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FERRITIN AS AN IRON TRANSPORT PROTEIN - FERRITIN UPTAKE AND IRON UTILISATION BY GUINEA PIG PLACENTA

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Animal Ethics Committee
Protocol Number 88/186/4

This dissertation is my own work. No part of it has been presented at this or any other University. The information used was obtained while I was employed by the University of the Witwatersrand, the Johannesburg Hospital and the Medical Research Council of South Africa (in the MRC Iron and Red Cell Metabolism Research Unit).



RDV Lamparelli

For My Wife Giuseppina, and My Parents

ABSTRACT

The guinea pig clears circulating tissue ferritin in a manner different from other mammals. Previous work in man and in rats has shown that injected tissue ferritin is removed from the plasma predominantly by the liver; however, in the guinea pig it is cleared predominantly by red cell precursors and furthermore, the ferritin iron is utilised directly for haem synthesis. During pregnancy, the foeto-placental complex also has a high requirement for iron.

The organ distribution of intravenously injected hepatic ferritin radiolabelled either singly with ^{59}Fe or doubly with ^{59}Fe and ^{125}I was studied in pregnant guinea pigs. At 5 hours, 71.2% of ^{59}Fe was present in the placenta and foetus. Transplacental transfer of ^{59}Fe was slow, with only 11.2% present in the foetus at 5 hours and reaching 38.6% at 21 hours. Analysis of placental cellular lysates for ^{59}Fe and ^{125}I revealed that the injected iron was present as intact ferritin at 2 hours but by 21 hours the ferritin had been catabolised, the ^{125}I excreted and the ^{59}Fe incorporated into endogenous ferritin. Most of the foetal ^{59}Fe counts were detected in the liver, with 35.3% of the transferred ^{59}Fe in ferritin, 30.4% in haemoglobin, 2.0% in transferrin and 10.6% in a low molecular weight pool. Placental uptake of labelled ferritin was significantly inhibited by a 300-fold molar excess of unlabelled ferritin ($P < 0.05$) but not by albumin, asialofetuin or by the injection of carbon particles. A reduction in ferritin uptake was noted after injection of mannosylated bovine serum albumin but this reduction did not reach

statistical significance. The mannosidase inhibitor swainsonine had no effect. Iron transfer to the foetus was not changed by 3 microtubular and microfilament inhibitors. The ferrous chelator 2,2' bipyridine significantly decreased the transfer of ^{59}Fe to the foetus whereas the ferric chelator desferrioxamine had no such effect. Electron microscopic analysis of placental tissue showed ferritin molecules being concentrated on invaginating pits on the cell membrane in keeping with an endocytic process.

In conclusion, these results indicate that the guinea pig placenta takes up homologous tissue ferritin and transfers the iron slowly to the foetus after reductive mobilisation.

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CONTENTS

Chapter			Page
1		INTRODUCTION	1
1.1		GENERAL INTRODUCTION	2
1.2		IRON METABOLISM	3
	1.2.1	Internal Iron Exchange	3
		1.2.1.1 Plasma transferrin and iron delivery to cells	4
		1.2.1.2 Ferritin	5
		1.2.1.3 Iron in pregnancy	10
	1.2.2	External Iron Exchange	10
1.3		THE PLACENTA	11
	1.3.1	Placental Structure and Development	11
	1.3.2	Placental Subtypes	13
1.4		PLACENTAL TRANSFER OF IRON	14
	1.4.1	Haemochorial Placenta	14
	1.4.2	Non-Haemochorial Placenta	16
1.5		SUMMARY	17
2		MATERIALS AND METHODS	18
2.1		ANIMALS	19
2.2		RADIO ISOTOPIC TECHNIQUES	19
	2.2.1	Radio Labelling of Guinea Pig and Rat Hepatic Ferritin	19
	2.2.2	Counting Techniques	22
2.3		ORGAN DISTRIBUTIONS OF INJECTED TISSUE ^{59}FE FERRITIN IN THE PREGNANT GUINEA PIG AND RAT .	23
2.4		ANALYSIS OF CELLULAR LYSATES	24

2.5 EFFECTS OF VARIOUS INJECTED AGENTS ON ^{59}FE FERRITIN UPTAKE AND ^{59}FE DISTRIBUTION IN PREGNANT GUINEA PIGS 25
2.5.1 Agents Affecting ^{59}Fe Ferritin Uptake 25
2.5.2 Agents Affecting Intracellular Iron Metabolism 26
2.5.2.1 Microtubular and microfilament inhibitors . 27
2.5.2.2 Mannosidase inhibition 27
2.5.3 Chelators and the Distribution of ^{59}Fe 28
2.6 EFFECTS OF A HIGH SATURATION OF TRANSFERRIN IN VIVO 29
2.7 ELECTRON MICROSCOPIC TECHNIQUES 29
2.8 STATISTICAL INTERPRETATION 30
2.9 ETHICAL CONSIDERATIONS 30
2.10 SUMMARY 30

3 RESULTS 31
3.1 ORGAN DISTRIBUTIONS OF ^{59}FE AFTER ^{59}FE FERRITIN INJECTION INTO PREGNANT ANIMALS 32
3.1.1 Distribution of ^{59}Fe after ^{59}Fe Ferritin Injection in Pregnant Rats and Guinea Pigs .. 32
3.1.2 Effect of Time on the Distribution of ^{59}Fe and ^{125}I after ^{59}Fe^{125}I Ferritin Injection into Pregnant Guinea Pigs 32
3.1.3 Effect of Time on the Intracellular Distribution of ^{59}Fe and ^{125}I after ^{59}Fe^{125}I Ferritin Injection into Pregnant Guinea Pigs. 37
3.1.4 Effects of Various Injected Substances on

Intravenously Injected ^{59}Fe Ferritin Uptake and ^{59}Fe Distribution in Pregnant Guinea Pigs 40
3.1.4.1 Effects of ferritin, albumin, asialofetuin or carbon particles on ^{59}Fe ferritin uptake 40
3.1.4.2 Effects of microtubular and microfilament inhibitors 40
3.1.4.3 Effects of mannosidase inhibition 42
3.1.5 Effects of Chelators on ^{59}Fe Distribution ... 42
3.1.6 Effects of a High Saturation of Transferrin . 42
3.2 ELECTRON MICROSCOPIC STUDIES 46
3.3 SUMMARY 46

4 DISCUSSION 49
4.1 DISCUSSION 50
4.2 SUMMARY 55

REFERENCES 57

LIST OF TABLES

Table No	Heading	Page
I (a)	The mean (± SD) organ distribution of ^{59}Fe 5 hours after injection of homologous hepatic ^{59}Fe ferritin into pregnant rats and pregnant guinea pigs ..	34
I (b)	The organ distribution of ^{59}Fe 5 hours after injection of rat hepatic ^{59}Fe ferritin into a pregnant guinea pig and guinea pig hepatic ^{59}Fe ferritin into a pregnant rat	34
II	The mean (± SD) percentage distributions of ^{59}Fe at progressive time intervals after intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs	36
III	The mean percentage distributions (a) and the ratios of ^{59}Fe to ^{125}I (b) at progressive time intervals after $^{59}Fe^{125}I$ doubly labelled ferritin injection into pregnant guinea pigs	38
IV	The effects of various substances injected intravenously on the mean (± SD) percentage distribution of ^{59}Fe 5 hours after the intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs ..	41

V The effects of 3 microtubular inhibitors [vincristine (0.15 mg/kg), vinblastine (0.6 mg/kg) or etoposide (10 mg/kg)] injected intravenously 24 hours prior to ^{59}Fe ferritin injection on the distribution of ^{59}Fe at 21 hours after intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs .. 43

VI The effects of 4 days of pretreatment with a mannosidase inhibitor [swainsonine (0.005 /umol/ml blood volume injected intraperitoneally 12 hourly) on the mean (± SD) percentage distribution of ^{59}Fe 21 hours after intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs 44

VII The effects of the chelators bipyridine or desferrioxamine (16 mg/kg) injected intramuscularly 30 and 2 minutes prior to and 2, 4, 6 and 8 hours after intravenous injection of ^{59}Fe ferritin, on the distribution of ^{59}Fe 21 hours after the intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs 45

VIII The effects of a high saturation of transferrin *in vivo* on the distribution of ^{59}Fe 21 hours after ^{59}Fe ferritin injection into pregnant guinea pigs. 47

LIST OF FIGURES

Figure No	Heading	Page
1	The organ distribution of ^{59}Fe 5 hours after the intravenous injection of homologous ^{59}Fe ferritin into pregnant rats and guinea pigs	33
2	The organ distribution of ^{59}Fe at various times after the intravenous injection of ^{59}Fe tissue ferritin into pregnant guinea pigs	35
3	AcA-44 ultrogel chromatographic analysis of placental cellular lysates 2, 5 and 21 hours after the intravenous injection of tissue ferritin labelled with ^{59}Fe and ^{125}I. The solid lines represent ^{59}Fe and the broken lines ^{125}I. A column calibration with ferritin and transferrin is shown in the top panel	39
4	Thin section views (x 40 000) showing control tissue (a) and tissue removed 1 hour after intravenous injection of 10 mg homologous tissue ferritin (b). Clustering and concentration of ferritin molecules in a coated pit on the cell membrane and in two large endocytic vesicles are arrowed	48

Chapter One

INTRODUCTION

1.1 GENERAL INTRODUCTION

All living organisms need iron. Iron is an integral component of many proteins essential to metabolic processes which maintain life. It is present in the circulating oxygen transport protein haemoglobin, in the tissue oxygen storage protein myoglobin, and in the enzymes involved in cellular respiration (cytochromes), and oxidation and reduction reactions (catalases and peroxidases).

Iron is stored in the intracellular iron storage protein ferritin and is transported to sites of tissue demand by the plasma protein transferrin. Small amounts of ferritin are normally secreted into the plasma in proportion to body iron stores (Walters et al, 1973). To date, no physiological function has been attributed to ferritin in the plasma.

Intravenous injection of tissue ferritin in man (Cragg et al, 1983) and in rats (Lipschitz et al, 1971) results in rapid hepatic clearance of the protein from the circulation (Hershko et al, 1973; Unger and Hershko, 1974) by specific receptors on the hepatocyte (Mack et al, 1983; Mack et al, 1985).

Recent studies *in vitro* (Pollack and Campana, 1981) and *in vivo* (Simon et al, 1987) and have demonstrated that the reticulocytes of guinea pigs which have been made anaemic, have receptors for tissue ferritin and are able to utilise the ferritin iron directly for haem synthesis (Blight and Morgan, 1983; Simon et al, 1987).

Apart from the erythron, the foeto-placental unit is another organ system which has a large demand for iron. Preliminary studies from this Unit had indicated that the placenta in the guinea pig may be analogous to the reticulocyte in its ability to interact with tissue ferritin and transfer the iron to the foe-

tus. This pathway would circumvent the conventional route of iron delivery by plasma transferrin. In this regard, it is possible that ferritin may have acted as an iron transport protein at some stage during evolution, and that the presence of the ferritin pathway in the guinea pig may be of phylogenetic significance.

A series of studies was carried out to elaborate on the possible uptake of ferritin by guinea pig placenta and the transfer of ferritin iron to the foetus.

Certain aspects of iron metabolism and placental function will now be reviewed so that the findings of these studies may be appreciated in the light of current knowledge in the field.

1.2 IRON METABOLISM

The body of an average 75 kg adult male contains approximately 3 800 mg of iron: ± 2 800 mg incorporated into a functional compartment (haemoglobin, myoglobin, cytochromes, non-haem enzymes) and 1 000 mg in a storage compartment (ferritin and haemosiderin) (Bothwell et al, 1979).

Biologically, iron metabolism follows two pathways, an internal one in which iron released from effete cells is re-utilised, and an external one where iron is gained from and lost to the exterior.

1.2.1 Internal Iron Exchange

Senescent erythrocytes are phagocytosed by cells of the reticuloendothelial system. The haemoglobin is catabolised to yield amino acids, bilirubin and ferrous iron (Jacobs, 1980). Depending on body requirements, this iron can either be stored intracellularly in ferritin or released from the cell, bound to the plasma protein transferrin and transported to its new destination (Bothwell et al, 1979).

1.2.1.1 Plasma transferrin and iron delivery to cells

This beta-1-globulin is a single chain glycoprotein (MW 79 550 daltons) synthesised as iron-free apotransferrin in the liver (Jacobs, 1980) at a rate which is, to a large degree, inversely proportional to the hepatic ferritin concentration (Morton and Tavill, 1978). Transferrin in the plasma acts to maintain iron soluble at physiological pH and as a means of transport for the element (Jacobs, 1980). Transferrin can bind 2 atoms of ferric iron, one at each of the N and C domains of the molecule (Chasteen, 1983) and can thus exist in four forms: diferric, apoferric and 2 types of monoferric transferrin (Huebers et al, 1981).

The reticuloendothelial system is the main source of iron entering the plasma (Morgan, 1981). How iron is released from these cells remains to be determined. Much more is known of the mechanism of iron delivery to cells. The cell systems best studied thus far include hepatoma cells (Dautry-Varsat et al, 1983), reticulocytes (Harding et al, 1983; Morgan, 1983) and erythroleukaemia cells (Klausner et al, 1983). Circulating transferrin binds on to the cell surface to a specific transferrin receptor, a transmembrane dimeric glycoprotein (MW 180 000 daltons) (Seligman et al, 1983). The transferrin-receptor complex is internalised by a process termed 'receptor-mediated endocytosis'. The first step in this process involves clustering of receptors over pits in the cell membrane underset by clathrin molecules (membrane structural proteins). These clathrin coated pits invaginate progressively to form an endosome which loses its clathrin coat. The receptors with their ligands become separated from the endosomes to form intracellular vesicles. These vesicles are transported in a saltatory fashion by microtubular and microfilament mechanisms (May and Cuatrecasas, 1985) which can be inhibited by

vinca alkaloids (Hemmaplardh et al, 1974). The interior of the vesicle then becomes acidified to pH ± 5.5 (van Renswoude et al, 1982) by an energy-dependent process with the result that ferric iron is released from transferrin and is reduced to the ferrous form. The acidified vesicle is termed the 'compartment of uncoupling of receptor and ligand' (CURL) (Goldstein et al, 1979). This ferrous iron traverses the vesicular membrane and enters the cytosol in the ferric form. How the iron is transported across this membrane and how the ferrous iron is oxidised remain to be elucidated. The intact apotransferrin-receptor complex within the vesicle returns to the cell surface where the vesicular membrane blends with the cell membrane. At physiological pH, apotransferrin dissociates from the receptor and re-enters the circulation.

Uptake of transferrin iron by the hepatocyte appears to be more complex. Rather than interacting with a single specific transferrin receptor, at least four pathways appear to operate. The most significant pathway under physiological conditions is a high-capacity low-affinity transferrin receptor mechanism (Page et al, 1984). A second specific pathway is via a low-capacity high-affinity transferrin receptor mechanism. (Cole and Glass, 1983; Page et al, 1984). Thirdly, transferrin may be taken up by the process of fluid phase endocytosis which is relatively inefficient (Sibille et al, 1982; Page et al, 1984; Trinder et al, 1986). Finally, transferrin may interact with a non-specific asialoglycoprotein receptor on hepatocytes (Young et al, 1983; Regoezci and Koj, 1985).

1.2.1.2 Ferritin

Ferritin is a water soluble intracellular iron storage protein (MW 430 000 - 480 000 daltons) present within all cells. It is made up of 24 subunits arranged to form a large, hollow, sphe-

rical shell (Harrison et al, 1980). The protein is synthesised as iron-free apoferritin. Human spleen apoferritin consists of 174 amino acids (Wustefeld and Crichton, 1982). Up to 4 500 atoms of iron can be stored in the central core of this molecule as hydrous ferric oxide-phosphate in microcrystal form (Harrison et al, 1980). The rate of apoferritin synthesis is regulated by the concentration of iron within the cell. An increase in intracellular iron induces the synthesis of new subunits via an increase in translation of inactive ferritin mRNA located as free messenger ribonuclear protein in the cytoplasm (Zahringer et al, 1975). Recently, an iron-responsive element (IRE), a sequence of nucleotides in the 5'-untranslated region (UTR) of mRNA, has been identified (Aziz and Munro, 1987; Hentze et al, 1987) which regulates translation of ferritin mRNA. The rise in intracellular iron may also lead to an increase in the rate of assembly of preformed ferritin subunits by a post-translational effect (Zahringer et al, 1976). Within the cell, the site of ferritin synthesis depends on whether the protein is destined for intracellular use or for export to the plasma. Ferritin for intracellular use is synthesised predominantly on ribosomes free in the cytoplasm whereas that for export is made mainly on ribosomes bound to the membrane of the endoplasmic reticulum (Hicks et al, 1969; Lee and Richter, 1977).

A number of different isoferritins have been identified. Ferritin contains two types of subunits which have been designated H (Heavy, Heart) and L (Light, Liver). H subunits (MW 21 000 daltons) predominate in the more acidic isoferritins (pI 4.5-5.0) which are present in the heart, erythrocytes, lymphocytes and monocytes whereas L subunits (MW 19 000 daltons) predominate in the more basic isoferritins (pI 5.3-5.8) found in the liver,

spleen and placenta (Worwood, 1986). The physiological significance of ferritin heterogeneity remains unexplained. Some descriptive differences are that H-rich ferritins turn over (Kohgo et al, 1980; Bomford et al, 1981) and take up and release iron more rapidly (Jones et al, 1978; Wagstaff et al, 1978) than L-rich ferritins. On the other hand, L-rich ferritins predominate in tissues rich in iron (Kohgo et al, 1980; Bomford et al, 1981). Acidic (H-rich) isoferritins may act as feedback regulators in myelopoeisis (Broxmeyer et al, 1981), though this has been disputed (Jacobs, 1983). Acidic ferritins may also be elevated in the plasma of patients with malignancies e.g. lymphomas (Cazzola et al, 1983).

One of the major functions of ferritin is to maintain iron in a soluble, non-toxic yet bio-available form. Access to the central core of the ferritin molecule is via 6 channels through the protein shell. Apoferritin appears to bind iron on its inner surface and to catalyse its oxidation to the Fe^{3+} form (Harrison et al, 1980). Hydrous ferric oxide is generated and forms a nucleus for further microcrystalisation (Harrison et al, 1980). Physiologically, release of iron from ferritin appears to be mediated by a reductive pathway involving ferrioxidases and $FMNH_2$ (Funk et al, 1983; Ulvik, 1983); however, this mechanism may be in doubt since it requires an oxygen-free environment not usually found intracellularly (Crichton, 1985). Other possible pathways of iron release from ferritin *in vivo* include non-enzymatic reduction by dihydroascorbate and free radicals as well as enzymatic reduction by xanthine oxidase (Crichton, 1985). Agents which can mobilise iron from ferritin *in vitro* include the chelators 2,2' bipyridyl, desferrioxamine, rhodotorulic acid, 2,3 dihydroxybenzoate, paphy (pyridine-2-aldehyde-2-pyridyl hydrazone)

(Crichton et al, 1980), and dithiols including DL-dihydrolipoate and DL-dihydrolipoamide (Bonomi and Pagani, 1986). More recently, human apotransferrin has been demonstrated to mobilise iron directly from spleen ferritin in the absence of either reducing or chelating agents, although the transfer of iron was enhanced by low molecular weight substances (ascorbate, citrate, bicarbonate, lactate) in the same concentrations as those found in human serum (Jin and Crichton, 1987). The mobilisation of iron atoms from ferritin seems to follow a last-in-first-out principle (Treffrey and Harrison, 1984).

Haemosiderin appears to be denatured ferritin (Harrison et al, 1980). Its iron is poorly mobilisable (Cook et al, 1982) and probably represents a cul-de-sac in cellular iron metabolism.

Ferritin may gain access to the plasma in one of two ways. Firstly, ferritin is secreted into the plasma in proportion to body iron stores, 1 /ug/l of plasma ferritin ordinarily representing 8-10 mg of storage iron (Walters et al, 1973). Secondly, large quantities of intracellular ferritin may be released into the circulation in the event of tissue damage and cell membrane disruption (Prieto et al, 1975). In most mammals, the released tissue ferritin is cleared rapidly from the circulation (Hershko et al, 1973; Unger and Hershko, 1974) by specific receptors on liver cells (Mack et al, 1983; Mack et al, 1985). In cultured rat hepatocytes, a high capacity low affinty mechanism has been identified with the ability of taking up 1.3×10^5 ferritin molecules/cell/hour (Osterloh and Aisen, 1989). Furthermore, in experiments incubating rat hepatocytes with doubly-labelled $^{59}Fe^{125}I$ ferritin, the protein fraction of the molecule (labelled with ^{125}I) has been shown to be present in lysosomes while the ^{59}Fe is being utilised in the mitochondria (Sibille et al, 1989); this is

in keeping with ferritin catabolism for iron release as compared with transferrin which can recycle after delivering its iron.

There are two major differences between plasma and tissue ferritin. Plasma ferritin has a much lower iron content (Worwood, 1976; Zuyderhout et al, 1978) and a higher proportion of glycosylated G-peptides (Worwood et al 1979; Cragg et al, 1981a,b) associated with the L-subunit (Santambrogio et al, 1987) than tissue ferritin. The higher degree of glycosylation prolongs the clearance half-life of plasma ferritin to about 30 hours, whereas less than 1% of tissue ferritin is present in the circulation 5 hours after injection (Cragg et al, 1983).

As alluded to earlier, the clearance of tissue ferritin injected into the guinea pig is different from other animals. In this regard, reference to previous work from this Unit is highly relevant. Almost 2/3 of ^{59}Fe ferritin injected into normal guinea pigs was taken up by red cell precursors within 24 hours of injection and the iron was utilised in haem formation. Furthermore, when erythropoiesis was stimulated either by repeated venesections or by the induction of a haemolytic anaemia by phenylhydrazine injection, 55% of injected ferritin iron was associated with circulating reticulocytes at 1 hour after ^{59}Fe ferritin injection (Simon et al, 1987). The rate of ^{59}Fe ferritin iron incorporation into haemoglobin was, however, much slower when compared with iron delivered to the cells by transferrin. By 1 hour, a mere 12.9% of ferritin iron taken up by the reticulocytes in phenylhydrazine-treated animals had been incorporated into haem, and only reached 83.4% 24 hours after ^{59}Fe ferritin injection. By contrast, the corresponding values for transferrin iron were 95.1% at 1 hour and 92.6% at 24 hours. Iron for utilisation in haem synthesis was released from ferritin equally well in

scorbutic and control animals, suggesting that ascorbic acid is not essential for iron release from ferritin (Simon et al, 1987).

1.2.1.3 Iron in Pregnancy

Pregnancy represents a period of rapid anabolism. The growing foetus, placenta and umbilical cord and the expanding maternal red cell mass result in a large overall iron requirement (± 1 200 mg) during pregnancy in human females (Bothwell et al, 1979). This must be met from the diet and from body iron stores. At term, the infant is born with approximately 80 mg of iron per kilogram body weight (Bothwell et al, 1979). The placenta scavenges iron and transfers it to the foetus irrespective of maternal iron stores (Murray and Stein, 1971) unless these are very low (Kelly et al, 1978; MacPhail et al, 1980). Since in humans, the upper limit of gastro-intestinal iron absorption under optimal conditions is ± 3.5 mg per day, large demands are placed on maternal body stores, especially in the second half of pregnancy (Bothwell et al, 1979). Transport of iron across the placenta to the foetus will be discussed in a later section (1.4).

1.2.2 External Iron Exchange

In relation to the total body iron, only a miniscule amount exchanges with the environment every day. Obligatory losses from the skin, and the gastro-intestinal and genito-urinary tracts amount to ± 1 mg/day in the adult male and approximately twice this value in the menstruating female. To remain in iron balance, this amount of iron must be absorbed from the daily diet. Iron requirements are increased further during periods of rapid growth which occur during infancy and adolescence, when daily requirements may reach 1.6 mg (Bothwell et al, 1979).

1.3 THE PLACENTA

During intrauterine life, the placenta represents the communication between the foetus and the outside world. The foetus derives its nutrition and gas exchange from, while also excreting its waste products through, the placenta which thus acts as gut, lung and kidney. It also acts as a selective barrier allowing certain large molecules to cross to the foetus while being impermeable to others. Finally, the placenta plays an endocrine role synthesising oestrogens, progesterones, placental lactogen and gonadotrophins. The description which follows relies heavily on the excellent works by Arey (1965), Steven (1975) and Kaufmann and Davidoff (1977).

1.3.1 Placental Stucture and Development

The placenta is a composite organ having a double origin. The foetal portion is the chorion frondosum consisting of the chorionic plate and chorionic villi. The maternal contribution is the decidua basalis. In between the two is the intervillous space.

Within a few days of fertilisation, the conceptus develops into a blastocyst with an inner cell mass (the future embryo proper) and the capsule-like wall, the trophoblast, which will evolve into a portion of the placenta. The inner cell mass gives rise to the embryonic disc, three layers of germ cells (entoderm, mesoderm, ectoderm) from which the organs will subsequently differentiate. The yolk sac then forms within the layers of the entoderm. The allantois forms later as a slender entodermal tube extending from the caudal end of the yolk sac into the mesoderm of the body stalk, one of the anlages of the umbilical cord. Wherever the trophoblast comes into contact with the uterine wall, it becomes thickened and many cells lose their boundaries

leading to the formation of a syncytiotrophoblast. The inner layers of the blastocyst retain their cell membranes and constitute the cellular cytotrophoblast. Cords of mesoderm then begin to extend into the trophoblast and this marks the beginning of the chorionic villi.

In pregnancy, three different regions of the endometrium are recognisable: (i) the decidua parietalis, the general lining of the uterus exclusive of the implantation of the embryo, (ii) the decidua capsularis, the region covering the conceptus and interposed between it and the uterine cavity and (iii) the decidua basalis, the region situated between the conceptus and the myometrium. The decidua basalis contains representatives of both the compact and spongy layers of the progravid endometrium. The portion of the decidua basalis most intimately incorporated into the basal plate is, in fact, the compact layer of this region.

The chorionic villi progressively attach to and erode into the decidua basalis. Structurally, the villi consist of a core of connective tissue in which are embedded blood vessels derived from the conceptus. This core is covered by a double layer of trophoblast, viz. cytotrophoblast and syncytiotrophoblast. As gestation progresses, the enlarging villus tree becomes separated into cotyledons by placental connective tissue septae.

Between the chorionic villi is the intervillous space. This cavernous space, primarily of foetal origin, also grows at the expense of decidual erosion. In the haemochorial type of placenta (see section 1.3.2), maternal blood enters the cotyledons via spiral terminations of the uterine arteries which thus open directly into the intervillous space converting it into a blood-filled sinus. Exchange of substances between the maternal and foetal circulations can now occur. Maternal blood then disperses,

escapes through venous outlets in the placenta and returns eventually to the uterine veins.

1.3.2 Placental Subtypes

Placentae can be subtyped according to the degree of contact between the chorion and the uterus and, more particularly, on the histological relations at the junction of these two tissues. Three broad subtypes are recognised:

(i) Epitheliochorial: In this least modified placental state the allantois and the chorion unite, become jointly vascularised by allantoic vessels and, by apposition only, the chorionic ectoderm becomes applied to the uterine lining. Nutrient substances and oxygen from maternal blood must pass out of the uterine vessels, through layers of connective tissue and epithelium of both the maternal decidua and the placental cotyledon before entering the allantoic vessels. Mammals which have this type of placenta include the sheep, pig, deer and horse.

(ii) Endotheliochorial: Here the chorionic villi erode into the uterine mucosa virtually down to the endothelium of its blood vessels. The syncytial chorionic epithelium then packs around these maternal vessels. Dogs, cats, racoons and bears have this type of placenta.

(iii) Haemochorial: More intimacy between the maternal and foetal circulations is achieved in this subtype of placenta through loss of the endothelium of the uterine vessels so that maternal blood circulates within cavernous spaces between the chorionic villi. Haemochorial placentae are found in the rabbit, rodents such as the gerbil and chinchilla, and primates including rhesus and green monkeys. Further loss of the layers of the chorionic villi results in the most intimate contact between the two circulations, where the barrier then consists only of a single

layer of trophoblast and the endothelial lining of the foetal blood vessels. This subcategory of placentation is termed 'haemo-endothelial' and is the type of placenta found in the guinea pig.

Although the guinea pig and human placentae resemble each other, there are 2 very significant differences. Firstly, in the guinea pig, blood flows in a clearly defined direction whereas in humans the flow is poorly defined and has been termed a 'pool-flow system.' Secondly, the human placenta has a far more extensive stroma of connective tissue which forms the preponderant volume when compared with the guinea pig, where connective tissue is minimal. Despite these differences, the other marked similarities may allow experimental results in the guinea pig to be transferable to human placental physiology.

1.4 PLACENTAL TRANSFER OF IRON

The mechanisms of iron transfer from maternal blood across the placenta to the foetus are different in haemochorial and non-haemochorial placentae and will be discussed separately.

1.4.1 Haemochorial Placenta

The source of maternal iron for transfer to the foetus was the subject of much speculation in the first half of this century; a popular theory surmised that the iron was derived from haemolysis of maternal erythrocytes in the placenta. Subsequent work in humans (Pommerenke et al, 1941) and guinea pigs (Vosburgh and Flexner, 1950) demonstrated rapid transfer of iron from the maternal circulation to the foetus, suggesting that the plasma rather than the erythrocytes was the source of the iron. This source was confirmed by work in rabbits by Bothwell and coworkers in 1958. They showed further that placental iron transfer to the foetus increased with foetal age and weight, that the majority of

iron is deposited as non-haem iron in the foetus, that iron transport to the foetus takes place against a concentration gradient, and that this transport is an active process and unidirectional (Bothwell et al, 1958).

Later investigations have shown that foetuses with this type of placenta derive all of their iron from maternal transferrin (Baker and Morgan, 1969; McArdle and Morgan, 1981). Wong and Morgan (1973) addressed several important points in their studies on guinea pigs. They demonstrated that the placenta took up maternal transferrin and was able to remove its iron and transfer it to the foetus. Of note were their findings that (i) on ferrokinetic data, plasma transferrin was an adequate source for foetal iron and that (ii) iron transfer could be inhibited by hypoxia and metabolic inhibitors, in keeping with an energy-dependent process (Wong and Morgan, 1973).

The chorio-allantoic placenta is the main route for iron transfer from the maternal plasma to the foetus. The mechanism of transferrin iron delivery to the placenta appears to be analogous to delivery to red cell precursors in many ways. Plasma transferrin binds to specific receptors on trophoblastic cells of the placenta (Seligman et al, 1979; Wade et al, 1979; Loh et al, 1980). The placental transferrin receptor is biochemically and immunologically similar to the receptor on the reticulocyte (Morgan, 1982). Autoradiographic studies using ^{125}I transferrin injected into pregnant rabbits have shown that the transferrin taken up by the trophoblastic cells enters an intracellular vesicular compartment (Baker et al, 1983). Iron uptake by rat placental cells in culture has been studied by McArdle and coworkers. As in erythroid cells, transferrin bound to the cell membrane is endocytosed, the ensuing vesicle is acidified by an

energy-dependent process, transferrin iron is released and apotransferrin is returned to the exterior (McArdle et al, 1985). The form in which iron is transferred across the trophoblastic cell, and what regulates the amount of iron transferred are still unclear. Van Dijk and coworkers (1985) postulate the presence of a low molecular weight iron pool to be responsible for iron transfer. The size of this pool may be regulated by the concentration of intracellular placental ferritin (van Dijk et al, 1986). How iron is accepted on the foetal side is also unresolved since to date, transferrin receptors on the foetal side of the placenta have not been identified.

Other points of note are that the major portion of iron transfer to the foetus occurs during the second half of pregnancy (Bothwell et al, 1958); removal of the foetuses does not alter the rate at which the placentae accumulate iron (Bothwell et al, 1958; Wong and Morgan, 1973); and iron transferred across the placenta is rapidly taken up by foetal tissues (Bothwell et al, 1958; Baker and Morgan, 1969).

1.4.2 Non-Haemochorial Placentae

Anatomically and physiologically, the barrier between the maternal and foetal circulations is much greater in these types of placentae. Injection of ^{59}Fe transferrin into a cat (which has an endotheliochorial placenta) resulted in minimal amounts of transferrin being bound to the placenta and ^{59}Fe transfer to the foetus when compared with the rabbit (which has a haemochorial placenta) (Baker and Morgan, 1973). Injection of homologous ^{59}Fe-labelled erythrocytes into pregnant cats resulted in ^{59}Fe transfer to the foetus in quantities which were calculated to be sufficient to satisfy all foetal iron requirements (Wong and Morgan, 1973).

The postulate in non-haemochorial placental iron transfer to the foetus is that maternal erythrocytes extravasated into the uterine lumen are phagocytosed by the chorionic epithelium, the haemoglobin is catabolised and iron thus released is transported to the foetus.

An interesting additional mechanism has been postulated in the pig epitheliochorial placenta. An iron-containing glycoprotein uteroferrin (MW 32 000 daltons) with acid phosphatase activity is secreted by the endometrial glandular epithelium into the uterine lumen in secretory granules (Schlosnagle et al, 1974) under the control of progesterone (Chen et al, 1973). The granules move across to the areolae (specialised regions of the chorion which develop opposite the uterine glands) where they are taken up (Raub et al, 1985). From there, uteroferrrin is either transported across the allantois or alternatively, the protein may be transported into capillaries and carried via allantoic and umbilical veins to the foetus (Buhi et al, 1982). Further details on how iron is released and transferred to the foetus are still undefined.

1.5 SUMMARY

In the introductory Chapter, a brief review of the literature is presented covering some aspects of iron metabolism, placental physiology and materno-foetal iron transfer. Furthermore, the rationale behind the series of experiments exhibited in this dissertation is demonstrated against the background of previous work done in the Iron and Red Cell Metabolism Research Unit Laboratory.

Chapter Two

MATERIALS AND METHODS

2.1 ANIMALS

Guinea pigs of the Dunkin-Hartley and rats of the Sprague-Dawley strains were used for the preparation of raw materials and for the studies on pregnant animals. For the latter, animals were time mated and housed in the Central Animal Unit of the University of the Witwatersrand Medical School until they were studied.

The gestation period for rats is 21 days (Rowett, 1974). The rats in these experiments were studied on day 18 of gestation.

The gestation period for guinea pigs is 63 $\pm$ 7 days (Sisk, 1976). In all experiments, guinea pigs in the last week of gestation were used.

All 'intravenous' injections were performed under light halothane (Fluothane, ICI Pharmaceuticals, Braamfontein, Johannesburg) anaesthesia. The route of injection in guinea pigs was intracardiac whereas in rats the lateral tail vein was used. After injection, the animals were housed in metabolic cages. At predetermined time intervals after injection, the animals were re-anaesthesised with halothane and killed by exsanguination via the intracardiac route. The organs were washed out *in situ* with normal saline at the time of cardiac puncture and were then removed. Unless otherwise stated, at least three animals were studied at each time interval.

2.2 RADIO ISOTOPIC TECHNIQUES

2.2.1 Radio Labelling of Guinea Pig and Rat Hepatic Ferritin

Five hundred /ug of iron as $FeCl_3.6H_2O$ were added to 500 /uCi $^{59}FeCl_3$ (Amersham International, Amersham, Buckinghamshire, England) in acid solution (pH 1.2). The iron was precipitated by the addition of sodium hydroxide to raise the pH above 4. The precipitate was washed twice with distilled water, suspended in

0.5 ml normal saline, heated to 50°C and solid citric acid added stepwise to allow dissolution of the iron. The solution was allowed to cool and the pH raised to 7.4 with sodium bicarbonate. The ferric citrate thus prepared was injected intravenously into animals as described in 2.1.

Twenty four hours later, the animals were killed, the livers removed and hepatic ferritin was isolated using a method described originally by Huebers and co-workers (1976), with minor modifications. The livers were weighed, placed in four times their weight of distilled water and homogenised in an Ultra-Turrax homogeniser (Jank & Hankel, IKA-Werk, D7813, Staufen, Germany). The homogenates were subjected to heat denaturation at 75-80°C for 10 minutes. After cooling, the homogenates were centrifuged at 2 000 g for 20 minutes, the pH of the supernatant was lowered to 4.9 with 25% acetic acid and the solution allowed to stand overnight at 4°C. Following centrifugation at 2 000 g for 20 minutes to remove debris, an equal volume of saturated ammonium sulphate was added to the supernatant to yield a solution containing 50% ammonium sulphate. This solution was allowed to stand overnight at 4°C. Centrifugation at 2 000 g yielded a pellet containing the ferritin fraction. The pellet was dissolved in normal saline and dialysed against several changes of normal saline to remove residual ammonium sulphate. Undissolved protein was removed by centrifugation.

The crude ferritin fraction was purified further by sequential column chromatography. The ferritin was dialysed overnight at 4°C against 0.25 M acetate buffer containing 0.05 M NaCl (pH 4.9) and applied to a column (2.5 x 47 cm) of carboxymethylcellulose (Whatman CM 52, Whatman Chemical Separation Ltd, Springfield Mill, Maidstone, Kent, England) equilibrated and eluted with

buffer identical to the dialysate. The flow rate was 13 ml/hour. The ultraviolet absorption of the effluent was measured continuously by a Uvicord spectrophotometer (LKB Instruments, Bromma, Sweden) at 280 nm. Ferritin-containing fractions collected from the first column were pooled, concentrated using polyethyleneglycol (PEG 20 000, Merck-Schuchard, Munich, Germany), dialysed overnight (against buffer for the second column) and applied to a column (1.0 x 91 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated and eluted with 0.054 M trisethanolamine/HCl buffer (pH 7.1) containing 0.125 M NaCl at a flow rate of 15 ml/hour. Ferritin fractions collected from the second column were pooled, concentrated, dialysed overnight (against buffer for the third column) and applied to a column (2.5 x 20 cm) of diethylaminoethylcellulose (Whatman DE52, Whatman Chemical Separation Ltd, Springfield Mill, Maidstone, Kent, England) equilibrated and eluted with 0.018 M trisethanolamine/HCl buffer (pH 7.1) containing 0.042 M NaCl at a flow rate of 17.4 ml/hour. Ferritin fractions were collected, pooled and dialysed overnight against normal saline prior to further analysis and usage. The purity of the ferritin was confirmed on flat bed 5% polyacrylamide gel electrophoresis.

In order to determine the fate of the protein and the iron components of injected ferritin separately, for certain studies single labelled guinea pig hepatic ^{59}Fe ferritin was labelled further using ^{125}I Bolton and Hunter reagent (Amersham International, Amersham, Buckinghamshire, England) using a modification of the method described by Goldie and Thomas (1978). Briefly, the charcoal trap was inserted into the vial of ^{125}I Bolton and Hunter reagent and the benzene solvent was allowed to evaporate in a fume cupboard. One ml of ^{59}Fe ferritin solution (12 mg

protein/ml) was added to the dried residue and allowed to mix with gentle agitation for 30 minutes in an ice/water bath. The solution was then applied to a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) coarse column (1.0 x 20 cm) eluted with 0.05 M Veronal buffer (pH 8.0). The column had previously been flushed with 0.5 ml of 5% bovine serum albumin (Sigma Chemical Company No A-6003, St Louis, Missouri, USA) to prevent non-specific adherence of ferritin to the gel. Ferritin fractions were collected, pooled and dialysed overnight against several changes of normal saline to remove residual free ^{125}I prior to further analysis or usage. The doubly labelled ligand was passed through the Sephadex G-25 column again 2 hours prior to use, to minimise the time-dependent deterioration of iodinated ferritin (Covell and Cook, 1987).

The protein concentration of the ferritin solution was measured by the Lowry method (1951) using bovine serum albumin as the standard. The iron content was estimated by a modification of the method recommended by the International Committe for Standardization in Haematology (1978). In order to denature the protein and release the iron, the ferritin solution was boiled for 10 minutes in a mixture of 20% (w/v) of trichloroacetic acid and 3% thioglycollic acid in 1 M HCl before addition of chromogen solution. The absorbance of the solution was measured at 595 nm on a Varian DMS 100 spectrophotometer (Varian Techtron Pty. Limited, Mulgrave, Australia) and the iron content was determined by comparison with a previously derived standard which had been treated in the same way.

2.2.2 Counting Techniques

The distributions of radio-isotopes in samples of maternal blood, urine, bile, liver, spleen and marrow, foetal blood and

liver, and placental cellular lysates were determined by counting the radio-activity in the samples in a 3 channel autogamma counter (Packard Instruments, Downers Grove, Illinois, USA). Where necessary, corrections were made for cross counts between ^{59}Fe and ^{125}I activity.

Since over 80% of recovered activity was present in the maternal blood, liver, spleen, marrow, placenta and foetus, interpretation of the distribution of radio-activity was facilitated by expressing the results in terms of percentage of recovered counts only.

Radio-activity in the total blood volume was calculated on the assumption that the total blood volume in the guinea pig is 65ml/kg (Sisk, 1976). Similarly, radio-activity in the bone marrow was calculated on the known ratios of femur marrow to total marrow (1:13) (Sisk, 1976).

2.3 DISTRIBUTION OF INTRAVENOUSLY INJECTED TISSUE ^{59}Fe FERRITIN IN THE PREGNANT GUINEA PIG AND RAT

The initial experiment was performed to establish differences in the organ distribution of ^{59}Fe ferritin when injected into pregnant animals of different species. Homologous hepatic ^{59}Fe ferritin was injected into pregnant guinea pigs and rats as described in 2.1. Five hours after injection, the animals were killed the organs washed and removed, and radio-activity in the various samples was counted (2.2.2). Significant differences were noted to be present between the two species. The possibility that these differences might be due to species differences in the ferritin or the placenta was considered; thus, studies were performed with rat ^{59}Fe ferritin injected into a pregnant guinea pig and guinea pig ^{59}Fe ferritin injected into a pregnant rat.

The distribution of ^{59}Fe ferritin in the pregnant rat was in many ways analogous to the non-pregnant animal in that the predominant fraction of ^{59}Fe was in the liver. However, in the guinea pig the largest fraction was in the foeto-placental unit. With this information, the time-related distribution of ^{59}Fe was studied at various time intervals after ^{59}Fe ferritin injection. The times chosen were 1/4, 1/2, 1, 2, 5 and 21 hours after ^{59}Fe ferritin injection.

The different fates of the iron and protein fractions of injected ferritin were determined by injecting $^{59}Fe^{125}I$ doubly labelled ferritin into the pregnant guinea pigs. The organ distribution as well as the urinary excretion of ^{59}Fe and ^{125}I were determined as described previously (2.2.2). Intraplacental distributions of ^{59}Fe and ^{125}I were determined on placental cellular lysates separated by column chromatography (2.4) and radio-activity counted in the customary fashion (2.2.2).

2.4 ANALYSIS OF CELLULAR LYSATES

The nature of intracellular iron in the placentae was determined on cellular lysates. At the appropriate time intervals after intravenous injection of the radio-labelled ferritin, the organs were removed, weighed, sliced, washed in normal saline, suspended in four times their weight of column buffer (see below) with 100 ug/ml soy bean trypsin inhibitor (Boehringer Mannheim GmbH, Mannheim, West Germany) (to prevent proteolysis by proteases released during cell breakdown), homogenised in an Ultra-Turrax to yield a near-single cell suspension and centrifuged at 1 000 g for 10 minutes to remove tissue debris. The supernatant was subjected to 3 x 15 second bursts of ultrasonication (Ultrasonicator, MSE Measuring and Scientific Equipment, London, Eng-

land) followed by ultracentrifugation at 100 000 g for 30 minutes to remove membrane fractions. The supernatent lysate was applied to an AcA 44 Ultrogel (LKB-Produkter AB, Bromma, Sweden) sizing chromatography column eluted with 0.05 M NaCl 0.02 M HEPES buffer (Pollack et al, 1985). The column had previously been calibrated with ^{59}Fe ferritin and ^{59}Fe transferrin. Fractions eluted from the column were collected, the radio-activity was counted (2.2.2) and the fractions identified graphically by comparison with the calibration peaks. In separate experiments, similarly prepared lysates of placenta and foetal liver were analysed 8 hours after the injection of ^{59}Fe ferritin. Here, the column was prewashed with 12 ml 1% Triton X-100 (Sigma Chemical Company, St Louis, Missouri, USA) and 12 ml 0.1 M EDTA to bind any chelateable iron (Pollack et al, 1985).

2.5 EFFECTS OF VARIOUS INJECTED AGENTS ON ^{59}FE FERRITIN UPTAKE AND ^{59}FE DISTRIBUTION IN PREGNANT GUINEA PIGS

2.5.1 Agents Affecting ^{59}Fe Ferritin Uptake

In order to elucidate the nature of the placenta-ferritin interaction, the effects of various compounds injected intravenously into guinea pigs was also studied. The mechanisms whereby hepatocytes acquire iron from transferrin have been described in section 1.2.1.1. In brief, these mechanisms include (i) a high-capacity low-affinity receptor, (ii) a low-capacity high-affinity receptor, (iii) non-specific fluid phase endocytosis and (iv) a receptor for asialoglycoproteins. From this, it was postulated that similar mechanisms might operate in the uptake of injected tissue ferritin by the placenta. To evaluate the specificity of placental ^{59}Fe ferritin uptake, a 50-fold molar excess of unlabelled guinea pig ferritin was injected either (i) concomitantly

with or (ii) 90 minutes prior to ($T_{1/2}$ x 2) and concomitantly with ^{59}Fe ferritin. Subsequent to the findings of this experiment, a 300-fold excess of unlabelled guinea pig ferritin was also studied.

An attempt was made to block non-specific fluid phase endocytosis by injecting a 300-fold molar excess of bovine serum albumin (Sigma Chemical Company No A-6003, St Louis, Missouri, USA) concomitantly with ^{59}Fe ferritin. Similarly, receptors for asialoglycoproteins were blocked by the intravenous injection of a 300-fold molar excess of asialofetuin (Sigma Chemical Company No A-4781, St Louis, Missouri, USA) concomitantly with ^{59}Fe ferritin.

Another mechanism whereby substances may gain access to cells is by non-specific reticuloendothelial-like phagocytosis. There is evidence that this entry mechanism can be blocked by carbon particles (van Snick et al, 1976). Accordingly, a suspension of carbon particles (India Ink, Pelikan, Hannover, Germany) was injected 30 minutes prior to (van Snick et al, 1976) ^{59}Fe ferritin.

A mannose-specific endocytic receptor mechanism has been identified on human placental tissue (Lennartz et al, 1987). On the premise that such a receptor may exist on guinea pig placenta and be responsible for ferritin uptake, an attempt was made to block this receptor by injecting mannosylated bovine serum albumin (EY Laboratories, San Mateo, California, USA) concomitantly with ^{59}Fe ferritin.

2.5.2 Agents Affecting Intracellular Ferritin Metabolism

As discussed previously (1.2.1.1), transferrin iron delivery to cells is by receptor mediated endocytosis, formation of endocytic vesicles which then become acidified, the iron released

from transferrin and return of the iron-free transferrin-receptor complex to the cell surface where the apotransferrin is released into the plasma. The endocytic vesicle moves along a system of intracellular microtubules and microfilaments.

2.5.2.1 Microtubular and microfilament inhibitors

Previous work has shown that iron utilisation by red cell precursors can be signficantly diminished by vinca alkaloids (Morgan, 1987). The effects of three microtubular and microfilament inhibitors vincristine (Oncovin, Eli Lilly, Isando, Transvaal), vinblastine (Periblastine, Lennon Ltd, Port Elizabeth) and etoposide (VP 16, Bristol Meyers Group, Braamfontein, Johannesburg) injected intravenously 24 hours prior to ^{59}Fe ferritin injection were assessed. The dose of vincristine used (0.15 mg/kg) and the timing of vincristine injection (24 hours prior to radio-iron injection) have been shown in rat experiments to depress profoundly the uptake of ^{59}Fe by the bone marrow (unpublished data from our Laboratory) where the ^{59}Fe is known to be almost exclusively in red cell precursors (Simon et al, 1987). The doses of vinblastine (0.6 mg/kg) and etoposide (10 mg/kg) were extrapolated from the dose of vincristine used.

2.5.2.2 Mannosidase inhibition

The experimental findings that ferritin uptake by the guinea pig foeto-placenta complex could be partially inhibited by mannosylated bovine serum albumin suggested that mannose residues on the protein might be of importance in the recognition of ferritin by placental uptake mechanism. Horse spleen ferritin has 9 mannose residues (Bomford and Munro, 1980). Furthermore, uptake and subsequent intracellular processing of glycoproteins by an hepatic mannose receptor has been described in the rat (Taylor et al, 1987). It was thought possible that intracellular ferritin cata-

bolism might depend, at least in part, on metabolism of ferritin mannose residues. Lysosomal alpha-mannosidase can be inhibited by the plant alkaloid swainsonine which, when ingested chronically by animals, results in a state analogous to the genetically-determined condition of mannosidosis (Dorling et al, 1978). In studies *in vitro*, swainsonine at a concentration of 1×10^{-6} M results in a tenfold reduction in alpha-mannosidase activity (from 100% to 10%). Feeding a special diet to pregnant animals presented some practical difficulties. To overcome these, we elected to inject an aqueous preparation of swainsonine (Sigma No. S-2064, Sigma Chemical Company, St Louis, Missouri, USA) intraperitoneally into the animals twice daily. The dose used was extrapolated from *in vivo* and *in vitro* studies by Dorling and coworkers (1978; 1980) and was calculated to be 0.005 /umol/ml of the total blood volume, injected twice daily. In trial guinea pigs, the animals aborted after 7 days of swainsonine treatment. It was therefore thought reasonable to study animals after 4 days of initiating swainsonine injections.

2.5.3 Chelators and the Distribution of ^{59}Fe

^{59}Fe transferrin injected intravenously into pregnant rats is cleared rapidly from the plasma predominantly by the placenta and the iron is rapidly transferred to the foetus (Baker and Morgan, 1969; Morgan, 1982). Intramuscular injection of iron chelators into these rats significantly altered the distribution of iron. Injection of the ferric chelators did not affect iron transport to the foetus but reduced iron uptake by the liver (Wong et al, 1987). On the other hand, injection of the ferrous chelator 2,2' bipyridine significantly decreased iron transfer to the foetus and increased iron excretion in the urine (Wong et al, 1987). An interesting property of 2,2' bipyridine is that in the

iron-free state, the chelator is hydrophobic (lipophilic) and is able to insert into membranes; once it has bound ferrous iron, this chelator loses its lipophilicity and becomes hydrophilic (Nunez et al, 1983).

With this background, and in order to study the nature of ferritin-derived iron in transit to the foetus, the effects of iron chelators on the distribution of ^{59}Fe were studied. Sixteen mg/kg body weight of 2,2' bipyridine (Merck Art 3098, Darmstadt, West Germany) or desferrioxamine (Desferal 500, Ciba-Geigy, Spartan, Kempton Park, Transvaal) were injected intramuscularly 30 and 2 minutes prior to (Wong et al, 1987) and 2, 4, 6 and 8 hours after ^{59}Fe ferritin injection.

2.6 EFFECTS OF A HIGH SATURATION OF TRANSFERRIN IN VIVO

In certain experiments, a persistent high in vivo saturation of the guinea pig transferrin was achieved physiologically by instilling a suspension of carbonyl iron (Huebers et al, 1986) [400 mg/kg in acidified saline (pH 2.0)] via a metal intra-oesophageal feeding canula into animals that had been starved overnight. ^{59}Fe ferritin was injected intravenously 2 hours after administration of the carbonyl iron. The carbonyl iron (particle size 3-4 μm) was obtained from the GAF Corporation (New York, USA).

2.7 ELECTRON MICROSCOPIC TECHNIQUES

The placental binding and uptake of ferritin was studied by electron microscopy. The placenta was removed 1 hour after the intravenous injection of 10 mg of homologous ferritin. A control animal was also studied.

Tissue for transmission electron microscopy was prepared

using a standard technique (Phillips et al, 1986). The tissue was fixed in 3% glutaraldehyde and 4% formaldehyde in Millonig's phosphate buffer (Millonig, 1961). After rinsing in buffer alone, post-fixation was carried out in 1% osmium tetroxide, buffered in Millonig's buffer for 1 hour. Following further rinsing in buffer, the tissue was dehydrated in a graded series of ethanols prior to infiltration with Spurr resin (Spurr, 1969). Polymerisation took place overnight at 70^{o}C. Thin sections (60 nm) were stained with alcohol, and saturated with alcoholic, saturated uranyl acetate and alkaline lead citrate (pH 13.5). Sections mounted on copper grids were examined in a Hitachi H600 transmission electron microscope.

2.8 STATISTICAL INTERPRETATION

Comparisons between groups of animals were by unpaired Student's t tests (SAS, 1985a,b). The differences were deemed to be significant if P was < 0.05.

2.9 ETHICAL CONSIDERATIONS

The studies had been approved by the Animal Ethics Committee of the University of the Witwatersrand Medical School. The Animal Ethics Protocol Numbers were 88/75/4 (updated from 86/89) for preparation of the raw materials and 88/186/4 for the pregnant animals.

2.10 SUMMARY

In this chapter, details are given of the animals, radiolabelled probes and laboratory techniques used to generate the data which form the basis of this dissertation.

Chapter Three

RESULTS

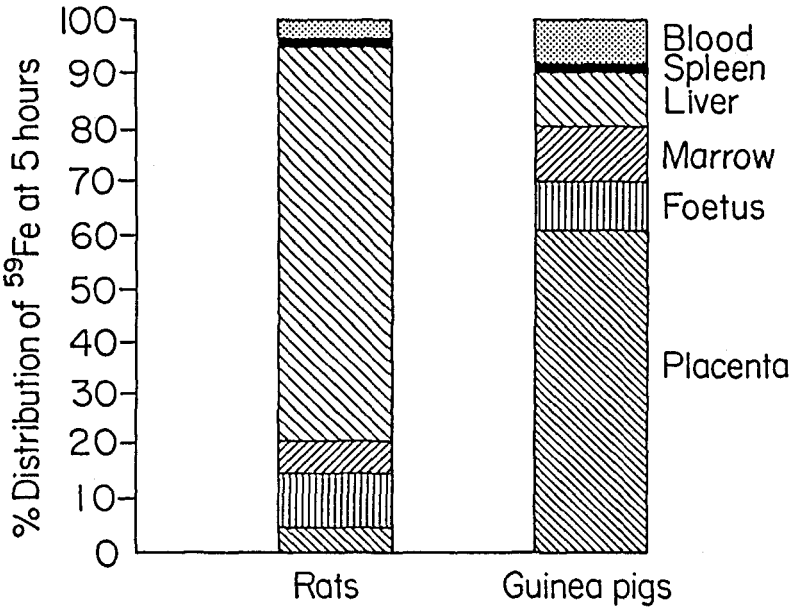


Figure 1 The organ distribution of ^{59}Fe 5 hours after the intravenous injection of homologous ^{59}Fe ferritin into pregnant rats and pregnant guinea pigs.

TABLE I (a) The mean (± SD) organ distribution of ^{59}Fe 5 hours after injection of homologous hepatic ^{59}Fe ferritin into pregnant rats and pregnant guinea pigs.

	Rats	Guinea pigs
	(%)	(%)
Blood	6.8 ± 2.8	7.8 ± 5.1
Liver	72.7 ± 7.4	9.4 ± 2.7*
Spleen	0.6 ± 0.1	0.9 ± 0.4
Marrow	5.6 ± 1.0	9.5 ± 4.5
Placenta	10.9 ± 1.2	60.8 ± 8.0*
Foetus	4.2 ± 1.1	11.2 ± 5.6
Foetus + Placenta	15.1 ± 1.5	72.0 ± 9.3*

* $P < 0.01$ when compared with pregnant rats

TABLE I (b) The organ distribution of ^{59}Fe 5 hours after injection of rat hepatic ^{59}Fe ferritin into a pregnant guinea pig and guinea pig hepatic ^{59}Fe ferritin into a pregnant rat.

	Rats	Guinea pigs
	(%)	(%)
Blood	0.3	11.8
Liver	83.0	17.2
Spleen	1.0	0.9
Marrow	4.3	13.7
Placenta	10.7	41.5
Foetus	0.5	14.7
Foetus + Placenta	11.2	56.2

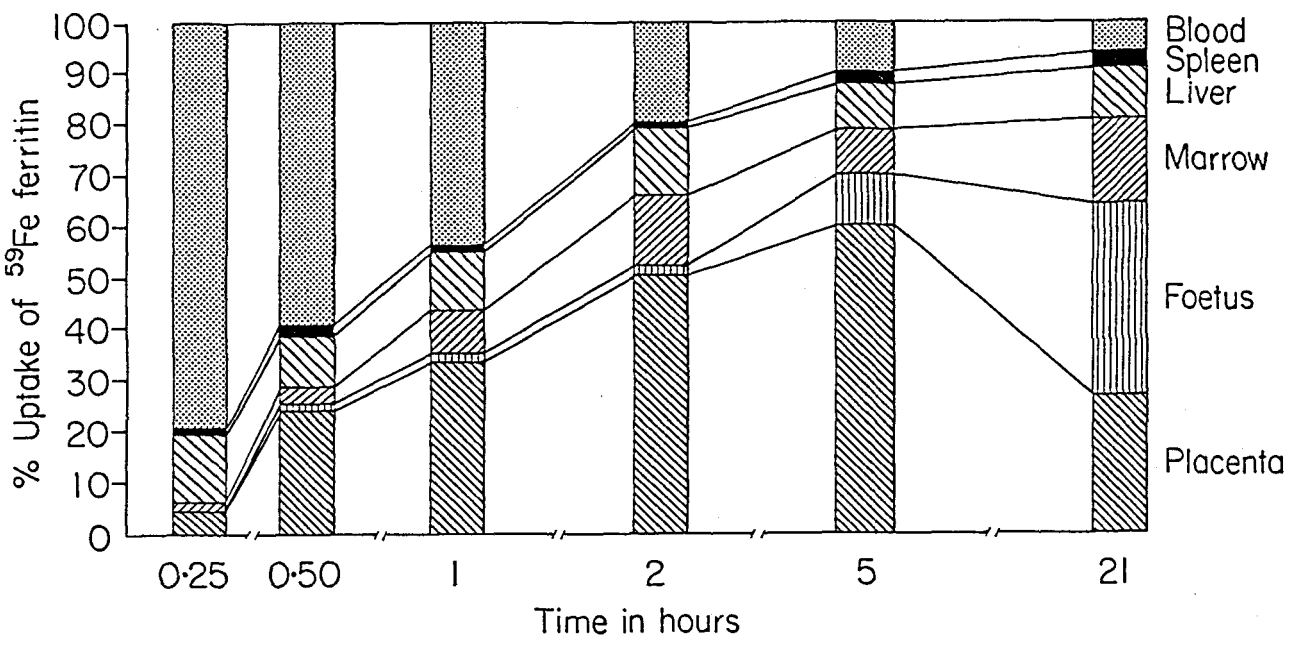


Figure 2 The organ distribution of ^{59}Fe at various times after the intravenous injection of ^{59}Fe tissue ferritin into pregnant guinea pigs.

TABLE II The mean (± SD) percentage organ distributions of ^{59}Fe at progressive time intervals after intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs.

	1 hour (%)	2 hours (%)	5 hours (%)	21 hours (%)
Blood	41.4 ± 5.6	17.6 ± 5.3	7.8 ± 5.1	2.2 ± 1.6
Urine	0	0	0.06 ± 0.05	0.04 ± 0.01
Bile	0	0	0.03 ± 0.01	0.01 ± 0.05
Liver	13.4 ± 4.1	13.5 ± 3.9	9.4 ± 2.7	12.3 ± 3.9
Spleen	0.5 ± 0.1	1.2 ± 0.1	0.9 ± 0.4	1.1 ± 0.7
Marrow	10.3 ± 4.7	13.7 ± 5.7	9.5 ± 4.5	22.1 ± 4.4
Placenta	33.4 ±11.2	51.2 ± 4.7	60.8 ± 8.0	23.7 ±11.1
Foetus	2.0 ± 0.7	3.2 ± 3.0	11.2 ± 5.6	38.6 ± 7.0
Foetus + Placenta	35.4 ±11.7	54.4 ± 5.7	72.0 ± 9.3	62.3 ±15.1

3.2% at 2 hours, 11.2% at 5 hours and 38.6% at 21 hours.

The time-related changes in the distribution of ^{59}Fe and ^{125}I are shown in Table III. Both ^{59}Fe and ^{125}I accumulated progressively up to 5 hours. Thereafter ^{59}Fe levelled off, while ^{125}I was lost from the placenta. The catabolism of ferritin was reflected by a rise in the placental Fe/I ratio from 1.06:1 at 1 hour to 3.0:1 at 21 hours. Virtually no ^{125}I entered the foetus and by 21 hours almost all of it had been passed in the maternal urine.

3.1.3 Effect of Time on the Intracellular Distribution of ^{59}Fe and ^{125}I after $^{59}Fe^{125}I$ Ferritin Injection into Pregnant Guinea Pigs

Analysis of the placental cellular lysates (Figure 3) revealed that at 2 hours the iron was still present intracellularly as injected $^{59}Fe^{125}I$ ferritin. By 5 hours, the pattern was similar but a second ^{59}Fe peak (identified by absorption spectrophotometry as ^{59}Fe haemoglobin) was also present. This was presumably present in contaminating maternal red cells. After 21 hours, none of the injected $^{59}Fe^{125}I$ ferritin remained in the placenta; some ^{59}Fe was found in ferritin representing ^{59}Fe in pre-existing or in newly-synthesised placental ferritin. A small amount of ^{59}Fe was present as transferrin iron. A plausible explanation for this is that the $^{59}Fe^{125}I$ ferritin may have been taken up by the maternal liver and the ^{59}Fe delivered to the placenta by endogenous transferrin. An alternative may have been that this was, in fact, foetal transferrin which had accepted ^{59}Fe already transported across the placenta. Neither of these theories was, however, pursued. Another small amount of ^{59}Fe was present in haemoglobin as described above. At all times, some ^{125}I activity was associated with a low molecular weight compound, in keeping

TABLE III The mean percentage distributions (a) and the ratios of ^{59}Fe to ^{125}I (b) in the organs at progressive time intervals after ^{59}Fe ^{125}I doubly labelled ferritin injection into pregnant guinea pigs.

(a)

Time	Urine	Placenta		Foetus		Marrow		Liver		Spleen		Blood	
(hours)	^{125}I	^{59}Fe	^{125}I	^{59}Fe	^{125}I	^{59}Fe	^{125}I	^{59}Fe	^{125}I	^{59}Fe	^{125}I	^{59}Fe	^{125}I
1	2.0	33.4	32.3	2.0	0.06	10.3	10.0	13.4	15.6	0.5	0.38	41.4	44.1
2	13.1	51.2	36.4	3.2	0.1	13.7	10.9	13.5	20.1	1.2	0.79	17.6	33.2
5	28.3	60.8	37.6	11.2	1.8	9.5	7.7	9.4	6.3	0.9	0.50	7.8	18.0
21	78.6	23.7	8.3	38.6	4.9	22.1	1.5	12.3	3.4	1.1	0.30	2.2	3.1

(b)

1	1:0.97	1:0.03	1:0.97	1:1.20	1:0.95	1:1.06
2	1:0.71	1:0.03	1:0.71	1:1.54	1:0.66	1:1.87
5	1:0.62	1:0.16	1:0.81	1:0.70	1:0.45	1:2.31
21	1:0.35	1:0.13	1:0.07	1:0.28	1:0.25	1:1.41

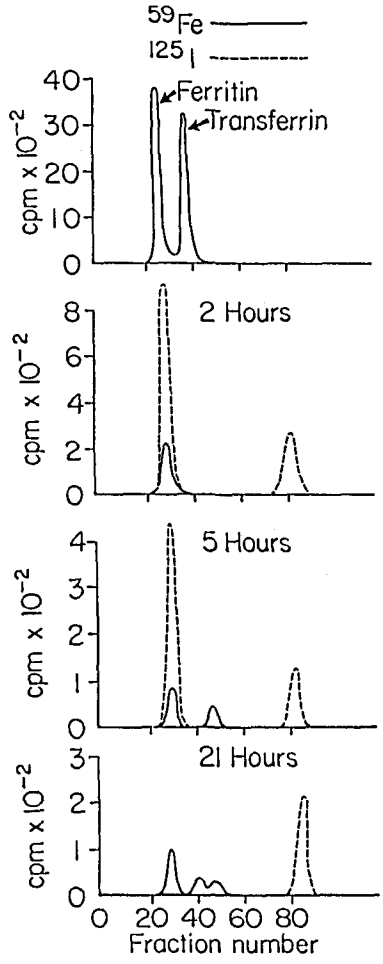


Figure 3 AcA-44 ultrogel chromatographic analysis of placental cellular lysates 2, 5 and 21 hours after the intravenous injection of tissue ferritin labelled with ^{59}Fe and ^{125}I. The solid lines represent ^{59}Fe and the broken lines ^{125}I. A column calibration with ferritin and transferrin is shown in the top panel.

with $^{59}Fe^{125}I$ ferritin being catabolised and ^{125}I excreted from the placenta bound to short polypeptides.

In a separate experiment, lysates of the placenta and foetal liver were analysed 8 hours after the injection of ^{59}Fe ferritin. Placental ^{59}Fe was again found to be present in ferritin (80%), transferrin (7.8%) and haemoglobin (1.3%) but a small amount (5.3%) was also present in a low molecular weight chelateable pool. Similarly, ^{59}Fe transferred to the foetal liver was in ferritin (35.3%), haemoglobin (30.4%), transferrin (2%) and a low molecular weight pool (10.6%).

3.1.4 Effects of Various Injected Substances on Intravenously Injected ^{59}Fe Ferritin Uptake and ^{59}Fe Distribution in Pregnant Guinea Pigs

3.1.4.1 Effects of ferritin, albumin, asialofetuin or carbon particles on ^{59}Fe ferritin uptake

The effects of a number of concomitantly injected substances on the mean (± SD) uptake and distribution of ^{59}Fe 5 hours after injection of ^{59}Fe ferritin are shown in Table IV. Significant reductions in foetoplacental ^{59}Fe were noted after 50-fold and 300-fold injections of unlabelled ferritin ($P < 0.05$; $T = 3.52$ and $T = 5.54$ respectively), with correspondingly more ^{59}Fe still being present in the blood ($P < 0.05$; $T = 3.55$ and $T = 3.89$ respectively). Injection of albumin, asialofetuin and carbon particles had no significant effect on ^{59}Fe distribution. Although mannosylated bovine serum albumin reduced foetoplacental ^{59}Fe, the reduction did not reach statistical significance.

3.1.4.2 Effects of microtubular and microfilament inhibitors

In a pilot study on single animals, inhibition of placental microtubular function by vincristine, vinblastine and etoposide had no effect on ^{59}Fe ferritin uptake or ^{59}Fe transfer to the

TABLE IV The effects of various substances injected intravenously on the mean (± SD) percentage distribution of ^{59}Fe 5 hours after the intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs. (Time 0 = Time of ^{59}Fe ferritin injection).

	Control	Ferritin (50x)	Ferritin (50x)	Ferritin (300x)	BSA (300x)	Mannosylated BSA (300x)	Asialofetuin (300x)	Carbon
Time of injection (minutes)		-90 + 0	0	0	0	0	0	-30
Blood	7.8 ± 5.1	32.8	27.8 ± 7.2*	29.2 ± 7.8*	8.2 ± 4.5	20.6 ± 2.5*	4.6 ± 0.1	4.9
Urine	0.06 ± 0.05	0	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.03	0.03 ± 0.01	0.01
Bile	0.03 ± 0.01	0	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.01
Liver	9.4 ± 2.7	7.9	16.9 ± 11.5	34.6 ± 4.4*	19.9 ± 2.7	16.9 ± 9.1	9.5 ± 4.4	11.6
Spleen	0.9 ± 0.4	0.5	0.54 ± 0.03	1.3 ± 0.8	1.1 ± 0.7	1.4 ± 0.3	0.33 ± 0.01	0.7
Marrow	9.5 ± 4.5	5.8	6.1 ± 0.4	7.1 ± 2.5	9.1 ± 3.9	7.8 ± 3.3	7.1 ± 0.1	7.3
Placenta	60.8 ± 8.0	36.5	38.8 ± 3.3*	22.4 ± 5.6*	49.1 ± 3.8	47.8 ± 7.6	63.4 ± 0.8	62.7
Foetus	11.2 ± 5.6	16.1	9.7 ± 3.0	5.1 ± 3.6*	12.5 ± 5.8	5.4 ± 0.8	13.2 ± 7.6	12.7
Foetus + Placenta	72.0 ± 9.3	52.6	48.5 ± 5.9*	27.5 ± 9.1*	61.6 ± 7.5	53.1 ± 7.7	76.6 ± 6.8	75.4

* $P < 0.05$ when compared with control animals

foetus. A marked reduction in marrow ^{59}Fe uptake was noted with all 3 agents (Table V).

3.1.4.3 Effects of a mannosidase inhibitor

Further insight into the possible role of mannose receptors and residues containing mannose in the uptake and utilisation of ferritin by the foetoplacental complex was obtained by studying the effect of 4 days of pretreatment with the mannosidase inhibitor, swainsonine, on the organ distribution of ^{59}Fe (Table VI). While it had no effect on foetoplacental uptake, there was a significant inhibition of erythroid marrow uptake from a mean (± SD) control value of 22.1% (± 4.4) to 10.4% (± 0.9) ($P < 0.05$; $T = 4.51$).

3.1.5 Effects of Chelators on ^{59}Fe Distribution

The effects of ferrous (2,2' bipyridine) and ferric (desferrioxamine) chelators on the distribution of ^{59}Fe 21 hours after ^{59}Fe ferritin injection are shown in Table VII. In animals injected with 2,2' bipyridine, the mean (± SD) uptake of ^{59}Fe by the foetoplacental complex was reduced significantly (30.6 ± 15.2%) when compared with controls (62.3 ± 15.1%) ($P < 0.05$; $T = 3.33$), with the major reduction being in transfer to the foetus. There was a corresponding rise in ^{59}Fe activity in the maternal liver, bone marrow and urine. In contrast, the injection of desferrioxamine had no significant effect on the distribution of ^{59}Fe.

3.1.6 Effects of a High Saturation of Transferrin

Presaturation of the guinea pig transferrin *in vivo* by the prior feeding of carbonyl iron (mean transferrin saturation 84%) resulted in significantly increased retention of ^{59}Fe by the foeto-placental complex (83.2 ± 0.9% vs 62.3 ± 15.2%; $P < 0.05$; $T = 3.54$) and decreased delivery to the marrow (5.3 ± 0.4% vs 22.1 ± 4.4%; $P < 0.05$; $T = 6.59$) at 21 hours after the injection of

TABLE V The effects of 3 microtubular inhibitors [vincristine (0.15 mg/kg), vinblastine (0.6 mg/kg) or etoposide (10 mg/kg)] injected intravenously 24 hours prior to ^{59}Fe ferritin injection on the distribution of ^{59}Fe at 21 hours after intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs.

	Control	Vincristine	Vinblastine	Etoposide
Blood	2.2 ± 1.6	2.0	1.7	4.9
Urine	0.06 ± 0.05	0.05	0.04	0.05
Bile	0.03 ± 0.01	0.01	0.02	0.07
Liver	12.3 ± 3.9	13.0	12.0	14.8
Spleen	1.1 ± 0.7	1.8	0.6	1.0
Marrow	22.1 ± 4.4	5.9	4.3	3.3
Placenta	23.7 ± 11.1	36.4	38.5	30.2
Foetus	38.6 ± 7.0	40.7	42.7	45.7
Foetus + Placenta	62.3 ±15.1	77.1	81.2	75.9

TABLE VI The effects of 4 days of pretreatment with a mannosidase inhibitor [swainsonine (0.005 μmol/ml blood volume injected intraperitoneally 12 hourly) on the mean (± SD) percentage distribution of ^{59}Fe 21 hours after intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs.

	Control	Swainsonine-treated
Blood	2.2 ± 1.6	2.9 ± 0.8
Urine	0.06 ± 0.05	0.05 ± 0.01
Bile	0.03 ± 0.01	0.01 ± 0.01
Liver	12.3 ± 3.9	17.4 ± 2.0
Spleen	1.1 ± 0.7	1.4 ± 1.2
Marrow	22.1 ± 4.4	10.4 ± 0.9*
Placenta	23.7 ±11.1	33.8 ± 7.8
Foetus	38.6 ± 7.0	34.5 ± 7.2
Foetus + Placenta	62.3 ±15.1	68.3 ±12.5

* $P < 0.05$ when compared with control animals

TABLE VII The effects of the chelators bipyridine or desferrioxamine (16 mg/kg) injected intramuscularly 30 and 2 minutes prior to and 2, 4, 6 and 8 hours after intravenous injection of ^{59}Fe ferritin, on the distribution of ^{59}Fe 21 hours after the intravenous injection of ^{59}Fe ferritin into pregnant guinea pigs.

	Control	Bipyridine-treated	Desferrioxamine-treated
Blood	2.2 ± 1.6	6.8 ± 2.4	4.3 ± 2.5
Urine	0.04 ± 0.05	4.6 ± 1.7*	0.05 ± 0.01
Bile	0.01 ± 0.01	0.06 ± 0.02	0.03 ± 0.01
Liver	12.3 ± 3.9	36.1 ±15.6*	8.0 ± 1.1
Spleen	1.1 ± 0.7	1.7 ± 0.5	1.2 ± 0.3
Marrow	22.1 ± 4.4	19.7 ± 4.2	8.5 ± 1.8
Placenta	23.7 ±11.1	23.7 ± 9.1	31.0 ±10.2
Foetus	38.6 ± 7.0	6.9 ± 4.3*	41.4 ± 6.5
Foetus + Placenta	62.3 ±15.2	30.6 ±15.2*	72.4 ±14.1

* $P < 0.05$ when compared with control animals

^{59}Fe ferritin (Table VIII).

3.2 ELECTRON MICROSCOPIC STUDIES

Electron microscopic findings of guinea pig placenta after the intravenous injection of homologous tissue ferritin suggested that it had been taken up by a process of endocytosis (Figure 4). An invaginating endocytic vesicle can be seen to be forming and electron-dense ferritin is present on the surface of the trophoblastic cell membrane and within intracellular vesicles (arrowed).

3.3 SUMMARY

In this Chapter, the results of a series of experiments involving the injection of radio-labelled ferritin into pregnant animals are presented. These included the effects of time on the distribution of the radio-labelled probe or of ^{59}Fe and ^{125}I alone, the effects of a variety of injected agents which affected cellular uptake and intracellular metabolism of the probe, the effects of presaturation of endogenous transferrin on ferritin uptake, and the redox state of the iron as determined by the injection of ferrous or ferric chelators. Finally, electron micrographs are presented showing uptake of ferritin by placental cells via endocytic vesicles.

TABLE VIII The effects of a high saturation of transferrin *in vivo* on the distribution of ^{59}Fe 21 hours after ^{59}Fe ferritin injection into pregnant guinea pigs.

	Control	High Transferrin Saturation
Blood	2.2 ± 1.6	1.9 ± 0.8
Urine	0.04 ± 0.05	0.02 ± 0.01
Bile	0.01 ± 0.01	0.02 ± 0.01
Liver	12.3 ± 3.9	8.9 ± 1.6
Spleen	1.1 ± 0.7	0.6 ± 0.1
Marrow	22.1 ± 4.4	5.3 ± 0.4*
Placenta	23.7 ±11.1	33.9 ± 7.0
Foetus	38.6 ± 7.0	49.3 ± 4.5
Foetus + Placenta	62.3 ±15.2	83.2 ± 0.9*

* $P < 0.05$ when compared with control animals

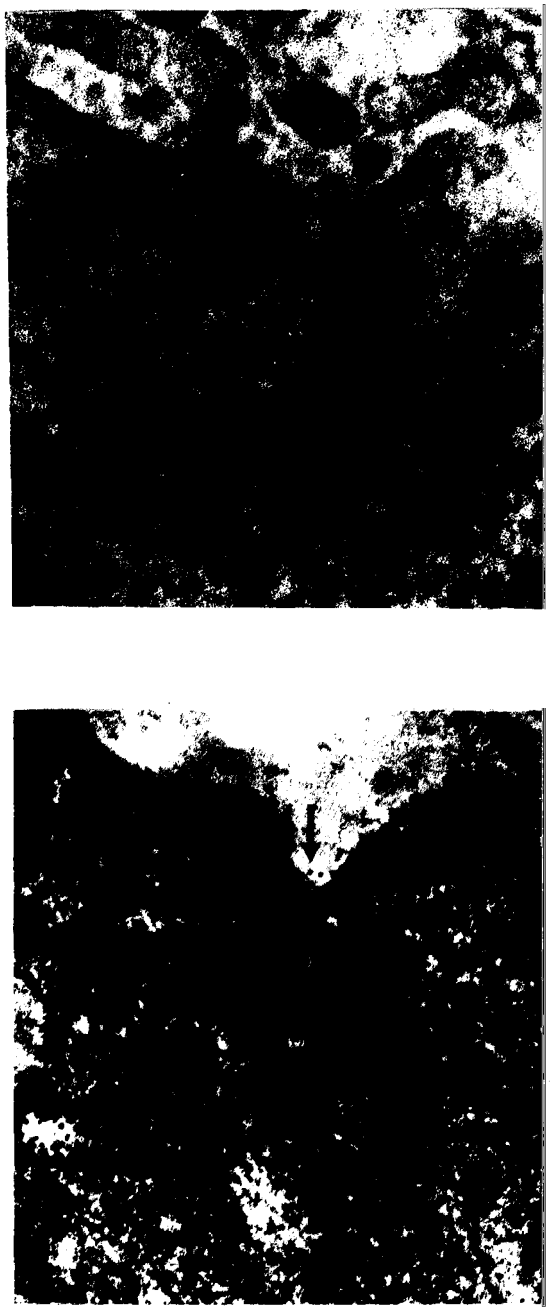


Figure 4 Thin section views of guinea pig placenta (x 40 000) showing control tissue (top) and tissue removed 1 hour after intravenous injection of 10 mg homologous tissue ferritin (bottom). Clustering and concentration of ferritin molecules in a coated pit on the cell membrane and in two large endocytic vesicles are arrowed.

Chapter Four

DISCUSSION

4.1 DISCUSSION

Iron is usually delivered to its target tissues by the circulating plasma protein transferrin (Aisen, 1980; Morgan, 1981) but is stored in the intracellular iron storage protein ferritin, which is found in virtually all animal and plant cells (Harrison et al, 1980). Small amounts of ferritin are normally secreted into the circulation in proportion to body iron stores (Walters et al, 1973).

Plasma ferritin differs from tissue ferritin both in its lower iron content (Worwood et al, 1976; Zuyderhout et al, 1978) and higher degree of glycosylation (Worwood et al, 1979; Cragg et al, 1981a,b). Large amounts of tissue ferritin only seem to enter the circulation when there is major organ damage, especially involving the liver. In most mammals, this ferritin is rapidly removed from the plasma by the liver (Lipschitz et al, 1971; Halliday et al, 1979) which is the major storage organ for iron. Glycosylated plasma ferritin has a much longer half life than tissue ferritin but it, too, is removed by the liver (Worwood et al, 1982).

Guinea pigs differ from rats and humans in that their red cell precursors are able to bind tissue ferritin (Pollack and Campana, 1981) and utilise its iron for haemoglobin synthesis (Blight and Morgan, 1983; 1987a,b). *In vivo* this has been shown to be a major pathway and in animals with stimulated erythropoiesis approximately 60% of injected tissue ferritin is removed by red cell precursors (Simon et al, 1987). The present findings indicate that during pregnancy the placenta, which is able to take up over 70% of injected tissue ferritin, is even more efficient than the erythroid marrow in removing tissue ferritin. The nature of the interaction between the placenta and tissue ferri-

tin is unclear; however, certain characteristics were defined. Uptake of ^{59}Fe ferritin by the placenta was inhibited by progressively larger excesses of unlabelled ferritin but not by albumin which suggests that the placenta has a specific though limited affinity for tissue ferritin. Furthermore, the interaction was not inhibited by blockade of the receptors for desialated proteins or of reticuloendothelial-like phagocytosis. Mannosylated bovine serum albumin appreciably reduced the placental uptake of ^{59}Fe ferritin (53.1 $\pm$ 7.7% vs 72.0 $\pm$ 9.3%; $P > 0.05$; $T = 2.71$) which suggested that mannose residues on ferritin may be involved in the uptake mechanism. In this regard, it is known that horse spleen ferritin has 9 mannose residues (Bomford and Munro, 1980), that a mannose receptor is involved in the uptake and processing of glycoproteins (Taylor et al, 1987) and that human placental tissue also possesses specific mannose receptors (Lennartz et al, 1987). However, mannosidase does not seem to be involved in the transfer of tissue ferritin iron to the foetus, since the mannosidase inhibitor, swainsonine, had no effect. In passing, it was noted that in swainsonine-treated animals, there was a significant reduction in ^{59}Fe in the marrow [known to be predominantly in haem (Simon et al, 1987)] and, hence, that intravesicular metabolism of mannose residues may be involved in ferritin catabolism and iron utilisation by guinea pig red cell precursors.

In the guinea pig, the clearance of tissue ferritin from the plasma in the present study and in a previous one (Simon et al, 1987) was found to be much slower than in the rat (Halliday et al, 1979). This may indicate that the guinea pig hepatocyte lacks the high-capacity ferritin receptors found previously in the rat (Mack et al, 1983, 1985). When placental uptake of ^{59}Fe ferritin was inhibited in different ways, there was a concomitant increase

in hepatic uptake. This suggests that the mechanisms of uptake by the two organs are different.

Experiments using ferritin which had been doubly labelled with ^{59}Fe and ^{125}I showed that it remained intact in placental cells for 2 to 5 hours. Thereafter there was breakdown of the protein with progressive loss of ^{125}I in the urine. Over the same time period, ^{59}Fe was slowly transferred to the foetus. A similar slow utilisation of tissue ferritin iron for haem formation by red cell precursors was noted in vivo in previous studies (Simon et al, 1987). In both instances, it seems to reflect the slow degradation of the protein prior to utilisation of the iron.

Since it was possible that iron released from ferritin catabolism might be released into the plasma, and then transported via transferrin to the foetus by the previously described normal pathway (Wong and Morgan, 1973), a further experiment was done. The endogenous guinea pig transferrin was saturated by feeding the animal a suspension of carbonyl iron prior to ^{59}Fe ferritin injection. In this setting, more ^{59}Fe was retained in the foeto-placental complex and iron delivery to the marrow was reduced. However, there was no change in foetal ^{59}Fe delivery.

The handling of ferritin differs markedly from what happens when transferrin iron is taken up by cells. In such circumstances, the carrier protein is rapidly returned to the exterior after donating its iron (Dautry-Varsat et al, 1983; Harding et al, 1983). The separate pathways of the two proteins were well demonstrated by Blight and Morgan (1987a,b) in guinea pig red cell precursors. While they were endocytosed together, their cellular transport differed, with many multivesicular endosomes containing ferritin but not transferrin. On electron microscopic studies, the placental uptake of ferritin also appeared to be via

an endocytic process (Figure 4).

The effects of iron chelators gave some insight into how iron is transported across the placenta and how iron is liberated from ferritin. While the ferric iron chelator desferrioxamine had no significant effect on the distribution of ^{59}Fe, the ferrous chelator 2,2' bipyridine inhibited ^{59}Fe transfer to the foetus and, as a result, there was more ^{59}Fe present in the maternal liver, bile and urine. These results are similar to those of Wong and coworkers (1987) who studied the effects of 2,2' bipyridine on the transport of transferrin iron to the foetus. Previous data suggest that this lipophilic ferrous chelator inserts into the membrane of the endocytic vesicle and is able to complex iron from transferrin once it has been reduced. The resulting iron-bipyridine complex is not lipophilic and therefore cannot traverse the unit membrane. Instead it leaves the cell within the recycling vesicle (Nunez et al, 1983; Morgan, 1983; Baynes et al, 1988a,b). The current data suggest that the chelator complexes ferrous iron released by reductive moblisation from ferritin (Funk et al, 1985) or already present in the ferritin molecule (Rohrer et al, 1987). If the situation is analogous to what happens with transferrin iron, then ferritin entering the placental cell must enter an endocytic vesicle which recycles, in keeping with previous findings using guinea pig reticulocytes (Blight and Morgan, 1987a,b). Ferritin uptake by guinea pig reticulocytes has also been shown to involve endosomal acidification (Blight, 1988). These various findings support previous in vitro evidence that acidification and reduction are necessary for the release of iron from the ferritin molecule (Harrison et al, 1980; Funk et al, 1985). Chromatographic analysis demonstrated that the iron transferred to the foetus was biologically

available: in the foetal liver, ^{59}Fe was found to be present in ferritin, transferrin, haem and a low molecular weight chelateable pool.

One unexplained finding in the present study was the failure of microtubular inhibiting agents [previously documented to inhibit transferrin endocytosis (Morgan and Iacopetta, 1987)] to inhibit placental uptake of tissue ferritin and ^{59}Fe transfer to the foetus. The lack of effect was not due to inadequate dosages since marrow ^{59}Fe uptake was markedly depressed (Table V).

The rapid hepatic clearance in most mammals of ferritin released by tissue damage probably represents a means of preventing loss of large quantities of iron from the body. In this regard, Siegenberg and co-workers have demonstrated in haemolytic rats, that a significant amount of iron can be being carried by ferritin in the plasma when the reticuloendothelial cell is loaded with iron and the circulating transferrin is already highly saturated (Siegenberg et al, 1989). In the guinea pig, the bone marrow appears to have taken on the role of the hepatocyte by being able to clear this tissue ferritin from the plasma and to incorporate its iron directly into haem, albeit at a rate which is slower than that for transferrin iron. In pregnancy, the guinea pig placenta has an even greater avidity for the tissue ferritin than the erythroid marrow; this may represent its greater need for iron. Of note is the fact that, on average, the iron content of a ferritin molecule is some three orders of magnitude greater than that of transferrin, and therefore is capable of delivering far more iron on a mole-for-mole basis than transferrin. This mechanism of iron delivery appears to be unique in the guinea pig and should be investigated further, including a search for it in mammals related to the guinea pig and possibly, in man.

5.2 SUMMARY

In the final Chapter, the findings of the experiments presented in the dissertation are discussed. Like erythroid precursors, the placenta is a major receptor for circulating tissue ferritin in the guinea pig. The uptake process is a specific one which involves the subsequent transfer of iron to the foetus after catabolism of the protein. Finally, its role in iron delivery is speculated upon.

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