FERRITIN IN MOTHER AND BABY

BY

ANNE MACLAREN KELLY, B.Sc., M.C.B.

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE FACULTY OF MEDICINE OF

THE UNIVERSITY OF GLASGOW

NOVEMBER, 1978

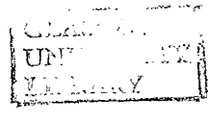


Table of Contents

Page

Chapter One(cont)

Page

Chapter Three (cont)

Page

Chapter Four(cont)

Page

Chapter Five(cont)

Page

Chapter Five (cont)

List of Plates

List of Figures

List of Tables

Table (Cont) Following Page

Table (Cont) Following Page

ACKNOWLEDGEMENTS

I wish to thank Professor M.C.Macnaughton for supervising this work and for helpful advice on the preparation of the manuscript. Dr.A.D.T.Govan has for the past seven years been a kindly mentor and without his advice and support this thesis could not have been undertaken. I acknowledge my great debt to him. The potential of a ferritin assay was first revealed by Dr.J.M.Scott and her encouragement and help in the collection of organs was most welcome. My thanks are also due to Dr.V.Madoc-Jones, Consultant Haematologist to Renfrew District who interpreted the bone marrow smears and photographed the appropriate slides and to Dr.J.C.Mclaurin and the Consultant Obstetricians of GRMH for access to clinical details. Dr.G.S.Fell, Royal Infirmary, Glasgow, made helpful critism of part of this manuscript. Dr.M.B.McNay and Dr.A.N.McDougall collated the clinical details for the normal pregnancy and cord studies. I also wish to thank the Biochemical Staff of GRMH particularly Dr.P.England, Mr.D.J.Macdonald, Mr.J.Muirhead, Mr.J.C.Ferguson and Mrs.D.Wilson for help and many acts of kindness. I am indebted to Mr.A.Robertson for photography and to Mr.M.MacKay who prepared the electron micrographs. Mrs.S.Lamb, GRMH and Mrs.J.Sweeney, Hawkhead Hospital, Paisley typed the manuscript and for their skill and patience I am most grateful. Mr.J.McCulloch kindly undertook the 2:3-DPG assays.

Summary

Reagents for a 2-site radioimmunoassay of ferritin have been prepared and the experimental conditions of the assay determined. Gestational changes in the ferritin concentration of maternal and fetal plasma, fetal organs and the placenta were found. No previous studies have shown plasma ferritin to decrease with advancing gestation in normal pregnant subjects maintained onprophylactic iron and that 30 per cent of mothers have depleted iron stores at term (Kelly et al, 1977). Patients with infections, intrauterine growth retardation and pre-eclamptic toxaemia had elevated ferritin concentrations and in the latter the severity of the condition was proportional to the increase in plasma ferritin.

Cord bloods had higher ferritin concentrations than maternal bloods and increased within 24 hours of delivery. In premature neonates the increase in ferritin continued over a period of seven days. The neonatal plasma ferritin was related to gestation, birth weight and the bilirubin concentration. There was no direct correlation between maternal and cord ferritin in the circulation but when the ferritin concentration of the mother signified that maternal iron stores were depleted, there was a significant reduction in the cord ferritin concentration (Kelly et al, 1978).

The total content and concentration of ferritin in liver, heart and spleen from 24 to 41 gestational weeks was determined and liver was found to contain the greatest amount of ferritin. The ferritin content of the three organs related to body weight and gestation.

The growth retarded livers had low concentrations and total contents of ferritin but only the latter occurred in spleens which were small for gestational age. The ferritin concentration of the liver and spleens of twins appeared to be lower than in singletons of equivalent gestation.

In the placenta the ferritin concentration increased until the eighteenth week before decreasing until 37 weeks when a second increase occurred at term. No difference in ferritin concentration was found between the peripheral and central areas but a concentration gradient was present between the maternal and fetal surface.

Electron micrographs of term placentae showed that ferritin was localised on the rough endoplasmic reticulum of the syncytiotrophoblast. The concentration of ferritin in term placentae associated with low maternal human placental lactogen concentrations or urinary oestriol excretions and in small term placentae was not significantly different from control organs but the total ferritin content of the small placentae was reduced (Kelly, 1978). There is evidence to suggest that the placenta can effectively prevent the deposition of excess storage iron in fetal organs.

Abbreviations

Ab-F-ImAd	Antibody-ferritin-immunoadsorbent
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
BDH	British Drug Houses
BSA	Bovine serum albumin
CHD	Congenital Heart Disease
CV	Coefficient of variation
d	Difference between duplicates
DAB	Diaminobenzidene tetrahydrochloride
DMS	Dimethylsulphoxide
1,3-DPG	1,3-Diphosphoglycerate
2,3-DPG	2,3-Diphosphoglycerate
EDTA	Ethylene-diamine-tetraacetic acid
F-ImAd	Ferritin immunoadsorbent
GAPD	Glyceraldehyde-3 phosphate dehydrogenase (1.2.1.12)
G-3-P	Glyceraldehyde-3-phosphate
Hb	Haemoglobin
IVH	Intraventricular haemorrhage
HCG	Human chorionic gonadotrophin
HCL	Hydrochloric acid
HMD	Hyaline membrane disease
HPL	Human placental lactogen
IRMA	Immunoradiometric assay
IU	International unit
IUGR	Intrauterine growth retardation
LFD	Light-for-dates

Mat	Maternal
m-RNA	Messenger ribonucleic acid
M.Wt	Molecular weight
N	Number
NAD	Nicotinamide adenine-dinucleotide
NADH	Nicotinamide adenine-dinucleotide-reduced
NRS	Neutralised rabbit serum
p	Probability
P	Inorganic phosphate
PBS	Phosphate buffered saline
PET	Pre-eclamptic toxaemia
3-PGA	3-Phosphoglycerate
PGK	3-Phosphoglycerokinase (2.7.1.30)
PGM	Phosphoglyceromutase (2.7.5.3)
PK	Pyruvate kinase (2.7.1.40)
r	Regression coefficient
RIA	Radioimmunoassay
Satn	Saturation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
2-site IRMA	2-site immunoradiometric assay
T	Term
TBS	Tris-buffered saline
TCA	Trichloracetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
WHO	World Health Organisation

Introduction

Iron plays a central role in mammalian metabolism and is present in every living cell. Man's need of iron was recognised by the ancient Egyptians and is listed in their pharmacopoeia (Hoppe, 1955). Iron is essential to life and therefore stores are required to protect against blood loss, a reduction in intake and for the feto-placental unit in pregnancy. The absorption of iron is not finely controlled and as no mechanism exists for the excretion of excess iron, man is protected from its toxic effects by its removal from plasma into storage depots.

Two iron storage proteins, ferritin and haemosiderin have been identified in animals and phyto-ferritin and phytosiderin in plants and fungi. The quantitation of iron stores was rarely applied routinely in the past as it was time consuming and often unacceptable to the subject. Storage iron has been measured by ashing the whole body and determining the iron content (Josephs, 1959), by repeated phlebotomy (Pritchard and Mason, 1964), by the histological or chemical quantitation of non-haem iron in bone marrow (Stevens et al, 1953) or liver (Weinfeld, 1964), by use of the iron chelator, desferroxamine (Wöhler, 1964) or by dilutional studies with radioiron (Finch, 1959). Recently a less traumatic method has been found and is dependent on the findings of Addison et al in 1972 that the concentration of ferritin in serum is proportional to body iron stores.

In 1894 Schmiedberg isolated a compound from pig liver which he called "ferratin" and further demonstrated its

presence in spleen and bone marrow. "Ferratin" is now known to have consisted of denatured ferritin and contaminating proteins. Pure ferritin was probably first isolated by Laufberger in 1937 as the cadmium salt of a crystalline protein which was stable in acid and alkaline solution and contained about 20 per cent of iron by weight. These findings were confirmed by Kuhn et al (1940) who reported the composition as 54.5 per cent protein, 12.1 per cent nucleic acid which has hever been confirmed and 35 per cent hydrous ferric oxide (FeO.OH) and suggested that there was an equimolar ratio of iron to peptide. Grannick (1942, 1946) has published prolifically on the isolation, structure and function of ferritin. He has shown it to be heat stable and to form distinctive octahedral or tetrahedral crystalline structures which are optically isotropic and have a degree of twinning. On ultracentrifugation dark brown particles with a molecular weight (M.Wt.) of 2,000,000 were formed and the colourless supernatant contained less than 30 per cent of protein in tne form of spherical molecules, M.Wt. 500,000. The ferritin solution was however homogenous on electrophoresis using a Tiselius apparatus but he noted that the content of iron, nitrogen and phosphorus was variable. By 1946 Grannick had isolated ferritin as its cadmium salt from bone marrow, spleen, liver and gasto-intestinal mucosa and shown its presence in the globus pallidus and the substantia nigra of the central nervous system. He demonstrated that in the presence of reducing agents iron could be removed from the molecule without denaturation of the protein which formed apoferritin. He revised the M.Wt. and found it to be 460,000 and described the space lattices

of ferritin and apoferritin as being identical. He reported the iron as being present in micelles, in the ferric form, $(FeO.OH)_8$ $(FeO\ PO_3H_2)$ and firmly bound to the protein which protected the cell from the toxic effects of ferric hydroxide. Ferritin has a magnetic dipole moment of 3.8 Bohr magnetons which is unusual for a compound of iron. Mazur and Shorr (1950) suggested that as ferritin and apoferritin had identical isoelectric points and electrophoretic mobilities that iron was sequestered inside the protein shell. This was confirmed by Farrant (1954) using electron-microscopy and by Harrison (1959) with X-ray diffraction studies and they further suggested that apoferritin was composed of sub-units forming a shell round the iron micelle.

The present estimate of the M.Wt. of apoferritin is 445,000 (Harrison, 1969 and Crichton, 1973). The iron content has been found to vary from zero to 4,500 atoms per molecule (Crichton, 1973) and based on amino-acid analyses there are probably 24 protein sub-units (Crichton, 1973).

Isoferritins were first reported by Richter (1964) when he isolated ferritin from neoplastic tissues which had a different mobility from that of normal tissues and later Alfrey (1967) isolated two types of ferritin from human bone marrow. Most tissues have been found to contain multiple isoferritins which seems to reflect different families of heteropolymers arising from different proportions of two sub-units. The isoferritin population of tissue can change with development and disease (Drysdale, 1977).

The synthesis of ferritin may be under the influence

of a number of genes as suggested by the presence of isoferritins and the frequent finding of a small amount of a polymeric form (Harrison,a 1974). Drysdale and Munro, (1966) have shown that synthesis is insensitive to inhibitors of mammalian m-RNA synthesis and that induction would appear to be at the post-transcriptional level.

Ferritin could be synthesised by the gradual accumulation of iron within the protein shell (Bielig and Bayer, 1955) or the iron micelle could be formed first, followed by the enveloping protein coat which would stabilise the molecule (Pape et al, 1968). The former pathway has gained most support. Radioisotopically labelled amino acids have been shown to be incorporated into the ferritin molecule which contained little or no iron and the metal content increased with time (Fineberg and Greenberg, 1955).

Iron would appear to induce ferritin synthesis and this theory was put forward by Grannick (1948) who fed iron to guinea pigs and found that ferritin accumulated in the mucosal cells of the intestine. This was later supported by the work of Crichton (1971). Reports suggest that apoferritin is a catalyst in the oxidation of iron and that when the ratio of iron to apoferritin is low the incorporation of iron into the molecule is proportional to the concentration of apoferritin (Harrison,b 1974). Once nucleated the iron core grows rapidly. Grannick and Michaelis (1943) earlier reported that iron in the ferric form was not incorporated into apoferritin. Zinc competitively inhibits the formation of ferritin as it is absorbed onto the iron micelle and is also bound to

apoferritin (Macara et al, 1973). This method of formation allows iron to be laid down and removed successively from the surface of the micro-crystals which have a relatively large surface area. The number of sites for deposition of iron increases as the core size increases. The iron core has a characteristic polyhedral form on electronmicroscopy (Harrison,b,1974). Many cores have a uniform density but some are divided into smaller particles and have a central space suggesting that iron adheres to the inner surface of the protein.

The mobilisation of ferritin iron is less well understood. Grannick and Michaelis,(1943) showed that the reduction of iron released it from the protein but biological reducing agents have been found inadequate in meeting physiological requirements (Bielig and Bayer, 1955). Mazur et al,(1958) suggested that xanthine oxidase may be important for this process in the liver but Crichton,(1973) has been unable to demonstrate the effect in vitro. Ferrireductase, a flavoprotein which requires reduced nicotinamide adenine dinucleotide may be the enzyme which reduces and mobilises ferritin iron (Oski and Sirivech, 1971).

Haemosiderin was considered by Grannick to be an insoluble breakdown product of ferritin which was visible histologically. Ferritin has a magnetic moment of 3.8 bohr magnetons and haemosiderin has similar although more variable magnetic properties (Harrison et al,b,1974.) Matioli and Baker (1963) suggested that ferritin was denatured by oxidising agents which were found in increasing concentration as ferritin accumulated causing the inhibition

of catalase activity. The denatured molecules may be sequestered in vacuoles, the protein removed by proteolytic enzymes, iron micelles polymerised and precipitated as large amorphous iron complexes.

The intestinal absorption of iron is not fully understood. Early studies suggested that the intestine could absorb and excrete iron (McCallum, 1894) but the latter function was soon rejected. In 1943 Hahn et al suggested the "mucosal block theory" and Granmick (1946) suggested that ferritin was in equilibrium with ferrous ions in the intestinal mucosa which were in turn in equilibrium with transferrin bound plasma iron. He described ferritin as aiding the absorption of iron and blocking excess absorption. Crosby (1963) suggested that when iron was bound to ferritin in the epithelial cells it was not available for absorption and this theory was supported and expanded by Smith et al, 1968. However in studying the subcellular distribution of radioiron in the intestinal mucosal cells of rats Norwood and Jacobs (1972) concluded that ferritin formation was not a major controlling factor in iron absorption.

In 1968 Buffe described a new fetal liver antigen, alpha 2-H globulin which was found in the serum in primary hepatoma and other malignant conditions. On biochemical and immunological grounds it was later thought that alpha 2-H globulin and ferritin were identical (Buffe, 1973). It has been suggested that embryonic tumours could be monitored by quantitating plasma ferritin (Buffe, 1973) but in a recent study of ovarian germ cell tumours (Pederson et al, 1976) it was found that only 50 per cent

had elevated levels which may have been due to liver metastases. Ferritin has been isolated from mammary carcinomas and in an investigation into the recurrence rates of this condition Wang (1976) found that 10 per cent of patients had grossly elevated serum ferritin concentrations and that they had marginally faster recurrence rates. Carcino-embryonic antigen was a more useful indicator of prognosis.

Ferritin has been quantitated in tissues and pathological sera for many years but it was not until 1972 that Addison et al detected its presence in normal serum by an immunoradiometric technique which has now been automated (Jones and Worwood, 1975). Since then a number of sensitive assays have been described including a 2-site immunoradiometric assay (Miles et al, 1974), a classical radioimmunoassay (Nütsu et al, 1974) which is now available in commercial kit form ("Travenol",) and an enzyme-linked immunoassay (Boenish, 1976).

Interest in the assay was aroused by reports that in disorders of iron metabolism the serum ferritin concentration reflected body iron stores. Jacobs et al (1972) showed that in the pathological conditions of iron deficiency and iron overload serum ferritin reflected iron stores assessed by bone marrow iron content and the differential desferrioxamine test. Walters et al (1973) investigated normal subjects and found a correlation between serum ferritin and body iron stores determined by repeated phlebotomy. Serum ferritin has also been shown to correlate with radioiron absorption studies in normal subjects (Lipschitz et al, 1974). The concentration of

ferritin in serum was found to be proportional to bone marrow iron deposits in patients with rheumatoid arthritis (Bentley and Williams, 1974), chronic renal failure on haemodialysis (Hussein et al, 1975) and the beta-thalassaemia trait (Hussein et al, 1976). Lipschitz et al, (1974) found that in anaemia from causes other than iron deficiency anaemia, particularly when associated with an inflammatory process the serum ferritin was above the normal range. They also found elevated levels in liver disease especially alcoholic cirrhosis and viral hepatitis. However when iron deficiency was present with inflammation or liver disease the serum ferritin concentrations were distinctly lower than that of the overall mean. In Hodgkin's disease and leukaemia serum ferritin has been found to be inappropriately raised in relation to the amount of iron stores but levels in the latter condition may indicate prognosis (Parry et al, 1975; Jacobs and Worwood, 1975).

Studies of infants and children have also shown that serum ferritin reflects iron stores. Simes et al, 1974 found in a normal population screen from birth until 15 years of age that the serum ferritin parallelled known changes in iron stores. He also found low concentrations in iron deficient children and high levels in children with thalassaemia major and sickle cell disease. Children with acute infections and acute lymphoblastic leukaemia can like adults have disproportionately raised serum ferritin levels. It has also been shown that in a study of infants at three and six months of age plasma ferritin reflects the iron content of the diet (Rios et al, 1975). In children with

thalassaemia major the serum ferritin was found to correlate with the liver iron concentration and the volume of blood transfused (Letsky, 1974).

The total body iron content in the adult male has been reported as 3 to 4g (Burman, 1974) and 4 to 5g (Pollycove, 1972). Haemoglobin contains about 70 per cent, stores 23 per cent, myoglobin 7 per cent, enzymes 0.2 per cent and plasma transferrin 0.1 per cent of the body's iron. Storage iron in the form of ferritin and haemosiderin, is found in quantity in the cells of the reticuloendothelial system of liver, spleen and bone marrow and in the parenchymal cells of the liver, In the adult male only about 3 per cent of total iron is found in the bone marrow (Zilva and Pannall, 1975). Estimated of the rates of iron release from ferritin and haemosiderin vary but the balance of evidence suggests that it is more rapidly released from the former (Harrison et al, 1974).

The adult female has only one third of the iron stores of the male and Scott and Pritchard (1967) have reported that 24 per cent of American college women have depleted iron stores. Butler (1967) found that only 30 per cent of Welsh women have optimum iron and folate stores. Burman, (1971) has described the considerable variations in the body iron content and distribution of iron which takes place during the first two years of life. At birth the distribution is similar to that of the adult male but by 6 weeks only 40 per cent of iron was found in haemoglobin and 36 per cent in storage form. However from six months until two years the distribution was similar to that at birth.

The functions of iron are mainly those of its compounds

which form two groups of proteins dealing with the transport and storage of iron on the one hand and metabolic and enzymatic functions on the other. Most of the compounds in the latter group are proteins with an iron-porphyrin prosthetic group and haemoglobin is quantitatively the most important. It carries oxygen to the tissues, removes carbon dioxide from them and is an erythrocyte buffer. Myoglobin is an oxygen reservoir for muscle at times of contraction. The mitochondrial cytochromes are electron transporting enzymes and play an essential role in the production of cellular energy. Cytochrome P-450 in the microsomal membrane of the liver is involved in the oxidative degradation of drugs and endogenous substrates (Dallman, 1974). Catalase and peroxidase reduce endogenously produced hydrogen peroxide. Some iron containing enzymes do not contain porphyrin and consist mainly of the iron containing flavoproteins associated with oxidative metabolism and include succinate dehydrogenase. Iron is also required as a co-factor for aconitase activity and for the hydroxylation of proline and lysine in collagen synthesis (Dallman, 1974). It has also been shown that iron is required for deoxy-ribonucleic acid synthesis and is essential for mitosis (Robbins and Pederson, 1970).

Iron deficiency anaemia is a world wide problem and is the commonest nutritional deficiency of industrialised countries. It has been defined in a number of ways including the absence of iron stores, an increase in the absorption of radioiron, an abnormally low serum iron and percentage transferrin saturation, a low haemoglobin level, the appearance of microcytosis, hypochromasia and

fragmentation and elevation of free protoporphyrin of the red cell. Most of these indices are associated with the abnormalities of the blood picture alone but the systemic manifestations of iron deficiency have recently received attention and include the decreased activity of cytochrome oxidase in the intestinal mucosa (Dallman, 1974). The abnormalities which arise in the blood appear successively and the number of people identified as iron deficient depends on the definition used which includes the level of cut-off (Crosby, 1977). At present there is no scientific approach to the definition and interpretive guidelines are arbitrary (Beaton, 1974). In one series the incidence of iron deficiency varied from 4 per cent to 72 per cent depending on the criteria selected (Beaton, 1974).

Severe iron deficiency has a high mortality (Ashford, 1934) but in general it is not associated with the mortality of other nutritional disease states. In the less severe form it is associated with lethargy, tachycardia, angular stomatitis and koilonychia but Elwood and Hughes (1970) have suggested that iron deficiency did not cause any important epidemiological evidence of disability until the haemoglobin was less than 8g per dl.

Iron is well conserved in the body and losses are small. 95 per cent of the iron required for haemoglobin synthesis is obtained from the breakdown of senescent red blood cells, from cellular wastage during erythropoiesis and from non-haem iron of viable erythrocytes (Cook et al, 1970). Only about 4 per cent of iron is obtained from intestinal absorption. In the adult male about 1mg of iron per day is lost in the urine, sweat, bile, faeces and

desquamated cells; but most is lost in the faeces. The loss of iron in exfoliated cells has been debated but it would appear that although 500g of cells are lost per day most of the iron has been reabsorbed (Jacobs, 1974). Infants have a relatively high surface area and the loss from the skin is considerably increased and represents one third of the total loss (Burman, 1971). In women of child-bearing age additional losses occur in menstruation. The mean overall loss of menstrual iron was 0.5mg per day or 12mg per period and the amount of blood lost in successive periods was consistent (Hytten et al, 1964). However if heavy periods are experienced the losses could be equivalent to 2 to 3mg per day (Jacobs and Butler, 1965). These losses are normally made up by increased absorption of iron from the diet.

In men and post-menopausal women 1mg of iron per day is recommended and for menstruating females 2mg per day. Iron absorption must not only cover losses but also the needs for growth and Moe (1963) has demonstrated the benefits obtained from iron fortified cereal fed to infants. It has been suggested that 1.5 to 2mg of iron per day must be absorbed by the growing child. Iron deficiency in the female at puberty was reported in 1554 in the "De Morbo Virgineo" (Lancet, 1941). At puberty the iron requirement is high due to continued growth and the onset of menstruation and 2.5mg are recommended. The iron requirement is also high in pregnancy due to the increased maternal blood volume and feto-placental requirements. An average intake of 2.5mg per day has been recommended during pregnancy.

The body responds to anaemia whether or not it is due to iron deficiency by increasing the absorption of iron from the diet (Zilva and Pannall, 1975). The average daily diet contains 10 to 15mg of iron but not all is available for absorption. Dietary iron is present in a number of forms including haemoglobin and myoglobin from which the intact haem molecule is absorbed into the mucosal cell after removal of the protein chain, as ferrous and ferric salts which are absorbed three times more easily in the reduced form (Brise and Hallberg, 1962) and as salts of phytate and phosphate which are poorly utilised. Foods rich in utilisable iron are green vegetables, eggs, red meat especially liver and bread made with iron fortified flour. Iron is absorbed in the duodenum and jejuneum.

Considerable demands are placed on the maternal iron stores during pregnancy due to an increase in the maternal red cell mass which demands about 500mg of iron, to the fetus which requires 250 to 300mg and for placental and uterine needs and to cover blood loss at delivery an additional 300mg are required. About 500mg of iron will be returned to the maternal stores in the purperium; despite this 1g of iron is required in the second half of pregnancy. If requirements were equal throughout gestation most subjects could meet the demands by increased absorption (Hahn et al, 1951) despite the low stores of many women. Storage iron was found to meet only one third of requirements in the average American woman (Pitkin et al, 1972). However most of the iron is required during the second half of pregnancy and it is doubtful if this large requirement could be met by absorption alone as the maximum amount of iron that can be

absorbed from the average diet is only 3 to 4mg.

The World Health Organisation in 1968 stated that iron deficiency anaemia in pregnancy is a world wide problem which has been recognised for many years. Today we may classify many more patients as iron deficient than in 1881 when a normal maternal haemoglobin at term was only 58 per cent of the non-pregnant level (Willcocks, 1881). However the problem was eventually recognised and by 1924 McGowan recorded a decrease in the condition which he considered to result from a better dietary knowledge and the "nearer approximation of the life of women to that of men". In 1936 Corrigan and Strauss recorded the successful treatment of pregnancy anaemia with ferrous sulphate and in 1942 Hamilton and Wright described the prevention of anaemia in pregnancy. It was advocated in the British Medical Journal in 1964 that iron should be given to all subjects throughout pregnancy to prevent ill-health in middle-life as a high proportion of post-menopausal chronic anaemias begin during the child-bearing years (Kilpatrick and Hardisty, 1961).

Recently the universal practice of prescribing iron during pregnancy which was recommended 25 years ago has been questioned. Hall (1974) has indicated that iron supplements are not required routinely in developed countries and Taylor and Lind (1976) have suggested that prophylactic iron therapy may lead to excess iron deposition in the bone marrow. However it has been shown that 47 per cent of women have low haemoglobin levels at term when no supplement has been prescribed but only 6 per cent were anaemic on iron supplements (Jacobs, 1974). Scott et al (1975) believe

that one of the factors which have reduced the incidence of anaemia in pregnancy in Glasgow is an iron supplemented diet. The divergence of opinion on the need for iron supplements during pregnancy is due to the absence of a definitive screening test for iron depletion. Tests used routinely in non-pregnant subjects include haemoglobin concentration, serum iron, total iron-binding capacity, free red cell protoporphyrin and packed cell volume which alter in pregnancy; the individual variation being considerable (Hytten and Duncan, 1956). It is therefore difficult to differentiate between physiological and pathological changes and some consider that as changes can be modified by iron supplements many patients are iron deficient. This view has been challenged by Hytten and Duncan (1956) who suggested that iron supplements should not be given solely on the basis of a fall in haemoglobin which reflects a disproportionate increase in plasma volume and red cell mass and not necessarily iron deficiency. Quantitation of the red cell mass would give a definitive diagnosis but such a test could not be performed routinely although Paintin (1962) after determining the calculated values for total red cell volume suggested that "the haemoglobin concentration may after all be a better index of anaemia". The benefits of an increased plasma volume with a low packed cell volume should not be ignored as the resulting decrease in plasma viscosity substantially reduces the work of the heart and contributes to the maintainance of normal blood pressure as cardiac output increases (Hytten and Duncan, 1956). It has been suggested that it is best to diagnose and treat iron deficiency

specifically and that subjects can best be identified by using the determination of haemoglobin concentration as a screening test and further investigate all patients with a haemoglobin of less than 11g per dl (Paintin et al, 1966). It is only by assessing the iron stores during pregnancy that this debate can be resolved.

The increase in the maternal red cell mass is the component most affected by inadequate iron intake but in all the most severe conditions the impairment is self-limiting. The affect of maternal anaemia on the fetus is unclear. It has been stated that still-births, neonatal deaths and malformations are frequent (Roszkowski et al, 1966) and Butler (1967) has suggested that a strong correlation exists between still births and maternal anaemia but it has been difficult to prove and some have suggested that moderate degrees of anaemia do not affect the fetus (Beaton, 1974). Klein (1962), MacGregor (1963) and Butler (1967) have described an increased incidence of premature labour in pregnancy anaemia.

The mature human fetus at birth has an iron concentration of 78mg per Kg which compares with 70mg per Kg of the adult male (Josephs, 1959). In the third trimester the fetus accumulates 4mg of iron per day (Pribilla et al, 1958). The total iron content of the term infant is 152 to 372mg which varies with birth weight, gestation, the time of clamping of the cord and the cord haemoglobin (Burman, 1971). The prime determinant of total body iron is fetal birth weight (Chang, 1973; Loria et al, 1977).

Haemorrhage before or during delivery decreased the

total body iron content (Burman, 1974). In a twin pregnancy, transfusion can occur from one twin to the other resulting in anaemia and polycythaemia respectively. Antenatally haemorrhage can occur from the fetal into the maternal circulation, during amniocentesis or at delivery from the placenta.

The distribution of iron between haemoglobin and storage sites at delivery is similar to that of the adult male (Burman, 1971). The fetal liver at birth contains the highest proportion of the non-haem iron and has a greater concentration than the adult male and may reflect that the liver is an erythropoietic organ in the second trimester which may still be active at birth (Chang, 1973). The liver non-haem iron content has been assessed mainly by histochemical methods or by chemical analyses (Chang, 1973; Loria et al, 1977). It has been shown that the non-haem iron content of the liver also correlates with the weight and gestational age of the infant; the former being more important. The total content doubles between 38 and 40 weeks (Chang, 1973) but the concentration did not show significant variation with gestation. In the kidney however both the concentration and the total non-haem iron content appeared to increase with advancing gestation. Loria et al (1977) showed that in light-for-dates premature and term babies that the non-haem iron content of the liver was lower than the equivalent eutrophic infant. They also reported that the hypertrophic mature infant had a depleted liver iron content and concluded that when the fetus attains a body weight of 4 Kg that the hepatic iron stores are mobilised.

Premature infants have low iron stores at delivery and it has been shown that they become anaemic more frequently and at an earlier age than their term counterparts (W.H.O., 1968). It has been reported that babies of severely iron deficient mothers developed anaemia in infancy although the neonatal haemoglobins were normal (Strauss, 1933) and Sissons and Lund (1958) suggested that the fetal iron content was decreased in maternal iron deficiency anaemia. However more recent studies have suggested that there is no correlation between the haemoglobin and serum iron of the mother and fetus at birth and at six months (Kessel and Sills, 1968) nor between the plasma ferritin concentrations (Rios et al, 1975).

Smith et al (1955) stressed the importance of fetal iron stores as during infancy the diet contains little iron and suggested that 70 per cent of the haemoglobin iron at one year and 40 per cent at two years was of maternal origin but this has been questioned by Burman (1971) who thought that the dependence was only apparent up to three months of age. However it has been demonstrated that premature and light-for-dates infants have higher haemoglobin levels when the diet is supplemented with iron (Burman, 1971).

Fetal iron is acquired from the maternal diet and iron stores. Iron is absorbed in increased amounts and is utilised by the fetus (Svandberg et al, 1975). Rapid depletion of maternal liver and spleen ferritin but not of haemosiderin take place together with a reduction in the maternal ferritin synthesis in the rat (Wyllie and Kaufman, (1971). Iron in the amniotic fluid can be absorbed through

the fetal intestine and be utilised by the fetus but this route is of minor importance (Orlic et al, 1974).

Maternal red blood cells were thought to be the source of fetal iron prior to the detection of iron in plasma in 1925 (Scholten and Veit, 1903). It was believed that erythrocytes disintegrated in the placenta liberating iron and the theory was supported by the presence of haemolysins and histochemically identifiable iron in the placenta. In 1942, Pommerenke et al demonstrated that within minutes of transferrin bound radioiron being present in human maternal plasma it appeared in the fetal circulation. This work was verified and expanded by Vosburg and Flexner, (1950) who showed that in guinea pigs the fetal iron content could be accounted for from the maternal circulation without the need to consider the maternal red blood cells as an alternative source of iron. It is now accepted that transferrin of the maternal circulation is the immediate source of iron transferred to the fetus (Morgan, 1974).

Transferrin is taken up by the rabbit placenta much faster than by the maternal bone marrow and the uptake increases per unit surface area and with advancing gestation. The process is independent of the fetus (Baker and Morgan, 1969). Receptor sites for the transferrin-iron complex are thought to be present on the maternal surface of the human placenta (Fletcher and Suter, 1969). Iron would appear to be taken up by the guinea pig placenta free of the maternal carrier protein before being transferred to the fetal circulation (Morgan, 1973). The rate of iron transfer across the placenta is very rapid and suggests that it does not pass through any biological pool of importance (Bothwell

et al, 1958).

The passage of iron across the human placenta increases ten fold from mid-pregnancy until term (Fletcher and Suter, 1969). The fetal iron and transferrin saturation are greater than that in the maternal circulation and placental iron absorption has been shown as an active process dependent on placental cellular metabolism (Wong and Morgan, 1973). The clearance of iron is the same whether it is bound to maternal or fetal transferrin and there would appear to be an unidirectional system for pumping iron through the placenta (Bothwell et al, 1958).

Histological studies of the distribution of iron in the human placenta throughout gestation were undertaken by McKay et al (1958). They demonstrated that non-haem iron reached maximum levels in the second trimester and thereafter decreased until just before term when iron was detected in the stromal macrophages. In rats it has been shown that in early pregnancy a higher percentage of iron is taken up by the yolk sac and placenta than in later gestation when iron appears to go directly to the fetus (Glasser et al, 1968). Fletcher and Suter (1969) have shown that although most of the radioiron was taken up by the fetus a significant amount was always retained by the placenta.

The present work was an attempt to study iron stores in pregnant women, their influence on iron utilisation and turnover in the fetus and the part played by the placenta in this process.

Plasma ferritin has not previously been determined in a Scottish population. A group of young males and females of reproductive age were selected to determine if the reported

sex difference in storage iron occurred and also to estimate the adequacy of the female iron stores to maintain a normal pregnancy. In order to determine the suitability of the present ferritin assay to assess iron stores, plasma ferritin and bone marrow iron content were quantitated in non-pregnant subjects.

Quantitation of plasma ferritin was used to define the changes which occur in iron stores during pregnancy and in the puerperium. Few reports exist on maternal iron stores throughout pregnancy but de Leeuw et al (1966) determined the bone marrow iron content in early and late gestation and in the puerperium of untreated and iron supplemented women. No publications are known which report the maternal plasma ferritin throughout pregnancy and initially it was hoped to correlate ferritin concentrations with bone marrow biopsies but the latter was regarded as unethical when not specifically merited by the patient's condition.

In disease states changes frequently occur in metabolism and a study of these changes often results in a better understanding of the normal physiological process. The effect of coincident disease during pregnancy on plasma ferritin was assessed. The pathologies included pre-eclamptic toxaemia of pregnancy, diabetes and pregnancy jaundice which occur relatively frequently in our population.

The influence of maternal iron stores on fetal stores was assessed by relating the maternal plasma ferritin to the cord ferritin concentration. The study had previously been undertaken by Rios et al (1975) who found no correlation between the two levels. It was felt this work merited repetition and supplementation by assessing factors known

to reduce maternal iron stores. Plasma ferritin of term babies in the neonatal period and in childhood have been investigated (Hiimes et al, 1974; Rios et al, 1975) but neither of the reports related the concentration to gestation, body weight or the clinical condition of the newborn. An investigation was undertaken to relate these factors to the daily changes in plasma ferritin during the neonatal period.

The gestational changes in the content of non-haem iron or ferritin iron of fetal organs has been reported (Fletcher and Suter, 1969; Burman, 1971) but little has been recorded of the specific quantitation of ferritin in the fetal organs. The varying rates of accumulation of ferritin in the liver, heart and spleen were investigated and the effect of body weight, gestational age and retarded growth assessed.

Ferritin has been identified in the placenta (Wöhler, 1955) and gestational changes in the non-haem iron content have been reported (MacKay et al, 1958; Glasser et al, 1963). It was thought that knowledge of the human placenta could be expanded by using a specific ferritin assay to relate the content to gestation, placental weight and the content of placental peptide hormones, human chorionic gonadotrophin and human placental lactogen.

Chapter One

PREPARATION OF REAGENTS AND METHODOLOGY

The quantitation of ferritin necessitated the isolation and purification of human ferritin for the production of rabbit anti-human ferritin and standard solution, the preparation of immunoadsorbent and radioisotopically labelled antibody.

Isolation and Purification of Human Ferritin from Spleen and Placenta

The isolation of ferritin is dependent on its high molecular weight and heat stability. Most methods involve the initial removal of contaminating proteins by heat coagulation followed by precipitation of ferritin with ammonium sulphate. Purification has been carried out by various methods including crystallisation of the protein from cadmium sulphate solutions (Grannick, 1942), chromatographic separation on columns of Sephadex and carboxymethyl cellulose (Drysdale and Munro, 1965) and by ultra-centrifugation (Penders et al, 1968). However, Penders et al (1968) have criticised the use of cadmium sulphate in the preparation of ferritin as poor yields were obtained and they also found that repeated columnchromatography could split ferritin into a number of fractions. The method chosen was based on the reports of a number of workers including Drysdale and Munro, 1965; Penders et al, 1968; Worwood, 1973; Aherne and Worwood, 1974 and Miles et al, 1974.

Reagents. 1. IM Acetic acid

170ml Acetic acid (BDH Analar) made up to 1 litre with distilled water.

2. Sodium acetate - BDH

3. Ammonium sulphate - BDH

4. 0.15M Sodium chloride.

   8.7g sodium chloride in 100ml distilled water.

5. 0.05M Barbitone buffer pH 7.5.

   10.3g of sodium barbitone in 1 litre distilled water and adjusted to pH 7.5 with 0.1M HCl.

6. Sephadex G200 - Pharmacia, Uppsala, Sweden.

7. BSA buffer. 10.3g sodium barbitone, 6g sodium chloride and 0.2g sodium azide in 1 litre distilled water and the pH adjusted to 8.0 with 5M HCl. Prior to use 0.5g of bovine albumin (Sigma) was added per 100ml buffer.

Method Normal human spleens removed at autopsy and normal placentae obtained by spontaneous vaginal delivery were homogenised with 4ml of water per g of tissue within an hour of arriving in the laboratory. The homogenate was heated (70 ± 2°C for 10 minutes) with constant stirring and then rapidly cooled to room temperature. It was then centrifuged at 2,500g for 15 minutes and filtered through Whatman No. 1 paper. This procedure removed the bulk of the contaminating heat coagulable proteins. Adjustment of the supernatant to pH 4.8 with 1M acetic acid precipitated mainly haemoglobin (Drysdale and Munro, 1965) (removed by centrifugation at 2,500g for 15 minutes). The supernatant was then made 0.05M with respect to sodium acetate by the addition of the solid salt and the pH adjusted to pH 5.5 with 1M acetic acid. The solution was maintained at 4°C and solid ammonium sulphate slowly added, with stirring, until the solution was 60 per cent saturated (310g per litre) and left overnight. By selecting such a concentration of ammonium sulphate both the iron rich ferritin and apoferritin were precipitated with the minimum of protein contaminants (Drysdale and Munro, 1965).

The precipitated ferritin was harvested after centrifugation at 1,500g for 10 minutes and dissolved in 40ml of 0.15M sodium chloride.

The dark brown solution was centrifuged at 100,000g for 2 hours in a Beckman L5-65 ultracentrifuge at 15°C. The dark brown precipitate was dissolved in the minimum volume (about 10ml) of 0.05M barbitone buffer pH 7.5.

The ferritin solution was further purified by chromatography on a Sephadex G200 column (90 x 2.5cm), equilibrated with 0.05M barbitone buffer pH 7.5. 5ml of the extract was carefully applied to the column and eluted with barbitone buffer (0.05M, pH 7.5). Ferritin is so large a molecule that it was almost excluded from the Sephadex and therefore appeared in the void volume. It was collected in 2ml fractions by an LKB fraction collector, 7000 Ultrorac and appeared as a single peak when the protein content was monitored at 280nm using an LKB Uvicord II recorder. The fractions were pooled and concentrated under positive pressure using an Amicon Model 202 apparatus and Amicon ultrafiltration membrane PM10. The concentrated ferritin solution was analysed for protein content by the method of Lowry, (1951) and by themethod of Laurell (1966). The purity of the preparation was determined by crossed-immunoelectrophoresis and by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. The yield of ferritin from a human spleen ranged from 52 to 75mg and from a human placenta from 19 to 49mg.

Preparation of Standard Ferritin Solutions The concentrated ferritin solution was diluted with BSA buffer to a concentration of 10mg per litre and dispensed in 500ul aliquots. The standard solutions were stored at -20°C and were stable over a two year period.

Crossed Immunoelectrophoresis

The purity of the preparation was checked by crossed immunoelectrophoresis (Ganrot, 1972). A chemically clean 8 x 8cm glass slide previously coated with 10ml 1.0 per cent agarose (w/v) (B.D.H.) in 0.075M barbitone buffer pH 8.6. Two hours later three holes (3mm diameter) were cut in the gel 2.5cm apart and 1.5cm from the end of the plate. The ferritin extract was added to two of the holes and human plasma to the third. The proteins were separated electrophoretically in a Shandon electrophoresis tank containing 0.075M barbitone buffer, pH 8.6, (10m amp., 180v for 2 hours). The gel was then cut into three sections, one hole per section and transferred, with care to three separate 8 x 8cm glass slides and positioned to one side of the plate. 7.5ml of 1.0 per cent agarose (w/v) in 0.075M barbitone buffer, pH 8.6 and containing 0.2ml of commercial anti-human ferritin (Hoechst) was poured onto the plate containing one of the ferritin extracts and care was taken to avoid overlapping the agarose slab. The second ferritin extract and the human plasma were set up against agarose containing 0.2ml anti-human plasma proteins (Hoechst). The plates were set aside in a humidified cabinet for 2 hours before being subjected to electrophoretic separation at right angles to the initial run. The run took place overnight in 0.076M barbitone buffer pH 8.6 at 10m amp. and 180v. The agarose slabs were washed lightly with distilled, running water and then placed in 0.15M sodium chloride for 30 minutes. They were again washed with water, covered with moistened Whatman No. 2 filter paper and dried in a stream of hot air. When the paper dried it was easily removed from the slide which was again lightly washed with water and dried rapidly in hot air. It was then placed in amido black (0.1%) for 3 to 5 minutes and rinsed in 0.8M acetic acid. The plates were then air dried and inspected. The plate containing the ferritin extract and anti-human ferritin had one large rocket - see Plate 1,1 but none were

visible in the plate containing ferritin extract and anti-serum against human plasma proteins. The control plate which contained human plasma and anti-human plasma proteins had upwards of thirty rockets and demonstrated a successful run - see Plate. 1,I

Investigation of Ferritin Extract by SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel has a high resolving power and molecules are separated on the basis of their net charge and molecular size.

Reagents

1. 30 per cent Acrylamide (BDH) in distilled water.
2. 1.5M Tris HCl (Sigma) containing 0.4 per cent sodium dodecyl sulphate (SDS) (BDH) adjusted to pH 8.8.
3. 10 per cent Ammonium persulphate (BDH) - prepared before use.
4. N,N,N^1,N^1, - tetramethylethylenediamine (TEMED) (BDH).
5. S D S Polyacrylamide-Lower Running Gel. To prepare two plates 6.25ml of Reagent 1, 6.25ml of Reagent 2, and 12.4ml of distilled water weremixed and degassed for 1 to 2 minutes. 83ul of Reagent 3 and 25ul of TEMED were then added.
6. 0.5M Tris HCl containing 0.4 per cent SDS and adjusted to pH 6.8.
7. SDS Polyacrylamide-Upper Stacking Gel. 5ml of Reagent 6, 3ml of Reagent 1 and 12ml of distilled water were mixed and degassed for 1 to 2 minutes. 60ul of Reagent 3 and 20ul of TEMED were than added and thegel mixed.
8. Electrode Running Buffer. 43.2g glycine and 3g SDS were dissolved in 3l 0.025M Tris base (Sigma).
9. SDS Sample Buffer. 40g of sucrose, 10g of SDS and 0.1g Bromophenol blue were dissolved in 100ml 0.1M Tris HCl, pH 6.8.

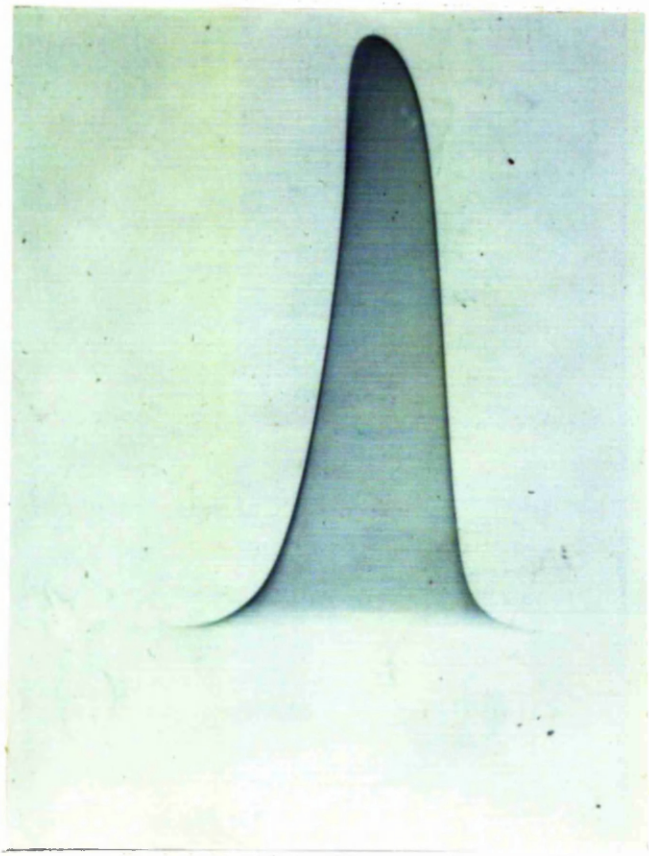


PLATE 1, I(a)

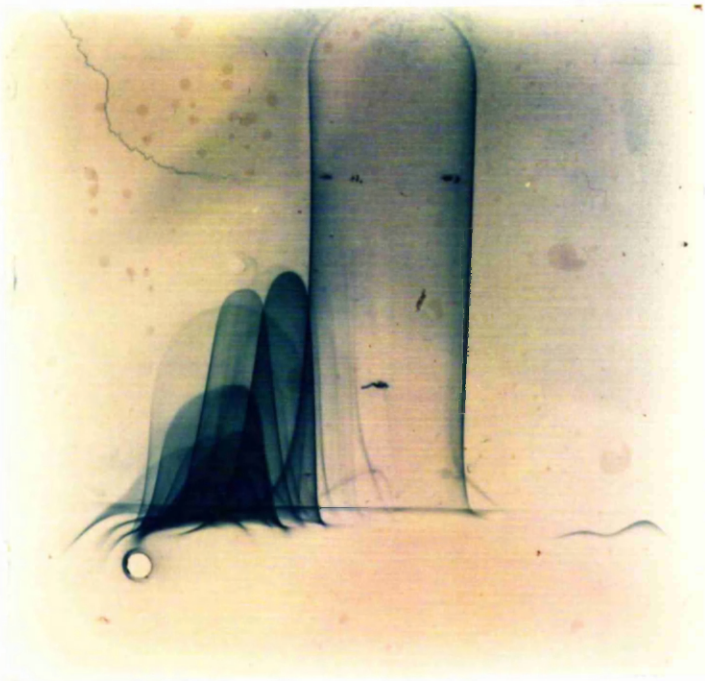


PLATE 1,I(b)

Plate 1 (a) The arc formed in a crossed immunoelectrophoretic analysis of a ferritin extract which precipitated with anti-ferritin plus anti-plasma proteins.

(b) Crossed immunoelectrophoretic analysis of human plasma precipitated with anti-plasma proteins.

10. Coomassie Blue. 1g of Coomassie blue and 12.5g of Trichloractic acid were dissolved in 100ml distilled water.

11. Destaining agent. Acetic Acid: ethanol: distilled water in ratio of 10:10:80.

Preparation of Plates The plates consisted of two 8 x 8cm glass slides separated by spacer arms and the whole assembly was taped together. The gel plates were filled to 2.5cm from the top with Reagent 5. The gels were gently overlayed with distilled water and left overnight to polymerise. Reagent 7, the upper stacking gel, was then layered carefully over the lower gels to fill the plates and the sample combs were then positioned. The gels were left aside for 1 hour to polymerise.

Mounting of Plates The gel plates were mounted in the Uniscil electrophoresis tank (Universal Scientific Ltd.) and sample combs removed. Running Buffer (Reagent 8) was added to the tank and allowed to enter the sample wells. Air bubbles were removed if necessary. The SDS Sample Buffer (Reagent 9) was mixed with sample and distilled water added to dilute the buffer 1:4. The samples were then carefully layered under the buffer into the sample wells using a Hamilton syringe.

The gels were run at 20m amp per plate for 5 hours to allow the bromophenol blue marker to reach the end of the plate. The tape was removed from the sides of the plates which were then gently prised apart. The gels were removed and stained in Coomassie blue overnight and were then destained with Reagent 11. Plate II shows three specimens of human sera run along side three spleen ferritin extracts containing 3.6mg protein per ml. Only one band is visible in the separation of the ferritin extract and it was judged pure. In Plate III can be seen the results of duplicate separations of human serum, two extracts of placental ferritin containing 0.6 and 0.1mg protein per ml and an

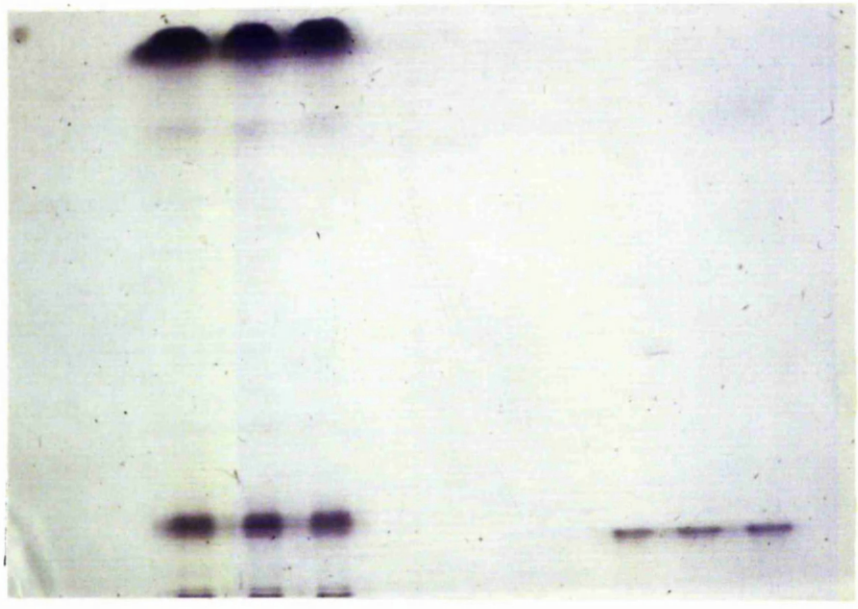


Plate 1, II Analysis of 3 specimens of human plasma and triplicate analysis of human spleen extract of ferritin (3.6mg/ml) by polyacrylamide-gel electrophoresis.

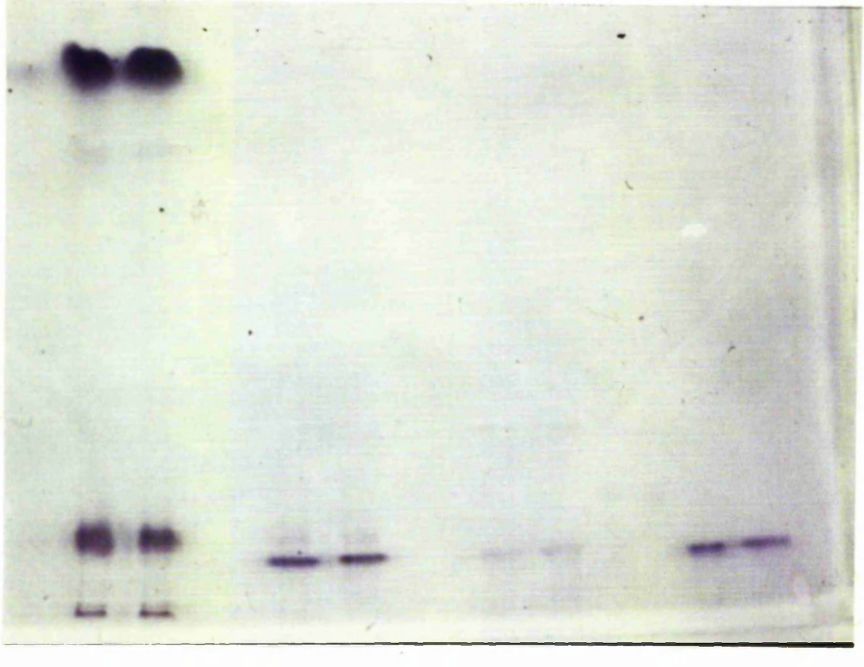


Plate 1, III Analysis in duplicate of human plasma, human placental extract I (0.6mg/ml), placental extract II (0.1 mg/ml) and human spleen extract (1.3 mg/ml)

extract of spleen containing 1.3mg per ml. The spleen extract gave only one band but both placental extracts contained contaminants which were later identified as alpha$_2$-macroglobulin and transferrin. Beta-lipoprotein has on one occasion been a contaminant in an extract of spleen ferritin.

Quantitation of Ferritin by the Method of Lowry (1951)

In the initial reaction protein is complexed with copper in alkaline solution which together with tyrosine and tryptophan reduce phosphomolybdic and phosphotungstic acids to molybdenum blue and tungsten blue.

Reagents 1) Alkaline tartrate reagent. 20g of sodium carbonate and 0.5g sodium tartrate in 1l 0.1M sodium hydroxide.

2) 0.004M copper sulphate. 0.1g copper sulphate ($CuSO_4 \cdot 5H_2O$) in 100ml distilled water.

3) Working alkaline copper reagent. 45ml of alkaline tartrate reagent and 5ml of copper sulphate solution mixed immediately before use.

4) Folin and Ciocalteau's Reagent: BDH. Reagent and distilled water (1:1) were mixed immediately before use.

5) Standard Protein Solution. A "Wellcontrol" serum of known protein content was diluted with 0.15M sodium chloride to a protein concentration of 4g per litre. Doubling dilutions were made of this solution to give protein concentrations from 250 to 2,000mg per litre.

Method 100ul of water, standard and test solutions were added to respective tubes and 5ml of working alkaline copper reagent added. The contents were mixed and left at room temperature for 15 minutes. 500ul of diluted Folin and Ciocalteau reagent was then added, mixing after each addition of reagent and the absorbance was read at 750nm after 30 minutes (Unicam SP 600).

Production of Rabbit Anti-human Ferritin

"The production of antisera is more of an art than a science" (Hurn and Landon, 1971). Methods which are successful in one laboratory often fail when repeated elsewhere and proven immunisation schedules suddenly give poor antisera. However a knowledge of the molecular weight and composition of the immunogen and the consideration of adjuvant, animal host, route of immunisation, dosage, timing of injections and harvesting of antisera aid the production of a good anti-serum (Hurn, 1974).

The immunogenicity of a compound is dependant on a molecular weight in excess of 5,000, properties of polymerisation, the rigidity of structure and accessibility of determinant groups, susceptibility to enzymatic degradation and content of phenylalanine, tryptophan, glutamic acid, lysine and tyrosine. Ferritin has a molecular weight of 450,000, is composed of 24 subunits and with the protein shell surrounding the metal core should theoretically be strongly antigenic.

The inclusion of adjuvants improves antibody response and the most useful was described by Freund in 1951 and consists of a neutral detergent, paraffin oil and killed mycobacteria. It is said to work by slowly releasing the antigen over a number of weeks, facilitating phagocytosis of the antigen by macrophages, causing the advantageous formation of local granulomatous lesions and aiding local and generalised stimulation of the reticulo-endothelial system (Hurn and Landon, 1971). It has been suggested that the best results are obtained when the adjuvant to aqueous antigen solution is in the ratio of 2 or 3:1 (Hurn, 1974).

Rabbits and guinea pigs have been frequently used to produce antisera but sheep, goats and horses yield larger volumes. The species used however is often dictated by local circumstances and could explain the popularity of the rabbit as host animal.

The route of immunisation has been most commonly intramuscular or

sub-cutaneous although lymph nodes, intra-articular, intradermal and intraperitoneal have also been used. Few scientific comparisons have been made but it has been suggested that the most avid antisera was obtained when lymph nodes were used although the number of animals responding was poor (Hurn and Landon, 1971). The multiple site intradermal regime (Vaitukaitis et al, 1971) has been recommended for the rapid production of antisera when antigen is scarce.

The amount of antigen administered is thought to be important and it has been suggested that the minimal effective concentration results in the most avid antiserum. It is common practice to give an initial dose of 1mg but more recently it has been suggested that 100ug initially and booster doses of 50ug could be more effective (Hurn and Landon, 1971). The time schedules for immunisation also vary considerably but are based on the fact that the lag phase is of the order of seven to eight days before immunoglobulin M appears and that maximum levels of the qualitatively and quantitatively important immunoglobulin G do not occur for four to six weeks. Response to booster doses differ in that the lag phase is reduced to four days and the maximum response is greater and achieved within ten days.

The purpose of the present investigation was to produce anti-human ferritin of sufficient avidity to assay plasma ferritin and therefore to save time it was decided to adopt a number of regimes which had been described in the literature. To make successful comparisons at least ten animals would have had to be allotted to each trial (Hurn and Landon, 1971) but this was not attempted and only one animal was used in each regime. It was therefore not possible to make valid comparisons.

Reagents 1. White, adult, female New Zealand Rabbits.

2. Freund's Complete Adjuvant - Difco.

3. Human spleen ferritin - 1.4mg per ml.

Method I A rabbit was immunised according to the schedule of Simes et al,(1974). 0.75ml of the ferritin solution was taken up into a syringe followed by 0.75ml of Freund's adjuvant and the contents shaken vigorously to form an emulsion. This dose was divided between two sites and injected intramuscularly. Three weeks later a booster injection of 0.25ml ferritin solution and an equal volume of the adjuvant was given. The animal was bled three weeks later by making a small incision to the ear and collecting the blood in a sterile Universal container. The serum was stored at -20°C.

II The protocol for the second rabbit was that of Miles et al (1974). The rabbit was injected in multiple subcutaneous sites with 0.5ml adjuvant containing 100ug ferritin and this was repeated twice at weekly intervals. Three weeks after the third injection a final injection of 100ug ferritin in 0.15M sodium chloride was given. The animal was bled ten days later.

III The third rabbit was treated by the regime described by Powell et al,(1975). 0.75ml ferritin solution was diluted with 1ml of adjuvant and injected intramuscularly at weekly intervals for three weeks. The rabbit was bled on the fourth, fifth and sixth week after the initial injection.

Assessment of specificity of rabbit anti-human ferritin The monospecificity of the anti-sera was checked by crossed-immunoelectrophoresis and by the Ouchterlony double diffusion method (1964).

The anti-sera was checked for monospecificity as described under the section "Preparation of Ferritin" by crossed-immunoelectrophoresis. Three plates containing 0.2ml anti-serum were set up against a ferritin extract, a ferritin extract diluted 1:1 with human plasma, and with whole human plasma. Only the first two plates gave a single rocket and nothing was seen on the third plate. These results suggested that the anti-serum was specific for human spleen ferritin.

The specificity of the rabbit anti-spleen ferritin was assessed by

the method of Ouchterlony (1964). Four wells were cut 0.5cm from the central well which was filled with anti-spleen ferritin. The surrounding wells were filled with human spleen ferritin, human liver, human heart and human placental extracts. There was a complete reaction of identity of all organ ferritins and only a single precipitin line was formed between each well.

Titre of rabbit anti-human ferritin Titre of antibody in the serum was determined by gel diffusion against purified human spleen ferritin. Doubling dilutions of the anti-serum were set up against spleen ferritin at a concentration of 0.6mg per ml. The titre was considered adequate for the assay if precipitation occured at dilutions of 1:64 of anti-ferritin.

The titre was also assessed by incubating the anti-human ferritin with horse ferritin immunoadsorbent - described under "Production of ^{125}Iodine Anti-Human Ferritin". Satisfactory anti-sera contained more than 1mg of specific antihuman ferritin per ml which could be bound by the ferritin immunoadsorbent.

Preparation of Protein Immunoadsorbent

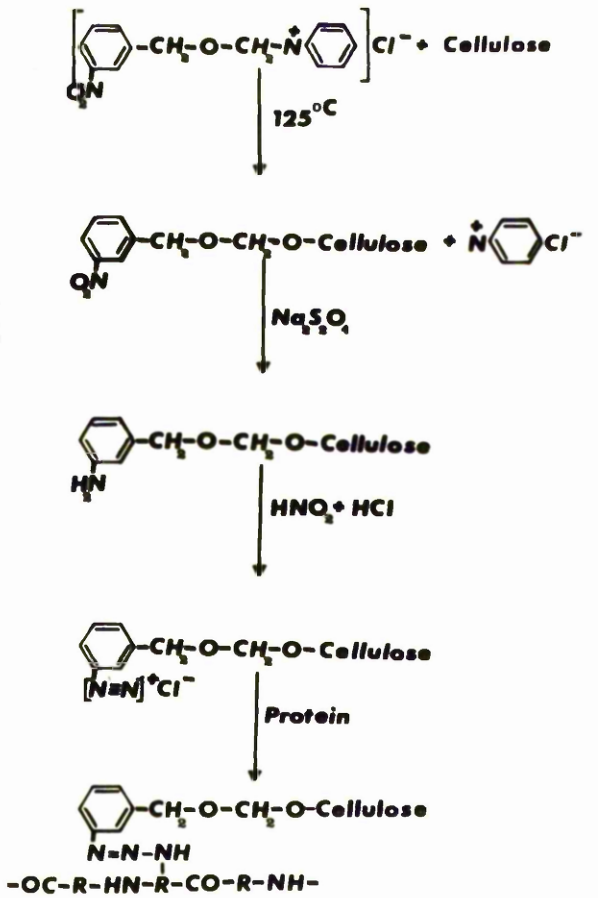


Fig. I, 1

Preparation of a protein immunoadsorbent

Preparation of Ferritin Immunoadsorbent

Soluble antigens coupled to insoluble supports are known as immunoadsorbents. The antigens retain the ability to combine with corresponding antibodies and have been used principally in the isolation of antibodies and in the quantitation of antibody titres. In the ferritin assay the immunoadsorbent is used to isolate specific anti-human ferritin and to protect it during iodination.

The insoluble support of the ferritin immunoadsorbent (F-ImAd) was 3-aminobenzyloxymethyl-cellulose and was synthesised by reacting cellulose with N(3-nitrobenzyloxymethyl)-pyridinium chloride (Gurevich, 1964). The compound was then diazotised with horse ferritin - see Figure 1,1..

The initial reaction was accelerated by drying the cellulose. This was accomplished by mixing it with sodium acetate and N(3-nitrobenzyloxy-methyl)-pyridinium chloride dissolved in ethanol, followed by evaporation of the solvent (Gurevich et al, 1961). On heating the reactants 3-nitrobenzyloxymethyl cellulose was formed and pyridine liberated. The nitro groups were then reduced to amino groups with sodium dithionite.

The amount of antigen bound by the immunoadsorbent is not directly related to the total number of amino groups present on cellulose but only to the number of groups on the surface of the immunoadsorbent. More antigen can therefore be bound by increasing the surface area of the carrier and this can be done by utilising a process developed in the manufacture of rayon (Fieser and Fieser, 1944). Cellulose is soluble in an ammoniacal solution of copper hydroxide, Schweitzer's reagent and on the addition of water, is precipitated in the form of a suspension which can then combine with one hundred and thirty-three times more antigen than before (Gurevich, 1964). The "amino-cellulose" in suspension was then reacted with nitrous acid to form a diazonium salt which then combined with ferritin. Diazonium salts can react with a

number of groups on the protein molecule including the phenol group of tyrosine, the imidazole group of histidine, the E-amino group of lysine, the sulphydryl group of cysteine, the indole group of tryptophan, the guanidine group of arginine and the α-amino group of glycine (Howard and Wild, 1957). The binding of the antigen to the immunoadsorbent is strong and is not destroyed by dilute acid.

Reagents All reagents were from BDH, Analar grade unless otherwise described.

1. Whatman CC41 Cellulose.
2. Ethanol - 90 per cent.
3. N(3-nitrobenzyloxymethyl)-pyridinium chloride.
4. Sodium acetate-anhydrous.
5. Benzene.
6. 0.96M Sodium dithionite.

   20g Sodium dithionite in 100ml distilled water.
7. 5M Acetic Acid.

   300ml Acetic acid diluted to 1 litre.
8. 0.04M Copper sulphate.

   5g copper sulphate ($CuSO_4.5H_2$)) in 500ml distilled water.
9. 0.75M Sodium hydroxide.

   2.25g Sodium hydroxide in 75ml distilled water.
10. 0.88 Ammonia.
11. Sucrose.
12. 1.84M Sulphuric acid.

    100ml concentrated Sulphuric acid diluted to 1 litre.
13. 2N Hydrochloric acid.

    166ml concentrated Hydrochloric acid diluted to 1 litre.
14. Urea.
15. Horse Spleen Ferritin - Calbiochem, Grade A.
16. 0.2M Borate buffer pH 8.0. 12.37g boric acid and 14.91g

potassium chloride were dissolved in 1l distilled water. 160ml of 0.1N sodium hydroxide was added to bring the pH to 8.0.

17. β-Naphthol.

18. Glycine.

19. 0.04M Phosphate buffer containing 0.1M sodium chloride, pH 7.4. 9.5ml 0.08M sodium di-hydrogen phosphate was added to 40.5ml 0.08M di-sodium hydrogen phosphate. 0.9g sodium chloride was added and the volume made up to 100ml with distilled water.

20. BSA buffer. 0.05M Barbitone buffer containing 0.1M sodium chloride and 5mg albumin per ml, pH 8.0. 10.3g sodium barbitone, 6g sodium chloride and 0.2g sodium azide were dissolved in 1 litre distilled water. The pH was adjusted to 8.0 ± 0.05 with 5M hydrochloric acid. 500mg bovine albumin (Sigma) was added to 100ml buffer before use.

Method: The preparation of aminocellulose 10g of Whatman CC41 cellulose powder was added with mixing, to 20ml of 90 per cent ethanol containing 1.4g N(3-nitrobenzyloxymethyl)-pyridinium chloride and 0.5g anhydrous sodium acetate in a porcelain evaporating basin. The mixture was initially heated and allowed to dry on a water-bath at 80°C and then in an oven maintained at 125°C for 40 minutes which produced pyridine and 3-nitrobenzyloxymethyl cellulose. The product was then placed in a Buchner funnel, washed with 600ml of benzene and air dried. The compound was further washed with 5l of distilled water to remove final traces of pyridine. The nitro groups were then reduced by adding the moist cellulose to 150ml of 0.96M sodium dithionite and heating at 60°C in a water-bath for 30 minutes. The amino-cellulose was freed from hydrogen sulphide by washing with 800ml distilled water, 600ml 5M acetic acid and

800ml distilled water and finally dried in a vacuum desiccator and ground with a mortar and pestle to give a creamy-yellow powder. The compound was stored in a vacuum dessicator at 4°C and was stable for more than one year.

Cupric hydroxide was precipitated when 500ml of 0.04M copper sulphate solution was mixed with 75ml of 0.75M sodium hydroxide. The precipitate was washed with 2l of distilled water and dissolved in 100ml of 0.88 ammonia. 0.33g of sucrose was then added followed, very slowly, by 1g of aminocellulose and the mixture stirred with a magnetic stirrer for 30 minutes. 300ml of distilled water was added and a flocculent white precipitate was formed when 250ml of 1.84M sulphuric acid was slowly added to destroy the copper complex and neutralise the solution which was indicated when the solution became pale blue. The precipitate was washed with 1l distilled water, suspended in 100ml of water and used within 30 minutes.

Diazotisation of Aminocellulose Immunoadsorbent and Coupling to Ferritin

The following reactions took place in a cold room at 4°C. 20ml of 2M hydrochloric acid was added to 10ml of the aminocellulose suspension (100mg aminocellulose) to produce a pink colour. The reaction took place in a glass beaker surrounded by a sodium chloride-ice mixture. 0.8ml of 0.15M sodium nitritewas then added and the pink colour disappeared. The mixture was left for 30 minutes. Solid urea was then added to remove nitrous acid which was indicated when starch iodide paper did not turn blue. The diazotised cellulose was then washed three times with 30ml of ice-cold distilled water and once with 30ml of 0.2M borate buffer, pH 8.3, in a refrigerated centrifuge MSE Model 6L, at 4°C and 2,000 r.p.m. for 5 minutes. The precipitate was then suspended in 4ml of the 0.2M borate buffer, pH 8.3 to give a concentration of 25mg diazotised cellulose per ml. The success of the diazotisation was confirmed when a few drops of the suspension gave a bright orange colour

when added to β-naphthol in 0.2M borate buffer.

Ferritin-immunoadsorbent (F-ImAd) was synthesised by the addition of 100mg of horse spleen ferritin crystals (Calbiochem, Grade A) to 4ml of the diazotised-cellulose suspension (100mg diazotised cellulose). The solution was mixed and left in the dark at 4°C for 3 days with occasional mixing. Excess diazo groups were combined with 0.4g glycine in 10ml of 0.2M borate buffer by stirring the mixture constantly for 60 minutes (Miles et al, 1974). Excess ferritin was removed by washing the F-ImAd complex ten times with 10ml 0.04M phosphate buffer, pH 7.4, in the refrigerated centrifuge at 4°C and 2,000 r.p.m. The ferritin concentration was determined in the supernatant by the method of Lowry, (1951) and thereby the amount of antigen bound to the diazotised cellulose was calculated. The complex was then washed three times with 10ml 0.05M barbitone buffer, pH 8.0 and finally suspend in 5ml of this buffer to give a concentration of 20mg cellulose per ml. The suspension was stored in the dark at 4°C and was stable for at least 12 months. It was found that 300 to 390mg of horse ferritin was bound by 1g of cellulose. A commercial preparation of aminocellulose (Miles Laboratories) which became available after the method was established was treated in the manner described above and was found to bind 210mg ferritin per g cellulose.

Theoretical Aspects of Ferritin Quantitation

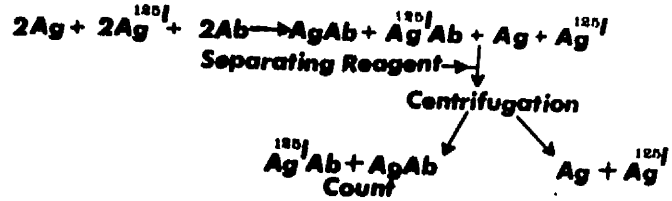
Radioimmunoassay (RIA) or saturation analysis was first described by Yalow and Berson and independently by Ekins in 1960. This technique has since been applied to the quantitation of more than two hundred substances present in low concentrations in biological fluids (Ekins, 1974) and is dependent on the competition of a compound and its radiolabelled derivative for a limited amount of anti-body. In 1968 Miles and Hales described an immunoradiometric assay (IRMA) which theoretically has improved sensitivity and precision (Rodbard and Weiss,

1973) when compared to RIA. IRMA measures compounds directly in the presence of excess radiolabelled antibody. A further development occurred in 1971 with the introduction of 2 site-IRMA also known as junction test, sandwich technique or direct radioimmunoassay (Addison and Hales, 1971). The test substance is first insolubilised on a solid matrix. An excess of radiolabelled antibody is then added and its uptake is directly proportional to the concentration of the antigen. The three methods have been compared by Woodhead et al, 1974 and Miles, 1976 and are diagramatically described in Figure 1,2.

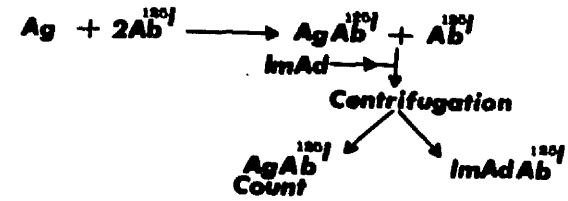
The presence of ferritin in normal serum was unrecognised until Addison et al in 1972 described an IRMA method with sufficient sensitivity to detect the compound. Radiolabelled anti-ferritin was added to serum and the free anti-body was then insolubilised by the addition of an immunoadsorbent of horse ferritin diazotised to amino-cellulose. The activity in solution was proportional to the ferritin concentration. Miles et al,(1974)applied 2 site-IRMA to the assay of serum ferritin which is dependent on the presence of at least two immunologically reactive groups on the antigen as it is first combined with rabbit anti-ferritin which coats the wall of the reaction vessel and then with highly specific radiolabelled anti-ferritin. After removal of the antibody the activity which remains bound to the side of the reaction vessel is directly proportional to ferritin concentration.

Preparation of Solid-Phase Antibody A number of substances have been used as matrices in the preparation of the solid phase antibody including agar gel, cyanogen-bromide activated cellulose, cellulose paper, polytetrafluoroethylene discs, polyethylene, polystyrene and polypropylene. The most popular are the plastic tubes which were first described by Catt and Tregar in 1967. Test tubes made of polypropylene or polyethylene take up buffered antibodies, probably by hydrophobic bonding (Catt and Tregar, 1967). Catt and Tregar, 1967 determined that

RIA



IRMA



2-site IRMA

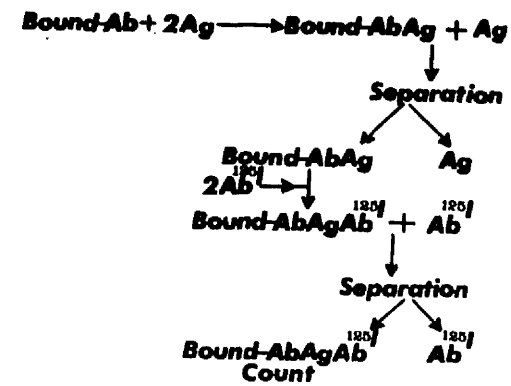


Fig. I, 2

Reaction steps in RIA, IRMA and 2-site IRMA

for optimum uptake the buffer should be alkaline. The incubation time was not critical as identical antibody uptakes were found at one minute and sixteen hours. Miles (1976) has reported that washing does not significantly remove buffer from the wall of tubes but poor replication and a reduced dose-response occurs on storing the tubes and it is generally accepted that two weeks is the maximum length for storage although this may be increased if they are stored frozen and containing buffer. However the buffered antibody itself is remarkably stable and in the present work has been successfully used in repeated assays over a four week period. The tubes have a limited capacity for immunoglobulin G (IgG) and although there is little alteration in the dose-response curve at widely varying antibody dilutions, maximum sensitivity is obtained at the highest dilution. Low titre anti-serum gives poor results (Miles, 1976) as albumin interferes with the uptake of IgG at low dilutions but this can be overcome by purifying the antibody, linking the specific antibody to the matrix with IgG or by using an alternative matrix. After removal of the buffered antibody the tubes must be washed with buffer containing albumin to saturate the remaining sites and remove trace amounts of free antibody. The extent to which the bound antibody decreases in activity varies with experimental conditions but considerable losses usually occur (Joustra, 1976). However as the antibody is taken up in excess these losses may not be important. These steps reduce the non-specific uptake of the labelled antibody in reaction two and the binding of antigen to free antibody.

First Reaction Ferritin of diluted plasma is insolubilised by solid-phase antibody in the first reaction. Serum and plasma are well known to inhibit assays non-specifically and this can be overcome by diluting standards in antigen free serum or more easily with serum from another species which does not cross react. The inhibition is probably due to high molecular weight proteins, alpha$_2$-macroglobulin and immunoglobulin

M present in serum and not to albumin (Reuter et al, 1973). At high concentrations of antigen dissociation occurs from the solid-phase and therefore a reduced amount of labelled antibody is taken up in the second reaction and a false low concentration is determined. This is known as the "high dose hook effect" and occurs when the antigen is in excess and the labelled antibody is present in limited concentration (Miles, 1976).

The incubation times of the reactions are dependent on the kinetics of the system and on the sensitivity required. The dose-response curve and sensitivity can be increased by carrying out the first reaction to equilibrium which is in the order of twenty-four hours (Woodhead et al, 1974) and thus obtain maximum uptake of antigen. The effect of longer incubation times on the dose-response curve is more evident in the second reaction but this is accompanied by an increase in the zero-dose response (Miles, 1976).

The serum is removed after the initial incubation with the solid phase and the tubes washed to remove free antigen, rapidly dissociating antigen and serum factors which can directly but non-specifically inhibit the uptake of radiolabelled antibody. This will increase the dose-response and minimise the high-dose hook effect (Miles, 1976) but if the washing process is repeated some firmly bound antigen will be dissociated, thereby reducing the dose-response.

Preparation of Radiolabelled Antibody The success of 2-site IRMA depends on a highly specific radiolabelled antibody for addition in the second reaction. As many anti-sera are raised to only partially purified immunogens the specificity required of the antibody can only be gained by isolation with an immunoadsorbent. The antigen of the immunoadsorbent must be of a high order of purity and this may be difficult to obtain in the required quantity. However as in the case of ferritin the antigen of another species may be used provided it has immunological identity.

The iodination of antibody as opposed to antigen offers a number of advantages (Woodhead et al, 1974). Antibodies have a known structure and when present in adequate concentration are easily iodinated. The problem of radiolabelling some antigens with a low tyrosine content is also eliminated. Configurational changes can occur in protein structure on iodination and in the case of labelled antigen may result in the antibody which reacts with the unlabelled compound failing to react with the labelled molecule. It has further been shown that labelled antibodies are more stable on storage than antigens which can be very unstable in physiological fluids. However an advantage of 2-site IRMA is that the serum factor is not important as incubation with iodinated antibody takes place in buffered solution.

Radiolabelling has been mainly undertaken using the gamma emitting isotopes of iodine; ^{131}I and more recently ^{125}I. The latter has a number of advantages including a longer half-life of sixty days in contrast to eight days and a better counting efficiency (Hunter, 1974). A number of methods are available for the radiolabelling of proteins. The classical method is that of Hunter and Greenwood,(1962)who oxidised Na ^{125}I with chloramine-T to ^{125}I which then combined with tyrosine and perhaps histidine (Hunter, 1974) in the protein molecule. The ^{125}I was then reduced with sodium metabisulphite. Reuter et al, 1976 has reported that radiation damage to protein can be reduced using smaller amounts of chloramine-T than the original method when it was present in excess amounts. Lactoperoxidase in the presence of hydrogen peroxide has also been used as an oxidising agent (Thorell and Johansson, 1971) but although it benefits from being a milder oxidising agent than chloramine-T the yields are poor (Reuter et al, 1976). Gaseous chlorine has been used to oxidise Na ^{125}I in presence of protein (Butt, 1972) but the percentage incorporation of iodine is less than with the classical chloramine-T method (Hunter, 1974). Bolton and Hunter (1972) have also

described a method which is independent of tyrosine in the protein molecule. N-Hydrosuccinimide ester of 3-(4-hydroxyphenyl) propionic acid is labelled with ^{125}I using chloramine-T and is then extracted and dried. The compound then conjugates with the free amino groups on the protein molecule but although the latter has not been in direct contact with the oxidising agent nor Na ^{125}I, the specific activity is low.

Iodination of the antibody linked to immunoadsorbent is advantageous as it protects one binding site of the immunoglobulin although the second may be destroyed as evidenced by Miles and Hales (1968) who showed that in the assay of insulin, antibody preparations did not bind unfixed insulin with one site and later bind to the insulin immunoadsorbent with the second antigen -- binding site. Hendrick and Franchimont (1976) digested ^{125}I-labelled antibody with papain and found that only the Fab portion of the molecule was iodinated.

The iodine uptake of the bound antibody is dependent on the amount of immunoglobulin present, as small amounts cannot be successfully iodinated (Woodhead et al, 1974). A satisfactory specific activity is indicated when one to three atoms of ^{125}I are bound per molecule of antibody. Excess iodination gives rise to poor immunological activity (Addison and Hales, 1971) and rapid degradation (Hendrick and Franchimont, 1976).

The iodinated antibody is freed from the immunoadsorbent by dilute acid, guanidine or urea. This can be done by simply washing in a paper lined filter funnel or using columns of Sephadex or Sepharose. Methods are aimed at removing free Na ^{125}I which may iodinate non-specific proteins, damaged material and low affinity antibodies from the specific ^{125}I-antibody. The eluted immunoglobulin is immediately diluted with albumin containing buffer or absorbed onto immunoadsorbent in buffer and stored frozen at -20°C.

Second Reaction In the second reaction ^{125}I-labelled antibody in buffer

is added to the reaction tube after removal of serum and standard solutions. The amount of activity taken up by antigen bound to the solid phase is directly proportional to its concentration. An advantage of this method is the addition of ^{125}I-labelled antibody in buffer solution after removal of the test solution as the non-specific serum effect is eliminated.

Increasing the amount of ^{125}I-antibody to the test increased the dose-response, extends the assay range, suppresses the high-dose hook effect and the optimum time of incubation (Miles, 1976). These advantages are gained at the expense of an increase in the zero dose response and mean detectable dose. However as the zero dose response or non-specific uptake is normally less than two per cent it is possible to detect very small changes in uptake particularly if counting time is extended. The sensitivity of the assay can also be increased by carrying out repeated extraction of the antigen provided that the binding capacity of the solid phase is not saturated (Woodhead et al, 1974).

Increasing the incubation time of the reaction increases the dose-response and the high-dose hook effect. A fall also occurs in the high-dose response but this occurs more slowly and is only significant after seven days (Miles, 1976).

Unbound ^{125}I antibody can be simply removed by decantation and the tubes washed to ensure complete removal of the free immunoglobulin. Repeated washing however causes a decrease in the dose-response and it has also been reported that small variations in the washing technique can impair the precision of the assay.

Interpolation of Data Data can be interpolated from the graph obtained by plotting the radioactivity of the solid phase against the logarithm of concentration. The dose-response approximates a sigmoid curve and any response indicating the high dose hook effect is omitted from the

standard curve.

Interpolation of data is time consuming, liable to operator error and therefore much thought has been given to producing programmes which would allow computerisation of data. RIA has received most attention and Rodbard (1969) has mathematically represented a calibration curve by expressing the logit transform of the percentage bound as a straight line with respect to the logarithm of the concentration. Burger (1972) and Healy (1971) have also produced programmes for evaluation of RIA data. Identical data processing methods can be applied to IRMA and 2-site IRMA (Miles, 1976) and Rodbard (1974) has shown that it can be analysed by fitting zero-dose response, infinite-dose response, slope and mid-point of the dose-response curve and carrying out a linear transformation by logit/log plot. England and Cain (1976) have applied progressive quadratic analysis to RIA data and this has been successfully applied to data obtained from a 2-site IRMA of ferritin.

Quantitation of Plasma and Tissue Ferritin

Ferritin was quantitated in human plasma and tissue extracts by the 2 site-immunoradiometric method of Miles et al,(1974). The solid phase antibody was formed by incubating diluted anti-human ferritin in polypropylene tubes. After removal of unbound antibody the standard and test solutions were added and incubation took place at $4^{\circ}C$ for twenty-four hours to allow maximum uptake of ferritin by the solid phase antibody. The tubes were then washed to remove free antigen prior to the addition of ^{125}I-anti-human ferritin which bound to the ferritin attached to the solid phase antibody during a forty-eight hour incubation at $4^{\circ}C$. Unbound material was removed, the tubes washed and their radioactivity measured by an automatic gamma counter. The amount of ^{125}I bound by the tubes was directly proportional to the ferritin concentration.

Reagents 1. Anti-ferritin in 0.2M sodium bicarbonate, pH 9.2 (1:10,000). This solution was stable for at least six weeks and could be reused in ten assays when stored at 4°C.

2. BSA buffer. 10.3g sodium barbitone, 6.0g sodium chloride and 0.2g sodium azide in 1 litre distilled water. The pH was adjusted to pH 8.0 ± 0.05 with 5M hydrochloric acid. 5g of bovine serum albumin (Sigma) was added immediately before use.

3. NIGR buffer was prepared as for BSA buffer but rabbit inactivated serum (Wellcome) was added in the ratio of 1:20.

4. Standard Solutions. Stock human spleen ferritin (10mg per litre) prepared as described under "Isolation and Purification of Human ferritin from Spleen and Placenta" was diluted 1:25 with BSA buffer to give a final concentration of 400ug ferritin per litre. This solution was further diluted to give standards from 2 to 400ug ferritin per litre which when diluted 1:20 with NIGR buffer gave values from 0.1 to 20ug per litre. The standards were prepared as follows:-

Actual Concn. (ug/l)	400	300	200	150	100	40	20	10	5	2
Stock Std. (ml)	2	1.5	1	3	1	1	0.5	0.1	0.1	0.05
BSA (ml)	-	0.5	1	5	3	9	9.5	3.9	7.9	9.95
Assay Concn. (ug/l)	20	15	10	7.5	5	2	1	0.5	0.25	0.1

5. ^{125}I Anti-ferritin. 1.0ml of stock ^{125}I rabbit anti-human ferritin in 5ml BSA buffer. The volume of solution which contained 20,000 counts per minute was calculated.

6. 0.04M Phosphate buffer pH 7.3. 10 tablets (Oxoid-Phosphate Buffered Saline - Dulbecco 'A') in 1 litre distilled water.

Preparation of Tissue Extracts Post mortem specimens of f etal spleens, livers and hearts were washed free of blood and stored at -20°C. Specimens of placentae were cut according to Plate [1], IV after the organ had been visually examined. The most normal areas were chosen in every case. Peripheral and central sections of the f etal and maternal surfaces were washed free of blood, lightly blotted to remove excess water and stored at -20°C.

Tissue ferritin, HPL and HCG in the placentae only were assayed by initially thawing the specimens overnight at 4°C. Approximately 1g sections were cut, lightly blotted and weighed. 10ml of 0.05M Barbitone buffer, pH 8.0 was added and the tissues homogenised using an Ultraturrax (Janke Kunkel) apparatus. The homogenate was filtered through Whatman No. 541 filter paper. The ferritin content was assayed by diluting the filtrate 1:5 with BSA buffer. Further dilutions were made with NIGR buffer using an automatic diluter (Micromedic Systems Inc.) to give final dilutions of 1:1,000 and 1:2,000 for placentae and fetal hearts and 1:2,000, 1:5,000 and 1:10,000 for fetal livers and spleens.

Placental HPL was assayed in the filtrate of the homogenate which contained approximately 1g tissue per 10ml 0.05M Barbitone buffer pH 8.0 and also in a 1:5 dilution of the filtrate.

Placental HCG was assayed in a 1:10 and 1:20 dilution of the filtrate of the tissue homogenate.

Method 1. Conical, polypropylene tubes (Sarstedt No. 39/10) were placed in plastic racks which held 100 tubes. The racks were designed in the laboratory and consisted of two sheets of perspex joined only at the four corners. Holes were drilled so that the tubes fitted snuggly; tops being one cm above the rack. The design allowed for rapid washing procedures as overflow material was contained in a drip tray and racks could be quickly decontaminated by washing in running water.

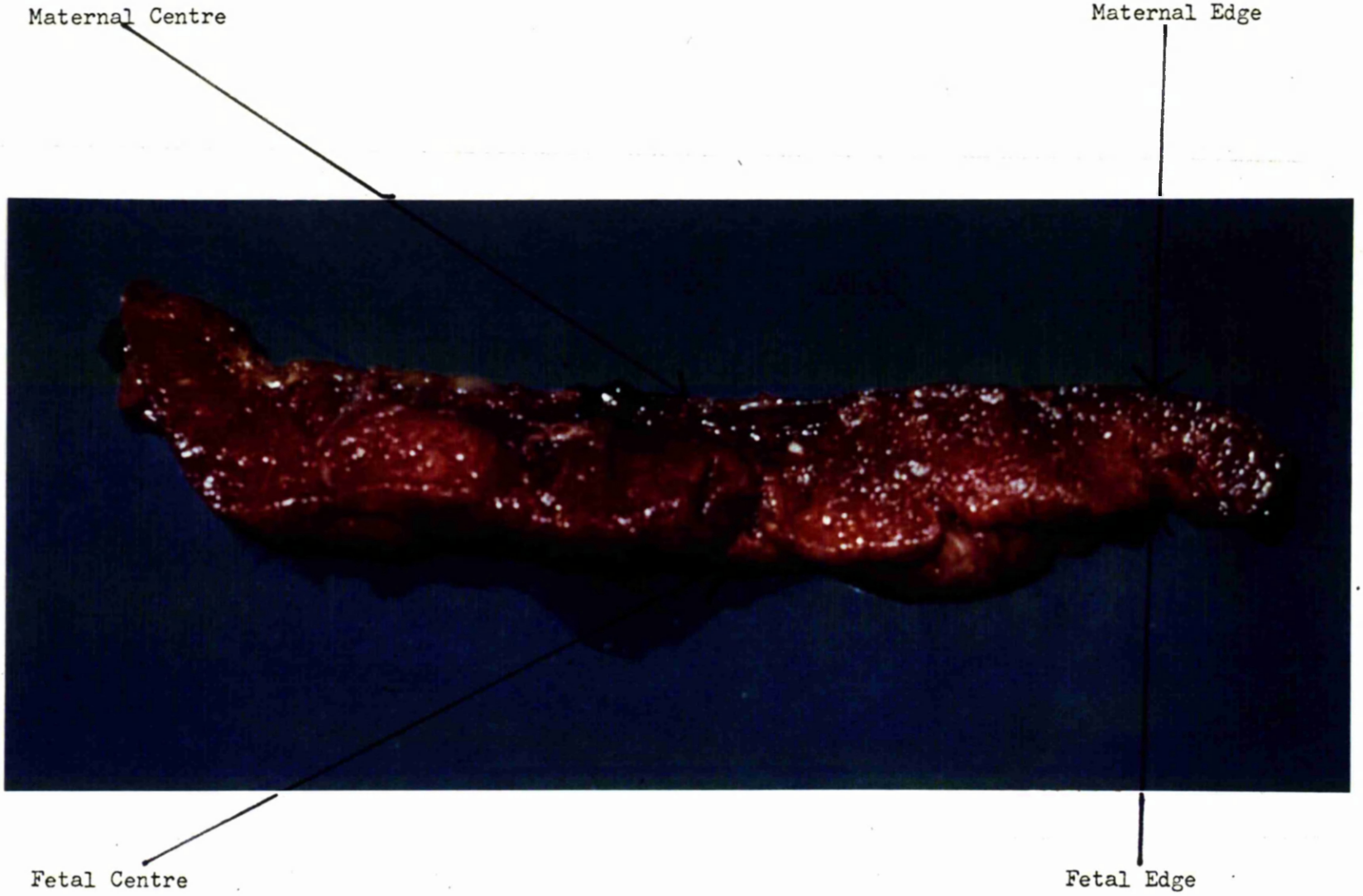


Plate 1. IV

0.5ml of 1:10,000 anti-human ferritin in 0.2M sodium bicarbonate, pH 9.2 was dispensed into each tube from an Oxford automatic pipette. The tubes were incubated for 24 hours at $4^{o}C$.

2. The anti-sera was aspirated from the tubes and stored for further use. The tubes were then washed twice with B.S.A. buffer and twice with glass distilled water and were finally sucked out twice with a Pasteur pipette attached to a water-pump but no attempt was made to totally remove fluid. They were used within 3 hours.

3. 200ul of NIGR buffer and diluted standard, control or test solutions were added to the anti-body coated tubes using a high speed automatic diluter (Micromedic Systems Inc.) Standard solutions, in triplicate were diluted 1:20 with BSA buffer and neonatal plasmas 1:100 with NIGR buffer. Appropriate dilutions of 1:1,000 to 1:100,000 of tissue extracts were made with NIGR buffer. Test samples were carried out in duplicate. Tubes were incubated at $4^{o}C$ for 24 hours.

4. Solutions were aspirated from the tubes and washed once with BSA buffer. They were then sucked out twice but were not completely dry.

5. An aliquot of ^{125}I-anti-ferritin was thawed and diluted in approximately 5ml of BSA buffer. The approximate amount of antibody which gave 20,000 counts per minute was taken up by the Micromedic diluter and dispensed into the tubes with the calculated volume of additional BSA buffer to give a total volume of 200ul. The tubes were incubated at $4^{o}C$ for 48 hours.

6. The free ^{125}I-anti-ferritin was removed from the tubes which

were then washed twice with 0.04M phosphate buffer pH 7.4. The tubes were loaded into an LKB (Wallac) gamma sample counter (Model 80000) and counted for 4 minutes.

7. A dose-response curve was prepared by plotting radioactivity against the logarithm of the ferritin concentration. The graph approximates a sigmoid curve. The concentration of the test solutions were initially interpolated from the graph but latterly a Hewlett Packard calculator (Model 9810A) was used which had been programmed for a progressive quadratic equation. Counts were typed into the machine and concentrations were calculated directly.

PRODUCTION OF ^{125}IODINE ANTI-HUMAN FERRITIN

^{125}I anti-human ferritin was produced by first isolating the specific anti-ferritin immunoglobulin from whole rabbit serum by incubating with horse spleen ferritin immunoadsorbent (F-ImAd) followed by iodination of the antibody-ferritin-immunoadsorbent (Ab-F-ImAd) complex with ^{125}I according to Hunter and Greenwood (1962). The elution of the labelled antibody was initially carried out by a modification of the method of Addison et al (1972). The Ab-F-ImAb complex was first washed in a double lined filter paper with buffer and followed by a dilute acid solution to remove low affinity antibodies. The ^{125}I anti-ferritin was then eluted with a stronger acid solution. Due to the specicity of the anti-serum which has been produced it was not necessary to re-isolate the labelled anti-ferritin on F-ImAd as described in the original paper as sensitivity was maintained by freezing the ^{125}I anti-ferritin at -20^{o}C immediately after elution. The anti-sera lost a little of its sensitivity after eighteen months storage at -20^{o}C but this was regained when the method of Miles et al (1974) was used to elute ^{125}I anti-ferritin from the ^{125}I-Ab-F-ImAb complex. Sephadex was used to support the complex which was washed successively with barbitone buffer and sodium acetate. The bulk of ^{125}I anti-ferritin was recovered by extraction with dilute acid and little with a linear hydrogen ion gradient which was there after omitted. This is in contrast to the ^{125}I anti-ferritin which was liberated from human F-ImAd with the gradient ion elution (Miles et al, 1974).

REAGENTS All reagents were from BDH-AnalarGrade unless otherwise stated.

Rabbit anti-human ferritin - produced as previously described.

Rabbit Serum - inactivated - Difco

Ferritin-Immunoadsorbent - (F-ImAd)-produced as previously described.

0.3M Phosphate Buffer pH 7.4 containing 0.15M sodium chloride.

^{125}I (Sodium ^{125}Iodide in dilute sodium hydroxide pH 8.11. IMS 30- Radiochemical Centre, Amersham)

Chloramine-T-Solution. 13mg of Chloramine-T dissolved in 5ml 0.3M phosphate buffer immediately before use.

Sodium meta bisulphite solution. 13mg of sodium metabisulphite dissolved in 2ml 0.3M phosphate buffer immediately before use.

Potassium Iodide Crystals.

0.1M Barbitone Buffer, pH 8.0 containing 0.22M sodium chloride and 10g per litre albumin.

2.06g sodium barbitone, 1.2g sodium chloride and 0.04g sodium azide - Dissolved in 100ml distilled water.

The pH was adjusted to pH 8.0 $\pm$ 0.05 with 5M hydrochloric acid and 1g of bovine serum albumin (Sigma) added.

BSA Buffer 0.05M Barbitone Buffer, pH 8.0 containing 0.11M sodium chloride and 5g per litre albumin.

10.3g sodium barbitone 6.0g sodium chloride, and 0.2g sodium azide. - Dissolved in 1 litre distilled water.

The pH was adjusted to pH 8.0 $\pm$ 0.05 with 5M hydrochloric acid and 5g of bovine serum albumin (Sigma) added.

Hydrochloric Acid pH 3.0

pH 2.0

Distilled water was adjusted to pH 3.0 $\pm$ 0.05 and pH 2.0 $\pm$ 0.05 with 5M hydrochloric acid.

0.5M Potassium Iodide 83g of potassium iodide dissolved in 1 litre distilled water.

0.1M Sodium Acetate pH 4.5 8.2g of sodium acetate dissolved in 1 litre distilled water and adjusted to pH 4.5 with 0.1M acetic acid.

0.001M Hydrochloric Acid

Method 1 (after Addison et al, 1972)

0.1ml of F-ImAd was washed four times in a centrifuge at 2,500g with 0.3M phosphate buffer, pH 7.4 to remove free ferritin. 2ML of anti-human ferritin serum was then added to 1 5ml capacity stoppered test tube. The non-specific binding was monitored by adding 2ml of rabbit serum to 0.1ml of washed F-ImAb. The tubes were left at 4^{0}C for four days with occasional mixing. The complexes were then washed four times with 0.3M phosphate buffer, pH 7.4 and finally suspended in 1ml of the buffer. The protein content was measured by the method of Lowry (1951) as described under "Ferritin Isolation". The non-specific binding was between 30 to 35 per cent of anti-ferritin binding.

Iodination was performed in a polystyrene tube (0.75cm diameter) cut to contain 1.0ml. A small glass mixing device was made from a sawn off portion of a paper clip which was then encased with glass by heating the metal in the thin portion of a Pasteur pipette and sealing at both ends. The tube and beetle were held in position on a magnetic stirrer with placticene and the whole was within a fume cupboard with extractor fan in operation. ImCi Ci of ^{125}I (10ul) was added to the tube followed in rapid succession by 50ul Ab-F-ImAd (50 to 100ug anti-ferritin) and 10ul Chloramine-T. The reactants were mixed for 30 seconds before the reaction was stopped with 20ul sodium meta bisulphite. A filter funnel (diameter 2.5cm) was lined with a double thickness of Whatman No.541 filter paper which was moistened with 0.3M phosphate buffer, pH 7.4 containing a few crystals of potassium iodide. A few drops of 0.05M barbitone buffer pH 8.0 was added to the reaction tube before transferring the total volume with a Pasteur pipette to the filter paper.

The complex was washed with 200ml 0.05M barbitone buffer, pH 8.0 and 100ml hydrochloric acid, pH 3.0. 4ml of hydrochloric acid, pH 2.0 was then added and the eluate collected in 4ml 0.1M barbitone buffer containing 0.4mg albumin. This process was repeated for a second time. Aliquots of the fractions giving 7 x 10^6 counts per minute were rapidly frozen at -20°C.

Method II after Miles et al (1974).

Anti-human ferritin was iodinated with ^{125}I while bound to the F-ImAd complex as described in the previous section. The ^{125}I IAb-F-ImAd complex was transferred to 10 cm column of Sephadex G50-fine which had previously been washed with 0.5M potassium iodide and 0.05M barbitone buffer containing 10g albumin per litre and equilibrated with the buffer. The complex was transferred to the column with a Pasteur pipette and was washed successively with 20ml 0.05M barbitone buffer, 10ml 0.1M sodium acetate, pH 4.5 and 10ml 0.001M hydrochloric acid. The ^{125}I anti-human ferritin was eluted with 20ml of a linear hydrogen ion gradient from 0.001M to 0.01M HCl. 1ml fractions of eluate were collected in 1ml BSA buffer and stored at -20°C. Figure I,3 demonstrates the elution pattern of the radioactivity.

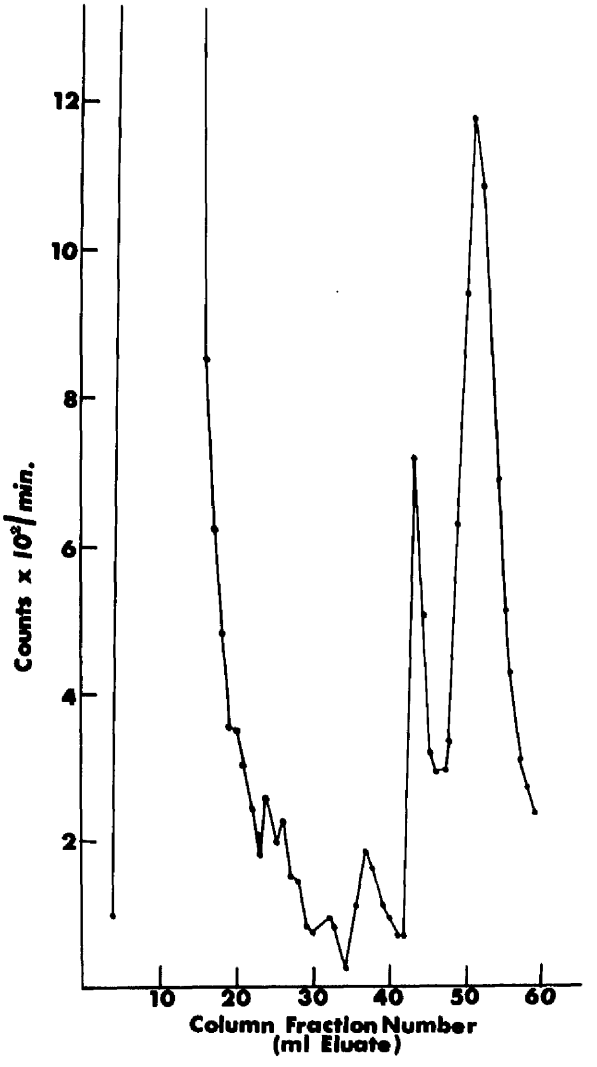


Fig.I, 3

The elution pattern of radioactivity from an Ab-F-Im Ad complex iodinated with ^{125}I.

The column was washed with BSA-buffer (No. 3-20), acetate buffer, pH 4.5 (No. 21-30), 0.001M HCL (No. 31-40) and hydrogen ion gradient 0.001 M HCL to 0.001 M HCL (No. 41-60)

Quantitation of Plasma Iron

Plasma iron was reduced to the ferrous form with ascorbic acid and liberated from transferrin by acid diluent. After protein precipitation with trichloracetic acid the colour was developed using a sensitive colour reagent Ferro-Zine. The method was adapted from Giovaniello et al (1967) and Stookey (1970).

Reagents

Acid Reagent 16.6ml of concentrated hydrochloric acid was added to 750ml distilled water followed by 1g neocuproin hydrochloride (2,9-Dimethyl-1,10-phenanthroline hydrochloride) (BDH) and made up to 1 litre. Stored at $4^{o}C$.

Acid Diluent 2g ascorbic acid (BDH-Analar) was dissolved in 200ml acid reagent immediately before use.

Colour Reagent 0.2g Ferro Zine Powder (3-(2-Pyridyl)-5,6-bis (4-phenylsulphonic acid)-1,2,4-Triazine) (Hach Diagnostics, USA) was dissolved in 1 litre distilled water and stored at $4^{o}C$.

Saturated Sodium Acetate 140g of sodium acetate (BDH-Analar) was added to 100ml distilled water and placed in a water bath at $56^{o}C$ until dissolved. Stored at $37^{o}C$.

1.2M Trichloracetic acid (BDH - Low in Iron for Haematology).

Stock Iron Standard (BDH) Ferric chloride solution containing 17.9mmol per litre.

Working Iron Standard Standards were prepared to cover the range 5.0 to 50.0umol per litre.

Method

0.2ml of distilled water (blank), plasma or standard solution was added to 0.2ml of acid diluent in a 2ml disposable tube. The contents were mixed in a Vortex mixer and left for 15 minutes. 0.2ml of 1.2M TCA was then added, the contents mixed and centrifuged at 2,000g for 10 minutes. 0.4ml of the supernatant was than added to 0.32ml of colour reagent

followed by 0.05ml of saturated sodium acetate. The contents were mixed and then left for 10 minutes. The colour was then determined in a micro cuvette (20mm light path, 0.8ml capacity) at 560nm on the Unicam SP 600.

Quantitation of Transferrin and the Percentage Iron Saturation

Plasma transferrin was quantitated using the immunological technique of single radial diffusion. The transferrin diffused radially from a small cylindrical well into a thin layer of agar gel which contained anti-transferrin. A precipitin ring was formed of which the diameter was directly proportional to the concentration of transferrin (Mancini et al, 1964). The percentage iron saturation was calculated from a knowledge of the iron and transferrin concentrations. It was assumed that the molecular weight of transferrin was 76,000 daltons (Morgan, 1974) and that one molecule of transferrin bound two molecules of iron.

Reagents

Barbitone buffer, pH 8.6 I = 0.05

Buffered Agar Gel - 0.9 per cent

Rabbit Anti-transferrin - Behringwerke.

Standard Human Transferrin - contained in Standard Human Serm - Behringwerke.

Method

A test tube of agar (10ml) was placed in a boiling water bath until the gel melted. It was then allowed to cool to 50-60°C. 0.1ml anti-transferrin was added and the solution immediately poured onto a glass slide (8 x 8cm) and allowed to set - about 30 minutes. Wells werecut 1mm in diameter and 1.5cm from the nearest well. Plasma was diluted with 0.16M sodium chloride 1:20 and 1:40 for maternal specimens and 1:20 for neonatal specimens. The wells were filled with standard and test solutions, in duplicate, using a micro-cap pipette (Drummond) until the meniscus just disappeared. The plates were left for 48 hours at room temperature in a humid atmosphere to allow complete diffusion. The diameter of the diffusion circle was read in mm using a MacSwinney rule and plotted linearly against the logarithm of the transferrin

concetration. The concentration of transferrin in the unknown was determined from the graph. After the concentration of iron and transferrin had been quantitated the percentage saturation was calculated using the formulae $3.8 \times 10^3 \times \frac{\text{plasma iron umol/l}}{\text{plasma transferrin mg/l}}$

Quantitation of Haemoglobin

Haemoglobin was converted by the action of ferricyanide to methaemoglobin which was then converted to cyanmethaemoglobin by the action of potassium cyanide.

Reagents

1. Isoton II - Coulter Electronics
2. Zap-oglobin - Coulter Electronics

Method

A well mixed specimen of whole blood (E.D.T.A.) was diluted 1:501 with isoton. 6 drops of Zap-oglobin was added to 20ml of the diluted blood and the haemoglobin concentration was determined directly on a Coulter Haemoglobinometer.

ASSAY of 2,3 - DIPHOSPHOGLYCERIC ACID (2,3-DPG)

Three enzymatic reactions were involved in the assay (Lowry, 1964 and Sigma, 1974).

1. 2, 3-DPG is hydrolyzed to 3-Phosphoglycerate (3-PGA) and inorganic phosphorus (P).

   The enzyme which catalyzes this reaction is present in purified preparations of phosphoglycerate mutase (PGM) and is termed 2,3-DPG phosphatase.

   2-Phosphoglycolic acid is needed as a stimulator for this reaction.

$$2,\ 3\text{-DPG} \xrightarrow[\text{2-Phosphoglycolic acid}]{\text{2,3-DPG phosphatase}} \text{3-PGA+P}$$

2. 3-PGA reacts with adenosine -5'-triphosphate (ATP) in the presence of 3-phosphoglycerate phosphokinase (PGK) to form 1,3-DPG and adenosine - 5'-diphosphate (ADP).

$$\text{3-PGA+ATP} \xrightarrow{\text{PGK}} \text{1,3 -DPG+ADP}$$

3. 1,3-DPG oxidizes nicotinamide adenine dinucleotide-reduced (NADH) to NAD in the presence of glyceraldehyde -3-phosphate dehydrogenase (GAPD) and is reduced to glyceraldehyde -3-phosphate (G-3-P).

$$\underset{(\text{HIGH } A_{340})}{\text{1,3-DPG+NADH}} \xrightarrow{\text{GADP}} \underset{(\text{LOW } A_{340})}{\text{g-3-P+NAD}}$$

The decrease in absorbance at 340nm caused by the oxidation of NADH to NAD reflects the amount of 2,3-DPG originally present.

REAGENTS

NADH pre-weighed vials containing 1mg NADH (Sigma) were kept in the dark at room temperature.

Triethanolamine Buffer Solution (Sigma Stock No.665-5)

0.2M triethanolamine, magnesium ions and EDTA. Stored at 4^{0}C.

ATP (Sigma Stock No. 35-5)

500mg of disodium salt of ATP were dissolved in 5ml buffer. This solution was stable when stored at -20^{0}C in 1ml aliquots.

GAPD/PGK enzyme mixture (Sigma Stock No.366-2)

A suspension in ammonium sulphate of glyceraldehyde-3-phosphate

dehydrogenase (rabbit muscle) 800u per ml and 3-phosphoglycerate phosphokinase (yeast), 450u per ml.

Phosphoglycerate mutase (Sigma Stock No. 665-3)

A suspension in ammonium sulphate of phosphoglycerate mutase (rabbit muscle), 2,400u per ml.

Phosphoglycolic acid (Sigman Stock No. 665-2)

50mg vial reconstituted with 5ml water. Stable at -20^{0}C and stored as 1 ml aliquots.

0.5M Trichloroacetic acid 8g of T.C.A. were dissolved in 100ml distilled water and stored at 4^{0}C.

Preparation Supernatant. 1.0ml of fresh blood was pipetted into 3.0ml of cold 8% TCA and shaken vigorously. After standing for 15 minutes at 4^{0}C the mixture was centrifuged at 2,000g. The clear supernatant was analysed. (Blood used should be extracted within 2 hours but the TCA supernatant was stable for 7 days).

Quantitation Haemoglobin. Haemoglobin was estimated on the blood specimen by adding 20µl blood to a test tube containing 5ml of 0.4% ammonium hydroxide. The contents were mixed and the absorbance read immediately at 542n.m. The haemoglobin content was calculated using the equation:

$$OD_{542} \times 28.2 = gHb/100ml$$

Analysis A 1mg vial of NADH was reconstituted with 8.0ml triethanolamine buffer.

Into 2 tubes marked BLANK and TEST the following were pipetted:-

	BLANK	TEST
NADH solution	2.5ml	2.5ml
ATP	0.1ml	0.1ml
Distilled Water	0.25ml	-
TCA supernatant	-	0.25ml

The tubes were mixed by inversion and 0.02ml GAPD/PGK and 0.02ml Phosphoglycerate mutase added to both tubes.

The contents were mixed and left for approximately 5 minutes.

The initial absorbance at 340n.m. of blank and test against water was determined on a Unicam SP 800.

The solutions were returned to tubes and 0.1ml Phosphoglycolic acid added to both. The contents were mixed by inversion and left for 30 minutes at room temperature to allow the reaction to go to completion. The final absorbance was then determined at 340 n.m against water.

Calculation

blank OD = initial OD - final OD.

test OD = initial OD - final OD

corrected test OD = test OD - blank OD

$$2,3\text{-DPG} = \text{corrected test OD} \times \frac{770}{\text{gHb/100ml}} \quad \text{umol/gHb}$$

Quantitation of Human Placental Lactogen (HPL)

HPL was assayed in plasma and tissue extracts by radioimmunoassay using ^{125}I labelled antigen and absolute alcohol to precipitate the antigen-antibody complex.

Reagents 0.06M Barbitone Buffer pH 8.6.

Assay Diluent 0.06M Barbitone buffer pH 8.6 containing 0.5mg/ml crystalline human serum albumin.

Antiserum Rabbit anti-H P.L raised by injecting a New Zealand White Rabbit with a saline solution (1mg/ml) pure HPL (National Biochemical Co.) emulsified with Freunds adjuvant.

The antisera was used at an initial dilution of 1:200 with assay diluent.

Stock Standard HPL 0.1ml of HPL in assay diluent at a concentration of 240mg per litre was stored at $-20^{\circ}C$.

Working HPL Standard 0.1ml of stock standard HPL was diluted 1:10 with assay diluent to give a concentration of 24mg per litre. Doubling dilutions were made to give concentrations from 0.375 to 24.0mg per litre.

^{125}I HPL ^{125}I HPL was prepared using the method of Hunter and Greenwood (1962).

Method

100ul of citrated plasma, standard or test solutions were dispensed into tubes followed by 100ul of ^{125}I-HPL, 600ul of assay diluent and 200ul of HPL anti-serum. The contents of the tubes were mixed and incubated for 1hr. at room temperature. 2ml of absolute alcohol were added to all except the "blank" tubes, before mixing and reincubating for 10 mins. The tubes were centrifuged at 1,300g for 10 mins. and the supernatant removed by inverting the tubes. The precipitate was counted

on an automatic gamma counter (Walloc, LKB) and the percentage of the fraction bound was calculated. The latter was plotted against concentration on semi-log paper.

Quantitation of Human Chorionic Gonadotrophin (HCG)

HCG was assayed in tissue extracts by radioimmunoassay using ^{125}I HCG as labelled antigen and a second antibody to precipitate the antigen antibody complex.

Reagents Phosphate Buffer, pH 7.3 containing 0.16M sodium chloride and 2g per cent albumin.

Stock Standard HCG A World Health Organisation preparation of HCG containing 5,300 iu per ampoule was used.

Working HCG Standards Ranged from 1.7 to 424m Iu per litre.

Anti-HCG Rabbit anti-human LH (HCG) obtained from Wellcome was made up to 10ml with Phosphate buffer, pH 7.3 (1:20,000).

^{125}I-HCG obtained from Prof. Bagshawe, Dept. Medical Oncology, Charing Cross Hospital, Fulham.

Working ^{125}I-HCG A 0.5ml aliquot of the stock material was diluted 1:50 with Phosphate buffer, pH 7.3 to give a concentration of 1ng per ml.

Rabbit Inactivated Serum Wellcome. Diluted 1:120 with Phosphate buffer, pH 7.3.

0.1M Ethylenediaminetetra-acetic acid 9.306g EDTA (BDH-Analar) were dissolved in 250ml distilled water.

Donkey Anti-Rabbit Serum (Wellcome) Diluted 1:12.5 with Phosphate buffer, pH 7.3.

Method

100ul of citrated plasma, standard or test solutions were dispensed into tubes followed by 100ul of ^{125}I-HCG, 400ul of assay diluent and 100ul of HCG antiserum. The contents of the tubes were mixed and incubated ay $4^{\circ}C$ for 48 hrs. 100ul of rabbit serum, 100ul of 0.1M EDTA solution and 100ul of donkey anti-rabbit serum were added to all except the "blank" tubes. The contents were mixed and incubated at $4^{\circ}C$ for

24hrs. After centrifugation at 2,000g for 45 mins. at 4°C, the supernatant was removed by suction and the precipitate counted on an automatic gamma counter (Wallac-LKB 8000). The percentage of the fraction bound was calculated and plottedagainst concentration on semi-log. paper.

PREPARATION OF PLACENTAL TISSUE FOR ELECTRON MICROSCOPY

The whole block immunoperoxidase technique of Nakane (1970) was used to visualise placental ferritin.

REAGENTS:

FORMALIN/SALINE: 10 per cent formalin in 0.15 M sodium chloride

TRIS BUFFER: 6.05g Tris Buffer (Sigma 1503) in 1 litre distilled water pH to 7.6 with HCL.

TRIS BUFFERED SALINE: (TBS) contained 20ml Tris Buffer (pH7.6) and 180 ml 0.15 M sodium chloride.

DIMETHYL SULPHOXIDE (DMS) 10 per cent in distilled water.

GLUTARALDEHYDE 5 per cent in distilled water.

DIAMINOBENZIDENE TETRAHYDROCHLORIDE (DAB) 3mg DAB in 10ml Tris buffer (pH7.6)

DAB/ peroxide 3 mg DAB in 10ml Tris buffer (pH7.6) + 0.1 ml 2 per cent H_2O_2

METHOD

1) The tissue was fixed for 8hrs in 10 per cent formalin in saline.
2) Washed overnight in TBS
3) Treated with 10% DMSO (1hr)
4) Washed TBS (5 min)
5) Treated with rabbit anti-human placental ferritin (24hrs)
6) Washed TBS (24hrs)
7) Treated with peroxidase labelled swine anti-rabbit serum (Mercia) (24hrs)
8) Washed TBS (24hrs)
9) Post fixed in 5 per cent glutaraldehyde (4hrs)
10) Washed overnight TBS
11) DAB (1-2hrs)
12) DAB/H_2O_2(15mins)
13) Washed with distilled water(2hr)
14) Treated with 1 per cent osmium tetroxide (4hrs)
15) Processed on resin-Epon

Chapter TWO

EXPERIMENTAL METHODOLOGY

A number of variable factors exist in the quantitation of ferritin which require evaluation for the assay to be performed at maximum sensitivity and precision.

Study of Factors Affecting the Ferritin Assay

Determination of Solid-phase Anti-ferritin Concentration

^{125}I-Human anti-ferritin was diluted in 0.05M barbitone buffer, pH 8.0 and 0.2M sodium bicarbonate, pH 9.2. The uptake of the antibody from the former diluent was unacceptably variable and the latter diluent was used in subsequent assays. Anti-ferritin was diluted 1:100, 1:1,000, 1:5,000, 1:10,000 and 1:20,000 in 0.2M sodium bicarbonate, pH 9.2. Standard ferritin solutions diluted 1:20 with NRS buffer and four plasmas diluted 1:20 with BSA were assayed using tubes coated with the varying antibody concentrations. Figure II,1 indicates that the dose response curve was inversely proportional to the antibody dilution up to 1:10,000 and that the best response was obtained at that dilution. The zero dose response was significantly lower only at the 1:100 dilution. However with a second anti-serum in which the optimum dilution was also 1:10,000 the zero dose response decreased with decreasing antibody concentration.

The mean of the plasma ferritin concentrations at solid-phase antibody dilutions of 1:1,000, 1:5,000 and 1:10,000 was 1.7 (± 0.4), 8.4 (± 0.2), 45.0 (± 2.6) and 213.3 (± 15.3) ug per litre. The coefficient of variation are within acceptable limits and support the results obtained by varying the solid-phase antibody concentrations. This suggests that the tubes have a limited capacity for antibody which is in excess of assay requirements and that alteration in antibody

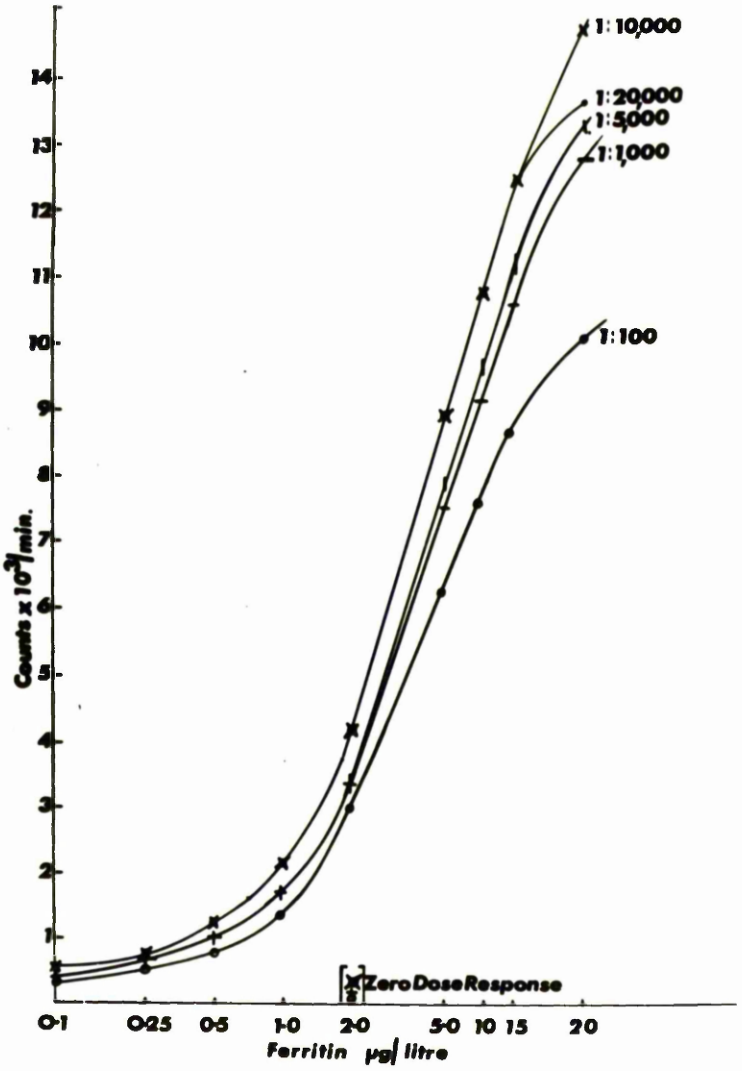


Fig.II, 1

The effect on the dose-response curve of the concentration of solid phase anti-ferritin at 1:100, 1:1,000, 1:5,000, 1:10,000 and 1:20,000 diluted with 0.2M sodium bicarbonate, pH 9.2

dilution had only a small effect on the dose-response curve.

Effect on solid-phase antibody uptake due to variation in reaction tube

The effect on solid-phase antibody uptake due to variation in batch number of clear polypropylene tubes (Sarstedt No. 39/10) was investigated using three different batches. Calibration curves were set up in the normal manner and no statistically significant changes were found in either the dose-response curve or the zero-dose response. The tubes were also manufactured in orange and purple polypropylene and as colour coding could have simplified the assay these tubes were also assessed. There was however a reduction in the dose-response curve, an increase in the zero-dose response and an unacceptable variation of the triplicate counts. Clear polypropylene tubes were therefore used for the investigation.

Variation in incubation time to coat reaction tubes

The effect of varying the incubation time in the uptake of anti-ferritin to form the solid phase antibody was studied by adding 0.5ml of a 1:10,000 dilution of anti-ferritin in 0.2M sodium bicarbonate, pH 9.2 for 10 minutes, 18 hours and 24 hours. The tubes were washed twice with BSA buffer and twice with distilled water and used immediately. After incubation with standard ferritin solutions for 24 hours, 20,000 counts per minute of ^{125}I anti-ferritin were added to each tube. Incubation took place for 48 hours before aspirating the labelled material and washing the tubes twice with phosphate buffer, pH 7.3. It can be seen from Figure II,2 that the zero-dose response shows a small increase when the incubation time is increased from 10 minutes to 18 hours but that the increase is minimal thereafter. There was a more pronounced alteration in the dose-response curve in relation to length of incubation time. The best response was obtained at 24 hours. There may be however factors other than length of incubation involved as in a

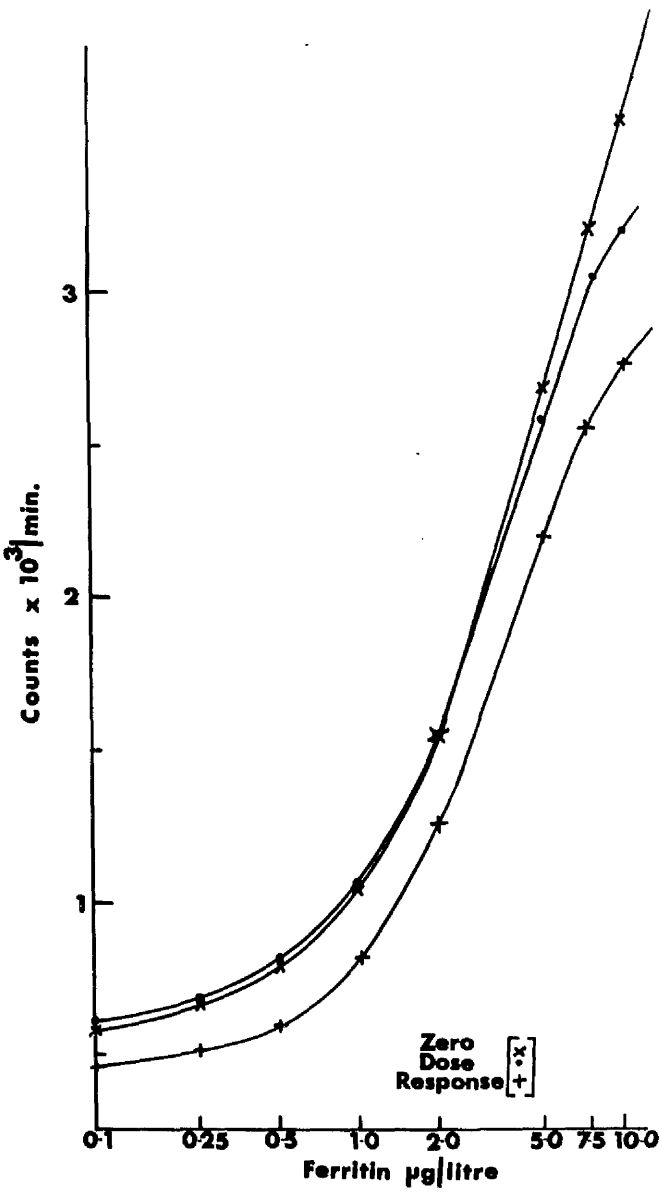


Fig. II, 2

The effect of time on the uptake of solid-phase anti-ferritin.

(+ 10 minutes, • 18 hours and X 24 hours)

previous experiment when 30 minute and 24 hour incubation times were compared the shorter time gave a statistically significant increase in the dose-response curve.

The effect of varying the incubation times of the two reaction steps

Standard ferritin solutions were incubated for 4, 24 and 96 hours in the first reaction but after the addition of ^{125}I anti-ferritin (20,000 counts per minute) all tubes were incubated for 48 hours. The second reaction step was investigated in the same manner in that standard solutions of ferritin were incubated for 48 hours but after the addition of ^{125}I anti-ferritin (20,000 counts per minute) the incubation times were 4, 24 and 96 hours. The tube activities were plotted against linear concentration and appear in Figure II,3. It can be seen that the zero-dose response is only significantly reduced in Reaction one during the 4 hour incubation time but that it is proportional to the incubation time in Reaction Two. In Reaction One there is a considerable increase in the dose-response curve between the 4 and 24 hour incubation times but that little is gained by prolonging the time to 96 hours. In Reaction Two the variation in incubation time on dose-response was more pronounced, increasing by 100 per cent between the 4 hour and 24 hour incubation times and by a further 50 per cent when extended to 96 hours.

Variation in the number of washes between reaction steps

The effect on the dose response curve by varying the number of times that tubes were washed between reactions was investigated. Tubes were washed 0, 2 or 10 times after the first reaction with BSA buffer and then twice with phosphate buffer, pH 7.3 after the second reaction. This was reversed in the second part of the study when tubes were washed twice after the first reaction and 0, 2 or 10 times after the second reaction. The results appear in Figure II,4 with tube radioactivity plotted against linear concentration where it can be seen that there is

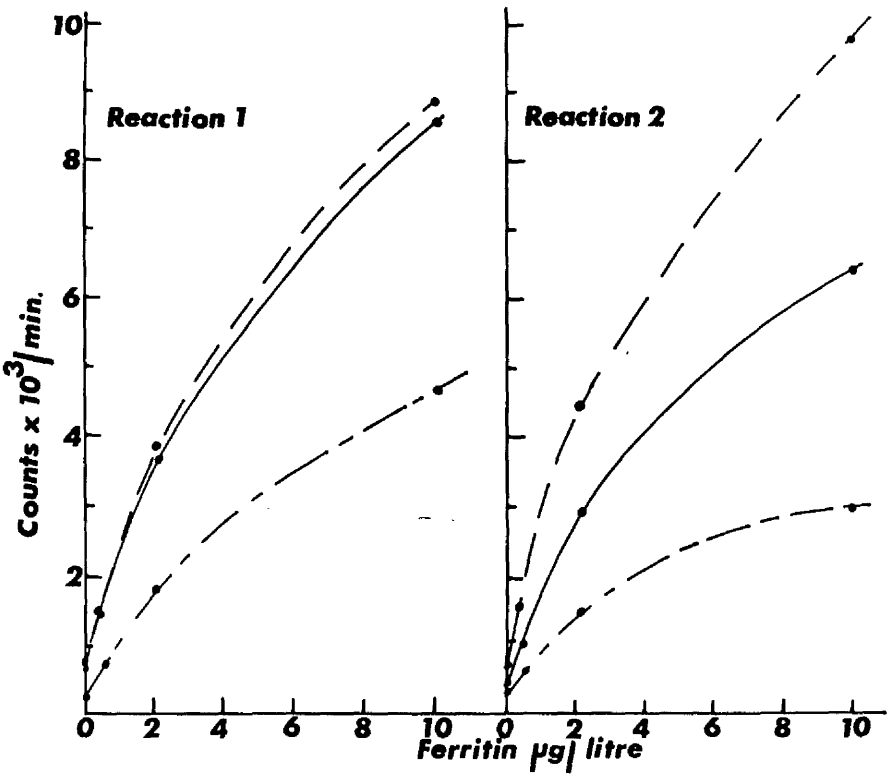


Fig. II, 3

The effect of time on Reaction 1 and Reaction 2, Tube were incubated for 48 hours in the alternate reaction.

(— — — 4hours, ——— 24hours and — — — 96 hours)

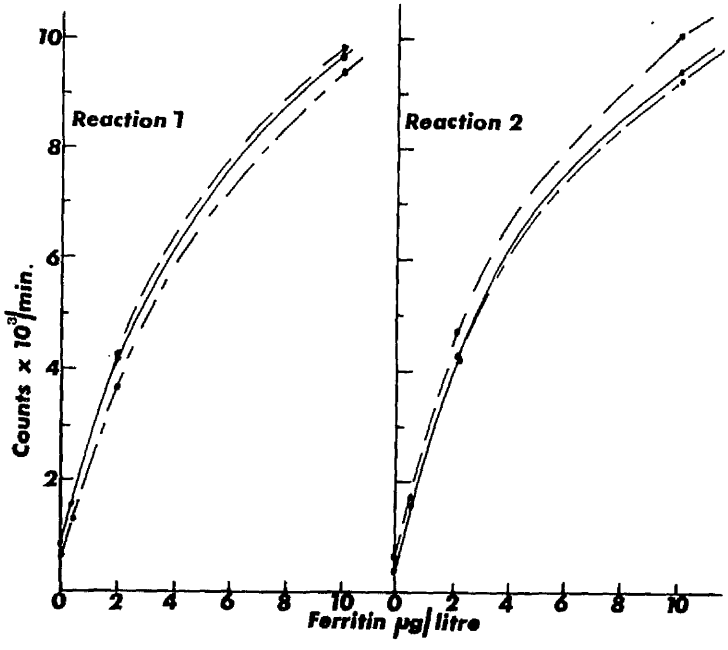


Fig. II,4

Effect of varying the number of tube washes in Reaction 1 and Reaction 2. In the alternate reaction tubes were washed twice.

(— — —0, —— 2, and — — — 10 washes).

a marginal decrease in the zero-dose response by increasing the number of washes. The effect of the washing procedure on the dose-response curve was less in Reaction one than Reaction two, the most obvious effect being between zero and 2 washes in the latter reaction.

The effect of storage and anticoagulant on ferritin concentration

Five specimens of plasma were assayed for ferritin content and then stored at -20°C for 12 months. The initial concentrations were 5.0, 10.8, 45.6, 192 and 230ug per litre and after 12 months 5.4, 10.5, 52.0, 181 and 228ug per litre which would indicate stability of ferritin over this period. Storage at room temperature for one week and at 4°C for 4 months had no effect on ferritin concentration. Tissue extracts were stored at 4°C for 6 weeks and were found to be stable.

Specimens of blood were collected in plain, universal containers and in the presence of lithium heparin and ethylediamine tetra-acetic acid. No difference was found between serum and plasma nor between plasmas with varying anti-coagulants.

Assessment of the effect of protein on the dose-response curve

The effect of bovine albumin and rabbit serum on the assay of ferritin was determined by diluting standards from 0 to 400ug ferritin per litre, in triplicate, 1:20 with BSA buffer, BSA buffer containing 40g bovine albumin per litre and NRS buffer to give final concentrations from 0 to 20ug ferritin per litre. Table2,I gives the mean of the triplicate counts together with the standard deviation (SD) and coefficient of variation (CV). There was no significant difference between the mean counts but the CV was less when dilutions had been made with NRS buffer and for this reason standards and test ferritin solutions in excess of 300ug per litre were diluted with NRS buffer.

Specificity

Human plasma, placental, liver, spleen and heart extracts were assayed

Table 2.1.

Effect of Protein on the Dose-Response Curve

Ferritin	BSA-Buffer			BSA + 4% Albumin			NRS Buffer		
ug/litre	Counts/ min.	SD	CV %	Counts/ min.	SD	CV %	Counts/ min.	SD	CV %
0.00	319	13	4.1	305	16	5.2	304	19	6.6
0.10	455	17	3.7	388	4	1.0	449	16	3.5
0.25	536	14	2.6	506	30	5.9	508	3	0.6
0.50	748	19	2.5	761	48	6.3	739	22	3.0
1.00	1,212	26	2.1	1,122	54	4.8	1,122	38	3.4
2.00	2,068	34	1.6	1,872	67	3.6	1,921	27	1.4
5.00	4,556	58	1.3	4,470	12	0.0	4,546	24	0.5
7.50	6,099	83	1.4	6,160	43	0.7	6,031	103	1.7
10.00	7,739	438	5.7	7,865	228	2.9	7,335	100	1.3
15.00	10,344	101	1.0	10,682	325	3.0	10,339	137	1.3
20.00	11,709	231	2.0	11,869	318	2.7	11,641	140	1.2

Standard solutions of ferritin diluted 1:20 to give final concentrations from 0 to 20 ug/per litre in 1). BSA buffer 2) BSA buffer +40g bovine albumin per litre and 3) NRS buffer.

The mean of triplicate counts together with standard deviation (SD) and coefficient of variation (CV) are given.

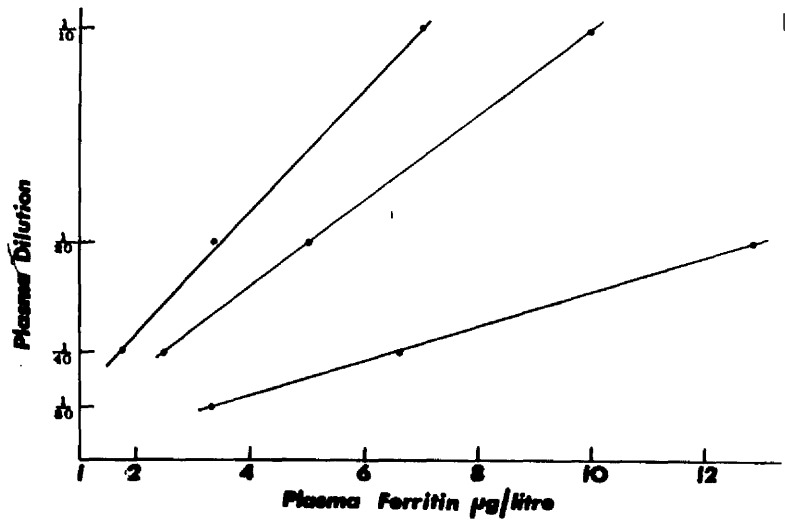
in triplicate at three dilutions the results were linear when plotted arithmetically and appear in Figure II,5. No significant difference was observed in the results obtained at the three dilutions.

Plasma containing 500ug ferritin per litre was diluted 1:20 with BSA buffer. Doubling dilutions in triplicate, were thereafter made with BSA buffer and MRS biffer. The difference obtained by diluting with the two buffers was not statistically significant.

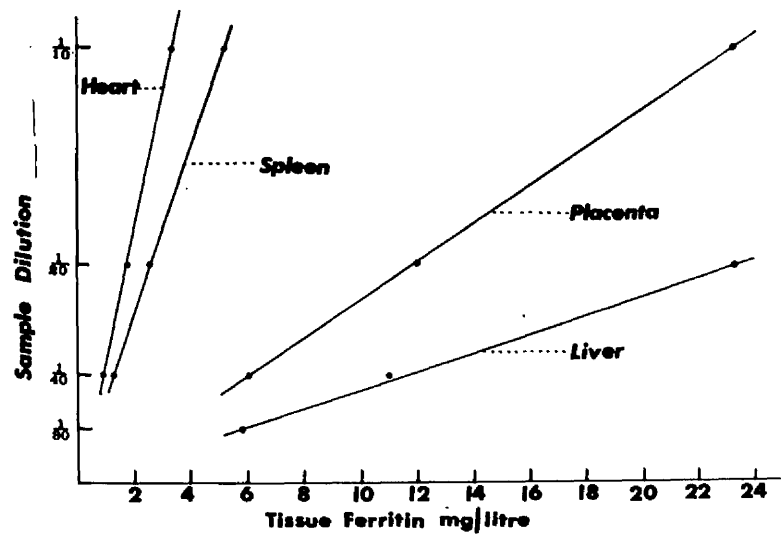
Effect on the assay of method of preparation of ^{125}I-anti-ferritin

Radiolabelled antiferritin was prepared on the same day by the method of Addison et al (1972) and Miles et al (1974). They were used in duplicate assays when 20,000 counts per minute were added to the tubes containing eleven calibration points, eight internal quality control standards whose values had been established in previous assays using ^{125}I anti-ferritin prepared by Addison's method and 27 test plasmas.

The calibration curves obtained showed a marginal decrease in the zero-dose response with the Miles's material (324 as compared to 440 counts per minute) and an increase in the dose-response curve using the same material. The 10ug per litre standard gave 6,454 counts per minute when labelled with the Miles's material and 5,918 counts per minute when labelled with Addison's material. The quality control specimens had mean concentrations of 3.9 ($\pm$ 0.6), 11.3 ($\pm$ 0.4), 10.0 ($\pm$ 2.8), 44.2 ($\pm$ 2.5), 82.5 ($\pm$ 2.1), 192.5 ($\pm$ 3.5) and 212.0 ($\pm$ 1.4) ug per litre when the two materials were compared. The values were also compared for test plasmas over the range of 3 to 500ug per litre. The standard deviation ($SD = \sqrt{\frac{\Sigma d^2}{N}}$ where d is the difference between pairs and N is the total number of estimations) between paired specimens was 4.4 for an overall mean of 81.0ug per litre.



(a)



(b)

Fig. II, 5

(a) Assay of ferritin in successive dilutions of three specimens of plasma.

(b) Assay of ferritin in successive dilutions of heart, spleen, placental and liver extracts.

Evaluation of Assay Performance

Analysis of Standard Curve

A standard curve appears in Figure II,6 in which the mean of triplicate counts together with 2 standard deviations are given. Radioactivity as counts per minute are plotted against the logarithm of the concentration. 20,000 counts per minute were added to each tube and the zero dose response was 345 counts per minute representing 1.8 per cent of the counts added. The tube of the lowest standard, 0.1ug per litre had radioactivity of 545 counts per minute or 2.8 per cent of added activity. The maximum of the working range was the 10ug per litre standard which had an activity of 12,425 counts per minute or 62.2 per cent of the added counts. There was therefore a twenty-three fold increase over the working range from 0.1 to 10.0ug per litre.

The coefficient of variation (CV) varies little throughout the calibration when standard concentrations are above 1ug per litre and average 1.2 per cent. This can be seen in Table 2,I when NRS buffer was used as diluent. The average CV when the standard ferritin concentration was 1ug per litre or less increased to 3.4 per cent.

Sensitivity of the Assay

"The term sensitivity as applied to an assay technique commonly refers to the ability of the system to measure small amounts with acceptable precision. This concept may be formalised by defining the sensitivity of an assay as the precision of measurement of a zero quantity" - Ekins & Newman, (1970) The sensitivity can more readily be defined as the mean detectable dose and was calculated using the standard curve shown in Figure II,6 after an 18 hour incubation of solid phase antibody. The zero dose response was 470, 0.1ug ferritin per litre 618 and 10.0ug ferritin per litre 13,045 counts per minute. Twice the root mean squared deviation was 59 counts per minute and the mean detectable dose

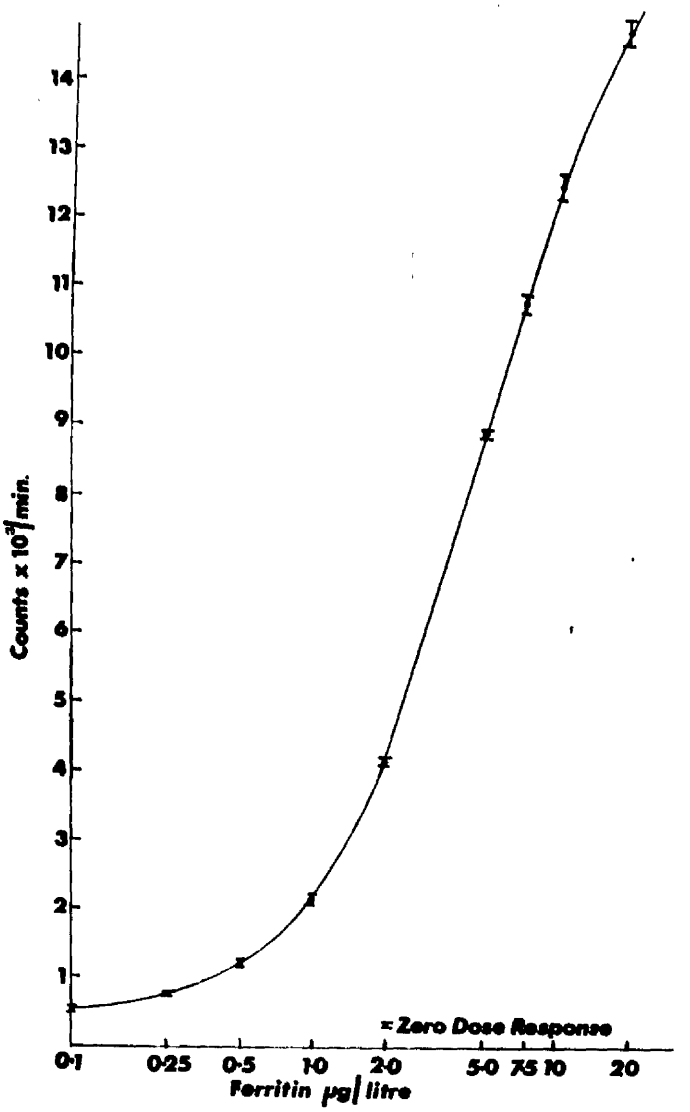


Fig. II,6

Standard Curve of Human Ferritin Assay. Each point represents the mean of triplicate counts $\pm$ 2SD

was found to be 38ng per litre equivalent to 529 counts per minute.

Accuracy of the Assay

The accuracy of the ferritin assay was determined by adding a known quantity of human spleen ferritin to a plasma with a known low ferritin content of 10ug per litre. A ferritin solution, containing 100ug protein per litre in NRS buffer was diluted 1:2, 1:4, 1:8, 1:16 and 1:32 with the plasma. The recoveries were 95 per cent ± 2.0 per cent at 55ug per litre, 97 per cent (± 1.8) at 32.5ug per litre, 95 per cent (± 3.4) at 21.3ug per litre, 96.4 per cent (± 1.5) at 16.1ug per litre and 92 per cent (± 3.1) at 12.8ug per litre.

Precision of the Assay

"Precision is the random error, the variation of results obtained by a method when the same sample is run repeatedly" - Henry,(1964). The precision of the ferritin assay was investigated within a single batch, between batches and between laboratories.

The results for the within batch variation when specimens were repeated between 4 to 18 times are shown in Table2,2 . The mean, standard deviation, and coefficient of variation have been determined and it would appear that there is little variation between 23 to 270ug per litre but that an increase does occur at lower concentrations. The precision was also investigated in the manner of Halliday et al (1975), by performing 112 duplicate analysis on plasma specimens of variable ferritin content in a single assay. The standard deviation (SD) of the different plasma concentrations were calculated according to Snecdecor (1952) from the differences between duplicate determinations. $SD = \sqrt{\frac{\Sigma d^2}{N}}$ where d is the difference between duplicates and N is the number of estimations. The precision of the overall assay by this method of analysis was found to be 3.8. Table2,3 gives a detailed breakdown over the various ferritin ranges.

Table 2,2.

Replicate determinations of plasma ferritin

"within batch"

No. determinations	Mean ug/l	SD ug/l	CV%
18	3.4	0.5	12.0
4	23.2	1.0	4.5
4	47.3	3.9	8.3
10	67.7	5.2	7.8
4	229.5	18.4	8.0
4	270.2	17.7	6.6

Table 2,3.

Duplicate determinations of 112 specimens of plasma

of varying ferritin concentrations in a single batch

Ferritin Range ug/l	No. Pairs	Mean ug/l	SD ug/l	CV %
1-10	11	6.2	0.9	14.7
11-20	13	15.5	1.4	9.2
21-50	36	37.3	2.7	7.4
51-100	31	66.2	3.5	5.3
101-200	14	131.5	5.2	4.0
200-400	7	272.0	8.7	3.2

The between batch variaiton was determined by quantitating ten specimens in duplicate in 9 to 30 separate assays over an eighteen months period. The means, standard deviations and coefficients of variation are shown in Table 2,4 and it would appear that although the standard deviation increases with the ferritin content the relative variation is constant over the range 10 to 250ug per litre. The variation is however considerable at concentrations less than 5ug per litre but clinically this is unimportant as it is well below the normal range of plasma ferritin concentrations.

The variation between laboratories was investigated when 4 plasma specimens and a ferritin standard solution were distributed to 11 laboratories from the Welsh National School of Medicine by Dr. Mark Worwood in 1977. Specimens were analysed by the method in current use using the standard spleen ferritin provided for the investigation and the laboratory's own standards. The results which were later returned appear in Table 2,5 . The author's results when using the standard ferritin provided and compared with those of the other 10 laboratories are in good agreement despite the different methodologies. A number of laboratories use an IRMA reaction and not the 2-site IRMA of the present work. When specimens were compared against the laboratory's own standards, 3 of the 4 results had acceptable precision. The 4th specimen was just within two standard deviations of the overall mean but when repeated on 13 later occasions the mean concentration was found to be 163.4ug per litre (SD ± 19.7).

Table 2,4

Replicate determinations of plasma ferritin between batches

No. Assays	Mean ug/l	SD ug/l	CV%
23	4.6	2.0	43.5
11	5.1	1.9	37.2
9	11.4	1.8	15.8
25	13.0	2.0	15.4
10	22.3	3.1	13.9
30	48.3	4.6	9.5
13	79.2	8.3	10.5
13	163.4	19.7	12.0
20	200.4	11.8	5.9
30	243.4	27.5	11.3

Table 2,5

Between laboratory variation in the quantitation of plasma ferritin (Author's work underlined)

a) Specimens assayed against ferritin standard supplied (A1)

(Results in ug ferritin per litre)

Laboratory	A2	A3	A4	A5
A	21	65	97	6
B	28	101	167	< 2
C	23	77	187	5
D	17	71	142	4
E	22	71	167	5
F	13	56	161	<10
G	20	66	132	3
H	21	69	152	4
K	-	-	-	-
L	38	78	113	9
M	52	128	195	18
Mean ± S.D.	26 ± 11	78 ± 21	151 ± 30	7 ± 5

b) Specimens assayed against laboratory's own ferritin standard

(Results in ug ferritin per litre)

Laboratory	A2	A3	A4	A5
A	30	90	118	9
B	35	134	208	2
C	15	60	142	5
D	15	67	136	2
E	26	75	192	4
F	17	56	161	9
G	28	88	170	7
H	26	87	230	6
K	33	91	175	7
L	34	73	111	9
M	29	80	128	13
Mean ± S.D.	26 ± 7	82 ± 21	161 ± 38	7 ± 3

Comparison of ferritin concentration by the Lowry method (1951) and the Laurell rocket technique (1966) After the first ferritin extract had been intensively purified and its concentration determined by the chemical method of Lowry (1951) it was used as a primary standard in the quantitation of subsequent ferritin extracts by the Laurell rocket method (1966). It was considered that if the concentration derived by the chemical method was greater than that by the immunological method contaminant proteins were present. This proved a simple and rapid method for indicating the success of the ferritin extraction but depended on the purity of the initial ferritin extract.

10ml of 1.0% agarose (BDH) (w/v) in 0.076 barbitone buffer, pH 8.6 and containing 0.2ml anti-human ferritin (Hoechst) was poured onto an 8 x 8cm glass slide. Holes were cut to contain 10ul volumes. The standard ferritin solution was diluted with 0.15M sodium chloride to a concentration of 100mg per litre. Doubling dilutions were subsequently made to give arange from 25 to 100mg per litre. A micro-cap pipette (Drummond) was used to fill the holes with test and standard solutions - the end point was taken when the liquid menscus disappeared when the plate was at eye level. The run took place overnight under the same conditions as described for crossed-immunoelectrophoresis. The peak heights of the standard solutions were measured and plotted against the concentrations to allow quantitation of the test solutions:-

see Plate 2,I

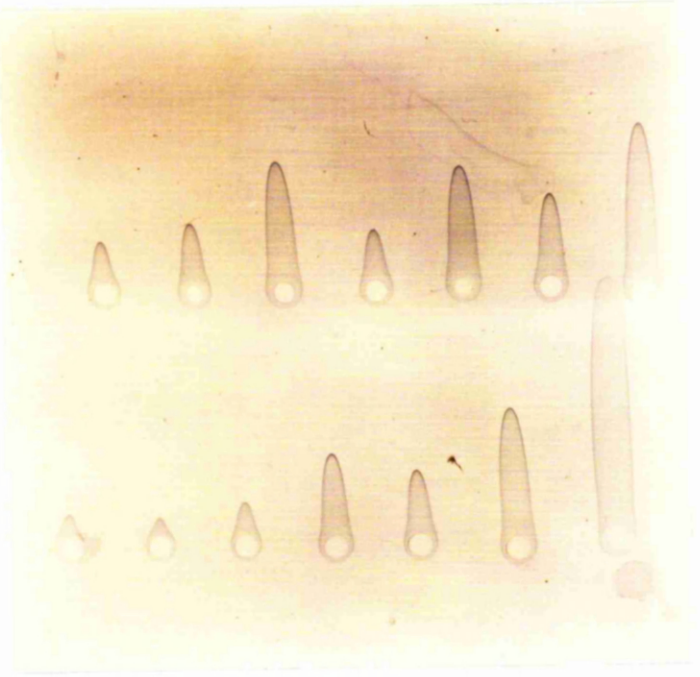


Plate 2.1.

Quantitation of Ferritin by the Laurell

Rocket Technique

Chapter Three

CLINICAL STUDIES OF NORMAL SUBJECTS

The variation in plasma ferritin concentration was investigated with relation to reproduction in a healthy population. Studies were made of male and non-pregnant female subjects in the reproductive age group, in patients throughout pregnancy and in the purperium and of paired mothers and babies at term. Ferritin concentrations were also related to the 2:3-diphosphoglycerate content of red blood cells of pregnant patients.

Variation in plasma ferritin between the sexes

The non-pregnant group consisted of 61 women between the ages of 18 and 33 years who were in good health and attending a family planning clinic for advice on contraception. None of the patients were taking oral contraceptives at the time of blood sampling. The male patients consisted of 24 fourth year medical students aged 19 to 21 years and 78 male partners of infertile marriages aged 23 to 46 years. All patients were clinically normal and were not receiving iron supplements.

The variation in plasma ferritin concentration in male and female subjects is shown in Figure III, 1, 2 and there was evidence of a skew distribution. There was no statistically significant difference in concentration between the 24 medical students (mean 92.4, SD ± 38.9ug per litre) and the 78 other male subjects (mean 92.0, SD ± 50.9ug per litre).

The arithmetic mean for the combined male subjects

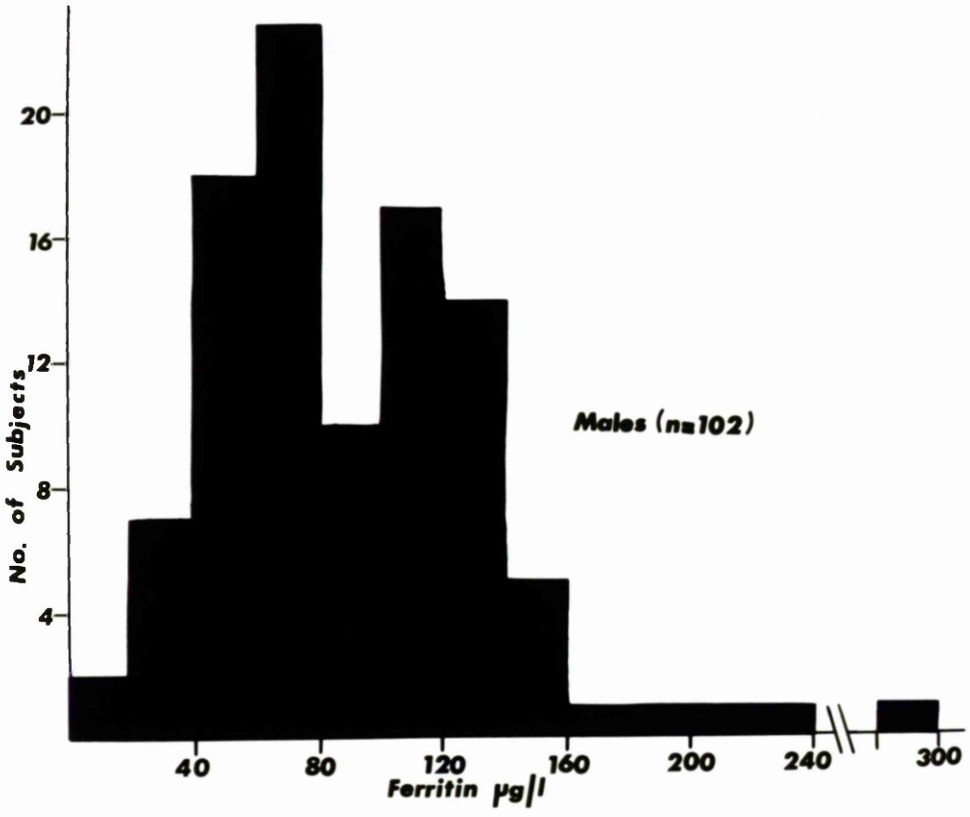


Fig.III,1

Distribution of Plasma Ferritin in Normal Male Subjects

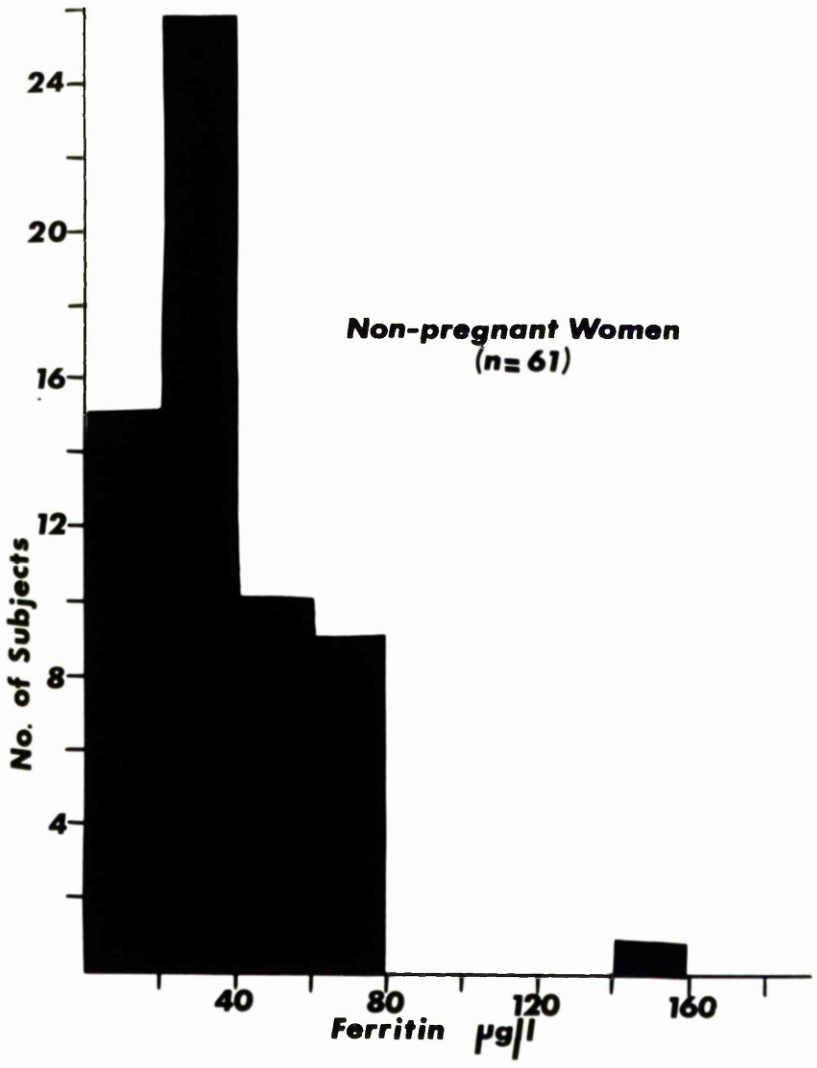


Fig III,2

Distribution of Plasma Ferritin in
Non-pregnant Female Subjects

was 92.3ug per litre with a standard deviation (SD) of 48.0 and the logarithmic mean 79.8 with a range of 24.0 to 265.0ug per litre. In the non-pregnant female the arithmetic mean concentration was 36.7 (SD ± 23.9ug per litre) and logarithmic mean 31.5, range 9.3 to 106.7 per litre. There was a statistically significant difference ($p < 0.001$) in ferritin levels between male and female subjects.

Variation in plasma ferritin in early pregnancy and in the puerperium

Seventy-one healthy pregnant patients between 15 and 20 weeks gestation who attended the antenatal clinic at the Royal Maternity Hospital were studied. They were aged between 17 and 34 years. Fifty-six of the patients had been started on prophylactic iron supplements (average 100mg of elemental iron per day) prior to referral to the clinic and all patients received iron supplements thereafter. Heparinised blood specimens were also obtained from 62 patients on their sixth postnatal day who were age matched with the early gestation group. No patient received iron therapy after delivery. Patients in both groups were judged retrospectively to have had normal pregnancies. They all had singleton pregnancies and normal babies were delivered at term.

Early pregnancy ferritin concentrations The arithmetic mean of plasma ferritin at less than 20 weeks gestation was 42.0 (SD ± 42.6)ug per litre and logarithmic mean 36.6ug per litre (range 4.9 to 270.0).

Postnatal ferritin concentrations On the sixth postnatal day the arithmetic mean ferritin concentration was 39.6 (SD ± 28.9)ug per litre and the logarithmic mean 32.5ug per litre (range 7.7 to 124.0).

Comparison of early gestation and postnatal ferritin concentrations The distribution of plasma ferritin concentrations in early pregnancy and on the sixth postnatal day are shown in Figure III,34. Comparison of the two groups indicate that when patients are maintained on iron supplements throughout pregnancy the iron stores, as indicated by the plasma ferritin, are not significantly altered between early pregnancy and the immediate postnatal period ($p<0.80$) and compare with concentrations obtained for non-pregnant, female subjects.

Variation in plasma ferritin and other indices of iron metabolism throughout normal pregnancy

Twenty healthy primigravidae who attended the antenatal clinic at Glasgow Royal Maternity Hospital consented to venepunctures at frequent intervals throughout their pregnancies and on the sixth postnatal day. The patients attended between 9am and 12 noon and after resting in the left lateral position for 15 minutes their blood pressure was recorded and 10ml of blood withdrawn into lithium heparin tubes. All patients had been started on prophylactic iron supplements (average 100mg of elemental iron per day) prior to referral to the antenatal clinic. These patients were judged retrospectively to be normal as they had maintained a normal blood pressure throughout

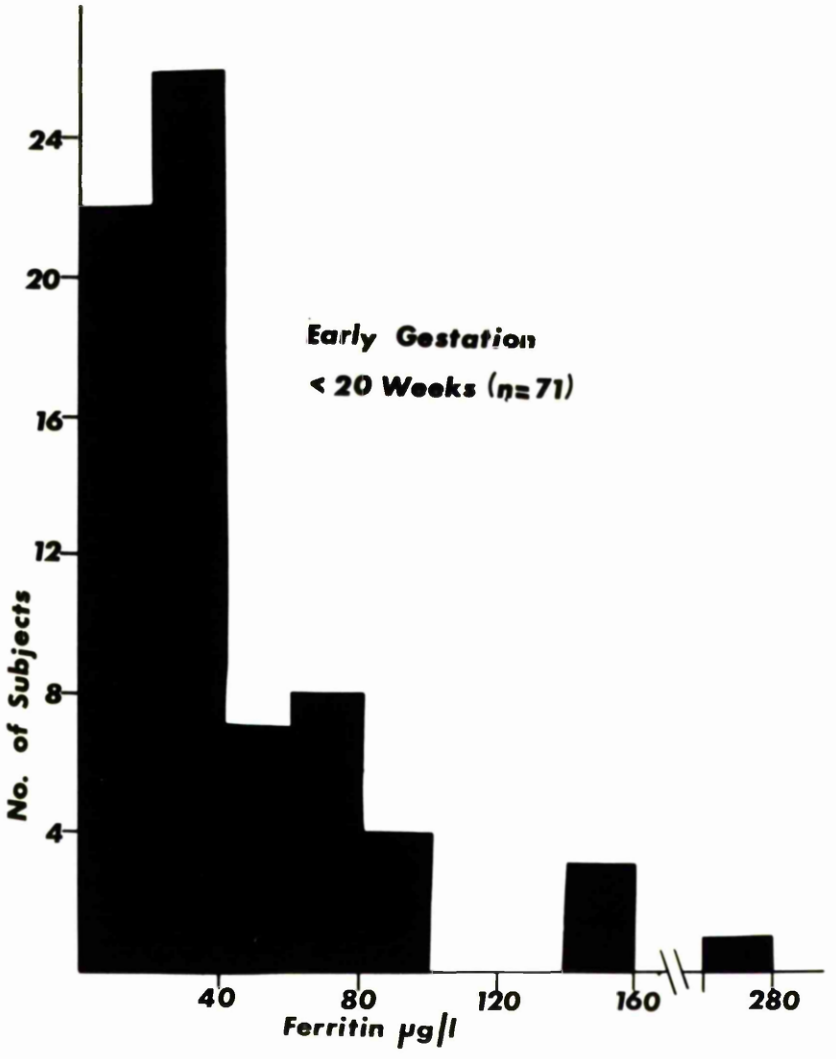


Fig III,3

Maternal Plasma Ferritin in Early Pregnancy

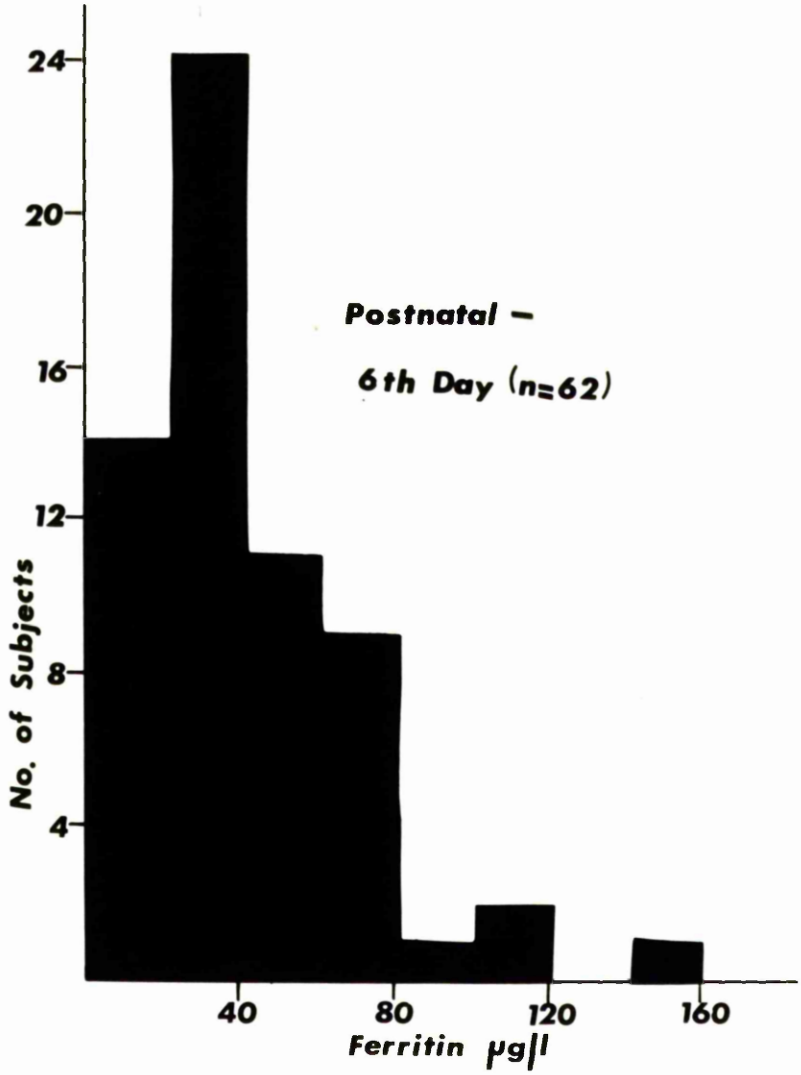


Fig III,4

Maternal Plasma Ferritin in Puerperium

their pregnancies and no glycosuria nor proteinuria had been detected. All patients were delivered between 38 and 41 weeks and had infants of normal birth weight.

The plasma ferritin variation with gestation The plasma ferritin concentrations related to gestation did not fit a normal distribution and a logarithmic transformation was made for comparisons. The logarithmic mean and the range related to gestation appear in Figure III,5. In Table 3,1 the arithmetic mean concentration of ferritin is given and included are values of the non-pregnant group previously described. There was no significant difference in plasma ferritin levels between non-pregnant women and iron-supplemented pregnant patients of less than 25 weeks gestation, or six days after delivery. However with increasing gestation the ferritin concentrations fell and highly significant differences were found at 26 to 30 weeks ($p < 0.001$), at 31 to 35 weeks ($p < 0.001$) and at term ($p < 0.001$) when compared to non-pregnant values.

Plasma iron concentration related to gestation The results indicate that with prophylactic iron therapy before 20 weeks there was no significant difference in plasma iron concentration throughout pregnancy, but on the sixth post natal day there was a fall which was statistically significant ($p < 0.005$).

Plasma transferrin concentration and saturation related to gestation Plasma transferrin concentrations were increased within the first 20 weeks when compared to the non-pregnant group ($p < 0.001$) and this significant increase continued

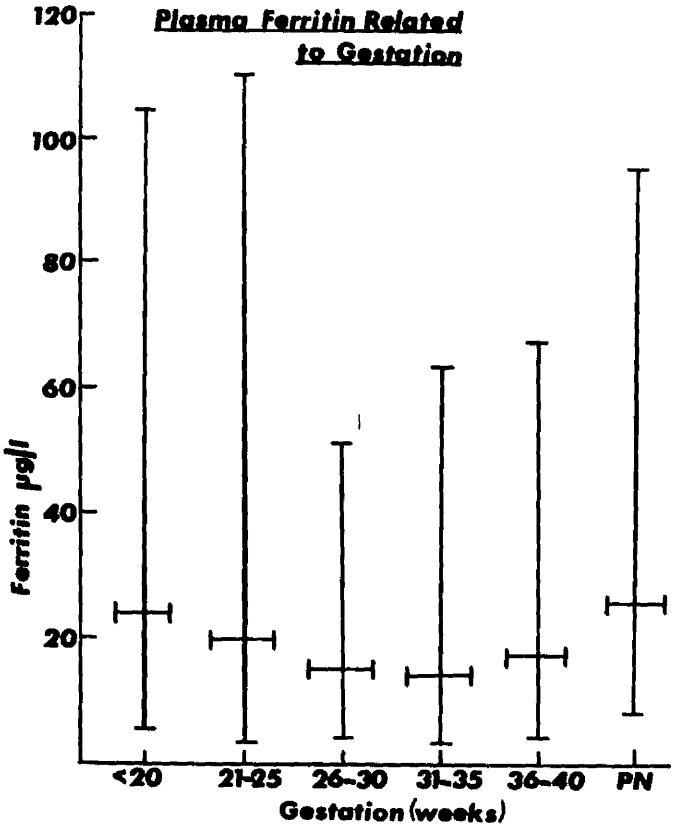


Fig III,5

Maternal Plasma Ferritin Related to Gestation

Table 3,1

Parameters of Iron Metabolism Related to Gestation in Normal Pregnancy

(Mean ± ISD. Number of subjects in parentheses)

	Non-Pregnant	Gestational age (weeks)					
		<20	21-25	26-30	31-35	36-40	Postnatal
Ferritin (ug/l)	37.1 ± 19.5 (61)	30.9 ± 24.1 (20)	27.0 ± 19.2 (20)	17.8 ± 10.6 (20)	17.9 ± 14.0 (20)	20.7 ± 11.2 (20)	32.9 ± 16.7 (20)
Iron (umol/l)	16.4 ± 7.7 (36)	19.3 ± 9.8 (20)	18.0 ± 7.8 (20)	15.3 ± 6.4 (20)	17.1 ± 8.1 (20)	17.6 ± 9.2 (20)	10.5 ± 5.1 (17)
Transferrin (mg/l)	2382 ± 548 (55)	3331 ± 620 (20)	3467 ± 546 (20)	3550 ± 473 (20)	3556 ± 486 (20)	3320 ± 580 (20)	3148 ± 627 (17)
Transferrin Saturation (per cent)	27.2 ± 14.1 (33)	32.0 ± 16.4 (20)	21.2 ± 9.8 (20)	17.8 ± 7.8 (20)	18.0 ± 6.5 (20)	21.8 ± 9.8 (20)	13.0 ± 6.5 (17)
Haemoglobin (g/dl)	-	12.7 ± 0.8 (20)	11.7 ± 0.7 (20)	11.6 ± 0.6 (20)	11.6 ± 0.7 (20)	11.9 ± 0.7 (20)	11.7 ± 1.1 (20)

until term ($p < 0.001$). The percentage saturation was calculated from the plasma iron and transferrin concentrations assuming a molecular weight of 76,000 daltons (Morgan, 1974) for the latter and that one molecule of transferrin binds two molecules of iron. There was no significant difference in percentage saturation between non-pregnant women and early pregnancy patients on iron ($p < 0.3$) but between 26 and 35 weeks there was a a highly significant fall when compared to less than 20 weeks gestation ($p < 0.005$). Between 36 to 40 weeks the percentage saturation increased and little difference was found between this group and the early pregnancy group ($p < 0.05$). A second fall in transferrin saturation was found on the sixth postnatal day ($p < 0.001$).

Haemoglobin concentration related to gestation Haemoglobin was significantly reduced between 21 to 35 weeks when compared to early pregnancy ($p < 0.001$); it increased at 36 to 40 weeks although the level remained lower than that found in early pregnancy ($p < 0.005$) and in the puerperium, the concentration was still significantly lower ($p < 0.005$). At term only one of the 20 patients studied serially had a haemoglobin of less than 11g per dl and concentrations ranged from 10.5g to 13.4g per dl.

No correlation was found between the concentrations of plasma ferritin and plasma iron, transferrin or haemoglobin.

Summary Normal pregnant women maintained on prophylactic iron supplementation showed a progressive fall in plasma ferritin to low levels in late pregnancy. Significant

falls in plasma transferrin saturation and whole blood haemoglobin were also found. The transferrin concentration remained elevated from early pregnancy until term.

Table 3,2

Ferritin Concentration (ug per l)

in the Post-natal Period

(Number of patients in parentheses)

Day	Total	Control	Estrovis	Bromocriptine
1	24.7 ± 17.6 (45)	25.8 ± 21.0 (15)	26.6 ± 16.0 (15)	23.2 ± 15.2 (15)
6	30.9 ± 17.1 (43)	34.3 ± 17.3 (14)	33.1 ± 19.2 (14)	25.7 ± 14.6 (15)
14	33.7 ± 24.3 (44)	39.3 ± 25.5 (15)	34.9 ± 23.9 (15)	26.5 ± 23.5 (14)
42	30.7 ± 22.8 (28)	23.2 ± 13.8 (10)	34.8 ± 22.1 (10)	34.8 ± 31.9 (8)

Factors Influencing Plasma Ferritin in the Puerperium

Forty-five women were studied at Day 1, 6, 14 and 42 of the puerperium. All patients were selected at delivery as having had pregnancies uncomplicated by major obstetrical emergencies, a normal haemoglobin at term and the delivery of a normal singleton baby. None of the patients were breast feeding and 15 in each group received bromocriptine, estrovis or ascorbic acid as a placebo to suppress lactation. The ante- and post-natal haemoglobins and blood loss at delivery were recorded.

The ferritin concentrations of the three treatment groups appear in Table3,2 together with a summation of all the results. There was no statistical difference between the groups although the bromocriptine group did seem to take longer than the others to attain a maximum. It was retrospectively found that a mild degree of iron deficiency anaemia (Hb $<$11.0g per dl) had occurred in early or mid-pregnancy in 2 of the patients in the control group, 4 in the estrovis group and 6 in the bromocriptine group. Post-natal haemoglobin levels of less than 11.0g per dl at 6 or 42 days occurred in one of the control group and six of the bromocriptine group. The ferritin concentrations for these patients who had ante- or post-natal anaemia compared to those with normal haemoglobin levels appear in Table3,3 and although ferritin concentrations are lower in the anaemic group and tend to fall in the post-natal period the scatter is such that the results are not statistically significant. Ferritin levels of less than 10ug per litre were found in 5 of the 12 patients on day 14, 5 out of 8 on day 42 of the antenatal anaemic group and 4 out of 7 on day 14 and all 3 patients on day 42 of the postnatal anaemic group. Only 2 of the patients with normal haemoglobin concentrations had low ferritin levels on day 14 but 3 had low levels on day 1. It can however be seen that a significant

Table 3,3

The Effect of Iron Deficiency Anaemia (Hb≤11.0g/dl)

in the Ante- and Post-natal Periods

on Ferritin Concentration (ug/l)

(Number of patients in parentheses)

Day Post-natal	Normal Hb > 11.0g/dl	Ante-Natal Hb < 11.0g/dl	Post-natal Hb < 11.0g/dl
1	24.1 ± 15.1 (28)	19.2 ± 12.3 (12)	21.8 ± 15.0 (7)
6	33.4 ± 17.8 (27)	24.3 ± 15.2 (12)	21.0 ± 10.5 (7)
14	34.2 ± 20.7 (28)	25.1 ± 21.7 (12)	18.6 ± 16.6 (7)
42	36.5 ± 23.7 (19)	17.5 ± 16.6 (8)	7.3 ± 1.5 (3)

increase in ferritin occurs in the normal group between the first and sixth day ($p < 0.05$) which is maintained on the fourteenth and forty-second day.

The effect of blood loss at delivery was correlated with the ferritin concentration and the results appear in Table3,4 . There was no statistical difference between the groups which was not entirely unexpected as the maximum blood loss was relatively small. This was unavoidable as the group were selected because of their normal deliveries. In order to keep sufficient numbers in the groups and to take account of individual iron stores the differences between Day 1 and Days 6, 14 and 42 were determined for each patient and these results appear in Table3,5 . Again there is a fall in the difference in ferritin concentration with increasing blood loss but due to the variability in the results it was not significant. However three of the five patients who had lost more than 200ml of blood at delivery had lower ferritin levels than on the first post-natal day, on Day 42.

The plasma iron on Day 1 was 13.1 ± 7.2 umol per l for 44 patients, on Day 6 was 6.6 ± 4.0 umol per l for 29 patients, on Day 14 was 10.7 ± 5.5 umol per l for 40 patients and on Day 42 was 14.1 ± 4.1 umol per l for 26 patients. The fall on Day 6 was statistically significant ($p<0.001$).

Summary In women with normal haemoglobin levels the plasma ferritin reaches a maximum on the sixth post-natal day and this concentration is maintained at least until the sixth week. The plasma iron appears to fall on the sixth day before increasing to first day post-natal levels on the fourteenth day of the puerperium. Relatively small losses of blood at delivery are often associated with low ferritin levels in the post-natal period

Table 3,4.

Ferritin Concentration (ug per l)

in Relation to Blood Loss

(Number of patients in parentheses)

Blood Loss (ml)	≤100	101-200	201-500
Day 1	25.1 ± 12.1 (14)	20.0 ± 14.0 (17)	29.9 ± 22.7 (11)
Day 6	37.1 ± 15.5 (13)	24.3 ± 14.7 (17)	34.0 ± 20.1 (11)
Day 14	37.2 ± 25.8 (13)	26.1 ± 17.8 (17)	39.5 ± 28.2 (11)
Day 42	40.1 ± 27.4 (9)	29.2 ± 21.0 (12)	21.7 ± 21.8 (5)

Table 3,5.

Difference in Ferritin Concentration (ug per l)

Related to Blood Loss

(Number of patients in parentheses)

Blood Loss (ml)	≤ 100	101-200	201-500
Day 6-1	13.7 ± 15.9 (11)	4.3 ± 11.4 (17)	6.7 ± 9.3 (11)
Day 14-1	12.7 ± 21.8 (12)	6.9 ± 18.0 (17)	8.4 ± 16.9 (11)
Day 42-1	16.1 ± 23.2 (8)	10.8 ± 27.3 (12)	-5.0 ± 25.3 (5)

GESTIONAL CHANGES IN PLASMA FERRITIN RELATED TO RED CELL 2:3- DIPHOSPHOGLYCERATE AND PYRUVATE KINASE ACTIVITY.

Serial studies were carried out to determine the relationship between plasma ferritin and red cell 2:3- diphosphogly cerate (2:3-DPG) concentration and pyruvate kinase (PK) (2.7.1.40) activity in normal pregnancies. Heparinised blood specimens were obtained from nine patients before 20 weeks and from an additional six patients from 25 weeks until term. Five patients were investigated post-natally. Patients were judged restrospectively to have had normal pregnancies and all were delivered between 38 and 41 weeks of infants of normal birth weight. Each patient had been started on prophylactic iron supplements (average 100mg elementaliron per day) prior to referral to the clinic.

The results of the study appear in Table 3,6. The plasma ferritin decreased until 33 to 36 weeks ($p<0.05$) which was followed by rise at term. 2: 3- DPG increased at 21 to 24 weeks ($p<0.1$) before falling to the lowest limitsfound in pregnancy at 25 to 28 weeks ($p< 0.001$). Haemoglob lin fell significantly until 25 to 32 weeks ($p< 0.02$) before rising until term. PK activities varied with gestation. The enzyme activity fell significantly until 25 to 28 weeks ($p< 0.05$) and remained at low activity until term ($p<0.02$).

In 8 of the 15 pregnancies studied serially low ferritin concentrations of less than 10 ug per litre were found on at least one occassion. In seven patients the 2:3-DPG levels were normal, but in the eighth, 2:3- DPG while initially low (mean-2SD) reached normal levels although the ferritin remained low. In three pregnancies low 2:3DPG levels were recorded on a single occassion. All three patients had normal haemoglobin levels and only the one described above had a low ferritin level. No correlation was found between plasma ferritin and 2:3DPG concentration nor PK activity.

TABLE 3,6

GESTATIONAL CHANGES IN PLASMA FERRITIN RELATED TO RED CELL 2:3- DPG, HAEMOGLOBIN AND PK

Gestation (weeks)	16-20	21-24	25-28	29-32	33-36	36-T	POST NATAL
No.of Patients	9	9	15	15	14	15	5
Ferritin umol/l	33.6 (26.0)	24.9 (14.2)	20.0 (10.3)	18.4 (17.0)	15.6 (8.0)	21.6 (13.1)	37.7 (21.2)
Hb g/dl	12.1 (0.7)	11.6 (0.6)	11.5 (0.5)	11.5 (0.8)	12.0 (0.7)	12.3 (0.7)	12.6 (0.6)
2:3 DPG umol/gHb	17.6 (3.1)	20.2 (2.1)	16.1 (2.5)	17.9 (2.5)	18.4 (2.7)	20.2 (2.3)	16.4 (1.9)
PK iu/g Hb	5.52 (0.84)	5.24 (0.59)	4.56 (1.11)	4.96 (0.97)	5.03 (0.85)	4.65 (0.75)	4.68 (1.38)

Correlation between Maternal and Cord Ferritin Concentrations and other Parameters of Iron Metabolism in Normal Pregnancies at Term

One hundred and ninety-one pregnancies were investigated and blood was obtained from the mother within 30 minutes of delivery and from the cord on delivery of the placenta. The patients were unselected other than appearing to be clinically between 38 to 41 weeks, had only minor complications throughout their pregnancies and were expected to produce normal, term babies by spontaneous vertex delivery. 76 patients were later removed from the series due to the baby being assessed as premature, light-for-dates or the sera being haemolysed. Finally 115 matched maternal and cord bloods were available from mothers who had completed a normal pregnancy and had been delivered of a normal baby at term. The mean baby weight was 3.72Kg (SD ± 0.45) and the mean placental weight 620g (SD ± 108). The mothers were aged between 16 and 41 years. The parity, social class, sex of the baby, gestation and the immediate maternal antenatal haemoglobin were recorded.

The biochemical parameters of iron metabolism of 115 paired cord and maternal sera appear in Table3,7 .

Maternal ferritin concentration at term The arithmetic mean of the maternal ferritin concentration, obtained from 115 sera was 40.4 (SD ± 33.1)ug per litre but the values obtained did not give a normal gaussian distribution. Logarithmic transformation of the data gave a logarithmic mean 30.3ug per litre with a range of 6.4 to 144.0ug per litre. There was no correlation between the maternal

Table 3,7

Parameters of Iron Metabolism in 115 paired Maternal and Cord Sera at Term

	Ferritin ug/l	Iron umol/l	Transferrin mg/l	% Saturation	Hb g/dl
Maternal Mean	40.4	16.6	3659	19.0	12.2
" SD	33.1	7.0	942	9.6	0.8
Cord Mean	200.0	27.0	1717	58.8	-
" SD	108.0	7.5	393	17.3	

TABLE 3,8

PARAMETERS OF IRON METABOLISM RELATED TO HIGH AND LOW MATERNAL FERRITIN

CONCENTRATIONS (SD IN PARENTHESES)

Group	No.	Maternal Ferritin ug/l	Maternal Iron umol/l	Maternal % Satn.	Maternal Haemoglobin g/dl	Cord Ferritin ug/l	Cord Iron umol/l	Cord % Satn.
Mat.Ferritin $\leq$10 ug/l	14	7.9 (2.0) $p<0.001$	13.4 (6.6) $p<0.02$	12.9 (10.8) $p<0.02$	11.80 (51.3) $p<0.2$	144.1 (51.3) $p<0.05$	26.3 (7.2) $p<0.8$	55.2 (9.1) $p<0.3$
Mat.Ferritin $<$12 ug/l	23	9.3 (2.5) $p<0.001$	13.0 (5.8) $p<0.005$	12.5 (9.2) $p<0.005$	11.85 (0.59) $p<0.05$	154.8 (63.3) $p<0.02$	27.1 (6.4) $p<0.4$	55.8 (11.9) $p<0.3$
Mat.Ferritin $>$30 ug/l	61	61.2 (32.7)	18.6 (7.1)	23.1 (14.6)	12.29 (0.87)	218.0 (119.5)	25.4 (8.3)	60.4 (17.8)

Significance of difference of the means of low maternal ferritin groups compared to high maternal ferritin group.

ferritin concentration and the weight of the baby or the placenta.

Cord ferritin concentrations of term babies The arithmetic mean ferritin concentration of the 115 cord sera was 200.0 (SD ± 108.0)ug per litre and the logarithmic mean 169ug per litre which gave a range of 55.0 to 296.0ug per litre. The cord ferritin concentrations, like the maternal ferritin levels did not correlate with either the baby weight ($r = 0.02$) or placental weight ($r = 0.05$).

Correlation between maternal and cord ferritin concentrations There was no direct correlation between the 115 paired cord and maternal ferritin concentrations (coefficient of correlation, $r = 0.07$). When the relationship was investigated at low maternal ferritin concentrations of less than 10ug per litre which we found to be two standard deviations below the non-pregnant mean (Kelly et al, 1977) or at less than 12ug per litre which included a larger population, the corresponding cord ferritin levels were 144.1 (SD ± 51.3) and 154.8 (SD ± 63.3)ug per litre respectively and were statistically lower ($p<0.02$) than cord ferritin of 218.0 (SD ± 119.5)ug per litre associated with maternal ferritins, greater than 30ug per litre. The results are summarised in Table 3,8 .

Cord ferritin concentration in male and female babies The 59 male infants had a mean cord ferritin of 216.1ug per litre (SD ± 120.2) which was greater than that of the 56 females with a mean concentration of 187.1ug per litre (SD ± 90.5) but the difference was not statistically

Table 3,9

Maternal and Cord Ferritin Concentrations Related to Parity

(Standard deviation in parentheses)

Parity	Number of Patients	Maternal Ferritin ug/l	Cord Ferritin ug/l
0+0	41	47.6 (± 33.3)	205.0 (± 129.3)
1 to 2+0	60	35.6 (± 30.1)	201.8 (± 97.8)
> 2+0	14	23.4 (± 18.3)	175.6 (± 85.5)

Table 3,10

Maternal and Cord Ferritin Concentrations in Relation to Gestation at Delivery (Standard deviation in parentheses)

Gestation (weeks)	Number of Patients	Maternal Ferritin ug/l	Cord Ferritin ug/l
41	26	32.6 (25.3)	213.8 (79.1)
40	46	37.0 (28.0)	203.9 (127.4)
39	19	35.0 (22.1)	190.3 (102.4)
38	24	53.6 (41.3)	194.5 (101.7)

significant ($p<0.2$).

The effect of parity on maternal and cord ferritin concentrations In Table 3,9 the ferritin concentrations are compared with regard to parity. There was no significant difference ($p<0.1$) when the maternal ferritin levels of the primigravid patients (mean 47.6 ± 33.3ug per litre) were compared to patients who had one or two previous pregnancies (mean 35.6 ± 30.1ug per litre) but when compared to those who had three or more previous pregnancies (mean 23.4 ± 18.3ug per litre) the difference was statistically significant ($p<0.02$). There was however no difference in cord ferritin concentrations when related to parity.

The effect of gestation on maternal and cord ferritin concentrations The maternal and cord ferritin concentrations were related to gestation at delivery and the results are shown in Table3,10. There was no statistically significant difference between maternal or cord ferritins of patients delivered between 38 and 41 weeks.

Effect of maternal age on maternal and cord ferritin concentrations The results were also analysed with respect to maternal age. Although there was some reduction in maternal ferritin up to the age of 30 years the only group which was significantly different from that of 16-18 years (mean 58.2 ± 34.8ug per litre) was the 26-30 years group (mean 36.4 ± 21.2ug per litre; $p<0.05$). There was no significant difference between the cord ferritin groups when related to maternal age.

The effect of social class on maternal and cord ferritin concentrations Patients were divided into social class. 93 patients were found to be in social class I to IV and 22 in class V. The results of maternal and cord ferritin concentrations appear in Table 3, 11. No statistical differences were found in maternal ferritin concentrations between social class I to IV, mean 42.4 (SD ± 33.0)ug per litre but social class V was significantly lower ($p < 0.005$) with a mean of 21.2 (SD ± 14.3)ug per litre. There was no difference in cord ferritin concentrations when related to social class.

Maternal haemoglobin concentration at term The mean maternal haemoglobin was 12.2 (SD ± 0.8)g per dl at term and the actual range was 10.5 to 14.8g per dl. Haemoglobin levels of less than 11.0g per dl were found in 3.5 per cent of mothers although patients had received iron supplements throughout pregnancy. When the maternal ferritin was low the haemoglobin concentration was also significantly reduced ($p < 0.05$) as shown in Table 3, 8.

Table 3,11

Maternal and Cord Ferritin Concentration

in Relation to Social Class

Social Class	Number of Patients	Maternal Ferritin ug/l	Cord Ferritin ug/l
1	4	38.6 (18.6)	172.7 (82.3)
2	24	41.3 (19.7)	218.6 (133.0)
3	39	43.4 (39.1)	197.5 (101.5)
4	26	44.5 (34.1)	202.0 (96.9)
5	22	21.2 (14.3)	196.1 (110.7)

(Standard deviation in parentheses)

Comparison of maternal and cord serum iron concentrations at term The mean maternal serum iron at term was 16.6 umol per litre (SD ± 7.0) and was significantly lower, $p < 0.001$ than the mean cord serum iron of 27.0 umol per litre (SD ± 7.5) as shown in Table 3,7 . There was no correlation between maternal and cord serum iron concentrations nor between these concentrations and baby weight.

Table 3,8 illustrates that when the maternal ferritin was less than 12ug per litre the corresponding maternal serum iron was 13.0 (SD ± 5.8) umol per litre and was statistically lower ($p < 0.005$) than the serum iron of 18.6 (SD ± 7.1) umol per litre associated with serum ferritin levels of greater than 30ug per litre. Although cord ferritin levels were significantly reduced when the maternal ferritins were low no difference was found in the corresponding cord serum iron concentrations.

Comparison of maternal and cord transferrin concentrations and percentage saturations The mean maternal transferrin concentration at term was 3,659mg per litre (SD ± 942) and was statistically greater ($p < 0.001$) than the corresponding cord transferrin concentration of 1717mg per litre (SD ± 393). The maternal percentage iron saturation of transferrin was 19.0 per cent (SD ± 9.6) and was statistically lower $p < 0.001$ than the cord percentage saturation of 58.8 (SD ± 17.3). Although low maternal ferritin concentrations were associated with the reduced maternal transferrin saturation of 12.5 per cent (SD ± 9.2) when compared to the higher maternal ferritin group with saturation of 23.1 per

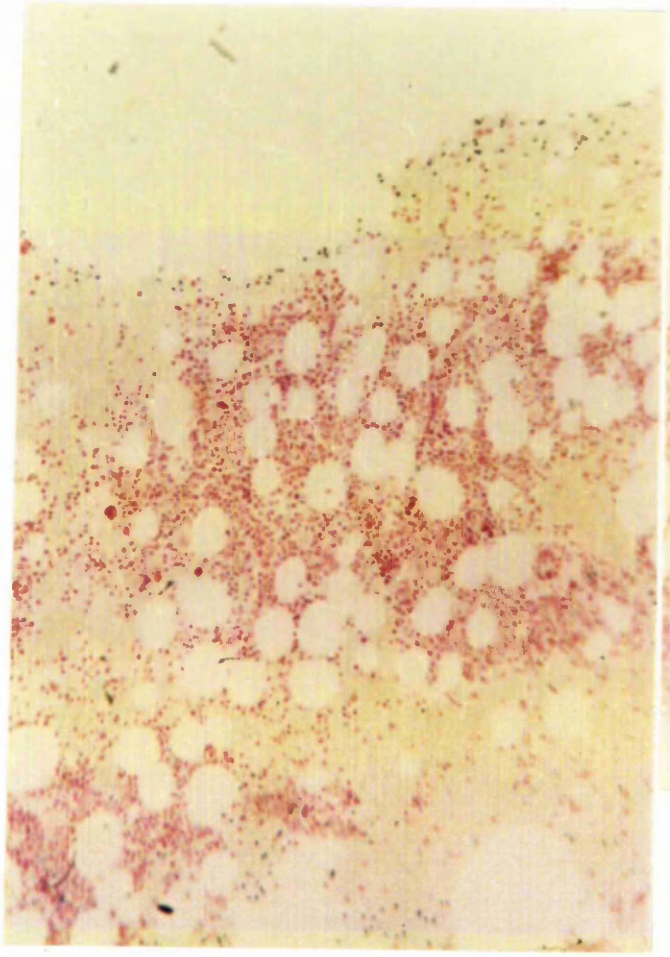
cent (SD ± 14.6) the corresponding cord transferrin saturations like the cord serum iron concentrations were not statistically different between the groups associated with low and high maternal ferritin concentrations. There was no correlation between maternal and cord transferrin concentrations nor the iron saturation of transferrin and baby weight.

Summary In subjects who had a normal pregnancy and were maintained throughout on iron supplements the cord serum ferritin, iron and percentage transferrin saturation are in excess of maternal concentrations at term but the transferrin concentration is lower. The maternal ferritins do not correlate directly with cord concentrations but low maternal levels are associated with low cord levels. The maternal ferritin concentration is statistically lower in multiparous and social class V mothers but is unaffected by maternal age, or gestation between 38 to 41 weeks. Cord ferritin concentrations are unaffected by the sex of the baby, or maternal parity, age, social class or gestation between 38 to 41 weeks.

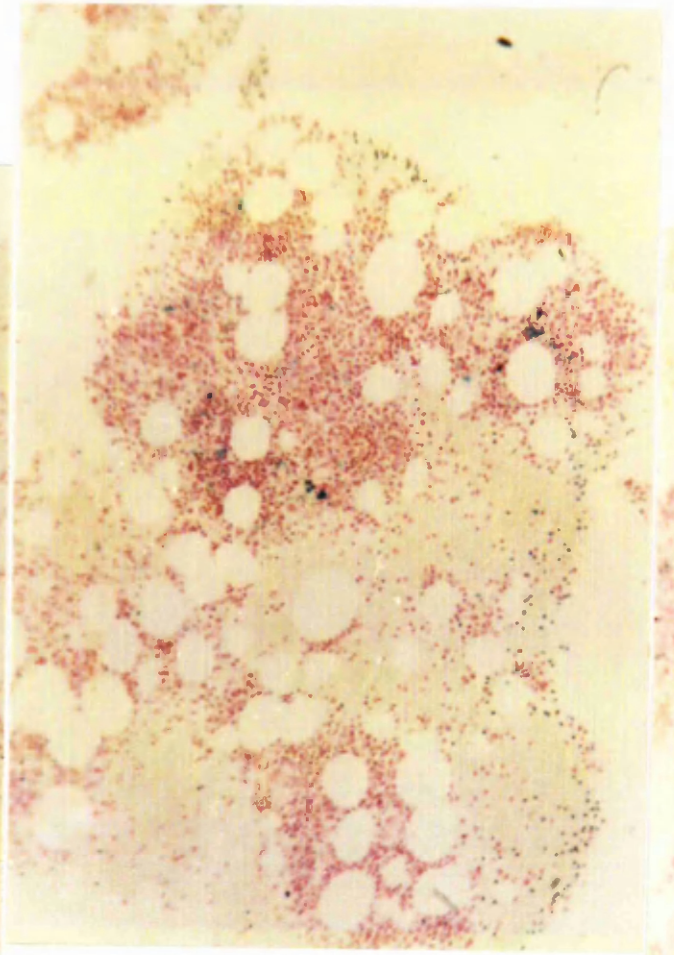
Relationship Between Serum Ferritin and Bone Marrow Iron Stores

Bone marrow biopsies were undertaken on male and non-pregnant female patients as part of the routine investigations of their clinical condition. Blood specimens were obtained at the same time for serum ferritin, iron and transferrin determinations. Bone marrow films were stained for iron (Perl's stain) and graded by one consultant haematologist as follows: 0, no stainable iron; $\pm$, sparse staining; + and ++, gradings of normal staining; and +++, gross staining see Plate 3,I. An iron deficient marrow was graded 0 or $\pm$, a normal marrow + or ++ and excessive deposition as +++.

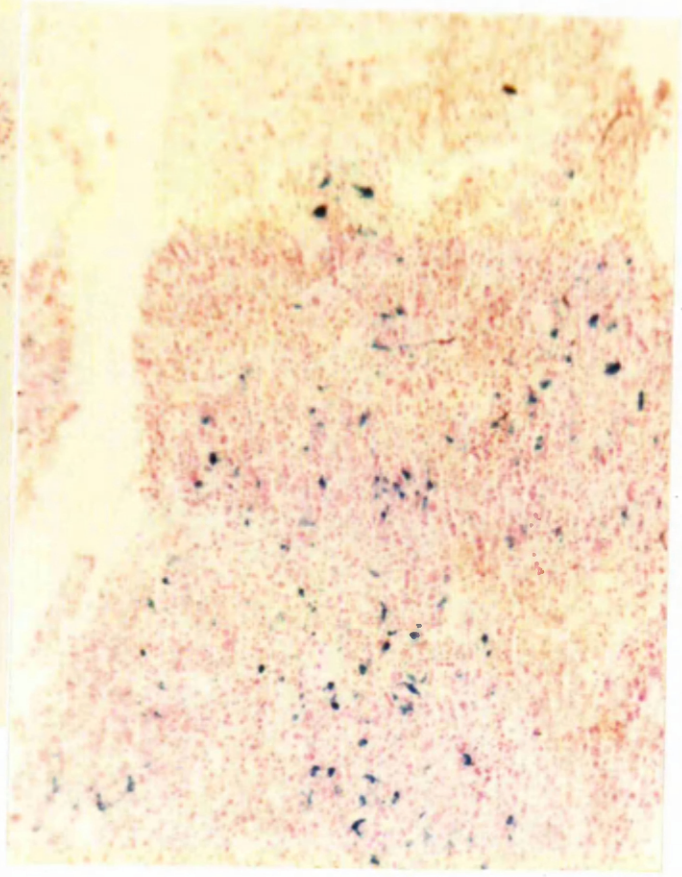
In 50 patients there was good correlation between the iron content of bone marrow and serum ferritin see Figure III,6 and Table 3,12. There was a significant difference between the ferritin groups ($p<0.001$). In cases of megaloblastic anaemia, chronic infections, inflammatory conditions or malignancies ferritin levels were higher than the controls for the respective bone marrow iron contents, see Table 3,13. In the control group with good correlation between bone marrow iron and ferritin concentration the serum iron and percentage transferrin saturation rose with increasing bone marrow iron content, but the groupings were best identified by serum ferritin. Twenty-Three patients had megaloblastic anaemia and all bone marrow biopsies contained iron. In this group the bone marrow iron content was associated with a higher plasma ferritin than in the corresponding control group and there was no difference in the ferritin level of the ++ and +++ marrows. Transferrin increased and the percentage transferrin saturation decreased with the increasing iron content of the marrow.



a) Iron deposit =0



b) Iron deposit = 1+



c) Iron deposit = 3+

Plate 3, (a-c)

Bone Marrow Stained for Iron

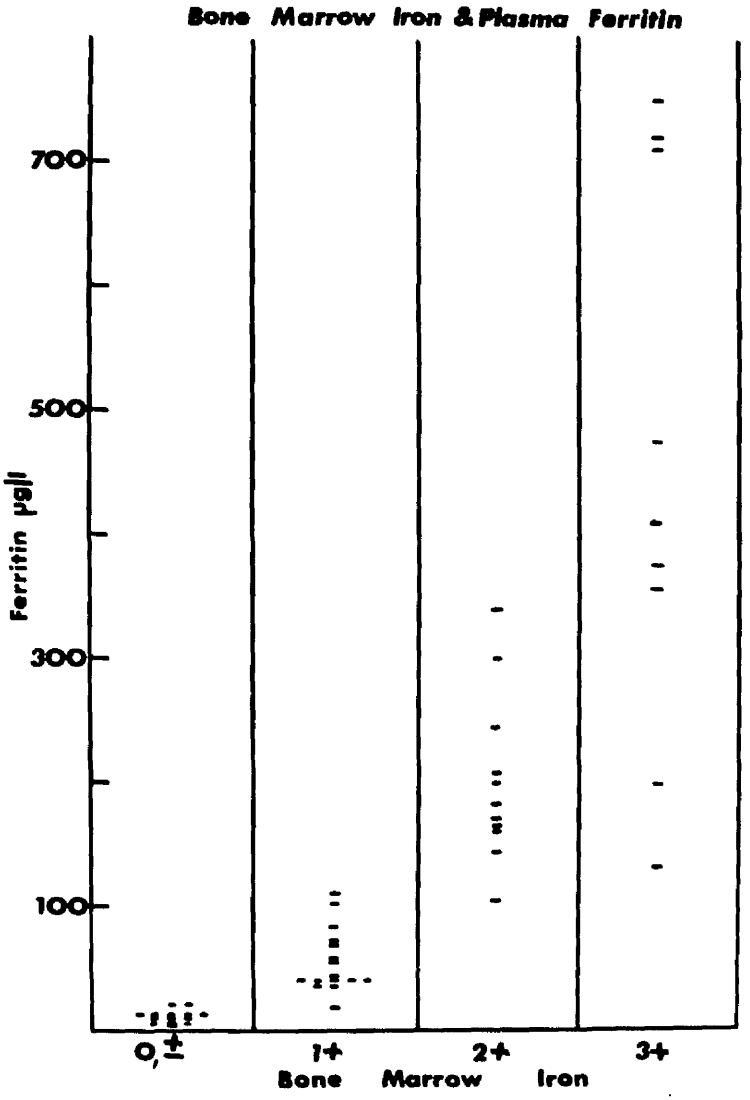


Fig III,6

Iron Content of Bone Marrow Related to Plasma Ferritin

TABLE 3,12

Indices of Iron Metabolism and Bone Marrow Iron

(Numbers in parentheses)

Bone Marrow Iron	0,±	+	++	+++
Ferritin ug/l	11.1 ± 6.0 (14)	55.3 ± 25.7 (16)	202.6 ± 69.1 (11)	458.7±226.4 (9)
Iron umol/l	5.5 ± 4.3 (13)	12.6 ± 9.3 (16)	18.2 ± 12.9 (11)	18.6 ± 9.6 (9)
Transferrin mg/l	2877 ± 655 (13)	1709 ± 345 (14)	1835 ± 321 (10)	1823 ± 622 (8)
Saturation %	8.3 ± 7.0 (13)	27.3 ± 21.8 (15)	32.2 ± 24.4 (10)	39.4 ± 13.7 (8)

Table 3,13

Bone Marrow Iron and Indices of Iron Metabolism in Group I: Megaloblastic Anaemia and Group II: Chronic Infection, Inflammatory Conditions and Malignancy

(Numbers in parentheses)

Group I	Bone Marrow Iron +	++	+++
Ferritin ug/l	116.8 ± 82.7 (6)	392.9 ± 244.2 (8)	318.1 ± 224.2 (9)
Iron umol/l	22.8 ± 7.3 (6)	22.1 ± 14.2 (7)	16.8 ± 8.0 (9)
Transferrin mg/l	1575 ± 304 (6)	1775 ± 424 (6)	1853 ± 669 (8)
Saturation %	53.5 ± 30.7 (6)	42.7 ± 25.5 (6)	35.6 ± 12.7 (8)
Group II	**0 ±**	**+**	**++**
Ferritin ug/l	137.5 ± 217.3 (13)	265.1 ± 243.3 (8)	314.7 ± 213.2 (6)
Iron umol/l	8.3 ± 5.0 (11)	15.3 ± 16.5 (8)	14.6 ± 15.3 (5)
Transferrin mg/l	2243 ± 561 (10)	1977 ± 632 (8)	1615 ± 352 (6)
Saturation %	15.3 ± 11.5 (10)	24.3 ± 26.0 (7)	22.8 ± 23.1 (4)

Twenty-seven patients had chronic infections, inflammatory conditions or malignancies and in this group the highest plasma ferritin levels were associated with the lowest amount of stainable iron in the bone marrow. The transferrin concentrations decreased with increasing marrow iron, but there was little variation in the other indices of iron metabolism in the plasma when the bone marrow contained increasing amounts of iron.

Summary

Bone marrow iron content correlates with the plasma ferritin concentration. However, in cases of megaloblastic anaemia, chronic infection, inflammatory conditions and malignancies increased plasma ferritin concentrations occur which do not correlate with the stainable iron in the bone marrow.

Chapter Four

STUDIES OF ABNORMAL PREGNANCIES, NEONATES, FETAL ORGANS AND PLACENTAE

The variation of plasma ferritin with regard to maternal pathology and neonatal development have been assessed. Factors influencing the ferritin content of fetal organs and the placenta were investigated.

Maternal Iron Metabolism and Disorders of Pregnancy

Plasma ferritin, iron, transferrin and percentage saturation were quantitated in a number of disorders specifically associated with pregnancy - toxaemia of pregnancy, intrauterine growth retardation, ante-partum haemorrhage, premature labour, hyperemesis, and multiple pregnancies; and in the more general clinical conditions of infection and the metabolic disorders associated with diabetes, abnormal liver function and anaemia. All patients were prescribed iron supplements (100 mg elemental iron) from early pregnancy.

Specific disorders of prenancy: Toxaemia

Toxaemia of pregnancy or pre-eclamptic toxaemia (PET) is a syndrome associated with an elevation of blood pressure (diastolic>90 mmHg) with either oedema and/or proteinuria and arises in the third trimester. In severe PET the diastolic blood pressure is greater than 110 mmHg or is 90 mmHg with proteinuria of more than 0.5g per 24 hours.

One hundred and twenty-four patients with clinically defined PET were studied between 26 weeks and term and included 19 women with severe PET. When the volume of plasma was sufficient iron and transferrin were also quantitated.

The results from the 124 PET patients appear in Table 4,1 and listed separately are the findings for the severe PET group. The mean plasma ferritin was higher in severe PET group when compared to the total PET group ($p \leq 0.01$). No significant difference was found between the groups in iron, transferrin or percentage saturation. Twenty-six patients, 24.8 per cent with nild to moderate PET had plasma ferritin concentrations of less than 10ug/per litre which compares with 30.0 per cent in normal pregnant women at term, but only two, 10.5 per cent of patients with severe PET had low ferritin levels.

At 36 to 40 weeks gestation the ferritin concentration of the 81 patients with mild/moderate PET was 25.3 ± 17.2 ug per litre and for the 12 patients with severe PET the elevated level was maintained at 65.1 ± 58.0 ug/per litre ($p < 0.001$).

TABLE 4,1

INDICES OF IRON METABOLISM IN PET

(Numbers in parenthesis)

GROUP	FERRITIN ug/l	IRON umol/l	TRANSFERRIN mg/l	SATURATION %
Total PET	37.1 ± 39.3 (124)	20.4 ± 9.7 (113)	3400 ± 833 (101)	24.8 ± 12.9 (95)
Severe PET	70.0 ± 50.7 (19)	21.3 ± 8.2 (12)	3149 ± 666 (18)	25.8 ± 13.8 (10)

Intrauterine Growth Retardation

Ninety-three patients who on clinical or biochemical grounds gave evidence of intra-uterine growth retardation were investigated. Thirty-eight women gave clinical evidence of carrying small babies (LFD), 22 had lost weight without any other complicating factor and 33 had low plasma HPL and/or urinary oestriol (O_3) concentrations. The results appear in Table 4,2. Plasma ferritin ($p \leq 0.01$) and iron ($p \leq 0.02$) appear to be lower and the transferrin higher ($p \leq 0.001$) in the "weight loss" group when compared to the other two groups. At 36 to 40 weeks the plasma ferritin was 52.4 ± 67.6 ug per litre in the LFD group, 26.3 ± 12.6 ug per litre in the weight loss group and 68.4 ± 67.2 ug per litre in the low HPL/O_3 group showing little variation when compared to the longer period of gestation. Considerable variability was encountered in the plasma ferritin concentration but it would appear to be elevated in the LFD ($p \leq 0.05$) and in the low HPL/O_3 groups ($p \leq 0.005$) when compared to normal pregnant patients with 20.7 ± 11.2 ug per litre at 36 to 40 weeks.

Antepartum haemorrhage

The effect of ante partum haemorrhage on iron metabolism was investigated in 21 patients who were admitted to hospital with varying degrees of blood loss. Specimens for analyses were obtained after treatment had commenced and therefore only reflect the adequacy of the medication. At 36 to 40 weeks the plasma ferritin was 26.3 ± 13.6 ug per litre, iron 10.9 ± 6.2 umol per litre, transferrin 3381 ± 612 mg per litre and transferrin saturation 12.1 ± 8.2 per cent. The ferritin and transferrin concentrations compare with the normal patients but iron ($p < 0.01$) and percentage saturation ($p < 0.01$) are lower.

TABLE 4,2

Indices of Iron Metabolism in Intrauterine Growth Retardation

(Number of Patients in parentheses)

Group	Ferritin ug/l	Iron umol/l	Transferrin mg/l	Saturation %
LFD	52.4 ± 67.6 (38)	19.7 ± 9.2 (20)	2988 ± 811 (37)	23.2 ± 9.5 (20)
Weight loss	26.3 ± 12.6 (22)	18.7 ± 8.4 (13)	3465 ± 537 (14)	18.5 ± 7.6 (13)
Low HPL/O_3	68.4 ± 67.2 (33)	20.3 ± 10.1 (23)	3182 ± 575 (27)	21.9 ± 10.9 (23)

Serial results which were available for one patient gave a plasma ferritin of 15.4 at 7 days, 19.0 at 14 days, 22.4 at 21 days and 32.0 ug per litre at 28 days after treatment had commenced. Only two of the twenty-one patients had ferritin concentrations of less than 10 ug per litre before delivery.

Premature Labour

Fifteen patients in premature labour, between 26 and 36 weeks gestation were investigated after admission to hospital. None of the patients were found to have low plasma ferritin levels on the first analysis and between 36 and 40 weeks the plasma ferritin was 20.6 $\pm$ 7.3 ug per litre, iron 16.6 $\pm$ 6.9 umol per litre, transferrin 3392 $\pm$ 533 mg per litre and transferrin saturation 13.2 $\pm$ 3.4 per cent. All results were within the limits of normality.

Hyperemesis

Fifteen women with hyperemesis between 21 and 36 weeks were investigated. On initial testing the plasma ferritin was 24.7 $\pm$ 32.9 ug per litre and six of the patients, 40 per cent, had concentrations of less than 10 ug per litre. At 36 to 40 weeks the plasma ferritin was 21.1 $\pm$ 17.1 ug per litre, iron 17.0 $\pm$ 6.1 umol per litre, transferrin 3096 $\pm$ 502 mg per litre and transferrin saturation 22.4 $\pm$ 9.4 per cent which compared with values found for control patients. Only three patients 20 per cent, had low ferritin concentrations before delivery.

Multiple pregnancies

Twins were diagnosed in 20 pregnancies. In the third trimester the plasma ferritin was 45.2 $\pm$ 46.5 ug per litre, iron 17.7 $\pm$ 12.3 umol per litre, transferrin 3072 $\pm$ 597 mg per litre and transferrin saturation 18.4 $\pm$ 12.1 per cent.

At 36 to 40 weeks only the plasma iron had marginally increased to 21.9 ± 17.1 umol per litre. Three patients had low ferritin levels in early pregnancy but at term only one had a low concentration and at 36 to 40 weeks the maternal ferritin of twins was higher than that of singletons ($p \leq 0.02$).

Infection associated with pregnancy

Urinary tract infections occur frequently during pregnancy and twenty of these patients were studied between 21 weeks and term. On initial testing the plasma ferritin was 89.3 ± 81.6 ug per litre which was higher than the normal control group ($p \leq 0.001$), iron 16.1 ± 10.2 umol per litre, transferrin 3354 ± 650 mg per litre and transferrin saturation 18.8 ± 11.8 per cent. Twelve patients were investigated before delivery and the plasma ferritin remained elevated 58.8 ± 38.6 ug per litre, ($p \leq 0.001$). No change was found in the other indices of iron metabolism. Only one patient had a low plasma ferritin of 10 ug per litre at term.

Metabolic disorders associated with pregnancy: Diabetes mellitus.

Eleven patients with diabetes mellitus were investigated from 26 weeks and one from 15 weeks until term. This latter patient had an initial plasma ferritin of 3 ug per litre, but by 30 weeks it was normal. 25 per cent of patients presented with low ferritin levels. At 36 weeks to term the plasma ferritin was 33.3 ± 16.4 ug per litre, iron 18.7 ± 8.4 umol per litre, transferrin 3485 ± 553 mg per litre and transferrin saturation 18.9 ± 7.6 per cent for seven patients. One patient had a low ferritin concentration. The indices of iron metabolism of pregnant diabetics are not significantly different from normal apart from a marginal increase in plasma ferritin ($p \leq 0.025$).

Disordered liver function.

Seventeen patients with abnormal liver function tests including elevated plasma bilirubin, alkaline phosphatase and alanine transaminase were investigated. The pattern of results and clinical symptoms suggested that most had "jaundice associated with pregnancy". One patient presented at 21 weeks, one at 26 weeks, two at 32 weeks and thirteen at 36 to 38 weeks. The initial plasma ferritin was 57.6 $\pm$ 60.6 ug per litre, iron 11.7 $\pm$ 10.3 umol per litre, transferrin 2986 $\pm$ 1021 mg per litre and transferrin saturation 13.2 $\pm$ 12.6 per cent. The plasma ferritin was elevated in one patient at 260 ug per litre, and the mean concentration was greater than the control group ($p<0.02$), but in five mothers (29 per cent) it was less than 10 ug per litre when they were first investigated.

Anaemia

A pregnant woman with a haemoglobin of 11.0 g per dl or less is considered anaemic. Thirty-eight women who attended the out-patient clinic had low haemoglobin levels when the determination was made on finger-prick specimens. Venous blood specimens were thereafter obtained for the determination of ferritin, iron, transferrin and haematological tests. The results were grouped according to the findings of the blood film which were undertaken by the haematologist. They were divided into three categories depending on whether they were purely iron deficient with a hypochromic, microcytic film, secondly megaloblastic or a mixed iron deficient megaloblastic picture or thirdly infection with polymorphs and leucocytosis identifiable in the film. The results appear in Table 4, 3 and the distribution of the ferritin concentrations is shown is Figure IV,1.

Table 4,3

Indices of Iron Metabolism in Anaemia of Pregnancy

(Numbers in parenthesis)

Anaemia	Hb g/dl	Ferritin ug/l	Iron umol/l	Transferrin mg/l	Saturation %
Iron Deficiency Anaemia	9.4±0.7 (21)	8.6±5.0 (22)	10.0±6.0 (18)	3652±1050 (12)	10.6±7.3 (17)
Megaloblastic Anaemia	9.6±0.6 (8)	35.2±31.5 (11)	17.2±6.4 (10)	2767±707 (8)	20.1±7.9 (9)
Anaemia + Infection	10.1±0.8 (4)	56.2±40.0 (5)	11.6±3.6 (5)	3132±1184 (3)	15.4±2.7 (4)

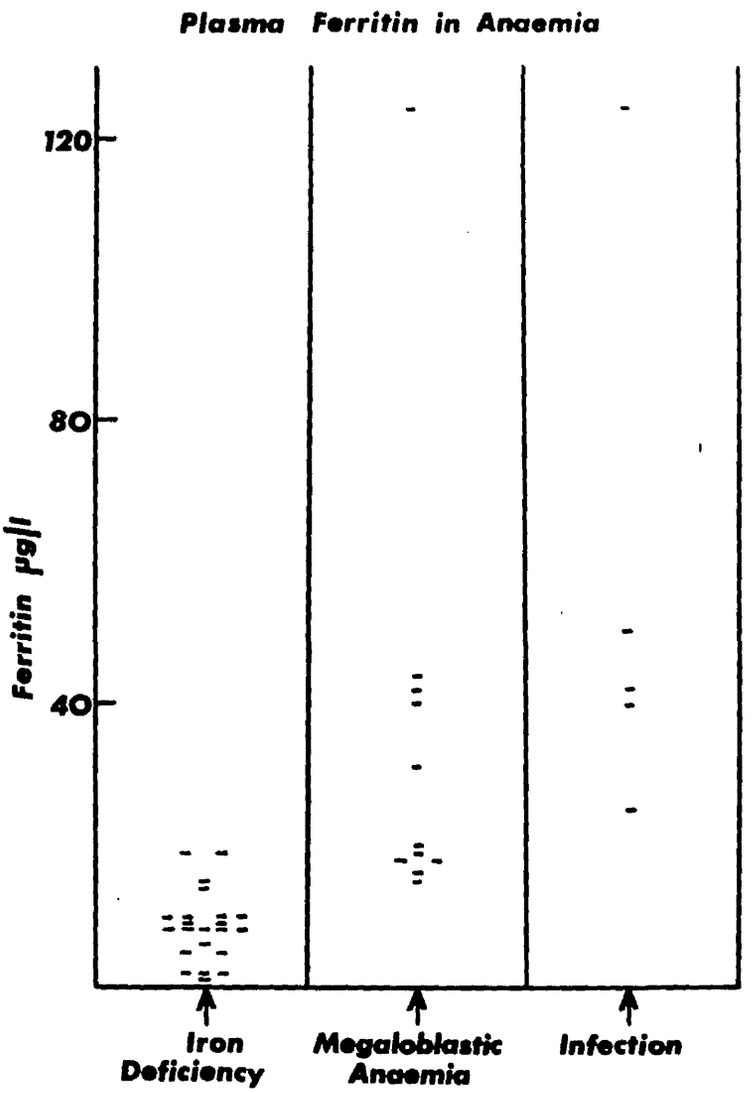


Fig. IV, 1

Plasma ferritin in iron deficiency and megaloblastic anaemia and infection

In the iron deficient group the ferritin, iron and transferrin saturation were reduced, but when megaloblastic anaemia was present the results were within normal limits. When the deficiency was purely due to folic acid or vitamin B_{12} deficiencies the ferritin concentration was higher than when iron deficiency was also present. The anemia of chronic infection presented results of elevated ferritin, but reduced iron concentration and were similar to the findings in patients with urinary tract infections.

Summary.

Elevated plasma ferritin concentrations were found in patients with severe toxaemia, urinary tract infections, those who were clinically small for dates or who had biochemical evidence of inadequate placental function. Premature labour was not associated with reduced iron stores as evidenced by normal plasma ferritin concentrations and although some patients with hyperemesis had low ferritin levels this was not statistically significant. In twin pregnancies mothers had raised ferritin concentrations which were also found occassionally when patients were jaundiced but low levels were also found. Anæemic patients had low ferritin concentrations when the condition was due to iron deficiency, but when it was caused by a megaloblastic condition or infection the levels were elevated.

Plasma Ferritin Concentration in the Neonate

Three hundred and sixty-five specimens of heparinised plasma from 158 patients between 27 and 41 weeks gestation were analysed to determine the plasma ferritin concentrations. The specimens were obtained by heel prick or venipuncture and the plasma used in the investigation was the volume remaining after the requested biochemical tests had been performed.

Plasma ferritin concentration in relation to gestation.

In Table 4, 4 the results of the plasma ferritin concentration are shown for premature and term babies in relation to their age in days. Logarithmic transformation was applied to the data as it gave a non-gaussian distribution. The mean ferritin concentration of term babies was greater than that of the prematures until the sixth day, but thereafter the results were greater in the latter group. Statistically significant differences were found between the two groups. There is a highly significant correlation between plasma ferritin and gestation ($r=0.57$; $p<0.001$).

The term babies demonstrated no statistical difference in the ferritin concentration from Day 2 to 36 when compared to Day 1 but wide variations were found for each day. The concentration on Day 1 was considerably greater than that of the term cord specimens with a mean of 169 (range 55 to 296) ug per litre reported on page 106 ("Cord ferritin concentrations of term babies").

The variation in ferritin concentration within the premature group was analysed in relation to Day 3 which had an arithmetic mean concentration of 185 ($\pm$ 76) ug per litre and from the seventh until the thirty-sixth day the concentrations were significantly elevated. On Day 7 the arithmetic mean concentration was 413 $\pm$ 55 ug per litre ($p<0.001$), Day 8-14 was 393 $\pm$ 147 ug per litre ($p<0.001$) and on Day 15-36 it was 419 $\pm$ 144 ug per litre ($p<0.001$).

TABLE 4,4

Plasma Ferritin in Term and Premature Babies in Relation to Age

Gestation	38-41 weeks			27-37 weeks			p
Day	No.	Mean ug/l	Range ug/l	No.	Mean ug/l	Range ug/l	
1	11	245	90-665	6	156	34-710	≤ 0.30
2	10	245	110-545	7	187	99-353	≤ 0.20
3	19	299	110-812	12	187	64-542	≤ 0.01
4	43	270	122-602	19	207	100-429	≤ 0.005
5	49	270	100-735	21	191	57-566	≤ 0.025
6	35	299	134-665	8	255	103-627	≤ 0.20
7	18	270	122-602	8	396	289-543	≤ 0.02
8-14	25	329	189-570	13	358	229-559	≤ 0.20
15-36	7	184	51-662	11	380	193-749	≤ 0.10

p is degree of significance when the 27-37 week group is compared to the 38-41 week group.

The ferritin concentration in the plasma of premature babies was analysed with respect to gestation and the results are shown in Table 4, 5. Statistical significance was found between 35 to 37 week group and 27 to 34 week group at Day 4-6 only ($p<0.001$).

Plasma Ferritin in relation to body weight

The plasma ferritin concentration was analysed with respect to the birth weight of the infant between Day 2 to 5 as during this time period little change occurred in plasma ferritin in either the premature or term group. The results are given in Table 4, 6 and suggest that there is a significant correlation between plasma ferritin concentration and body weight ($r=0.47$; $p<0.001$). No significant difference in the plasma ferritin concentration of twins was found when compared to singletons. Nine twins between 28 and 33 weeks gestation had a level of 185 $\pm$ 46 (range 120 to 258) ug per litre on Day 4 or 5 and five twins of 37 to 38 weeks gestation had a concentration of 285 $\pm$ 110 (range 202 to 460) ug per litre over the same period. In general, normal levels were also found when the baby was light-for-dates and examined over an identical time scale. Ten babies between 39 and 40 weeks gestation had low birth weights and the plasma ferritin was 291 $\pm$ 138 (range found 37 to 500)ug per litre and of the eleven premature babies who were also light-for-dates the concentration was 209 $\pm$ 116 (range found 80 to 460)ug per litre. Only one of the twenty one babies had a level outwith the normal limits. Low plasma ferritin concentrations were rarely found and were not identified with one clinical condition. Nine of the 158 babies examined had low levels and three were premature and one mature, but light-for-dates, five infants had levels of less than 60 ug per litre and four were between 60 and 100 ug per litre.

TABLE 4,7

Plasma Ferritin in Relation to Plasma Bilirubin

(Number of specimens in parentheses)

Group	Bilirubin µmol/l < 150	151–200	201–250	251–350
Mature	243 ± 77 (22)	276 ± 97 (39)	315 ± 102 (45)	353 ± 110 (21)
Premature	135 ± 55 (8)	237 ± 98 (21)	271 ± 72 (13)	394 ± 104 (6)
Total	214 ± 86 (30)	262 ± 98 (60)	314 ± 121 (58)	362 ± 104 (27)

TABLE 4,5

Plasma Ferritin in Premature Infants

(Number of specimens in parentheses)

Gestation weeks	Day 1-3 Ferritin µg/l	Day 4-6 Ferritin µg/l
35–37	231 ± 105 (15)	271 ± 102 (14)
31–34	125 ± 70 (4)	206 ± 92 (18)
27–30	180 ± 78 (6)	178 ± 51 (14)
27–34	158 ± 76 (10)	194 ± 77 (32)

TABLE 4,6

Plasma Ferritin in Relation to Body Weight

Body Weight kg	Numbers	Plasma Ferritin ug/l
1.0 - 1.5	11	197 ± 54 $p \leq 0.01$
1.6 - 2.0	14	215 ± 128 $p \leq 0.05$
2.1 - 2.5	16	278 ± 92 $p \leq 0.2$
2.6 - 3.0	22	271 ± 104 $p \leq 0.2$
3.1 - 3.5	22	293 ± 89 $p \leq 0.2$
3.6 - 4.0	9	351 ± 142

p is degree of significance when compared to
3.6 - 4.0 Kg group

When serial specimens were available the concentrations were found to increase but remained lower than the normal gestational range. The plasma ferritin of 24 premature infants who were asphyxiated at birth, Apgar 6 and 1 minute was 235(±106) ug per litre on Day 4 to 6 and of 14 term babies was 266(±71) ug per litre. No difference was apparant between the asphyxiated infants and the overall gestational equivalent. Six babies had infections and the mean plasma ferritin was 348(± 218) ug per litre. Three of the babies had levels of 375, 540 and 650 ug per litre, but the number of subjects was small and the findings could not be considered statistically.

Plasma ferritin in relation to plasma bilirubin concentration.

The plasma ferritin concentrations of premature and term babies in whom there was no evidence of a blood group incompatibility, are given in Table 4,7 in relation to the maximum plasma total bilirubin. In both groups the ferritin concentration increased in proportion to the rising bilirubin level. The mature infants had higher ferritin concentrations when the bilirubin was less than 250 umol per litre, than premature infants, but this was not maintained when there was a further increase in bilirubin concentration.

Plasma ferritin in babies with blood group incompatibilities

Thirteen babies had ABO or Rhesus incompatibilities and gave a positive Coombs' test. Seven babies required exchange transfusions at 4hrs-7 days after birth. Five babies whose birth weight ranged from 2.16 to 3.79 Kg (mean 3.10 ± 0.50 Kg) and who did not require transfusions (plasma bilirubin 111± 73 umol per litre) had elevated plasma ferritin concentrations of 409 ± 81 ug per litre. At birth the blood of a baby who had three transfusions in utero was almost entirely of donor origin.

The birth weight was 2.30 Kg at 33 + weeks and the baby did not require an exchange transfusion. The bilirubin remained low, 72 umol per litre, but the plasma ferritin was grossly elevated at 1,000 ug per litre on Day 1 and 1,270 ug per litre three hours later.

The results of the pre- and post-exchange ferritin concentrations for seven babies are given in Table 4,8. The three donor bloods which were analysed contained 3,13 and 54 ug of ferritin per litre. The plasma ferritin of the pre-exchange specimens was significantly greater than the normal range ($p<0.005$) but the level decreased on exchange transfusion due to low concentration of donor plasma. However, further specimens showed that the ferritin did not remain at the lower level. In Patient 1 the plasma ferritin was 300 ug per litre at 28 hours, in Patient 2 it was 490 ug per litre at 60 hours, in Patient 5 it was 325 ug per litre at 82 hours and in Patient 7 it was 450 ug per litre at 72 hours. The highest ferritin concentration in this group was found in Patient 3 at 19 days when the level was 1,850 ug per litre and the bilirubin 52 umol per litre.

Plasma Iron Concentration in the Neonate: Table 4,9

The plasma iron decreased rapidly in term babies within the first 48 hours and thereafter a rapid increase followed and this concentration was maintained during one month which was the period of study. There was considerable individual variation. No cord levels were available for the premature group but similar low concentrations were found in the first two days to be followed by a rapid increase on Day 3 which was maintained until Day 36. Due to the lack of numbers daily changes in transferrin concentration could not be analysed but the concentration of term infants from Day 1 to 7 was $1,432 \pm 330$ mg per litre (N=39) which compared with term cord concentrations of $1,717 \pm 393$ mg per litre (N=115).

TABLE 4,8

Rhesus-isoimmunised Babies Requiring Exchange Transfusion

Patient	Weight Kg	Age at Exchange	Pre-Exchange		Post-Exchange	
			Ferritin ug/l	Bilirubin umol/l	Ferritin ug/l	Bilirubin umol/l
1	3.37	< 4 hrs	365	260	277	128
2	3.36	48 hrs	620	392	205	250
3	2.81	~ 4 hrs	990	152	268	104
4	3.04	4 days	420	388	325	260
5	3.81	~ 4 hrs	258	164	140	85
6	2.98	7 days	740	472	332	248
7	-	~ 4 hrs	610	335	208	165
Mean			572	309	251	177
± 1SD			249	121	70	75

TABLE 4,9

Plasma Iron μmol/l in Term and Premature Babies

(Number of specimens in parentheses)

Group	Premature	Term
Cord	-	27.0 ± 7.5 (115)
Day 1-2	11.5 ± 9.4 (8)	14.7 ± 9.5 (10)
Day 3	31.1 ± 29.2 (6)	26.0 ± 23.6 (9)
Day 4	37.8 ± 17.8 (9)	24.6 ± 19.0 (25)
Day 5	31.8 ± 23.0 (9)	25.2 ± 16.6 (22)
Day 6	60.2 ± 29.5 (4)	19.2 ± 18.7 (18)
Day 7	24.2 ± 6.5 (4)	13.7 ± 11.8 (11)
Day 8-14	35.7 ± 27.7 (8)	26.1 ± 21.2 (18)
Day 15-36	24.0 ± 16.4 (4)	22.1 ± 16.5 (8)

During the first week the transferrin concentration of the premature group was 1,303 ± 388 mg per litre (N=19) and was not significantly different from the term group.

Summary

The concentration of plasma ferritin in the neonate is related to gestational age, birth weight and plasma bilirubin level. In the premature infant the ferritin increase with age. In this series twins, light-for-dates and asphyxiated babies had normal ferritin concentrations, but there were indications that infected babies may have raised levels.

Ferritin Deposition in Fetal Organs

The ferritin content of hearts, livers and spleens from 26 fetuses obtained at autopsy were quantitated as described under "Quantitation of Plasma and Tissue Ferritin", page 65 . Six of the babies were stillborn, 16 died in the neonatal period and four at 13, 20, 30 and 36 days after delivery. Clinical details of the babies appear in Table 4,10. There were 14 males and 12 females in the study. Twenty-two babies were born prematurely and four were delivered at term, between 38 and 40 weeks. Five infants were cosidered to be light-for-dates according to the criteria of Battaglia and Lubchenco (1967). Nineteen babies were from singleton pregnancies and seven from twin pregnancies. The weights of the fetuses and their organs related to gestation are summarised in Table 4, 11.

Fetal organ ferritin related to gestation

The concentration and total organ content of ferritin related to gestation at death are shown in Table 4, 11. A term infant with gross congenital abnormalities including hypoplastic lungs, oesophageal atresia and absence of kidneys and gall-bladder was omitted from the results as the ferritin content of the liver (13.3mg) and spleen (234ug) appeared much reduced and may have resulted from the malformations described. There was considerable variation in organ concentration and content at all stages of gestation.

The total ferritin content of the liver increased with advancing gestation ($r=0.65$; $p \leq 0.001$) as shown in Figure IV, 2. There was a statistically significant increase in ferritin content between 24 weeks and term ($p \leq 0.005$).

Table 4,10

Clinical Details of Fetuses

Fetus	Sex	Weight (Kg)	Gestation (Weeks)	Age at Death	Weight Liver (g)	Weight Heart (g)	Weight Spleen (g)
1	F	0.68	24	SB	35	5	1
2	F	0.78	25+	10 hrs	36	7	1
3	M	0.75	25+	12 hrs	28	5	1
4	F	0.93	25+	SB	45	5	1
5	F	1.06	28	9 hrs	41	4	1
6	F	1.06	28+	4 days	40	8	2
7	F	1.36	29	SB	77	10	5
8	M	1.58	29	30 days	60	11	5
9	F	1.48	29+	35 days	73	12	7
10	F	1.09	31	6 days	52	10	3
11	F	0.76	31	SB	48	6	2
12	M	1.29	31	1 day	41	8	2.5
13	M	1.88	32	1 hr	65	14	3
14	F	1.66	32	4 days	71	11	5
15	M	2.01	33	20 hrs	80	16	6
16	M	1.78	33+	13 days	66	15	5
17	M	1.60	35	SB	32	10	4
18	M	1.90	35	SB	56	10	7
19	F	2.38	35	12 hrs	155	20	20
20	M	1.89	36	8 mins	90	11	10
21	M	2.13	36	18 hrs	78	9	5
22	F	2.08	36+	4 days	72	13	6
23	M	2.94	38	20 days	240	17	8
24	M	3.68	40	4 days	185	22	11
25	M	2.40	40	1.5 hrs	127	19	10
26	M	2.38	41	15 mins	90	16	10

Table 4,10 (cont)

Pathology

1. PET, Hydrops, CHD
2. IVH, Atelectasis, Asphyxia, Twin Ia
3. Asphyxia, Atelectasis, Twin IIa
4. Fetal Bleed
5. IVH, Atelectasis
6. Pneumothorax, IVH, HMD
7. Intra-uterine pneumonia, Premature Rupture Membranes
8. Recurrent Pneumonia, Anaemia
9. Pulmonary fibrosis, Pneumonia, Pneumothorax
10. HMD, Pulmonary Haemorrhage, Pneumothorax, Twin Ib
11. Anencephaly, Twin IIb
12. IVH, Atelectasis, HMD
13. Asphyxia, Potter IIb
14. HMD, Atelectasis
15. IVH, Asphyxia
16. IVH, Pneumonia, Twin IIc
17. Polycythaemic Twin Id
18. Anaemic Twin IId
19. Rhesus-isoimmunisation
20. Asphyxia, Anencephaly
21. Tentorial tear, Subdural haemorrhage
22. IVH, HMD
23. Pneumonia, Pyelonephritis
24. HMD, Pneumothorax
25. Asphyxia, Osteogenesis imperfecta
26. Multiple Congenital Abnormalities

TABLE 4,11

FETAL ORGAN FERRITIN RELATED TO GESTATION

(± ISD IN PARENTHESES)

Gestation	No.	Fetal Weight Kg	LIVER			HEART			SPLEEN		
			Organ Wt.g	Ferritin ug/g	Ferritin ug/liver	Organ Wt.g	Ferritin ug/g	Ferritin ug/Heart	Organ Wt.g	Ferritin ug/g	Ferritin ug/spleen
24-29	7	0.95 (0.24)	43.1 (15.9)	427 (166) p<0.10	19,874 (14,579) p<0.005	6.3 (2.1)	46 (30) p<0.70	291 (225) p<0.05	1.7 (1.5)	111 (97) p<0.10	264 (385) p<0.05
30-32	5	1.34 (0.45)	55.4 (12.3)	701 (337) p<0.50	39,256 (21,958) p<0.05	9.8 (3.0)	28 (26) p<0.20	236 (148) p<0.05	3.1 (1.1)	143 (60) p<0.20	460 (277) p<0.10
33-35	7	1.82 (0.31)	74.6 (38.6)	625 (564) p<0.40	48,817 (43.386) p<0.05	13.4 (3.7)	41 (40) p<0.60	619 (667) p<0.50	7.7 (5.5)	301 (234) p<0.50	2,311 (2,009) p<0.40
36-40	6	2.62 (0.78)	132.0 (67.5)	987 (612)	118,521 (57,074)	15.2 (5.0)	53 (25)	879 (532)	8.3 (2.4)	476 (421)	4,007 (3,663)

p = degree of significance when compared to group 36-40 weeks gestation.

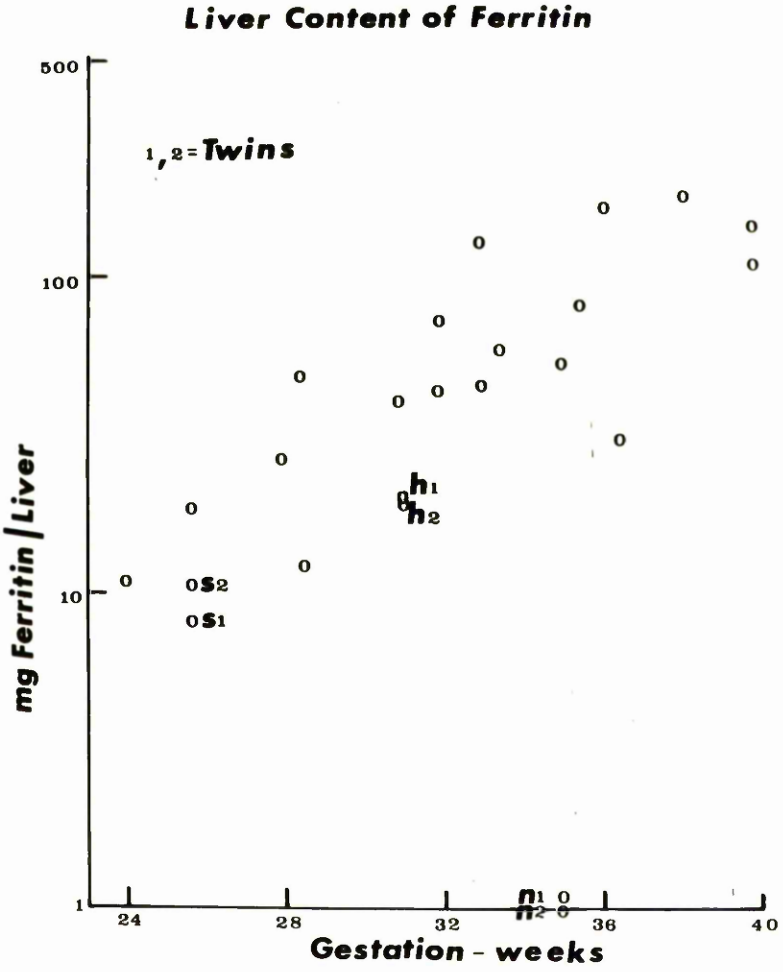


Fig. IV, 2

Fetal liver ferritin content related to gestation

The liver ferritin concentration did not increase with gestation ($r=0.34$; $p<0.20$). The mean concentration between 33 to 35 weeks suggested that no increase had taken place but as this group of seven contained twins with the lowest levels found in the series, this finding would appear to be false. The liver of the Rhesus-isoimmunised fetus was twice the normal weight but the ferritin content was only 9.2 per cent above the mean as the concentration was only 55 per cent of the gestational mean. Five singleton fetuses were asphxiated at birth and three had increased concentrations and total contents of ferritin in the liver which were two to four times the gestational mean. The values for the remaining two fetuses were within normal limits.

The total ferritin content of the heart appeared to increase with organ weight ($r=0.66$; $p<0.001$) see Figure IV,3 and like the concentration was not related to advancing gestation (Table 4,11). There was a statistically significant increase in the total ferritin content of heart between the 24 to 29 week group and the term group ($p<0.05$) but no significant difference was found in concentration. Between 24 to 32 weeks no increase was found in the total ferritin content although the weight of the heart increased. This appeared to be explained by the fall in concentration which occurred between 30 and 32 weeks.

The total ferritin content of the spleen increased with gestation ($r=0.60$; $p<0.005$) between 24 weeks and term ($p<0.05$) see Table 4,11. The most significant increase of 402 per cent occurred between 32 and 35 weeks and coincided with the largest increase in weight, 148 per cent and concentration, 110 per cent. The concentration of ferritin also increased with advancing gestation ($r=0.538$) and is illustrated in Figure IV,4.

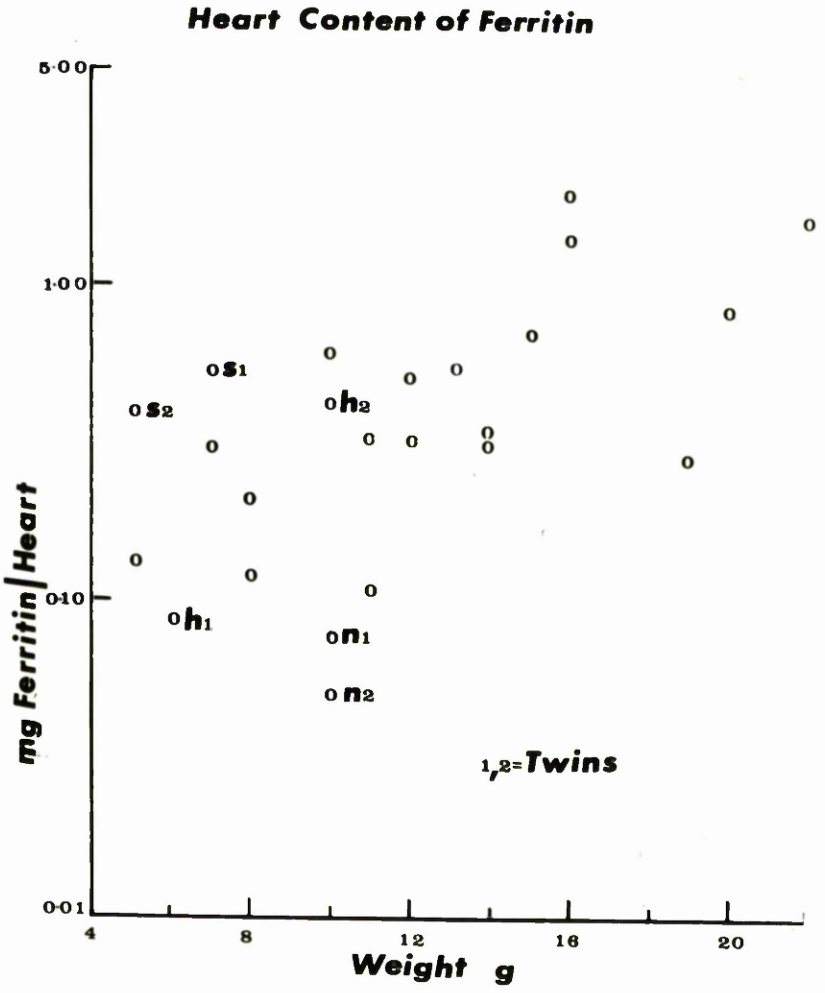


Fig. IV, 3

Fetal heart ferritin content related to organ weight

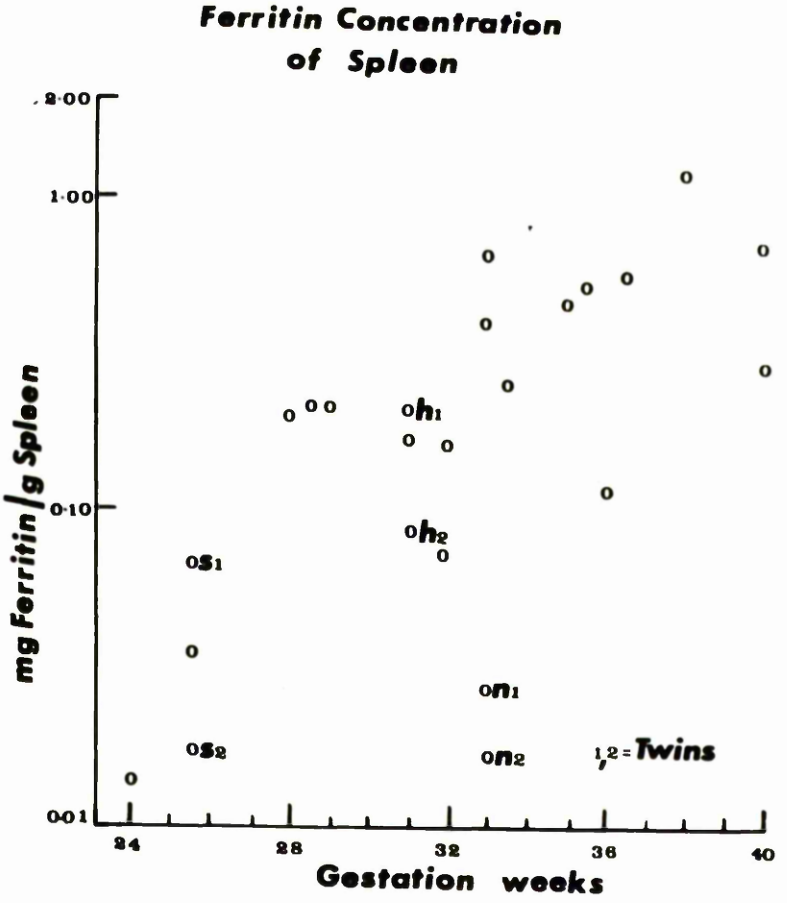


Fig. IV, 4

Fetal spleen ferritin concentrations related to gestation

N_1 and N_2 equivalent to 35 weeks gestation.

The twins who had low concentrationsof ferritin in the liver also had decreased concentrations in their spleens. The spleen ferritin concentration of the Rhesus-isoimmunised fetus was normal but as the organ was three times the normal weight, the content was grossly elevated. No clinical condition was consistently associated with any gross variation of spleen ferritin.

Fetal organ ferritin related to body and organ weight

The ferritin concentration and total content of liver, heart and spleen related to body weight of the fetus at the time of death are given in Table 4, 12. The weights of all three organs were proportional to the increase in total body weight although no account was taken of gestation. Liver $r=0.90$; $p<0.001$; heart $r=0.87$, $p<0.001$; spleen $r=0.71$, $p<0.001$). The total content of ferritin correlated with body and organ weight, but only the ferritin concentration of spleen related to body weight ($r=0.70$; $p<0.001$) However between 1,100 to 1,500g and 1,600 and 2,000g no increase in concentration occurred in any of the organs and in the heart and spleen it decreased although the weights increased. The clinical and biochemical data are given in Table 4, 13 for 5 light-for-dates babies. Only 1 of the 5 had a small spleen (-1SD) but 3 had low total contents of ferritin. Two babies had small hearts, 3 had low ferritin concentrations and contents but 2 had elevated concentrations and contents of ferritin. One infant had a small spleen but 4 had low concentrations and total contents although one was only marginally reduced and one baby had in increased concentration but a normal content.

Six livers and five spleens were small for the gestational age of the fetus. The ferritin concentration and content of these organs are contrasted in Table 4, 14. with normal weight organs of equivalent gestation.

TABLE 4,12

FETAL ORGAN FERRITIN RELATED TO BODY WEIGHT

(± ISD IN PARENTHESES)

Fetal weight g	No.	Liver			Heart			Spleen		
		Organ Wt. g	Ferritin ug/g	Ferritin ug/liver	Organ wt.g	Ferritin ug/g	Ferritin ug/heart	Organ wt. g	Ferritin ug/g	Ferritin ug/spleen
680-1,000	5	38.4 (8.1)	353 (79) p<0.10	13,771 (5,771) p<0.005	5.6 (0.9)	48 (32) p<0.60	287 (216) p<0.05	1.2 (0.4)	45 (32) p<0.05	63 (67) p<0.02
1,100-1,500	6	54.0 (16.9)	620 (242) p<0.30	33,860 (16,301) p<0.01	8.7 (2.7)	36 (26) p<0.20	279 (192) p<0.02	3.4 (2.2)	247 (105) p<0.20	999 (1,119) p<0.05
1,600-2,000	7	62.9 (17.4)	618 (439) p<0.30	43,512 (32,273) p<0.02	11.7 (2.0)	24 (18) p<0.05	306 (250) p<0.02	5.6 (2.3)	181 (230) p<0.10	929 (1,142) p<0.025
2,010-3,680	7	133.9 (63.5)	996 (669)	116,008 (56,690)	16.6 (4.4)	63 (36)	1083 (604)	9.4 (5.2)	497 (355)	4,501 (3,147)

p = degree of significance when compared to group 2,100-3,680 g.

TABLE 4,13

ORGAN FERRITIN IN LIGHT - FOR - DATES BABIES

Parameters which are ± ISD of the gestational mean are marked ↑ or ↓

Gestation weeks	Body Weight Kg	LIVER			HEART			SPLEEN		
		Wt. g	Ferritin Concn ug/g	Ferritin Content ug	Wt. g	Ferritin Concn ug/g	Ferritin Content ug	Wt. g	Ferritin Concn ug/g	Ferritin Content ug
31	0.76	48	395	18,950↓	6↓	73 ↑	437 ↑	2↓	88 ↓	175 ↓
31	1.09	52	391	20,332	10	9 ↓	88 ↓	3	216 ↑	648
35	1.60	32↓	14 ↓	438 ↓	10↓	5 ↓	51 ↓	4	28 ↓	112 ↓
40	2.40	127	1,135	144,145	19	15 ↓	285 ↓	10	248	2,480
40+	2.38	90	148↓	13,320 ↓	16	89 ↑	1,430 ↑	10	23 ↓	234 ↓

TABLE 4,14

FERRITIN IN NORMAL AND LIGHT WEIGHT ORGANS

Gestation weeks	Normal Weight Liver			Light Weight Liver		
	Organ Wt.g	Ferritin ug/g	Ferritin mg/liver	Organ Wt.g	Ferritin ug/g	Ferritin mg/liver
28	41	660	27.1	28	386	10.8
29	77	645	49.6	40	315	12.6
31	71	612	43.5	41	988	40.5
32	65	1,120	73.0	48	395	19.0
33	80	1,620	129.6	52	391	20.3
36	90	911	82.0	72	406	29.2
Mean	70.7	928	65.5	46.8	480	22.1
± ISD	16.8	392	36.4	14.8	251	11.1

	Normal weight Spleen			Light Weight Spleen		
	Organ Wt.g	Ferritin ug/g	Ferritin ug/spleen	Organ Wt.g	Ferritin ug/g	Ferritin ug/spleen
29	5	213	1,065	1	207	207
31	3	216	648	2	88	175
32	5	166	828	2.5	171	427
33	6	400	2,400	3	75	225
37	11	688	7,568	6	568	3,408
Mean	6.0	337	2,502	2.9	222	888
± ISD	3.0	216	2,915	1.9	201	1,412

As numbers are small no definite conclusions can be drawn but it would appear that when liver growth is retarded both the total content and concentration of ferritin are reduced. The former is not unexpected as in the group as a whole there was a strong correlation between ferritin content and organ and body weight. However in one liver at 31 weeks both concentration and total content were well within normal limits. Three of the five small spleens had normal concentrations but the contents were 50 per cent or less of the normal weight gestational equivalent.

Organ Ferritin of twins

The ferritin concentration in the livers and spleens of three sets of twins was considerably lower than in singletons but this difference was not found in the heart. The hepatic weight, ferritin concentration and content were all less than the gestational mean values. The lowest liver ferritin contents of the series were found in the "N" twins previously described under "Fetal organ ferritin related to gestation", page 126, who had a placental transfusion in utero. The seventh fetus whose twin survived and was not available for comparison had values greater than the mean for all three organ parameters and was of average body weight. The ferritin content of the spleens of the three sets of twins was 10 to 56 per cent of the gestational mean and the concentration in five of the six was 7 to 63 per cent of the gestational mean. The seventh twin who had a normal total ferritin content in the liver also had a normal level in the spleen.

Table 4, 15 contains the findings of the three sets of twins who were available for comparison. Twins SI and II were the most immature of the series and died from asphyxiation and atelectasis. Despite the larger liver of SI the total ferritin content was 28 per cent less than SII.

TABLE 4,15

ORGAN FERRITIN IN TWINS

FETUS	S1	S11	H1	H11	N1	N11
Gestation weeks	25+	25+	31	31	35	35
Body Weight Kg	0.78	0.75	1.09	0.76	1.60	1.90
Survival Time	10 hr	12 hr	6/365	SB	SB	SB
Liver Weight g	36	28	52	48	32	56
Liver Ferritin ug/g	234	386	391	395	14	20
Liver Ferritin mg/organ	8.4	10.8	20.3	19.0	0.4	1.1
Heart Weight g	7	5	10	6	10	10
Heart Ferritin ug/g	77	62	9	73	5	8
Heart Ferritin ug/organ	540	310	88	437	51	78
Spleen weight g	1	1	3	2	4	7
Spleen Ferritin ug/g	18	70	216	88	28	17
Spleen Ferritin ug/organ	18	70	648	175	112	120

The heart of SI was 24 per cent heavier than SII and the concentration was similarly increased with a resultant 74 per cent rise in the total ferritin content. No differences were found in the spleens which were of identical weights. The body weights of Twins HI and HII were below the tenth percentile for their gestational age. HI had hyaline membrane disease and a bilateral pneumothorax and HII was an anencephalic fetus. No difference was found in the liver ferritin concentration and that of total content was due to the discrepancy in the organ weights. HII had the smaller heart but the ferritin concentration was such that the total content was 396 per cent greater than that of the larger heart. This did not occur in the spleen as HI had the larger spleen which had the higher concentration. The "N" twins were still-born resulting from a placental transfusion. NI was polycythaemicand the body weight was less than the tenth percentile for gestational age, and NII anaemic. The latter was the heavier twin which reflected in the weights of the liver and spleen. The heavier liver had a higher ferritin concentration but this was reversed in the spleen although the total ferritin content of both liver and spleen was greater in the heavier twin.

Fetal organ ferritin in relation to placental ferritin.

Four placentae of fetuses made available for investigation were also analysed for ferritin content and the results are shown in Table 4,16. As expected little variation was found in the placental ferritin concentration within the 24 to 35 week gestation range, but "Placenta 3" was the exception. It was a very large organ for the weight of the baby with a grossly elevated ferritin concentration which was twice the 95th centile for term placentae and five times the gestational mean.

TABLE 4.16

FETAL ORGAN FERRITIN IN RELATION TO PLACENTAL FERRITIN

Fetus	Gestation weeks	Fetal Wt.Kg	Placental Wt. g	Placental Ferritin		Liver Ferritin		Heart Ferritin		Spleen Ferritin	
				ug/g	mg/organ	ug/g	mg/organ	ug/g	ug/organ	ug/g	ug/organ
1	24	0.68	310	26	8.1	318	11.2	2	8	14	14
2	28	1.06	340	38	12.9	660	27.1	75	298	207	207
3	29	1.36	440	220	96.8	645	49.6	62	621	213	1,065
4	35	1.89	375	37	13.9	911	82.0	47	519	63	632

The fetus, which was still-born, had contracted intrauterine pneumonia and had an enlarged liver and spleen although the heart was of normal size. The ferritin concentration of the liver was grossly elevated but the ferritin content was normal. Normal concentrations and contents of ferritin were found in heart and spleen. The ferritin studies on the remaining three fetuses were all within the normal gestational limits.
The placental weights were correlated with the organ ferritin content in 26 pregnancies and no correlation was found with liver content ($r=0.07$) or heart content ($r=0.04$) but a degree of correlation was recorded with spleen content ($r=0.52$); $p<0.05$).

Summary.

The total ferritin content of liver, heart and spleen is proportional to increasing gestation and organ weight, but only the spleen ferritin concentration increases with advancing gestation. The results suggests that a growth retarded liver has a decreased ferritin concentration and total content but onlu the latter would appear to occur in the spleen. The ferritin concentration in the livers and spleens of twins appears lower than the singleton of identical gestation but this was not found in the heart. There is some evidence to suggest that enlarged placentae with increased ferritin concentrations are associated with elevations in the total ferritin content of the fetal liver.

Ferritin in Normal and Abnormal Placentae

The ferritin concentration of normal and abnormal placentae was determined and related to that of human placental lactogen (HPL) and human chorionic gonadotrophin (HCG).

Eleven placentae which were normal macroscopically and were associated with uneventful pregnancies were obtained between 38 and 40 weeks gestation and formed the control group. Eight term placentae of less than 500 g and 10 term placentae associated with low maternal plasma HPL's and urinary oestriols were also investigated. Sixteen placentae between 24 to 37 weeks were obtained from pregnancies terminated by spontaneous premature labour or by induction or Caesarean section prompted by the maternal or fetal condition. Sections from the maternal and fetal surfaces at the centre and periphery of each organ were obtained from all placentae of more than 24 weeks maturity and treated as described under "Preparation of Tissue Extracts". On termination of normal pregnancies at less than 20 weeks gestation 6 placentae were obtained. The organs were generally incomplete and as only small amounts of material were available one section was obtained from each organ.

Ferritin in Normal Term Placentae

The normal term placentae weighed 627 ($\pm$ 70)g and the total ferritin content was 38.9 ($\pm$ 15.6) mg per organ. There was little difference between the maternal peripheral concentration 69.1 ($\pm$ 21.7; range 44.0-110.0) ug per g and the maternal central concentration 65.0 ($\pm$ 18.2; range 42.0-109.0) ug per g or between the fetal peripheral 59.1 ($\pm$ 23.9; range 28.0-111.0) ug per g and the fetal central ferritin concentration 52.6 ($\pm$ 23.8; range 27.0-102.0) ug per g. In both the peripheral and central areas however,

however, a concentration gradient existed between the maternal and fetal surfaces in nine placentae but in two no difference was found. The mean concentration difference between the surfaces at the periphery was 18.5 ($\pm$ 20.4) ug per g and at the centre was 22.7 ($\pm$ 28.1) ug per g. The results are illustrated in Figure IV,5.

Ferritin in Abnormal Term Placentae

The ferritin concentration of the small placentae and placentae of normal weight but associated with low HPL and oestriol concentrations during pregnancy are contrasted with normal placentae in Table 4,17. No difference was found in the ferritin concentration of the two abnormal groups when contrasted with the control group. Concentration gradients occured between the maternal and fetal surfaces in 6 of the low weight placentae but in the remaining two no difference was found. The mean concentration gradient at the periphery was 19.0 ($\pm$ 19.3) ug per g and at the centre 9.3 ($\pm$ 13.1) ug per g and did not differ significantly from the control group ($p<0.98$ and $p<0.3$, respectively). The abnormal placentae of normal weight showed concentration gradients in 7 of the 10 organs and no variation in 3. The concentration gradient at the periphery was 14.8 ($\pm$ 9.9) ug per g and 9.6 ($\pm$ 10.1) at the centre and did not vary statistically from the control group ($p<0.7$ and <0.2 respectively). The ferritin content of the low weight placentae was 22.1 ($\pm$ 8.4) mg which was significantly lower than the control placentae ($p<0.01$) and in contrast to that of the abnormal placentae of normal weight which contained 39.3 ($\pm$ 15.4) mg and compared with the control group with 38.9 ($\pm$ 15.6) mg.

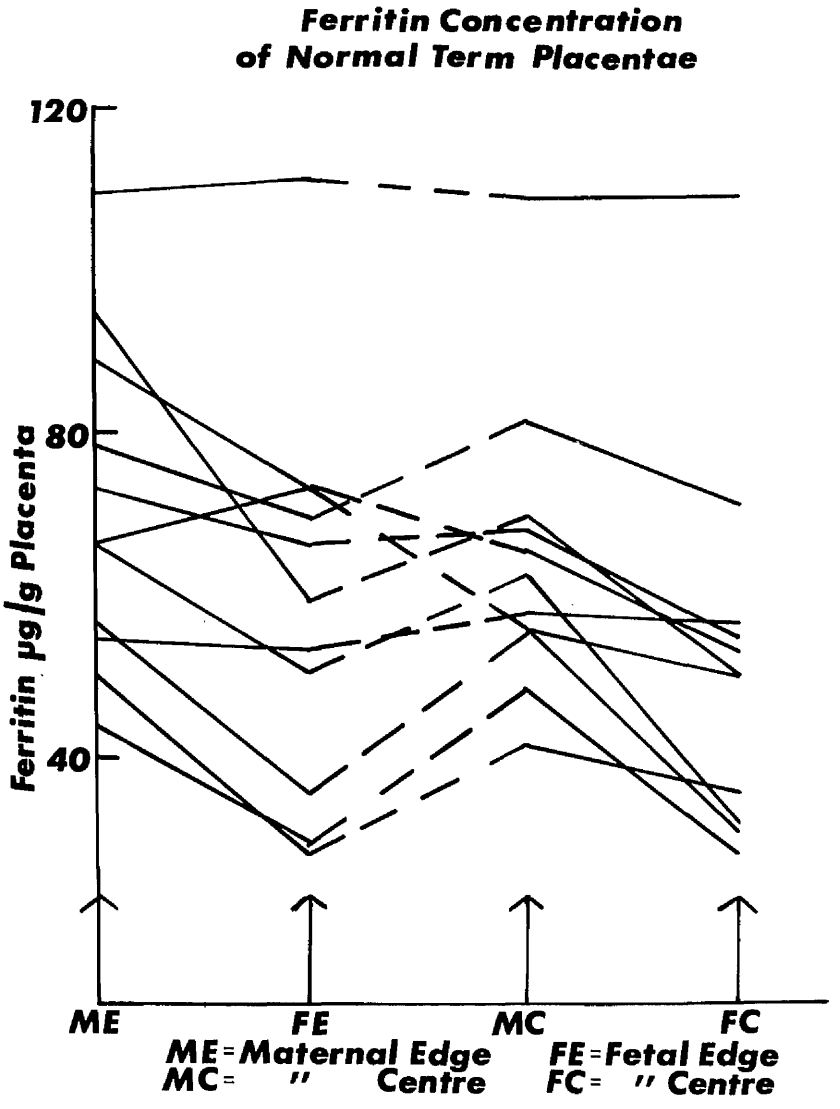


Fig. IV, 5

Ferritin concentration gradient of normal term placentae.

TABLE 4,17

FERRITIN CONCENTRATION OF PLACENTAE AT TERM (ug/g)

(± 1 SD in parentheses)

GROUP	WEIGHT Placenta (g)	MATERNAL EDGE	FETAL EDGE	MATERNAL CENTRE	FETAL CENTRE
Normal (n=11)	627 (± 70)	69.1 (± 21.7)	59.1 (± 23.9)	65.0 (± 18.2)	52.6 (± 23.8)
Abnormal (n = 10)	640 (± 75)	66.2 (± 27.2)	52.3 (± 25.6)	65.8 (± 23.5)	59.5 (± 21.4)
LFD (n=8)	394 (± 51)	67.9 (± 30.0)	49.9 (± 21.7)	56.9 (± 24.8)	48.3 (± 18.0)

Ferritin in 24 to 37 weeks Placentae

The ferritin concentrations of placentae between 24 to 37 weeks gestation appear in Table 4,18 and were found to be significantly lower than normal, term placentae ($p<0.05$ at 36-37 weeks, $p<0.02$ at 33-35 weeks and <0.05 at 24-28 weeks). Concentration gradients between the maternal and fetal surfaces were found in all 16 placentae and at the periphery was 13.1 ($\pm$ 12.8) ug per g and at the centre 14.5 ($\pm$ 18.7) ug per g. There was no significant difference between the concentration gradients in these premature placentae and normal term placentae. Table 4,18 gives the ferritin content of the premature placentae which contained only 39 per cent of the ferritin of normal term placentae. These placentae cannot however be considered normal as there was evidence of placental insufficiency, indicated by low maternal HPL and oestriol concentrations and minor degrees of infarction (less than 10 per cent) were noted in a number of organs.

Placental Ferritin in Relation to HPL, HCG and Gestation

The concentration and content of placental ferritin, HPL and HCG in relation to gestation is summarised in Table 4,19. The ferritin concentration, the mean of four concentrations for each placenta of more than 24 weeks gestation, was found to be low at 8 weeks, but, reached term levels between 11 and 18 weeks. Thereafter the concentration fell significantly until 37 weeks ($p<0.02$) when a second increase occurred. This pattern of variation with gestation did not occur for HPL nor HCG.

The concentration of HPL in the placenta was found to increase rapidly between 18 and 28 weeks and then at a slower rate until 35 weeks when a small fall occurred which was not significant ($p<0.6$) and was maintained until term.

TABLE 4,18

FERRITIN CONCENTRATION OF 24 to 37 WEEK PLACENTAE (ug/g)

($\pm$ ISD IN PARENTHESES)

Gestation weeks	Number	Weight Placenta g	Maternal Edge	Fetal Edge	Maternal Centre	Fetal Centre	
24-28	2	325 (21)	46.6 (5.9)	37.7 (2.6)	40.4 (24.6)	35.2 (5.9)	
34-35	5	376 (107)	41.4 (20.7)	34.8 (23.9)	43.3 (10.0)	33.1 (10.8)	
36-37	9	375 (104)	45.0 (31.3)	39.9 (16.2)	41.2 (29.6)	33.5 (10.6)	
38-40	11	627 (70)	69.1 (21.7)	59.1 (23.9)	65.0 (18.2)	52.6 (23.8)	

TABLE 4,19

PLACENTAL FERRITIN IN RELATION TO HPL, HGG AND GESTATION

(± ISD IN PARENTHESIS)

Gestation weeks	Number	Placental weight g	FERRITIN		HPL		HGG	
			Concn. ug/g	Content mg	Concn ug/g	Content mg	Concn u/g	Content UX 103
8	2	-	27.0 (5.0)	-	104 (122)	-	498 (679)	-
11	2	-	79.0 (15.0)	-	184 (210)	-	199 (83)	-
18	2	-	68.7 (29.4)	-	121 (91)	-	26 (29)	-
24-28	2	325(± 21)	40.0 (9.8)	13.1 (4.0)	*325 (129)	115 (40)	*292 (439)	85 (141)
34-35	5	376(±107)	39.0 (13.4)	14.1 (3.1)	367 (193)	127 (57)	27 (21)	9 (6)
36-37	9	375(±104)	40.6 (18.6)	15.0 (6.9)	316 (88)	117 (40)	17 (21)	6 (6)
38-40	11	627(± 70)	61.5 (20.8)	38.9 (15.6)	315 (105)	194 (68)	11 (8)	7 (6)

* Number = 3

The fall in HPL between 11 and 18 weeks was not significant as there was considerable variation in early pregnancy. There was little variation in the placental content of HPL between 24 weeks and term. The concentration of HPL in the four placental regions are given in Table 4,20. There was wide variation both within and between placentae at all stages of gestation. HPL, like ferritin shows a concentration gradient between the maternal and fetal surface in the majority of organs. However, in two normal term placentae and in one abnormal term placenta the concentration at the periphery was in the reverse direction to that of ferritin which followed the normal pattern. Reverse HPL gradients in both peripheral and central areas occurred in one normal term placenta, two abnormal term placentae and two at 36 to 37 weeks and again either the normal ferritin concentration gradient was present or no difference in concentration was found. The HPL content of control, term placentae 194 $\pm$ 68 mg was no different from that of small term placentae, 149 $\pm$ 41 mg and abnormal term placentae of normal weight, 238 $\pm$ 96 mg.

The HCG concentration of placentae varied widely in early pregnancy when very high levels could occur. At 33 weeks low levels were found which gradually declined until term. The placental content of HCG did not vary between 33 and 40 weeks. The concentration of HCG in the four placental areas is shown in Table 4,21.

A concentration gradient only existed in the 25 to 29 weeks group when high levels were found and decreased from the maternal to the fetal surface.

TABLE 4,20

PLACENTAL HPL CONCENTRATION ug/g

(± ISD IN PARENTHESES)

Gestation weeks	No.	Mean Concentration	Maternal Edge	Fetal Edge	Maternal Centre	Fetal Centre
8	2	104 (122)	-	-	-	-
11	2	184 (210)	-	-	-	-
18	2	121 (91)	-	-	-	-
25-29	3	-	376 (141)	308 (128)	352 (160)	264 (137)
34-35	5	-	390 (185)	385 (176)	413 (234)	338 (179)
36-37	9	-	373 (110)	329 (149)	320 (102)	241 (114)
38-40 (≤500g)	8	-	407 (189)	391 (199)	430 (179)	348 (89)
38-40 (>500g)	10	-	358 (129)	316 (181)	354 (115)	375 (163)
38-40 Control	11	-	308 (112)	273 (156)	348 (108)	329 (105)

TABLE 4,21

PLACENTAL HGG CONCENTRATION UNITS/g

(± ISD IN PARENTHESIS)

Gestation weeks	No.	Mean Concentration	Maternal Edge	Fetal Edge	Maternal Centre	Fetal Centre
8	2	498 (679)	-	-	-	-
11	2	199 (83)	-	-	-	-
18	2	26 (29)	-	-	-	-
25-29	3	-	365 (545)	287 (437)	306 (451)	213 (323)
34-35	5	-	25 (13)	27 (16)	26 (29)	25 (23)
36-37	9	-	18 (17)	18 (23)	17 (21)	16 (26)
38-40 <500g	8	-	20 (14)	21 (14)	13 (6)	15 (8)
38-40 ≥500g	11	-	16 (15)	16 (10)	21 (21)	15 (11)
38-40 Control	11	-	9 (6)	12 (12)	13 (11)	11 (8)

The HCG concentration at this gestestation varied from 12 to 799 units per g. The total HCG content of the control term placentae was $7 \pm 6 \times 10^3$ units, of the low weight term placentae was $6.9 \pm 3.7 \times 10^3$ units and of the abnormal placentae of normal weight was 11.0 ± 9.1 units.

Localisation of Placental Ferritin Synthesis.

Ferritin was localised in the placenta by electron microscopy using rabbit anti-human placental ferritin in an immunoperoxidase method. Ferritin was found on the rough endoplasmic reticulum of the syncytiotrophoblast see Plates 4,I and 4, II.

A diagramatic representation of placental structure is given in Fig IV,6 (Garrey et al, 1971) which indicates that the syncytiotrophoblast, the outer trophoblastic layer is a multinuclear syncytium with no distinct cell boundaries and is in direct contact with maternal blood. The inner layer of the trophoblast, the cytotrophoblast or Langhan's layer forms a single layer of cuboidal cells. The placental organ is made up almost entirely of a multitude of chorionic villi most of which protrude into the intervillous blood spaces - Fig IV, 7 (White et al, 1952). The detailed structure of a placental villus from a 2 cm human embryo is shown in Fig IV,8 but the brush border on the syncytiotrophoblast is not visible. The fetal vessels are filled with primitive erythrocytes (Maximow and Bloom, 1958).

Ferritin would appear to be synthesised on the rough endoplasmic reticulum at the surface of the syncytiotrophoblast. Even at high magnification there islittle evidence that it is synthesised in the deeper layers — Plate 4,II.

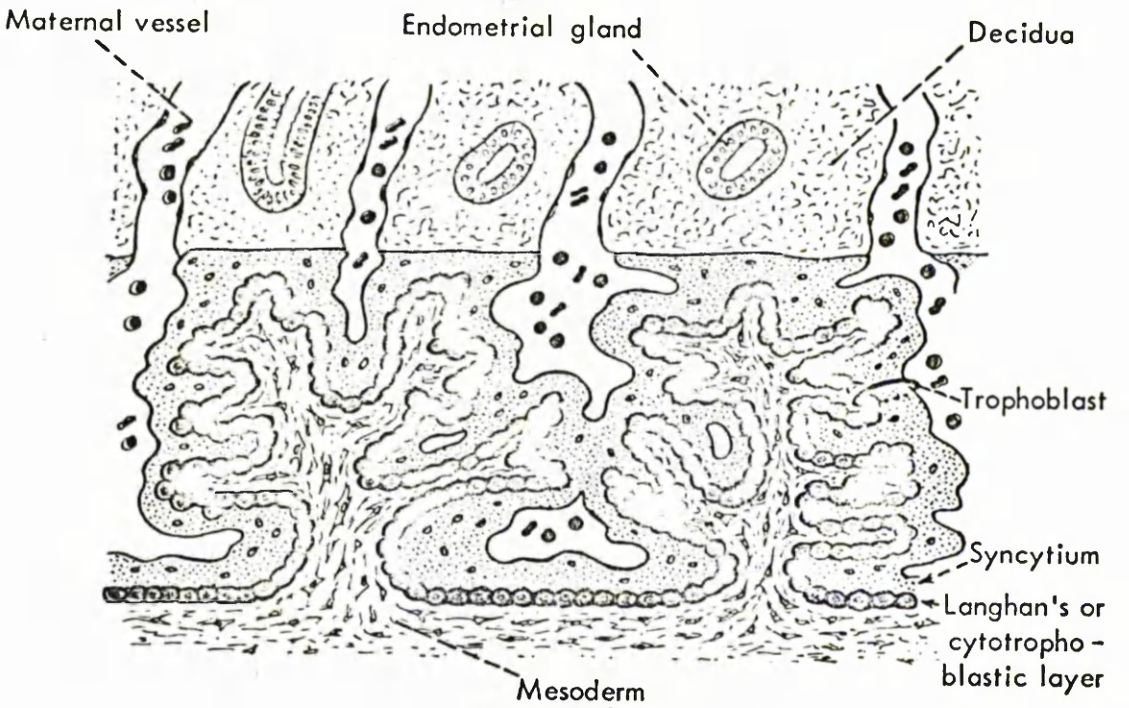


Fig. IV,6

Diagramatic representation of placental structure

Fig. IV,7

Capillaries in the placenta

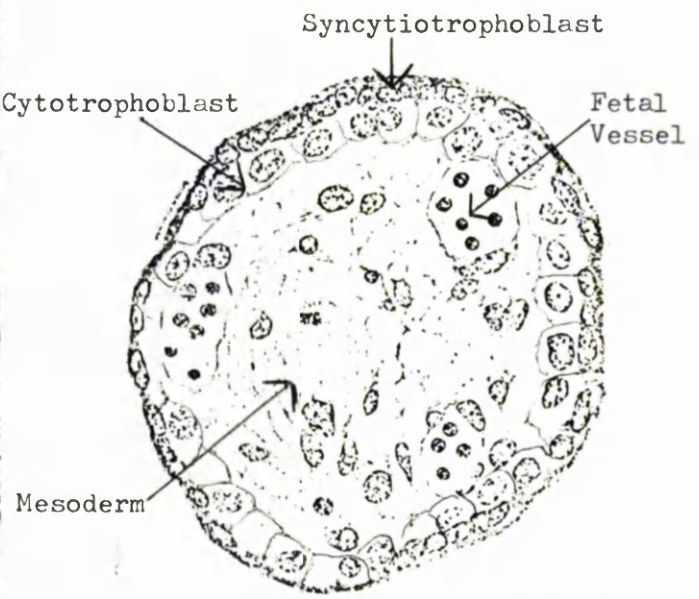


Fig. IV,8

Section through placental villus

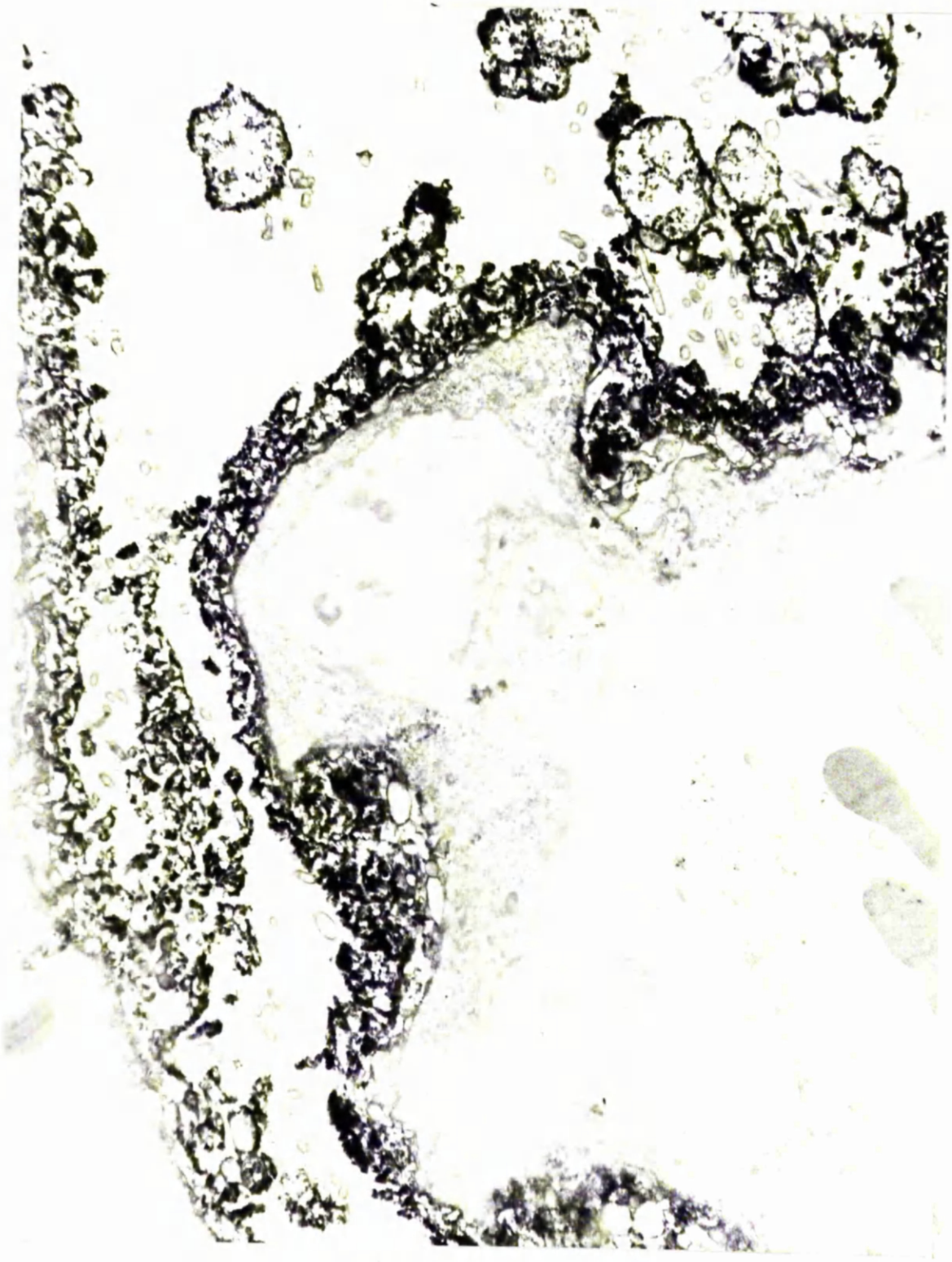


Plate 4,I

Electron Micrograph of Normal Placenta

(Magnification x 6,000)

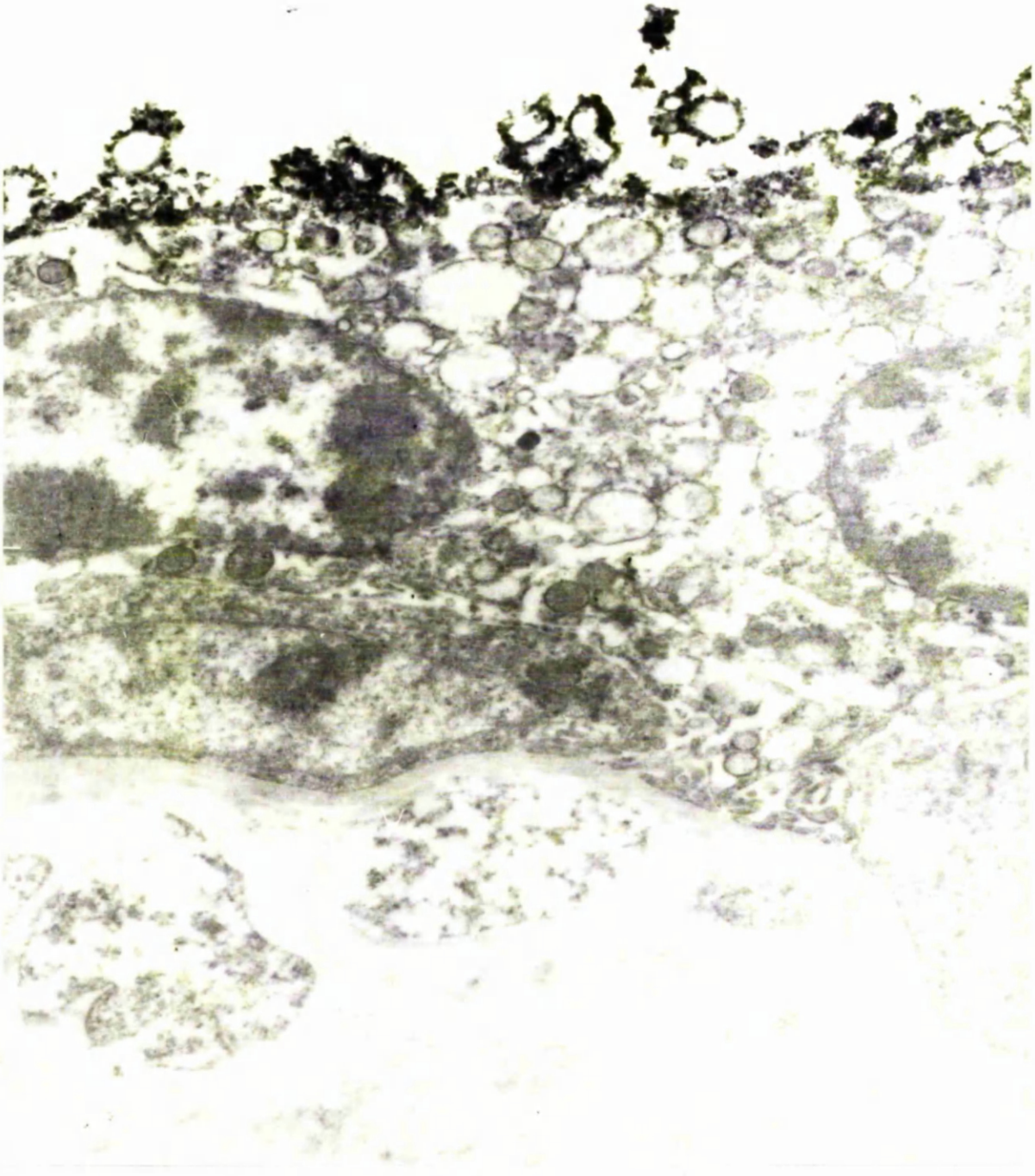


Plate 4,II

Electron Micrograph of Normal Placenta

(Magnification x 17,500)

This picture could arise from the failure of reagents to penetrate the deeper layers and further work will be required to investigate this possibility.

Summary

The ferritin concentration of placentae increased until 18 weeks when a fall took place which was maintained until 38 weeks when a second increase occurred. No difference was found in the ferritin content of the normal and abnormal term placentae but when the organ weight was low the ferritin content was much reduced. A concentration gradient was present between the maternal and fetal surfaces but no difference was found in any of the groups between the peripheral and central areas. The gestational changes in placental ferritin concentration did not correlate with those of HPL nor HCG. The concentration gradients of HPL and HCG between the maternal and fetal surfaces were similar to ferritin.

In normal term placentae ferritin was located in the granular endoplasmic reticulum of the syncytiotrophoblast.

Chapter Five

Discussion

"Perhaps the critical information needed to decide the interpretation of the 'physiological anaemia of pregnancy' is whether or not the average young woman has a sufficiency of 'storage iron' : the falling concentration of haemoglobin could hardly be attributed to iron deficiency if there were demonstrable stores in the body of unused iron" - Hytten and Leitch (1971).

The assessment of iron stores throughout gestation would determine the adequacy of iron intake and the variability in the utilisation of iron by the mother and the feto-placental unit. The concentration of ferritin in serum has been found to give a quantitative measure of iron stores in normal, iron-deficient and iron-overloaded subjects (Jacobs et al, 1972). Iron stores can be precisely assessed by repeated phlebotomy and the results correlate well with those obtained by plasma ferritin (Walters et al, 1973). A simpler technique is to measure urinary iron excretion after administration of desferrioxamine but there is considerable overlap between normal and iron-deficient subjects. Estimates of iron stores obtained by this method have been shown to correlate with results obtained by repeated venesection (Olsson , 1972). Storage iron has also been evaluated semi-quantitatively by staining the bone marrow for iron and estimates correlate with plasma ferritin levels (Lipschitz et al, 1974). The most sensitive indirect measure of iron stores is by assessment of iron absorption and Cook et al (1974) found good correlation with plasma ferritin concentrations in normal subjects.

Plasma ferritin is therefore established as a measure of storage iron in normal, iron-deficient and iron-overloaded subjects (Jacobs and Worwood, 1975).

An Assessment of Iron Stores in Normal Subjects.

The evaluation of iron stores in pregnancy has been limited and mainly determined, only semi-quantitatively and in a small number of subjects by staining for non-haem iron in bone marrow. The most comprehensive report is that of de Leeuw et al (1966) who studied women in early and late pregnancy and in the puerperium. However Beaton (1974) has suggested that this method of assessing iron stores is "less precise and potentially misleading" when the subjects is not in iron balance. Svandberg (1975) studied iron absorption in pregnancy using a sensitive whole body counter. He found that in early pregnancy decreased absorption took place in comparison to non-pregnant subjects with equivalent bone marrow content of iron. In the puerperium increased iron absorption occurred which was independent of storage iron. There is therefore a need for further assessment of storage iron during pregnancy by a method which can be widely applied and does not require the administration of radioisotopes. The quantitation of ferritin in maternal serum being non-invasive and requiring only a simple venesection would appear to be the method of choice.

Normal male and non-pregnant female subjects

The published ferritin concentrations in serum or plasma are consistently 2 to 4 times higher in normal men than in non-pregnant women and support similar ratios which were found using repeated phlebotomy measurements (Pritchard and Mason, 1964; Walters et al 1973):-

Reference	Ferritin ug/l	
	Male	Female
Addison et al (1972)	52 (12-128)	29 (10-56)
Jacobs et al (1972)	69 (6-186)	35 (3-162)
Walters et al (1973)	103 (36-224)	36 (2- 83)
Cook et al (1974)	94 (27-329)	34 (9-125)
Hussein et al (1975)	188 (110-330)	58 (15-135)
Finch et al (1977)	127 (66-244)	46 (20-107)
Kelly et al (1977)	80 (24-265)	32 (9-107)

(Mean with range in parentheses)

The reported ranges vary considerably particularly for males which could reflect a difference in methodology but perhaps more importantly reflects the different criteria used to select the normal population studied. However the results reported in the present series agree well with those of Cook et al (1974) using the same assay.

The ferritin concentrations of men and non-pregnant women in the present series were found to be proportional to the non-haem iron content of bone marrow and statistically significant differences were found between the levels associated with each grading. Lipschitz et al (1974) also used four gradings in categorising the iron content of bone marrow and similar ferritin concentrations were identified with equivalent gradings in both surveys. In patients with megaloblastic anaemia, chronic inflammatory conditions or malignancies disproportionately elevated plasma ferritins were found in relation to the iron content of bone marrow. Similar findings have been reported by others - (Jones et al, 1973; Lipschitz et al, 1974).

On the basis of the plasma ferritin concentration and on the finding of Walters et al (1973) that 1ug of ferritin per litre of plasma represents about 8mg of storage iron, the average Glasgow woman in the reproductive age group has a store of 256mg of iron which is equivalent to the iron content of one unit of blood. This figure compares with that of 254mg obtained by Pritchard and Mason (1964) and 210mg by Walters et al (1973) in different populations and using the repeated phlebotomy technique.

10.2 per cent of our women had plasma ferritin levels of less than 10ug per litre (mean-2SD) and were deemed to be iron deficient. A study of Lancashire women aged 16 to 50 years revealed that 15 per cent were iron deficient on the basis of their plasma ferritin levels (Fairhurst et al, 1977). These figures are lower than that for American college women of whom 24 per cent were found to have depleted iron stores on the basis of bone marrow iron contents (Scott and Pritchard, 1967). In Sweden 20 per cent of female subjects were considered iron deficient (Garby, 1973) although in a later Swedish series when all women had haemoglobins greater than 12g per dl only 10 per cent were iron deficient using the same method of investigation (Svandberg, 1975). The percentage of women who are truly iron deficient varies with the different populations studied and also with the criteria used for identification (Beaton, 1974). It is however apparent that iron stores are small in women of child-bearing age and are inadequate to meet the requirements of pregnancy which have been estimated to be 1,000mg. Svandberg (1975) has determined that 6.5mg of iron is required daily in the last ten weeks of pregnancy and could not be met from the diet. The plasma ferritin concentrations found in our non-pregnant female

population supports the present practice of prophylactic iron therapy during pregnancy.

Iron requirements in pregnancy.

Iron supplements are generally prescribed at a first clinic visit. A slightly higher incidence of congenital abnormalities was found in babies born to mothers who received iron during the first 56 days of pregnancy but there was no evidence of adverse effects when iron was taken after this time (Forfar, 1973). It is a practice in Glasgow Royal Maternity Hospital that patients receive an average of 100mg of elemental iron per day but in this series no check was kept on whether or not the patient kept to this regime.

In the initial survey on age matched patients in early pregnancy and in the puerperium no difference was found between these groups and non-pregnant female subjects. A higher proportion of patients of less than 20 weeks gestation had lower ferritin levels despite iron therapy than non-pregnant subjects. This suggested that iron stores were being utilised early in pregnancy although erythropoiesis is not thought to increase until the fifth month of gestation (de Leeuw et al, 1966). It has been suggested that iron of the bone marrow is mobilised early in pregnancy (Hancock et al, 1968). Svandberg (1975) has shown decreased absorption of both dietary iron and iron salts when compared to non-pregnant subjects. He concluded that absorption of food iron is less than the basal daily requirements at this time. In patients in the puerperium the distribution of ferritin values was similar to that in non-pregnant subjects and indicated that iron supplements prevented a net loss of iron. There was no evidence of iron overloading as a result of therapy, which has been suggested by Taylor and Lind, (1976.

Plasma ferritin in normal pregnancy.

The basis of the physiological anaemia of pregnancy indicated by a progressive fall in haemoglobin concentration and haematocrit which are independent of iron supplementation, can only by answered by determining maternal iron stores throughout pregnancy. The expansion of the plasma volume which begins in the first trimester has been recognised for many years (Willcocks, 1881) and many have debated the importance of haemodilution in the production of anaemia.

In the present work (Kelly et al., 1977) serial plasma ferritin concentrations of normal pregnant patients on iron supplements indicated that iron stores decreased statistically from 26 weeks until term. Earlier falls were found to occur in some subjects. The lowest concentrations were found between 31 to 35 weeks. The changes in ferritin paralleled haemoglobin levels as both fell until 35 weeks before rising at term. Similar findings were later reported by Fenton et al (1977) who studied untreated and iron supplemented patients. They found that ferritin concentrations fell in both groups from 12 weeks gestation but in the former group an increase at term did not occur. Serum ferritin during pregnancy was also determined by van Eijk et al (1978) and they too found that concentrations fell with advancing gestation irrespective of iron therapy. Their patients who received supplements had higher levels than the untreated group. They did not record an increased ferritin concentration at term in the iron supplemented group, found in the other two studies although the haemoglobin increased.

The early fall in ferritin is common to all three investigations suggesting an early mobilisation of iron stores. It is unlikely at this stage of gestation that mobilisation can be a result of fetal or placental requirements. It has been suggested that the increase in maternal red cell mass occurs in mid-pregnancy (de Leeuw et al, 1966; Pritchard and Scott, 1970). Taylor and Lind (1976) have suggested that the decrease in mean red cell volume at 6 to 15 weeks gestation could reflect a period of marrow suppression. These findings suggest that maternal iron stores should not be utilised in early pregnancy. Alternatively Svandberg (1975) has shown that at this period of gestation iron absorption is less than in non-pregnant women and that pregnant women are in negative balance. Increased levels of erythropoietic activity have been found in plasma from 13 weeks (Manasc and Jepson, 1968). At less than 10 weeks 62.5 per cent of requirements are obtained from the diet and at 11 to 20 weeks 38.5 per cent (Svandberg, 1975). The inadequate absorption of iron could stimulate the mobilisation of iron and reduce the stores. The fall in plasma ferritin reflects the reduction of iron stores and is supported by the work of Hancock et al (1968) who studied the bone marrow iron content. They found that in early pregnancy 75 per cent of women had depleted iron stores and 55 per cent lacked storage iron. They concluded that their findings reflected early mobilisation rather than exhaustion of iron.

In mid-pregnancy there is increased erythropoiesis which mobilises iron stores (de Leeuw et al, 1966). The hormonal effect on erythropoiesis is not clear by HPL, present in increasing concentration at this time, enhances the effect of erythropoietin on erythropoiesis (Jepson and Friesen, 1968).

Estrogen however impairs erythropoiesis although the effect is partly prevented by progesterone (Jepson, 1968). The fetus and placenta grow at an accelerated rate and therefore iron demands are high. Absorption of dietary iron increases and between 31 and 40 weeks 17 per cent of dietary iron is absorbed. However Svandberg (1975) has calculated that despite increased absorption, at 21 to 30 weeks and at 31 to 40 weeks only 32 per cent and 46 per cent respectively of requirements are obtained from the diet. It would appear from the ferritin concentration in our subjects that despite iron supplement stores were increasingly utilised until 35 weeks.

After 35 weeks and until term the plasma ferritin levels increased in our iron supplemented subjects suggesting that either requirements had decreased and/or iron absorption could meet demands and partially replenish stores. Patients not receiving iron supplements do not show increased plasma ferritin concentrations between 35 and 40 weeks (Fenton et al, 1977; van Eijk et al, 1978) and as already noted absorption of dietary iron is inadequate at this time (Svandberg, 1975). MacKay et al (1956) have demonstrated iron in the placenta in early pregnancy and just prior to term which may suggest that requirements are less than in mid-pregnancy. Changes in plasma volume could also be responsible for the increased ferritin concentrations. If these occur independently of iron therapy the increase in ferritin should also be unrelated to supplements. This does not occur. Despite the term increase in ferritin the concentrations are still significantly lower than in early pregnancy and support the earlier findings of gross depletion in bone marrow non-haem iron at term (de Leeuw, 1966; Svandberg, 1975).

At term only one out of the 20 subjects studied serially had a haemoglobin level of less than 11g per dl. These results agree with previous reports (Jacobs, 1974) which stated that 6 per cent of patients maintained on oral iron had haemoglobin concentrations which were under 11g per dl at term. It is therefore concluded that adequate iron was present to cover the requirements of pregnancy. Serial ferritin concentrations however indicated that stores were severely depleted in six of the patients in whom the level was less than 7.4ug per litre. This concentration was taken to indicate depleted iron stores. It was arrived at by adjusting the ferritin concentration of 9.3ug per litre which was found to be the lowest limit of normal in the non-pregnant group for the increase in plasma volume which is maximum at 34 weeks. Weir et al (1971) reported that in our population the mean maximum volume increase was from 3 to 3.75 litres. Jacobs et al (1972) have reported that in both sexes "a concentration below 10ug per litre is associated with a low transferrin saturation and iron deficient erythropoiesis". It is recognised that the alteration in concentration of plasma constituents during pregnancy is rarely explained by dilution (Lind, 1975). Ferritin may be the exception. It appears to be present in plasma as an overflow from and proportional to organ stores. The results suggests that despite iron therapy many patients in late pregnancy may have poor iron reserves and would be unable to respond to haemorrhage in the perinatal period.

Haemoglobin and plasma iron changes in pregnancy

The haemoglobin levels determined in the serial study decreased until 35 weeks before rising until term and agreed with the report of de Leeuw et al (1966). Haemoglobin levels followed the variation in ferritin concentrations although there was no direct correlation between the two parameters. In similar studies of normal pregnant subjects receiving prophylacticiron the early pregnancy haemoglobin levels were identical to our mean value of 12.5g per dl but after the initial fall the increase occurred earlier at about 28 weeks (Svandberg 1975; Taylor and Lind, 1976; Sjöstedt et al, 1977; van Eijk et al, 1978). The later increase in haemoglobin in the present series may be explained by the early pregnancy ferritin concentrations which were much lower than those reported by Fenton et al, 1977 and van Eijk et al, 1978. Our population may have such poor reserves of iron, as shown by the much lower ferritin levels in early pregnancy that despite iron supplements the maximum maternal erythropoiesis cannot be attained at a time of rapid fetal growth.

The plasma iron concentrations did not vary throughout pregnancy but a significant fall was found in the sixth postnatal day in our patients which was also found in the serial study in thepuerperium. Plasma iron therefore did not reflect the changes in iron stores throughout pregnancy. Similar findings have also been reported (Hancock et al, 1968; Fenton et al, 1977) but Svandberg (1975) and van Eijk et al (1978) described a fall in early pregnancy of serum iron. Transferrin was found to increase significantly very early in pregnancy and be maintained until the sixth postnatal day.

van Eijk et al (1978) also quantitated transferrin specifically and reported similar findings. However when transferrin was measured as total iron binding capacity a steady increase was found throughout pregnancy (Hancock et al, 1968; Svandberg, 1975; Fenton et al, 1977). A fall was recorded in the iron content of transferrin from 26 weeks until 35 weeks but thereafter an increase occurred. Similar findings were reported by Hancock et al (1968) but Svandberg (1975) demonstrated a rapid fall until 24 weeks when a constant level was maintained until term. Fenton et al (1977) reported little change in their subjects maintained on iron.

Iron stores in The puerperium.

Serial studies in the puerperium demonstrated that plasma ferritin increased until the sixth postnatal day to non-pregnant concentrations and that no statistically significant change occurred thereafter. The findings suggested that the iron contained in the excess haemoglobin synthesised during pregnancy was rapidly returned to the stores after delivery. This is supported by the findings of Shepp et al (1972) who reported that the iron content of red blood cells was present in reticulo-endothelial ferritin within two days.

It was not possible to predict from the presenting ferritin concentration the subjects who would become iron deficient during pregnancy. The patients who had ferritin levels of less than 10ug per litre at term had early pregnancy ferritin levels of 20.1 $\pm$ 11.2ug per litre and patients with normal ferritin at term had initial concentrations of 32.2 $\pm$ 26.3ug per litre ($p \leq 0.10$). Similar findings were reported by Fenton et al (1977) and Hancock et al (1968) stated that adequate iron in bone marrow in early pregnancy did not preclude iron deficiency anaemia in pregnancy.

The increase in maternal haemoglobin mass accounts for about 500mg of iron (de Leeuw et al, 1966) if iron stores are adequate during the period of maximum erythropoiesis. The average blood loss at delivery in the present series was 197ml and contained approximately 78mg of iron. Basal losses and iron lost in lochia must also be considered, the total loss in thepuerperium could be 100mg during the first postnatal week. 400mg of iron should therefore be returned to the stores. Walters et al (1973) determined that 8mg of storage iron was equivalent to 1ug ferritin per litre of plasma and the sixth day postnatal ferritin should therefore be minimally 50ug per litre. However one must also account for stores existing at delivery. The plasma ferritin was 13.9ug per litre at 31 to 35 weeks, the period of maximum plasma volume expansion. The plasma ferritin should therefore be 63.9ug per litre in the puerperium. The concentration found was only 50 per cent of this value and suggested that only 280mg of iron was recovered from red blood cells. Iron metabolism is in a labile state and the non-haem iron content of bone marrow is an unreliable idicator or iron stores at this time (Beaton, 1974). Plasma ferritin may be similarly affected. However the postnatal ferritin concentration was identical to that of the non-pregnant control group. It is possible that the maximal increase in red cell mass did not occur. This is supported by the finding that our patients had lower haemoglobin levels than have been reported by others and that the increase in haemoglobin did not occur until 36 to 40 weeks whereas others have reported increases occurring at 28 weeks (Sjostedt et al, 1977).

It is also possible that iron released in the purperium is not taken up initially by the normal storage depots but there is no supportive evidence.

Fenton et al (1977) also reported that 5 to 8 weeks after delivery that serum ferritin was not significantly different from first presentation and was twice that at term. Their ferritin concentrations throughout pregnancy and in thepuerperium were about 50 per cent higher than our values. In thepuerperium the serum ferritin was 55ug per litre and accounts for the return of 500mg of iron to the stores from a maximal increase in red cell mass. van Eijk et al (1978) reported that patients maintained on 100mg elemental iron daily throughout pregnancy had ferritin concentrations 12 weeks after delivery which were identical to term values and only 53 per cent of the level at 14 weeks gestation. It is difficult to relate this finding to our own and that of Fenton et al (1977) other than by a high blood loss at delivery. It is unlikely to result from sampling later in the postnatal period.

Minor degrees of anaemia (Hb 9.2-10.8g per dl) were found in 12 patients antenatally and 50 per cent had ferritin concentrations which indicated depleted iron stores on the fourteenth postnatal day. This could indicate that despite correction of anaemia, stores were never formed and that expansion of the red cell mass had not been maximal and equivalent to the blood lost at delivery. These patients had normal haemoglobin levels at term and an average blood loss of 231ml, equivalent to an iron loss of 92mg which was not significantly different from subjects who had maintained normal haemoglobin levels.

In accounting for basal losses and iron content of lochia the total loss was in the region of 115mg. In order that the haemoglobin be normal despite a reduced red cell mass the expansion of plasma volume must have been reduced (de Leeuw et al, 1966).

15 per cent of patients had postnatal anaemia but blood loss at delivery was similar to that of normal patients. 57 per cent of this group had ferritin concentrations less than 10ug per litre. The plasma ferritin fell steadily throughout the entire postnatal period and suggested that absorption of dietary iron was not keeping pace with basal losses which are low at this time (Svandberg, 1975). The failure to maintain positive iron balance could be due to a low content of available iron in the diet or a fault in the intestinal absorption of iron. Almost 30 years ago Scott and Govan (1949) estimated that the average dietary iron content of our pregnant population was adequate at 16.7mg but its availability for absorption was not reported. The absorption of iron is normally increased postnatally and is approximately 11 per cent of dietary iron and independent of iron stores (Svandberg, 1975). Assuming that with improved social-economic conditions the diet of our present day patients is at least as good as in 1948 and that the intake is maintained in the puerperium, adequate iron should be absorbed.

There was no correlation between blood lost at delivery and plasma ferritin concentration but the blood loss of the group was small. When assessment was made of iron stores at delivery by taking account of plasma ferritin on the first postnatal day, the iron store size on the 6th to 42nd day of the puerperium was proportionally smaller in the higher blood loss groups which is to be expected.

Gestational changes in 2:3-DPG, related to plasma ferritin.

Oxygen transport to tissues is dependent on red cells haemoglobin concentration and its affinity for oxygen. The latter is determined by pH, carbon dioxide, temperature and 2:3-diphosphoglycerate (2:3-DPG) (Brewer, 1974). There is an inverse correlation in normal and anaemic subjects between 2:3-DPG and haemoglobin concentration (Torrance et al, 1970). An increased concentration of 2:3-DPG moves the oxygen dissociation curve to the right thus releasing more oxygen to the tissues at a given oxygen tension (Benesch and Benesch, 1969).

Conflicting reports have been published regarding the concentration of 2:3-DPG in the red cell during pregnancy. In the present series the level increased between 16 and 24 weeks before falling until 28 weeks and thereafter increasing until term. Postnatal values are lower than at term but similar to early pregnancy concentrations. These values are in agreement with those of Rorth and Bille Brahe (1971) who found that levels increased with gestation but as they did not measure 2:3-DPG between the twelfth and twenty-eighth week they could have missed the increase which occurs between the twenty-first and twenty-fourth week. Weiss et al (1976) also reported an early increase in 2:3-DPG between 19 and 22 weeks but found the decrease which followed was maintained until term. A third study (MacLennan et al, 1976) also reported an increase in 2:3-DPG betweenthe second and third trimester. Their second trimester group was not sub-divided and was reported as the average concentration between the weeks 14 to 26, they too could therefore have missed the peak at 23 weeks.

MacDonald and MacDonald (1977) have reported decreasing 2:3 DPG levels when they investigated patients serially at each trimester.

Compensatory increases in 2:3-DPG occur in anaemia (Torrance et al, 1970) and it was thought that changes in the organic phosphate in pregnancy may relate to changes in iron stores as indicated by plasma ferritin. The plasma ferritin concentrations in this group of patients followed the same pattern as that described earlier for 20 normal pregnancies studied serially. The concentration fell from early pregnancy until 36 weeks. A small increase then occurred and was maintained until term. An inverse relationship would be expected between plasma ferritin and 2:3-DPG were iron stores to influence 2:3-DPG but no correlation was found. It would therefore appear that the changes in iron stores which take place in normal pregnancy do not control the demonstrated changes occurring in 2:3-DPG during gestation. The major factors regulating 2:3-DPG in non-pregnent subjects are pH and the oxygenation state of haemoglobin which regulate the glycolytic enzymes required to synthesise the organic phosphate (Gerlach and Duhm, 1972). These factors were not studied but as pH is known to increase in pregnancy as a result of the respiratory alkalosis this may be an important regulating factor(Seeds et al, 1964).

The present results suggest that in subjects receiving iron supplements during a normal pregnancy that iron stores will be restored to pre-pregnancy levels in the purperium.

Plasma Ferritin in Pathological Pregnancies

In a number of pathological conditions including liver disorders, chronic inflammation and malignancy, iron metabolism is disturbed and raised plasma ferritin concentrations occur in non-pregnant subjects which are disproportionate to iron stores (Lipschitz et al, 1974). A high perinatal mortality and premature labour are associated with the low social-economic class who also have anaemia. It was therefore decided to determine plasma ferritin concentrations in pregnant patients with intercurrent pathology and to assess iron stores in antepartum haemorrhage and premature labour.

Liver disorders.

Plasma ferritin concentrations were raised and the difference was statistically significant from the control group in patients with idiopathic jaundice of pregnancy - recurrent intra-hepatic cholestatic jaundice of pregnancy. All patients had a mild degree of hyper-bilirubinaemia with raised aspartate and alanine transaminase and alkaline phosphatase activities. Most patients had pruritus. Individual cases of hepatocellular disease have been shown to have changes in serum transaminase activities accompanied by corresponding changes in serum ferritin (Jacobs and Worwood, 1975) but no correlation between serum ferritin and serum alkaline phosphatase and bilirubin have been found (Lipschitz et al, 1974). No correlation between plasma ferritin and the indices of liver function were found in the present series. Sherlock (1968) has described the hepatic histology as mild focal and irregular bile stasis. Although the mean ferritin was elevated 29 per cent of patients had iron deficient levels on first presentation.

The plasma iron, transferrin and percentage transferrin saturation were not significantly different from the control group.

Lipschitz et al (1974) have described elevated serum ferritin levels in non-pregnant patients with liver disease, mainly viral hepatitis and alcoholic cirrhosis. The concentrations in their patients were ten times higher than in our pregnant patients who had a different and milder type of liver disorder. Elevation of serum ferritin has also been found in drug induced hepatic necrosis and in patients with surgical obstructive jaundice (Jacobs and Worwood, 1975).

It has been suggested that the increase seen in serum iron some days after the onset of viral hepatitis arises from damaged hepatocytes (Sussman, 1974). It would seem reasonable that in our patients the ferritin is also excreted excessively into the circulation by damaged hepatic parenchymal cells. The latter has been thought to occur in non-pregnant, jaundiced patients and the plasma ferritin concentration no longer reflects the iron stores (Lipschitz et al, 1974).

Pre-eclamptic toxaemia.

The toxaemic patients had increased plasma ferritin levels and in the severe group the concentrations were significantly higher. Only 10 per cent of patients had depleted iron stores compared to 30 per cent in the control group. The other parameters of iron metabolism were within normal limits.

Toxaemia can be complicated by jaundice (Sherlock, 1968) and we have shown in a separate study that gamma-glutamyl transferase (EC2.3.2.2) is elevated in this condition (Kelly and McEwan, 1978).

The liver may therefore by the source of the raised plasma ferritin in toxaemia. Shorr (1954-55) in an extensive review of the vasoactive and anti-diuretic activities of ferritin has shown that in experimental and essential hypertension that ferritin is released by the liver under aerobic conditions into the blood stream. Liver ferritin is readily utilised in pregnancy (Wyllie and Kaufman, 1971) and we have shown that in normal pregnancy iron stores are low from mid-pregnancy (Kelly et al, 1977). There could however have been a shift in iron from the red cell to the reticuloendothelial compartment which would increase the plasma ferritin concentration (Jacobs and Worwood, 1975). The raised ferritin could also be explained by the disseminated intravascular coagulation and haemoconcentration which is known to occur in toxaemia (Scott, personal communication) but could also arise as result of retarded feto-placental growth and the resultant reduction in the fetal demand for iron.

Chronic infections

Patients with infections, mostly of the urinary tract, had also significantly elevated plasma ferritin concentrations when initially investigated and these values were maintained until term. The results are in agreement with investigations of non-pregnant patients with inflammatory conditions (Lipschitz et al, 1974) but the concentrations in the present series were much lower. Only 8 per cent of patients had depleted iron stores at delivery. All othersindices of iron metabolism were identical to the control group unless anaemia was also present. The ferritin in these cases remained elevated although the plasma iron was reduced in comparison to normal pregnant patients.

The raised plasma ferritin concentrations could be due to a shift of iron from the red cell to the reticuloendothelial compartment as already suggested in cases of idiopathic jaundice of pregnancy. Alternatively a number of conditions including acute and chronic infections and chronic inflammatory states are accompanied by a mild anaemia and an impaired release of iron from the reticuloendothelial system. These conditions are also associated with a low serum iron concentration, percentage transferrin saturation and iron-binding capacity. In addition to a reduction of iron release there is also a shortened red cell survival time due to an extracorpuscular factor and an inadequate response to erythropoietin (Lynch, 1974).

Anaemia

Patients with iron deficiency anaemia or megaloblastic anaemia had respectively low and normal ferritin concentrations and both groups had comparable haemoglobin levels. In iron deficiency anaemia in non-pregnant patients a mean plasma ferritin of 5ug per litre was reported by Jacobs et al (1972) and 4ug per litre by Lipschitz et al (1974). Our value of 8.6ug per litre was higher but the degree of anaemia was lower - 9.4g per dl. Plasma iron and the percentage transferrin saturation were lower and transferrin concentration higher than the control group as expected. In patients with megaloblastic anaemia the ferritin concentration was 35.2ug per litre and similar to that of normal patients.

Patients with an increased red cell turnover, including those with megaloblastic anaemia have been reported as having elevated serum ferritin levels which were disproportionately increased in relation to bone marrow haemosiderin (Lipschitz et al, 1974). The blood films of a number of our patients indicated that iron deficiency was also present and may explain our normal ferritin values. Patients with megaloblastic anaemia but no evidence of iron deficiency had higher plasma ferritin levels which were not significantly different from normal.

One could speculate that the difference in ferritin levels between our pregnant patients and non-pregnant patients with megaloblastic anaemia is that vitamin B12 deficiency in pregnancy is usually of dietary origin. Primary B_{12} deficiency is unusual in women of child-bearing age (Scott, 1962). Folic acid deficiency results more from fetal demands than dietary deficiency as a higher incidence is found in multiple pregnancy (Scott, 1962). It is therefore possible that when megaloblasic anaemia is detected after mid-pregnancy the condition may have arisen over a relatively short time scale and that the resultant disturbance in iron metabolism is not maximal.

Intra-uterine growth retardation.

In patients with clinical signs of intra-uterine growth retardation (IUGR) and those who had biochemical signs of IUGR, such as a low plasma HPL or urinary excretion of oestriol, elevated plasma ferritin concentrations were found when compared to the control group.

Patients with weight loss but no other signs of IUGR had normal plasma ferritin levels and may reflect the fact that not all patients were delivered of an LFD baby. In patients with clinical or biochemical signs of IUGR the feto-placental unit may not maxinally utilise iron and maternal stores may be augmented by prophylactic iron. This is supported by the evidence that there is a high incidence of iron deficiency anaemia in LFD infants irrespective of gestational age (Fomon, 1967) and might suggest that maternal iron deficiency is not a contributing factor but that there may be a failure in the placental uptake or fetal utilisation of iron.

Antepartum haemorrhage and premature labour.

Evidence from the literature suggests that APH and premature labour may be associated with iron deficiency (Scott,1962; Butler, 1967). The patients with APH whom we investigated had normal plasma ferritin and transferrin concentrations although the plasma iron and percentage transferrin saturation were low. This may be due to the fact that patients had been admitted to hospital and treated for a number of days before blood was obtained for analyses. A serial study revealed a gradual increase in ferritin with time at a stage of gestation when plasma ferritin is decreasing. However although one could use this evidence and extrapolate back to the APH and suggest that iron stores were low there is no justification on the present findings. The reason for the low plasma iron and percentage transferrin saturation in the presence of a normal ferritin in APH subjects may be explained by the variation in degree of APH which was not assessed.

There was no evidence of iron deficiency in any of our patients admitted in premature labour. Specimens were however obtained some time after admission and the results in both the APH and premature labour groups may only reflect prompt and adequate iron therapy.

Hyperemesis.

Low iron stores were expected in subjects with hyperemesis due to the patient's inability to maintain an adequate iron intake. Although the mean plasma ferritin concentration was within normal limits, 40 per cent of patients had low ferritin levels indicating depleted iron stores. At delivery only 20 per cent of patients were iron deficient indicating that treatment had been successful in half the group.

Multiple pregnancies.

It is suggested from the results in twin pregnancies that iron intake was increased to cope with the increased requirements as the plasma ferritin was significantly elevated compared to normal singleton pregnancies. It is the practice when twin pregnancies are recognised in early pregnancy and the haemoglobin levels are at the lower limits of normal to consider the patients as high risk candidates for anaemia. These patients then receive 300mg of elemental iron and 15mg of folic acid per day. In early pregnancy 15 per cent and at term only 5 per cent of patients had low ferritin levels and the mean term level was 44.0ug per litre compared to the normal singleton term value of 17.3ug per litre and reflects the adequacy of the regime.

Diabetes.

No abnormalities of iron metabolism were detected in diabetic pregnancies.

It would appear that in pregnancy low concentrations of plasma ferritin may be interpreted as iron depletion but in toxaemia, recurrent intra-hepatic cholestatic jaundice of pregnancy, infections and megaloblastic anaemia the increased level may not reflect increased iron stores. The higher plasma ferritins found in pregnancies which show clinical or biochemical evidence of IUGR may reflect reduced utilisation of iron by the feto-placental unit.

The Relationship between Maternal and Cord Ferritin Concentrations at Term.

An investigation was undertaken into the correlation of maternal and cord ferritin concentrations as there are conflicting reports on the influence of maternal iron stores on fetal stores (Sturgeon, 1959; Lanzkowsky, 1961; Rios et al, 1975). The prevalence of frank iron deficiency anaemia between 6 months and two years of age is well recognised (Siimes et al, 1974) and additional infants have normal haemoglobin levels but haematological evidence of anaemia which can be corrected by iron supplements (Guest, 1948). It is therefore of importance to determine the effect of maternal iron stores on fetal stores and to find a parameter which will predict infantile iron deficiency anaemia.

Variability in indices of iron metabolism.

The biochemical indices of iron metabolism are elevated in cord serum. The serum iron and percentage transferrin saturation are 162 per cent and 310 per cent respectively of maternal values and are in agreement with the work of Rios et al, (1975). The cord serum ferritin concentration of 175ug per litre is five times the maternal level of 30.3ug per litre. The cord ferritin levels were greater than that of Siimes et al (1974) and Rios et al (1975), lower than that of Fenton et al (1977) and in agreement with Hussain et al (1977) and van Eijk et al (1978) although the same order of magnitude between maternal and cord ferritin exists in all six studies.

There was no direct correlation between maternal and cord ferritin concentrations which is in agreement with the findings of Rios et al (1975) and Hussain et al (1977).

However when the patients were grouped according to whether the mother had low or above average ferritin levels there was a significant difference between the respective cord concentrations. Similar findings have recently been reported by Fenton et al (1977). Hussain et al (1977) found no correlation between maternal and cord ferritin levels but none of the mothers had deficient iron stores and the plasma ferritin concentrations were in excess of 16ug per litre. van Eijk et al (1978) studied 30 patients, 50 per cent of whom received iron supplements and found no significant difference in cord ferritin between the two groups. Low maternal ferritins were found by these authors but the small number of patients studied may account for the absence of correlation.

In the present work no difference was found in serum iron between cord sera associated with low or normal maternal ferritin concentrations and this is in agreement with others (Shott et al, 1972; Rios et al, 1975 and van Eijk et al, 1978). In contrast, Sisson and Lund (1958) in a study of circulating haemoglobin mass in the newborn concluded that the more severe the anaemia in the mother the more this will be reflected in the infant at birth. It was calculated that in severe maternal anaemia the infant could be deprived of one quarter of its readily available iron store. It would therefore appear on the balance of evidence from the present work and that of the others described that there is probably no direct correlation between maternal and fetal iron stores unless the mother is severly depleted. The placenta may play a controlling role in limiting the passage of iron from mother to fetus.

Infantile iron deficiency anaemia has been associated with children of high parity mothers (Shulman, 1961), light for dates and premature infants (Fomon, 1967) and infants of mothers who are a high medical risk and belong to the low socioeconomic class (Shott et al, 1972). Scott et al (1975) have also shown that unmarried mothers aged 15 to 22 years and all mothers over 40 years have a high incidence of iron and folic acid deficiencies. It was therefore thought that maternal and cord ferritin concentrations should be analysed with respect to the known "at risk" groups.

The effect of parity on serum ferritin.

In our serial study of 20 patients throughout pregnancy we found no difference in ferritin concentrations between primigravidae and parous patients at under 20 weeks gestation but in the investigation of women at term there was a significant reduction in ferritin when patients with three or more previous pregnancies were compared to those of lower parity. The discrepancy between these findings is probably due to the larger number of parous patients in the study at term rather than the difference in gestation at the time of ferritin analyses. The cord ferritin decreased with increasing maternal parity but this was not significant when analysed statistically. Shott et al (1972) found that parity did not correlate with cord serum iron, percentage transferrin saturation, haematocrit or haemoglobin.

The effect of gestational age on serum ferritin.

Shulman, 1961 has shown that premature infants have an increased incidence of poor iron stores which is in keeping with fetal iron stores being acquired in the third trimester (Fletcher and Suter, 1969).

Our study investigated only . . . infants born between 38 and 41 weeks and over this short period no statistical difference was found between the maternal or the cord ferritin concentrations.

The effect of social class on serum ferritin.

The highest incidence of infantile iron deficiency anaemia is associated with the "high medical risk, low socioeconomic class" (Shott et al, 1972). No difference was found in maternal ferritin between the social classes 1 to 1V but it was significantly lower in class V. This may have been due to the high parity (three or more pregnancies) in the latter group which was 22.7 per cent in contrast to 9.7 per cent in the former group. 50 per cent of mothers in the low social class group had ferritin concentrations of less than 12ug per litre which was the percentage quoted by Shott et al, (1972)for iron deficiency anaemia in a comparable American population. We are also in agreement with the same authors who found no correlation between parameters of iron metabolism in cord serum with respect to social class.

The effect of baby weight and maternal age on serum ferritin.

No correlation was found between baby weight and cord or maternal ferritin, serum iron or percentage transferrin saturation. Kessel and Sills (1968) found a similar lack of correlation between cord serum iron and term baby weights. Placental weight did not correlate with either maternal or cord ferritin concentration.

In the maternal age group 26 to 30 years there was a significant reduction in serum ferritin. No explanation for this finding was determined when results were analysed for high parity and low social class.

The age group 36 to 41 years had the highest percentage of patients with high parity (60 per cent) and belonging to social class V (40 per cent).

Our results indicate that when the maternal ferritin is less than 10ug per litre 28.6 per cent of babies have cord ferritin concentrations of less than 100ug per litre in contrast to 15.8 per cent when the maternal ferritin is normal. Low maternal ferritin levels as expected, are associated with high parity and low social class but these factors are not solely responsible for low fetal iron stores. Although all patients received iron supplements Bonner et al,(1969)have shown that a number neglect to take the tablets. In this series patients ferritin concentrations were not monitored throughout pregnancy and a lapse in taking the tablets early in the third trimester could not be ruled out. If this were followed by a period of increased iron intake when admission to hospital for delivery was imminent the maternal ferritin could well be normal at term but fetal stores depleted.

Plasma Ferritin in The Neonate

The physiological anaemia of infancy describes the fall in haemoglobin concentration over the first few weeks of life which occurs irrespective of iron supplements. It results from a fall in red cell mass and decreased erythropoietic activity rather than haemodilution or iron deficiency (Stockman and Oski, 1978). At 8 to 12 weeks the haemoglobin increases but if the infant has not received iron supplemented feeds the iron reserves will be depleted when the birth weight is doubled. The time at which iron deficiency limits the rate of haemoglobin synthesis is a function of the neonatal haemoglobin concentration and the rate of growth (Stockman and Oski, 1978). As iron deficiency anaemia of childhood is the most common nutritional deficiency of industrialised countries a knowledge of iron stores in the neonatal period could be valuable in determining the babies at risk and with this objective plasma ferritin was determined in the neonatal.period.

Plasma ferritin in relation to gestation.

Plasma ferritin increases with advancing gestation and was found to be significantly elevated in term infants when compared to premature babies and both groups were much increased in relation to adult levels. In term infants the plasma ferritin rose rapidly between delivery and 24 hours later but thereafter the level was constant. A similar initial increase occurs in haemoglobin and haematocrit if the cord has not been clamped immediately and reflects a compensatory fall in plasma volume in response to the transfusion induced hypervolaemia and also the poor oral intake at this time (Sisson and Lund, 1958; Mentzer, 1978).

Within the first few days of life haemoglobin returns to the cord level but we found the ferritin concentration increased slightly. It has been shown that the Fe^{59} label of heat treated red cells appeared in serum ferritin 30 minutes after injection which suggests that circulating ferritin originates in the cells associated with red cell breakdown (Jacobs and Worwood, 1975). The breakdown of red cells which have a half-life of 80 to 100 days in the term infant compared to 120 days in the adult, could lead to an increase in plasma ferritin which may be modified by a readjustment of plasma volume.

No cord blood specimens were available from premature babies but the plasma ferritin increased gradually throughout the first week of life although it was not statistically significant until Day 7, thereafter the concentration was maintained until Day 36. Initially premature babies had lower ferritin concentrations than term infants but by Day 7 the situation was reversed. The rapid increase in ferritin in the preterm group could be due to rapid red cell breakdown; the half-life of the red cell is 60 to 80 days and higher bilirubin concentrations are attained. Plasma volume changes may also be important as oedema which occurs in premature babies often disappears fairly rapidly. The plasma volume of term infants has been quoted as 42.7ml per Kg (Mollison et al, 1950) and alternately as 61.0ml per Kg (Sisson and Lund, 1958) and of premature babies as 62ml per Kg (Schulman et al, 1954) but it is accepted that hypo- and hyper-volaemia occur in term and premature babies (Sisson and Lund, 1958).

Plasma ferritin in relation to body weight.

There is a highly significant correlation between plasma ferritin and body weight but in the present series all but one of the twenty-one term and premature babies who were also light-for-dates had normal ferritin concentrations for gestation. These babies however are known to have low iron stores at birth and a large percentage develop iron deficiency anaemia in infancy (Shott et al, 1972). A significant increase in haemoglobin was found in premature light-for-dates infants when compared to non-retarded babies (Burman, 1974). Increased erythropoiesis in utero has been said to account for the polycythaemia seen in babies associated with placental insufficiency and intra-uterine hypoxia (Walker and Turnbull, 1953; Mentzer, 1978). Catabolism of the increased red cell mass could temporarily give rise to a normal plasma ferritin despite depleted organ stores. These infants are classically long, thin and wrinkled and appear to be clinically dehydrated and therefore haemoconcentration could also account for the normal plasma ferritin concentrations.

Publications on baby plasma ferritin concentrations are few. Siimes et al (1974) found a significant increase in ferritin at one month when compared to the cord level and Rios et al (1975) found that in term infants the level increased sharply during the first 24 hours and remained at high levels for the next six weeks. Only at three months did the ferritin significantly decrease. These results are similar to our own for term infants which differed from the values of premature babies who attained maximum concentrations on the seventh day.

Neonatal plasma iron.

The plasma iron concentration did not follow the ferritin pattern of the neonatal period as low levels occurred in the first 48 hours before increasing rapidly to levels which were maintained for one month. The cord levels of 27.0umol per litre were similar to those of de Leeuw et al (1966), Shott et al (1972) and Rios et al (1975) but lower than that of Sturgeon (1954). However the latter also found low serum iron concentrations within the first 48 hours in term infants followed by increasing levels for the next 11 days. A subsequent fall in serum iron was found at 4 months of age. As previously described we found no correlation between maternal and cord iron concentrations, Sisson and Lund (1958) reached similar conclusions when studying maternal levels at term and their babies at 3 to 5 days of age although a number of mothers had severe iron deficiency anaemia. They concluded that the determination of serum iron in the newborn did not indicate iron deficiency, "since it relates to current iron turnover, and is in such a sensitively balanced state that many factors can influence the values found at any one time." They reported that the maternal iron state did control the red cell volume and circulating haemoglobin mass and therefore the demands of the fetus were not entirely met at the mother's expense if she were anaemic. However there is evidence to suggest that the fetus does take precedence over the mother to obtain iron for haemoglobin synthesis and the formation of iron stores (de Leeuw et al, 1966).

Factors influencing iron stores in the neonate.

The plasma ferritin was found to increase with advancing gestation and birth weight. Weight has been shown to be an important factor in determining the total iron content at birth (Josephs, 1959) and in term infants the total body iron can vary by 80 per cent depending on body weight (Burman, 1971). Cord haemoglobin and the time of clamping the cord have also been said to influence iron stores but Sisson and Lund (1958) found that neither immediate or delayed clamping of the cord influenced the total blood volume or the red cell volume by the fourth day.

The plasma ferritin would appear to differ from haemoglobin concentration in relation to gestation. It is claimed that haemoglobin, haematocrit and red cell count increase from 12 to 34 weeks (Lubin, 1978). Others have suggested that the cord haemoglobin does not change significantly during the last trimester quoting term haemoglobin levels of 17.1 $\pm$ 1.8g per dl and premature levels of 17.5 $\pm$ 1.6g per dl (Stockman and Oski, 1978). However they have also found gestation to be reflected in the progressive fall in haemoglobin over the first one to three months when the rapidity of the decline and magnitude of the decrease depends on immaturity. The ferritin concentration in neonates of 27 to 30 weeks gestation was only 60 per cent of term values and in the 1.0 to 1.5 Kg babies the ferritin was 56 per cent of the concentration in the 3.6 to 4.0 Kg infants. Many premature babies appear oedematous and a dilutional effect may be partly responsible for the lower levels.

The ferritin concentrations however do appear to reflect the total body iron content at birth as a value of 50mg for a 1.00 Kg infant and 372mg for a 4.11 Kg infant have been reported (Josephs, 1959). The iron content of the bone marrow does not reflect iron stores in the neonate as little or no iron is found at birth in premature or term babies (Seip and Halvorsen, 1956).

5.7 per cent of babies had ferritin concentrations lower than the calculated limits. Only 3 per cent had values of less than 60 ug per litre at birth and would appear to reflect the ability of the fetus to accumulate iron irrespective of maternal stores. It has been reported that there is a reduction in plasma volume in the newborn of anaemic mothers (Sisson and Lund, 1958) therefore false elevations of plasma ferritin may occur in these babies.

Relationship between plasma ferritin and bilirubin.

The plasma ferritin concentration was found to correlate with the maximum bilirubin concentration. The serum ferritin did not correlate with bilirubin in adults with hepatitis (Lipschitz et al, 1974) but in the latter the source of circulating ferritin would appear to be the liver whereas in the neonate it arises from red cell catabolism. In term infants the ferritin increased rapidly within twenty-four hours with only small increases occurring thereafter but in premature neonates the initial levels were significantly lower than in the mature group but rose throughout the first week reaching higher levels than at term.

The variation in plasma ferritin concentration particularly in the premature infant reflects the changes known to occur in bilirubin.

Physiological hyperbilirubinaemia of term infants is characterised by maximum concentrations of 200 umol per litre at 4 days and in premature babies maximum levels of 260 umol per litre attained at 7 days. Hyperbilirubinaemia results from the increased circulating red cell mass at birth, the decreased red cell survival time, the increased enterohepatic circulation of bilirubin and the reduced activity of the hepatic glucuronyl transferase (Lubin, 1978). The fact that the increase in plasma ferritin is greater and peaks later in premature infants than term babies could be explained on a similar basis to bilirubin metabolism. The uptake of the circulating ferritin by the liver, the main iron storage organ could be decreased at this time and unable to cope with the ferritin liberated from the breakdown of the increased red cell mass. This could result from immaturity of enzyme systems, a deficiency of intermediate compounds involved in iron storage or the system could simply be overwhelmed by the amount of ferritin presented. The latter may be more important as the infant has already a high concentration of ferritin in the liver at birth. However in a series of four patients a rapid plasma ferritin turnover has been found in the neonate (Siimes et al, 1975).

Plasma ferritin of the neonate appears to reflect previous studies of iron stores and both have been found to increase with advancing gestation and birth weight. However rapid changes in plasma volume and hepatic function which occur particularly in premature and light-for-dates infants, appear to be reflected in the marked variation in serial studies of individual babies.

The term infant has a relatively stable ferritin concentration between Day 3 and Day 7 which may therefore give a more precise measure of iron stores. This however could only be confirmed by a long term follow up of these babies which was outwith the scope of the present work.

The Ferritin Content of Fetal Organs

Little information is available on the storage of non-haemoglobin iron during gestation (Loria et al, 1977) although almost ninety years ago Lapicque and Guillemonat (1889) described the "life curve" of the iron content of human and animal livers which they found to be high in the newborn, low in childhood and higher in adults.

The total iron content of the neonate has been found to be proportional to birth weight (Josephs, 1959) and as in the adult between 60 per cent (Burman, 1971) and 75 per cent (Schulman, 1961) is found in the haemoglobin mass. Storage iron in the term neonate accounts for 14 to 20 per cent of the total iron (Schulman, 1961; Burman, 1971) and 10 per cent is found in the tissue or parenchymal mass as myoglobin iron (Schulman, 1961).

Adult storage iron in the form of ferritin and haemosiderin is found in significant amounts in the liver, spleen and bone marrow but in both term and premature infants little or no iron is found in the latter at birth (Stockman and Oski, 1978). Brückman and Zondek (1939) found that the non-haem iron content of the liver of the newborn was 1.77g per Kg dry weight of tissue which is equivalent to 442ug per g of wet weight tissue calculated on their finding of a 75 per cent water content. He summarised the findings of other workers on the non-haem hepatic iron concentration determined by a variety of chemical means and found comparable results. Chang (1973) determin ed the non-haem iron content of term and preterm infants and found that the content increased by 50 per cent after 35 gestational weeks.

The content of the non-haem iron was 42mg in the term infant and the concentration was in agreement with Bruckman and Zondek (1939). A late accumulation of storage iron which constitues 50 per cent of the total body iron also occurs in the rat (Morgan, 1961). Loria et al (1977) have recently completed a study of the accumulation of hepatic non-haem iron throughout gestation and at 39 to 41 weeks the content was 40.5 (±36.0) mg. A limited number of reports are also available on the non-haem iron content of kidney. It is said to be present in the ratio of liver to kidney as 9 to 1 in the newborn and 5 to 1 in the adult (Bruckman and Zondek, 1939) but Chang (1973) found that in term and premature infants the ratio was 40 to 1.

Previous studies of storage iron in the developing fetus have been limited to determining the non-haem iron content of liver and kidney by quantitating the iron content after acid digestion. In the present study we applied a specific radioimmunoassay technique to determine the ferritin content of liver, heart and spleen. Ferritin was quantitated as it could be analysed with specificity, the iron content is more easily mobilised than haemosiderin (Wyllie and Kaufman, 1971)and it may have other functions as yet unknown (Powell et al,1975).

The ferritin content of fetal liver.

The concentration of ferritin in the liver of 36 to 40 week neonates was 1mg per g and the total ferritin content 118.5mg. Horse spleen ferritin contains 20 per cent and adult rat liver 27.5 per cent iron (Harrison et al, 1974).

The iron content of liver ferritin depends on the state of iron balance and can fall from 29 per cent in the newborn rat to 4.6 per cent at day 18. Assuming an iron content for ferritin of 20 per cent the liver ferritin iron of our term infants is 0.2mg per g tissue, 23.7mg per organ or 9mg per Kg body weight. On the basis of earlier reports of total non-haem iron content of the human term liver (Brückman and Zondek, 1939; Chang. 1973; Loria et al, 1977) the ferritin iron content would appear to represent about 50 per cent of the total non-haem iron, the figure reported by Harrison et al (1974) as the percentage of ferritin to haemosiderin in storage organs. Our ferritin iron content represents 69.7 per cent of the 34mg of total body storage iron calculated to be found in a 3.3Kg term neonate (Schulman, 1961). All studies have reported similar findings for the storage iron content of neonatal liver including Rhodesian African fetuses whose mothers had excess iron stores (Buchanan, 1968). Chang (1973) has stated that such uniformity could suggest a mechanism in the placenta to prevent iron overload. He also considers that the variability in all reports of non-haem iron content of liver, irrespective of whether they are grouped according to body weight or gestation, indicates the variable iron status of the mother. Burman (1974) has said that total body iron of the fetus is independent of maternal iron stores and is determined by body weight, gestation and the time at which the cord is clamped. The two statements are not incompatible as most of the total body iron is contained in the circulating haemoglobin and would be maintained at the expense of building up iron stores which are quantitatively much smaller and represent material not immediately required.

The liver ferritin content correlated with body weight, organ weight and gestation. The weight of the baby appeared to be the most important factor. This has been reported in a study of premature and term babies who were of normal birth weight, light-for-dates (LFD) or hypertrophic (Loria et al, 1977). The premature and LFD groups had low non-haem iron contents in the liver compared to normal weight infants. It was also found that hypertrophic babies had low levels and the suggestion was made that on reaching a normal gestational age the fetal storage iron was mobilised and lowered mainly by growth rather than gestation. The ferritin content of the liver was at least twenty-four times greater than in spleen and eighty times greater than heart, irrespective of gestation and would therefore appear to be the main iron storage organ. The fetus synthesised most ferritin between 36 weeks and term and infants born before this time would have less than 41 per cent of the term ferritin content. Chang (1973) found that premature babies born between 28 and 34 weeks had less than 50 per cent of the liver iron stores of term babies. Loria et al (1977) found that preterm infants at 30 to 32 weeks had only 60 per cent of the liver weight and non-haem iron content of term babies. These results support the long held view that premature babies are at risk from iron deficiency anaemia arising in infancy due to low iron contents at birth. Iron supplements given earlier and in greater amounts than those given to term babies in order to keep pace with the accelerated growth rate can prevent the condition (Schulman, 1961; Lundström et al, 1977).

The concentration of ferritin in the liver unlike the total content did not relate to body weight, gestation nor organ weight. The ferritin concentration fell between 30 and 35 weeks and in relation to body weight the concentration remained constant between 1.1 and 2.0 Kg. Loria et al.(1977) also found no correlation between the non-haem iron concentration of liver and gestation and reported a falling concentration between 28 and 38 weeks. These results could not be due to an accelerated hepatic weight gain as this was relatively constant between 30 weeks and term. During this time however iron could be actively required and would not be available for storage at a rate commensurate with organ growth. The red cell mass of 46ml per Kg for premature infants of birth weight 1.07 to 2.30 Kg was similar to that found in term babies (Schulman and Smith, 1954). The maximal rate of erythropoiesis could therefore be gained at the time when we note a fall in the rate in the accumulation of iron stores. The liver is the main erythropoietic organ in the second trimester and is still active at term so both maternal and fetal hepatic iron could be mobilised. There is evidence that the fetus can utilise its own iron stores in utero (Loria et al, 1977). Fletcher and Suter (1969) have suggested that increased concentrations of iron might be found in the fetal liver in early pregnancy when fetal blood volume is small and at term when fetal erythropoiesis is decreased. The increase in the maternal red cell mass could also lead to smaller amounts of iron being available to the feto-placental unit as fetal parasitism is not total (Chang, 1973; Fenton et al, 1977).

Ferritin content of fetal heart.

Little information is available on the iron stores of the fetal heart. The isoferritins of heart are more acidic than liver but would appear to contain equivalent amounts of iron (Powell et al, 1975). The heart ferritin content correlated with organ and baby weight but not with gestation. Little increase was noted in heart weight between 33 weeks and term but organ weight did correlate strongly with baby weight ($r=0.87$; $p<0.001$). The concentration of ferritin in the heart, unlike liver and spleen remained constant between 24 weeks and term and suggests that in this organ ferritin may not serve as an iron storage compund but have an alternate function. Little variation was noted in the non-haem iron concentration of maternal and fetal heart after the acute and chronic administration of intravenous iron (Wöhler, 1964). Pancreatic isoferritins contain little or no iron and therefore do not serve as iron storage proteins and have led to the consideration of alternative functions for ferritin (Powell et al, 1975).

Ferritin content of fetal spleen.

In the newborn the spleen is second only to liver in storage iron content. The total ferritin content of spleen was found to correlate with baby and organ weight and gestation. At term the spleen contained only 4 per cent of the ferritin content of liver. The spleen was found to contain only 1 per cent of the activity of liver after the administrations of plasma containing labelled iron to the mother (Fletcher and Suter, 1969).

On the basis of human fetal spleen containing equivalent amounts of iron to horse spleen ferritin the iron content at 36 to 40 weeks was 0.8mg or 0.3mg per Kg body weight. Fetuses of less than 36 weeks contained less than half the ferritin content of term infants although the maximum rate of synthesis occurred between 30 and 35 weeks when the ferritin content increased five fold. The spleen was the only organ in which the concentration of ferritin correlated not only with gestation but also with body weight ($r=0.70$; $p<0.001$) suggesting that there may be a more controlled synthesis of ferritin in spleen than in liver or that the two organs may receive iron from different sources and at varying rates. In support of the former it has been shown that excess iron is taken up preferentially by the fetal liver (Wöhler, 1964). The iron accumulating in the ferritin of the fetal spleen could result from the breakdown of fetal red cells, an amount which would increase with fetal growth. In the rat the fetal liver is the principal site of iron deposition and erythropoiesis. It has been suggested that the transfer of iron from the liver to other tissues is probably minimal but that erythroid cells entering the circulation could contribute to the iron content of the spleen (Orlic et al, 1974).

Possible functions of fetal organ ferritin.

Liver and spleen ferritin contain the greatest number of isoferritins in common but the iron content of a particular isoferritin varies with the tissue of origin (Powell et al, 1975). It has been suggested that the isoferritin distribution and iron content may be related to metabolic differences in tissue ferritins (Gabuzda and Pearson, 1969).

Shepp et al (1972) has shown that two metabolically distinct pools of ferritin exist in bone marrow. The anabolic form accepts iron from transferrin and is an isoferritin characteristic of erythroid cells. The catabolic form accepts iron from haemoglobin and is an isoferritin of reticulo-endothetial cells. However both cells take up iron during erythroid cell maturation, regardless of the form of the administered iron. It is uncertain whether erythroid ferritin is a precursor in the synthesis of haem or a form of storage iron which is not an intermediate in the synthetic process. Powell et al (1975) consider that the major iron containing isoferritin in spleen may be the catabolic ferritin of bone marrow whereas the major component in liver may represent anabolic ferritin in parenchymal cells. When isotopically labelled ferrous citrate was administered to rats in utero the major fractions appeared in the parenchymal cells of liver which constituted 83 per cent of the total volume of adult rat liver and lesser amounts in the erythroid cells of spleen (Orlic et al, 1974). "the differences in iron content in isoferritins common to different tissues may therefore reflect different cell populations in different tissues and organ variation in iron metabolism" - Powell et al, (1975). It is open to conjecture whether these studies of adult tissues can be applied to f etal organs. It would appear reasonable to assume that as the liver is a site of erythropoiesis in the fetus much iron would be found in the anabolic isoferritin. The only report of fetal spleen ferritin suggests that active iron uptake is associated with the erythroid cells of the organ (Orlic et al, 1974) and would therefore be in the anabolic form unlike the major isoferritin of adult tissue which is in the catabolic form.

There is some evidence to suggest that under certain circumstances the spleen has erythropoietic activity.

Variation in ferritin content of fetal organs and relationships to the placenta.

The ferritin content of liver correlated with the content in spleen ($r=0.60$; $p<0.005$) and in the heart ($r=0.66$; $p<0.001$). Chang (1973) found a significant correlation between total storage iron in liver and kidney with body weight and gestational age. He suggested that they reflected similar findings between total body iron and body weight and gestation. However of the three organs only the concentration of ferritin in the spleen correlated with baby weight and gestation. These findings would also suggest that ferritin synthesis in these three fetal organs may be controlled by different mechanisms. There is evidence to suggest that the control of mobilisation of non-haem iron in the adult is organ specific. The iron content of adult kidney is little influenced by pathological consequences which exhaust the hepatic iron stores (Bruckman and Zondek, 1939). Morgan (1961) has reported that in normal lactating rats the non-haem iron of the spleen but not the liver is utilised. In contrast Wyllie and Kaufman (1971) found that the uptake and utilisation of iron was much greater in liver than spleen in the pregnant rat. The female rat in comparison to the male contains more ferritin iron in the liver and kidneys but the same concentration in the heart. The rate of ferritin synthesis in the female rat is twice that in the male but the breakdown is similar in both sexes.

These differences are thought to result from the action of oestrogens on ferritin synthesis but others have suggested that hormones may have an indirect affect on iron absorption. Sex differences are not found in all species (Harrison et al, 1974). The formation of iron stores and ferritin synthesis therefore appears to be organ and species specific.

The placental weight correlated only with the spleen ferritin content although the content of all three organs related to total body and organ weight. It has been reported that total body iron content related to body weight and gestational age but not to placental weight (Burman, 1974). When the ferritin content of four placentae was compared to that in the relevant fetal organs normal values for the appropriate gestation were found in three. The fourth placenta was larger than normal and contained a gross content of ferritin. The fetal liver was enlarged but the spleen and heart were normal. The ferritin content of liver and spleen were $\pm$2SD of the gestational mean but well within normal limits for the weight of the baby. As the ferritin(p126-7) content correlates more closely with baby weight than gestation the ferritin content was considered normal in all three organs. The pregnancy resulted in a still-birth caused by intrauterine pneumonia. Bothwell et al (1958) have shown that infection in the rabbit impairs the transport of iron across the placenta and reduces the total turnover of plama iron and the percentage of iron going to the fetus. The latter statement was not in agreement with the present findings. Adults with chronic infections have increased plasma ferritin concentrations (Lipschitz et al, 1974).

In the present study increases associated with acute maternal infections have been found which could be explained by a shift in iron from red cell to reticulo-endothelial sites (Jacobs and Worwood, 1975). The haemoglobin concentration was not quantitated in this fetus so there is no evidence to support the theory. No reasons could be found to explain the high placental ferritin content. Wöhler (1964) has shown that despite acute and chronic administration of intravenous iron to rabbits that fetal organs were not "inundated" with iron. The placenta appeared to act as a barrier to the passage of excess iron to the fetus by storing the metal. African babies whose mothers were commonly found to have excess storage iron did not demonstrate any abnormalities of iron metabolism (Buchanan, 1968). Wöhler (1964) has suggested that excess non-haem iron in the rabbit placenta is present as haemosiderin and that there is little variation in the ferritin content. However he quoted the work of Göltner and Stark (1960) who have shown that in the human placenta the ferritin content increases but haemosiderin remains unchanged after 14 days of intravenous iron therapy. Our evidence supports the finding that increased ferritin can occur in the human placenta.

The effect of IUGR on organ ferritin content.

Intrauterine growth retardation can best be studied by comparing a group of light-for-dates (LFD) infants with a normal weight group of equivalent gestation. The five LFD babies in the present series varied widely in gestational age and therefore an alternative approach was adopted.

Individual LFD fetuses were compared with the mean for the equivalent gestation.

The body weight of all 26 fetuses correlated closely with the weights of liver, heart and spleen. In only one of the LFD group was the liver or spleen small and in two the heart was reduced in weight. This may reflect the wide variation in the group as a whole. Three of the five had low total ferritin contents of liver, heart and spleen but two had increased contents of heart and one an increased concentration but normal total content of the spleen.

In contrasting the content of normal and low weight organs independently of baby weight all but one of the six livers had low ferritin contents and concentrations and the sixth had a normal concentration but low total content. The five low weight spleens all had reduced total contents although two had normal concentrations. It would appear from the small numbers studied that in intrauterine growth retardation the liver weight is frequently normal but the concentration and total content of ferritin are reduced. In small livers irrespective of baby weight, similar findings occur. LFD babies and normal weight babies with low weight livers would therefore require earlier and larger iron supplements than the normal weight group to prevent iron deficiency anaemia in infancy. The former group is easily identified but the latter would normally be unrecognised. The latter may explain the development of anaemia in a normal weight, term infant who had received a routine diet.

Low total body iron contents and low hepatic non-haem iron contents have been reported respectively by Burman (1974) and Loria et al (1977) in LFD babies but both have suggested that the concentration is normal. Our results support the former but we also found concentrations to be reduced. Two LFD fetuses had increased ferritin contents of the heart which quantitatively contribute little to the total body iron content. No heart abnormalities were noted in either baby.

The organ ferritin content of twins.

In the present series twins had lower liver and spleen ferritin contents than singletons but this may only reflect that three of the seven were LFD and one set was extremely premature. The fetus whose twin survived was of average birth weight and the ferritin content of liver and spleen were normal. When discrepancies existed in organ weights the larger organ frequently contained the higher ferritin content. However in one set of twins the spleens were of equivalent weight but one organ contained four times as much ferritin and in two paired hearts the one which weighed only 50 per cent of the other contained four times as much ferritin. The twins who had a twin-to-twin transfusion *in utero* had similar organ ferritin contents. This suggested that the process was not gradual or one would have found evidence of increased erythropoietic activity in the anaemic twin and a lower ferritin content in the liver. An increased ferritin content in the liver and spleen of the plethoric twin would have suggested increased red cell catabolism. It was therefore concluded that the episode was acute and resulted in the still-birth of both twins.

Vosburgh and Flexner (1950) in a study of guinea pig litter mates showed that there could be considerable differences in the amount of iron transferred to members of the same litter which was not related to body weight. He suggested that this reflected a complex mechanism which regulated iron transport across the placenta. The total amount of iron transferred from the mother to all fetuses is not affected by the size of the litter and newborn rats from large litters have low hepatic iron contents (Munro and Linder, 1978).

PLACENTAL FERRITIN

The presence of ferritin in the placenta has been reported by a number of authors (Tecce and Tecce, 1950; Latham and Vosburgh, 1950; Mazur et al, 1955). A placental protein, PP_2, was isolated and purified from human placenta by Bohn (1973) and was identified as ferritin. It had a molecular weight (M.Wt.) of 450,000 to 500,000 and was composed of subunits of M.Wt. 20,000. The iron content was found to vary from 12.8 to 25.6 per cent depending on the Rivanol precipitate analysed.

Ferritin in normal, term placentae.

The concentration of ferritin in normal term placentae was 61.5ug per g of wet tissue. Our results agree with those of Mazur et al (1955) who quantitated the ferritin of three placentae by an immunochemical method and found them to contain 62.5 and 75ug ferritin per g of tissue. Bohn (1973) also used an immunological technique and determined the concentration to be 29ug per g tissue, but suggested that the result may be low due to the single extraction procedure used. We found no difference in the ferritin concentration between the peripheral and central regions. A concentration gradient was however found between the maternal and fetal surfaces.

The non-haemoglobin iron concentration of human placentae has been reported as 10.5ug per g of wet tissue (MacKay et al,1958) and Apte et al, (1971) have found that the amount of non-haem iron as a proportion of the total placental iron to increase with iron intake. Control placentae were found to contain 10.4ug non-haem iron per g and after 60mg per day of iron supplements the placentae contained 22.3ug per g. Our normal term placentae contained 12.3 ug ferritin iron per g of tissue calculated on the basis of placental ferritin containing 20 per cent.

It would therefore appear from the findings of MacKay et al (1958) and Apte et al (1971) that in our placentae a high proportion of storage iron was present as ferritin. In contrast Wohler (1964) found the ferritin iron concentration to be 17 ug per g which constituted only 17 per cent of the total non-haemoglobin iron. The latter would appear to be in conflict with the non-haemoglobin iron concentration reported by others. The rat term placenta contains 76.3 per cent of non-haem iron as ferritin, termed non-haem water soluble iron (Morgan, 1961) and 71.0 per cent as determined by Kaufman and Wyllie (1972). These reports on rat placentae are more in keeping with our findings that a high proportion of non-haem iron is present as ferritin in the human placenta.

Placental ferritin in relation to gestation.

The present investigations determined that placental ferritin concentrations varied with gestation. It rose during the first trimester, remained constant throughout the second trimester and decreased to low levels between 24 and 37 weeks. A second increase took place at term. The total ferritin content remained constant between 24 and 37 weeks despite the increase in placental weight. A 50 per cent increase followed between 37 weeks and term. At parturition the placental ferritin concentration was 6 per cent of the fetal liver and contained 34 per cent of the total ferritin content of liver.

MacKay et al (1958) in a histological study of the developing human placenta found similar changes in the non-haem iron content in the first and second trimesters by observing Prussian Blue stainable iron. In the third trimester low levels were found which were maintained until term.

At delivery there was an increase in macrophages containing haemosiderm. The histological findings corresponded to the results they obtained by chemically quantitating non-haemoglobin iron. In the rat the non-haem iron content and concentration increased from day 13 until day 17 and thereafter the concentration decreased although the content remained constant (Morgan, 1961). The ratio of what was considered to be ferritin and haemosiderin remained constant throughout gestation. Kaufman and Wyllie (1972) extended the study of rat placental storage iron until parturition on day 21. They likewise found that ferritin iron concentration increased until day 17 and decreased thereafter until day 20. The total ferritin content followed a similar pattern. However, they further reported that between days 20 and 21 a rapid increase in organ weight occured. The ferritin concentration was found to remain constant and there was 140 per cent increase in placental ferritin content. Haemosiderin was also deposited in amounts between 17 and 22 per cent of ferritin during the latter half of gestation, but in contrast to ferritin did not fall on day 19. The amount of ferritin iron in the placenta at parturition was 19 per cent of the total content of the feto-placental unit. In comparison we found that human placental ferritin constituted 24 per cent of the ferritin of the feto-placental unit. The rat fetus is known to contain more storage iron than the human fetus (Morgan, 1961).

The placental ferritin content at term did not relate to the ferritin content of liver or heart, but did correlate with spleen.

The total fetal iron content has been found to relate to gestational age and body weight rather than placental weight (Burman, 1974) and as most non-haem iron is found in the fetal liver our findings would appear to agree with the statement.

Ferritin in abnormal placentae.

Nylander (1953) has suggested that the passage of iron to the fetus is controlled by placental ferritin. The ferritin content of term placentae which weighed less than 500g was significantly lower than the term control group. The concentration and concentration gradient between the maternal and fetal surfaces were similar in both groups. There would therefore appear to be normal iron transfer per gramme of placental tissue but the total amount of iron transferred to the fetus would be reduced. Low iron stores would be found in these infants. In term placentae of normal weight but considered abnormal due to the low maternal HPL concentration or urinary oestrogen excretion the ferritin concentration, concentration gradient and total ferritin content were similar to the control group.

Transfer of iron.

The mechanism by which iron is transferred from mother to fetus is ill defined. In studying placental transfer a knowledge of the placental type is of prime importance. It is much greater in the haemochorial than the epitheliochorial or endotheliochorial and therefore findings in animals cannot always be applied to humans. It has been suggested that it is possible to correlate iron transfer and placental types to phylogenetic assemblages of separate ancestry extending back to the late Cretaceous period about 75 million years (Seal et al,1972).

In 1942, Pommerenke et al, suggested that in humans there was a direct and rapid transfer of iron and that the maternal plasma was the source of fetal iron. These findings were later substantiated by work in the guinea pig (Vosburgh and Flexner, 1950; Wong and Morgan 1973) in the rat (Nylander, 1953; Kaufmann and Wyllie, 1972) and in the rabbit (Bothwell et al, 1958; Baker and Morgan, 1969).

The uptake of maternal transferrin bound iron is thought to be similar in the placenta and developing red cell (Laurell and Morgan, 1964). It has been shown in vitro that transferrin is taken up by placental binding sites in the rat and that the amount bound is proportional to the concentration and independent of degree of saturation with iron (Laurell and Morgan, 1964). Similarly iron uptake was also found to be proportional to concentration and independent of transferrin concentration. In contrast it has been reported in the rabbit that iron saturated transferrin is taken up more readily than iron free transferrin by the placenta in vivo and that similar findings have been described in human reticulocytes (Baker and Morgan, 1968). It has also been suggested that human placental binding sites may work more efficiently at lower levels of iron saturation than those of red cell precursors and therefore iron was directed to the fetus when the mother was iron deficient (Fletcher and Suter, 1969). In the rabbit the ability of the placenta to bind transferrin in vivo increased with advancing gestation, but the uptake of the maternal liver, spleen and bone marrow was unaltered. (Baker and Morgan, 1968).

The two binding sites on transferrin are generally accepted as having similar affinities for iron, but not all agree as physico-chemical differences between sites have been reported (Fletcher and Huehns, 1967; Jacobs, 1977). Functionally different binding sites have been suggested by Fletcher and Suter, (1969) as the reason for most of the placental iron being delivered to the liver before being mobilised for erythropoiesis. However, work on the rat has shown no evidence of selective unloading of iron as a result of selective uptake even in iron depleted or iron overloaded animals (Pootrakul et al, 1977). The passage of proteins from mother to fetus is highly selective and in the human, transferrin can cross the placenta in some instances, but little or no maternal transferrin is present in fetal plasma at birth (Glasser, 1968). Morgan (1964) found rat placenta to be more permeable to transferrin than human or guinea pig, but Fletcher and Suter (1968) and Wong and Morgan (1973) have reported that transferrin does not cross the human or guinea pig placenta.

Iron uptake is an important function of the placenta, but the stimulus for the removal of iron from the maternal plasma to the fetus is unknown (Glasser et al, 1968). The uptake of iron does parallel fetal demands but it has been shown to continue in the absence of the fetus (Bothwell et al, 1958). Placental hypertrophy occurs in severe iron deficiency and has been considered advantageous to the fetus who may initiate the stimulus but the response is dependent on maternal nutrition (Beischier et al, 1970).

When iron supplements are given the placental uptake is increased and may therefore be influenced by the availability of iron (Apte et al, 1971). Following haemorrhage the placenta responds like reticuloendothelial and mucosal cells by increasing iron transport. (Bothweel et al, 1958). The accumulation of fetal iron would therefore appear to be controlled by complex mechanisms and influenced by fetal size, a placental factor and maternal iron metabolism. Glasser et al, (1968) found that in the rat, placental growth and the rate of iron transfer were major factors in determining the amount of iron found in the placenta. They concluded that the control of iron clearance from the maternal circulation could be due to the maturation of an independent placental iron transfer system. In addition there was a mechanism which allowed a more rapid rate of removal of iron from the placenta by the fetus.

Results from the present study show that the placenta contains a significant proportion of the ferritin of the feto-placental unit and by deduction that it also constitutes much of the non-haem iron content of the placenta. The rapid decrease in the concentration of placental ferritin in mid-pregnancy occurred at a time when ferritin was being rapidly synthesised by the fetal liver.

Regulation and localisation of placental ferritin.

A ferritin concentration gradient was found in most placentae between the maternal and fetal surface, irrespective of pathology. HPL throughout gestation and HCG at 25 to 29 weeks were also noted to have similar concentration gradients. Other workers have reported similar findings for HPL (Gau and Chard, 1975).

These were related to the amount of trophoblast per unit area. Trophoblast was found to decrease towards the fetal surface and the blood flow was similarly reduced. They concluded that the rate of HPL synthesis was primarily controlled by the rate of blood flow past the trophoblast cells. Ferritin synthesis could likewise be controlled by the rate of blood flow which would vary the amount of iron available for uptake. Electron micrographs in the present work demonstrated that ferritin of the normal term placenta was localised in the syncytiotrophoblast in the granular or rough endoplasmic reticulum of the villi. Wohler (1964) using a specific method also identified ferritin in the syncytiotrophoblast of human placentae. Bothwell et al (1958) reported iron in the deeper tips of the rabbit fetal villi which were in contact with the maternal venous sinusoids. These results are in contrast to the work of McKay et al (1958) who, using the classical Prussian Blue staining method for iron, found placental non-haem iron to be greatest in the second trimester and localised in large granules in the villi at the junction of the cytotrophoblast and Langhan's layer. Histochemical observations by McKay et al (1958) determined a rapid fall in iron content during the third trimester although at term there was an increase in macrophages or Hofbauer cells, which contained haemosiderin. They suggested that as the preparations also stained for phosphate that the iron staining compound was ferritin. Haemosiderin also contains phosphate and therefore they could not differentiate the compounds on this basis. The finding of iron in large granules also suggests that they were visualising haemosiderin rather than ferritin which gives a diffuse staining pattern with a Prussian Blue stain.

They localised iron at maximalconcentrationas single particles in the connective tissue of the villous stroma. Regular lines of granules at the junction of the cytotrophoblast and connective tissue were found in only one third of the villi. One expects to find a more uniform distribution of any substance which plays an active role in fetal development. This type of easily stainable iron is frequently found in placentae and is probably haemosiderin from necrotised tissue (Govan, 1978).

We found that a high proportion of placental ferritin was attached to the ribosomes of the endoplasmic reticulum in contrast to the findings in rat liver tissue (Redman, 1969; Munro and Linder 1978). Redman (1969) found that free ribosomes made 7 to 20 times more ferritin than did attached ribosomes which appeared to synthesise all secretory proteins including albumin. His work was confirmed by Munro and Linder (1978) who also found that the smaller portion of ferritin which may be secreted into the circulation was synthesised by membrane bound ribosomes. They also found that only free ribosomes could be induced by iron to synthesise ferritin and raised the question as to why the secreted ferritin which was not induced by iron, reflected iron stores. Electron micrographs of human liver and intestinal mucosa of subjects with haemochromatosis have shown vesicles packed with ferritin (Munro and Linder, 1978).

The siting of ferritin on the rough endoplasmic reticulum suggests that in common with other proteins synthesised on the same site in the liver, it may be secreted by the placenta.

The tissue is bathed by maternal blood and ferritin could therefore be released into the maternal circulation. Such a mechanism would have no place in the formation of fetal iron stores although it could be important in preventing excess iron deposition in the feto-placental unit. However, in the rat liver the ribosomes of endoplasmic reticulum do not synthesise ferritin in response to iron and in both the liver and the intestinal mucosa excess iron appears in ferritin packed vesicles. Thornburg and Faber (1976) have demonstrated in the rabbit placenta that there was no mechanism for the active transport of ferritin although it could diffuse passively from both the fetal and maternal circulations into the organ. Discrimination of molecular size was a function of the endothelium of the fetal capillary. We found no evidence of ferritin in the placenta other than in the surface layer of the syncytiotrophoblast-see p 137.

Ferritin iron can be released by reducing agents including sodium dithionite at acid pH and by the chelating agent, desferrioxamine at physiological pH but there is no evidence that the primary control mechanism utilises a chelator for the biological release of iron (Harrison et al, 1974). An enzyme, ferrireductase, present in liver has been shown to catalyse the anaerobic reduction of ferritin iron in the presence of a flavin mononucleotide and NADH. The enzyme is inhibited by oxygen (Osaki and Sirivech, 1971). Shorr (1955-56) showed that anoxia caused the release to the circulation of iron from ferritin in liver and suggested that the low oxygen tension in the placenta (Walker and Turnbull, 1953) provided ideal conditions for the storage of iron from the maternal plasma and its transfer across the placental membranes to fetal blood.

The siting of ferritin in the syncytiotrophoblast at least in term placentae suggests that in order that ferritin iron can reach the fetal circulation it must first be liberated in the ferrous form and transferred in a low molecular weight compound. If maternal hypoxia were to occur it would appear from the siting of ferritin that iron could enter the maternal plasma more directly than fetal blood but this is thought not to occur (Bothwell et al, 1958). However, we did find that in some types of maternal pathology, particularly severe PET high ferritin concentrations were present in the maternal circulation.

Placental ferritin in the control of iron transport.

The function of ferritin in plasma is unknown (Kaufman and Wyllie, 1972) but it has been suggested as a controller of iron transport across the placenta (Nylander, 1953; Wöhler 1955; Heilmeyer 1958), as a temporary store for fetal use (Kaufman and Wyllie, 1972) and as a barrier to prevent excess iron being transported to fetal tissues (Wöhler, 1964).

In a small study Fletcher and Suter (1969) have shown that iron transfer from mother to fetus is very rapid in early and late human pregnancy although decreased rates have been shown to occur at term in the guinea pig (Wong and Morgan, 1973). This rapid rate of transfer would suggest that iron did not mix with a large placental pool (Bothwell et al, 1958). However, Goltner and Stark (1960) have shown that in human pregnancy, after a single intravenous injection of iron to the mother, placental ferritin is increased in relation to the time elapsed and concomitantly the fetal serum iron increased.

When the placental ferritin began to fall the fetal serum iron continued to increase suggesting that ferritin played an active role in iron transport. Wong and Morgan (1973) have suggested that non-haem iron may represent the transit iron pool in the guinea pig. Recently it has been suggested that a fraction of the total cell ferritin takes part in the absorption of iron in the rat. The ferritin in the cytosol of the mucosal cells represents only one-third of the total cell ferritin but due to the constancy of iron distribution between this ferritin fraction and "free" iron, cytosol ferritin could represent an extension of the free iron pool and would therefore take part in the active transfer of iron to the serosal surface (Munro and Linder, 1978).

In studying the gestational changes of placental ferritin we found that in the first trimester placental ferritin concentrations attained term levels and by the end of the second trimester, which was the earliest assessment of fetal ferritin, the concentration in the liver was only half that at term. In contrast, the fetal organ ferritin increased most significantly when the placental concentration was lowest. Between 34 and 36 gestational weeks the hepatic ferritin content increased by 136 per cent when the placental concentration was only 57 per cent and the total content was 39 per cent of term levels. After 38 weeks the placental ferritin concentration doubled, the hepatic concentration was not increased but the content rose by 57 per cent and gained the same amount of ferritin as in the previous period when the placental concentration was low. If as would seem logical the size of a transit pool were inversely proportional to the rate of transfer our findings prior to term could be explained by ferritin being part of the iron transit pool.

However, at term the placental ferritin concentration increased which could have resulted from a reduction in iron transport to the fetus or to an increase in iron from the maternal circulation. The former hypothesis would appear to be false as the rate of ferritin synthesis in the fetal liver was similar to that in the proceeding period. Placental ferritin may therefore not be part of the iron transport pool and this conclusion is supported by the lack of correlation between placental and fetal liver ferritin. However, it has been suggested that in the rat mucosa only a fraction of the total ferritin is part of the free iron pool and a similar situation may also exist in the placenta (Munro and Linder, 1978).

Placental ferritin as a fetal iron store.

The function of ferritin as a fetal iron store would be a better explanation of the gestational changes found in the placenta. The mechanism for the placental uptake of iron would appear to mature earlier than the fetal utilisation and lead to increasing ferritin concentrations in the first half of pregnancy. When fetal uptake increased the placental concentration fell until late pregnancy. Although the placental ferritin content increased at term when it appears from our limited figures that the rate of iron transfer remains maximal, more iron may be available to the feto-placental unit at this time. This is suggested by the decrease in maternal plasma volume (Hytten and Paintin, 1963) while the haemoglobin remains constant and the increase in maternal plasma ferritin (Kelly et al, 1977). The intestinal absorption of iron remains elevated (Svanberg, 1975) and therefore more iron is made available to the fetus unless it is stored by the mother.

This does not appear to happen as the maternal bone marrow is often depleted of iron at term (de Leeuw et al, 1966; Svanberg, 1975).

Wohler (1964) reported that when iron was given over a 14 day period the placental ferritin increased but the placental haemosiderin and fetal serum iron were unaltered. Similar results showing increased placental non-haem iron have been reported after iron supplements have been taken by the mother in the last trimester (Apte et al, 1971). Morgan (1961) has shown that a decrease in the non-haem iron concentration of the placenta occurred at the same time as the iron transfer to the rat fetus was maximal. Kaufman and Wyllie (1972) also showed that the amount of ferritin and haemosiderin in the rat placenta varied with gestation, but only ferritin decreased when storage iron began to accumulate in the fetus. They concluded that the placenta appeared to retain iron in excess of fetal needs because the capacity of the fetal plasma and tissue receptors were exceeded or fetal utilisation lagged behind the availability of iron. It would also appear from these studies that placental ferritin is in equilibrium with transit iron as suggested by Wöhler (1964).

The stimulus for the utilisation of placental iron is unknown. It is unlikely to occur by passive diffusion as fetal plasma iron exceeds the maternal level from 20 weeks. The percentage iron saturation of fetal transferrin also remains high throughout fetal life due to a persisting low transferrin concentration (Fletcher and Suter, 1969). In the adult intestinal absorption is regulated by total body iron stores and the rate of erythropoiesis (Jacobs, 1978).

These states are unlikely to regulate mobilisation of placental iron as the fetal organ ferritin concentration and total content increase with advancing gestation. However, unlike the liver with large iron stores the bone marrow of the neonate contains no iron (Stockman and Oski, 1978) and may therefore exert a constant and increasing stimulus for iron transfer as the fetus increases in size.

In the present work the placenta of a severly rhesus sensitised fetus with evidence of an increased rate of erythropoiesis, was enlarged but had a normal ferritin concentration. The rate of red cell formation did not appear to influence the placental ferritin concentration. Factors which regulate iron absorption in the adult do not appear to play a significant role in the fetal utilisation of placental ferritin iron(Table 4,16-Fetus No.4).

Fetal and maternal transferrin bind iron at the same rates (Fletcher and Suter, 1969). In the rabbit the fetal protein does not appear to bind to the placental surface (Baker and Morgan, 1969) although it has been shown that transfer of iron from the placenta is slower into a transferrin free solution than into plasma (Laurell and Morgan, 1964). The passage of iron from the placenta is active and is not merely a passive transfer down a concentration gradient.

The rate of placental iron transfer is greatly inhibited by maternal hypoxia (Wong and Morgan, 1973) a condition which Shorr (1955-56) has suggested would be ideal for the reduction of ferritin iron and the release of iron into the circulation.

Walker and Turnbull (1953) have shown that there is a decreased fall in oxygen content and saturation of fetal blood near term and it is therefore possible that ferritin iron could be utilised at a time when the normal route for iron transfer is inhibited.

It is unlikely that ferritin, due to the size of the molecule could be transported to the fetal vessels before liberating iron. It is possible that iron would be mobilised on site in the syncytiotrophoblast before being transported in a smaller molecular form. If the latter part of the pathway was also that of the normal transfer of iron then after mobilisation further transport would be inhibited by hypoxia. However, if a high concentration accumulated iron could pass into the fetal circulation by simple diffusion.

Placental ferritin in the prevention of fetal iron overload.

The function of the placenta as a barrier to the passage of excess iron was suggested by Wohler (1957) who visualised that the barrier could only be overcome by a gross excess of iron although Pribilla (1954) had reported true penetration of administered iron. Chang (1973) suggested that a mechanism existed to prevent fetal iron overload and sited Rhodesian African fetuses who had no excess iron stores although they occurred frequently in the mothers. Burman (1974) also concluded that a placental iron barrier existed although iron in the organ was available to the fetus.

Wohler (1964) gave large amounts of iron in the form of ^{59}Fe iron saccharate and sorbitol to rabbits and found that despite high plasma iron concentrations in the mother and increased iron deposits in maternal organs, the accumulation in the fetal liver was small.

He concluded that penetration of the placental barrier had not occurred.

It is possible that the mechanism which prevents the passage of excess iron from the placenta to the fetus is similar to that in the small intestine. The electron micrographs which identified ferritin in the syncytiotrophoblast resembled the distribution of ferritin in the small intestine of iron loaded subjects. The control of intestinal iron absorption is not completely understood but it would appear to be related to the iron content of mucosal cells (Turnbull, 1974). It had earlier been suggested that to prevent iron absorption in excess of requirements iron was incorporated into ferritin in the mucosal cells (Crosby, 1963; Worwood and Jacobs, 1972). When iron first enters the mucosal cells it is present in a labile pool and is probably protein bound. Alternatively it may be transferred across to the serosal surface, possibly as a small molecular weight chelate (Munro and Linder, 1978) or synthesised into haem compounds and incorporated into ferritin (Jacobs, 1977). The regulation of iron absorption by serosal transfer is not understood but it would appear to be part of an equilibrium between plasma and the exchangeable iron in all tissues. It therefore appears that when iron stores are large, the serosal transfer of iron is decreased and intracellular iron is incorporated into ferritin. This mechanism may well operate in the placenta. The localising of ferritin in the syncytiotrophoblast of the villi would support this theory as excess iron would be immediately incorporated into ferritin, thus preventing further penetration of the organ.

The possibility of a placental mechanism existing to prevent the passage of excess iron to the fetus was illustrated by a 29 week (p.131) placenta which contained a gross excess of ferritin. Although the fetal organs contained above average amounts of ferritin the content was not excessive. Flexner (1954) demonstrated that the placenta had a large reserve capacity to retain iron when he found that of all the substances studied only iron and phosphate saturated the placenta.

The gestational variation in placental ferritin concentration also suggests that iron in excess of fetal needs is being taken up by the placenta in the first trimester and at term. In order to prevent overloading the fetus, iron is retained by the placenta. Although the ferritin concentration in fetal liver is low in early gestation compared to term, the ability of the fetus to store iron may be limited by the ability of the liver to remove iron from the circulation, or by the immaturity of the pathway for ferritin synthesis (Siimes et al, 1975). The plasma iron remained relatively constant throughout gestation but more iron may be presented to the placenta in early and late pregnancy as both the maternal iron saturation of transferrin and plasma ferritin concentration decreased between these stages of gestation.

It is therefore possible that iron may be presented in excess of fetal needs in early pregnancy when systems for the accumulation of iron stores are immature, and also at term when excessive amounts of iron are presented due to decreased maternal requirements. Placental ferritin may therefore operate as a "store" and as a "sink" by taking up iron at a time of plenty for the future use of the fetus and also by retaining iron which would otherwise overload the fetal organs.

These studies of the ferritin concentration in the maternal and fetal circulations, in fetal organs and in the placenta do no more than describe for the first time in human pregnancy the collective picture of the changes which occur in relation to gestation. The factors which control the uptake of iron by the placenta, its mobilisation by the fetus and the variation in relation to maternal pathology remain unknown.

Ferritin would now appear to be more than a storage protein. The cytosol ferritin of the rat mucosal cell is in equilibrium with iron in transit to the serosal surface and a heat stable factor is present in high concentrations in the plasma of subjects with haemochromatosis (Munro and Linder, 1978). These factors suggest that ferritin in the circulation and in the cell may regulate iron metabolism.

It is tempting to suggest, from the localisation of ferritin in the placenta that the syncytiotrophoblast functions in a similar fashion to the small intestine in the uptake and transfer of iron. The newer techniques of translating messenger ribonucleic acid of the free and bound ribosomes and recovering the ferritin fractions by gradient elution could determine the intracellular sites of ferritin synthesis and the subunit type. A study of the isoferritins of the placenta and their relation to gestation could also yield information on the changing role of ferritin in the placenta.

References

Addisonm G.M., Hales, C.N. (1971): "Two Site Assay of Human Growth Hormone". Hormone Metabolic Research, 3, 59-60.

Addison, G.M., Beamish, M.R., Hales, C.N., Hodgkins, M., Jacobs, A., Llewellin, P. (1972): "An Immunoradiometric Assay for Ferritin in Serum of Normal Subjects and Patients with Iron Deficiency and Iron Overload". Journal of Clinical Pathology, 25, 326-329.

Aherne, W., Worwood, M. (1974): "Some Properties of Human Ferritin". Biochemical Society Transactions, 2, 429-432.

Alfrey, C.P., Lynch, E.C., Whitley, C.E. (1967): "Characteristics of Ferritin Isolated from Human Marrow, Spleen, Liver and Reticulocytes". Journal of Laboratory and Clinical Medicine, 70, 419-428.

Apte, S.V., Iyengar, L., Nagarajan, V. (1971): "Effect of Antenatal Iron Supplementation on Placental Iron". American Journal of Obstetrics and Gynecology, 110, 350-351.

Ashford, B.K. (1934): "A Soldier in Science". Published by W. Morrow, New York, p 400.

Baker, E., Morgan, E.H. (1969): "The Role of Transferrin in Placental Iron Transfer in the Rabbit". Quarterly Journal of Experimental Physiology, 54, 173-186.

Battaglia, F.C., Lubchenco, L.O. (1967): "A Practical Classification of Newborn Infants by Weight and Gestational Age". Journal of Pediatrics, 71, 159-163.

Beaton, G.H. (1974): in "Iron in Biochemistry and Medicine". Edited by A. Jacobs and M. Worwood. Published by Academic Press, London. p 477-525.

Beischer, N.A., Sivasamboo, R., Vohra, S., Silpisornkosal, S. (1977): "Placental Hypertrophy in Severe Pregnancy Anaemia". Journal of Obstetrics and Gynaecology of the British Commonwealth, 77, 398-409.

Benesch, R., Benesch, R.E. (1969): "Intracellular Organic Phosphates as Regulators of Oxygen Release by Haemoglobin". Nature, 221, 618-622.

Bentley, D.P., Williams, R. (1974): "Serum Ferritin Concentration as an Index of Storage Iron in Rheumatoid Arthritis". Journal of Clinical Pathology, 27, 786-788.

Bielig, H.J., Bayer, E. (1955): quoted in "Iron in Biochemistry and Medicine". Edited by A. Jacobs and M. Worwood. Published by Academic Press, London, p 100, 104.

BMJ-Leader (1966): "Prophylactic Folic Acid in Pregnancy". British medical Journal, 2, 1543.

Boenish, T. (1976): "Improved Enzyme Immunoassay for Trace Proteins". in Protides of Biological Fluids. Edited by H. Peters. Published by Pergammon, Oxford and Paris, 24, 743-749.

Bohn, H. (1973): "Isolierung des Placenta-Proteins PP_2 und seine Identifizierung als Ferritin". Archiv für Gynäkologie, 215, 263-275.

Bolton, A.E., Hunter, W.M. (1972): "A New Method for Labelling Protein Hormones with Radioiodine for Use in Radioimmunoassay". Journal of Endocrinology, 55, 30-31.

Bonnar, J., Goldberg, A., Smith, J.A. (1969): "Do Pregnant Women Take Their Iron?". Lancet, 1, 457-458.

Bothwell, T.H., Pribilla, W.F., Mebust, W., Finch, C.A. (1958): "Iron Metabolism in the Pregnant Rabbit".

American Journal of Physiology, 193, 615-622.

Brewer, G.J. (1974): "2,3-DPG and Erythrocyte Oxygen Affinity". Annual Review of Medicine, 25, 29-38.

Brise, H., Hallberg, L. (1962): "The Influence of Meals on Iron Absorption in Oral Iron Therapy". Acta Medica Scandinavica, 171, Supplement 376, 1-38.

Brückmann, G., Zondek, S.G. (1939): "Iron, Copper and Manganese in Human Organs at Various Ages". Biochemical Journal, 33, 1845-1857.

Buchanan, W.M. (1968): "Storage Iron in Liver and Spleens of Rhodesian African Children". Transactions of the Royal Society of Tropical Medicine and Hygene, 62, 353-358.

Buffe, D. (1968): "Presence d'une Proteine d'origine tissulaire, α_2 H-globuline, dans le serum de sujets atteints d'affections malignes". International Journal of Cancer, 3, 850-853.

Buffe, D. (1973): "Fetoproteins and children's tumors". Gann Monograph Cancer Research, 14, 117.

Burger, H.G., Lee, V.M.K., Rennie, G.C. (1972): "A Generalised Computer Programme for the Treatment of Data from Competitive Protein-Binding Assays". Journal of Laboratory and Clinical Medicine, 80, 302-312.

Burman, D. (1971): "Iron Requirements in Infancy". British Journal of Haematology, 20, 243-247.

Burman, D. (1974): in "Iron in Biochemistry and Medicine". Edited by A. Jacobs and M. Worwood. Published by Academic Press, London. p 543-558.

Butler, E.B. (1967): in Ph.D. Thesis, University of Wales.

Butt, W.J. (1972): "The Iodination of Follicle-Stimulating Hormone and Other Hormones for Radioimmunoassay". Journal

of Endocrinology, 55, 453-454.

Catt, K.J., Tregar, G.W. (1967): "Solid Phase Radioimmunoassay in Antibody - Coated Tubes". Science, 158, 1570-1575.

Chang, L.L. (1973): "Storage Iron in Foetal Livers". Acta Paediatrica Scandinavica, 62, 173-175.

Cook, J.D., Marsaglia, G., Eschbach, J.W., Funk, D.D., Finch, C.A. (1970): "Ferrokinetics: a Biologic Model for Plasma Iron Exchange in Man". Journal of Clinical Investigation, 49, 197-205.

Cook, J.D., Lipschitz, D.A., Miles, L.E.M. (1974): "Serum Ferritin as a Measure of Iron Stores in Normal Subjects". American Journal of Clinical Nutrition, 27, 681-687.

Corrigan, J.C., Strauss, M.B. (1936): "The Prevention of Hypochromic Anaemia in Pregnancy". Journal of the American Medical Association, 106, 1088-1090.

Crichton, R.R. (1971): "Studies on the Structure of Ferritin and Apoferritin". Biochimica Biophysica Acta, 229, 75-82.

Crichton, R.R. (1973): "The Biochemistry of Ferritin". British Journal of Haematology, 24, 677-679.

Crichton, R.R., Millar, J.A., Cumming, R.L.C., Boyce, C.F.A. (1973): "The Organ Specificity of Ferritin in Human and Horse Liver and Spleen". Biochemical Journal, 131, 51-59.

Crichton, R.R. (1974): in "Iron in Biochemistry and Medicine". Edited by A. Jacobs and M. Worwood. Published by Academic Press, London. p 87.

Crosby, W.H. (1963): "The Control of Iron Balance by the Intestinal Mucosa". Blood, 22, 441-449.

Crosby, W.H. (1977): "Who Needs Iron?". New England Journal of Medicine, 297, 543-545.

Dallman, P. (1974): in "Iron in Biochemistry and Medicine". Edited by A.Jacobs and M.Worwood. Published by Academic Press, London. p 437-472.

Drysdale, J.W., Munro, H.R. (1965): "Small-scale Isolation of Ferritin". Biochemical Journal, 95, 851-858.
Drysdale, J.W., Munro, H.N. (1966): "Regulations of Synthesis and Turnover of Ferritin in Rat Liver". Journal of Biological Chemistry, 241, 3630-3637.
Drysdale, J.W. (1977): "Human Isoferritins in Normal and Disease States". in Seminars in Haematology, 14, 71-88.

van Eijk, H.G., Kroos, M.J. Hoogendoorn, G.A., Wallenburg, H.C.S. (1978): "Serum Ferritin and Iron Stores during Pregnancy". Clinica Chimica Acta, 83, 81-91.

Ekins, R.R. (1960): "The estimation of Thyroxine in Human Plasma by an Electrophoretic Technique". Clinica Chimica Acta, 4, 453-459.
Ekins, R.P., Newman, G.B. (1970): "Statistics in Endocrinology", Edited by J.W.McArthur, T.Colton. Published by MIT Press, Cambridge, p345-378.
Ekins, R.R. (1974): "Basic Principles and Theory". British Medical Bulletin, 30, 3-11.

Elwood, P.C., Hughes, D. (1970): "Clinical Trial of Iron Therapy of Psychomotor Function in Anaemic Women". British Medical Journal, 1, 254-255.

England, P., Cain, O. (1976): * A simple Method for the Interpolation of Radioimmunoassay Data". Clinica Chimica Acta, 72, 241-244.

Fairhurst, E., Dale, T.L.C., Ridge, B.D. (1977): *A Comparison of Anaemia and Storage Iron Deficiency in Working Women". Proceeding of the Nutritional Society 36, 98A.

Farrant, J.L. (1954): "An Electron Microscopic Study of Ferritin". Biochimica Biophysica Acta, 13, 569-576.

Fenton, V., Cavill, I., Fisher, J. (1977): "Iron Stores in Pregnancy". British Journal of Haematology, 37, 145-149.

Fieser, L.F., Fieser, M. (1944): in "Organic Chemistry". Published by D.C. Heath, Boston. p 891.

Finch, C.A. (1959): "Body Iron Exchange in Man". Journal of Clinical Investigation, 38, 392-396.

Finch, C.A., Cook, J.D., Labbe, R.F., Culala, M. (1977): "Effect of Blood Donation on Iron Stores as Evaluated by Serum Ferritin". Blood, 50, 441-447.

Fineberg, R.A., Greenberg, D.M. (1955): "Ferritin Biosynthesis". Journal of Biological Chemistry, 214, 91-106.

Fisher, A.M. (1969): "Anaemia in Pregnancy". British Journal of Hospital Medicine, 2, 1460-1469.

Fletcher, J., Suter, P.E.N. (1969): "The Transport of Iron by the Human Placenta". Clinical Science, 36, 209-220.

Flexner, L.B. (1954): "Gestation". Transactions of the First Conference of Gestation. Edited by L.B. Flexner. Published by Macy, New York. p 174.

Fomon, S.J. (1967): in "Infant Nutrition". Published by W.B. Saunders, Philadelphia. p 170.

Forfar, J.O. (1973): "Drugs to be Avoided During the First Three Months of Pregnancy". Prescriber's Journal, 13, 130-134.

Freund, J. (1951): "The Effect of Paraffin Oil and Mycobacteria on Antibody Formation". American Journal of Clinical Pathology, 21, 645-656.

Gabuzda, T.G., Pearson, J. (1969): "Metabolic and Molecular Heterogeneity of Marrow Ferritin". Biochimina et Biophysica Acta, 194, 50-54.

Ganrot, P.O. (1972): "Crossed Immunoelectrophoresis". Scandinavian Journal of Clinical and Laboratory

Investigation, 29, Supplement 124, 39-47.

Garby, L. (1973): "Iron deficiency". definition and prevalence". Clinical Haematology, 2, 245-250.

Garrey, M.M., Govan, A.D.T., Hodge, C.H., Callander, R. (1971): in "Obstetrics Illustrated". Published by E. and S. Livingstone, Edinburgh and London. p14.

Gau, G., Chard, T. (1976): "The Distribution of Placental Lactogen in the Human Term Placenta". British Journal of Obstetrics and Gynaecology, 83, 790-793.

Glasser, S.R., Wright, C., Heyssel, R.M., (1968): "Transfer of Iron Across the Placenta and Fetal Membranes in the Rat". American Journal of Physiology, 215, 205-210.

Gerlach, E., Duhn, J. (1972): "Regulation of the Concentration of 2:3-diphosphoglycerate in the Erythrocyte". Abstracts of the 8th International Congress on Clinical Chemistry, 5, 4.

Giovaniello (1967): in "Automation in Analytical Chemistry: Technicon International Symposium". Published by Technicon, New York, p 185-188.

Gitlin, D., Kumate, J., Urrusti, J., Morales, C. (1964): "The Selectivity of the Human Placenta in the Transfer of Plasma Proteins from Mother to Fetus". Journal of Clinical Investigation, 43, 1938-1951.

Göltner, E.C., Stark, G. (1960): quoted by Wöhler, F. (1964): in "Intermediary Iron Metabolism of the Placenta". Current Therapeutic Research, 6, 473.

Govan, A.D.T. (1978): Personal Communication.

Granick, S. (1942): "Ferritin". Journal of Biological Chemistry, 146, 451-461.

Grannick, S. (1946): "Ferritin: Its Properties and Significance for Iron Metabolism". Chemical Reviews, 38, 379-403.

379-403.

Granick, S., Michaelis, L. (1943): "Apoferritin of Horse Spleen". Journal of Biological Chemistry, 147, 91-97.

Granick, S. (1948): "Protoporphyrin 9 as a Precursor of Chlorophyll". Journal of Biological Chemistry, 172, 717-727.

Granick, S. (1951): "Structure and Physiological Functions of Ferritin". Physiological Reviews, 31, 489-511.

Guest, G.M. (1948): in "Nutritional Anaemia". Published by Robert Gould Research Foundation, Cincinnati, 1, p 144.

Gurevich, A.E., Kuzovleva, O.B., Tumanova, A.E. (1961): "Production of Protein-Cellulose Complexes in the Form of Suspensions Able to Bind Great Amounts of Antibodies". Biokhimiya, 26, 803-809.

Gurevich, A.E. (1964): in "Immunological Methods". Edited by J.F. Ackroyd. Published by Blackwell Scientific Publications, Oxford. p 113-136.

Hahn, P.F., Bale, W.F., Rose, J.F., Balfour, W.M., Whipple, G. (1943): "Radioactive Iron Absorption by Gastro-intestinal Tract". Journal of Experimental Medicine, 78, 169-188.

Hahn, P.F., Carothers, E.L., Darby, W.J., Sheppard, C.W., Cannon, R.D., Bean, A.S., Densen P.M., Peterson, J.C., McClellan, G.S. (1951): "Iron Metabolism in Human Pregnancy as Studied with ^{59}Fe". American Journal of Obstetrics and Gynecology, 61, 477-490.

Hall, M.H. (1974): "Pregnancy Anaemia". British Medical Journal, 2, 661-663.

Halliday, J.W., Gera, K.L., Powell, L.W. (1975): "Solid Phase Radioimmunoassay for Serum Ferritin". Clinica Chimica Acta, 58, 207-214.

Hamilton, H.A., Wright, H.P. (1942): "Development of Hypochromic Anaemia During Pregnancy". Lancet, 2, 184-186.

Hancock, K.W., Walker, P.A., Harper, T.A. (1968): "Mobilisation of Iron in Pregnancy". Lancet, 2, 1055-1058.

Harrison, P.M. (1959): "The Structures of Ferritin and Apoferritin". Journal of Molecular Biology, 1, 69-80.

Harrison, P.M. (1969): in "Iron Metabolism and Anaemia". Pan American Health Organization Scientific Publications. p 2-20.

Harrison, P.M., Hoy, T.G., Macara, I.G., Hoare, R.J. (1974):a, "Ferritin Iron Uptake and Release". Biochemical Journal, 143, 445-451.

Harrison, P.M., Hoare, R.J., Hoy, T.G., Macara, I.G. (1974):b, in "Iron in Biochemistry and Medicine". Edited by A. Jacobs and M. Worwood. Published by Academic Press, London. p 73-109.

Hendrick, J.C., Franchimont, P. (1976): "RIA with Labelled Antibodies: Sandwich Method". in Proceedings of the Protides of Biological Fluids. Edited by H. Peters. Published by Pergammon, Oxford and Paris, 24, 735-737.

Henry, R.J. (1964): in "Clinical Chemistry, Principles and Technics". Edited by R.J. Henry. Published by Harper and Row. p 124.

Holly, R.G., Grund, W.J. (1959): "Ferrodynamics During Pregnancy". American Journal of Obstetrics and Gynecology, 77, 731-742.

Hoppe, J.O., Marcelli, G.M.A., Tainter, M.L. (1955): "A Review of the Toxicity of Iron Compounds". American

Journal of Medical Science, 230, 558-571.

Howard, A.N., Wild, F. (1957): "The Reaction of Diazonium Compounds with Amino Acids and Proteins". Biochemical Journal, 65, 651-659.

Healy, M.J.R. (1972): "Statistical Analysis of Radioimmunoassay Data". Biochemical Journal, 130, 207-210.

Heilmeyer, L. (1958): quoted by Morgan, E.H. (1961): "Transfer of Iron from the Pregnant and Lactating Rat to Foetus and Young". Journal of Physiology, 158, 582.

Hows, J., Hussein, S., Hoffbrand, A.V., Wickramasinghe, S.N. (1977): "Red Cell Indices and Serum Ferritin Levels in Children". Journal of Clinical Pathology, 30, 181-183.

Hunter, W.M., Greenwood, F.C. (1962): "Preparation of ^{131}I Labelled Human Growth Hormone of High Specificity". Nature, 194, 495-496.

Hunter, W.M. (1974): "Preparation and Assessment of Radioactive Tracers". British Medical Bulletin, 30, 18-23.

Hurn, B.A.L., Landon, J. (1971): in "Radioimmunoassay Methods". Edited by K.E. Kirkham and W.M. Hunter. Published by Churchill and Livingstone, Edinburgh. p 21.

Hurn, B.A.L. (1974): "Practical Problems in Raising Antisera". British Medical Journal, 30, 26-28.

Hussein, S. Prieto, J., O'Shea, M., Hoffbrand, A.V., Baillod, R.A., Moorhead, J.F. (1975): "Serum Ferritin Assay and Iron Status in Chronic Renal Failure and Haemodialysis". British Medical Journal, 1, 546-548.

Hussein, S., Hoffbrand, A.V., Laulicht, M. (1976): "Serum Ferritin Levels in Beta-Thalassaemia Trait". British

Medical Journal, 2, 920.

Hussein, M.A.M., Gaafar, T.H., Laulicht, M., Hoffbrand, A.V. (1977): "Relation of Maternal and Cord Blood Serum Ferritin". Archives of Disease in Childhood, 52, 782-784.

Hytten, F.E., Paintin, D.B. (1963): "Increase in Plasma Volume During Normal Pregnancy". Journal of Obstetrics and Gynaecology of the British Commonwealth, 70, 402-407.

Hytten, F.E., Cheyne, G.A., Klopper, A.I. (1964): "Iron Loss at Menstruation". Journal of Obstetrics and Gynaecology of the British Commonwealth, 71, 255-259.

Hytten, F.E., Duncan, D.L. (1970): "Iron Deficiency Anaemia in the Pregnant Woman and its Relation to Normal Physiological Changes". Nutrition Abstracts and Reviews, 26, 855-869.

Hytten, F.E., Leitch, I. (1971): in "The Physiology of Human Pregnancy" - 2nd Edition. Published by Blackwell Scientific Publications, Oxford and Edinburgh. p 42.

Jacobs, A., Butler, E.B. (1965): "Menstrual Blood Loss in Iron - Deficiency Anaemia". Lancet, 2, 407-409.

Jacobs, A., Miller, F., Worwood, M., Beamish, M.R., Wardrop, C.A. (1972): "Ferritin in the Serum of Normal Subjects and Patients with Iron Deficiency and Iron Overload". British Medical Journal, 4, 206-208.

Jacobs, A. (1974): in "Iron in Biochemistry and Medicine". Edited by A. Jacobs and M. Worwood. Published by Academic Press, London. p 405-432.

Jacobs, A., Worwood, M. (1975): "Ferritin in Serum". The New England Journal of Medicine, 292, 951-956.

Jacobs, A. (1977): "Low Molecular Weight Intracellular Iron

Transport Compounds". Blood, 50, 433-439.

Jepson, J.H., Friesen, H.G. (1968): The Mechanism of Action of Human Placental Lactogen on Erythropoiesis". British Journal of Haematology, 15, 465-471.

Jepson, J.H. (1968): "Endocrine Control of Maternal and Fetal Erythropoiesis". Canadian Medical Association Journal, 98, 844-847.

Jones, P.A.E., Miller, F.M., Worwood, M. (1973): "Ferritinaemia in Leukaemia and Hodgkins Disease". British Journal of Cancer, 27, 212-217.

Jones, B.M., Worwood, M. (1975): "An Automated Immunoassay for Ferritin". Journal of Clinical Pathology, 28, 540-542.

Josephs, H.W. (1959): "The Iron of the Newborn Baby". Acta Paediatrica, 48, 403-418.

Joustra, M. (1976): "Current Trends in Solid Phase RIA". in Protides of Biological Fluids. Edited by H. Peters. Published by Pergammon Press, Oxford and Paris, 24, 711-714.

Kabat, E.A. (1961): in "Experimental Immunochemistry". Edited by E.A. Kabat and M.M. Mayer. Published by Springfield, Illinois. p 26.

Kaufman, N., Wyllie, J.C. (1972): "A Study of Storage Iron in the Placental and Foetal Tissues of the Rat". British Journal of Haematology, 22, 287-297.

Kelly, A.M., MacDonald, D.J., McNay, M.B. (1977): "Ferritin as an Assessment of Iron Stores in Normal Pregnancy". British Journal of Obstetrics and Gynaecology, 84, 434-438.

Kelly, A.M., MacDonald, D.J., McDougall, A.N. (1978):

"Observations on Maternal and Fetal Ferritin Concentrations at Term". British Journal of Obstetrics and Gynaecology, 85, 338-343.

Kelly, A.M. (1978): "The influence of Maternal Iron Stores and Placental Function on Cord Ferritin". Abstracts of the Xth International Congress of Clinical Chemistry. No.157, p 96.

Kelly, A.M., McEwan, H.P., (1978): "Renal Tubular Function in Toxaemia of Pregnancy". Proceedings of the 1st Congress of the International Society for the Study of Hypertension in Pregnancy - In Preparation.

Kessel, I., Sills, D.J. (1968): "Neonatal and Maternal Serum Iron Levels at Birth". Journal of Obstetrics and Gynaecology of the British Commonwealth, 75, 752-753.

Kilpatric, G.S., Hardistry, R.M. (1961): "The Prevalence of Anaemia in the Community". British Medical Journal, 1, 778-782.

Klein, L. (1962): "Premature Birth and Maternal Prenatal Anaemia". American Journal of Obstetrics and Gynecology, 83, 588-590.

Kuhn, R., Sörensen, N.A., Birkofer, L. (1940): "Structural Plan of Ferritin: Iron Protides of the Spleen". Berichte der deutschen chemischen Gesellschaft, 73B, 823-837.

Lancet Leader (1941): "The Green Sickness". Lancet, 1, 345-355.

Lanzkowsky, P. (1961): "The influence of Maternal Iron-Deficiency Anaemia on the Haemoglobin of the Infant". Archives of Disease in Childhood, 36, 205-209.

Lapicque, J., Guillemonat, A.L. (1889): quoted by Bruckmann, G., Zondek, S.G. (1939): "Iron, Copper and Manganese in Human Organs at Various Ages". Biochemical Journal, 33, 1845-1857.

Latham, E.F., Vosburgh, G.J. (1950): Unpublished. Quoted by Vosburgh, G.J., Flexner, L.B. (1950): "Maternal Plasma as a Source of Iron for the Fetal Guinea Pig". Americam Journal of Physiology, 161, 202-211.

Laufberger, M.V. (1937): "Sur La Cristallisation de La Ferritin". Bulletin de La Societe de Chimie Biologique, 19, 1575-1582.

19, 1575-1582.

Laurell, C.B., Morgan, E. (1964): "Iron Exchange Between Transferrin and the Placenta in the Rat". Acta Physiologica Scandinavica, 62, 271-279.

Laurell, C.B. (1966): "Quantitative Estimation of Proteins by Electrophoresis in Agarose Gel Containing Antibodies". Analytical Biochemistry, 15, 45-52.

de Leeuw, N.K.M., Lowenstein, L., Hsieh, Y.S. (1966): "Iron Deficiency and Hydraemia in Normal Pregnancy". Medicine, 45, 291-315.

Letsky, E.A., Miller, F., Worwood, M., Flynn, D.M. (1974): "Serum Ferritin in Children with Thalassaemia Regularly Transfused". Journal of Clinical Pathology, 27, 652-655.

Lind, T. (1975): "The Assessment of Normal Pregnancy". British Journal of Hospital Medicine, 14, 253-258.

Linder, M.C., Munro, H.N. (1972): "Assay of Tissue Ferritin". Analytical Biochemistry, 48, 266-278.

Linder, M.C., Munro, H.N. (1977): "The Mechanism of Iron Absorption and its Regulation". Federation Proceedings, 36, 2017-2023.

Lipschitz, D.A., Cook, J.D., Finch, C.A. (1974): "A Clinical Evaluation of Serum Ferritin as an Index of Iron Stores". New England Journal Medicine, 290, 1213-1216.

Loria, A., Aymore, V.P., Arroyo, P. (1977): "Fetal Hepatic Storage of Metabolites in the Second Half of Pregnancy". The Journal of Pediatrics, 91, 569-573.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951): "Protein Measurement with the Folin Phenol Reagent". Journal of Biological Chemistry, 193, 265-275.

Lowry, O.H., Passonneau, J.V., Hasselberger, F.X., Schultz,

D.W. (1964): "Effect on Ischaemia on Known Substrates and Cofactors of the Glycolytic Pathway in Brain". Journal of Biological Chemistry, 239, 18-42.

Lubin, B. (1978): "Neonatal Anaemia Secondary to Blood Loss". Clinics in Haematology, 7, 19-34.

Lundström, U., Siimes, M.A., Dallman, P.R. (1977): "At what Age Does Iron Supplementation become Necessary in Low-birth-weight Infants". Journal of Pediatrics, 91, 878-883.

Lynch, S.R. (1974): in "Iron in Biochemistry and Medicine". Edited by A. Jacobs and M. Worwood. Published by Academic Press, London and New York. p 578.

Macara, I.G., Hoy, T.G., Harrison, P.M. (1973): "The Formation of Ferritin from Apoferritin". Biochemical Journal, 135, 343-348; 785-789.

Manasc, B., Jepson, J. (1969): "Erythropoietin in Plasma and Urine during Human Pregnancy". Canadian Medical Association Journal, 100, 687-691.

Mancini, G., Vaerman, J.P., Carbonara, A.G., Heremans, J.F. (1963): in "Proceedings of the Protides of Biological Fluids". Edited by H. Peters. Published by Pergammon, Oxford and Paris, 11, 370-376.

Matioli, G.T., Baker, R.F. (1963): "Denaturation of Ferritin and its Relationship with Haemosiderin". Journal of Ultrastructure Research, 8, 477-490.

Maximow, A.A., Bloom, W. (1958): in "A Textbook of Histology". Published by W.B. Saunders and Co., Philadelphia and London. p 524.

Mazur, A., Shorr, E. (1950): "A Quantitative Immunochemical Study of Ferritin and its Relation to the Hepatic

Vasodepressor Material". Journal of Biological Chemistry, 182, 607-627.

Mazur, A., Baez, S., Shorr, E. (1955): "The Mechanism of Iron Release from Ferritin as Related to its Biological Properties". Journal of Biological Chemistry, 213, 147-160.

Mentzer, W.C. (1978): "Polycythaemia and the Hyperviscosity Syndrome in Newborn Infants". Clinics in Haematology, 7, 63-74.

Miles, L.E.M., Hales, C.N. (1968): "Labelled Antibodies and Immunological Assay Systems". Nature, 219, 186-189.

Miles, L.E.M., Lipschitz, D.A., Bieber, C.P., Cook. J.D. (1974): "Measurement of Serum Ferritin by a 2-Site Immunoradiometric Assay". Analytical Biochemistry, 61, 209-220.

Miles, L.E.M. (1976): in "Proceedings of Protides and Biological Fluids". Edited by H. Peters. Published by Pergammon Press, Oxford and Paris, 24, 695.

Moe, P.J. (1963): "Iron Requirements in Infancy". Acta Paediatrica, Supplement 150.

Mollison, P.L., Veall, N., Cutbush, M.(1950): "Red Cell and Plasma Volume in Newborn Infants". Archives of Disease in Childhood, 25, 242-253.

Morgan, E.H. (1961): "Transfer of Iron from the Pregnant and Lactating Rat to the Foetus and Young". Journal of Physiology, 158, 573-586.

Morgan, E.H. (1964): "Passage of Transferrin, Albumin and Gamma Globulin from Maternal Plasma to Foetus in the Rat and Rabbit". Journal of Physiology, 171, 26-41.

Morgan, E.H. (1974): in "Iron in Biochemistry and Medicine".

Edited by A.Jacobs and M.Worwood. Published by Academic Press, London. p29-71.

Munro, H.N., Linder, M.C. (1978): "Ferritin Structure, Biosynthesis and Function". Physiological Reviews, 58, 317-396.

McCallum, A.B. (1894): "On the Absorption of Iron in the Animal Body". Journal of Physiology, 16, 268-280.

MacDonald, R.C., MacDonald, H.N. (1977): "Erthrocyte 2,3-Diphosphoglycerate and Associated Haematological Parameters During the Menstrual Cycle". British Journal of Obstetrics and Gynaecology, 84, 427-433.

McGowan, J.P. (1924): "Iron Deficiency and its Possible Relationship to Human Disease". Lancet, 2, 1060-1061.

MacGregor, M.W. (1963): "Maternal Anaemia as a Factor in Prematurity and Perinatal Mortality". Scottish Medical Journal, 8, 134-140.

McKay, D.G., Hertig, A.T., Adams, E.C., Richardson, M.V. (1958): "Histochemical Observations on the Human Placenta". Journal of Obstetrics and Gynaecology, 12, 1-36.

MacLennan, A.H., Emerson, P.M., Hunter, D.J.S., Darby, J.H. (1976): "Tissue Oxygenation and Red Cell 2:3-Diphosphoglycerate in Normal and Abnormal Pregnancy". British Journal of Obstetrics and Gynaecology, 83, 378-382.

Nakane, P.K. (1970) "Classifications of Anterior-Pituitary Cell Types with Immunoenzyme Histochemistry". Journal of Histochemistry and Cytochemistry, 18, 9-20.

Neville, A.M., Grigor, K., Heyderman, E. (1976): "Clinicopathological Role of Tumour Index Substances in Paediatric Neoplasia". Journal of Clinical Pathology, 29, 1026-1032.

29, 1026-1032.

Nutsu, Y., Listowsky, I. (1973): "Mechanisms for the Formation of Ferritin Oligomers". Biochemistry, 12, 4690-4695.

Nutsu, Y., Kohgo, Y., Yokata, M., Urushizaki, I. (1974): "Radioimmunoassay of Serum Ferritin in Patients with Malignancy". Annals of the New York Academy of Sciences, 259, 450-452.

Nylander, G. (1953): "On the Placental Transfer of Iron". Acta Physiologica Scandinavica, 29, Supplement 107, 1-105.

Olsson, K.S. (1972): "Iron Stores in Normal Men and Male Blood Donors as Measured by Desferrioxamine and Quantitative Phlebotomy". Acta Medica Scandinavica, 192, 401-407.

Orlic, D., Lev, R., Rosenthal, W.S. (1974): "Fetal Rat Utilization of ^{55}Fe Absorbed by Fetal Intestine from Swallowed Amniotic Fluid". Blood, 43, 429-436.

Osaki, S., Sirivech, S. (1971): "Identification and Partial Purification of a Ferritin Reducing Enzyme in Liver". Federation Proceedings, 30, 1292-1296.

Ouchterlony, O. (1964): in "Immunological Methods". Edited by J.F. Ackroyd. Published by Blackwell Scientific Publications, Oxford. p 55.

Paintin, D.B. (1962): "The Size of the Total Red Cell Volume in Pregnancy". Journal of Obstetrics and Gynaecology of the British Commonwealth, 69, 719-723.

Paintin, D.B. (1963): "The Haematocrit Ratio in Pregnancy". Journal of Obstetrics and Gynaecology of the British Commonwealth, 70, 807-810.

Paintin, D.B., Thomson, A.M., Hytten, F.E. (1966): "Iron

and Haemoglobin Level in Pregnancy". Journal of Obstetrics and Gynaecology of the British Commonwealth, 73, 181-190.

Pape, L., Multani, J.S., Stitt, C., Saltman, P. (1968): "In vitro Reconstitution of Ferritin". Biochemistry, 7, 613-616.

Parry, D.H., Norwood, M., Jacobs, A. (1975): "Serum Ferritin in Acute Leukaemia at Presentation and during Remission". British Medical Journal, 1, 245-247.

Pedersen, N.S., Axelsen, N.H., Bock, E., Nørgaard-Pedersen, B. (1976): "A Paper Disc 2-Site Immunoradiometric Assay for Ferritin". Protides of Biological Fluids, 24, 659-662.

Penders, T.J., de Rooij-Dijk, H.H., Leijnse, B. (1968): "Rapid Isolation of Ferritin by Means of Ultracentrifugation". Biochimica Biophysica Acta, 168, 588-590.

Pitkin, R.M., Kaminetzky, H.A., Newton, M., Pritchard, J.A. (1972): "Maternal Nutrition". Obstetrics and Gynecology, 40, 773-785.

Pollycove, M. (1972): in "The Metabolic Basis of Inherited Disease". - 3rd Edition. Edited by J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson. Published by McGraw-Hill, New York. p 1051.

Pommerenke, W.T., Hahn, P.F., Bale, W.F., Balfour, M.C. (1942): "Transmission of Radioactive Iron to the Human Fetus". American Journal of Physiology, 137, 164-170.

Pootrakul, P., Christensen, A., Josephson, B., Finch, C.A. (1977): "Role of Transferrin in Determining Internal Iron Distribution". Blood, 49, 957-966.

Porter, F.S. (1974): "The Effect of Iron Content on the

behaviour of human ferritin in an inhibition-type radioimmunoassay". Journal of Laboratory and Clinical Medicine, 83, 147-152.

Powell, L.W., Alpert, E., Isselbacher, K.J., Drysdale, J.W. (1975): "Human Isoferritins: Organ Specific Iron and Apoferritin Distribution". British Journal of Haematology, 30, 47-55.

Pribilla, W., Bothwell, T.H., Finch, C.A. (1958): in "Iron in Clinical Medicine". Edited by R.O. Wallerstein and S.R. Mettier. Published by Berkeley University California. p 58-64.

Pritchard, J.A., Mason, R.A. (1964): "Iron Stores of Normal Adults and Replenishment with Oral Iron Therapy". Journal of the American Medical Association, 190, 897-901.

Pritchard, J.A., Scott, D.E. (1970): in "Iron Deficiency". Edited by L. Hallberg, H.G. Harweth, A. Vannotti. Published by Academic Press, London. p 173.

Redman, C.M. (1969): "Biosynthesis of Serum Proteins and Ferritin by Free and Attached Ribosomes of Rat Liver". Journal of Biological Chemistry, 244, 4308-4311.

Reuter, A.M., Hendrick, J.C., Sulon, J., Franchimont, P. (1973): "Interference by Serum Proteins with LH Assay Using Immunosorbent". Acta Endocrologica (Kbh), 72, 235 - 242.

Reuter, A.M., Briemont, Y., Tixhon, C., Franchimont, P. (1976): in "Proceedings of the Protides of Biological Fluids". Edited by H. Peters. Published by Pergammon, Oxford and Paris, p 705.

Richter, G.W. (1964): "Electrophoretic and Serologic Properties of Ferritin". British Journal of Experimental

Pathology, 45, 88-94.

Rios, E., Lipschitz, D.A., Cook, J.D., Smith, N.J. (1975): "Relationship of Maternal Iron Stores as Assessed by Determination of Plasma Ferritin". Pediatrics, 55, 694-699.

Robins, E., Pederson, T. (1970): "Iron: Its Intracellular Localisation and Possible Role in Cell Division". Proceedings of the National Academy of Sciences, 66, 1244-1251.

Rodbard, D., Bridson, W., Rayford, P.L. (1969): "Rapid Calculation of Radioimmunoassay Results". Journal of Laboratory and Clinical Medicine, 74, 770-781

Rodbard, D., Weiss, G.H. (1973): "Mathematical Theory of Immunoradiometric Assays". Analytical Biochemistry, 52, 10-44.

Rodbard, D. (1974): "Statistical Quality Control and Routine Data Processing for Radioimmunoassays and Immunoradiometric Assays". Clinical Chemistry, 20, 1255-1270.

Rörth, M., Bille-Brahe, N.E. (1971): "2:3-Diphosphoglycerate and Creatine in the Red Cell during Human Pregnancy". Scandinavian Journal of Clinical Laboratory Investigation, 28, 271-276.

Roszkowski, I., Wojcicka, J., Zaleska, K. (1966): "Serum Iron Deficiency during the Third Trimester of Pregnancy; Maternal Complications and Fate of Neonate". Obstetrics and Gynecology, 28, 820-825.

Schmiedberg, O. (1894): "Ueber das Ferratin und seine diätetische und therapeutische". Achive Experimenti Pathologie, 33, 101-116.

Scholten, R., Veit, J. (1903): Zeitschrift fur Geburtshilfe

und Gynäkologie, 49, 210-232.

Schulman, I., Smith, C.H. (1954): "Studies on Anaemia of Prematurity". American Journal of Disease of Children, 88, 567-595.

Schulman, I., (1961): "Iron Requirements in Infancy". Journal of American Medical Association, 175, 118-123.

Scott, D.E., Pritchard, J.A. (1967): "Iron Deficiency in Healthy Young College Women". Journal of the American Medical Association, 199, 897-900.

Scott, J.M., Govan, A.D.T. (1949): "Anaemia of Pregnancy in Glasgow and District". British Medical Journal, 2, 1083-1085.

Scott, J.M. (1962): "Anaemia in Pregnancy". Post Graduate Medical Journal, 38, 202-213.

Scott, J.M., Goldie, H., Hay, S.H. (1975): "Anaemia in Pregnancy: The Changing Postwar Pattern". British Medical Journal, 1, 259-161.

Scott, J.M. (1978): Personal communication.

Seal, U.S., Sinha, A.A., Doe, R.P. (1972): "Placental Iron Transfer". Relationship to Phylogeny of the Mammals". American Journal of Anatomy, 134, 263-269.

Seeds, A.E., Battaglia, F.C., Hellegers, A.E. (1964): "The effects of Pregnancy on pH, pCO_2 and Bicarbonate Concentrations of Peripheral Venous Blood". American Journal of Obstetrics and Gynecology, 88, 1086-1089.

Seip, M., Halvorsen, S. (1956): "Erythrocyte Production and Iron Stores in Premature Infants during the First Months of Life". Acta Paediatrica (Stockholm), 45, 600-617.

Shepp, M., Toff, H., Yamada, H., Gabuzda, T.G. (1972): "Heterogenous Metabolism of Marrow Ferritins during Erthroid Cell Maturation". British Journal of Haematology, 22, 377-382.

Sherlock, S. (1968) in "Diseases of the Liver and Biliary

System" - 4th Edition. Published by Blackwell Scientific Publications, Oxford and Edinburgh. p 548.

Shorr, E. (1954-55): "Intermediary Metabolism and Biological Activities of Ferritin". Harvey Lectures. Published by Academic Press, New York. p 112-153.

Shott, R.J., Andrews, B.F. (1972): "Iron Status of a Medical High-Risk Population at Delivery". American Journal of Disease in Childhood, 124, 369-371.

Sigma Technical Bulletin (1974): "2,3-Diphosphoglyceric Acid". 35-UV (12-74), 1-12.

Siimes, M.A., Addiego, J.E., Dallman, P.R. (1974): Ferritin in Serum: "Diagnosis of Iron Deficiency and Iron Overload in Infants and Children". Blood, 43, 581-590.

Siimes, M.A., Koerper, M.A., Licko, V. (1975): "Ferritin Turnover in Plasma: An Opportunistic Use of Blood Removed During Exchange Transfusion". Pediatric Research, 9, 127-129.

Sisson, T.R.C., Lund, C.J. (1958): "The Influence of Maternal Iron Deficiency in the Newborn". American Journal of Clinical Nutrition, 6, 376-385.

Sjöstedt, J.E., Manner, P., Nummi, S., Ekenved, G. (1977): "Oral Iron Prophylaxis during Pregnancy". Acta Obstetricia et Gynecologica Scandinavica, Supplement 60, 1-9.

Smith, J.A., Drysdale, J.M., Goldberg, A., Munro, H.N. (1968): "The Effect of Enteral and Parenteral Iron on Ferritin Synthesis in the Intestinal Mucosa of the Rat". British Journal of Haematology, 14, 79-86.

Smith, N.J., Rosello, S., Say, M.B., Yeys, K. (1955): "Iron Storage in the First Five Years of Life". Pediatrics, 16,

166-173.

Snedecor, G.W. (1952): "Queries". Biometrics, 8, 85-88.

Stevens, A.R., Coleman, D.H., Finch, C.A. (1953): "Iron Metabolism; Clinical Evaluation of Iron Stores". Annals of Internal Medicine, 38, 199-205.

Stockman, J.A., Oski, F.A. (1978): "Physiological Anaemia of Infancy and the Anaemia of Prematurity". Clinic in Haematology, 7, 3-18.

Stookey, L.L. (1970): "Ferrozine - A New Spectrophotometric Reagent for Iron". Analytical Chemistry, 42, 779-781.

Strauss, M.B. (1933): "Anaemia of Infancy from Maternal Iron Deficiency in Pregnancy". Journal of Clinical Investigation, 12, 345-353.

Sturgeon, P. (1954): "Studies of Iron Requirements in Infants and Children - Normal Values for Serum Iron; Copper and Free Erythrocyte Protoporphyrin". Pediatrics 13, 107-204.

Sussman, M. (1974): in "Iron in Biochemistry and Medicine". Edited by A.Jacobs and M.Worwood. Published by Academic Press, London and New York. p 669.

Svanberg, B. (1975): "Absorption of Iron in Pregnancy". Acta Obstetrica et Gynecologica Scandinavica, Supplement 48, 7-108.

Taylor, D.J., Lind, T. (1976): "Haematological Changes During Normal Pregnancy": Iron Induced Macrocytosis". British Journal of Obstetrics and Gynaecology, 83, 760-767.

Tecce, di G., Tecce, T. (1950): "Pieprefenza di Ferritina Nella Placenta Umana a Termine di Gravidanza". Quaderni nutriz, 11, 42-48.

Thorell, J.I., Johansson, B.G. (1971): "Enzymatic Iodination of Polypeptides with ^{125}I to High Specific Activity". Biochimia Biophysica Acta, 251, 363-369.

Thornburg, K.L., Faber, J.J. (1976): "The Steady State Cocentration Gradients of an Electron-Dense Marker (Ferritin"). Journal of Clinical Investigation, 58, 912-925.

Torrance, J., Jacobs, P., Restrepo, A., Eschbach, J., Lenfant, C., Finch, C.A. (1970): "Intraerythrocytic Adaptation to Anaemia". New England Journal of Medicine, 283, 165-169.

Turnbull, A. (1974): in "Iron in Biochemistry and Medicine". Edited by A.Jacobs and M.Worwood. Published by Academic Press, London. p 369.

Vaitukaitis, J., Robbins, R., Nieschlag, E., Ross, G.T. (1971): " A method for Producing Specific Antisera with Small Doses of Immunogen". Journal of Clinical Endocrinology and Metabolism, 33, 988-991.

Vosburgh, G.J., Flexner, L.B. (1950): "Maternal Plasma as a Source of Iron for the Fetal Guinea Pig". American Journal of Physiology, 161, 202-211.

Walker, J., Turnbull, E.P.N. (1953): "Haemoglobin and Red Cells in the Human Foetus". Lancet, ii, 312-318.

Walters, G.O., Miller, F.M., Worwood, M. (1973): "Serum Ferritin Concentration and Iron Stores in Normal Subjects". Journal of Clinical Pathology, 26, 770-772.

Wang, D.Y. (1976): "Plasma CEA and Ferritin as Prognostic Markers in Human Breast Cancer" in Protides of Biological Fluids. Edited by H.Peters. Published by Pergammon Press, Oxford and Paris, 24, 481-483.

Weinfeld, A. (1964): "Storage Iron in Man". Acta Medica Scandinavica, 177, Supplement 427.

Weir, R.J., Paintin, D.B., Brown, J.J., Fraser, R., Lever, A.F., Robertson, J.I.S., Young, J. (1971): "S Serial Study in Pregnancy of the Plasma Concentrations of Renin, Corticosteroids, Electrolytes, Proteins and of Haematocrit

and Plasma Volume". Journal of Obstetrics and Gynaecology of the British Commonwealth, 78, 590-602.

Weiss, R.R., Roginsky, M.S., Mann, L.I., Melber, A., Bachoric, J., Tejani, N., Bhakthavathsalan, A., Evans, M.I. (1976): "Erythrocyte 2,3-diphosphoglycerate in Normal and Hypertensive Gravid Women and their Newborn Infants". American Journal of Obstetrics and Gynecology, 124, 692-695.

White, C., Cook, F., Gilliatt, W. (1952): in "Midwifery". Published by Edward Arnold & Co., London. p 15.

Willcocks, F. (1881): "On Some Points in the Pathology of Anaemia and on the Action of Iron and Arsenic". Lancet, 2, 94.

Woodhead, J.S., Addison, G.M., Hales, C.N. (1974): "The Immunoradiometric Assay and Related Techniques". British Medical Bulletin, 30, 44-54.

Wöhler, F. (1955): quoted by Wöhler, F. (1964): in "Intermediary Iron Metabolism of the Placenta". Current Therapeutic Research, 6, 464.

Wöhler, F. (1964): "Intermediary Iron Metabolism of the Placenta with Special Consideration of the Transport of Therapeutically Administered Iron through this Organ". Current Therapeutic Research, 6, 464-48.

Wong, C.T., Morgan, E.H. (1973): "Placental Transfer of Iron in the Guinea Pig". Quarterly Journal of Experimental Physiology, 58, 47-58.

World Health Organisation (1968): "Nutritional Anaemias". WHO Technical Report, No. 405, 14-37.

Worwood, M., Jacobs, A. (1972): "The Subcellular Distribution of ^{59}Fe in Small Intestinal Mucosa". British

Journal of Haematology, 20, 321-327.
Wyllie, J.C., Kaufman, N. (1971): " A study of Storage Iron in the Pregnant Rat". British Journal of Haematology, 20, 321-327.

Yallow, R.S., Berson, S.A. (1960): Immunoassay of Endogenous Plasma Insulin in Man". Journal of Clinical Investigation, 39, 1157-1175.
Yamada, H., Gabazuda, T.G. (1974): "Erthroblast Ferritin". Journal of Laboratory and Clinical Medicine, 83, 478-488.
Zilva, J.F., Pannall, P.R. (1975): in "Clinical Chemistry in Diagnosis and Treatment" - 2nd Edition. Published by Lloyd-Luke, London. p 389.