensity of TfR staining along the full length of the villus-crypt unit to ascertain if TfR plays any role in mucosal iron transport and whether calcium can modulate this role. Transferrin receptors expression were found on basal, lateral and intracellular membranes of duodenal villous and lamina propria in ID + Ca tissues to varying intensity (0 - 3) in all treatments (Plate 6.3 and Plate 6.4.). Enterocytes of IS+Ca rats showed level 2 grading of TfR localisation in the basal, lateral membrane and the lamina propria (6.3b). In contrast ID + Ca tissues showed level 3 expression in the duodenal basal- lateral membrane, crypts and lamina propria of rats on iron deficient calcium sufficient diets (Plate 6.4c). There was very weak to no transferrin receptor expression (0 - 1) detected in the basal membrane and lamina propria of animal on ID -Ca diets (6.4d). In general, transferrin receptor localisation was more intense in the lamina propria region in all tissues (Plates 6.3 and Plate 6.4).

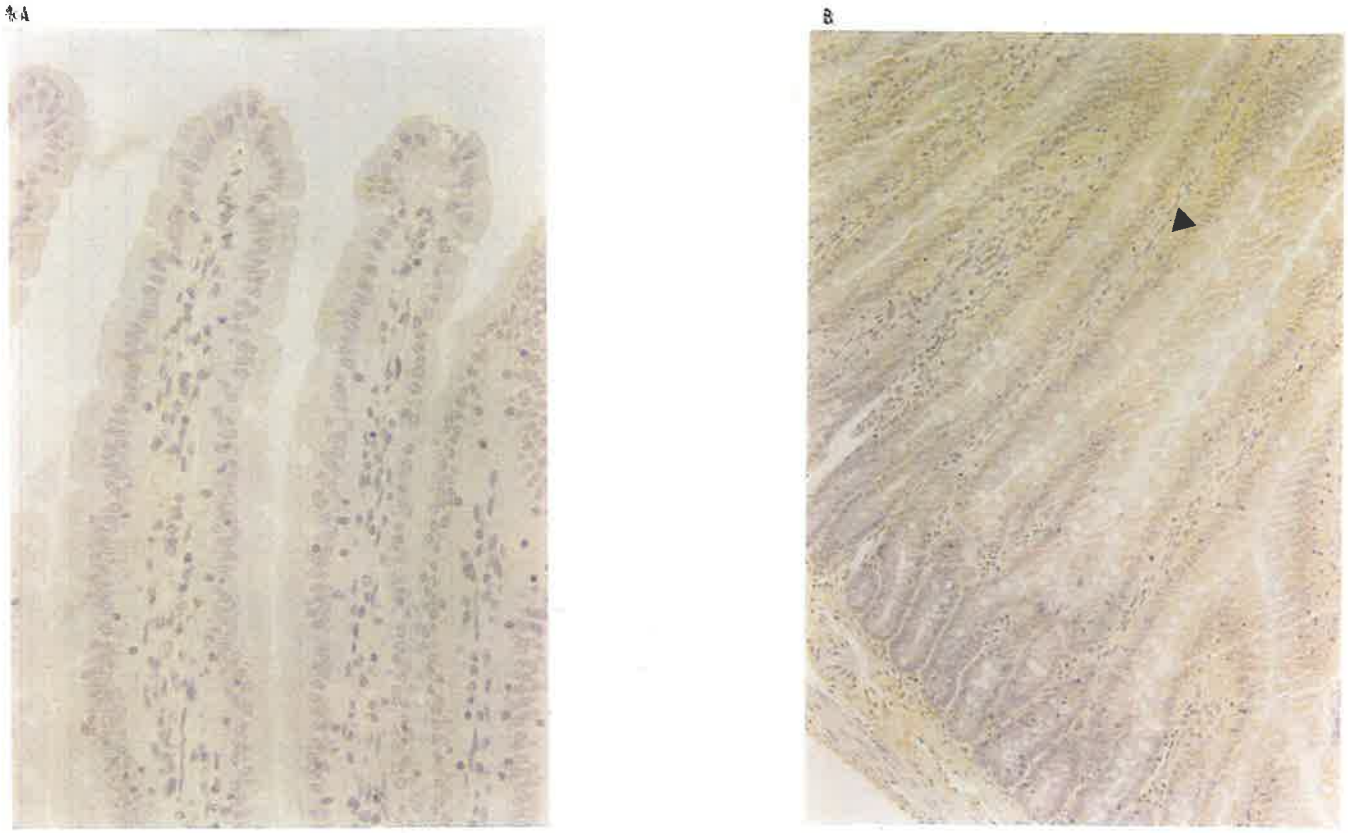


Plate 6.3. Immuno peroxidase staining of duodenal villus crypt enterocytes with monoclonal antibody (mouse anti rat) directed against rat transferrin receptors in rats feed IS + Ca diets showing (A) Negative control and (B) IS+Ca; control diet rats showed low level of TfR localisation in the basal region of the enterocyte and the lamina propria (Magnification 25X). Arrows show sites of intense expression.

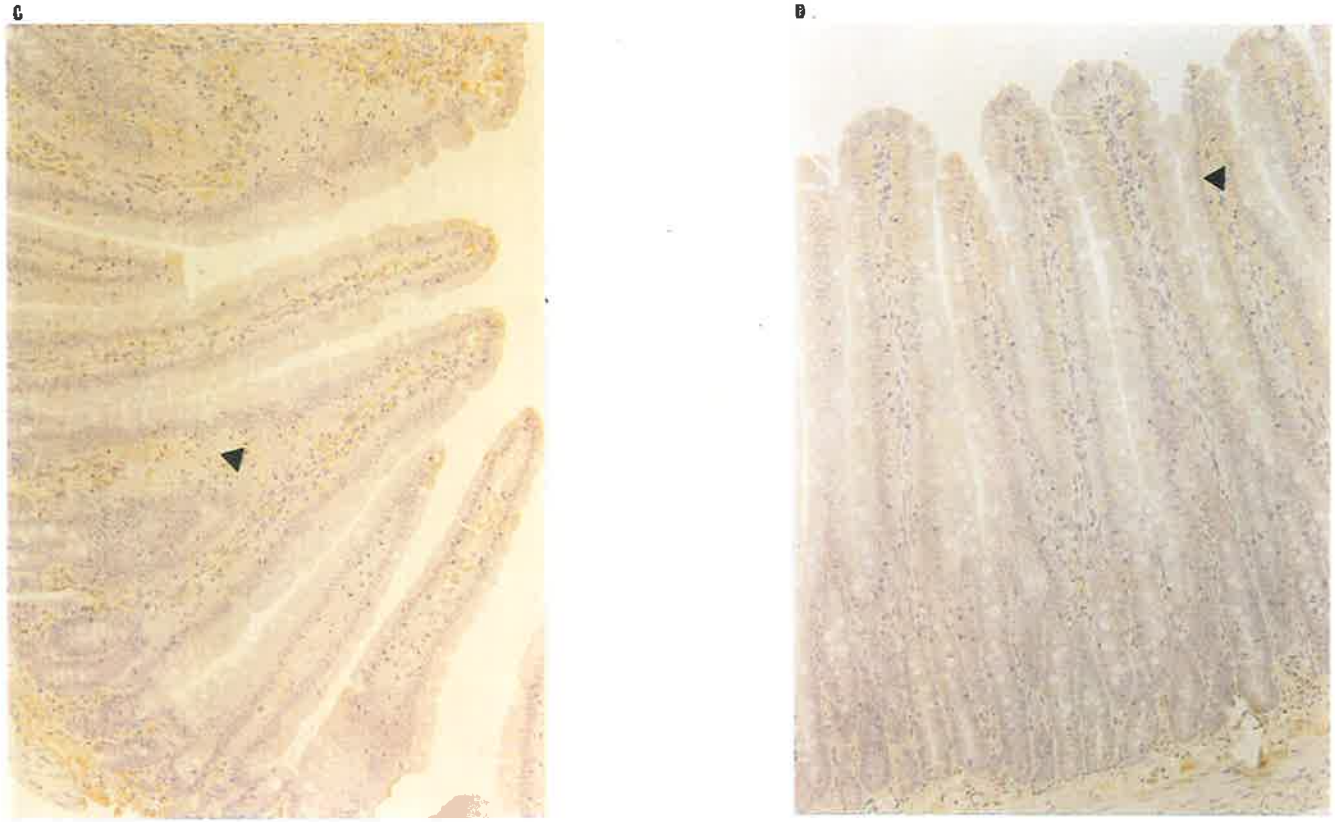


Plate 6.4. Immuno peroxidase staining of duodenal villus crypt enterocyte with monoclonal antibody (mouse anti rat) directed against rat transferrin receptors in rats feed (C) ID+ Ca diets and (D) ID - Ca diets. Duodenal sample of ID + Ca shows higher expression levels than ID -Ca found on basal, lateral and intracellular membranes of duodenal villous and crypt cells and the lamina propria. Receptor localisation was more intense in the crypt region and decreased along the villus to the tip. No receptor expression was detected in the apical brush border membrane in the ID - Ca (D) (X25 Magnification). Arrows show sites of intense expression.

6.4 DISCUSSION

Ferritin and transferrin receptors are vital for iron homeostasis. Ferritin is synthesised to sequester iron for storage during positive iron balance while transferrin receptors play a reverse role by mediating iron transport into cells when there is negative iron status or a high iron requirements eg. for mitosis (Gavin *et al.*, 1994; Anderson *et al.*, 1991; 1996). Previous studies in chapters 3, 4 and 5 have shown that iron deficient diets containing high calcium can induce negative iron status, but the mechanism of interaction was not know. The model proposed in chapter 5 suggests that calcium may be impeding intracellular iron transport from apical side of the epithelial cells to the basolateral region by modulating iron binding proteins, such as ferritin, that sequester iron or prevent iron transport to the peripheral blood. Alternatively, calcium may impede the expression and translation of transferrin receptor gene which internalises transferrin-bound iron by receptor-mediated endocytosis thus inhibiting epithelial iron transport. Although recent studies have shown an involvement of transferrin receptors in intestinal iron absorption (Anderson *et al.*, 1990; Oates *et al.*, 1997), the details of how this works is yet to be fully elucidated. The effects of calcium on intestinal TfR expression under the four dietary regimes during the weaning period were therefore investigate using imunohistochemical and *in situ* hybridisation techniques.

6.4.1 Calcium modulates L-ferritin gene expression in duodenal enterocyte during weaning

The current observation confirm earlier results in chapter 5 which showed that musocal ^{59}Fe uptake was high despite positive iron status. The high mucosal ^{59}Fe iron uptake and intense ferritin expression in the enterocytes of iron sufficient weaning rats support the proposal that duodenal ferritin may function as an iron acceptor to block transfer of excess iron from the enterocyte to blood (Wheby, 1970; Kozma *et al.*, 1994). It is also possible that ferritin is not playing an active role, but rather soaking up any iron which is around as a result of the blockage. Localisation of the hybridisation signals for L-ferrtin mRNA to the entire villus epithelium was consistent with results obtained by

Pietrangelo *et al.* (1992) who detected both L-and H-ferritin mRNA expression in the epithelia cells of the villus in normal iron sufficient human duodenum. In addition, the present study showed localisation of mRNA expression in the crypts epithelium which conforms with observations of Oates and Morgan (1997) who found both L-and H-ferritin expression in the crypts epithelium of rats. The expression of L-ferritin mRNA in the villus enterocytes suggests an upregulation of ferritin synthesis which may sequester excess iron. The hybridisation of ferritin mRNA in the crypts may not only be regulating intestinal iron uptake but could be linked to cell proliferating, since there is a high turnover of cells as they differentiate from crypt to villous cells, coupled with high iron needs for both lateral and spatial gut growth.

Although ferritin iron binding proteins are synthesised to sequester iron for storage during positive iron balance as indicated above, strong hybridisation signals were localised at the villus tips with the antisense mRNA for L-ferritin in iron deficient with calcium (ID+Ca) duodenal samples, with no expression detected under ID-Ca conditions. The apparent discrepancy in the intensity of L-ferritin mRNA expression observed in this current study compared to significantly lower L-ferritin mRNA accumulation in duodenal samples from IDA patients detected by Pietrangelo *et al.* 1992 may be due to differences in calcium concentrations in the diets of IDA patients which were not assessed. Results from this present study and those in chapter 5, further confirm that differences in the level of intestinal mRNA expression were due to variations in dietary calcium particularly under iron deficiency condition during weaning. This was indicative when no L-ferritin mRNA expression was detected in the enterocytes of rats fed ID-Ca diet. These distinct differences were however not significant in the Hb and serum ferritin concentrations of ID + Ca and ID - Ca.

The relationship between the inclusion or reduction of calcium in the diet and mRNA expression was further evident in this current study. *In situ* hybridisation of L-ferritin mRNA in enterocyte from iron deficient rats (ID-Ca) further suggests an

intracellular inhibitory effect of calcium on the induction of iron binding proteins that can block duodenal mucosal iron uptake. Ferritin gene expression has been shown to be modulated by iron at both the transcriptional and posttranscriptional levels (Kozma et al., 1993; Hubert *et al.*, 1993; Hentze and Kuhn, 1996). The differences seen in the villus localisation of L-ferritin mRNA expression in ID + Ca and ID - Ca suggest a high dietary calcium content during iron deficiency modulates ferritin mRNA which blocks lumenal iron uptake.

6.4.2 H-ferritin gene expression in duodenal enterocytes during weaning

In the assessment of the effects of calcium on ferritin mRNA expression, *in situ* hybridisation of H-ferritin mRNA expression was more diffuse than that obtained using the corresponding L-ferritin reagent. These data suggests that L-ferrtin may be more important in intestinal iron regulation as it quickly sequesters and stores excess iron from the lumen to prevent oxidative damage to the cell, while H-ferritin could be pertinent to prolonged iron storage for cell growth and proliferation in the gut. It is also possible that L-ferritin under ID + Ca condition may only be playing a passive role by soaking up iron into stores as a consequence of upregulation of DCT1 gene (Gunshin *et al.*, 1997). The interaction between calcium, ferritin and DCT1 together under iron deficiency will need to be investigated. The observations seen in the pattern of L-ferritin mRNA expression relative to H-ferritin mRNA in the intestine in the present study, however, were consistent with other findings (Leibold *et al.*, 1984; Hubert *et al.*, 1993; Oates *et al.*, 1997).

In contrast to these findings, Yeh and coworkers (1996) have shown a 50 % higher expression of H-ferritin mRNA (than L-ferritin mRNA) in 12 day old neonatal rats fed 6 mL of 56 μL/mL ferrous sulphate solution for 18 hours. Although H-ferritin increased with age, the L-ferritin subunit showed no further developmental changes between 12 to 32 days. The low H-ferritin expression seen in the current study contrasts with the high H-ferritin found by Yeh *et al.* (1996) can be attributed to the differences in

the age of the animals and the extremely high iron dose that these authors used which was in excess of physiological needs. However, the stability in the expression of L-ferritin mRNA in spite of age, further suggests that L-ferritin is functionally associated with intestinal iron regulation as shown in this present study.

6.4.3 Calcium effects on transferrin receptor gene expression in duodenal enterocyte during weaning

Like human infants, the weanling rat absorbs considerably more iron than the adult. Transferrin receptor plays a major role in cellular iron acquisition by facilitating the uptake of iron laden transferrin and TfR increases under iron deficiency (Anderson *et al.*, 1991; Kozma *et al.*, 1994; Oti-Boateng *et al.*, 1996). Immunohistochemical staining showed transferrin receptors to be distributed in basal-lateral membrane villus cells and in crypts cells in sections obtained from the four dietary treatment. However, there were differences. The enterocytes of IS+Ca rats showed the least transferrin receptors expression in the crypts while ID+ Ca showed the most. The differences in TfR intensity found between IS and ID animals may be linked to cell proliferation and not necessarily regulation of TfR in response to low body iron needs. The staining observed in the lamina propria also suggests a potential caveat for peroxidase activity which may be associated with engogenous activity of eosinophil. If so, dietary calcium could be regulating the number of eosinophils as well as transferrin receptor numbers particularly, under iron deficiency. Transferrin receptor expressions, even under iron deficiency was however not found at the villus tip cells, but in the cell differentiating zone of the duodenum. No TfR mRNA expression was detected in any of the treatment groups.

The detection of polarised distribution of transferrin receptor in the villus epithelium and the absence of transferrin receptor mRNA are consistent with two published findings of Anderson *et al.* (1991) and (Oates and Morgan 1997). Anderson *et al.* (1991) have shown that increased villous transferrin receptor expression occurred during weaning (21 day olds rats), but that these changes were inversely relating to iron

absorption. They suggested that there was no direct association between transferrin receptor in mediating serosal efflux of iron from the intestinal epithelium. Although there may be no direct links between TfR expression and regulation of intestinal iron transit, the current study, however, showed that calcium modulates mucosal TfR expression and the role of TfR with intestinal iron transfer during weaning remains a dilema.

6.5 CONCLUSION

This current study has further confirmed the earlier findings in chapter 3-5 that dietary calcium inhibits intestinal iron uptake by iron binding proteins. These can modulate intra- and inter-cellular iron absorption to peripheral blood thus inducing iron deficiency. The increased expression of L-ferritin mRNA in upper villus epithelial cells of animals fed ID + Ca suggests that calcium may be blocking intracellular transfer of iron from the lumen to peripheral blood by upregulating ferritin synthesis. These observations further support the perception that ferritin deposition in the enterocytes regulates duodenal enterocyte iron movement by maintaining a steady state of ferritin by a feed back mechanism during sufficient or excess iron status.

The current study did not demonstrate an effect of calcium on TfR mRNA expression although calcium limited TfR protein expression under iron sufficiency. It is, however, likely that transferrin receptors do not play a direct role in intestinal iron transport. Findings by Anderson and coworkers (1990) have been recently supported by Oates and Morgan (1997) who have shown that the detection of transferrin receptors in duodenal crypts may be more a consequence of the high iron needs of the enterocyte for cell growth and differentiation than for iron transport. *In vivo* studies are needed to elucidate the role of transferrin receptors in the gut, particularly during the period of rapid growth which coincides with gut growth and adaptation in infancy and childhood. Appreciation of the inhibitory effects of calcium on intestinal iron uptake at the molecular level is essential when selecting weaning diets for optimal iron bioavailability, since both nutrients are essential important during weaning.

CHAPTER 7

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSIONS

This thesis presents comprehensive data and analysis on the prevalence of iron deficiency and anaemia and factors that influence iron status in young children during the period of weaning, in two geographically diverse regions of the world. Iron deficiency and anaemia are associated with lethargy, irritability, increased risk of infection, impaired intestinal nutrient absorption and reduced attention span particularly in young children. There is compelling evidence that iron deficiency anaemia before the age of two years can have long term and perhaps irreversible effects on behaviour and school performance (Walter, 1993). Two epidemiological studies, experiments using animal models and molecular methods were used to investigate the causes and consequences of inadequate iron status on physical growth, evaluate links between dietary practice and haematological and biochemical indicators of iron nutrition and examine the mechanism of interaction of other nutrients, particularly calcium on iron absorption in children during weaning.

The studies conducted in Ghana and Australia provided more evidence that the ingestion of bioavailable iron by infants and toddlers during weaning is essential for maintaining positive iron balance during this period of high iron needs.

7.1 Clinical epidemiological studies

The comparative clinical studies in Ghana and Australia are the first in both countries to document weaning practices and their effects on iron status in 6-24 month old children, using strict haematological and biochemical parameters and dietary intakes to assess iron status, in addition to anthropometric measurements. The two clinical studies clearly showed that iron deficiency is still a major nutritional problem in young children, in both developed and developing countries, despite decades of political rhetoric to eradicate iron deficiency by the year 2000 (Berg, 1992). Infants were most at risk of iron deficiency than toddlers in Ghana ($p < 0.01$). The prevalence of IDA was more common in 6-18 than >18 -24 month olds ($P < 0.01$). Overall, two out of every

three Ghanaian children between the age of 6 - 24 months had inadequate iron status with half of the population sampled diagnosed as having iron deficiency anaemia. By comparison, Australian children had lower incidence of iron deficiency anaemia (7%) relative to their Ghanaian counterparts (55%) (Oti-Boateng *et al.*, 1994, 1997). Only one-third of the Australian population sampled had inadequate iron status with older children at most risk.

The high prevalence of iron deficiency in Australian and iron deficiency anaemia in Ghanaian children was ascribed to exclusive breast feeding beyond six months and the ingestion of weaning diets low in bioavailable iron at a vulnerable period of high iron needs. Duration of breast feeding inversely correlated with SF ($p < 0.05$), a finding consistent with work by Siimes and Saarinen (1984) who showed that iron balance in infancy is more vulnerable to depletion between six to eight months in breastfed children. Australian infants on average, were exclusively breastfed for four months relative to an average of 10 months of breast feeding in Ghanaian children.

Both the Australian and Ghanaian clinical studies further demonstrated that although breast milk can be relied on for maintaining adequate iron status at 6 months, the timing and type of weaning diets is crucial in tipping the balance between adequate and inadequate iron status. For example, the consumption of certain diets such as cow's milk or soyflour, with excess calcium or soy protein respectively, have been associated with inhibitory effects of on iron absorption (Hurrell *et al.*, 1992; Gleerup *et al.*, 1995; Reddy *et al.*, 1996).

7.1.1 Regional differences in weaning practice

While prolonged breast feeding was the most common risk factor for a reduction in body iron stores in infants and toddlers, the type of solids introduced to children during the weaning period in the two geographically diverse regions were distinct and this contributed to the differences in iron status.

The common solids introduced during the weaning period to Australian children were iron fortified infant or adult cereals and vegetables while Ghanaian children consumed unfortified adult cereals, vegetables and soyflour diets. Although the bioavailability of electrolytic iron (EI) fortified adult cereals is lower than haem iron or ferrous iron, it (EI) can provide enough iron to maintain Hb levels above 110g/L (Morck *et al.*, 1981; Howard *et al.*, 1993). This present study however, showed that the ingestion of iron fortified cereals with cow's milk can further inhibit the availability of non-haem iron absorption in young children. Cow's milk which is a high source of protein and calcium was consumed as a primary beverage and in large volumes by a majority of children in Australian children, which probably accounted for the high prevalence of iron deficiency. In contrast, Ghanaian children, particularly infants 6-12 months ingested soyflour meals frequently and continued to receive their milk source from breast milk.

The associations between prolonged breast feeding and reduction in body iron stores in Australian children was masked in the Ghanaian children whose diets were deficient in many essential nutrients such as vitamins, iron and zinc, which are essential for both physical, mental and psychomotor development (Mokni *et al.*, 1993; Lozoff *et al.*, 1987; Walter *et al.*, 1989; Walter 1993). The enhancing relationship between iron and vitamins was obvious in the positive correlations found between dietary iron, vitamin C and thiamine levels ($r = 0.55$; $P < 0.001$: $r = 0.7$, $P < 0.001$) respectively. The limited consumption of these essential nutrients particularly iron, was also apparent in the high prevalence of iron deficiency anaemia diagnosed in Ghanaian infants and toddlers who consumed only a limited range of foods with excess soyflour meals. Older Australian children, >18-24 months, who had limited food choices in their diets and ingest excessive amounts of cow's milk a day were also at risk of developing NAID and IDA.

7.1.2 Factors influencing nutritional iron status

One of the main factors influencing the high incidence of NAID and IDA in young children in both countries, was determined to be inadequate ingestion of dietary iron. Majority of children in Ghana and Australian consumed less than 50% of the recommended daily allowance to conserve body iron stores. The recommended daily dietary iron intake was low in all the three age groups with Ghanaian infants 6-12 months consuming only a third of the RDA (3.6 ± 3 mg) for iron, while toddlers 18-24 months had 64% of the RDA (6.7 ± 4 mg Fe) of iron a day ($P < 0.001$). Australian infants (6-12 months) on the other hand ingested more iron than their older counterparts (60% vs 42% of RDA, respectively). There was an association between dietary iron intake and Hb concentration ($r = 0.4$; $p< 0.01$) in the Ghana study, with no such association found between Hb and dietary iron intake in the Australian study. Probably a more comprehensive dietary analysis, similar to that used in the Ghana study, could have detected an association. There was a negative association between SF and Hb in the Australian study, but no such links were found in the Ghana study. The absence of any correlations between SF and Hb and the high mean SF values found in Ghanaian children diagnosed as IDA suggests that SF may not be specific for diagnosing iron deficiency in areas where the prevalence of common childhood diseases may be rampant. There was however, a negative association between transferrin receptor (TfR) concentration with Hb, thus indicating a reverse association between TfR and Hb. The use of this new TfR assay to diagnose iron deficiency is a more useful tool in distinguishing the difference between nutritional iron deficiency and anaemia of chronic disease.

Total energy intake of Ghanaian infants and toddlers was over 3000 kjoules on average with daily protein intake exceeding the RDA at all ages. The children were not protein energy malnourished but they had inadequate micronutrient intakes. Mean daily calcium intake decreased with age with 18 - 24 month olds ingesting only a quarter of the RDA for age. The mean daily consumption of other nutrients such as vitamin A and

D, zinc, retinol and B complex vitamins such as nicotinic acid, thiamine and riboflavin also decreased as children got older and was a third of the recommended daily allowance by 24 months of age. The high cow's milk intakes of Australian children probably provided them with adequate vitamins with excess calcium intakes.

Socio-economic analysis of parents of children in Ghana and the broad socio-economic background of children in Adelaide suggest that the prevalence of iron deficiency and anaemia was not only limited to any given socio-economic background in either country. The incidence and severity of anaemia were more prevalent in Ghanaian children compared with Australian children, probably due to different eating habits. Most Ghanaian diets were cereal and vegetable based with fish providing the main animal protein, whereas in Australia red meat and cow's milk were the main animal protein sources.

There was also lack of suitable weaning guidelines for mothers in either countries. It was interesting to note that the weaning advice given by health workers in both countries were based on old weaning guidelines which have since been modified. Health workers in either country have not kept abreast with new national and international recommendations based on scientific findings in food trends to enhance nutrient absorption. For example, it appears that in the 1990's the weaning practice of Adelaide infants and toddlers were generally compatible with the recommendations and guidelines set out by CAFHS booklet, contrary to the advice by the Australian College of Paediatrics (1990). Parents may have received conflicting information on the age at which solids and cow's milk can be introduced. Similarly in Ghana, there was an ardent promotion of soyflour as weaning food by health workers because of its high protein content which was evident in the current study. Although soyflour may be effective in providing adequate protein for infants and toddlers, this present study has cautioned excessive use of soyflour diets based on evidence from the literature that soyflour has anti-nutritive factors that can inhibit iron absorption (Cook *et al.*, 1981) and its potential

link with intestinal mucosal damage (Liener, 1986; Calam *et al.*, 1987). It can be inferred from these findings that Ghanaian children who consumed large amounts of partially processed soyflour porridges have the potential to developed intestinal mucosal damage and allergies which are similar to that observed with cow's milk intake.

The adverse effects of introducing cow's milk, which is a rich source of calcium, before the age of 12 months have been well documented. Cow's milk is known to inhibit iron absorption, as well as increase gastrointestinal blood loss, but it is a common drink for infants and toddlers in industrialised countries (Fomon *et al.*, 1981; Hurrell *et al,* 1989; Ziegler *et al.*, 1990; Fuchs *et al.*, 1993; Karr *et al.*, 1996). The potential risk associated with ingesting cow's milk and depletion of body iron stores was demonstrated in the Australian study in chapter 3. Analysis of the calcium content in the cow's milk consumed by all children in the Australian study showed a 12% risk associated with cow's milk intake and serum iron reduction.

7.2 Mechanism of interaction of calcium on iron absorption during weaning

In recent years research has been directed towards understanding the mechanism of intestinal iron regulation as a means of optimising or reducing exogenous iron absorption during periods of iron deficiency or iron overload (Anderson *et al.*, 1991; Gunshin *et al.*, 1997; Fleming *et al.*, 1997). However, knowledge underlying cellular mechanism of iron absorption particularly in the presence of other nutrients during the weaning period when the gut is undergoing temporal and spatial growth and maturation remains fragmented (Barton *et al.*, 1983).

The objective of the second part of this research was, therefore, devoted to elucidating the effects of dietary components, such as calcium, on the assimilation of dietary iron during the weaning period. Calcium was chosen based on the importance of both calcium and iron during periods of rapid growth and the effects of excess consumption of calcium on iron status during the period of introduction of solids as

clearly shown in chapter 3. An Animal model of weaning was used to investigate the effects of varying calcium and iron contents of weaning diets on intestinal iron absorption and peripheral transport of iron from the gut to the blood. Similar haematological and biochemical analyses to those used in the clinical surveys were employed to make reliable comparisons between the animal and human studies.

This is the first comprehensive study to use four diets (Section 2.2) of varying proportions of iron and calcium contents (section 5.2) to assess the haematological, biochemical iron status, brush border and intact tissue radio-iron (^{59}Fe) uptake, digestive enzyme analyses, morphometric, anthropometrics immuno-cytochemical and molecular techniques to assess the interaction of these nutrients on intestinal iron absorption.

Results showed no differences in body-weight gain per dietary intake in three of the treatment groups (IS+Ca, IS-Ca and ID-Ca), but animals on diets with no iron but calcium supplement (ID + Ca) had arrested growth for up to six days. There were significant differences ($p < 0.05$) in Hb, Hct, serum ferritin, transferrin and iron concentrations between the iron sufficient and iron deficient treatment groups. A reduction in dietary calcium significantly increased ($p < 0.01$) body iron stores and mucosal iron uptake under ID compared to IS conditions. There were inverse effects of calcium on vesicle and mucosal iron uptake under iron deficiency. Dietary calcium inhibited mucosal iron uptake, but enhanced dietary calcium uptake into vesicle. There were significant differences in lactase and sucrase activity in brush-border membrane vesicles in the four treatment groups with rats in the ID + Ca showing the least sucrase activity. Duodenal morphometry also showed an increased mucosal villus height and crypt depth in the three treatment groups compared with controls ($p < 0.01$).

These results confirmed the hypothesis that marginal iron diets with adequate calcium contents increase the risk of iron deficiency by inhibiting duodenal mucosal iron transport from the basolateral to peripheral blood. There is a critical period during

weaning when the consumption of a diet with high calcium content and low iron can retard growth potential. The consumption of high calcium with low iron diets significantly increased brush-border membrane vesicle iron uptake in rats but caused a three-fold decrease in iron uptake by the intact intestine. The high calcium low iron diets also delayed gut maturation by decreasing digestive enzyme activity in brush border membrane vesicles. A reduction of calcium in the diet improved Hb concentration by 7% and serum ferritin by 40% under iron deficiency conditions. This supports earlier findings from the clinical study reported in chapters 3 and 4 that dietary calcium inhibits intestinal iron uptake.

These apparent interaction of calcium on intestinal iron uptake suggested that there is a complex interaction between calcium and ferritin (and possibly transferrin) which has been shown to regulate intestinal iron uptake. Further investigation of the mechanism of interaction of dietary calcium on non-haem iron absorption in the proximal intestine in children was therefore initiated to provide valuable information which is particularly relevant to the formulation of weaning diets for optimal iron bioavailability.

7.4 Effects of dietary calcium on ferritin and TfR proteins and gene expression

Previous clinical studies have shown that iron deficiency and iron deficiency anaemia are prevalent in infants and toddlers in both industrialised and developing countries. (Oti-Boateng *et al.*, 1994; 1997). The high prevalence of iron deficiency and anaemia in Australian children was associated with a low intake of bioavailable non-haem iron from weaning diets which were often high in calcium. An experimental animal model of varying iron and calcium concentrations also showed that dietary calcium inhibited iron transport in duodenal enterocytes (Oti-Boateng *et al.*, 1995; 1996). The mechanism by which calcium regulates iron binding proteins associated with mucosal iron movement from the basolateral membrane of duodenal enterocytes to peripheral blood has not been investigated. Although TfR may not play a role in

intestinal iron uptake regulation (Anderson *et al.*, 1990), the hypothesis that dietary calcium may affect the expression and function of ferritin and transferrin receptor genes in duodenal enterocytes during the weaning period, based on findings from chapter 5, was investigated using immuno-histochemistry and *in situ* hybridisation techniques.

Duodenal samples from rats fed four semi-purified rat diets with varying iron and calcium contents (Section 5.2.1) were compared. Intense ferritin ferritin mRNA expression at the apical membrane, crypt-villus junction, enterocyte cytosol and muscularis of tissues from IS+Ca and ID+Ca treated animals was observed using *in situ* hybridisation technique. Immunocytochemistry showed localisation of the TfR in the crypts of IS+Ca and ID+Ca, thus indicating actively proliferating cells, with no expression seen in either IS-Ca or ID-Ca treated animals. Since TfR has not been shown to be actively playing a role in the regulation of intestinal iron absorption, it can be speculated that ferritin may by playing a passive role. These results suggest that dietary calcium significantly inhibit intestinal non-haem iron absorption at the intra-cellular level by up-regulating villus enterocytes ferritin binding proteins under iron deficiency conditions. It is, however, proposed to further investigate the mechanism of interaction of these nutrients on gene expression to draw emphatic conclusions. The results obtained using molecular techniques do confirm earlier clinical findings and animal work that marginal iron diets with high calcium contents increase the risk of iron deficiency especially during weaning. This finding is of special significance when formulating weaning diets for optimal iron bioavailability.

7.5 Conclusion

Iron deficiency and anaemia are still prevalent during weaning in both industrialised and developing countries, in spite of improved knowledge in iron metabolism. The long term adverse effects of iron deficiency anaemia during the weaning period on physical and intellectual capacity of a child makes the prevention of iron deficiency anaemia in young children an important public health issue. Current

universal weaning recommendations, based on present knowledge of human infant's nutritional and developmental requirements, suggest exclusive breastfeeding for the first four to six months in developed countries and the extension of regular breast feeding for at least two years in developing countries. These need evaluating. Although breast milk is the best source of nutrients for neonates, this study has shown that, gradual introduction of weaning foods to infants at four and no later than six months should be encouraged in both developed and developing countries because the iron content of breast milk becomes inadequate to supply all iron needs of the rapidly growing child. Measures to improve intake of dietary iron both in content and bioavailability, such as those listed below, are recommended especially as iron deficiency anaemia has been associated with impaired cognitive functions and behaviour.

Complementary staple weaning foods and other grains that are caloritically dense and adequate in protein is important and variety is essential in providing for complete nutritional needs. Iron, zinc, vitamin B complex, vitamin D and vitamin A rich foods should be encouraged. Fruits, vegetables, meat and other protein rich foods must be introduced in moderation without compromising bioavilability of iron which is highly susceptible to inhibition. Excess consumption of cow's milk, plant protein which are high in polyphenols, tannins, phytate and soyflour must be avoided particularly during the vulnerable period of high iron needs from 6-24 months of age.

This present study has shown that over 90% of the iron source of children is in the non-haem iron form which are highly subjected to inhibition. Although food iron fortification may provide additional dietary iron and prevent iron deficiency, this form of iron is equally subjected to inhibition when it enters the common non-haem iron pool. Iron fortified foods are also expensive and unaffordable by most people from developing countries. A long-term goal in resolving iron malnutrition in children would be to genetically select cereals with high bioavailable iron attributes because cereals form more than 70% of the diets of young children.

Finally while this study has elucidated some of the effects of dietary calcium on intestinal iron absorption and iron status, the mechanism by which calcium interferes with gene expression of iron binding proteins such as ferritin, iron regulatory proteins (IRP) or DCT1 that control the rate of mRNA translation or stability, needs further investigation.

APPENDICES

APPENDIX 1 CLINICAL TRIALS

Appendix 1.1 Parents information

PARENT INFORMATION SHEET

THE IRON STATUS AND INTAKES OF 6-24 MONTHS OLD CHILDREN IN GHANA

CARRIED OUT BY :

1. *Departments of Animal Science, University of Adelaide, South Australia and*

2 *Department of Paediatrics and Child health, University of Science and Technology, Kumasi, Ghana*

Purpose of study

You are invited to take part in a survey to find out how the diets of children affect the iron levels in their blood. Iron is a very important, being neccessary for the production of the red blood cells which are required to carry oxygen in the body. Iron also affects growth and development includidng behaviour and school performance. Iron defciency appears to be a worldwide problem. Our aim is to determine the incidence of iron deficiency in Ghanain infants and young children and if necessary to improve the iron content of weaning foods.

What do I have to do

If you agree to participate, it will be neccessary to take a small sample of your baby's blood (1 ml, which is about a quarter of a teaspoon) from the vein using a clean and strile needles and syringes The blood will be taken, only once, by a Registered Nurse, who will take all necessary steps agaist infection. The are no major side effects although your baby may experience some discomfort, and there is a slight possibility of bruising.

We also want to know what your child eats to enable us compare child's diet with his/her blood iron levels.

Your Rights

You are under no obligation to participate in the survey and if you choose not to concsent, your future relationship with this clinic or future reatment at the UST hospital will not be affected in any way.

Any information you give will be treated with confidence.

Benefits

A complete screening of your child's blood will be done . If there is any problemin your child's blood he/she will be offered appropriate treatment. You will also be able to discuss any concerns with the specialist involved in the study. Curent advice on feeding practices will be available if you desire.

Any questions

If you have any questions do not hestitate to contact the people listed below:

Peggy Oti-Boateng, Nutrition Biochemist, TCC, UST, Kumasi or University of Adelaide, South Australia.

Dr Ben Baffoe Bonnie, Paediatrician, Department of Child Health, School of Medical Sciences, UST, Kumasi.

Mrs Rosanna Agble, Nutritionist, Ministry of Health, Accra.

Appendix 1.2 Dietary Questionnaire

FLINDERS MEDICAL CENTRE
DEPARTMENT OF PAEDIATRICS & CHILD HEALTH

FOOD RECORD

NAME OF CHILD.....

DATE OF BIRTH .....

ADDRESS.....

WEIGHT.....

HEIGHT/LENGTHS .....

HEAD CIRCUMFERENCE .....

ANY ILLNESS IN THE PAST THREE DAYS?.....

.....

EXPLANATORY NOTE

Children need food to grow well and stay healthy. We are going to study iron intake of babies and children in Adelaide. We would like to know how much iron your child gets from his/her diet.

To allow us to do this we need you to keep a record of what your child eats and drinks a day. It is important that we get an accurate idea of your child's intake. Please do not change the normal way you feed your child on the day you fill in the form.

DIRECTIONS

1. Fluids

Record how much your child drinks and how much you put in the bottle or cup. Record every breast feed, and tell us about how long you feed for. Remember to record the night feeds.

Write down the amount and type of **infant formula** and baby drinks. Tell us how you make up the formula on page 4.

Record how much cow's milk the child has with or in the bottle or cup. If you add anything to the cow's milk write it down on page 4.

Record the amount of juice or water your baby drinks. If you dilute juice or use baby syrup eg Delrosa or Ribena tell us how much you prepared them on page 4.

2 Solids

Remember to record what the child eats and not what is offered. If you like write down what you offer, what is left and we will work it out. Write down all foods eaten at meals and between both at home and away from home. Tis includes snacks such as fruit finger, potato crisps, ice creams and biscuits.

Give amounts as closely as possible. Use measuring spoons. Give number and brand of commercial biscuits, slides bread, weetbix, cheese sticks etc. If using commercial tinned or packet baby food tell us the brand type and weight of the tin or the jar which is written on the label. Separate out packaged and home made foods. Babies and toddlers: give each solid separately (eg tbs potato, tbs carrot, tbs pumpkin, 3 oz meat etc. Record how much butter you add to vegetables or milk or sugar to cereal. Tell us if you add egg yolk or grated cheese and how much.

If you give homemade mixed dish such as casserole, stew, custard sauce, please give the recipe and tell us how much that recipe made.

Record any illness that your child had in the past three days and record any medication given. If you have any questions, please phone Peggy Oti-Boateng on 204 4383.

DRINKS

INFANT FORMULAE

For 1 day

For 1 bottle

Type of formula.....

Number of scoops:.....

Amount of water : .....

Other additives:

Type.....

Amount:.....

COWS'MILK

Type of formula.....

Amount of milk:.....

Amount of water : .....

Other additives:

Type.....

Amount:.....

OTHER FLUIDS eg Juice, Delrosa, Cordial, Water, Ribena.

Type .....

Amount :.....

Added water : .....

Total volume.....:

FLINDERS MEDICAL CENTRE

COMMITTEE ON CLINICAL INVESTIGATION/DRUG & THERAPEUTICS ADVISORY COMMITTEE

ANNUAL REVIEW OF INVESTIGATIONS -1/1994

15:38. 2/9/94

Protocol No: 144/92

Title: The iron status of 6-24 month old children in relation to weaning practices in South Australia.

Investigator: Ms. P. Oti-Boateng, Paediatrics & Child Health

Date of Approval: 23.11.93 Minute 2837

Status of the Project - Is this project

Complete [ ] In progress [ ] Abandoned [ ] Not commenced [ ]

Do you seek CCI re-approval? Yes [ ] No [ ]

(Please TYPE or PRINT response)

Give a brief statement of results (if complete), reason not commenced or abandoned, OR progress report and anticipated completion date:

Results stated in the 21 February 1994 review main unchanged. The research project for which the ethic approval was sought for has been completed. We were however, unable to assess Aboriginal children due to difficulties encountered with the Aboriginal Community Health and Recreation Services, in assisting us to access Aboriginal children from baby clinics and homes in Adelaide.

Give details of adverse effects noted (e.g. subjects withdrawn from trial, complications of procedures, adverse drug effects, etc.

Due to difficulties encountered in solely trying to recruit 300 healthy babies from immunisation and baby clinics in Adelaide into the study coupled with ,lack of funding to recruit assistants, more emphasis has now been placed on animal models for the phase 2 of my Ph.D research project.

Has the base on which the Committee made its decision changed?, e.g. is there new or additional information that the Committee should be made aware of? Are risks of harm or risks of not securing a benefit the same as when the original application was made. Were there any unforeseen events:

Yes. I have transferred my work to the University of Adelaide. I have also changed Dr R Gibson and Dr Karen Simmer as my Supervisors.

Have adverse drug effects been reported to : DTAC [ ] DHCS [ ] ADEC [ ]

I certify that this research project, of which I am the principal investigator, has complied with the conditions (and special conditions if specified) of ethical approval including security of records and procedures for consent.

Name Peggy Oti-Boateng (printed) Signature.....

RETURN WITHIN 60 DAYS TO: Carol Hakof, Department of Risk Management, Level 3

Appendix 2. Composition of rat diet

American Institute of Nutrition (AIN 89) diet composition, Modified by CSIRO, Human Nutrition Division, Adelaide, Australia.

	%
Casein	20
Sugar	10
Cornstarch	50.15
Sunflower Seed Oil	10
Cellulose (Sulkaflok)	5
Choline Chloride	0.2
Methionine	0.15
Mineral mix	3.5
Vitamin mix	1.0

Mineral mix (per 15kg diet)

	grams
Calcium Carbonate	187
Tri potassium citrate	231
Sodium chloride	39
Magnesium oxide	12.6
Manganous Sulphate	3.68
Iron Chloride	6.3
Zinc Sulphate	1.36
Copper Sulphate	0.296
Potassium Iodate	0.009
Sodium Selenate	0.009

Plus sugar up to 525g

Vitamin mix (per 15kg diet)

	mg
Nicotinic acid	450
Panothenic acid	240
Thiamine	90
Riboflavin	90
Pyridoxine	105
Folic acid	30
Biotin	3

A Palmitate	120
dl alpha tocopherol	1500
B12 Cyanocobalamine	60 mL (of a 5mg/mL soln)
K Menadione	300 mL " " "
D Dupharal 1000	150 mL " " "

Plus sugar up to 150g

Appendix 3. Notes on Stains and Fixatives for Histology

3.1 *10% Buffered Formalin*

18L Distilled water

2L 98-100% Formalin (AnalaR; Hopkin and Williams, Chadwell Health, Essex England)

130g Di-sodium hydrogen orthophosphate anhydrous (AnalaR; BDH Chemicals, Ausrtalia Pty Ltd, Kilsyth, Vic)

80g Sodium dihydrogen orthophosphate (Univar; Ajax Chemicals, Sydney, Australia)

3.2 *Tissue Processing Protocol*

60 minutes in 70% ethanol (x2)

60 minutes in 80% ethanol

30 minutes in 95% ethanol

90 minutes in 95% ethanol

120 minutes in absolute ethanol (x2)

60 minutes in 1:1 absolute ethanol and histoclear

120 minutes in Histoclear (x2)

120 minutes in wax (x2)

Appendix 4 Buffers and Solutions for Electrophoresis

Urea load buffer (1x)

3g Urea

1mL 0.5M Tris, pH6.5

Method: Dissolve urea in Tris solution and pure water to 8mL. Store in aliquots at -200C

Appendix 4.2 *Electrophoresis buffer (10x)*

3g Tris

14.4g Glycine (electrophoresis reagent; Sigma, St. Louis, USA)

Method: Dissolve Tris and glycine in nanopure water and bring final volume to 100mL. Dilute 1:10 prior to use.

Equilibrium solution (0.05M Tris, pH6.5, 8M urea, 1% SDS)

20mL 0.5M Tris, pH6.5

72g Urea

60mL Glycerol

10mL 20% (w/w) SDS

Method: Dissolve urea in the solutions and bring the final volume to 200mL with nanopure water.

4.4 *SDS Load buffer (2x)*

2.5mL 0.5M Tris, pH6.5

2mL 20% (w/v) SDS

4mL 50% (v/v) Glycerol

0.1mL 1% (w/v) Bromophenol blue

Method: Combine solutions and bring final volume to 10mL with nanopure water. Freeze in aliquots at -200C. Just before use add 5µL 1M DTT to each 100 µL SDS load buffer.

4.5 *Coomassie Blue Stain*

1g Coomassie Brilliant Blue R250 (Sigma, St. Louis, USA)

500mL Methanol

400mL Nanopure water

100mL Acetic acid

Method: Combine stain with water and methanol and mix well for one hour then acetic acid and mix again. Filter before use.

4.6 *Electrophoresis Buffers and Solutions*

10% Ficoll Load Buffer

10mL Ficoll buffer (x10) is made by combining 0.025g Bromophenol blue, 0.025g Xylene Cyanole FF and 2.5g Ficoll 400 with water to 10mL. Stored in 1mL aliquots at -200C.

1x TAE Buffer

40mM Tris acetate

20mM sodium acetate

1mM EDTA, pH 8.2

4.7 *RNA Formamide Loading Buffer*

95% Formamide (v/v), 20mM EDTA, 0.06% xylene cyanol and 0.06% bromophenol blue was prepared as follows:

A 25x stock was prepared containing 25mg Xtlene cyanol, 25mg Bromophenol blue, and 1mL 0.25M EDTA, pH8. To make 1x FLB, 190 µL deionosed formamide was combined with 10 µL of the stock 25x stock.

TE Buffer with 5mM ?-Mecaptoethanol

10mM Tris-HCL, pH 8

0.1M EDTA

5mM b-Mercaptoethanol

4.8 *Prehybridisation/Hybridisation Solution*

To give final concentrations of 50% deionised formamide, 5x SSC, 0.5% SDS, 1x Denhardt's solution, 10% (w/v) dextran sulphate and 100 µg/mL Salmon Sperm DNA the following were combined.

125mL 100% Deionised formamide (AnalaR, BDH Laboratrory Supplies, Poole, England)

62.5mL 20x SSC

12.5mL 100x Denhardt's solution

1.25mL Salmon sperm DNA (20mg/mL) (Sigma, ST. Louis, USA)

50ml 50% Dextran sulphat (sodium salt; Pharmicia LKB Biotechnology, Uppsala, Sweden)

100x Denhardt's Solution

2% (w/v) Bovine serum albumin

2% (w/v) Ficoll (Sigma, St. Louis, USA)

2% (w/v) Polyvinyl pyrrolidone

SCC

150mM sodium chloride

20mM Sodium citrate

1mM EDTA, pH 7

SSPE

150mM sodium chloride

20mM sodium dihyrogenphosphate

1mM EDTA, pH 7.4

4.9 *Krebs' Hensliet Salt Mixture*

0.350g Potassium chloride

0.160g Potassium phosphate monobasic (anhydrous)

6.90g Sodim chloride

2g D-Glucose

0.288g Magnesium sulphate (.7H2O)

Method: Dissolve in 1L RO water, adjust pH to 7.5, filter sterilise and store at 40C

Appendix 5 cDNA Tissue In Situ Hybridisation Buffers and Solutions

5.1 *8.1 1x TBE Buffer*

130mM Tris

50mM Boric acid

2.5mM EDTA, pH 8.3

5.2 *Prehybridisation/Hybridisation Solutions*

Prehybridisation/Hybridisation Solutions

Per 30 µL reaction Volume:

28.8 µL Premix

3.6 µL cDNA/DEPC water

1.8 µL Blotto

1.8 µL 0.4M b-Mercaptoethanol

5.3 *Premix*

(final concentrations of 2x SSPE, 1mg/mL yeast tRNA, 0.5mg/mL Slamon sperm DNA, 1% SDS and 50% formamide/10% PEG 6000)

12.5 µL 20x SSPE

12.5 µL Yeast tRNA (10mg/mL)

62.5 µL Salmon sperm DNA (10mg/mL)

6.25 µL 20% (w/v) SDS

62.5 µL 100% (v/v) Formamide 20% (w/v) PEG 6000

5.4 *Post Hybridisation Wash Solutions*

Tissue Wash Solutions

75 mL 100% (v/v) Formamide

75 mL 4xSSPE

107 µL b-Mercaptoethanol

1.5 mL 10% (w/v) SDS

Rnase Solution

To make a 1mL solution add:

10 µL Rnase A (10mg/ml) (Boehringer Mannheim Australia Pty. Ltd, Rose Park, SA)

0.8 µL Rnase T1 (1.3mg/ml)) (Boehringer Mannheim Australia Pty. Ltd, Rose Park, SA)

990mL 2xSSPE.

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