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THE ROLE OF FERRITIN IN IRON ABSORPTION.

By

J.D. TORRANCE, M.5c. (Rand)

A Thesis presented for the
degree of Doctor of Philosophy in the
University of the Witwatersrand.

Johannesburg,
January, 1967.

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This is to certify that the thesis "The Role of Ferritin in Iron Absorption" presented for the degree of Doctor of Philosophy at the University of the Witwatersrand is my own work and has not been presented at any other University.

J.D. TORRANCE.
M.Sc. (Rand).

TO

MY WIFE AND PARENTS

ACKNOWLEDGEMENTS

It is with pleasure that I record my thanks to Dr. T.H. Bothwell for his continued help and encouragement during the preparation of this thesis. It was he who introduced me to the study of iron metabolism and I am much indebted to him for the practical advice and constructive criticism he has been able to give me on account of his wide knowledge and experience in this field. This investigation was made possible by grants to Dr. Bothwell from the National Institutes of Health, Bethesda, Md. (AM 04912-03), The Council for Scientific and Industrial Research, South Africa, and the Atomic Energy Board, South Africa.

My thanks are due to Prof. P. Levy for the interest he has shown in my work and the help he has given me.

To Prof. R.M. Charlton I owe sincere thanks for his invaluable advice throughout the investigation and in the preparation of this thesis.

I wish to thank my colleagues, Dr. R. Green, Dr. P. Jacobs, Dr. S.R. Lynch and Dr. S.S. Zail for many stimulating discussions and the members of the Department of Medicine Photographic Unit for their painstaking work in preparing the diagrams.

My grateful thanks go to my mother for her care and accuracy in typing the manuscript.

Finally, I should like to thank Prof. G.A. Elliott, in whose department this work was carried out, for his encouragement.

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CHAPTER I.

INTRODUCTORY REVIEW

OF

IRON METABOLISM.

CHAPTER I.

I. Introduction.

Although reports of the medicinal use of iron date back to ancient times it was not until the present century that the many functions of iron in the body were studied. Once started, the investigation received impetus from the seriousness of iron deficiency anaemia, a major cause of ill health throughout the world. The introduction of radio-isotope tracer techniques in 1939 greatly facilitated investigation of absorption, excretion and the metabolic pathways of iron. The tremendous amount of work already carried out has led to a fairly comprehensive knowledge of the various aspects of iron metabolism. Nevertheless, there remain wide gaps in the overall picture. In particular little is known of either the actual process of iron absorption from the small intestine or of the mechanism by which absorption changes to meet the body's iron requirement. Investigation of these two aspects of iron metabolism and more particularly of the role played in absorption by the iron storage protein ferritin form the basis of this dissertation.

Since it is important to consider absorption as an integral part of the overall metabolism of iron, a brief outline of the general principles of iron metabolism is necessary. Although all the experimental work in this study was carried out on rats, a large proportion of our present knowledge of iron metabolism relates to humans.

For this reason the outline given here deals necessarily with human metabolism. It seems likely, however, that metabolism in the rat will be very similar; indeed the reason for animal studies is to advance the understanding of human metabolism.

II. Nature of iron-containing compounds in the body.

The body of a normal adult male contains between 3 and 4 grams of iron. Little, if any, of this is present in an inorganic or ionised form. The solubility of ferric iron is so low at the pH of the body fluids (solubility product = 10^{-36}) that it is necessary for this element to be chelated during transportation and metabolism. The iron which is toxic in its free form no longer displays toxicity in its various physiologically bound forms. In these compounds iron is present either in a porphyrin ring as haem, or is associated directly with a protein. Some of these substances are described below.

A. Haemoproteins.

The haemoproteins include haemoglobin, myoglobin, the cytochromes and various iron containing enzymes. These compounds have a variety of functions ranging from oxygen transport in the case of haemoglobin to electron transfer by the cytochromes and cleavage of peroxide by catalase and peroxidase.

In these compounds four of the six co-ordination positions of the iron atom are occupied by the nitrogen

atoms of a porphyrin ring. This is shown by haem (Figure 1) which is the iron protoporphyrin complex. The two remaining positions are directed on a line perpendicular to the plane of the porphyrin ring (Figure 2). These positions may be filled either by two donor groups of a protein or by one such group and a small molecule.

1. Haemoglobin.

Three-quarters of the iron in the body is present in the form of haemoglobin. It is hardly surprising that this protein attracted interest at an early stage since its oxygenated form is so markedly coloured.

Indeed as early as 1843 Liebig postulated that combustion in the animal body was related to the presence of iron and took place in the blood, where haemoglobin oxidised biological substrates. Crystalline haemoglobin was prepared in 1867 by Hoppe-Seyler, this being the first protein to be obtained in a crystalline form.

Haemoglobin is a conjugated protein of molecular weight about 67,000. It consists of a protein, globin, which accounts for 96% of the molecule, and four haem groups which make up the remaining 4%. It is possible to separate the globin from the haem. Because of this the two moieties have been investigated separately. The accent was initially placed on the elucidation of the haem structure since this was more

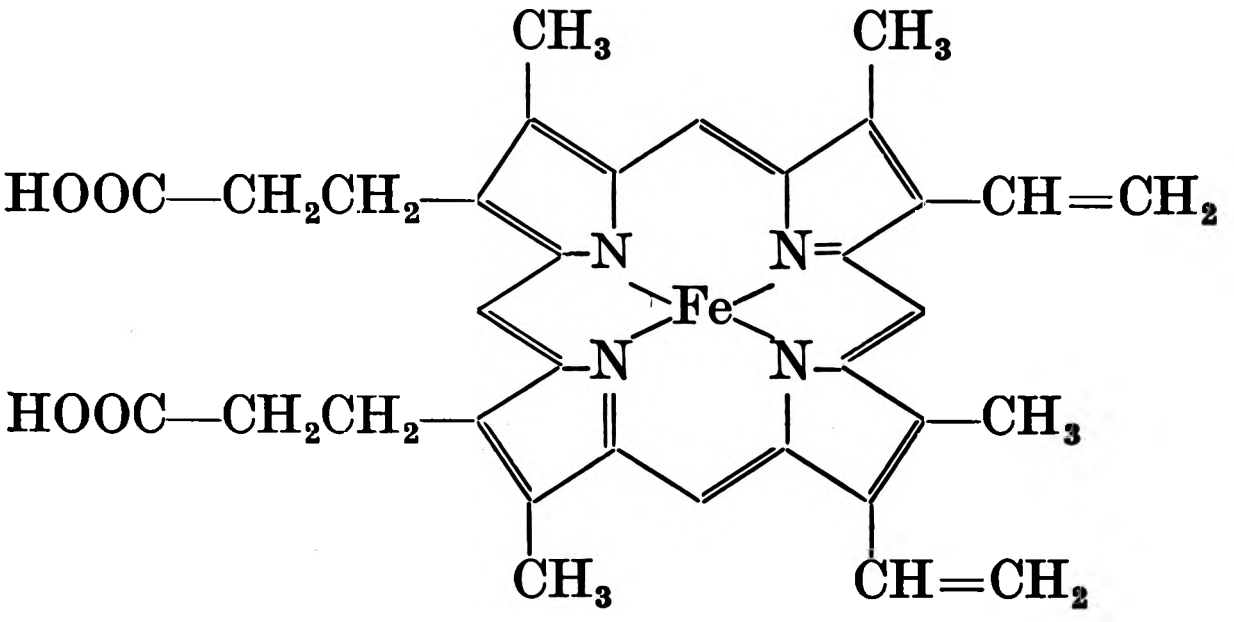


Figure 1.

The structure of haem.

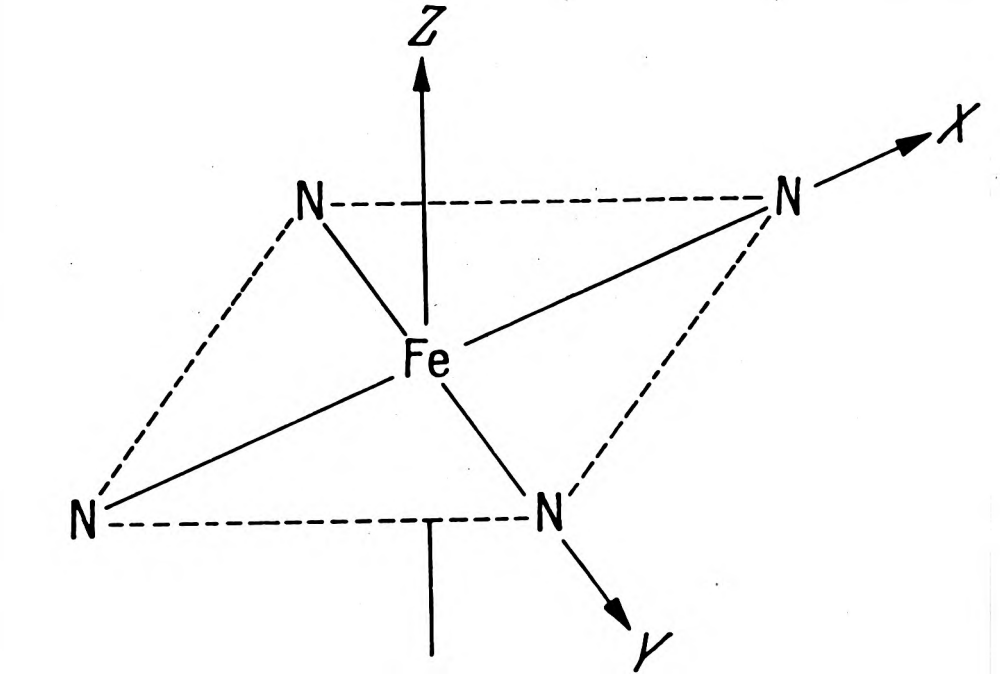


Figure 2.

The stereochemistry of iron

amenable to attack by classical organic chemical techniques.

Investigations into the structure of haem were closely associated with the study of the plant pigment chlorophyll, since removal of the iron atom from haem and the magnesium atom from chlorophyll yielded the same product, porphyrin. Several workers were engaged in exhaustive studies extending over a period of forty years before the structure of haem was finally elucidated. Two of these investigators, Willstäter and H. Fischer, received Nobel prizes for their contributions. Further advances into the understanding of haem biochemistry occurred in 1945 when Shemin and Rittenberg investigated the biosynthesis of haem using labelled glycine. Due largely to their work, the origin of all the atoms in the haem moiety and the various intermediates in its biosynthesis from glycine and succinyl coenzyme A are now known. This work was closely associated with the investigation of the porphyrias, a group of metabolic disorders in which excessive amounts of porphyrins or abnormal porphyrins are excreted.

The haem moiety of the various haemproteins in the body is synthesised by a single pathway. Although several haemoglobins, differing in crystal form, solubility, and affinity for oxygen, have been identified, the haem moiety is always the same, the variations being due to small differences in the

globin moiety. The globin of human adult haemoglobin is composed of two pairs of polypeptide chains which have been called α and β respectively. In the fetal haemoglobin the second pair of chains differs from the β chain and is designated γ. Almost the entire amino acid sequence of the α and β chains has been elucidated, as well as a large proportion of the γ chain. Each polypeptide chain consists of about 140 amino acid residues.

Another Nobel prize was earned in the field of haemoglobin chemistry by Perutz who, together with his co-workers, used X-ray diffraction techniques to obtain some idea of the shape and dimensions of the haemoglobin molecule. This work showed that the molecule has dimensions of 64 x 36 x 48 A^{o} and probably consists of four layers of peptide chains, each layer being 9 A^{o} thick and composed of four folded peptide chains. The four haem groups lie parallel to each other on the globin surface. The various chains are held together by non covalent forces to form a three-dimensional globular protein.

The haem group is attached to the globin molecule at three places - by a co-ordination bond and two bonds on to the protoporphyrin itself. The latter bonds are probably between the two propionic acid residues and two basic groups in the globin molecule.

2. Myoglobin.

The haemoprotein myoglobin, which is structurally and functionally related to haemoglobin, occurs in striated muscle. It differs from haemoglobin in having a molecular weight of only 16,500. Due to the existence of only one haem group in the molecule and the consequent absence of haem-haem interaction, myoglobin has a greater affinity for oxygen than haemglobin, particularly at low oxygen pressures. Because of this it is able to accept oxygen from the circulating haemoglobin. In this way it serves as an oxygen reservoir in the muscle. It is the presence of this protein in muscle which gives it its brown colour. Whales which are able to remain submerged for some time have so high a concentration of myoglobin that their muscle appears black.

3. Haem enzymes.

(a) Catalase and peroxidase.

Catalase and peroxidase are haem enzymes which liberate oxygen from peroxides. In both these enzymes the iron remains permanently in the ferric form. Catalase from beef liver has a molecular weight of 250,000 and contains 0.09% iron. Catalase has four iron atoms per molecule, whilst peroxidase has only one. Catalase specifically decomposes hydrogen peroxide, but it may also function as a peroxidant for larger molecules, especially various phenols.

(b) Cytochromes.

The cytochromes are haem enzymes occurring in the mitochondria of cells. The iron in these enzymes undergoes reversible oxidation enabling them to act as an electron transfer system.

Although these enzymes are obviously a most important facet of iron metabolism, the amount of iron involved in their metabolism is small. For this reason they are not important in the present investigation.

B. Non-haem iron compounds.

In contrast to the haem proteins which are chiefly concerned with oxygen transport and oxidative metabolism, the non-haem iron proteins are concerned chiefly with iron transport and storage.

1. Transferrin.

Iron is carried round the body in the plasma firmly bound to a specific iron binding protein, transferrin, which constitutes 3% of the plasma proteins. Although the presence of non-haem iron in plasma was described in 1925 by Fontes and Thiovolle (1925), it was not until 1952 that transferrin was obtained in a pure crystalline form by Koechlin (1952). This glycoprotein, which has a molecular weight of about 83,000, has also been called siderophilin, iron binding protein and β-metal-combining globulin.

Until 1957 transferrin was assumed to be a single molecular species; with the advent of starch-gel electrophoresis in that year, however, two bands of

transferrin were obtained (Smithies, 1957). Subsequently, further genetically controlled variants were found so that by 1964 fifteen distinct species of transferrin had been demonstrated (Bearn and Parker, 1964). No fundamental physiological differences between normal and mutant transferrins have been demonstrated and they are all antigenically related. Under physiological conditions each molecule of the protein is able to bind two atoms of iron by means of an essentially ionic bond. In this process the transferrin, which is initially colourless, develops a salmon-pink colour (absorption maximum 470-480 mμ). The bound iron is present in the ferric form and there is conflicting evidence as to the nature of the groups involved in the complex. Some workers (Fiala and Burk, 1949; Schade et al, 1949) consider that two phenolic groups of the protein and a bicarbonate ion are involved, whilst Hazen (1963) suggests that tyrosine and histidine are the sites on the transferrin molecule to which iron is bound. The tenacity of the binding at physiological pH is greater even than that of synthetic chelates. Davis et al. (1962) have determined the formation constants for the two iron atoms as 10^{30} and $10^{27.7}$. If these figures are correct it is apparent that only negligible quantities of free iron (5.6×10^{-26} μg./100 ml.) could exist in the plasma. The iron transferrin complex can be

completely and reversibly dissociated by lowering the pH below 5 (Surgenor et al., 1949).
Although it is possible to measure transferrin directly by an immunochemical technique, for practical reasons it is usually estimated by determination of its total iron binding capacity. In humans there are normally 200-350 mg. of transferrin per 100 ml. plasma, and this is capable of binding 270-470 µg. of iron. The iron binding capacity of transferrin in the plasma is normally about one-third saturated.

The iron binding properties of the transferrin in the plasma is such that there is an extremely low free Fe^{+++} activity despite a much higher absolute concentration. This is important because many biologically active substances in the plasma are readily inactivated by oxidation in the presence of heavy metal ions. The iron-transferrin complex may be regarded as a small readily available nontoxic circulating iron depot.

2. Storage iron.

The body contains a variable amount of storage iron, which generally represents about one-quarter of the total. This storage iron is readily mobilized when the need for iron is increased, as after an acute blood loss. The stores are present chiefly in the reticulo-endothelial system, which is a diffuse organ situated mainly in liver, marrow, spleen and lymph

glands. Two physically different forms of storage iron are found - a diffuse soluble fraction, in which the molecules are dispersed, and the remainder as insoluble aggregates. These two forms, which are known respectively as ferritin and haemosiderin, are closely related both structurally and functionally.

(a) Ferritin.

Ferritin, a water soluble crystalline protein, was isolated from horse spleen by Laufberger (1937). Since this time it has been found to occur in a variety of cells in both vertebrate and non-vertebrate animals (Richter, 1963) as well as in some plants (Hyde et al., 1962). Isolation of ferritin is relatively simple, since unlike most proteins it is not denatured by heating to 80°C. On addition of cadmium sulphate to a solution of ferritin, crystals of the protein separate. The ease with which ferritin can be obtained in a pure crystalline state has facilitated investigation of its structure by physico-chemical techniques.

Ferritin consists of a well defined and constant protein moeity known as apoferritin and micelles consisting of a variable amount of colloidal hydrated ferric oxide - phosphate complex. The amount of iron in horse spleen ferritin varies between 17 and 33% of the dry weight. This iron,

which is present in the ferric state, can be removed from the ferritin to yield apoferritin (Granick, 1946c). For this to occur the iron has first to be reduced to the ferrous state. Physiological substances such as cysteine, glutathione and ascorbic acid are able to carry out this reduction. Experimental evidence suggests that reduction of ferritin iron in the liver may involve the enzyme xanthine oxidase (Mazur et al., 1958).

Apoferritin also crystallises from cadmium sulphate. The crystals obtained are colourless, octahedral and isomorphous with those of ferritin. The molecular weight of apoferritin determined by sedimentation-diffusion (465,000) (Rothen, 1944) and by X-ray diffraction in conjunction with crystal-density measurements (480,000) (Harrison, 1963), agree well. Amino-acid compositions determined in apoferritin from various species are similar, and suggest the presence of about twenty identical peptide chains.

A model for the structure of apoferritin has been proposed by Harrison (1963). According to this model, which is in agreement with the chemical and X-ray diffraction evidence, apoferritin consists of twenty sub-units each composed of a single peptide chain and situated at the vertices of a pentagonal dodecahedron. Until 1954 the inorganic

component of ferritin was thought to be attached to the surface of the protein. In this year, however, Farrant (1954) obtained electron micrographs of anhydrous ferritin which clearly demonstrated that the micelles, which have a diameter of 55-60 A^{o}, are situated inside a shell of protein having an external diameter of 94-110 A^{o}. Using wet crystals rather larger dimensions (74 A^{o} for the micelle and 122 A^{o} for the protein shell) were obtained by X-ray diffraction and low angle X-ray scattering in solution (Harrison, 1963). Various structures have been proposed for the micelle to account for its characteristic 'tetrad' appearance in electronmicrographs. Farrant (1954) proposed that there were four roughly spherical sub-units each with a diameter of about 27 A^{o}, but more recent work has favoured a structure of six spherical units situated at the vertices of a regular octahedron. The exact chemical nature of the micelle has not been elucidated. The composition can be represented by the formula $(FeOOH)_8(FeO:PO_3H_2)$. The iron present in the micelles has been shown by Michaelis et al. (1943) to have a characteristic and relatively constant magnetic moment corresponding to 3.8 Bohr magnetons. The molecular weight of ferritin containing the maximum amount of iron was calculated by Harrison (1964) to be 860,000. In this state 26% of the weight would be due to iron and 44% to the micelles.

Crystalline ferritin is not homogeneous and several workers have separated it into a number of fractions by various techniques. Thus in ultracentrifuge studies 20-25% of apoferritin has been found. The fact that iron-rich fractions sediment first enables them to be separated from the iron-poor fractions. Kopp et al., (1963) and Theron et al (1963) using starch-gel electrophoresis were able to demonstrate the presence of two or three minor components having a more rapid ultracentrifugal sedimentation rate than the main component. Using disc electrophoresis in acrylamide-gels Richter (1963) demonstrated that ferritin from different species can be separated into up to five bands. He called the bands α , β , γ , δ and ϵ in order of descending mobility. The fastest migrating band, α , accounted for 65-85% of the ferritin, the β band for 10-15% and the γ band for less than 10%. The remaining bands accounted for only a small proportion of the ferritin. Richter ascribed the different mobilities of these various ferritins to differences in their apoferritin moieties.

There is evidence that the presence of iron in a cell initiates the rapid de novo synthesis of apoferritin (Fineberg and Greenberg, 1955). Once the protein shell has been formed the iron is incorporated to give ferritin. The iron can

only be incorporated into ferritin if it is present in the ferrous state (Loewus and Fineberg, 1957). It has been suggested that ferric ions cannot pass between the sub-units at the centres of the pentagonal faces. Once inside the protein shell the iron is reconverted to the ferric state and remains in this form until reduced prior to release from the ferritin.

Mazur et al. (1955) have demonstrated that in addition to the ferric iron within the ferritin molecule there are traces of ferrous ions attached to the surface. Mazur (1956) suggested that this iron may be responsible for the vasodepressor and antidiuretic effects of ferritin (Mazur et al., 1948, 1950).

(b) Haemosiderin.

The amount of iron stored in the form of haemosiderin is normally slightly less than that stored by ferritin (Morgan and Walters, 1963). Both these forms of iron are easily mobilised. Although haemosiderin is most easily distinguished from ferritin by its insolubility in water there are differences in the physical, biochemical and physiological properties of these two compounds. The properties of haemosiderin have not been as thoroughly investigated as those of ferritin, chiefly due to the difficulty in its purification. Ferritin, a soluble crystalline protein, was very

amenable to attack by biochemical and physiochemical techniques. Haemosiderin on the other hand was not readily attacked by these methods and because (unlike ferritin) it can be seen *in situ* under light microscopy it has been studied chiefly by histochemical techniques. Recently, however, improved methods for the purification of haemosiderin have been developed and it has been possible to investigate the chemistry of this compound. The work of McKay and Fineberg (1964a 1964b) refuted Michaelis' hypothesis that haemosiderin consisted of aggregated ferritin. These authors found that although apoferritin was present in haemosiderin, it accounted for only a minor proportion of the protein content. In addition, haemosiderin contained 2-7 times as much phosphorus and twice as much iron per atom of nitrogen as ferritin. This suggested a difference in micelle composition. They concluded from these data that haemosiderin did not result purely from degradation and aggregation of ferritin. The purified haemosiderin granules were shown to contain protein, fatty acids, cholesterol, sugar, one or more haem pigments, iron, phosphorus, sulphur, calcium and perhaps magnesium. McKay and Fineberg (1964b) suggest that haemosiderin is essentially an iron-loaded cell organelle such as a mitochondrion.

(c) Other iron compounds.

In addition to transferrin-bound iron and storage iron, small amounts of non-haem iron are found in the flavoproteins, succinic dehydrogenase and xanthine oxidase. The quantity of iron in this form is however negligible and little is known of the part played by the iron in these enzymes.

III. Amount and distribution of body iron.

Although difficult to measure, the total iron content of the human body is usually given as about 4 grams. This figure is based on complete chemical analysis of four cadavers by Widdowson et al. (1951). The distribution of this iron between the various iron complexes has been described (Bothwell and Finch, 1962). About 65% of the total body iron is present in erythrocytes in the form of haemoglobin. Roughly 30% is usually present in the iron-storage compounds ferritin and haemosiderin, whilst the myoglobin of the muscles accounts for between 3 and 5%. The remaining iron containing compounds, in spite of their importance in iron transport and enzymatic processes, contain only a small percentage of the body iron. There is at any one time about 0.1% attached to transferrin and roughly the same amount in catalase, the cytochromes and other iron-containing enzymes. This distribution of iron is probably the same in the rat and other animals.

IV. Dynamic aspects of iron metabolism.

Under normal circumstances only 3 to 4 mg. transferrin-bound iron is present in the plasma at any time. This small pool of iron, however, has a rapid turnover rate. After intravenous injection of a tracer dose of transferrin-bound radioiron not only can the rate of removal of iron from the plasma be measured but the subsequent distribution of the radioisotope can be followed. The isotope leaves the plasma very rapidly, the half time being normally between one and two hours. Measurement of the half time in this way together with the serum iron and plasma volume enables the amount of iron passing through the plasma per day to be calculated using the formula:

Plasma iron turnover mg./day/100 ml. whole blood *

$$= \frac{\text{Serum iron/}\mu\text{g/100 ml.}}{T_{\frac{1}{2}}\text{ (minutes)}} \times \frac{100 - \text{haematocrit}}{100}$$

---

* This simplified formula was derived by Bothwell and Finch (1962) as follows:

Plasma iron turnover (mg./day/100 ml. plasma)

$$= \frac{\text{Plasma iron } (\mu\text{g./100 ml})}{T_{\frac{1}{2}}} \times 0.693 \times 1440$$

where $0.693 = \text{Log}_e 2$ 1440 = minutes/day

$T_{\frac{1}{2}}$ = time in minutes for clearance of 50% of activity from plasma

Since $0.693 \times 1440 \simeq 1000$

plasma iron turnover (mg./day/100 ml. plasma)

$$= \text{plasma iron } \frac{(\mu\text{g/100 ml.})}{T_{\frac{1}{2}}\text{ (minutes)}}$$

In terms of whole blood this becomes
plasma iron turnover (mg./day/100 ml. whole blood)

$$= \frac{\text{plasma iron } (\mu\text{g/100 ml.})}{T_{\frac{1}{2}}\text{ (minutes)}} \times \frac{100 - \text{haematocrit}}{100}$$

In normal subjects the plasma iron turnover amounts to about 0.6 mg./day/100 ml. of whole blood, or 26-36 mg. per day. Twenty-four hours after injection of radioiron a negligible amount of activity is still present in the blood. At this time 80-90% of the labelled iron is situated in the bone marrow whilst the remaining 10% is in the storage organs such as the liver and spleen.

The iron which goes to the marrow is incorporated into haemoglobin in the developing red cells. These cells then pass into the circulation. The red cells gradually age until after 110-120 days they are removed from the circulation by the reticulo-endothelial system. In this organ the haemoglobin is broken down into globin, iron and the porphyrin ring. The globin is catabolized, and the amino acids enter the amino acid pool. The porphyrin undergoes a sequence of reactions and is eventually excreted as bile pigment, whilst most of the iron re-enters the blood where it is bound by the transferrin and is ready to start the cycle once more. Since red cells survive about 120 days, the daily requirement of iron for haemoglobin synthesis is 1/120th of that present in the circulating haemoglobin. This amounts to about 25 mg. per day. This is also the amount of iron released per day from erythrocyte destruction.

The iron leaving the plasma is replaced not only by iron from destroyed red cells but also by iron from stores and iron absorbed from the lumen of the small intestine. It

is noteworthy that calculations of the amount of iron leaving the plasma per day derived from the half time of injected tracer and from the daily turnover of haemoglobin iron are almost identical. This indicates that the major pathway of iron is from the plasma via the marrow to red cells and back to the plasma again via the reticulo-endothelial system. (Figure 3). Iron entering the body from the intestine is therefore necessarily limited and contributes only a small amount to the daily turnover.

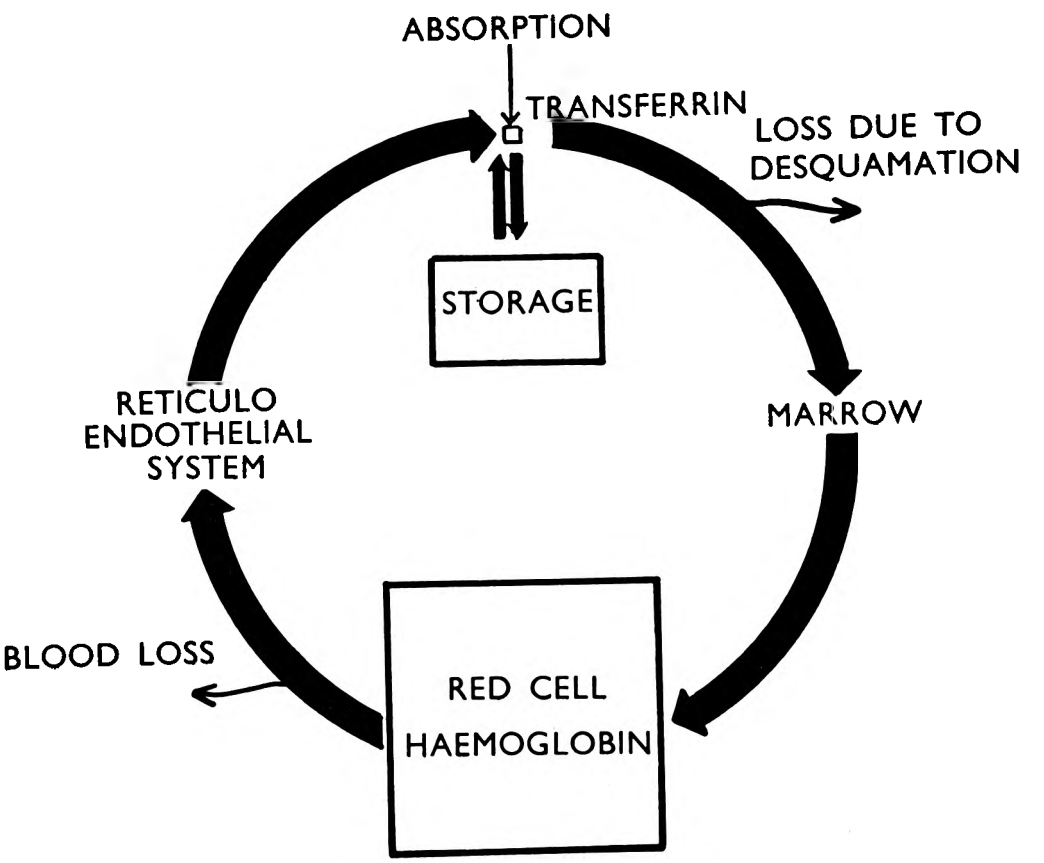


Figure 3.

The major cycle of iron in the body.

CHAPTER II.

IRON BALANCE

AND

ABSORPTIVE MECHANISMS.

CHAPTER II.

IRON BALANCE.

I. Iron deficiency and iron overload.

The importance of an adequate iron balance is apparent from a consideration of the effects of iron deficiency and iron overload. In the adult, iron deficiency results from either abnormal iron loss, such as occurs in acute or chronic bleeding, or from inadequate absorption. This may be due to impaired intestinal function, as in sprue and other malabsorptive disorders, but is more commonly due to a dietary insufficiency. This occurs frequently in young children since a milk based diet provides insufficient iron to meet the requirements of the rapidly expanding red cell mass.

When iron loss exceeds absorption the body mobilises iron from the storage depots to maintain a constant haemoglobin level. If the negative balance persists after stores have been depleted, the serum iron falls and the amount of circulating transferrin increases. Both factors result in a lowering of the percentage saturation of the transferrin. When the saturation falls below 15%, haemoglobin synthesis is impaired. This leads to a lowered haemoglobin level and the production of microcytic and hypochromic red cells. If not corrected, tissue iron depletion occurs, and this results in changes in mucous

membranes and various epithelial cells. Beutler (1965) has recently reviewed the effects of tissue iron depletion and indicates that they are not due to the anaemia but to the iron deficiency per se. It is generally accepted that iron-deficiency anaemia precedes tissue iron depletion (Bothwell and Finch, 1962) but Beutler (1965) has suggested that tissue depletion can occur at a stage when haematological indices are normal.

In certain circumstances abnormal amounts of iron are absorbed. This leads to the slow accumulation of iron in the body. Two forms of iron overload, idiopathic haemochromatosis and Bantu siderosis, have been extensively investigated. Idiopathic haemochromatosis is generally regarded as an inherited metabolic disorder involving excessive iron absorption from the gut (Bothwell, 1965; Bothwell et al. 1965). The excess iron is deposited in several tissues such as liver, pancreas and heart and associated with this there is portal cirrhosis, diabetes and cardiac failure. The second type of overload is that found in the adult Bantu of Southern and Central Africa. This condition is attributable to the consumption of a diet containing excessive amounts of iron (Bothwell, 1964). It is noteworthy that a major portion of this iron is derived from the consumption of alcoholic beverages (Walker and Arvidson, 1953; Bothwell et al., 1964). Not only do these beverages contain appreciable amounts of iron but the alcohol itself has been shown to enhance iron absorption (Charlton et al., 1964). Several studies have shown a

close correlation between the degree of iron overload and the presence of portal fibrosis or cirrhosis (Higginson et al., 1957; Isaacson et al., 1961). Usually the iron is in reticuloendothelial cells and liver parenchyma, but when portal cirrhosis is present iron is also found in epithelial deposits in a number of organs (Isaacson et al., 1961), e.g. the pancreas, thyroid, adrenals, pituitary and myocardium. The high pancreatic deposits are associated with insulin-dependent diabetes in a proportion of Bantu subjects with iron overload (Seftel et al., 1960).
Iron overload of a different type is caused by repeated blood transfusions or parenteral treatment with colloidal iron preparations (Cleton and Blok, 1964). Injected colloidal iron or iron from erythrocytes accumulates chiefly in reticulo-endothelial cells. Large amounts of reticulo-endothelial iron are well tolerated without any apparent tissue damage. It is therefore apparent that excessive quantities of iron may be toxic only when present in parenchymal cells.

II. Normal iron balance.

From the foregoing discussion it is evident that both a deficiency and a surfeit of iron are deleterious to the body. In normal circumstances neither of these conditions arises since the iron content of the body remains within fairly narrow limits throughout adult life. This is achieved by adjustments in the rate at which iron is absorbed by the gut. There is also evidence that the rate

of excretion may vary, but this mechanism is a limited one.

Meticulous iron balance studies carried out by McCance and Widdowson (1938) established that the body's capacity for excreting iron is extremely limited.

Although these balance studies were not without technical problems (Josephs, 1958), the results obtained were confirmed by several other workers (Dubach et al., 1955; Finch, 1959) when radioiron became available. The isotopic studies indicated that between 0.6 and 1 mg. of iron is lost per day in males and approximately twice this amount in menstruating females. Several studies have been carried out in order to establish the principal routes by which these small amounts of iron are lost from the body. Radiochromium studies have shown that a small amount of blood accounting for between 0.05 and 0.6 mg. of iron is lost in stools daily (Bothwell and Finch, 1962). In addition to this there are small losses due to desquamation of iron-containing epithelial cells from the skin and from the gastrointestinal and renal tracts. Particularly important in this respect are the cells of the intestinal mucosa which have a rapid turnover. Recent work by Crosby et al., (1963) indicates that in iron overload, iron excretion may be increased to some extent by the deposition of increased amounts of iron in these epithelial cells which subsequently slough off and are excreted. Nevertheless the ability of the body to excrete iron is extremely limited. It is therefore apparent that the absorptive process is of paramount importance in the regulation of the body iron

content. For this reason much attention has been focussed on the process of iron absorption and the mechanisms by which absorption is adjusted to meet changing body requirements. Although much information has been obtained regarding the response of the absorptive process after various manoeuvres, little is known of the mechanisms evoking these changes in absorptive behaviour.

III. Factors influencing absorption.

The factors known to influence absorption may be rather roughly divided into luminal and mucosal factors.

A. Luminal factors.

The luminal factors are particularly important from a dietary point of view since these are factors which can be altered readily.

A prime aim in studying iron absorption is to obtain a knowledge of the way in which food iron is handled. Several investigators have studied this problem using isotopically labelled foodstuffs. Moore and Dubach (1956) and Chodos et al. (1957) have investigated absorption from labelled egg, liver, muscle and leafy vegetable while absorption of labelled haemoglobin has been studied by Callender et al., (1957). Labelled milk prepared by Schulz and Smith (1958) and labelled bread prepared by Steinkamp et al. (1955) have been similarly investigated. These studies show that iron is absorbed fairly well from muscle and haemoglobin but only poorly from eggs and leafy vegetables. In particular these studies have demonstrated that the various forms of

iron are not handled in the same way. Absorption of iron from haemoglobin, for instance, is not affected by substances such as ascorbic acid, food or phytate (Callender et al., 1957; Turnbull et al., 1962) which are known to alter the absorption of ionised salts (Bothwell and Finch, 1962). Because of the number of variables and the wide range of absorptions in studies of this kind, most investigations have been carried out using simple iron salts. Some of the factors influencing the absorption of simple iron salts are discussed below:-

1. Size of the dose.

The amount of iron absorbed is dependent on the size of the administered dose. Although the percentage absorption decreases with increasing dosage, there is a progressive rise in the amount retained (Bothwell, Pirzio-Biroli and Finch, 1958; Smith and Pannacciulli, 1958; Bonnet et al., 1960). This is true for quantities of iron ranging from physiological doses to those used therapeutically.

2. Effect of reducing agents.

When large doses of ferric iron are given, absorption is less than half that found with ferrous salts (Moore et al., 1944; Hahn et al., 1945; Brise and Hallberg, 1962). Various reducing agents such as ascorbic acid (Moore et al., 1939), inosine (Cheney and Finch, 1960), succinic acid (Brise and Hallberg, 1962), fructose (Stitt et al., 1962) and cysteine (Jacobi et al., 1956),

all increase iron absorption. Several different hypotheses have been offered to explain the action of these substances. Ascorbic acid maintains the iron in the more readily absorbed ferrous state. It has been suggested that succinic acid stimulates oxidative metabolism with which iron transport is linked, while fructose and cysteine chelate iron, thus maintaining it in a soluble form at the alkaline pH of the small intestine. Since all these substances are able to act as reducing agents and in one way or another change the redox balance of the absorbing cell it seems possible that they have a common action. Pollack, Kaufman, Crosby, and Butkiewicz (1963) have demonstrated a similar enhancement of absorption by oral administration of hydroquinone: a reducing agent without known or postulated metabolic function. This enhanced absorption occurred even in the presence of sufficient ascorbic acid to maintain the iron in the ferrous state. In addition, it has been shown that large amounts of ascorbic acid increase the absorption of ferrous iron. This suggests that the ascorbic acid has some function in addition to maintaining the iron in the reduced form. It is possible that it acts by reducing the luminal pH (Jacobs et al., 1966).

3. Factors reducing absorption.

Phosphates and phytates form insoluble compounds with iron and both these substances have been shown to reduce iron absorption markedly (McCance et al., 1943; Sharp et al., 1950). Studies in which

ionisable salts were given with food indicated that absorption was reduced by about 50% (Pirzio-Biroli et al., 1958). There is evidence that absorption decreases progressively as the bulk of the meal increases (Sharpe et al., 1950). Nevertheless it seems that the composition of the diet is more important than the size of the meal.

4. Other factors.

Various other luminal factors have also been shown to effect iron absorption. Gastric juices can solubilise and reduce iron in vitro (Pollack, Kaufman, Crosby, and Butkiewicz, 1963; McCance et al., 1943). The importance of hydrochloric acid has recently been demonstrated by Jacobs et al., (1964). They demonstrated that administration of hydrochloric acid with iron enhanced absorption of a test dose of iron in subjects with histamine-fast achlorhydria. The increased absorption of ferric iron was more marked than that of ferrous iron, and absorption of haemoglobin iron was not increased at all. It has been suggested that pancreatic juices may reduce iron absorption, since they are alkaline and have a high phosphate content. Although there is some evidence in favour of this (Davis and Biggs, 1965), some doubt remains as to the significance of pancreatic secretions. It has been suggested that absorption may be facilitated by the secretion of some chelating substance into the lumen, which then binds iron before its entry into the cells

(Pirzio-Biroli, 1956). Beutler (1963) has some evidence for the presence, in gastric juice, of a non-protein component able to maintain iron in a soluble form up to pH8. This observation has, however, not been fully reported. Other workers have also attempted to demonstrate the presence of iron chelates in intestinal or bile secretions, but with limited success.

B. Mucosal factors.

Iron deficient subjects absorb more iron from both salts and food than do iron replete subjects. This observation, together with the fact that body iron stores remain roughly constant through adult life in normal subjects, suggests that the absorptive mechanism is under some form of regulatory control. There is little evidence for this control being exercised by luminal factors. It therefore seems probable that absorption is controlled chiefly by factors which exert their influence upon the absorbing mucosal epithelium.

The chief factors known to influence absorption are the state of body iron stores and the rate of erythropoeisis. In addition there is evidence that other factors, such as anoxia and erythropoeitin, may influence absorption. A number of studies have demonstrated enhanced absorption from both iron salts and labelled food iron when iron stores are decreased (Hahn et al., 1943; Dubach et al., 1948; Bothwell, Pirzio-Biroli and Finch, 1958). On

the other hand when stores are increased there is a reduction in absorption (Bothwell, Pirzio-Biroli, and Finch, 1958; Pirzio-Biroli et al., 1958). This was well demonstrated in a study in which the iron stores of two groups of normal subjects were altered (Pirzio-Biroli and Finch, 1960). In the group in which stores were depleted by phlebotomy the mean absorption increased from 6% to 20% while in the group given 1.5 g. of iron by parenteral injection the mean absorption fell from 6% to 3%.

The single factor which appears to exert the greatest control over iron absorption is the rate of erythropoeisis. Increased erythropoeisis due to bleeding (Bothwell, Pirzio-Biroli, and Finch, 1958), haemolysis (Chappelle et al., 1955; Krantz et al., 1959; Stewart et al., 1953; Bothwell, Pirzio-Biroli,and Finch, 1958) or relative anoxia (Reynafarje et al., 1959) results in enhanced absorption, while the opposite occurs when erythropoeisis is depressed (Krantz et al., 1959; Reynafarje et al., 1959; Bothwell, Pirzio-Biroli and Finch, 1958). The increased iron absorption caused by acute bleeding cannot be prevented by the parenteral administration of iron either before, at the same time, or after bleeding (Beutler et al., 1963; Moore et al., 1941). It seems strange however that normal iron absorption occurred in a case of marrow aplasia without large iron stores and that only a small decrease in absorption was brought about by experimentally induced aplasia (Beutler et al., 1963).

Mendel (1961) has evidence that both anoxia and erythropoeitin administration result in increased absorption of iron. In the case of erythropoeitin administration the increased absorption was dependent on the presence of an intact marrow organ. Ablation of the marrow by administration of radiostrontium and splenectomy eliminated the effect. The response to anoxia, on the other hand, was still present in the absence of the marrow. Erythropoeitin must therefore exert its effect by stimulating the erythropoeitic process while anoxia increases iron absorption by some other mechanism.

There are thus at least three different types of stimulus which may exert a significant effect on iron absorption. These include the level of body iron stores, the rate of erythropoeisis and anoxia. How these factors exert their effects and whether they do so through some common final pathway is not known. Some mechanisms proposed to account for several aspects of iron absorption are discussed in the following section.

MECHANICS AND MECHANISMS OF IRON ABSORPTION.

IV. Relative importance of various areas of intestine in iron absorption.

Before discussing the various aspects of absorption itself, it is necessary to establish the relative importance of the various areas of the gastrointestinal tract in iron absorption. Although absorption can occur at any level of the gastro-intestinal tract, when iron is presented to it in an absorbable

form (Vahlquist et al., 1945), absorption is most effective in the duodenum and gets progressively less distally. Evidence for this came initially from several studies. Endicott et al., (1949) prepared autoradiographs of the intestinal tract of guinea pigs after radioiron administration. Brown and Justus (1958) counted washed segments of small intestines from rats after feeding radioiron. Both investigations showed activity to be maximal in the duodenum and to diminish distally. A similar result was obtained by Wack and Wyatt (1959) using autoradiography. These authors showed that chronic anaemia or protein deficiency in the experimental rat increased the absorptive capacity of the intestinal tract far beyond the duodenal region which was the zone of maximal absorption under normal conditions. The site of absorption extended progressively down the intestinal tract corresponding with the drop in circulating haemoglobin level. It should however be noted that in these experiments the anaemia was produced by raising weanling rats on a low iron diet, and that this in itself may alter the mucosal behaviour.

It is important to note that these studies do not measure absorption but rather mucosal uptake of iron. There is, however, experimental work demonstrating that absorption into the body is also maximal in the duodenum and diminishes distally. This evidence was obtained from both <u>in vitro</u> and <u>in vivo</u> studies. Dowdle et al. (1960) showed that iron transport in everted gut segments prepared from rat intestine was invariably maximal with segments removed near

the pylorus. The efficiency of transport decreased in more distal segments. Duthie (1964) determined the absorption of various doses of iron injected with ascorbic acid into different areas of rat small bowel. He found that absorption from the duodenum was not superior to absorption of a dose injected 5 cm. distal to the duodeno-jejunal flexure when 10 µg. was administered. At higher doses, absorption was superior in the duodenum and he observed a gradient of absorption from duodenum to ileum. Repeated bleeding or iron deficiency resulted in a greater increase in absorption from jejunum or ileum than from duodenum, but the gradient remained the same. It is thus evident that the duodenum is the most effective area for iron absorption.

V. Role of lymphatics in absorption.

It has been well established that absorption of iron occurs directly into the blood stream rather than through the lymphatics (Reizenstein et al., 1960; Endicott, 1949). Further evidence in support of these findings has been obtained in our own laboratories. After oral administration of radioiron no activity was found in lymph collected from a patient with a thoracic duct fistula (Green, 1965).

VI. Dynamics of small intestine.

A brief outline of the dynamics of the small intestine and the structure of the absorbing cell is necessary for an understanding of the importance of some of the time relationships operative when absorption is altered by various manoeuvres. The small intestine is an organ with an enormous

surface area. It consists of a vast sheet of columnar epithelial cells, there being inward projections, the crypts, and outward projections, the villi. It stands on a basement membrane beneath which is an intricate network of capillaries. On histological examination it appears as a permanent structure, but in reality it is in motion. The crypts of Lieberkühn are the 'regeneration' or 'production' zones which supply the whole mucosa. The crypts are lined with immature cells which are undergoing frequent mitosis. The cells leaving the crypts mature into adult cells and ascend the villus. How they do this is unknown, since electron microscopy shows no obvious motile apparatus. It is thought that the cells simply glide over the surface of the basement membrane. In order to maintain a steady state as the cells mount the villi an equal number must be lost from the tip, or more precisely, the rate of production must equal the rate of loss (Leblond and Stevens, 1948). The cells reaching the tip of the villus are lost from specific localised sites known as 'extrusion zones'. This loss of cells can be seen under the dissecting microscope (Creamer et al., 1961). Electron microscopy shows that cells undergo a change before desquamation (Curran and Creamer, 1963). These changes suggest that it is not a healthy cell but rather a husk which is shed. This means that much of the intracellular material may be recirculated into the villus before shedding. There is a relatively constant rate of epithelial replacement which does not vary much from species to species. The rates for

various areas of small bowel in man and rat are shown below.

Table 1.

The turnover rate of mucosal cells.

	Rat.	Man.
Duodenum	1.6 days (Leblond and Stevens, 1948)	2 days (Bertalanffy and Nogy, 1961)
Jejunum	1.3 days (Bertalanffy, 1960)	
Ileum	1.4 days (Leblond and Stevens, 1948)	3 days (Lipkin, Sherlock and Bell, 1963).

The rest of the gastrointestinal tract is similarly in motion, but by comparison the small intestine has by far the fastest turnover. On the basis of data obtained in the rat Leblond and Walker estimated that in man the daily loss of cells into the lumen of the gut would weigh half a pound (Leblond and Walker, 1956).

The situation in the gut is well summarised by Leblond and Stevens (1948):-

> "This procession of cells involves a slow, massive motion of the whole intestinal epithelium gliding upwards to its death".

But as Creamer (1965) points out, the term 'slow' is somewhat misleading since the volume of cells involved makes the small intestine one of the most dynamic organs in the body.

VII. Structure of the absorbing cell.

The columnar absorbing cell is the predominant type within the epithelium that lines the small intestine. These tall cells are as high as the epithelium itself, extending from the basement membrane to the lumen of the gut. Electron

microscopy shows the presence of a specialised structure on the apical surfaces of these absorbing cells (Figure 4). This surface consists of a series of fingerlike cytoplasmic projections or microvilli. These projections are approximately 5000A° high and 500A° wide. On each surface of the cell facing the lumen there are about 300 of these microvilli. It is probably through the protoplasmic membranes of these projections that iron absorption occurs. The question arises as to how and in what form iron is transported through the cell from the membrane to the capillary of the villus.

VIII. Mechanisms of iron transport across mucosal cells.

As mentioned previously, any hypothesis concerning the mechanism of absorption of iron must consider how iron is transported across the mucosal cell. Few workers have concerned themselves with this specific problem. The mechanism proposed by Granick is both ingenious and possible (Granick, 1956). According to this hypothesis, the iron moves into and through the mucosal cell along a concentration gradient (Figure 5). This gradient is made possible by the ease with which iron can be converted from the ferrous to the ferric form. Granick postulates the existence of an oxidation-reduction potential, or rate of oxidation, that is greater in the front part of the cell, and a reducing mechanism which is more active in the rear part of the cell. This establishes a continuous concentration gradient in the following way:- Ferrous iron entering the mucosal cell from the lumen is immediately oxidised to the ferric state. In this way a low ferrous iron concentration is maintained



Figure 4.

Electronmicrograph of an epithelial mucosal cell showing the microvilli.

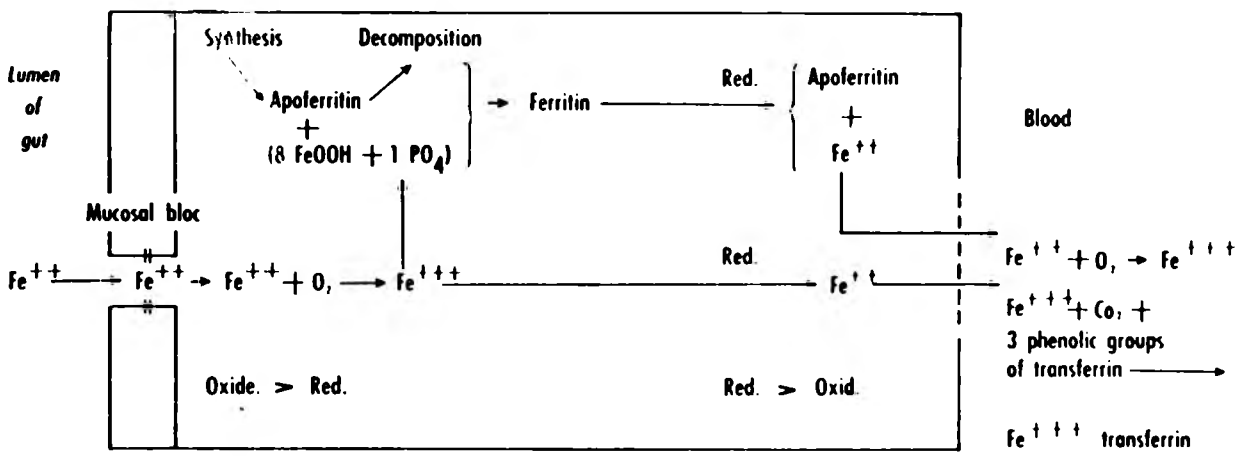


Figure 5.

Granick's scheme for iron transport across the mucosal cell.
(from Granick, S. 1956)

in this portion of the cell, establishing a concentration gradient resulting in the continuous movement of ferrous ions into the cell. The ferric iron then migrates to the other end of the cell where the activity of ferric iron is kept low by reducing the iron and passing it on to the blood. Once in the blood stream the iron is chelated by transferrin and the concentration of ferrous iron is maintained at a low level enabling ferrous iron to migrate continuously from the cell down a concentration gradient into the plasma.

Although possible, this explanation seems unlikely, since the avidity with which ionic iron binds to proteins and many other substances suggests that iron in the mucosal cells is probably present in a bound form. It seems more likely that iron passes through the cell in one or more bound or chelated forms. Several authors have suggested that amino-acid iron complexes may play an important part in iron absorption (Groen et al., 1947; Jacobi et al., 1956; Rummel and Candon, 1956; Brown and Rother, 1963a). However only Brown and Rother (1963a) have produced significant evidence supporting this postulate. At the present time, therefore, the idea that iron is carried through the mucosal cell in a chelated form is an attractive theory with insufficient experimental backing.

IX. Mechanisms controlling iron absorption.

The earliest hypothesis concerning the mechanism of control of iron absorption was the 'mucosal block' theory proposed

by Hahn et al., (1943). This theory was based on experiments carried out on three dogs. The authors observed that when a large dose of iron was followed within 1.3 to 6 hours by a dose of radioiron, less activity appeared in the red cells than would have been expected. It was concluded that the initial dose of non-radioactive iron blocked the uptake of the radioiron.

Following his demonstration of the presence of ferritin in the mucosa of guinea pigs fed large doses (10 mg./day) of iron Granick elaborated the 'mucosal block theory'. (Granick, 1949, 1956). According to this theory apoferritin is constantly being synthesised and broken down in the mucosal cell. If large amounts of iron are fed, ferritin accumulates in the cell preventing further iron absorption. Iron incorporated in this way into ferritin eventually becomes available for absorption when the reducing mechanisms reconvert the ferric iron of ferritin to the ferrous state. The reduced iron then moves into the plasma. Granick envisaged the apoferritin as an integral part of the mucosal cell membrane. In place of the iron hydroxide clusters present in ferritin, he suggested that the apoferritin molecule had tiny holes 100A^{o} in diameter. Ferrous iron present in the lumen passed through the pores into the mucosal cell where it was oxidised to the ferric state. When the amount of iron in the lumen was large, ferric ions accumulated near the luminal surface of the cell. These ions were then incorporated into the apoferritin in the membrane, gradually clogging the pores

with deposited ferric hydroxide micelle. This provided the 'mucosal block'. The block was gradually removed as the reducing mechanism of the cell reduced the iron in the ferritin. In anaemia the reduced oxygen tension increased the reducing ability of the cell, thus enabling the mucosal cell to absorb increased amounts and shortening the duration of any 'mucosal block' which might be set up. Attractive as this theory may seem, the evidence against it is overwhelming. The work of the Heilmeyer's group (Wöhler et al., 1957; Heilmeyer et al., 1957; Heilmeyer, 1958; Keiderling and Wöhler, 1954) showed that a progressive rise in the ferritin iron content of the gastrointestinal tract occurs after administering 15 mg. ferrous iron orally to guinea pigs. The ferritin content of the stomach and small intestine was maximal after 10 hours and of the duodenum after 20 hours. The ferritin content then declined reaching normal levels approximately 40 hours after the oral dose. Throughout this period, even at peak ferritin values, absorption of iron continued. In a long term study in which 15 mg. ferrous iron was given daily for 28 days, absorption continued even when the ferritin content of the gastrointestinal tract was maximal. The authors were unable to find evidence suggesting that a high ferritin iron content in the intestinal mucosa reduced iron absorption. They suggested that ferritin in the mucosal cells serves as a mediator rather than a regulator in absorption:-

> "If much iron is absorbed, there is much ferritin; if less is taken up, there is less ferritin".

A most telling point against the mucosal block theory is

its failure to explain the excessive absorption and heavy tissue iron deposits found in subjects after prolonged ingestion of dietary or medicinal iron (Bothwell et al., 1964). Indeed increasing amounts of iron are absorbed with increasing dosage (Smith and Pannaccialli, 1958). This relationship has also been shown in mice for dosages up to lethal amounts (Beutler and Kelly, 1962). Duthie and co-workers administered three doses of radioiron into the same segment of dog bowel at two-hour intervals. They found practically no difference in the absorption from these doses, indicating the absence of a mucosal block (Duthie, Code and Owen, 1962).

Brown et al., (1958) extensively investigated the existence of a mucosal block in humans. They found that oral doses of 2-4 mg. per kilogram of body weight decreased absorption of a tracer dose administered some hours later. The block was however only partial and of short duration, lasting roughly six hours in normal subjects and three hours in cases of iron deficiency. The possibility of luminal mixing of labelled and non-labelled iron cannot be altogether excluded in these experiments. In addition the authors emphasised the artificial nature of the block produced by such large quantities of inorganic iron, pointing out that dietary iron could not produce this effect. They were also unable to determine the site or the nature of this block. From these various data it is apparent that the controlling mechanism which regulates absorption of dietary iron according to requirement is unlikely to be

the 'mucosal block' as described by Granick.
The 'mucosal block' concept was so widely accepted that few further investigations into the nature and control of iron absorption were carried out until recently. After demonstrating that the 'mucosal block' theory was not able to explain the control of iron absorption, Brown turned his attention to various aspects of the absorptive process itself. His studies indicated that radioactivity can be detected in the blood and in all fractions of the mucosa within one minute after introduction of 20µg. radioiron in the form of ferrous sulphate (Brown and Rother, 1963a, 1963b). The mucosal activity increased to a maximum at 15 minutes and then declined. The nature of the non-particulate fraction of the mucosal extract was studied in detail. The radioiron in this fraction was shown to be present in one of two forms - either protein-bound or dialysable. Although the protein-bound form was not identified, the authors concluded from their experiments that it was not ferritin and that transferrin might be one of the components of the mucosal iron-binding protein. The dialysable form was thought to be amino acid-bound iron. The identification of these two forms of mucosal iron by Brown and Rother is discussed later in the light of the findings of the present investigation. After the oral administration of radioiron the proportion of activity in the dialysable fraction of the non-particulate extract decreased with time, while the proportion of protein-bound iron increased.

The dialysable form predominated during the initial 15-30 minutes, after which the major fraction of iron was present in the protein-bound form. These results were interpreted as being due to the presence of two pathways of absorption - an early rapid transit amino-acid bound form and a more slowly mobilised protein-bound form. These authors also investigated absorption in rats in which absorption was enhanced either by induced iron deficiency or by administration of ascorbic or succinic acids. In both cases the enhanced absorption was associated with a shift to a predominance and persistence of the rapid-transit fraction of mucosal iron. In iron deficient rats this non-protein fraction accounted for the major fraction throughout the period studied (48 hours). To account for the paradox of the long persistence of the rapid-transit form of iron Brown suggested that:-

> "Under conditions of enhanced absorption, entry of iron into the mucosal cell is so greatly facilitated by amino-acid binding that it overwhelms the subsequent process of iron release from the cell to the blood stream.".

This two-stage mechanism of absorption has also been suggested by Gitlin and Cruchaud and by Bannerman et al. Gitlin and and Cruchaud (1962) studied the absorption of radioiron in iron-deficient, normal and iron-loaded mice. The kinetics of absorption was studied in these animals using a wide range of dosage. Radioiron administered orally to normal mice was lost rapidly in the faeces for 24 hours. A slower loss then occurred until, after 3-4 days, the daily loss was only 0.5-3% of the total body activity. This is

about the same as the daily intestinal loss in mice (Stevens et al., 1953). Although the percentage retained within the body dropped with increasing dosage, the pattern of loss was the same at all levels. Iron-deficient and iron-loaded animals showed the same pattern of isotope loss except that the former retained more and the latter less iron than normal animals. The authors found that when doses of iron greater than 100 µg. were administered, absorption increased in a linear fashion with increasing dosage. This behaviour was described by the equation:

$$An = \frac{A2 - A1}{D2 - D1} \cdot Dn + A$$

Where An = Amount of iron absorbed from dose Dn (Dn $>$ 100 µg.), A1 and A2 are absorptions from doses D1 and D2 (D1 and D2 $>$ 100 µg.).
A = intercept with the axis at zero dose. An exaggerated form of the curve obtained is shown in Figure 6.
If A is set at zero then a straight line parallel to the linear portion of the absorption curve is obtained. (Dotted line in figure). The curve obtained by subtracting this from the absorption gives a curve B which resembles those encountered in enzymatic or carrier reactions. From this treatment it is apparent that absorption occurs by two simultaneously operating processes. One is a first order process in which there exists a linear relationship between the amount absorbed and the dose administered - this is shown by the dotted line. The second process appears to have the kinetics of an enzyme reaction in which the amount

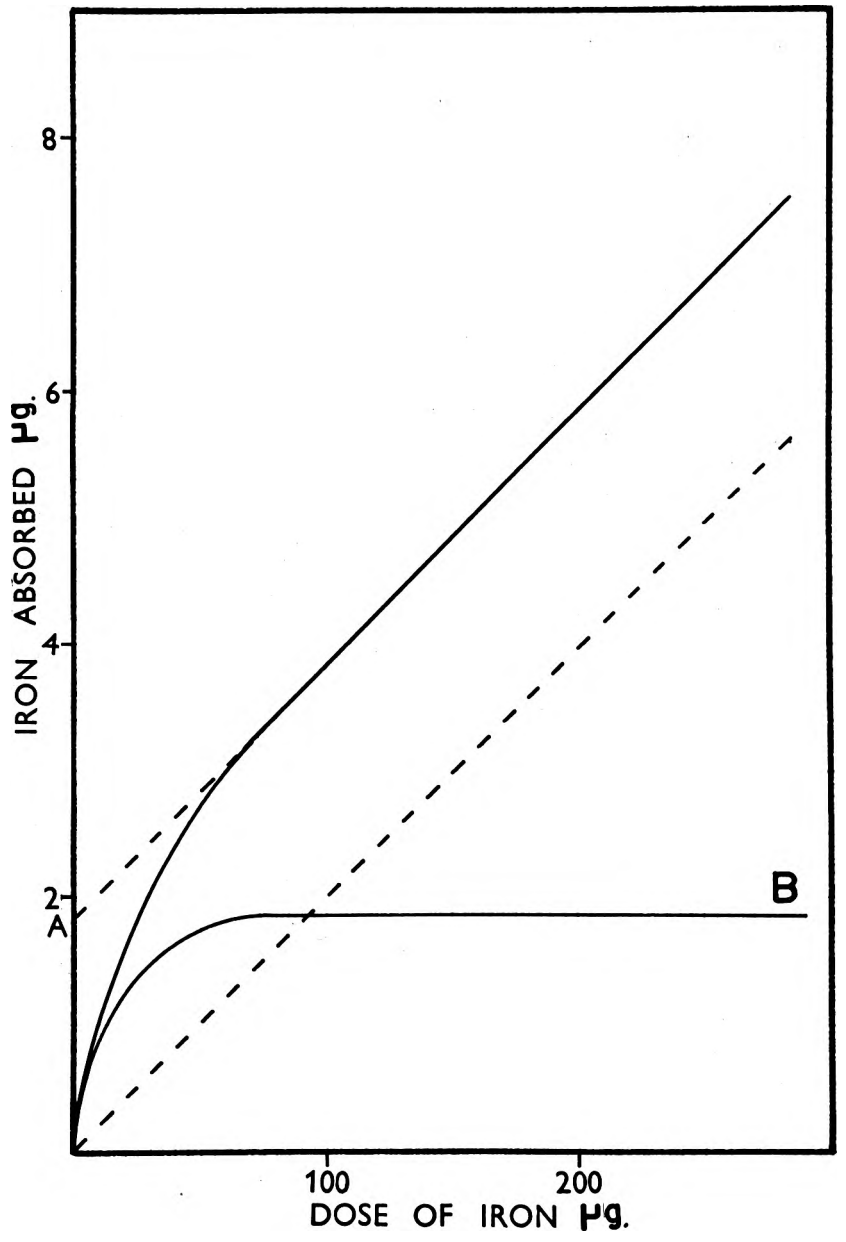


Figure 6.

An exaggerated graph showing the findings of Gitlin and Cruchaud.

of enzyme or carrier present is the limiting factor. The authors studied the contribution of these two forms of transport in normal, iron-deficient and iron-loaded animals. Both processes, but more particularly the second, were found to be affected by the variation in body iron stores. The value of A (the intercept on the ordinate) provides a measure of the contribution of the second process to the total absorption. A had a value of 1.6 in normal rats compared with 3.4 in iron-deficient rats and 0.45 and 0 in two groups of iron-loaded animals. The slope of the linear portion of the absorption curve had the greatest gradient in iron-deficient animals and the least in iron-loaded animals, indicating that absorption of this type was also dependent on body iron stores. The nature of this linear component could not be deduced from the experiments, but the kinetic data were in keeping with either passive diffusion or absorption mediated by a carrier system present in large excess.

Bannerman et al., (1962) demonstrated a similar relationship between absorption and iron dosage in rats. The amount of iron absorbed was proportional to the size of the dose over a wide range but at low dosage levels the relationship was curvilinear. This was found in both iron-deficient and iron-supplemented rats; the slope of the linear section of the curve was again very steep in the iron-deficient animals and almost negligible in iron-supplemented rats.

These authors also showed that the level of dietary iron itself is able to modify the absorptive behaviour of the

epithelium. This was demonstrated by maintaining rats on a semisynthetic low-iron diet and on an iron-supplemented diet. On the low-iron diet animals developed an uncomplicated iron-deficiency anaemia. These anaemic rats absorbed 80% of a test 50 µg. dose of radioiron whilst the iron-supplemented animals absorbed only 10% of such a dose. At this stage the iron-deficient rats had a haemoglobin level of 6g./100 ml. compared with 18g./100 ml. in the supplemented group. The diets were then changed so that the iron-deficient animals now received the supplemented diet while the iron-replete animals were fed the low-iron diet. The haemoglobin level and the absorption of a 50 µg. test dose were measured for several days after switching the diet. The haemoglobin level of the replete animals showed no change until the fifth day, when it started to fall very slowly. The level in the deficient rats started to rise on the first day, reached 12g./100 ml. by the fifth day and continued to increase until the tenth day, when it reached 18g./100 ml. The change in absorption from the test dose was more dramatic. The percentage absorption in the iron-deficient group dropped sharply from 80% to 40% by the second day whilst absorption in the iron-replete animals increased from 10% to 40% in the same period. These trends continued until by the fifth day the formerly iron-deficient animals absorbed less than 10% of the dose while the animals which had initially been iron supplemented absorbed more than 50%. The results showed that the change in the absorptive behaviour of the

mucosal cell is too rapid to be due to a change in the haemoglobin level or the body iron stores. It seems likely that the tenfold increase in absorption which these authors demonstrated in iron-deficient animals is due more to their low iron diet than to the iron deficiency anaemia per se. The authors suggested that the iron content of the mucosal tissue might be important in controlling iron absorption - a theory which was later developed further by Crosby. Further evidence for a dual pathway in iron absorption was provided by the in vitro studies carried out by Schachter and co-workers. Using everted gut sacs prepared from segments of proximal small intestine from rats, Dowdle, Schachter and Shenker (1960) demonstrated transport of radioiron from mucosal to serosal surface against a concentration gradient. This active transport mechanism was dependent on oxidative metabolism and the generation of phosphate bond energy. The capacity of the transport mechanism was limited. Further work by Manis and Schachter (1962) using the same system showed that the iron is transported against both potential and concentration gradients. Their studies also indicated that two steps were involved in the active transfer. Iron was first taken up by the mucosa and subsequently transferred to the serosal surface. They demonstrated that oxidative metabolism was necessary for both steps. The net transfer to the serosal surface was the slower of the two processes and was more readily rate limited. They showed that both ferric and ferrous iron were taken up by the mucosa but net

transfer to the serosal surface was specific for divalent iron.

A similar two-step process of mucosal uptake and transport to the tissues was also shown in loops of duodenum *in vivo*. Both *in vivo* and *in vitro* the slower second step was shown to have a greater relative dependence on oxidative metabolism. In addition it was shown that, although mucosal uptake was approximately the same throughout the small intestine, the serosal transfer process was more limited to the proximal duodenum. It decreased markedly in the upper small intestine and in the ileum was only 9% of the duodenal value. This was also demonstrated *in vivo*. In a loop prepared from duodenum 83% of the radioiron absorbed from the lumen was subsequently transferred to the blood stream, whereas the corresponding value in the midjejunum was only 15%.

For active transfer of iron *in vitro* the authors found it necessary to include a metabolisable hexose in the bathing medium. Addition of monosaccharide hexoses which the mucosa was not able to metabolise, e.g. 3-methyl-D-glucose, resulted in inhibition of iron transport. Although mucosal uptake was influenced by these sugars, the effect on the mucosal transfer mechanism was more marked. This was also the case when metabolic inhibitors were used to depress absorption from loops *in vivo*.

By oral administration of either ferric or ferrous iron to rats before preparation of the everted segment, Manis and Schachter were able to demonstrate a decrease in both

stages of active transport. This effect was observed with doses as small as 100 µg. Although they were unable to explain the mechanism of the inhibition, they obtained evidence that it was due to a direct effect of the iron on the intestine. They suggested that the decrease in mucosal uptake might be due to saturation of an essential carrier compound by the increased amount of iron in the mucosal cells. Since this did not explain the reduction in net transport to the serosal surface, they postulated an effect of iron which inhibited and thereby regulated the second step in the absorption. They found that the amount of iron in their normal rat diet was sufficient to produce to some degree a block to absorption. Increased requirement for iron (as in late pregnancy) resulted in a stimulation of the second transfer step both in everted sac and in vivo loop experiments. (Manis and Schachter, 1962b).

In a subsequent communication (Manis and Schachter, 1964) these authors report measurements concerning the magnitude and the dynamic relationship between ferrous and ferric pools in the mucosa during absorption of iron. Unfortunately their results are not only difficult to follow but do not seem to be consistent with their previous reports. Nevertheless it is apparent that in the course of active transport in vitro ferrous iron is absorbed into a mucosal ferrous pool which has a relatively rapid turnover rate. Iron in this pool is either transported to the serosal surface or is diverted into a ferric iron pool. The ferric iron pool appears to be a storage depot, since in iron-loaded animals not only is the mucosal uptake of ferrous iron

depressed but increased amounts of ferrous iron are incorporated into the ferric pool with a concomitant reduction in transport to the serosal surface. The authors found that a similar process occurred in vivo. From their experiments they concluded that ferritin accounted for only about 8% of the mucosal ferric iron pool.

Although the work of Brown and Rother (1963a), Bannerman and co-workers (1962), Gitlin and Cruchaud (1962) and Schachter and co-workers (Dowdle et al., 1960; Manis and Schachter, 1962a) all suggest the probability of a two-step process of absorption, they each reach this conclusion from a different angle. It is difficult to be sure whether in fact these authors are referring to the same thing in proposing this dual pathway. In examining the non-particulate mucosal extract during absorption of radioiron Brown found the iron changing from one form to another with the passage of time. In the in vitro system of Schachter et al. the two processes were mucosal uptake and transfer across the cell. In addition to this, however, these workers showed the formation of a ferric pool in the mucosa during absorption - this process probably corresponds to the changes found by Brown and Rother. The work of Schachter et al. tends to indicate a two-phase process rather than a double pathway. The work of Bannerman et al. and Gitlin and Cruchaud, on the other hand, indicates absorption of iron by two separate routes.

In addition to these studies of the mechanism of the

absorptive process, several workers have investigated the factors governing the control of iron absorption.

X. Humoral factors in iron absorption.

The way in which iron absorption from the gastrointestinal tract responds to changes in erythropoeitic activity (see page 31) suggests the existence of a humoral link between the marrow and the intestinal mucosa. However, in spite of several attempts, no such humoral mechanism for control of iron absorption has yet been demonstrated.

Beutler and Buttenweiser (1960) tried unsuccessfully to stimulate iron absorption in mice and rats by administration of plasma from iron-deficient animals. They also considered the possibility that the normal regulatory mechanism was an inhibitory one. This was disproved by the failure of normal rat plasma to depress absorption when injected into iron-deficient rats. They were also unable to alter absorption in normal animals by intravenous or intraperitoneal injection of homogenates of liver, kidney, spleen or bone marrow from iron-deficient rats. Although suggestive, these experiments certainly do not exclude the possibility of a humoral controlling agent. It must be remembered that the plasma is in continuous circulation and that the substance responsible for controlling absorption may be continuously removed or degraded and replaced. If this is true then the injection of 0.5 ml. plasma from an iron-deficient rat would probably be insufficient to demonstrate the presence of a humoral factor. A better way to show such a factor would be by

cross circulation between two animals but this has not yet been attempted. A further search for a humoral regulator of iron absorption was made by Fischer and Price (1963) but their studies yielded no decisive information on this score.

Two of the chief factors controlling iron absorption are the rate of erythropoeisis and the state of iron stores. The link between the sites of storage, utilisation and absorption of iron seems obvious - the circulating transferrin. This iron transport protein conveys iron between these various iron compartments and its iron content reflects chiefly the balance between iron received from storage depots and iron removed at sites of erythropoeisis. Transferrin by way of its saturation or some other property might therefore be able to carry information concerning the state of these two important controlling organs. There are several possible variables - the absolute concentration or total iron-binding capacity, the level of serum iron, the unsaturated iron-binding capacity, the percentage saturation of the transferrin, and finally the rate at which iron is removed from the plasma or clearance time. Each of these factors is, in general fairly stable and does not vary much between individuals. In man the serum is normally about one-third saturated, having a total iron-binding capacity of 300 µg. per 100 ml. and a serum iron level of 100 µg. per 100 ml. The half time for injected isotope is usually 90-120 minutes. In view of this it is not surprising that

interest in a humoral controlling agent has centred around the iron-binding protein of the serum.

The very efficient absorption of iron in iron deficiency - a condition characterised by an elevated total iron-binding capacity - led to the obvious conclusion that a high level of unsaturated transferrin might promote iron absorption (Laurell, 1951, 1952). This theory received impetus from the work of Greenberg et al. (1961) who found no correlation between absorption and any of the following factors:- the serum iron level, the plasma iron clearance and the utilisation of injected radioiron or anaemia. Excellent correlation was found between absorption and the unsaturated iron binding capacity. This led the authors to conclude that absorption is primarily influenced by the concentration of unsaturated transferrin in circulation. Further apparent support for this theory came from the very elegant studies of Hallberg and Sölvell (Hallberg and Sölvell, 1960; Sölvell, 1960). These authors modified the method of Saylor and Finch (1953) to study iron absorption in humans. Radioiron was given orally either as a single dose or as a constant intragastric infusion. A different isotope of iron was injected intravenously before giving the oral dose. Determination of the amount of each isotope in the blood at various intervals enabled the passage of iron both into and out of the plasma to be followed. Using this technique they found that subjects who absorbed iron normally no longer absorbed any of the oral isotope when the transferrin was saturated by intravenous administration of non-radioactive

iron. Intravenous administration of transferrin to patients whose absorption appeared to have ceased due to transferrin saturation caused an immediate increase in absorption. This interpretation of their data is dependent on several assumptions. Their assumption "that all absorbed iron is bound to transferrin" was found to be incorrect. Wheby and Jones (1963) demonstrated that when the transferrin saturation is over 60% in rats or 70% in dogs, most of the absorbed iron is deposited in the liver on its first circulation through the portal system. The fact that plasma bound radioiron infused into the portal circulation was not removed in this way suggested that iron entering the plasma sites of intestinal absorption is not already bound to transferrin under the circumstances. In a subsequent investigation Wheby (1963) made similar observations in iron absorptions studies in humans. Experiments in which the mesenteric blood vessels of an isolated loop of dog intestine containing radioiron were perfused (Pollack, Balcerzak and Crosby, 1963) provided further evidence that the iron entering the plasma was not transferrin bound. At normal serum iron levels radioiron absorbed into the plasma was bound to transferrin, and migrated as a discrete band on starch-gel electrophoresis. When the saturation of the transferrin was increased by an intra-arterial infusion of iron, however, there was a diffuse distribution of the radioiron in the effluent, indicating that much of the absorbed iron was not bound to transferrin. This study also showed the

absorption continued unchanged even though the saturation of the circulating transferrin was increased to near-saturation. The in vivo experiments of Wheby and Jones (1963) also indicated that raising the saturation of the iron binding protein above 60%, by intravenous infusion of ionic iron, had no significant influence on the amount of iron absorbed in rats. The experiments outlined above show that Hallberg and Sölvell were unable to detect absorption of isotope from the lumen when the transferrin was saturated because isotope absorbed into the plasma was removed with its first passage through the liver. The authors should have suspected this from one of their own experiments. When the saturation of the circulating transferrin, previously labelled with ^{59}Fe, was raised to saturation by constant intravenous infusion of iron the rate of clearance of ^{59}Fe remained unchanged whilst the rate of clearance of non-radioactive iron increased considerably.

Yuile et al. (1950) measured absorption of radioiron in three normal and four anaemic dogs, before and after artifically saturating the transferrin by repeated intravenous injection of small amounts of iron. Absorption was unaffected in the normal and in two of the anaemic animals. In the other two anaemic dogs absorption was slightly depressed, but remained greater than in normal animals. Increasing the plasma iron to normal levels in the anaemic animals had no effect on absorption.

Similar results were obtained in humans by Brown, Dubach and Moore (1958). They were not able to block absorption in normal volunteers by artifically saturating the circulating transferrin with intravenous saccharated iron oxide. The chief effect of this procedure was to decrease the proportion of absorbed radioiron incorporated into haemoglobin from 60% to 20%.

Bothwell, Pirzio-Biroli and Finch (1958) found no correlation between plasma iron concentrations and the amount of iron absorbed. They also demonstrated that saturating the serum iron of rabbits by injection of non-viable cells had no significant effect on absorption. In addition, absorption was not depressed by this procedure in phlebotomised humans.

It would appear from all this evidence that at the time of absorption the plasma iron-binding protein and its relative saturation play little or no part in controlling the amount absorbed. It is not surprising therefore that Heilmeyer et al. (1961) found that iron absorption was not decreased in a patient with congenital atransferrinaemia. A further demonstration that transferrin is not necessary for absorption is provided by the <u>in vitro</u> system of Jacobs et al. (1966). In this system radioiron was introduced into an isolated loop of rat duodenum and physiological saline solution containing dextran was perfused through the mesenteric blood vessels. The perfusing fluid contained no plasma and yet significant absorption was obtained. This system also provided evidence that the ultimate

mechanism controlling iron absorption resided in the small intestine itself. Increased absorption occurred in isolated loops from rats which had been venesected, while intravenous injection of iron 24 hours before preparation of the loop resulted in depressed absorption. The amount of iron absorbed at any time is therefore dependent upon some property of the small intestine itself.

XI. Recent theories of iron absorption.

The nature of the changes in the intestinal mucosa which control absorption remain to be elucidated.

Some light has however been shed on this by the work of Crosby's group. A theory concerning the mechanism responsible for the control of body iron balance was presented by Crosby (1965) at the Tenth Congress of the International Society of Haematology. His hypothetical treatment of the problem, based on results obtained by himself and his associates, is to a degree a return to the 'mucosal block' theory. The main feature in this theory is the major role played by the iron content of the mucosal epithelium in regulation of body iron equilibrium. It achieves this by playing a double role. It controls directly the absorption of dietary iron. In addition, at the end of the cell's life it is desquamated and a variable amount of iron is lost with it. In this way the mucosa operates as an organ for selective excretion of excess iron.

In order to assess the value of this theory it is necessary

to describe the experimental results on which it is based. Using a whole-body liquid scintillation counter Conrad and Crosby (1963) measured the retention of activity in iron-deficient and normal human volunteers after the oral administration of radioiron. They found that iron-deficient subjects not only absorbed a greater proportion of the test dose, but also excreted all the unabsorbed activity more rapidly than normal subjects. Since a carmine red marker was excreted at the same rate by both groups, the delay in normals could not be related to differences in intestinal motility. The authors suggested that this delay was due to retention of radio-iron within the intestinal epithelial cells. This sequestered iron remained in these cells and was lost from the body with the physiological desquamation of the cells. In iron-depleted subjects, on the other hand, all the iron entering the mucosal cells was absorbed into the body. These subjects therefore achieved equilibrium after the time required for the passage of unabsorbed iron from the body.

The presence of this sequestered iron within the mucosal cells was directly demonstrated in rats by autoradiography (Conrad and Crosby, 1963). Normal adult rats were killed between 2 and 48 hours after the oral administration of radioiron. Autoradiographs showed activity in the columnar epithelium of duodenum and jejunum. In the early stages most of the epithelium covering the villi was radioactive. With the passage of time the activity

moved distally so that between 12 and 24 hours only the distal half of the villus and by 36 to 40 hours only the tip was radioactive. Forty-eight hours after the oral dose no activity remained in the columnar epithelium. When the same procedure was carried out on either iron-loaded or iron-deficient animals, the sections showed that little radioactivity accumulated in the epithelial cells at any time.

A similar autoradiographic technique was used to demonstrate accumulation of parenterally administered iron in mucosal cells (Conrad, Weintraub and Crosby, 1964). Normal rats were killed at various times after intravenous injection of a dose of radioiron which was well within the binding capacity of the circulating transferrin. Accumulation of activity was demonstrated in the epithelium of the stomach, small intestine and colon, whereas little activity was found in the submucosa or muscular layers of these organs. In the animals killed 4 to 16 hours after injection, only the crypts and the proximal portion of the villi were radioactive. At later times the activity moved down the villi until by 72 hours all epithelial cells in the villi and crypts were labelled. 2 hours after injection 4.2% of the activity was localised in the small intestine. The activity increased to 5.8% at 24 hours and then declined rapidly until 72 hours when only 1.3% of the injected activity still remained. Radioiron similarly administered to iron-loaded rats gave

approximately the same results. However, when iron-deficient rats were used, the small intestine contained about one-half the amount of injected radioiron detected in normal rats. In addition, the percentage did not increase up to 24 hours as in normal and iron-loaded animals, but reached a maximum of 3.6% at 4 hours and then declined. They also noted that the percentage of the injected isotope going to the colon was dependent on the state of iron stores. 24 hours after injection 0.85% of the dose was found in this region in normal rats, whilst 1.32% was present in the colon in iron-loaded animals and 0.43% in iron-deficient animals.

These various experiments undoubtedly demonstrate that iron from the lumen is sequestered in the columnar epithelial cells in normal animals but not in iron-loaded or iron-deficient animals. This was confirmed by studies in which direct measurement of sequestered iron showed much lower values in iron-deficient and iron-loaded than in normal animals (Wheby and Crosby, 1963). The postulate that little iron enters the mucosal cells of iron-loaded animals and that all iron entering these cells in iron-deficient rats is transferred to the plasma seems reasonable enough. Iron from the plasma is incorporated into mucosal cells when they are formed in the crypts of Lieberkühn. This iron remains in the cell throughout its lifetime and eventually is lost from the body when the cell is desquamated. The percentage of injected radioiron incorporated into these cells in iron-depleted animals is

roughly one-half of that found in normal or iron-loaded animals.

The authors (Conrad et al., 1964) then demonstrated that absorption of iron from the lumen was depressed by parenteral administration of non-radioactive iron. The depression was most marked at the time when the concentration of parenteral iron in the mucosa was maximal (24 hours after administration). This was demonstrated using an intravenous dose of 250µg. of iron. They found that when larger amounts of iron were injected the quantity of iron sequestered by the small intestine increased, but not in proportion to the dose. There was a linear inverse relationship between the log of the quantity of iron administered between 10 and 1,000µg. and the percentage sequestered by the small intestine. This inverse exponential relationship resembled absorption studies where the percentage absorbed decreases, although the total absorption increases (Conrad and Crosby, 1962; Bothwell, Pirzio-Biroli and Finch, 1958).

Absorption studies carried out 24 hours after the intravenous administration of non-radioactive iron showed that absorption was inversely proportional to the quantity of iron administered intravenously. A linear relationship was demonstrated between the percentage absorption from the lumen and the log of the intravenous dose.

On the basis of these results Crosby and his group proposed a hypothesis to explain the intestinal control of iron absorption:-

> "Depending upon information concerning the body's requirement for iron, more or less of what may be called 'messenger iron' is incorporated into newly forming absorptive epithelial cells of the small intestine. A large dose of messenger iron reflects an iron-loaded state; such epithelial cells are able to reject almost all of the available dietary iron. In iron deficiency the cells are formed with little or no messenger iron, and through such cells there is relatively free passage of dietary 'iron' into the body. Intermediary states are represented by various amounts of messenger iron according to the requirements of the body.".

The demonstration that radioiron administered parenterally is incorporated into and remains in mucosal cells until they desquamate indicated that endogenous iron can be lost from the body via this route. Crosby et al. (1963) believe that this provides a mechanism for eliminating excess unwanted iron. Conrad et al. (1964) measured the rate at which injected radioiron is eliminated. Their results show a loss of 9% in the first week followed by a slower linear loss of radioactivity amounting to 0.2% per day. Iron-depleted rats lost less and iron-loaded rats more activity than normal animals in any period. In balance studies, using a low iron milk diet, normal animals had a mean daily loss of 11.6 µg. more iron in the stools than was consumed in the diet. The corresponding figure in iron-overloaded animals was 49.4 µg. This variation was shown to be due to non-haem iron losses since all animals, irrespective of their state of iron repletion, lost approximately the same amount of haemoglobin. Further evidence in support of the presence of an active mechanism for iron excretion was obtained from a case of transfusional siderosis. When the patient had recovered from his

anaemia he was venesected repeatedly to remove the excess accumulated iron. The patient became anaemic after removing only half the iron received from transfusions. The authors calculated that the rate of iron loss up to the time of phlebotomy must have been about 4 mg. per day. It should however be remembered that it is difficult to remove all the body iron by bleeding.

XII. Aim of the present investigation.

This investigation was undertaken to study the mechanism by which iron passes from the intestinal lumen through the mucosal cell to the plasma and the way in which various factors affect this mechanism. There has been some doubt concerning the role of ferritin in this process. The presence of ferritin in the mucosa of guinea pigs after massive doses of iron has been demonstrated by both Granick (1946a, 1946b) and Wöhler et al (1957). Gabrio and Salomon (1950) demonstrated the presence of ferritin in the small intestine of the horse, after oral administration of 30 g. of iron, as ferrous ammonium sulphate,

In all these studies the ferritin was shown to accumulate chiefly in the duodenum. The amount decreased distally along the length of the small intestine. Granick found traces of ferritin in the duodenum of untreated guinea pigs by immersing mucosal scrapings in 10% cadmium sulphate and counting the number of ferritin crystals formed. Gabrio and Salomon (1950), using a sensitive immunochemical technique, found ferritin in the duodenum of an untreated

horse. It should however be mentioned that these authors determined the nitrogen content of their precipitate and were, therefore, measuring both ferritin and apoferritin. The precipitate which they obtained in the untreated horse was very pale in colour and, as they point out, probably consisted mainly of apoferritin. Beutler (1963), using methods similar to those of Granick, and Brown and Rother (1963a), using an immunochemical technique, were unable to demonstrate the presence of ferritin in mucosa of rats during the absorption of physiological quantities of iron.

The first aim of the present study was to resolve the uncertainty concerning the presence or absence of ferritin in the mucosa during absorption of small doses of iron. Apart from establishing its mere presence, participation of ferritin in the absorptive process had to be demonstrated before moving to the next stage of the investigation, which involved a detailed study of its behaviour during absorption. This stage also involved a study of other forms of labelled iron present in mucosal cells during the absorption of radioiron.

The study was also instigated in order to obtain some idea of the way in which iron absorption is controlled to meet the body's requirements. A search was therefore made for the mechanisms by which factors such as the state of iron stores and the rate of erythropoeisis affect the absorptive process.

It must be stressed that the objectives of the present study were very similar to those of Crosby's group and that a number of the experimental designs were in some ways similar. Crosby's findings were published during the course of this investigation and our own results serve to complement and supplement those which he has reported. This is, however, fortuitous since the present study was undertaken independently at a time before any of Crosby's work had been published.

CHAPTER III.

DEVELOPMENT OF THE IMMUNOCHEMICAL METHOD AND SELECTION OF DOSAGE FOR ABSORPTION DETERMINATIONS

DEVELOPMENT OF THE IMMUNOCHEMICAL METHOD.

The present study was designed chiefly to reinvestigate the role, if any, of ferritin in the absorption of physiological doses of iron. To conduct such an investigation it is necessary to have both an effective means of isolating ferritin in a pure form from intestinal tissue and an extremely sensitive technique for measuring the very small amounts of iron involved. The high specific activity radioiron now available makes the accurate determination of such small quantities of iron possible. Isolation of ferritin is most easily accomplished immunochemically.

Such an immunochemical isolation involves the preparation of an antiserum to rat ferritin. This would theoretically involve the isolation of pure crystalline rat ferritin and preparation of an antiserum to it in some suitable species. Since this would require the sacrifice of many rats in order to obtain enough pure ferritin, it is indeed fortunate that rat and horse spleen ferritin have been shown to display excellent immunological cross-specificity (Mazur et al., 1960). It is therefore possible to use an antiserum to horse spleen ferritin for the preparation of rat ferritin. A brief outline of the isolation of crystalline horse spleen ferritin is given below (the detailed method of preparation is given on page 210)

I. Preparation of ferritin.

Horse ferritin was prepared from spleens. The spleens

were homogenized in water and the homogenate heated to 80°C to precipitate the majority of the non-ferritin proteins. After centrifugation the pH of the solution was adjusted to 4.6 and a further precipitate of proteins removed. The ferritin in the supernatant solution was crystallised with cadmium sulphate. The mother liquor was dark brown and still appeared to contain ferritin - this fraction was called non-crystallisable ferritin by Granick (1946c). The crystalline ferritin was dissolved in 2% ammonium sulphate and further purified by four subsequent crystallisations with cadmium sulphate. The cadmium sulphate was then removed by repeated precipitation with 50% ammonium sulphate, which in turn was removed by exhaustive dialysis.

The various stages in this preparation were studied by starch-gel electrophoresis. A sample of the heat supernatant solution was submitted to electrophoresis, together with the purified ferritin, and the so-called non-crystallisable ferritin. The starch-gel was divided in half by slicing through its thickness. One half was stained for protein with amidoschwarz and the remaining half was stained for iron with ferrocyanide (Figure 7). The final product was not homogenous. Three distinct bands could be seen with both amidoschwarz and ferrocyanide staining. The fastest migrating band accounted for most of the ferritin whilst the slower bands became progressively fainter each time by a factor of about ten. The proteins present in the initial heat supernatant solution which did not stain for iron were eliminated

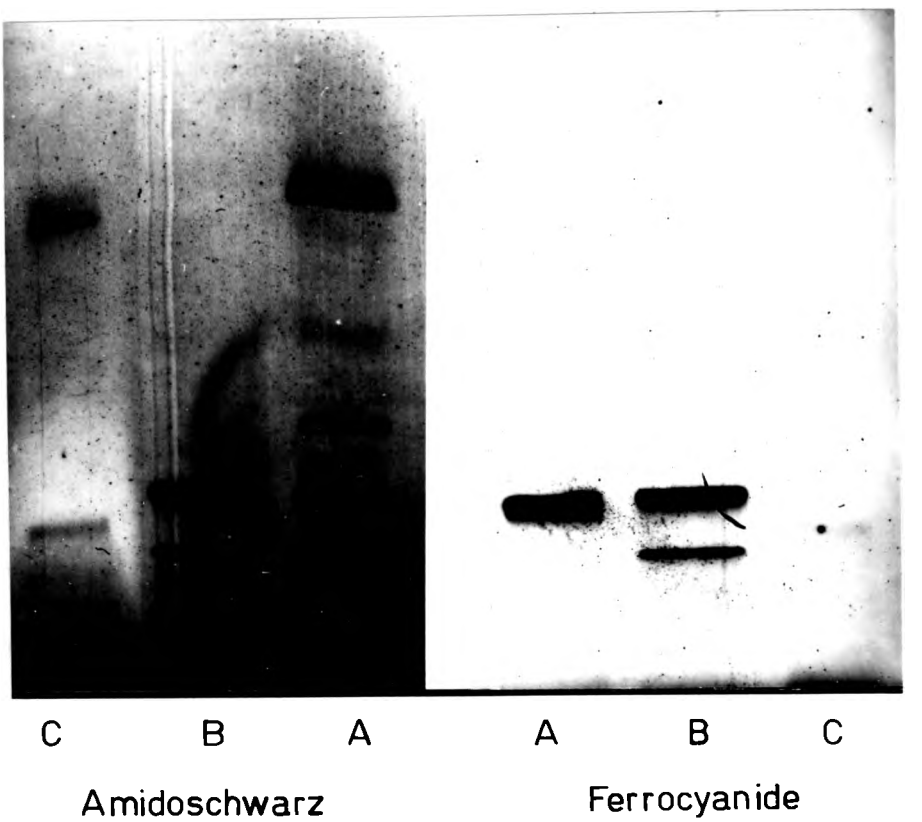


Figure 7.

Various stages in the isolation of ferritin (A = supernatant solution after heating to 80° C, B = purified ferritin and, C = non-crystallisable fraction) were examined by starch-gel electrophoresis. The gel was divided lengthwise and one-half was stained with the protein dye amidoschwarz, whilst the remaining half was stained for iron with ferrocyanide. The purified ferritin was not homogenous and three bands were visible with both stains.

during the purification. The non-crystallisable ferritin fraction showed two distinct protein staining bands, one of which also stained with ferrocyanide. This band had a mobility intermediate between the first and second ferritin bands.

The multiplicity of bands produced by starch-gel electrophoresis of purified ferritin has also been found by Theron et al. (1963) and Kop et al. (1963). In both reports the authors established that the various bands are not artefacts. This was done by extracting the bands from the starch-gel and submitting them to starch-gel electrophoresis a second time. In each case a single band having the same mobility as before was obtained. By using various concentrations of ammonium sulphate, Kop et al. were able to prepare from the pure ferritin a sample having only the major band. A sample in which the minor bands were considerably enriched was also prepared by these authors. They obtained the minor bands by precipitation at comparatively low ammonium sulphate concentrations (less than 22% at room temperature) and the main band chiefly by precipitation at higher concentrations (more than 22%). These authors pointed out that Mazur et al. (1950) (1955) found that ferritin fractions precipitating at lower ammonium sulphate concentrations have a higher iron content. This means that the major band has a lower iron content than the minor bands. Kop et al. confirmed this by showing on ultracentrifugation that the preparation enriched in the minor bands had a

distinctly higher sedimentation coefficient than the preparation containing only the major band. They also investigated the serological properties of these two preparations and found them to be the same.

II. Preparation of antiferritin.

The purified ferritin was prepared for injection into rabbits by alum precipitation (Proom, 1943) or by mixing with Freund's complete adjuvant (Freund et al., 1948). The rabbits received an initial intramuscular injection and booster doses prepared in the same way after each venesection. They were bled bimonthly and the plasma separated and stored at -15°C until required for use.
The antiserum produced in this way was shown to precipitate rat ferritin quantitatively. The other major iron-containing proteins, haemoglobin and transferrin were not precipitated.

III. Quantitative aspects of the precipitin reaction.

It is well established that in order to obtain quantitative precipitation it is necessary to have the antigen and antibody present in the correct proportions. This is particularly true when using horse antisera. This can be ascribed to the fact that when more than a certain amount of horse antiserum is added to an antigen no precipitate is produced since soluble compounds are formed. Such a serum is said to exhibit a prezone. Rabbit antibodies do not show a prezone; they do however, in common with other precipitating systems, exhibit the phenomenon of a 'postzone' or 'inhibition zone'. This

is the zone in which there is insufficient antibody to precipitate all the antigen present. In this instance the excess antigen inhibits precipitation.

The antihorse spleen ferritin prepared in rabbits behaved as expected for rabbit antiserum. When increasing amounts of ferritin antigen were added to a fixed amount of antiserum, the amount of ferritin precipitated rose to a peak and then decreased. When the amount of ferritin was kept constant and increasing volumes of antiserum were added, the amount of ferritin precipitated again increased to a maximum, but this amount was precipitated in all succeeding samples (Figure 8).

The antiserum was always used in excess in these studies. The titre was determined for the pooled plasma from each batch. The smallest volume to give maximum precipitation was taken as the titre for that batch of antiserum.

In carrying out the precipitin reaction in the radioactive studies carrier ferritin was added to the solution before adding the antiserum. This was necessary because the amount of ferritin present in the intestine under physiological circumstances is very small. The addition of carrier not only facilitated the handling of the precipitate but also provided a convenient method of checking that precipitation was quantitative. This was done by the chemical determination of iron on the final washed precipitate. This should be the same as that in the added ferritin carrier since the contribution of ferritin iron from the gut extract was very small in proportion to

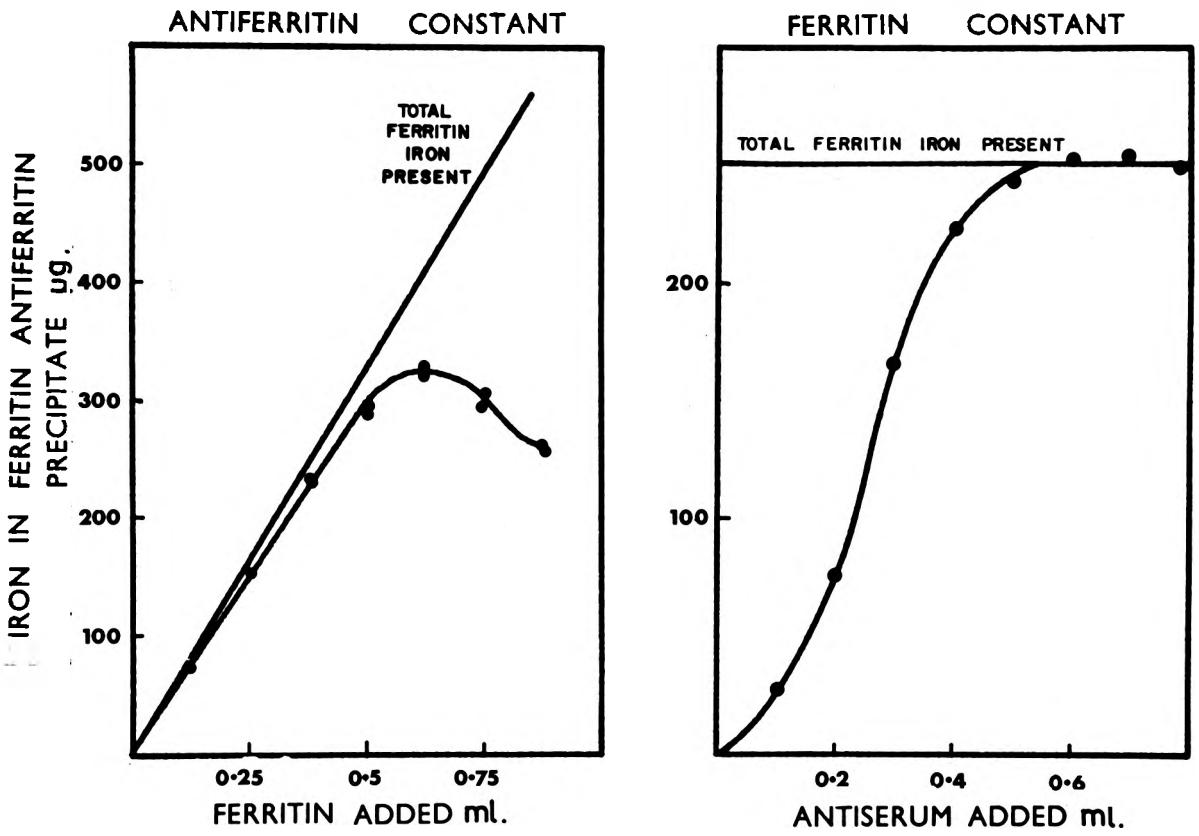


Figure 8.

The effect of antibody antigen ratio on the amount of ferritin iron precipitated.

that of the added carrier.

IV. In vitro labelling.

The addition of carrier was however not without disadvantages since, on investigation, it was found that radioactivity became attached to ferritin when free tracer radioiron was present. The tracer picked up in this way was fairly firmly bound and remained attached to the ferritin during electrophoresis, column chromatography and, most important, during precipitation with antiserum. Ferritin which had become labelled in this way was referred to as in vitro labelled ferritin.

A. Significance to present study.

Since the iron administered to the animals in these studies was in the form of ferric chloride, it seemed possible that, even after washing, the mucosal extract might contain free iron which could be taken up by the ferritin. It was therefore imperative to exclude in vitro labelling when assessing the true in vivo incorporation of iron into ferritin during absorption.

B. Prevention of in vitro labelling.

1. Citrate.

It was found that the addition of trisodium citrate to ferritin prevented to some extent the in vitro labelling. The degree to which in vitro labelling was prevented was dependent on the concentration of the sodium citrate (Figure 9). This prevention of in vitro labelling was real since quantitative

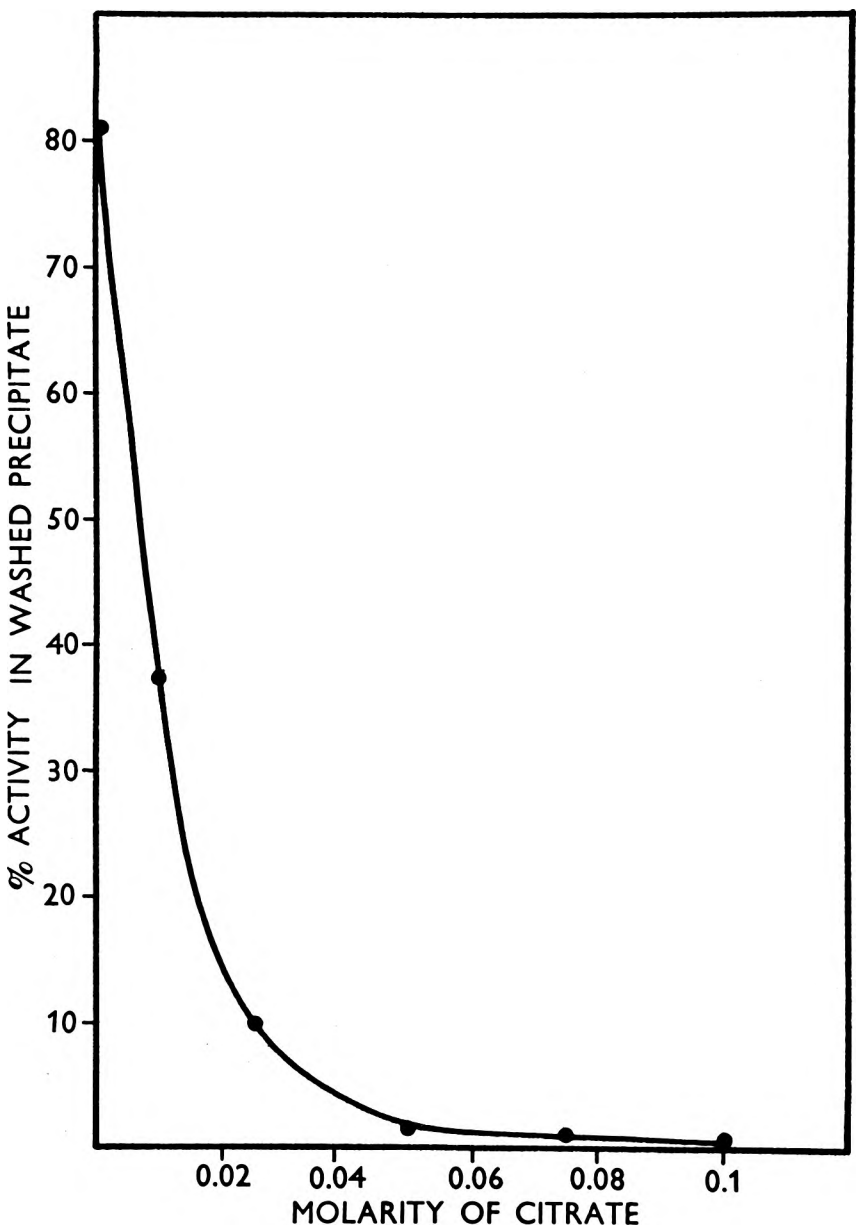


Figure 9.

The prevention of *in vitro* labelling by citrate.

precipitation of the ferritin occurred even in the presence of 0.1M sodium citrate.

2. Ethylenediaminetetra acetic acid (EDTA).

Since the prevention of *in vitro* labelling by citrate was so dependent on the citrate concentration, the effect of a powerful iron chelate, EDTA (K = 26.1), was investigated. This compound adjusted to either pH6 or pH7 was able to prevent completely the uptake of radioiron by ferritin at concentrations from 0.01M to 0.1M. This is shown in Table 2. in which *in vitro* labelling in the presence of various molarities of citrate and EDTA pH7 are compared.

Table 2.

Prevention of *in vitro* labelling by citrate and EDTA.

MOLARITY	% of counts attached to ferritin	
	Citrate	EDTA
0	81	95
0.01	36.6	0
0.025	9.7	0.25
0.05	1.8	0
0.075	1.1	0
0.1	0.6	0

V. Stability of *in vivo* labelled ferritin in presence of citrate and EDTA.

Since an agent was used in these studies to prevent *in vitro* labelling of ferritin, it was important to establish that radioiron incorporated into ferritin *in vivo* was not removed

by the chelate. Ferritin labelled with ^{59}Fe *in vivo* was therefore prepared (see methods) and crystallised together with carrier non-radioactive horse spleen ferritin. It was shown that concentrations of citrate or EDTA up to 0.1M did not remove any of the activity. Either of these chelates could therefore be used to prevent *in vitro* labelling without interfering with *in vivo* labelling. In the present investigation EDTA was chosen because it was effective at low concentrations.

VI. Removal of *in vitro* label from ferritin.

Although not germaine to this study, the ability of citrate and EDTA to remove ^{59}Fe from ferritin previously labelled *in vitro* was investigated. It was found that citrate in concentrations from 0.01M to 0.1M removed about 9% of such a label. The amount removed was independent of the concentration within this range. On the other hand, when EDTA pH6 was used the amount removed increased with increasing concentrations from 0.01M to 0.1M (Figure 10). The effect of pH on the removal of tracer from ferritin labelled both *in vitro* and *in vivo* was examined. Two samples of in vitro labelled ferritin were prepared, the tracer being added to the ferritin 24 hours and 15 minutes respectively before carrying out the experiment. The EDTA was adjusted to various pH values in the range 8 to 4.5 and added to the ferritin to give a final concentration of 0.05M. The percentage of activity remaining attached to the ferritin-antiferritin precipitate was determined and the result is shown in Figure 11.

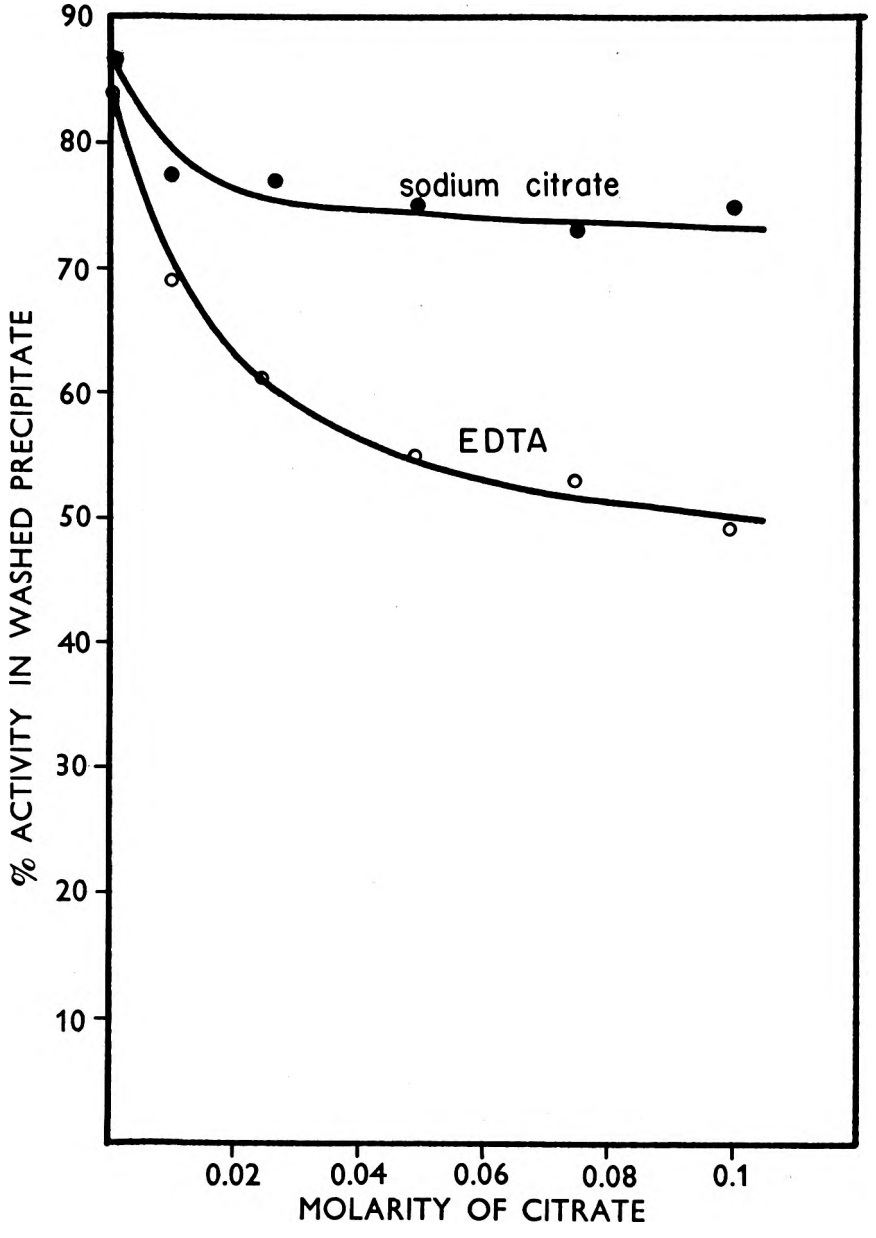


Figure 10.

The loss of radioactivity from *in vitro* labelled ferritin in the presence of various molarities of sodium citrate or EDTA pH6.

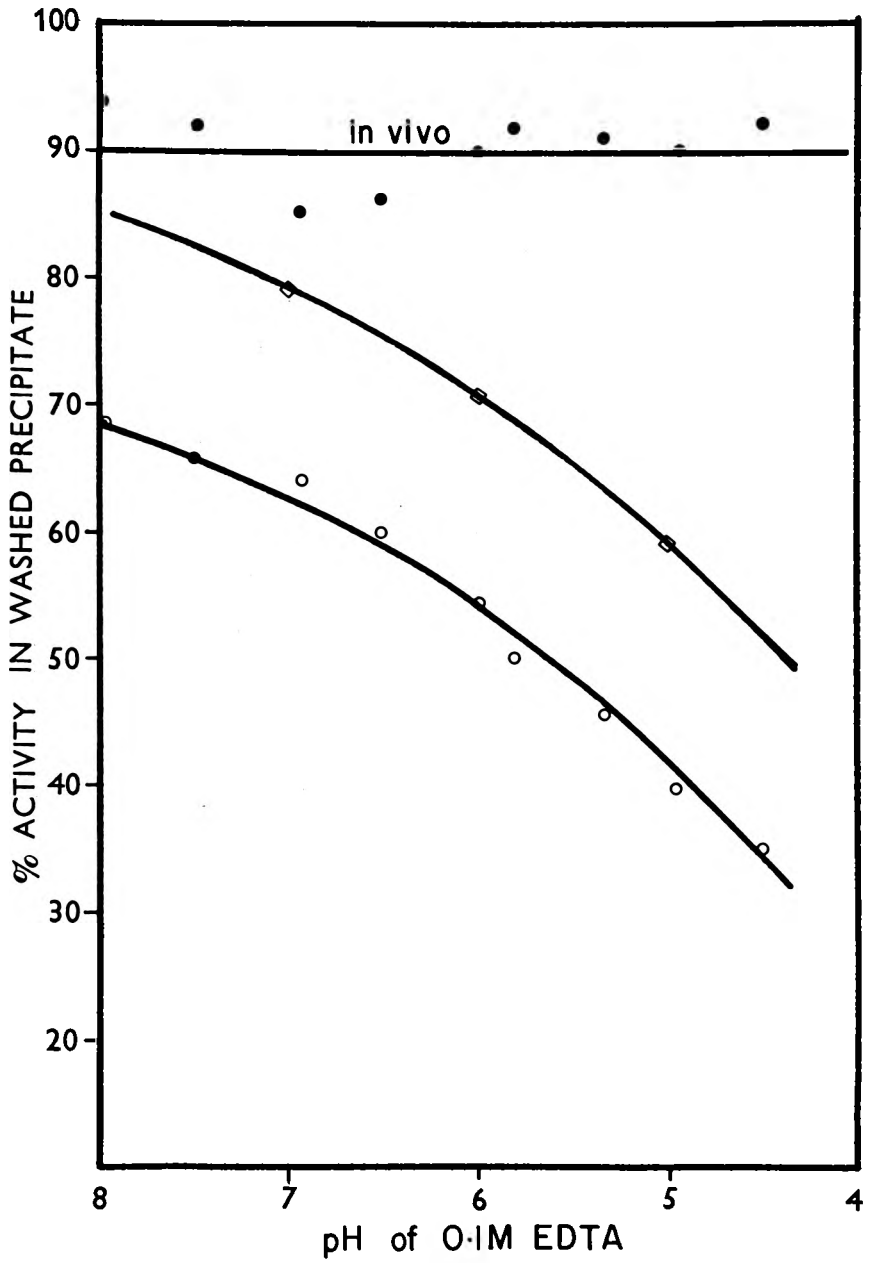


Figure 11.

The effect of pH on the ability of 0.05M EDTA to remove radioactivity from in vivo and in vitro labelled ferritin. Two samples of in vitro labelled ferritin were used. These were prepared by adding tracer radioiron to the ferritin 24 hours (diamonds) and 15 minutes (circles) before adding EDTA.

No activity was removed from *in vivo* labelled ferritin between pH4.5 and pH8. However the removal of tracer from *in vitro* labelled ferritin by EDTA increased with decreasing pH in both the 15 minute and 24 hour specimens.

VII. Summary of final method.

It has been established that rat ferritin is quantitatively precipitated by anti-horse spleen ferritin. In carrying out such a precipitation the formation of artefact, due to the presence of free radioiron, can be avoided by the presence of EDTA or citrate, neither of which are able to remove tracer incorporated into ferritin *in vivo*. The method used for the precipitation in these studies was based on these experiments. The small intestine was washed with 0.01M EDTA pH7 in physiological saline and homogenised in 0.1M EDTA pH7 in water. The homogenate was centrifuged at 9,000 x g and the ferritin in an aliquot of the supernatant solution was precipitated by addition of antiserum. Ratioactivity present in the washed precipitate provided a measure of ^{59}Fe- labelled ferritin.

VIII. Determination of iron absorption in this study.

Only part of the orally administered radioiron taken up by the mucosal cells is transferred via the plasma to the various parts of the body. In this investigation only the activity which reached the plasma was considered as 'Absorption'. Previous studies carried out by Hyde (1957) have included the mucosal radioiron content in calculating absorption. Both the present study and

those of Crosby and his co-workers have shown that this procedure cannot be justified, since a variable proportion of this mucosal iron is lost with the desquamation of these cells.

In view of the difficulties involved in investigating absorption of food iron this was not attempted; instead, small doses of inorganic iron were used. The work of Gitlin and Cruchaud (1962), described in the previous Chapter, indicated that the mechanism by which iron is absorbed is dependent on the magnitude of the oral dose. At low dosages active transport predominates, whilst the passive process becomes more important with increasing dosage. In most of the studies a dose of 10 µg. iron in a form of ferric chloride was selected as the standard dose and this was used throughout the investigation. When different doses were used for some particular reason the results obtained were, of course, not comparable with those obtained with the standard dose. Gitlin's curve for active transport is asymptotic at low dosages, whereas the curve for passive diffusion is linear; thus at the 10 µg. dosage level the contribution of passive absorption is negligible and can be completely disregarded.

An important criticism of isotopic techniques for measuring iron was raised by Garby and Sjölin (1959). These authors suggested that the exchange of iron across the intestinal barrier may not be entirely a one-way process. If two-way exchange does occur, the distribution of isotope would be affected by the relative size of the pools in lumen and

mucosa, and the amount of isotope gaining access to the body would not necessarily reflect the actual quantity of iron absorbed. There is, however, no evidence to support this suggestion. Indeed experimental work, although not conclusive, suggests that iron absorption is unidirectional. The movement of iron in only one direction is not unique since Bothwell et al. (1958) have demonstrated that iron transport across the placenta is definitely a one-way process.

Chappelle et al. (1955) found that the rate at which radioactivity was lost from animals whose storage iron was tagged was not increased when the luminal pool was raised by constant iron feeding. In a similar experiment carried out more recently, Pollack et al. (1964a) also found that a high iron intake did not accelerate the loss of activity from more or less randomly labelled rats. These experiments appear to exclude the possibility of iron moving simultaneously in two directions across the mucosal cell. These authors also carried out an experiment in which dog gut was labelled by intravenous injection of ^{59}Fe. The distal duodenum was perfused with saline in control dogs and with an iron-containing solution in others. The proximal duodenum was undisturbed. The activity in the proximal and distal segments was compared. No significant difference was found between the experimental and the control animals.

Another consideration relates to the quantity of iron administered. Pollack et al (1964a) carried out their absorptions using an oral dose of 250 µg. iron. In the

present investigation only 10 µg. of iron was normally given. It seemed possible that at this low dosage level an error might arise by isotope dilution in the iron pool within the mucosal cell itself. The present investigation showed that between 100 µg. and 150 µg. non-haem iron are present in the small intestine of the rat. Preliminary investigations suggest that only about one-third of this iron is present in the mucosal cells. The iron within these cells, mean life of which is one to two days (Leblond and Stevens, 1948) is lost when they desquamate. When absorption is determined with a 10 µg. dose and absorption is 10%, only 1 µg. iron is absorbed. If this iron mixes with the relatively large mucosal pool false results may be obtained since some of the non-radioactive iron initially present in the mucosal cell may pass into the animal. The amount of isotope entering the plasma would not therefore provide a true measure of the amount of iron 'absorbed'. This would lead to a similar error to the one postulated by Garby and Sjölin. The effect would be particularly noticable in experiments in which the animals had received an intravenous dose of inorganic iron on the day previous to determination of the absorption, since this would tend to increase the iron pool in the mucosal cells.

An experiment was designed to investigate this possibility. 40 rats were injected intravenously with radioiron. Half the animals received tracer only; whilst the other animals were given isotope, together with 100 µg. carrier iron.

16 hours later an oral dose of 250 µg. non-radioactive iron as ferric chloride was administered to half the animals in each group. After a further 2 hours had elapsed the animals were killed, and the activity present in the small intestine was determined. If iron entering the mucosal cell from the lumen had displaced iron already present in the mucosa, there should have been less radioactivity in the small intestine of the animals which had received the oral dose of non-radioactive iron than in those which had not. This was found not to be the case. The results, which are summarised in Table 3, show that feeding iron orally did not significantly reduce the percentage of injected activity present in the small intestine. Whilst not conclusive, this experiment supports the thesis that iron absorbed into the animal does not mix significantly with the mucosal iron pool en route.

Table 3.

Demonstration that absorbed iron does not mix with the mucosal iron pool.

Intravenous injection	Mean percentage of injected counts in small intestine	
	No oral dose of non-radioactive iron	250 µg. oral iron 2 hours before killing
Tracer only	2.790 (SE±0.162)	2.696 (SE±0.151)
100 µg. carrier	3.024 (SE±0.91)	2.968 (SE±0.093)

CHAPTER IV.

INVESTIGATION OF THE ROLE OF FERRITIN IN IRON ABSORPTION.

CHAPTER IV.

I. Demonstration of ferritin in the absorbing mucosa.

An attempt was made to demonstrate the presence of ferritin labelled with ^{59}Fe in the small intestine of a rat 1 hour after the oral administration of 1 µg. radioiron. The iron in the form of ferric chloride, labelled with 20 µc. ^{59}Fe was administered by means of an oesophageal cannula to a rat which had been starved for 24 hours. An hour after administration of the radioiron the upper 10 cm. of small intestine was removed and washed well with 0.01M EDTA pH7 in physiological saline. The washed segment was homogenised in 1.5 ml. 0.1M EDTA pH7 in water. The homogenate was carefully heated to 70°C in a water-bath and, after cooling, was centrifuged at 99,000 x g for 10 minutes. The presence of minute amounts of ferritin labelled with ^{59}Fe in the heat supernatant solution was indicated by the following observations.

A. Sephadex G 200 chromatography.

Columns of Sephadex G 200, a molecular sieve compound, are able to separate molecules of differing size up to a maximum known as the exclusion limit, which for this grade of Sephadex is molecules having a molecular weight of about 200,000. Molecules larger than the exclusion limit, such as ferritin (MW 490,000 - 800,000 according to the iron content) pass through the column unhindered and emerge after the void volume of the column has been collected.

Both pure crystalline ferritin and the heat supernatant solution were chromatographed on the same Sephadex G 200 column. This was carried out by placing 0.5 ml. ferritin solution on a column which had been equilibrated with M/150 phosphate buffer pH8. The ferritin was washed into the column with 6 ml. phosphate buffer before putting an aliquot of heat supernatant solution on to the column. The column was then eluted with phosphate buffer. The activity in the heat supernatant solution emerged from the column 6 ml. later than the pure ferritin, which could easily be seen due to its colour. It appears therefore that some of the activity in the heat supernatant solution was associated with a large molecular weight molecule, which might be ferritin.

B. Starch-gel electrophoresis.

Starch-gel electrophoresis was carried out on an aliquot of the heat supernatant solution together with two other samples. These were *in vitro* labelled ferritin (prepared as described in Chapter III.) and a sample prepared by mixing the heat supernatant solution with an amount of *in vitro* labelled ferritin having approximately the same radioactivity. The electrophoresis was carried out for 7 hours at 6 volts/cm. using the buffer system described by Haut et al. (1962). An autoradiograph of the starch-gel showed a single band of radioactivity in the same position in each of these samples (Figure 12). It was therefore established that the radioactivity present in the heat supernatant

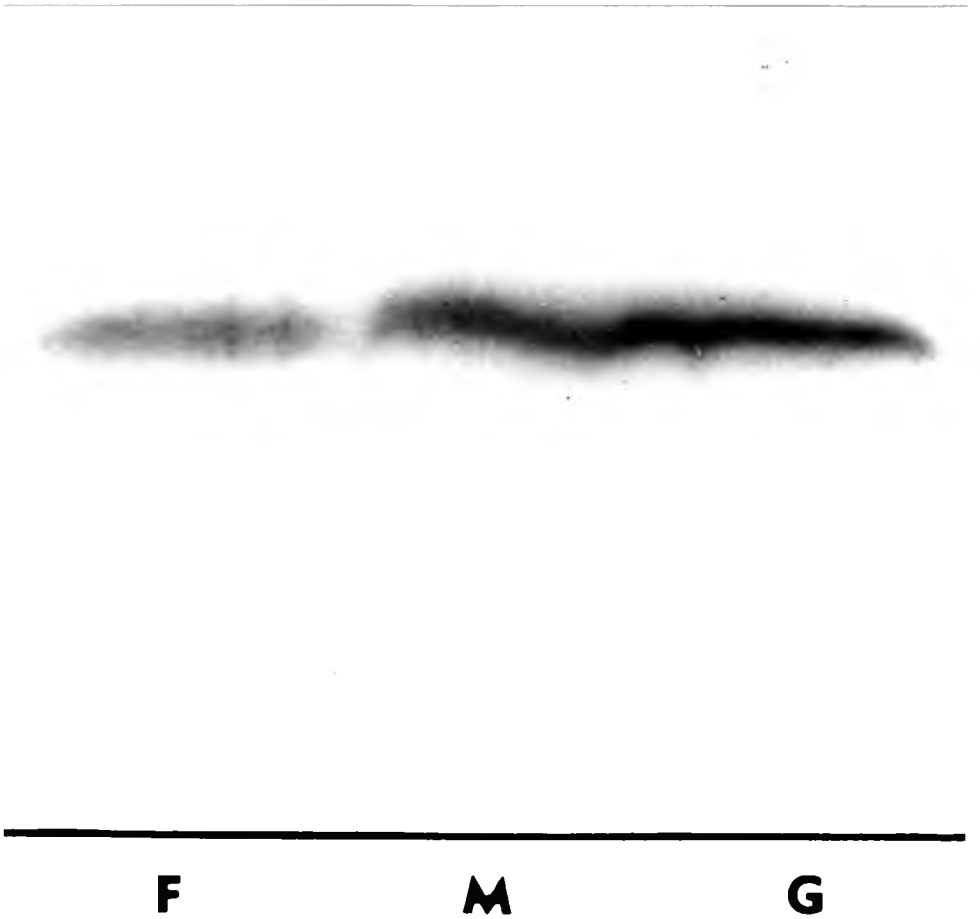


Figure 12.

Autoradiograph showing the behaviour of ferritin and mucosal heat supernatant solution on starch-gel electrophoresis.

F. *in vitro* labelled ferritin.

G. mucosal heat supernatant solution.

M. a mixture of F and G.

solution was associated with a substance whose electrophoretic characteristics, under these conditions, were identical with those of ferritin. It was of interest that only a single band of radioactivity was seen since ferritin normally exhibits three or four discrete bands on starch-gel electrophoresis (Figure 7). This was probably due to the fact that the major band accounts for 90% of the ferritin. In addition, evidence presented later in this Chapter indicated that ferritin radioactivity in the mucosal heat supernatant solution was associated preferentially with the relatively iron-poor fraction which constitutes this major band.

C. Gel diffusion.

A further sample of the heat supernatant solution was used in a modified Ochterlony agar-gel procedure. In this technique antibody and antigen placed in separate wells in an agar plate diffuse towards each other through the gel. Where the diffusing fronts meet a precipitin band is formed. The position and shape of this band is determined by the rates of diffusion of the respective proteins. In addition, when more than one antigen well is used, the way in which the two precipitin bands meet enables one to tell whether the antigens in the two wells are identical or not.

In this experiment antiserum to horse spleen ferritin was placed in the central well (Figure 13) and aliquots of the heat supernatant solution were placed in two of

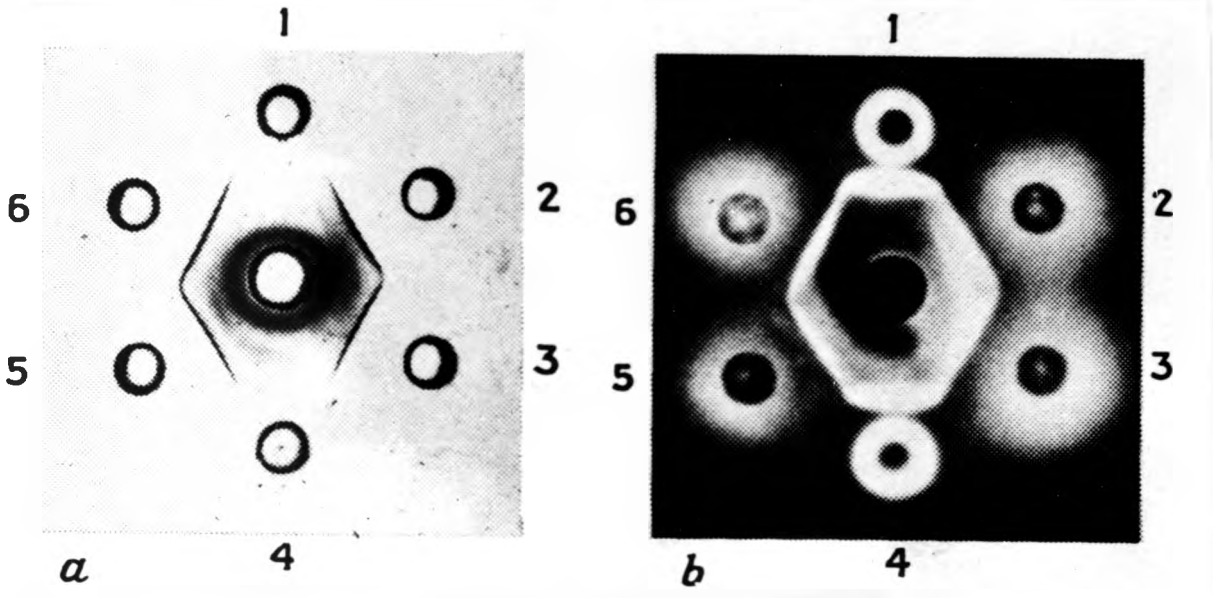


Figure 13.

Agar-gel diffusion plate, wells 1 and 4 contain duodenal homogenate; wells 2, 3, 5 and 6 contain ferritin. Although no lines can be seen opposite the wells containing heat supernatant solution from the duodenal homogenate (a), the autoradiograph (b) reveals bands of radioactivity coalescing with the bands opposite the ferritin wells. This demonstrates the presence of radioactive ferritin in the mucosa.

the peripheral wells. The remaining four wells were filled with a solution of *in vitro* labelled horse spleen ferritin having roughly the same amount of radioactivity as the heat supernatant solution. After 3 days visible lines of precipitation could be seen opposite to the wells containing the horse spleen ferritin, but no such lines were visible opposite those containing the heat supernatant solution. At this stage the radioactivity not present in the precipitin bands was removed by washing the gel for 3 days in repeated changes of distilled water. The gel was then allowed to dry, giving a very thin transparent film An autoradiograph prepared by exposing the dried gel to X-ray film revealed the presence of bands of radioactivity opposite the wells which had contained heat supernatant solution. These bands coalesced with the bands opposite the cups which had contained horse spleen ferritin. In spite of the absence of a visible precipitin band, therefore, this autoradiograph provided very strong evidence for the presence of minute amounts of ferritin labelled with ^{59}Fe in the heat supernatant solution

II. Dependence of iron incorporation into ferritin on metabolic energy.

The validity of these observations was established in a further experiment in which 10 µg. ^{59}Fe was introduced into the lumina of guts which had been removed from 3 rats 24 hours previously and stored at 4°C. After

incubation at 37°C for 30 minutes the guts were subjected to the usual procedures. Although 28%, 7% and 35% of the radioactivity was present in the washed guts, only 0.1%, 0.0% and 0.03%, respectively, of this activity was attached to ferritin. When radioiron was injected into a duodenal loop of a living rat in the same way, 40% of the activity was associated with the washed gut and 5.5% of this was attached to ferritin.* These results indicated that incorporation of ^{59}Fe into ferritin is an active metabolic process. The need for metabolic energy in iron absorption was also demonstrated, in our laboratory, by using an artificial solution to perfuse an isolated loop of gut (Jacobs et al., 1966).

III. Ultracentrifuge studies.

Mazur, Litt and Shorr (1950) have shown that centrifugation of a solution of ferritin results in its separation into iron-rich and iron-poor fractions. An experiment was therefore devised to determine the relationship between the ^{59}Fe-labelled ferritin in a mucosal extract and these fractions after centrifugation.

A mucosal extract was prepared by harvesting the mucosal cells from the upper 20 cm. of small intestine 30 minutes after administering an oral dose of 10 µg. labelled iron

* This high mucosal uptake of iron occurs only when the radioiron is placed in a loop and is not accompanied by any increase in either absorption or incorporation into ferritin. It seems likely that the additional iron is non-specifically adsorbed to membranes. (Jacobs et al. 1966).

to a starved rat. These cells were washed with 0.01M EDTA in physiological saline pH7 and homogenised in 0.1M EDTA pH7. The homogenate was heated to 75°C and centrifuged at 9,000 x g for 20 minutes. The supernatant solution from this centrifugation was divided into two aliquots and carrier ferritin was added to each aliquot. One aliquot was centrifuged for one hour at 35,000 r.p.m. in the 40 rotor of a Beckman Spinco Model L ultracentrifuge which produces the following r.c.f. values:-

Top of tube	52,000 x g
Middle of tube	86,730 x g
Bottom of tube	110,800 x g

After centrifugation the pale supernatant solution was separated from the dark brown pellet. The precipitate was redissolved in water. The ferritin in the aliquot which had not been ultracentrifuged and in both the supernatant solution and redissolved pellet was then precipitated with antiserum. The antiferritin-ferritin precipitate was dark brown in the 9,000 x g supernatant solution and in the redissolved pellet and the precipitate from supernatant solution was almost white . The results obtained from three rats treated in this way are shown in table 4.

Table 4.

Distribution of ferritin activity after ultracentrifugation.

	Percentage of mucosal counts present as ferritin.		
R A T	100,000 x g	110,000 x g precipitate	110,000 x g supernatant solution
1	44	29	12.5
2	40	22	20
3	36	16	17.5

When pure ferritin was centrifuged in the same way 80% of the ferritin iron content was present in the precipitate. Since a mean of only 57% of the radioactive ferritin was found in the ultracentrifuge precipitate, it follows that the activity must preferentially attach to ferritin molecules having a low iron content as such molecules do not sediment as easily as the iron-rich ferritin molecules. It is thus apparent that the radioactive iron entering the mucosal cell is largely incorporated into ferritin molecules having a low iron content. An alternative explanation would be that the iron entering the mucosal cell stimulates apoferritin formation, and that the iron is then incorporated into the newly formed apoferritin molecules.

IV. Change in the distribution of mucosal radioactivity with time.

The proportion of radioactivity present as ferritin in

the mucosal extract was then studied at various times after oral administration of radioiron.

To compare the ferritin content soon after ingestion with findings at a later stage, a short length of small intestine was used. This was necessary because if a long length were used the slow passage of the radioiron down its length would mask short term changes occurring in individual mucosal cells. It has been reported that the duodenum is the most active area in iron absorption (Duthie, 1964). To test this the mucosal uptake and the proportion precipitable with antiferritin were determined in the various anatomical regions of the small intestine. The intestines were removed from 7 rats 7 hours after the administration of a 10 µg. dose of labelled ferric chloride. After washing, the proximal 10 cm. of small intestine was removed, and the remainder divided into two, the upper 25% and the lower 75%. The mean uptakes were 9.3% in the first 10 cm., 4.1% in the upper quarter of the remainder and 3.0% in the rest of the small intestine. Respectively, 63%, 66% and 37% of this activity was attached to ferritin. This is shown in Table 5.

Table 5.

Relative capacities of various area of small intestine to take up oral iron and incorporate it into ferritin.

	Mean uptake (%)	Percentage of total uptake in small intestine	Percentage of mucosal activity in ferritin
Upper 10 cm.	9.3	57	63
Proximal $\frac{1}{4}$ of remainder	4.1	25	66
Distal $\frac{3}{4}$ of remainder	3.0	18	37

Since more than half the total mucosal uptake occurred in the proximal 10 cm. of small intestine this section was selected to characterise radioactivity in ferritin at various times after the oral administration of radioiron. A 10 µg. dose of radioiron was administered to 5 groups of 9 rats. The animals were sacrificed after 10, 30 and 60 minutes, 4 and 24 hours respectively, and 10 cm. lengths of gut from the pylorus distally were removed. After washing with 0.01M EDTA (pH7.0) in saline the specimens were homogenised in 0.1M EDTA (pH 7.0) and the labelled ferritin content was determined on aliquots of heat supernatant solution. The mean proportion of the radioactivity present in the washed intestine precipitable with the ferritin antiserum in the animals killed 10 minutes after administration was 31%. By 30 minutes this figure had risen to 58% and by 60 minutes to 75%. During this period there was little change in the total mucosal activity. The increase in labelled ferritin was thus

accompanied by a corresponding diminution in the non-ferritin component. At 4 hours the pattern resembled the 1 hour distribution, the mean percentage identifiable as ferritin being 78% (Figure 14). However, by 24 hours there was little residual radioactivity in the washed intestine. This experiment established that after the oral administration of radioiron the proportion of activity in the duodenal mucosa associated with ferritin increased with time (up to 1 hour) at the expense of the non-ferritin fraction. Studies were then carried out in order to investigate whether the proportion of mucosal activity present as ferritin changes when absorption is stimulated or depressed.

V. The effect of various factors on mucosal ferritin formation.

Absorption of iron is markedly influenced by various factors discussed in Chapter II. The way in which these factors altered the proportion of oral iron incorporated into ferritin is discussed in the following section.

A. Size of dose.

In the normal human subject it has been established that, although the percentage absorption drops with increasing dosage, there is a progressive rise in the absolute amount of iron absorbed (Bonnet et al., 1960; Bothwell, Pirzo-Biroli and Finch, 1958; Smith and Pannacciulli, 1958). This relationship holds for a wide range of doses (15 µg.-400 mg.). This pattern of absorption was examined in the rat using doses from 10 to 10,000 µg.

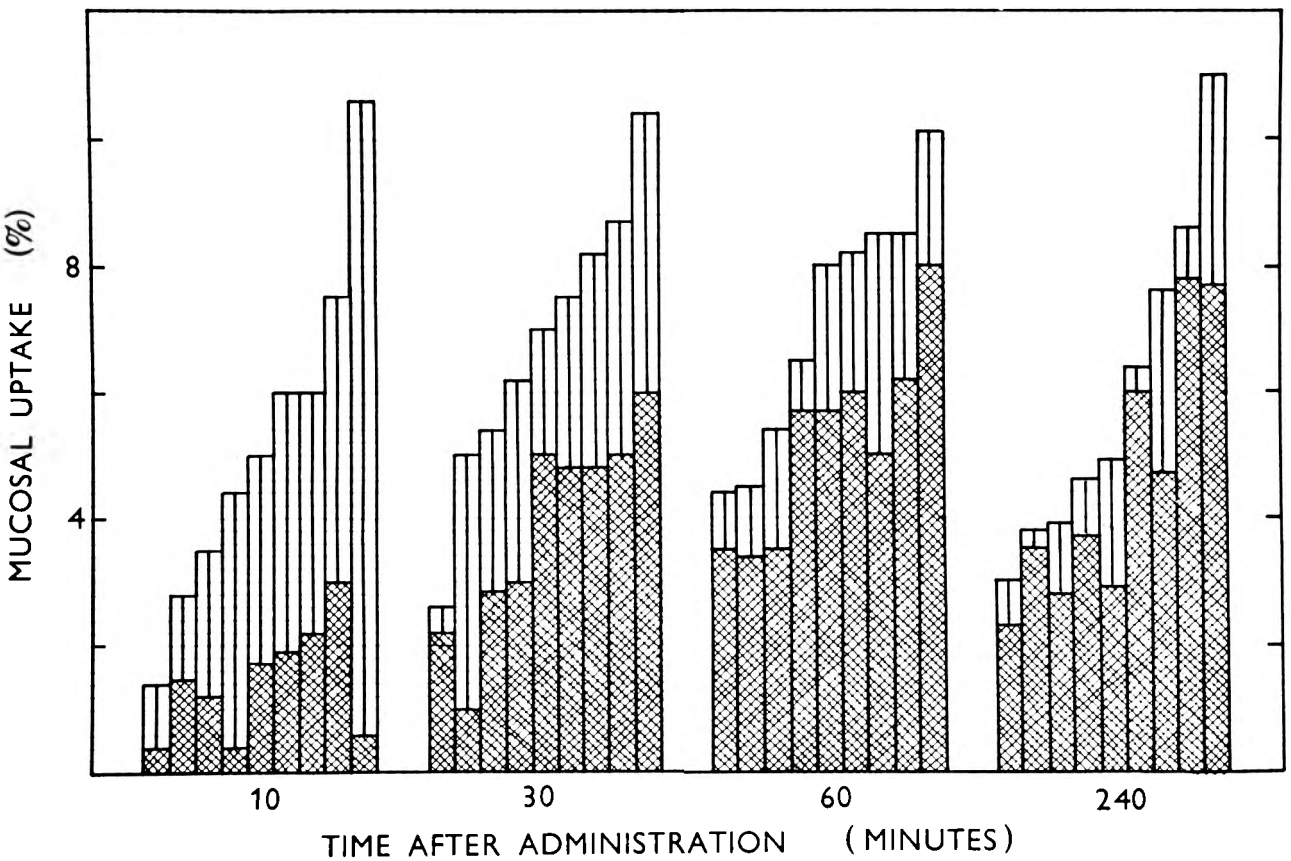


Figure 14.

Labelled ferritin (hatched) and non-ferritin activity in the upper small intestine at various intervals after administration of 10 µg. of radioiron.

the result obtained was essentially the same as in humans (Figure 15); the mean absorptions increased from 1.2 µg. at the 10 µg. dosage to 8 µg., 35 µg. and 500 µg. at the 100, 1,000 and 10,000 µg. dosages respectively. These represent percentage absorptions of 12, 8, 3.5 and 5 respectively. Similar results have been obtained in mice by Beutler and Kelly (1962).

The pattern of mucosal radioiron distribution was studied on 2 groups of 10 rats. The groups received doses of 100 µg. and 10,000 µg. iron respectively. The animals were sacrificed after 5 hours and the whole small intestine removed, opened and washed with 0.01M EDTA pH7 in 0.9 NaCl solution. The mean percentages of mucosal activity precipitable as ferritin were 45% (S.E. 4.7) with the 100 µg. dose and 12% (S.E. 1.3) with 10,000 µg. Although the percentage of the administered dose present in bowel at this time was less with the larger dose (a mean of 3.1% (S.E. 0.3) compared with 8% (S.E. 0.8)), this figure represents a much greater quantity of iron (310 µg. compared with 8 µg). Similarly the mean labelled ferritin content of the intestine was 30 µg. in the group given 10,000 µg. of iron, and 3.5 µg. in the animals given 100 µg. The corresponding mean percentages of the administered dose absorbed beyond the mucosa were 3.7% (S.E. 0.7) and 19.9% (S.E. 2.7) (370 and 19.9 µg.). The results are summarised in Table 6.

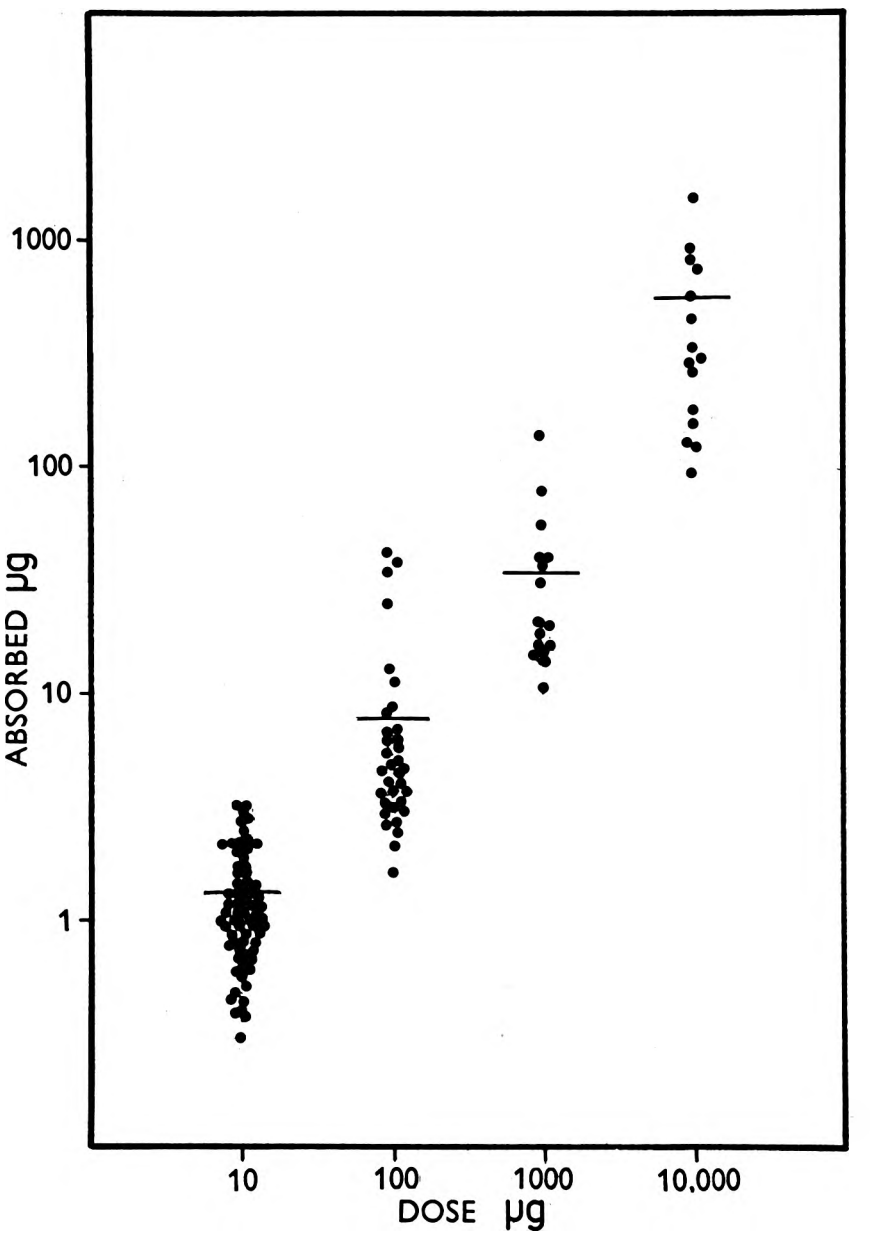


Figure 15.

The relationship between the amount of iron absorbed and the dose given.

Table 6.

The effect of dosage on incorporation of oral iron into ferritin.

Dose µg.	Percentage of dose in mucosa	Percentage of mucosal activity in ferritin	µg. Fe in gut	µg. Fe as ferritin in gut	Absorption (%)	µg. Fe absorbed
100	8	45	8	3.5	19.9	19.9
10,000	3.1	12	310	30	3.7	370

B. Chemical form of administered iron.

Several authors have shown ferrous salts to be better absorbed in man than ferric salts (Moore et al., 1944; Hahn et al., 1945; Brise and Hallberg, 1962). This applies only to fairly large doses since no difference in absorption was demonstrable at low dosage (1 µg./Kg.) (Bonnet et al., 1960; Bannerman et al., 1962).

In the present study absorption was most frequently assessed using an oral dose containing 10 µg. labelled iron in the form of ferric chloride.

Experiments carried out using 10 µg. doses of ferrous sulphate, ferrous ascorbate and ferric citrate showed that both the percentage absorption and the distribution of the mucosal activity at various times after administration were not significantly different from those obtained with ferric chloride at this dosage level.

C. State of iron stores.

Enhanced absorption of iron from both salts and food has been demonstrated when tissue iron stores are low. On

on the other hand decreased absorption occurs when iron stores are high (Chapter II - pages 30 and 31).
In the present study rats raised on a low iron diet showed a marked enhancement in absorption accompanied by depression of the proportion of a test dose incorporated into ferritin. 10 rats were raised from the weanling stage on a low iron diet. 5 of these animals received a 10 µg. dose of radioiron and the remaining 5 received a 100 µg. dose. At the same time 5 animals raised on a standard laboratory diet (19 mg. Fe/100 g.) were given a 10 µg. dose. The amounts absorbed were determined by killing the animals 2 hours after the oral administration of the iron and counting the carcasses after removing the entire gastrointestinal tract, care being taken not to spill the contents. The small intestines were washed with 0.01M EDTA in saline, and the activity present in ferritin determined using the precipitin reaction. The result of this experiment is shown in Figure 16.
The mean absorption in the control group was 8.6% (S.E. 1.5) and 20.6% (S.E. 4.2) of the administered dose was present in the washed mucosa. This mucosal activity was made up of 15% (S.E. 2.5) ferritin and 5.6% (S.E. 5.2) non-ferritin. The iron-deficient rats on the other hand had a mean absorption of 77.2% (S.E. 2.4) and only 1% (S.E. 2) of the dose was present as ferritin in the mucosa. The remaining non-ferritin activity in the mucosa accounted for 3.2% (S.E. 2.0) of the administered dose. The iron-deficient animals which received the

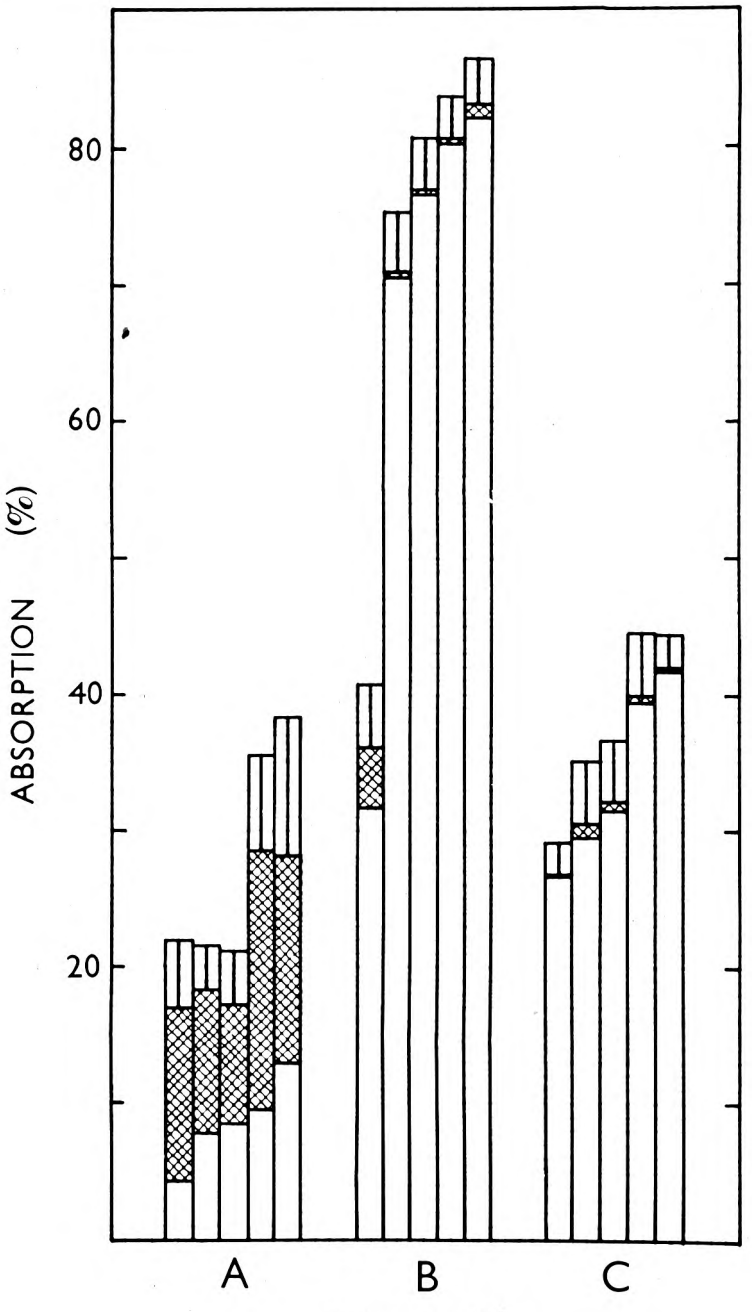


Figure 16.

The absorption and distribution of gut radioactivity after oral administration of radioiron to normal and iron-deficient rats.

Clear columns — percentage in carcass.

Cross hatching — mucosal ferritin activity.

Vertical line — mucosal non-ferritin activity.

A. Normal rats given 10 µg. of iron

B. Iron-deficient rats given 10 µg. of iron

C. Iron-deficient rats given 100 µg. of iron

100 µg. dose absorbed 33.6% (S.E. 2.4), and the activity in the washed mucosa consisted of ferritin 1% (S.E. 0.3) and non-ferritin 3.5% (S.E. 0.3). These results are summarised in Table 7.

Table 7.

Absorption and distribution of gut radioactivity after oral administration of radioiron to rats fed normal and low iron diets.

DIET		Normal	Low iron	Low iron
Dosage		10 µg.	10 µg.	100 µg.
		Percentage of administered dose		
Absorption		8.6	77.2	33.6
Washed gut	Ferritin	15	1	1
	non-ferritin	5.6	3.2	3.5

D. Changes in erythropoeitic activity.

The proportion of orally administered radioiron incorporated into mucosal ferritin was investigated in rats whose rate of erythropoeisis had been altered. Erythropoeisis was stimulated either by venesection or by injection of acetyl-phenylhydrazine. The latter treatment stimulates erythropoeisis by causing the haemolytic destruction of a proportion of red cells. Depression of the erythropoeitic rate was achieved by hypertransfusion of compatible viable packed red cells. The absorption and distribution of mucosal activity 30 minutes after an oral dose of 10 µg. radioiron in normal, venesected and

polycythaemic rats is shown in Figure 17. The mean absorption at 30 minutes was 6.5%, 43% and 1% for the control, venesected and polycythaemic groups respectively. Of the administered dose 4.5%, 0.5% and 2% was present in the mucosa as ferritin. This experiment again demonstrated decreased incorporation of a test dose into ferritin when the absorption is enhanced. It is also interesting to note that a lower percentage of the administered dose was found in the mucosa of the polycythaemic rats as compared with the normal control animals.

VI. Fate of labelled mucosal ferritin.

In order to investigate the role of mucosal ferritin in the absorptive process, several experiments were performed. Firstly, an attempt was made to determine whether iron incorporated into ferritin within mucosal cells can subsequently enter the plasma. Ten µg. iron as $^{59}FeCl_3$ was administered to 25 rats. After 4 hours, 13 animals were sacrificed, the whole small intestine removed, washed, and the labelled ferritin content of the mucosa determined. The radioactivity of the carcasses was also assessed to discover how much iron had passed beyond the mucosa at this time. The remaining 12 animals were killed twenty-four hours after administration of the iron, and the gastro-intestinal tracts removed, The radioactivity still present in the washed small intestines was of negligible amount, but that present in the carcasses was determined as before. The mean proportion of the administered dose present in the

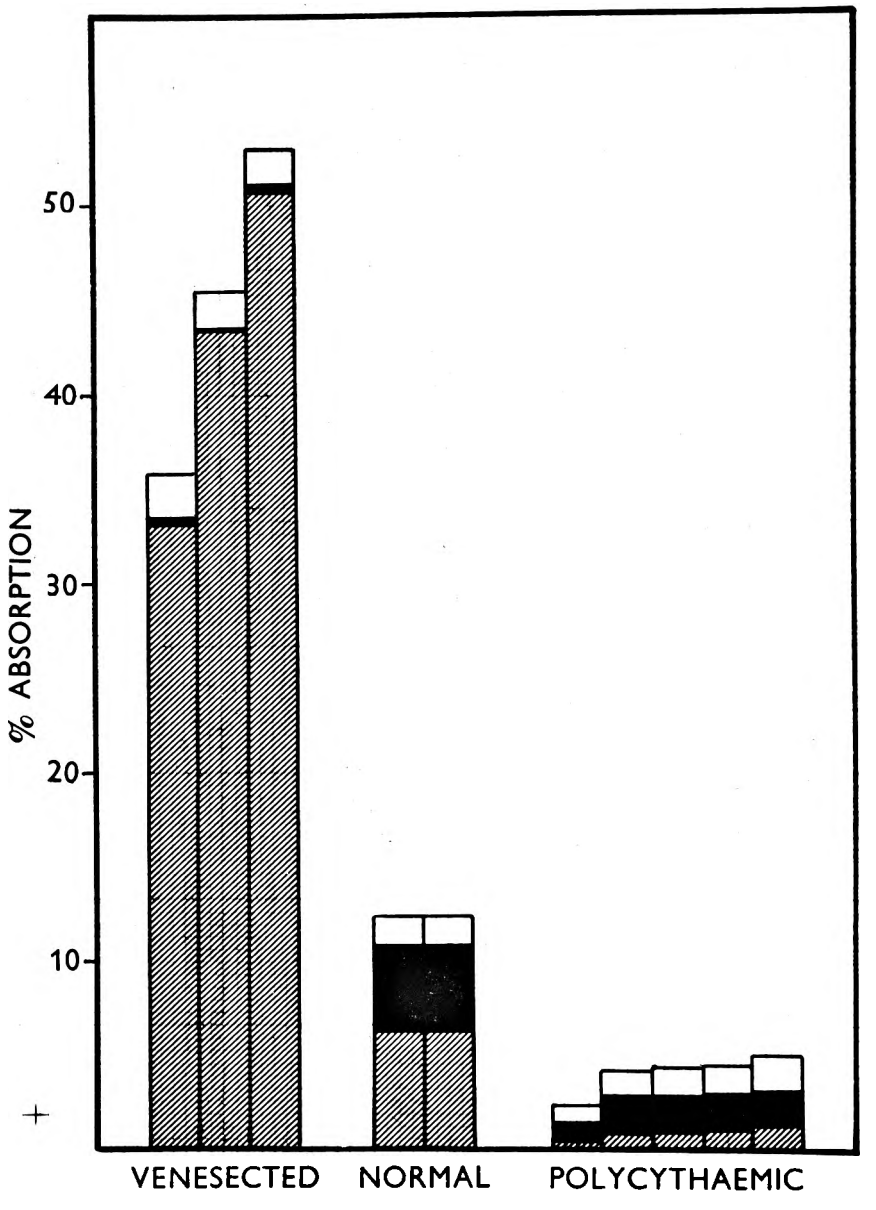


Figure 17.

The effect of venesection or induced polycythaemia on absorption and distribution of mucosal radioactivity 30 minutes after the oral administration of 10 µg. radioiron.

Cross hatched	-	Radioactivity absorbed.
Shaded	-	mucosal radioactivity in ferritin.
Unshaded	-	non-ferritin mucosal radioactivity.

carcasses at 4 hours was 23.9% (S.E. 5.6) compared with 39.1% (S.E. 5.6) at 24 hours. The mean percentage present in the washed small intestine at 4 hours was 8.5% (S.E. 1.0), and of this 69% (S.E. 5.2) was ferritin. These results, although inconclusive ($p < 0.1$), suggest that some of the labelled iron within the mucosa 4 hours after administration may subsequently pass into the body. The fact that the sum of the mean mucosal and carcass activity at 4 hours was less than the mean activity present in the carcass at 24 hours may be partly ascribed to the loss of mucosal cells during the initial washing procedures (see Methods).

When a larger dose of iron was given (100 µg.) the results were somewhat different. A mean of 28.8% (S.E. 4.3) of the administered dose was in the carcasses of 9 animals at 4 hours, a mean of 6.3% (S.E. 1.0) being in the washed intestines; 74% (S.E. 6.0) of the intestinal activity was precipitable as ferritin. However, after 24 hours only 30.2% (S.E. 5.0) was in the remaining 9 carcasses. On repeating this experiment at a later date a similar result was obtained. On this occasion the mean activity of 11 carcasses was 10.5% (S.E. 3.8) at 4 hours, compared with 11.0% (S.E. 3.5) in the remaining 10 animals at 24 hours. At 4 hours 3.7% (S.E. 0.4) was in the washed intestines and of this 75% (S.E. 3.7) was present as labelled ferritin. To demonstrate more convincingly this incomplete transfer of mucosal activity, the absorption of 10 µg. iron was studied in 8 animals overloaded with iron by intramuscular

injection (see Methods). The last injection of iron-sorbitol citrate was given the day before the labelled dose was administered. The mean activity in the carcasses of the 4 animals sacrificed at 2 hours was 3.3%, compared with 35% in the washed small intestines. Sixteen and a half percent of the oral dose was present as labelled ferritin. However, in the remaining 4 animals killed at 24 hours, the mean activity in the carcasses was still only 4.7%, although the intestinal activity had fallen to 2.9% (Figure 18). These total absorptions at 24 hours were considerably reduced when compared with carcass figures obtained in a control group of 4 normal rats, which were also killed at 24 hours. The mean absorption in these animals was 19.6%. In further studies it became apparent that marked depression in iron absorption only occurred in iron overloaded animals which had received parenteral iron up until 24 hours prior to study. When several days had elapsed after the last injection less striking differences were noted. For example, in one experiment absorption was studied in 10 animals which had received their last injection 7 days previously. The mean absorption was 8.3% (S.E. 1.7) as compared with 12.6% (S.E. 1.3) in a control group of 10 animals. It seems therefore that the fate of iron sequestered within mucosal cells is dependent on various factors. When the amount of iron given orally is small, some of this iron may pass into the body between 4 and 24 hours. When larger doses are administered to normal animals or small doses to animals that have recently received parenteral iron,

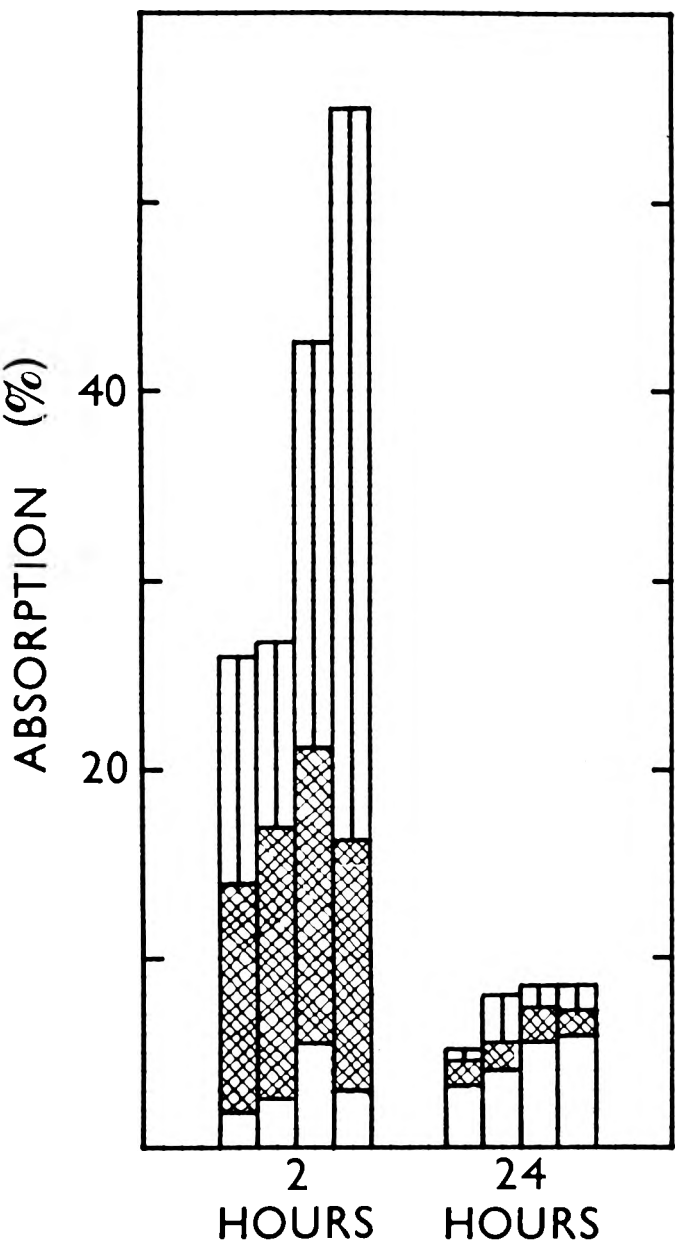


Figure 18.

The absorption (clear columns) and distribution of mucosal uptake (hatched = ferritin; vertical line = non-ferritin) of 10 µg. radioiron given orally to rats loaded with iron by repeated intramuscular injections. In these animals iron taken up by the mucosa did not pass into the animal but was lost with the desquamation of the mucosal cells.

no late movement of iron into the body could be detected. These findings indicate that the sequestration of iron within mucosal cells may subserve an important regulatory function when physiological amounts are given. In situations where the need for iron is increased (e.g. after repeated venesections), absorption is increased and little iron is retained in mucosal cells. In contrast, increased amounts of iron are trapped when the need for iron is reduced, and this iron is lost from the body as the mucosal cells exfoliate. These findings are summarised in Table 8.

Table 8.

The fate of isotope incorporated into ferritin.

Hours after Administration	Dose µg.	Percentage of dose in Carcass	Intestine	Percentage of intestine counts in ferritin.
4	10	23.9	8.5	69
24		39.1		
4	100	28.8	6.3	74
24		30.2		
4	100	10.5	3.7	75
24		11.0		
2	10	3.3	35	47
4	loaded	4.7	2.9	

CHAPTER V.

INVESTIGATION OF THE NON-FERRITIN MUCOSAL ACTIVITY.

CHAPTER V.

In the previous chapter it was demonstrated that radio-iron entering the mucosa from the lumen is incorporated into two fractions. One of these fractions decreased with time whilst the other, which was shown to be ferritin, increased. Investigations concerning the nature of the non-ferritin fraction are reported in this chapter.

The non-ferritin component in the present study behaved in a similar manner to the early rapid-transit fraction described by Brown and Rother (1963a). In addition, two-dimensional paper chromatography of an ultrafiltrate of gut homogenate showed the presence of leucine, phenyl alanine, tyrosine, β-alanine, alanine, histidine, lysine, glutamic acid, glutamine, cysteine, methyl serine and particularly serine and glycine. These are very similar to the amino acids found in mucosal extracts by Brown and Rother (1963a). For this reason attempts were made to identify an iron amino acid complex in the homogenate.

I. Dialysis.

The rapid-transit fraction of amino-acid bound iron described by Brown and Rother was dialysable. In the present study a dialysable fraction was found only when EDTA had been used in washing or homogenising the gut. The activity in a supernatant solution from a gut washed with physiological saline and homogenised in water had no dialysable fraction. This was definitely established

by dialysing under various conditions:

(a) against running tap water;

(b) against distilled water;

(c) against various buffers.

Provided the pH of the system remained between 5.5 and 8.2, no dialysable radioiron fraction was present.
When EDTA was used in preparing the gut extract, a dialysable fraction was invariably found.

II. Demonstration of an Fe-EDTA complex in the presence of EDTA.

EDTA was frequently used to prevent *in vitro* labelling of ferritin during the preparation of mucosal extracts. In these studies the non-ferritin activity (which was not dialysable in the absence of the chelate) was rendered dialysable by addition of EDTA. It seemed probable that this dialysable fraction consisted of an Fe-EDTA complex. This was established by paper chromatography and paper electrophoresis. The mucosal supernatant solution from a gut which had been homogenised in 0.1M EDTA pH7 was submitted to paper chromatography using the phenol system, and to paper electrophoresis using barbital buffer. A synthetic ^{59}Fe-EDTA complex was submitted to these procedures at the same time. The results (Figures 19, 20) show that in both separations radioactivity in the mucosal extract migrated to the same position as the synthetic Fe-EDTA complex. It is therefore apparent that when the mucosa is handled in this way the activity

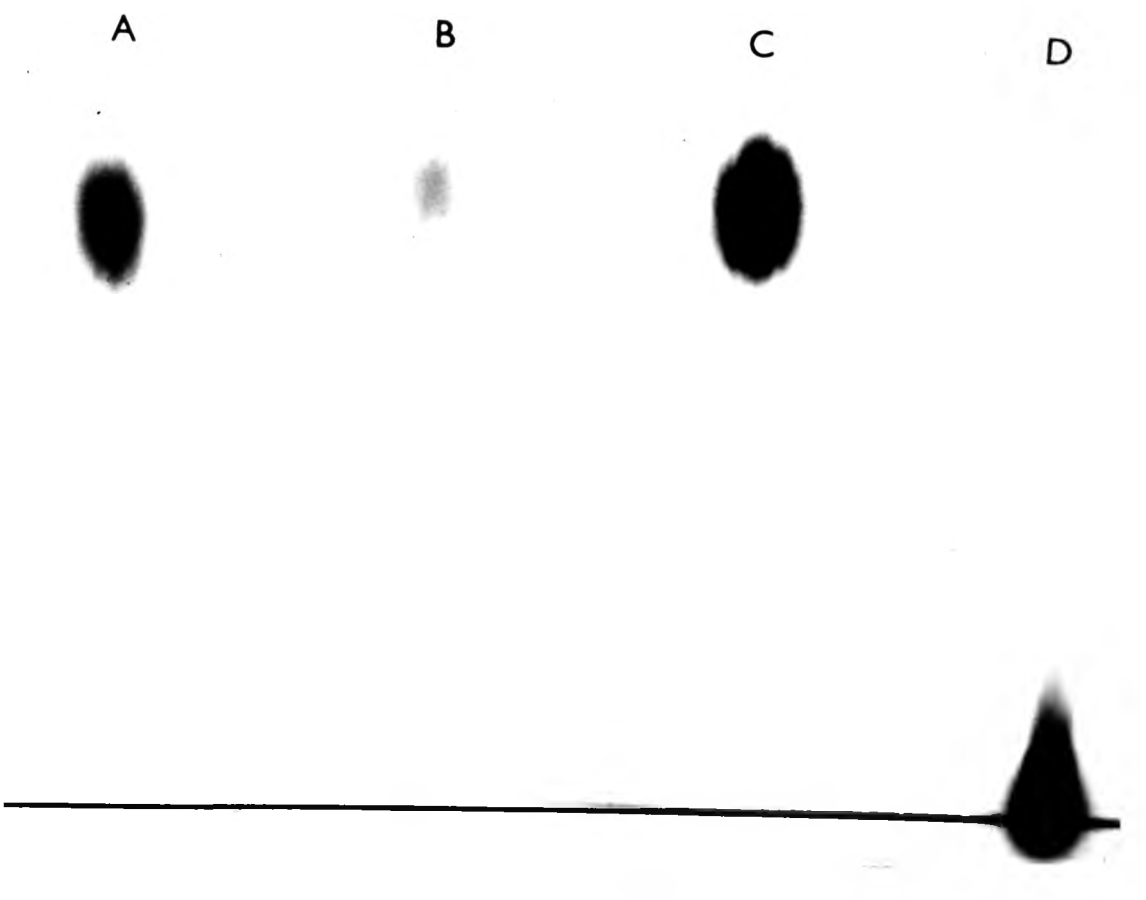


Figure 19.

An autoradiograph showing the chromatographic demonstration of an iron-EDTA complex in mucosal extracts prepared with this chelate.

A. Extract prepared in 0.01M EDTA.

B. Extract from a mucosa washed with 0.01M EDTA.

C. ^{59}Fe-EDTA.

D. Mucosal extract prepared without chelate.

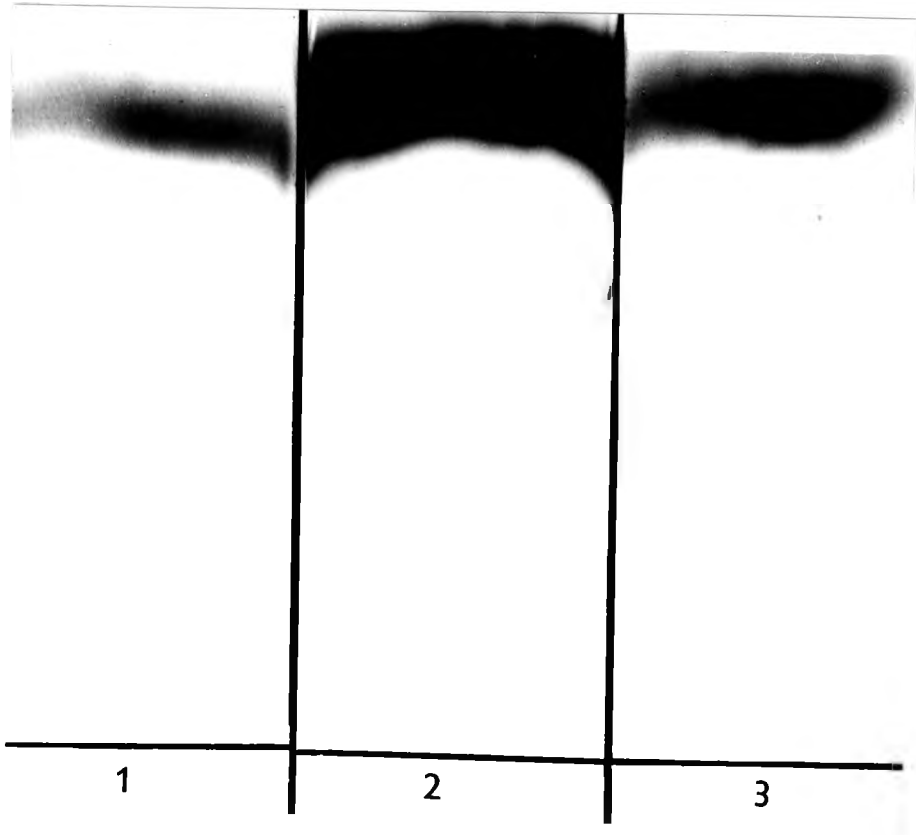


Figure 20.

An autoradiograph showing the electrophoretic demonstration of an iron-EDTA complex in mucosal extracts prepared with this chelate.

1. Extract prepared from gut washed with 0.01M EDTA.
2. ^{59}Fe-EDTA in non-radioactive mucosal extract.
3. Extract prepared in 0.01M EDTA.

in the mucosal extract consists of a mixture of ferritin and EDTA bound radioiron.

III. Absence of an iron-amino acid complex.

Although the lack of a dialysable fraction in the absence of EDTA appeared to exclude the possibility of an iron-amino acid fraction, paper chromatography and paper electrophoresis were carried out with the systems which Brown and Rother used to demonstrate the labelled amino acids. Extracts were prepared from rat mucosa 10 minutes after an oral dose of radioiron. The solvent system described for paper chromatography (96% Ethanol:water: cyclohexane 70: 30: 10) was found to be biphasic. The upper layer, which was very small, was discarded. Using the lower layer, the amino acids present in the radioactive mucosal supernatant solution migrated, but the activity remained at the origin. When chromatographed in the two-dimensional system (Butanol : acetic acid followed by phenol) the activity in the mucosal extract also remained at the origin, whilst the amino acids, including serine and glycine, migrated to their appropriate positions. Electrophoresis of the supernatant solution in 0.05 M sodium acetate buffer pH 4.8 also failed to show the presence of migrating amino acid-bound radioactivity. In this system the activity once again remained at the origin.

IV. Explanation of Brown's results.

The experiments described above showed that when mucosal cells harvested during the absorption of ^{59}Fe are washed

with saline and homogenised in water, no labelled dialysable fraction can be found in the supernatant solution. In addition, paper electrophoresis and paper chromatography failed to demonstrate the presence of amino acid-bound radioiron.

What then is the explanation for Brown and Rother's observations? The possibility of the chelate 'Fe-3-Specific', which they used to wash the mucosa, acting in a manner similar to EDTA was considered. 'Fe-3-Specific' is the monosodium salt to N-N Dihydroxy-ethylglycine, an amino acid derivative, which gives a purple colour with ninhydrin. The complex ^{59}Fe-Fe-3-Specific had almost the same Rf value as serine and glycine in Brown's Alcohol:water:cyclohexane system. Mucosal cells harvested during the absorption of iron were washed with this chelate and rinsed with saline before homogenising. Chromatography of the extract prepared in this way gave ninhydrin-staining radioactive spot having the same Rf value as serine and glycine (Figure 21). Paper electrophoresis of this extract, using an 0.05 M Sodium Acetate buffer pH 4.8, produced a radioactive ninhydrin-staining band migrating to the same position as a ^{59}Fe-Fe-3-Specific complex produced synthetically. In a similar aliquot of mucosal cells washed with saline and homogenised in water, the activity remained at the origin (Figure 22). The rapid-transit fraction described by Brown as an amino acid iron complex appears to be an artifact produced by his experimental procedure. The nature of the true rapid-transit fraction therefore still remains unidentified.

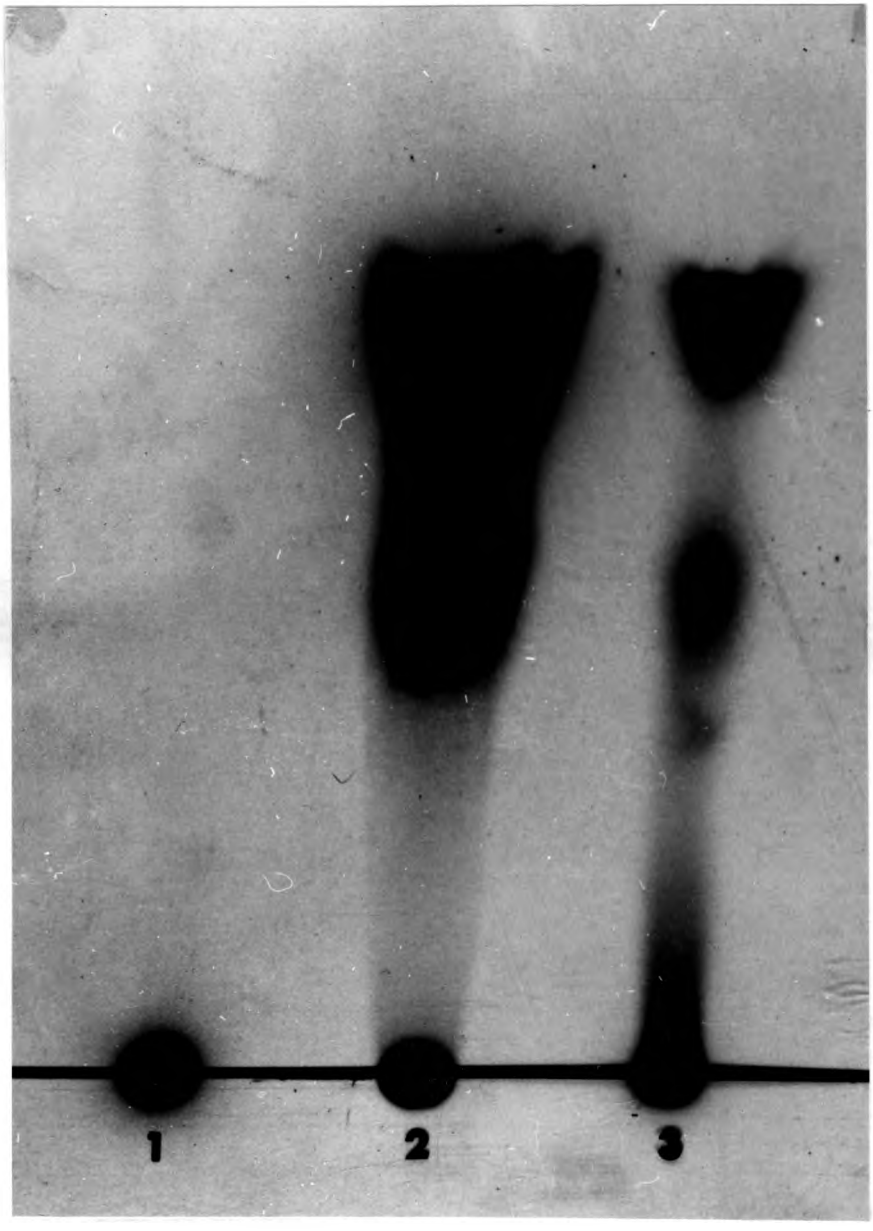


Figure 21.

Chromatographic demonstration of an iron-versene-Fe-3-Specific complex in an intestinal loop washed with this chelate - An autoradiograph showing the paper chromatographic separation of the following solutions:

1. The supernatant solution from a loop washed with saline and homogenised in water.
2. A ^{59}Fe-Versene Fe-3-Specific complex.
3. The supernatant solution from a loop washed with 0.05 M Versene-Fe-3-Specific followed by saline and homogenised in water.

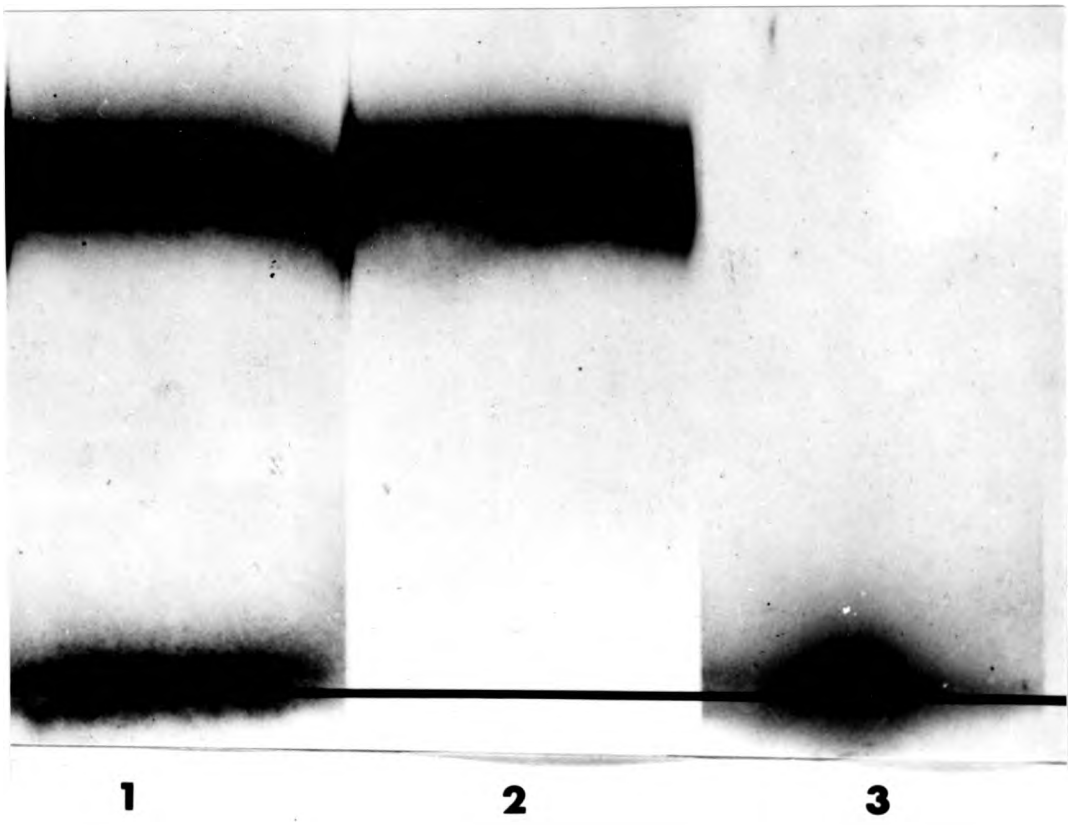


Figure 22.

Electrophoretic demonstration of an iron-Versene Fe-3-Specific complex in an intestinal loop washed with this chelate - An autoradiograph showing the paper electrophoretic separation of the following solutions:

1. The supernatant solution from a loop washed with 0.05M Versene Fe-3-Specific followed by saline and homogenised in water.
2. The ^{59}Fe-Versene-Fe-3-Specific complex.
3. The supernatant from a loop washed with saline and homogenised in water.

Washing the intact mucosa with 'Fe-3-Specific' also produced a dialysable fraction not present in its absence. Chelation by this substance was further demonstrated by chromatography on Sephadex G75 columns. When the chelate was present, two distinct peaks were obtained whereas in its absence only a single peak due to ferritin was found.

V. Column chromatography.

The nature of the radioactive complexes formed in mucosal cells during the absorption of radioiron was investigated by chromatographic fractionation of mucosal extracts. Sephadex G 200 should be ideal for the separation of the various labelled components in the mucosal extract, since such a column was shown to separate mixtures of ferritin, transferrin and haemoglobin (Figure 23), the major known soluble iron-containing compounds. The incomplete separation of haemoglobin from transferrin was not an obstacle in the present investigation because iron is not likely to be incorporated into haemoglobin during absorption. However, Chromatography of a mucosal extract on a sephadex G 200 column gave only a single peak of radioactivity, which emerged after the 'hold-up' volume of the column. If the counts per fraction are plotted on semilogarithmic graph paper (Figure 24) it is apparent that the single eluted peak was not homogeneous, but consisted of three distinct fractions. It is obvious, however, that the main fraction accounts for almost all the activity present in the eluate.

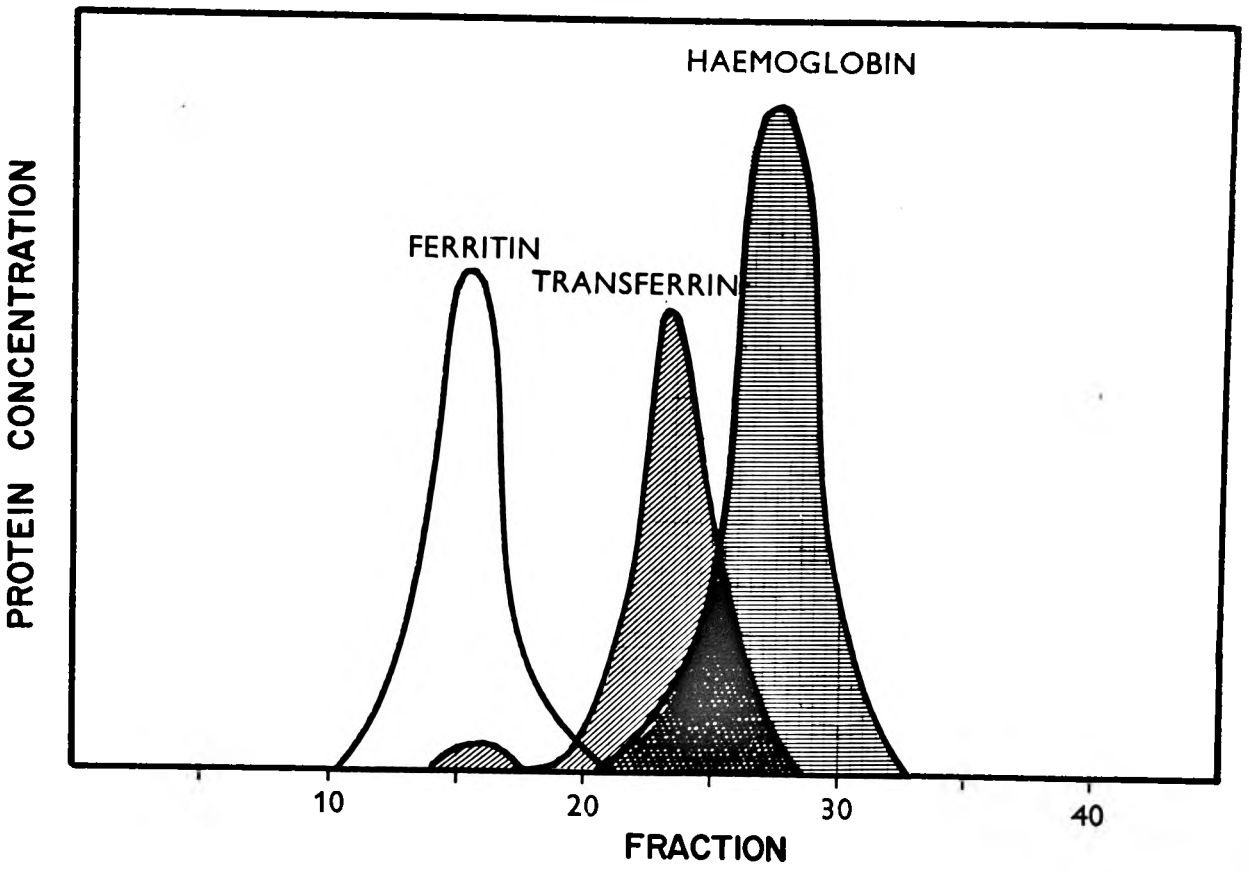


Figure 23.

Separation of iron containing proteins on a Sephadex G 200 column. Ferritin and transferrin are well separated although the ferritin appeared to pick up about 3% of the radioactive iron originally bound to transferrin. The haemoglobin is not completely separated from transferrin but this is not important in these studies since radioiron is not likely to be incorporated into haemoglobin during absorption.

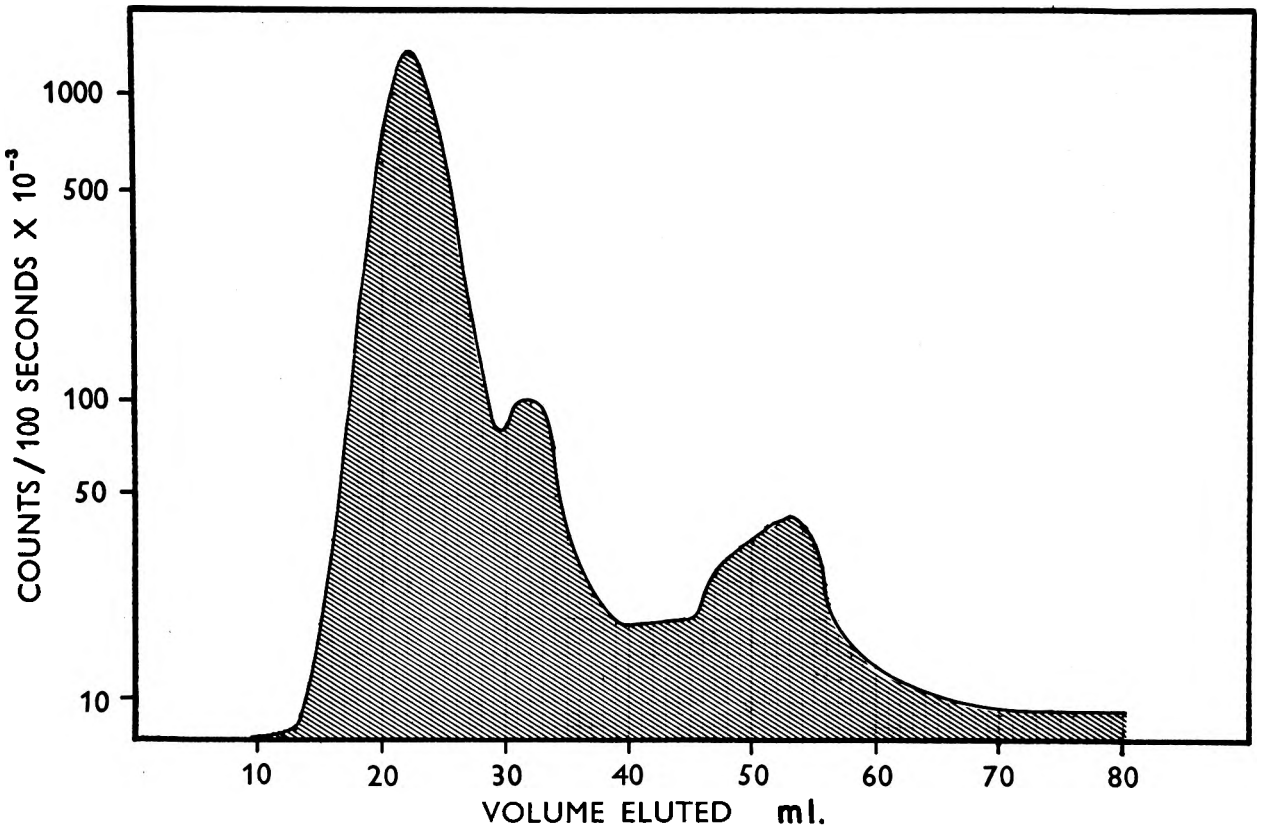


Figure 24.

Chromatography of a mucosal extract on a Sephadex G 200 column. Although three peaks were obtained it is apparent that the minor peaks are negligible compared with the main peak.

In a similar separation the Sephadex G 200 column was characterised before applying the mucosal extract. This was carried out by passing non-radioactive ferritin, together with labelled transferrin, through the column before the extract. The exact volume of solvent required to elute these two proteins from the column was thus determined. Although the two proteins were separated, some overlapping occurred, and it appeared that the ferritin took up a small amount of activity from the transferrin (Figure 25). When the mucosal extract was placed on this column, a single sharp peak, corresponding exactly with the expected position for ferritin, was obtained. The peak was not symmetrical, and had a shoulder corresponding to the position in which transferrin should emerge. Further evidence for the presence of small amounts of transferrin-bound radioiron in the mucosal extract is presented at a later stage.

Only about 70% of the radioactivity put on to Sephadex G 200 column was recovered in the eluate. This was strange in view of the fact that 100% recoveries, in a single peak, were obtained with Sephadex G 75 columns. This observation was checked by simultaneous chromatography on Sephadex G 75 and G 200 columns. An extract prepared from mucosal cells, ten minutes after an oral dose of 10 µg. of iron, gave a single peak in both cases. These peaks represented 91% of the radioactivity put on to the G 75 column but only 70% of counts from the G 200 column (Figure 26). The percentage of counts present as ferritin in each of the

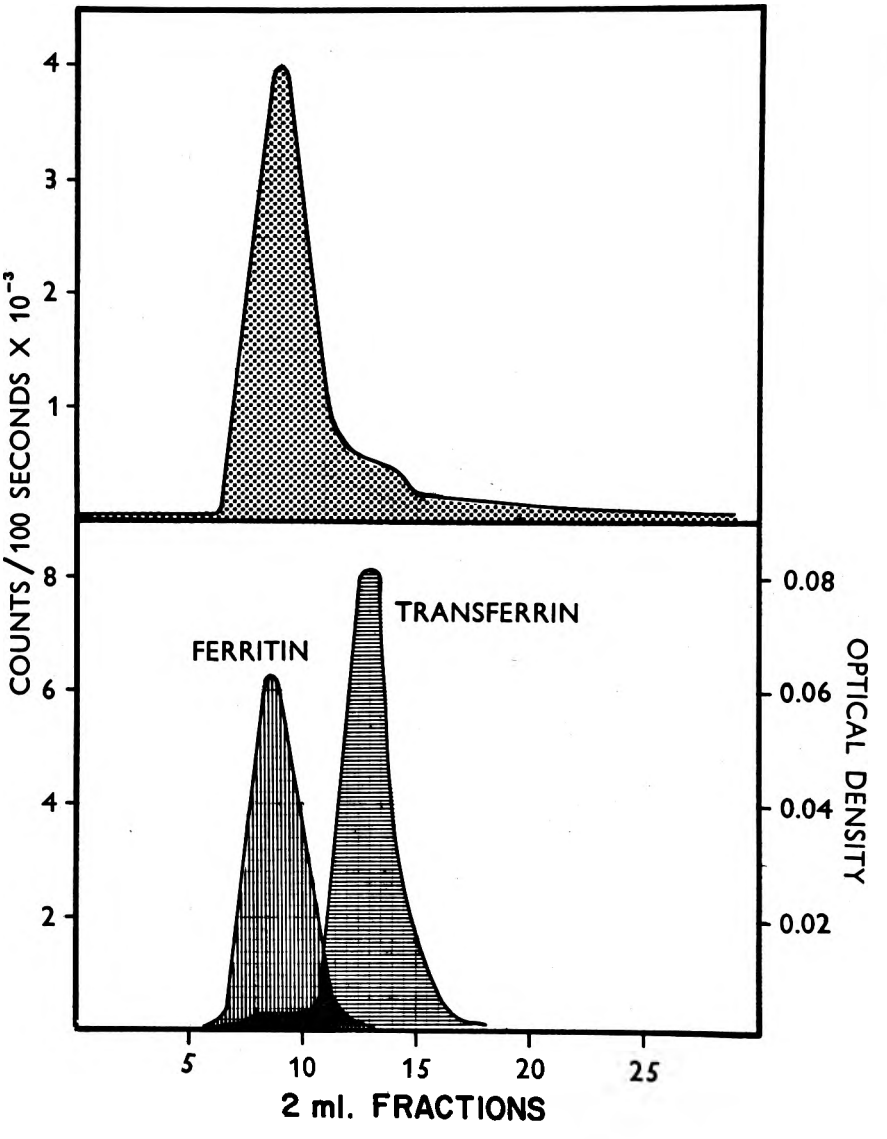
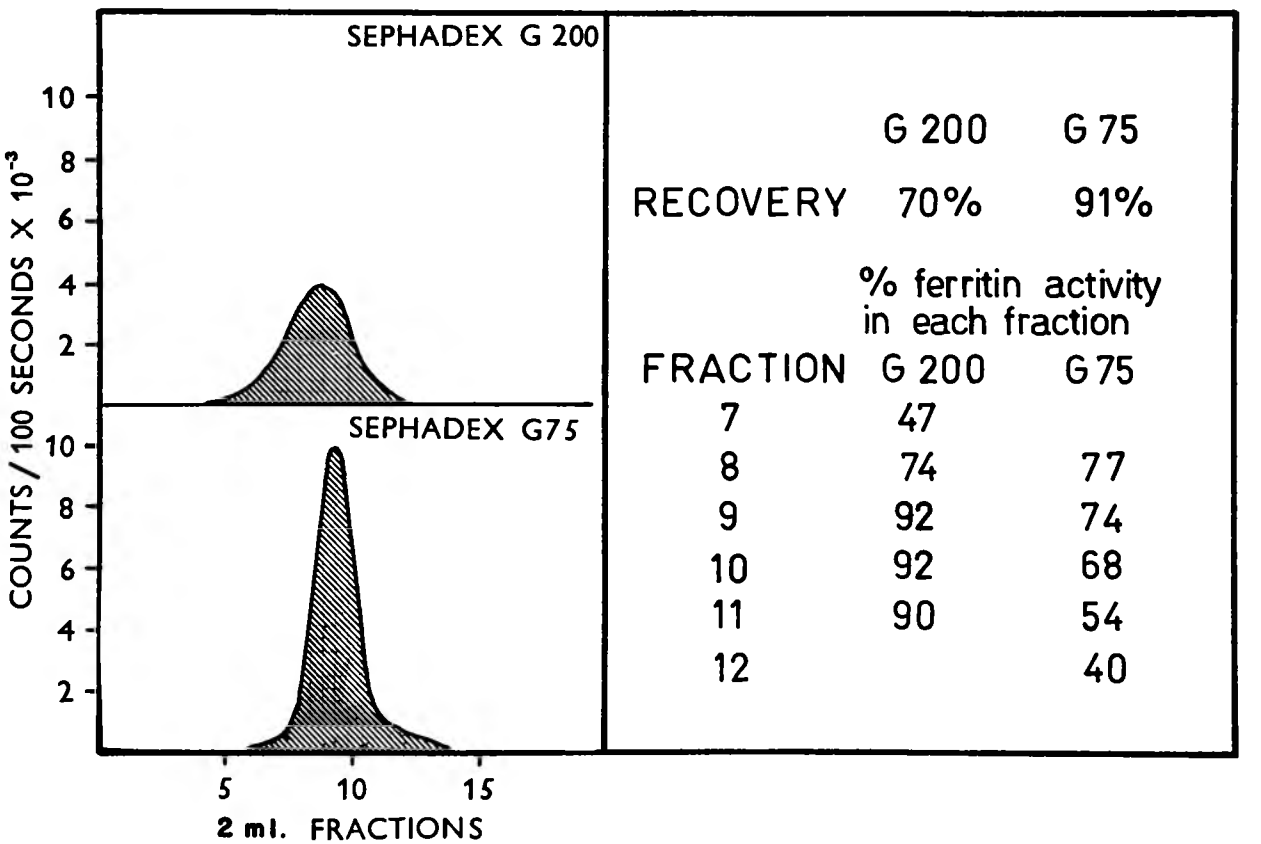


Figure 25.

Chromatography of a radioactive mucosal extract on a Sephadex G 200 column (Top). The behaviour of transferrin and ferritin on the column was first determined using transferrin labelled with radioiron and unlabelled ferritin (Bottom). Most of the radioactivity eluted from mucosal extract corresponded to the position of ferritin but a shoulder corresponding to transferrin was also present.



	G 200	G 75
RECOVERY	70%	91%

	% ferritin activity in each fraction	
FRACTION	G 200	G 75
7	47	
8	74	77
9	92	74
10	92	68
11	90	54
12		40

Figure 26.

Comparison of the recoveries of radioactivity obtained when the same radioactive mucosal extract is chromatographed on Sephadex G 200 and G 75 columns. The table also shows the proportion of radioactivity in each fraction which is precipitated by antiferritin. It seems that the peak obtained from the G 75 column contains a non-ferritin radioactive component not present in the peak from the G 200 column.

fractions comprising these peaks was determined. These results, which are shown in Figure 26, indicate that whereas the activity eluted from the G 200 column consisted almost entirely of ferritin, that eluted from the G 75 column contained some additional radioactive constituent. This would occur if activity were associated with a protein fraction having a molecular weight between the exclusion limits of these two grades of Sephadex, that is between 50,000 and 200,000. In this event a second peak of activity should be eluted from the G 200 column. However, in this investigation a second peak was not observed.
When the gut extract, prepared without chelate, was chromatographed on a DEAE cellulose column, using gradient elution, a peak was obtained which contained nearly all the eluted activity. In addition to this main peak, a minor peak varying from 0-10% of the eluted activity was obtained. These two peaks accounted for only about half the activity originally put on to the column. The remaining activity was retained on the column - chiefly at the top. Precipitation of most of the activity in the major fraction with the antiserum established that ferritin was the main radioactive compound in this fraction. The minor peak appeared to be due to transferrin-bound activity, since it could be considerably increased by the addition of rat plasma (Figure 27).
Washing the mucosa with saline containing 0.01M EDTA before homogenising in water resulted in the elution of three instead of two peaks from DEAE cellulose. The

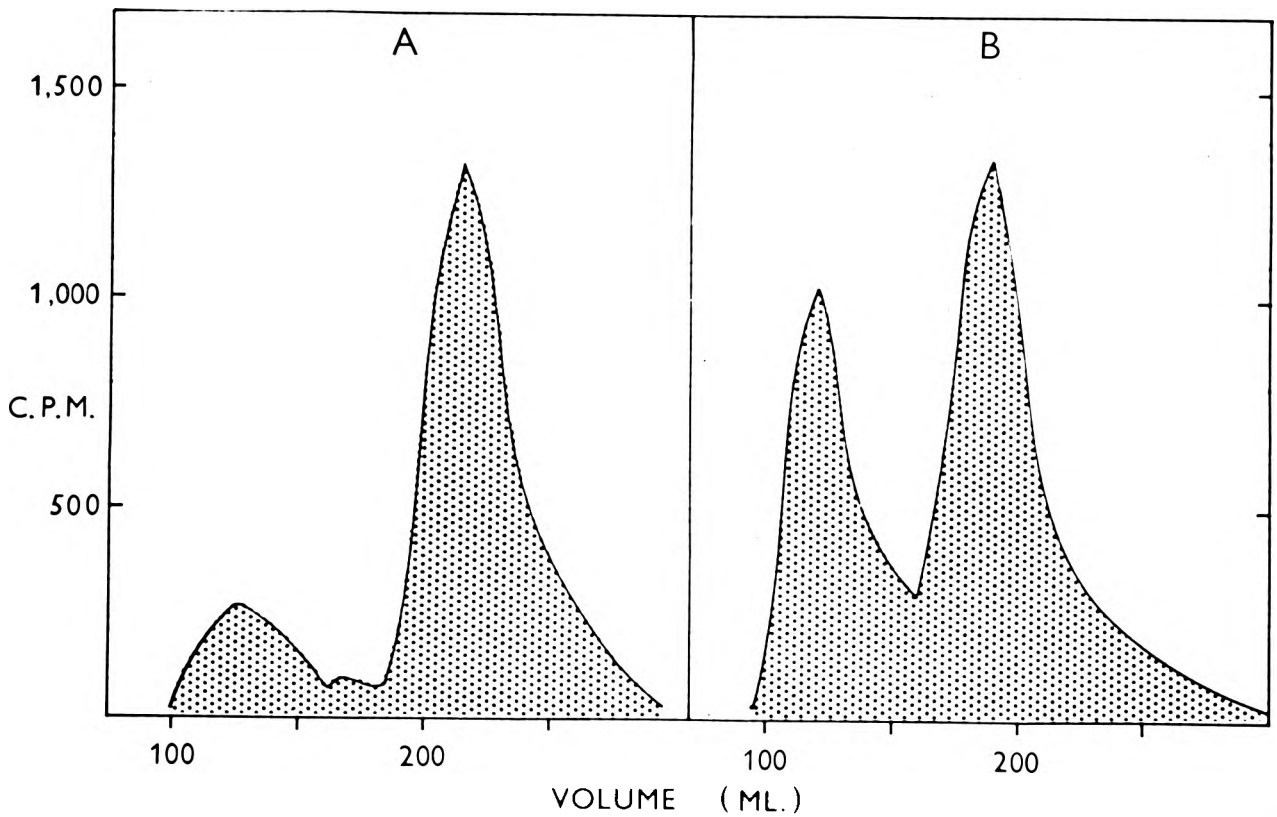


Figure 27

DEAE cellulose chromatography : A. mucosal supernatant solution; B. mixture of supernatant solution with unlabelled plasma. Although some activity was eluted in the transferrin position when supernatant solution only was chromatographed, the amount was considerably increased by the addition of plasma (24% of the activity placed on the column compared with 6%). The second peak consisted of labelled ferritin in each instance.

mucosal extract was chromatographed on a DEAE cellulose column equilibrated with M/150 Sorensen phosphate buffer pH8. An elution gradient was applied to the column by increasing the ionic strength of the developing solvent with sodium chloride. The pH was kept constant at pH8 to prevent dissociation of iron from transferrin, which occurs at more acid pH values. The pattern of protein elution from the DEAE column was determined by reading the optical density of the fractions at 280 mμ. This is shown in Figure 28. The first band to emerge from the column accounted for only 3% of the initial activity, whilst the remaining bands accounted for 15% and 48% respectively. Forty-three per cent. of the activity was retained on the column, mainly near the top. There was not sufficient material for further investigation of the first peak but the position in which it emerged from the column is that expected for transferrin. The second peak was the dialysable EDTA-Fe complex, and the final peak represented ferritin. This pattern was not reproduced if the gut was homogenised in 0.1M EDTA. In this case only two peaks were obtained (Fe-EDTA and ferritin), and no activity remained on the column. The three-peak pattern described above presumably depended upon the entry of only a small amount of chelate into mucosal cells during washing. Under these conditions the transferrin competed with the EDTA for iron.

Similar results were also obtained more conveniently using stepwise elution on a short DEAE cellulose column (Figure 29).

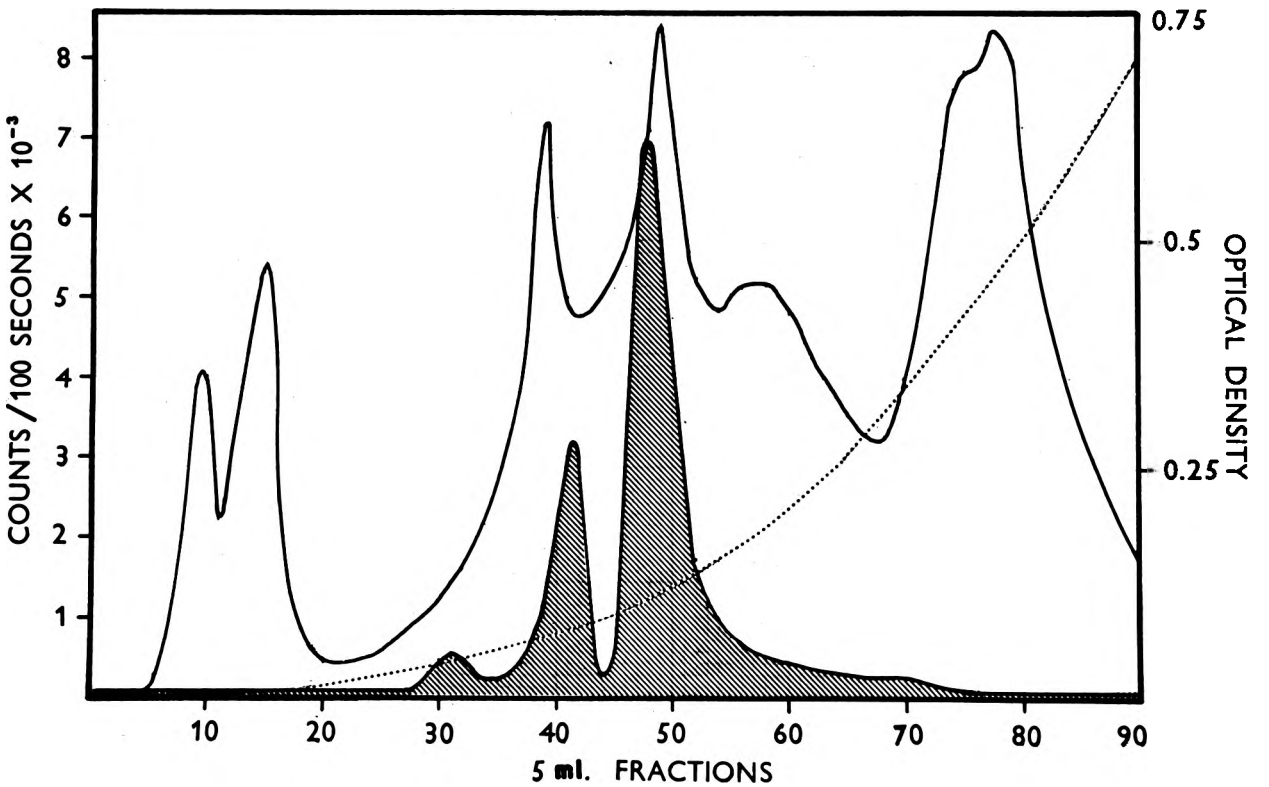


Figure 28.

Gradient elution chromatography on DEAE cellulose of a mucosal extract from an intestinal loop washed with 0.01M EDTA pH7 in saline and homogenised in water.
Cross hatched area represents eluted radioactivity.
Solid line indicates the optical density at 280 mμ.
The dotted line indicates the shape of the sodium chloride gradient.
The first peak of radioactivity corresponds to the expected position for transferrin.
The second peak is due to the ^{59}Fe-EDTA complex whilst the main peak consisted of ferritin.

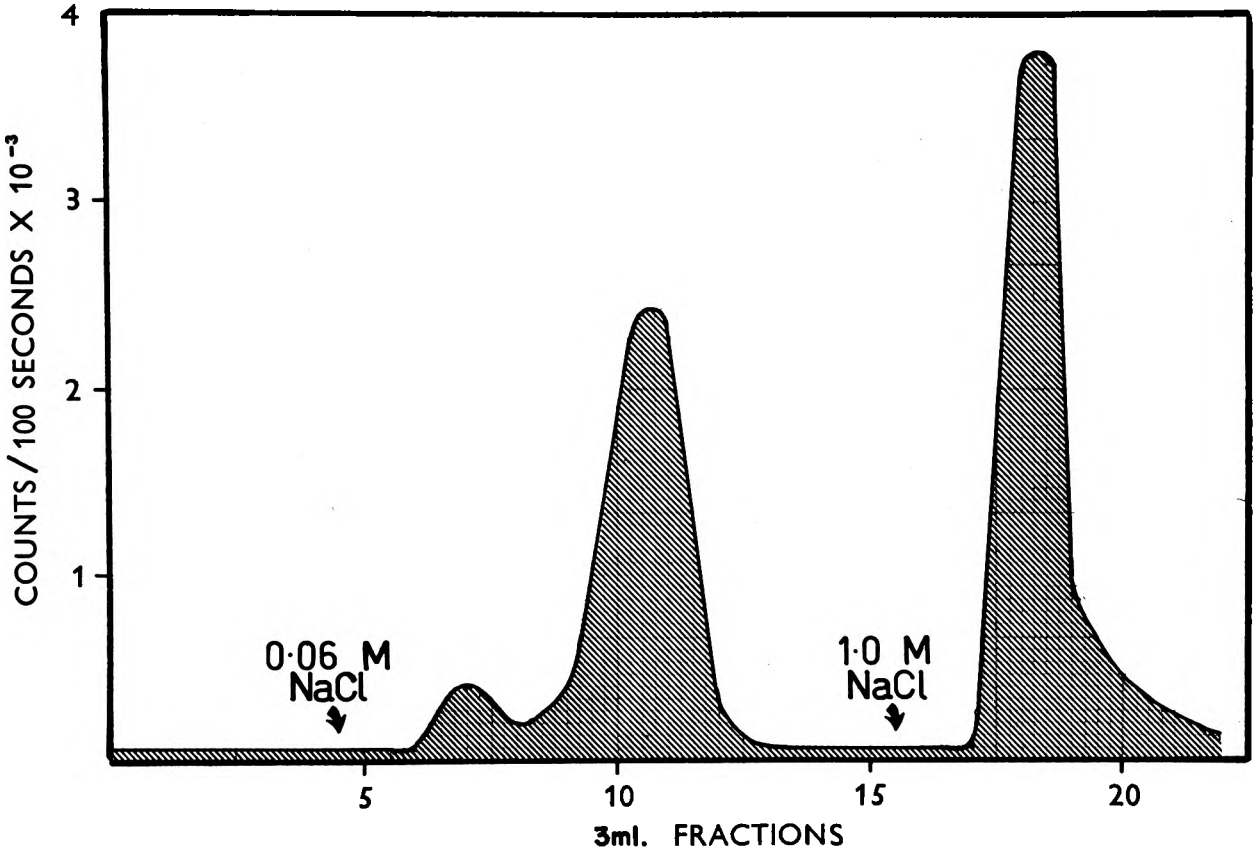


Figure 29

DEAE cellulose chromatography of a mucosal extract prepared from an intestinal loop which had been washed with 0.01M EDTA pH7 in saline and homogenised in water. Stepwise elution was used. No activity was eluted by the starting buffer M/150 Sorensen phosphate pH7. When sufficient sodium chloride was added to the buffer to give an 0.06M solution transferrin was eluted first followed by the iron-EDTA complex. The salt concentration was raised to 0.5M in order to elute the ferritin.

no activity was eluted from the column until the ionic strength of the starting buffer ($^{M}/150$ Sorensen's phosphate pH7) was increased by addition of sufficient sodium chloride to give an 0.06M solution. At this ionic strength both transferrin-bound iron and the iron EDTA complex were eluted and emerge as two distinct bands. This was demonstrated using a snythetic mixture of ^{59}Fe bound to both compounds. The ferritin fraction was eluted from the column by increasing the sodium chloride concentration to 0.5M. With mucosal extracts the initial band due to the transferrin-bound iron is not always present.

The effect of adding the EDTA chelate to a mucosal extract was investigated using stepwise elution. The result obtained is shown in Figure 30. Without chelate only a single peak consisting of ferritin was obtained. The non-ferritin activity remained at the top of the column. When EDTA was added to the mucosal supernatant solution the ferritin peak was unchanged but the activity which previously remained on the column was eluted by the addition of 0.06M NaCl. This corresponds to the fraction which was not dialysable until the chelate had been added. The presence of the dialysable fraction produced by addition of chelate to mucosal extracts was also well demonstrated by the use of Sephadex G 75, G 50 or G 25. (Figure 32). When mucosal extract was chromatographed on Sephadex G 75 using $^{M}/150$ phosphate buffer pH7 most of the activity placed on the column was eluted in a

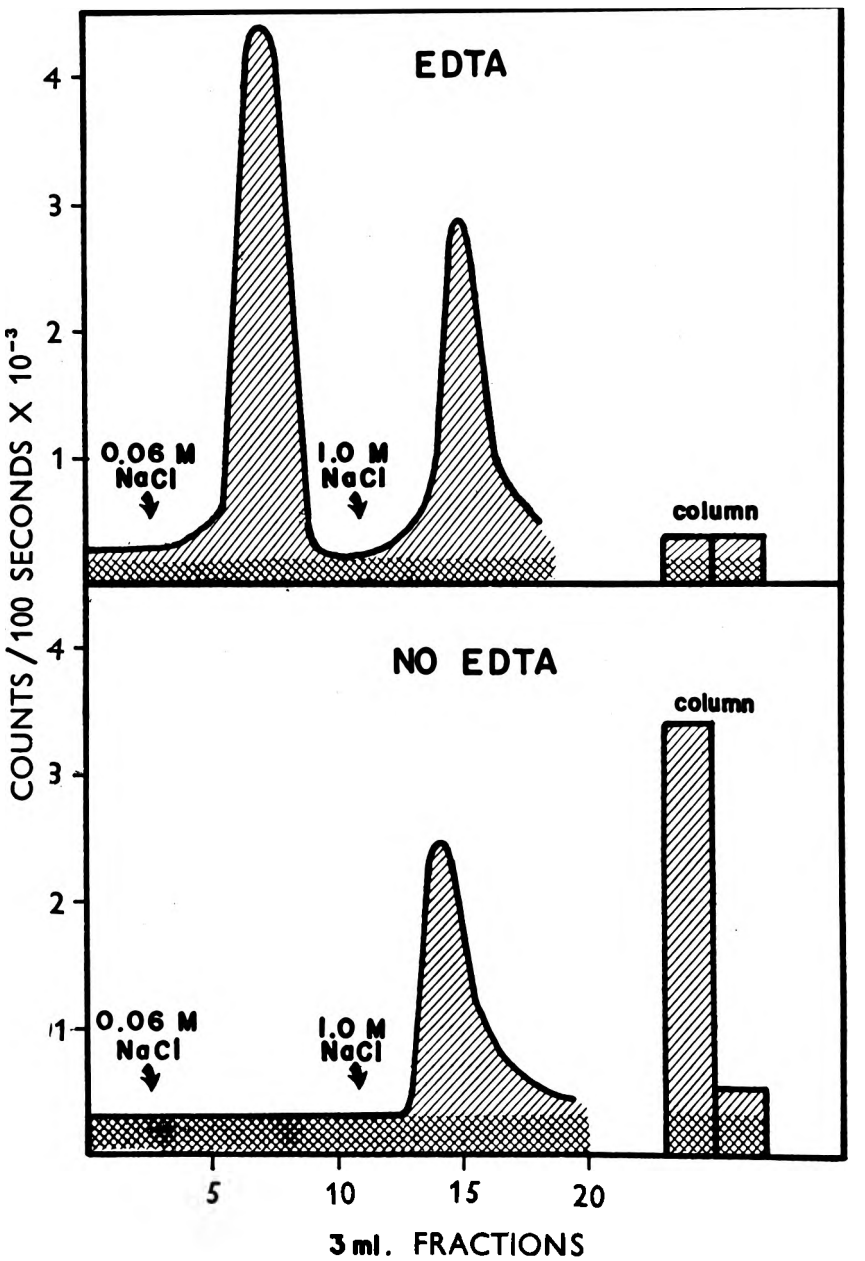


Figure 30.

The different behaviour, on DEAE cellulose, of mucosal extracts prepared with and without EDTA. When the chelate is absent more than half the radioactivity is trapped at the top of the column. In the presence of EDTA this activity is eluted as the iron-EDTA complex by 0.06M sodium chloride in the starting buffer.

single band emerging after the 'break through' volume. When a chelate - either EDTA or Fe-3-Specific - was present, the activity was eluted in two separate peaks, as the ferritin and the iron chelate peaks.

Since the possibility existed that iron is transported through the cell in an inorganic form, as suggested by Granick, the behaviour of inorganic iron on Sephadex columns was investigated. Sephadex G 75 was selected since this had been found to give the best recoveries with mucosal extract. The columns were prepared in and eluted with physiological saline. A sample of radioiron and a similar sample to which non-radioactive mucosal extract had been added, were chromatographed at the same time. Less than 1% of the pure radioiron was recovered whereas 54% was eluted in a single peak when mucosal extract was present. In another experiment a ferric sol, prepared by heating a ferric chloride solution, was placed on a Sephadex G 75 column. This was retained at the top of the column as a dark brown narrow band. It required 2N HCl to elute this band.

The behaviour of tracer radioiron was determined on Sephadex G 75 columns prepared in and equilibrated with barbital buffer at various pH values. The recoveries of tracer $^{59}FeCl_3$ placed on these columns and developed with the buffer used to equilibrate the column are shown in Figure 31. Above pH 6.9 less than 3% of the counts were eluted by 65 ml. of buffer. As the pH decreased the recovery increased to 8% at pH 5.7 and 44% at pH 4.4.

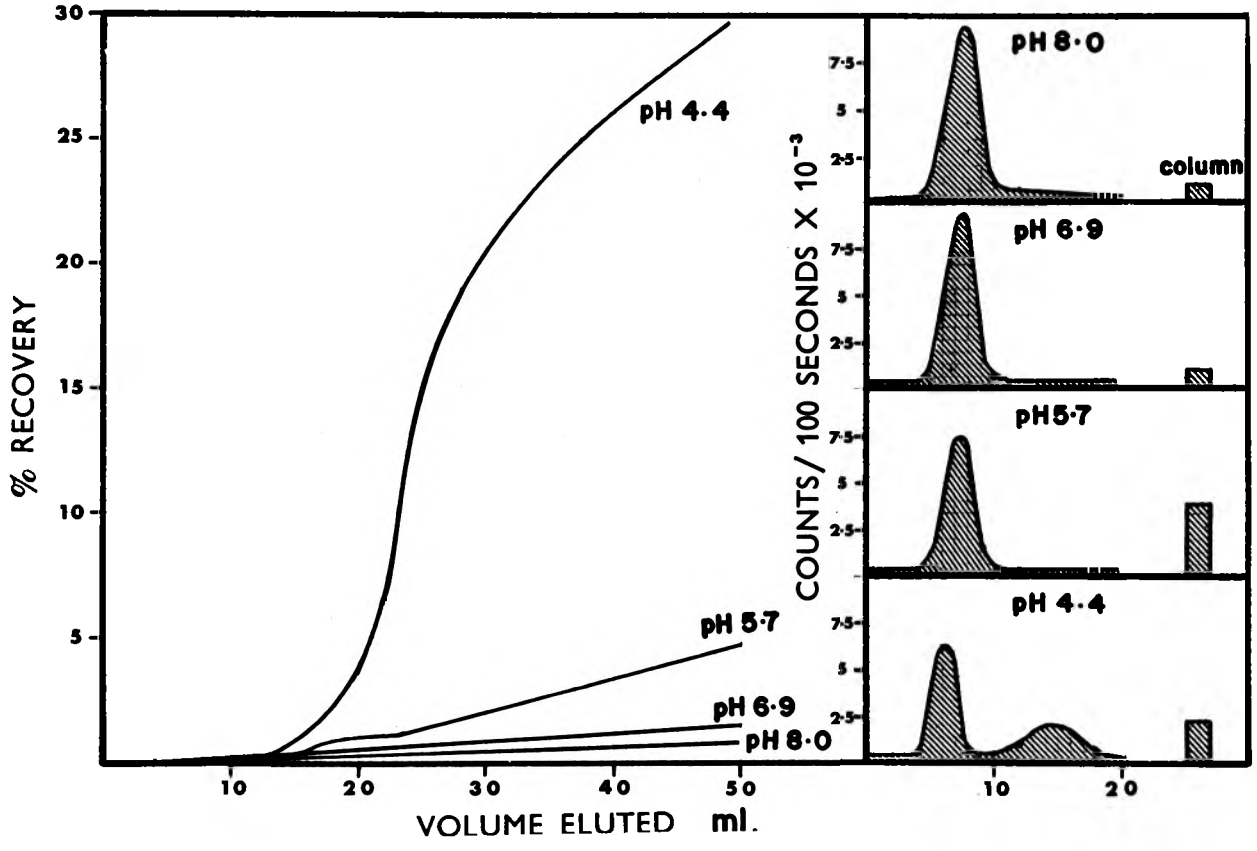


Figure 31.

The effect of pH on the elution of tracer radioiron and mucosal extracts from Sephadex G 75 columns. The graph on the left shows the cumulative recovery of tracer radioiron. The shaded areas on the right show the behaviour of a mucosal extract under similar conditions. Above pH 6.9 all the activity is eluted in a single peak whilst at pH 5.7 some of the radioactivity remains on the column. At pH 4.4 two peaks of radioactivity are eluted and some activity remains on the column. This suggests the presence of transferrin which binds iron at the higher pH values and releases it when the pH falls below 6.

Columns prepared in the same way were used to chromatograph a mucosal supernatant solution prepared from saline washed mucosal cells homogenised in water. As before, the columns were developed with the buffer with which they had been equilibrated. The result is shown in Figure 31. Since all the activity passed unhindered through the column at pH8, it is apparent that at this pH all the radioiron was fairly firmly bound to protein or other molecules of MW greater than 50,000. As the pH decreased more activity remained on the column and at pH 4.4 some of the radioactivity emerged as a second band. This result could be explained by the presence of transferrin-bound radioiron. As the pH decreased iron was released from transferrin. At pH 6.3 the iron transferrin complex was partly but not completely dissociated and at pH 4.4 the dissociation was complete. The iron released from the transferrin would behave as inorganic iron. The iron released from protein would therefore be retained on the column at pH 5.7 and be eluted at pH 4.4. This experiment suggests that free ionic iron is not present in mucosal extracts having a pH above 7.

Sephadex G 75 was also used to demonstrate the increase in protein-bound mucosal radioiron which occurs with time after administration of an oral dose. Two rats were anaesthetised and, after opening the peritoneal cavity, radioiron was injected directly into duodenal loops. The first rat was killed 10 minutes after placing the iron in the loop, and the second after 2 hours. The mucosa of each was removed, half of it

washed in saline and the remainder washed with EDTA in physiological saline (0.01M pH7). The saline washed portion was homogenised in water, whilst the EDTA washed portion was homogenised in aqueous EDTA (0.1M pH7). The supernatant solutions obtained by centrifuging these homogenates were chromatographed on Sephadex G 75 columns. The result of this experiment is shown in Figure 32. In the absence of EDTA a single peak was obtained at both 10 minutes and 2 hours. When EDTA was present two peaks were obtained at these times. At 10 minutes the second (iron-EDTA) peak accounted for 53% of the eluted activity. By 2 hours this peak accounted for only 17% of this activity. The peak due to protein-bound radioiron increased from 40% to 83% over the same period. Similar results demonstrating the time relationship between the ferritin and non-ferritin fractions were obtained using DEAE cellulose columns. The results of this separation were correlated with dialysis and precipitation data. Four groups of 9 rats were starved for 48 hours during which time they had free access to glucose water. The rats were then given the standard 10 µg. dose of labelled iron. After 10, 30, 60 and 240 minutes the rats were killed and the mucosal cells from the upper 10 cm. small intestine harvested. The cells from all the animals in a group were pooled and washed twice with 0.01M EDTA pH7 in saline and then homogenised in 20 ml. 0.1M EDTA pH7 in water. The homogenate was centrifuged at 9,000 x g for 15 minutes. The radioactivity present in the deposits was measured

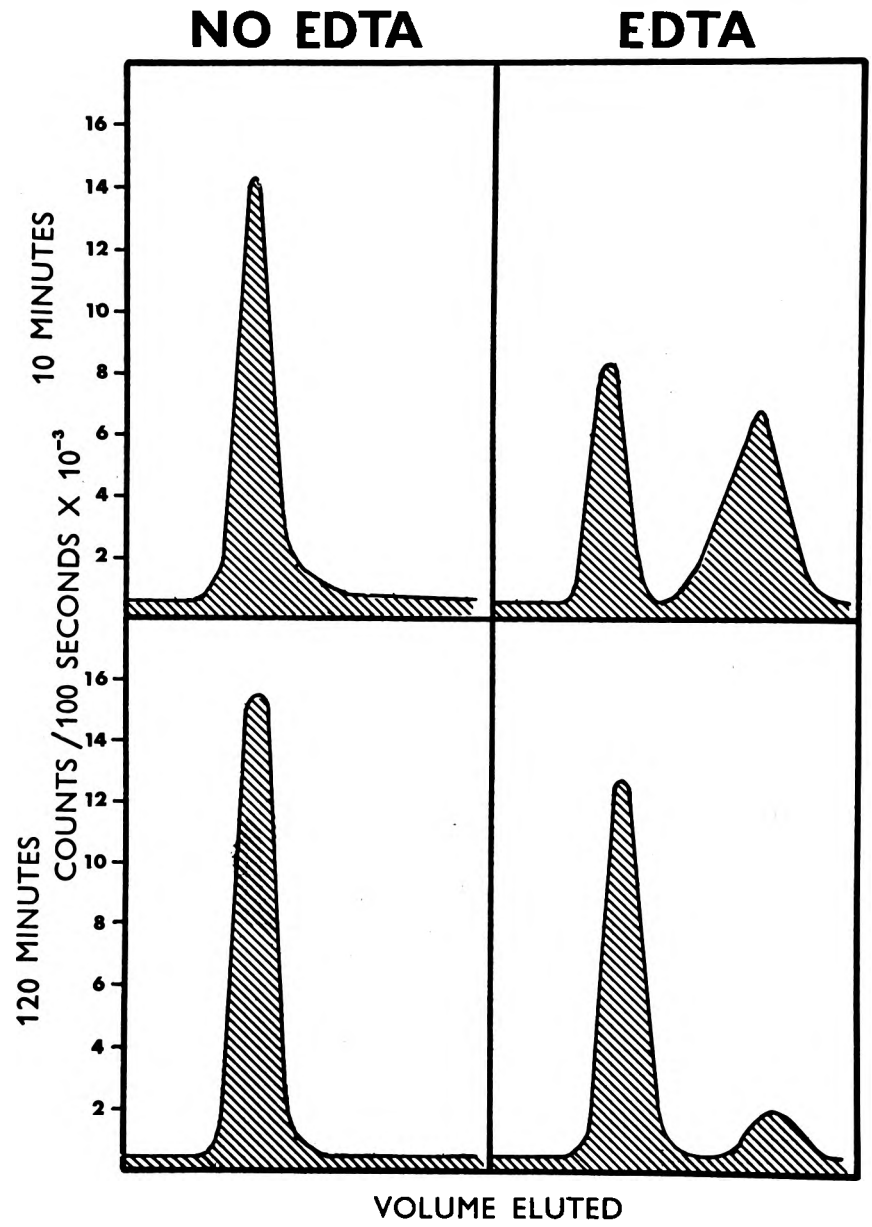


Figure 32.

The effect of chelates on the chromatography of mucosal extracts on Sephadex G 75 columns. In the absence of chelate almost all the radioactivity is eluted in a single peak. In the presence of EDTA ferritin is eluted first and this is followed by chelated iron. This is used to demonstrate that the proportion of mucosal radioactivity associated with ferritin increases with time after the oral administration of radioiron.

and aliquots of the unheated supernatant solutions were submitted to precipitation by perchloric acid, ammonium sulphate (pH 7) and the ferritin antiserum, to dialysis for 48 hours against running tapwater, and to column chromatography on DEAE cellulose. At 10 minutes, 30 minutes, 60 minutes and 4 hours, 28%, 17%, 23% and 24% of the activity was present in the deposit from the initial centrifugation. Thirty per cent, 52%, 60% and 80% respectively of the activity present in the supernatant solutions was precipitable with perchloric acid. The fraction precipitable with perchloric acid seemed to consist entirely of ferritin, since corresponding figures with antiferritin serum were 32%, 48%, 68% and 76% respectively. Somewhat higher proportions were precipitated with ammonium sulphate (43%, 65%, 83% and 91%). Dialysis resulted in losses of 66%, 50%, 31% and 12% of the supernatant activity at the different times. On chromatography using a DEAE cellulose column with stepwise elution, two peaks of radioactivity were obtained, the first with 0.06M NaCl and the second with 1.0M NaCl. The proportion of the radioactivity present in the first fraction (iron-EDTA) fell progressively with time, whilst that in the ferritin fraction rose. At 10 minutes, 30 minutes, 60 minutes and 4 hours, 47%, 33%, 15% and 8% respectively were in the first fraction, and 29%, 39%, 52% and 60% in the ferritin.

These results are summarised in Table (9) and represented graphically in Figure 33.

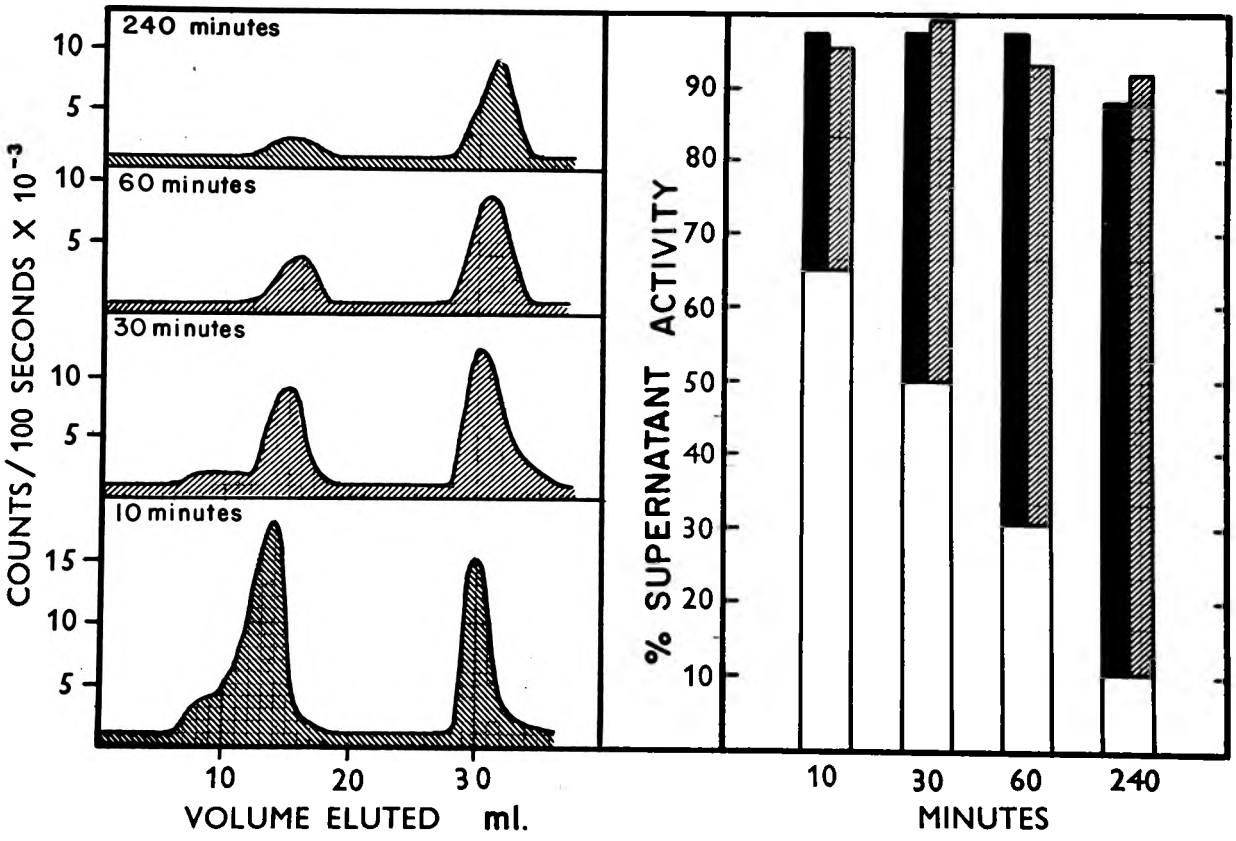


Figure 33.

The change in the distribution of mucosal radioactivity with time. The increasing proportion of radioactivity associated with ferritin (second peak) is shown on the left by DEAE cellulose chromatography and on the right by the increase in activity precipitated by antiferritin (black) or perchloric acid (hatched). The unshaded portion of the columns represents dialysable activity.

Table 9.

Correlation of ferritin DEAE cellulose fractions, protein precipitable activity, and non-dialysable fractions.

Time (Minutes)	10	30	60	240
	Percentage of supernatant counts			
Ferritin	32	48	68	76
$HClO_4$	30	52	60	80
$(NH_4)_2SO_4$ precipitable	43	65	83	91
2nd DEAE peak	29(38)	39(54)	52(78)	60(88)
Non-dialysable	34	50	69	88
Dialysable	66	50	31	12
Non $(NH_4)_2SO_4$ precipitable	57	35	17	9
1st DEAE peak	47(62)	33(46)	15(22)	8(12)

Both the table and the graph show excellent correlation between the ferritin precipitable activity, the non-dialysable fraction and the perchloric acid precipitable material. The figures shown in brackets are obtained by correcting the initial results to give 100% recoveries from DEAE cellulose columns. Such a correction is valid since recoveries are virtually 100% when EDTA is present (Figure 30). The incorrect figures must have been due to poor geometry in counting the eluant fractions. Ammonium sulphate adjusted to pH 7.4 precipitated rather more activity than was present in the ferritin fraction. The reason for this was not discovered.

VI. Paper electrophoresis.

Excellent separation of the radioactive constituents in the mucosal extract was achieved by paper electrophoresis. Autoradiographs prepared from two such separations, using barbital buffer pH 8.6, are shown in Figures 34 and 35. In the first experiment mucosal extract containing non-radioactive rat plasma, *in vitro* labelled ferritin, rat plasma labelled with ^{59}Fe, and a mixture of the labelled ferritin and non-radioactive plasma were all subjected to electrophoresis. The autoradiograph showed that ferritin remained at the origin in the absence of plasma but with plasma migrated ahead of transferrin. There were bands of activity corresponding to the positions of ferritin and transferrin in the mucosal extract strip. In addition to these bands there was activity at the origin and two poorly defined bands between the origin and the transferrin position. There was also a sharply defined but faint band having a migration rate much faster than any of the other bands. This band was still present when plasma was added to the mucosal extract, but the activity at the origin and between the origin and the transferrin band disappeared. These changes were accompanied by an increase in the intensity of the transferrin band.

The second experiment demonstrated the increase in the proportion of activity associated with the ferritin fraction with time. Mucosal extracts were prepared 10 minutes and 2½ hours after injecting radioiron into

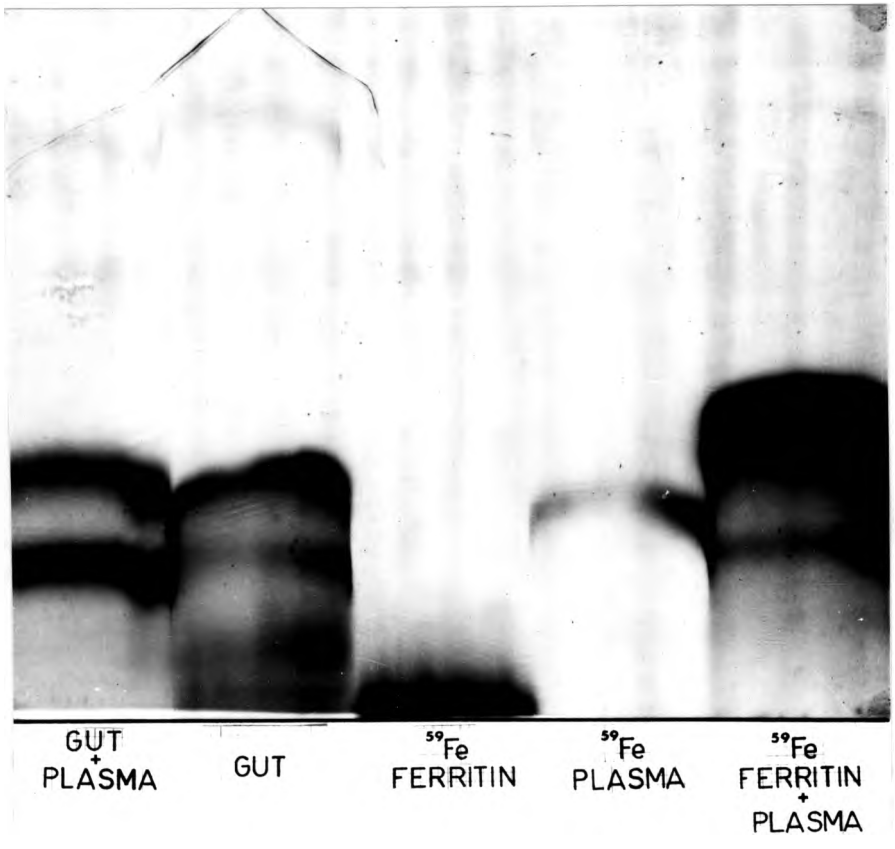


Figure 34.

Autoradiograph of the paper electrophoresis of a mucosal extract with and without added plasma. The mucosal extract has bands of radioactivity corresponding to the position of ferritin and transferrin. Activity is also present at the origin and two bands are present between the origin and the transferrin position. A faint band having a faster rate of migration was also present. On adding plasma to the mucosal extract the intensity of the transferrin band increased at the expense of the radioactivity between the origin and the transferrin band itself.

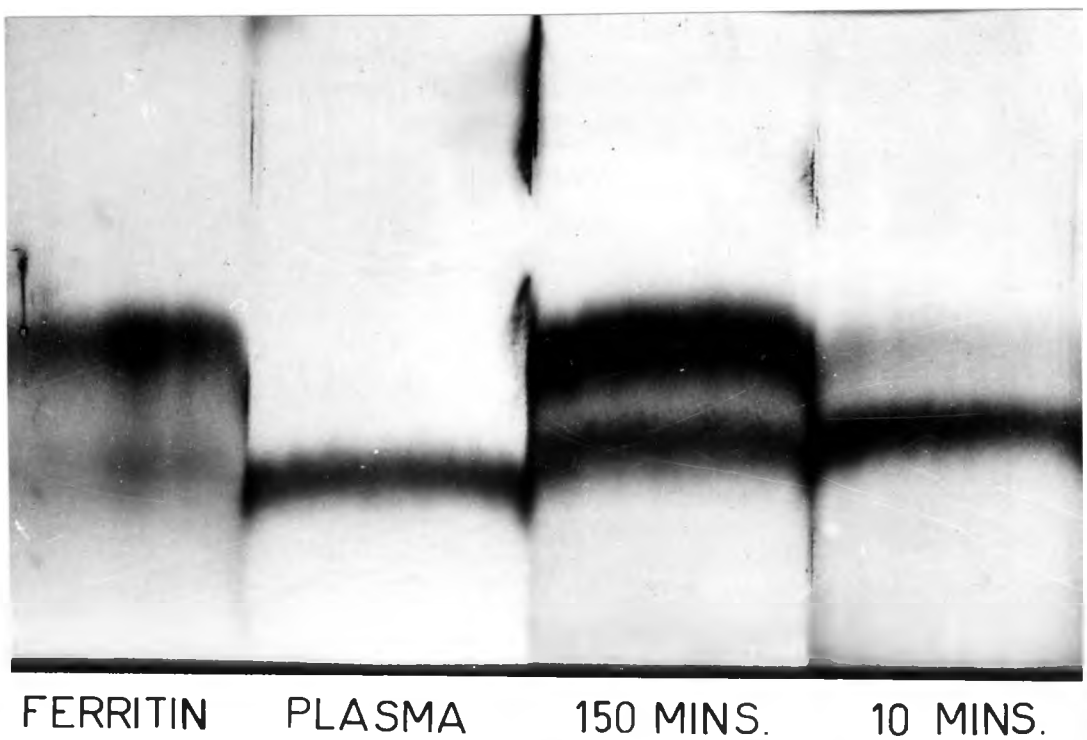


Figure 35.

Autoradiograph of the paper electrophoresis of mucosal extracts prepared 10 and 150 minutes after administration of radioiron. The increase in the proportion of activity associated with ferritin at the later time can be seen.

the duodenal loop as before. These extracts were submitted to electrophoresis along with *in vitro* labelled ferritin and rat plasma labelled with ^{59}Fe. Prior to electrophoresis non-radioactive plasma was added to the ferritin and both gut extracts. The autoradiograph showed that the plasma removed a small amount of activity from the *in vitro* labelled ferritin. At 10 minutes little of the mucosal activity was associated with the ferritin band, most of it being present in the transferrin band. By 2½ hours this situation was reversed, most of the activity at this stage being in ferritin. Although the fast migrating band described in the previous experiment cannot be seen in Figure 34, it was visible on the original X-ray plate, being denser at 10 minutes than at 2½ hours. Another autoradiograph in which the strips were left in contact with the X-ray plate for a longer period showed these bands quite clearly, however the more intense bands were fogged after this time.

VII. *Starch-gel electrophoresis*.

Starch-gel electrophoresis of mucosal extract yielded no additional information. Nevertheless it provided yet another demonstration of the change in distribution of mucosal activity with time. Mucosal extracts, prepared from a duodenal loop 10 minutes and 2½ hours after injection of radioiron into the loop, were submitted to starch-gel electrophoresis at the same time as *in vitro* labelled ferritin and plasma labelled with ^{59}Fe. The starch-gel was divided horizontally into two. One

half was cut into narrow strips, and the radioactivity of serial segments of these strips determined. The results are shown in Figure 36. In the early stages most of the activity was associated with transferrin whilst in the later stages ferritin activity predominated. An autoradiograph prepared from the remaining half of the gel confirmed this result.

The starch-gel electrophoresis patterns of extracts of whole gut and of separated mucosal cells from the same animal were compared. This experiment showed that the transferrin peak was present only when whole gut was used. This favours the idea that the transferrin-bound radioactivity derives from contaminating plasma, and not from the mucosal cells themselves.

VIII. Ammonium sulphate precipitation.

An attempt was made, by ammonium sulphate precipitation, to characterise the nature of the radioactivity in a mucosal extract prepared from an *in vivo* duodenal loop which had contained activity for 15 minutes. Before precipitation the pH of the ammonium sulphate was adjusted to 7.4 in order to prevent release of transferrin-bound iron in the procedure. The proportion of radioactivity precipitated at various saturations of ammonium sulphate was determined for the radioactive mucosal extract, plasma labelled with ^{59}Fe, *in vitro* labelled ferritin, and tracer ^{59}Fe added to a non-radioactive extract of duodenal mucosa. The experiment showed (Figure 37) that transferrin-bound radioiron precipitates sharply between

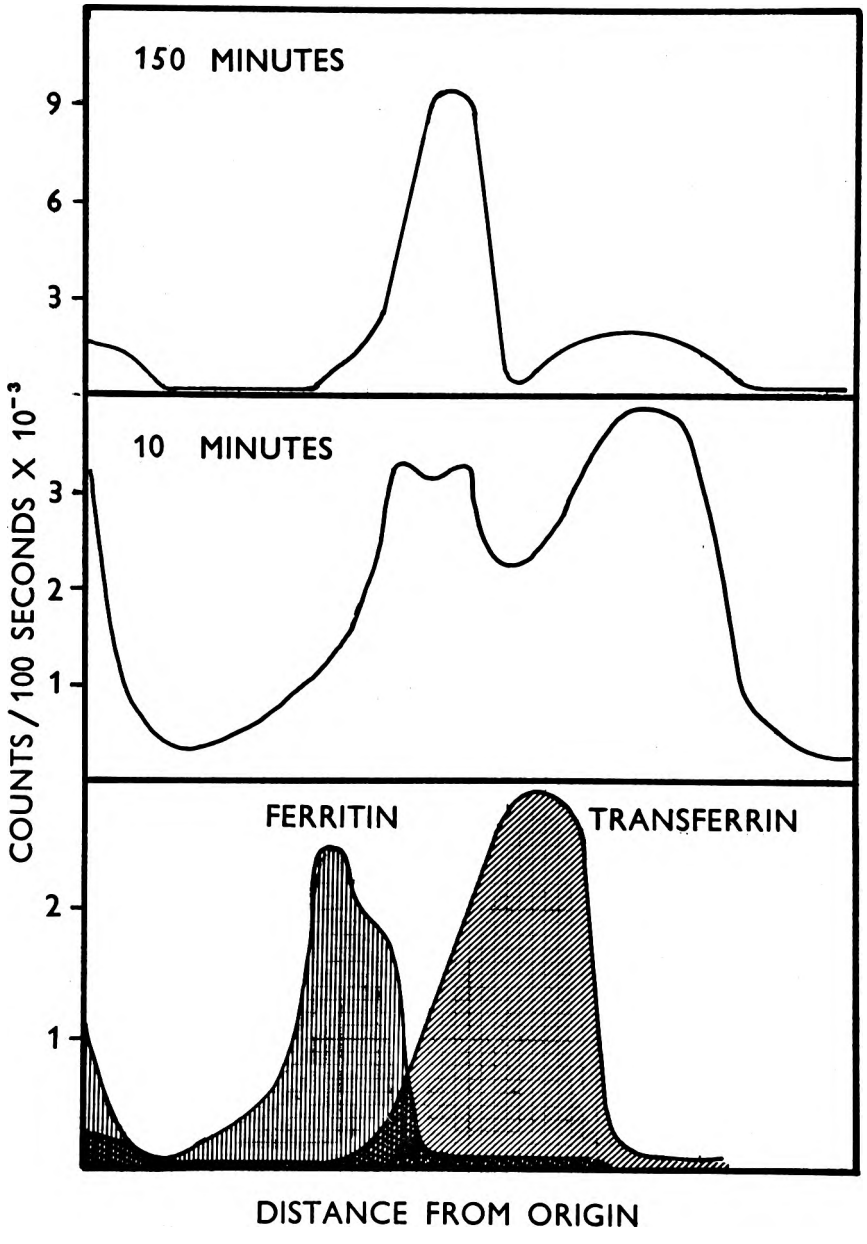


Figure 36.

Starch-gel electrophoresis of mucosal extracts prepared 10 and 150 minutes after administration of radioiron. It is apparent that proportion of mucosal radioactivity associated with ferritin (first peak) increases with time.

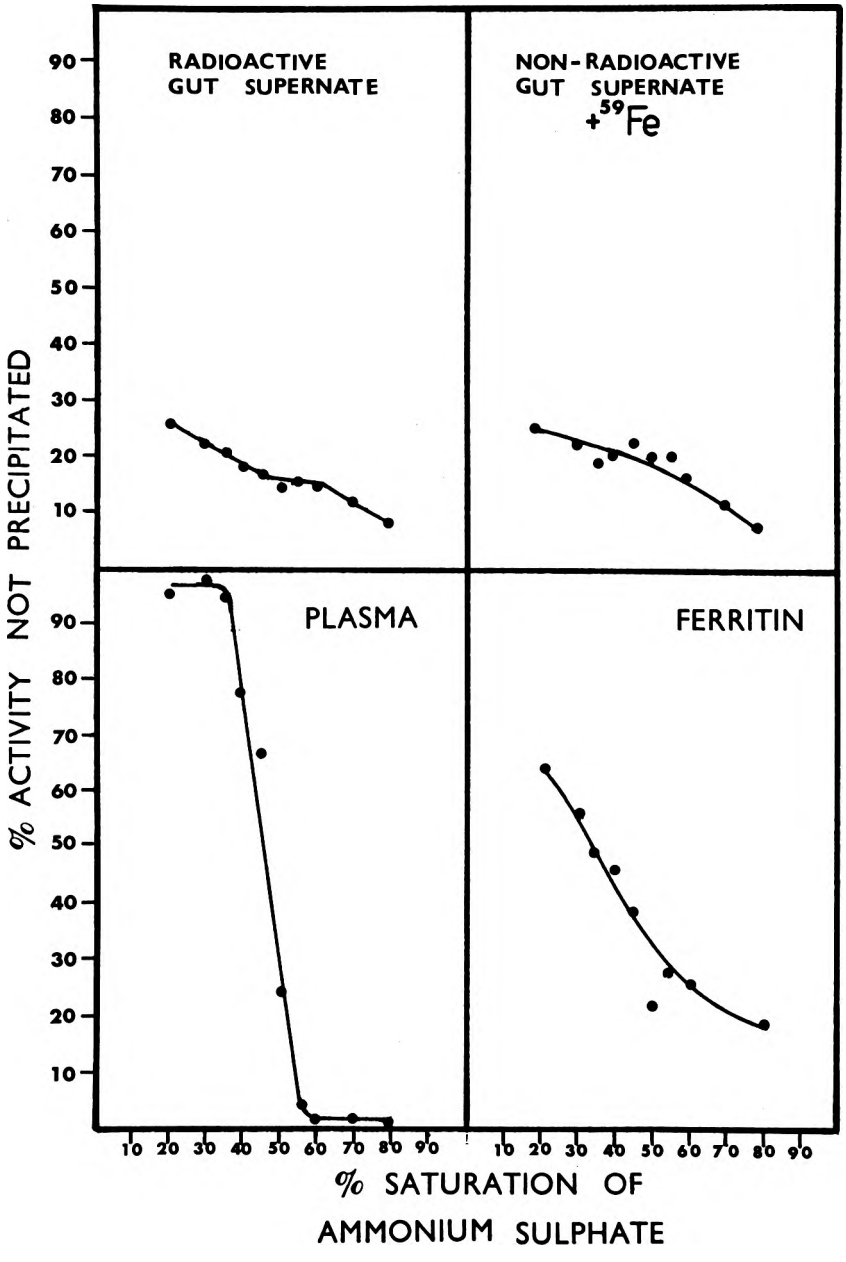


Figure 37.

The precipitation of ferritin, transferrin, radioactive mucosal extract, and radioiron in non-radioactive mucosal extract by ammonium sulphate.

ammonium sulphate saturations of 35% and 55%. The ferritin-bound activity, on the other hand, precipitates over the entire range of saturations. Both *in vitro* and *in vivo* labelled mucosal extracts gave approximately the same precipitation pattern. At the lowest concentration of ammonium sulphate used (20% saturated), 75% of the radioactivity was precipitated. The remaining radioiron came out of solution over the entire range in the same way as ferritin. This experiment appeared to indicate that inorganic iron added to a mucosal extract behaved similarly to iron which had entered the cells during absorption. It seems possible therefore that radioiron is present in the mucosal cell in an inorganic form.

If inorganic iron is present it should be possible to determine its state. This was attempted using a specific ferrous chelate, $\alpha\alpha'$-dipyridyl, and EDTA, which chelates both ferrous and ferric ions. The proportion of radioactivity rendered dialysable by addition of these chelates was measured. The result obtained, however, was dependent on the amount of chelate used. This was shown by an experiment in which a fasted rat was killed 10 minutes after administration of an oral dose of radioiron. The mucosa was stripped from the upper 20 cm. of small intestine, washed in saline, homogenised in water and centrifuged at 9,000 x g. for 20 minutes. Varying amounts of $\alpha\alpha'$-dipyridyl and EDTA pH7 were added to aliquots of the supernatant solution. These aliquots

were then allowed to stand for 2 hours after which they were exhaustively dialysed against running tap water. The result of this experiment is shown in Table 10.

Table 10.

The dialysable fraction produced by addition of $\alpha\alpha'$-dipyridyl or EDTA to a mucosal extract.

MOLARITY	Percentage dialysable		
	$\alpha\alpha'$-dipyridyl	EDTA	$\alpha\alpha'$-dipyridyl and EDTA
0.08	56	75	74
0.002	42	66	68
0.0002	32	46	48
24% of supernatant solution was ferritin radioactivity			

It is apparent that EDTA is able to render a greater proportion of activity dialysable than $\alpha\alpha'$-dipyridyl and that at a given concentration the amount rendered dialysable by EDTA alone cannot be enhanced by adding the same concentration of $\alpha\alpha'$-dipyridyl. Another technique was used to try and arrive at the amount of free ferric iron present in the supernatant solution. 0.01M citric acid was added and this was followed by Amberlite IRA 401 anion exchange resin. The mixture was shaken and the resin separated, washed and counted. The procedure successfully removed all $^{59}Fe^{+3}$ from an aqueous solution but was unable to remove any activity from a mucosal extract.

These results,together with the lack of a dialysable fraction in a mucosal extract prepared without chelate, suggest that there is no free inorganic iron present in the mucosal extract. The non-ferritin iron is probably protein bound and chelates added to the extract can compete for this iron. It seems that most of this iron is available to a specific ferrous chelate. The significance of this observation is doubtful since it was demonstrated that a mucosal homogenate is able to convert added ferric ions to the ferrous state when $\alpha\alpha'$-dipyridyl is present.

CHAPTER VI.

INVESTIGATION OF FACTORS INFLUENCING MUCOSAL BEHAVIOUR.

CHAPTER VI.

When dietary iron is present in normal amounts, absorption is adjusted to meet the changing body requirements. Some insight into the way in which the mucosa of the proximal small intestine is able to modify the amount of iron retained by the body was obtained from experiments described in Chapter III. Further experiments were carried out to investigate the factors which influence mucosal behaviour. This was done by studying the uptake of orally administered iron after manipulating the animals in various ways calculated to modify absorption.

1. The effect of intravenous ferric ammonium citrate.

Since results obtained in an earlier experiment suggested that the parenteral administration of iron affected mucosal behaviour 24 hours later, a study was carried out to define this effect in more detail.

The percentage saturation of the circulation transferrin was raised for a protracted period by 3 intravenous injections of 50 µg. iron given at intervals of 5 hours. (Preliminary studies established that following this procedure the saturation remained over 80% for 12 hours, whereas in rats given only saline injections the percentage saturation varied between 12% and 44%.). The iron in the form of ferric ammonium citrate was dissolved in 1 ml. 0.9% NaCl solution, and administered via the tail veins. The injections were given at various times before

and after the oral administration of 10 µg. labelled iron. Animals from a group of 50 matched rats were subdivided at random into 5 groups of 10 each. The first iron injection was given 48 hours, 18 hours and 6 hours respectively before administration of the labelled oral dose to the first 3 groups of rats. The fourth group received the first injection immediately before the oral iron, while the control group was given saline at this time. The percentage absorption of the oral iron was then assessed by whole body counting 7 days later. Mean absorptions were 28.3%, 12.0% and 30.6% in the 48 hour, 18 hour and 6 hour groups respectively, compared with 54.1% in the zero time group and 54.2% in the control group (Figure 38).

Since it seemed possible that the depression in absorption noted in this study might relate to changes in the iron concentrations of mucosal cells, further studies were done in 6 animals to find out how much of the labelled ferric ammonium citrate was taken up by the gastrointestinal tract. In 3 animals all the ferric ammonium citrate injections were labelled with ^{59}Fe, while the other 3 received the label in either the first, second or third injection. Results were very uniform. At 18 hours between 6.5% and 7.9% of injected radioactivity was in the gastrointestinal tract, with 1.4% to 1.8% present in the upper 10 cm. of small intestine. Perfusion of the whole gut showed that only negligible amounts of radioactivity were present in the lumina.

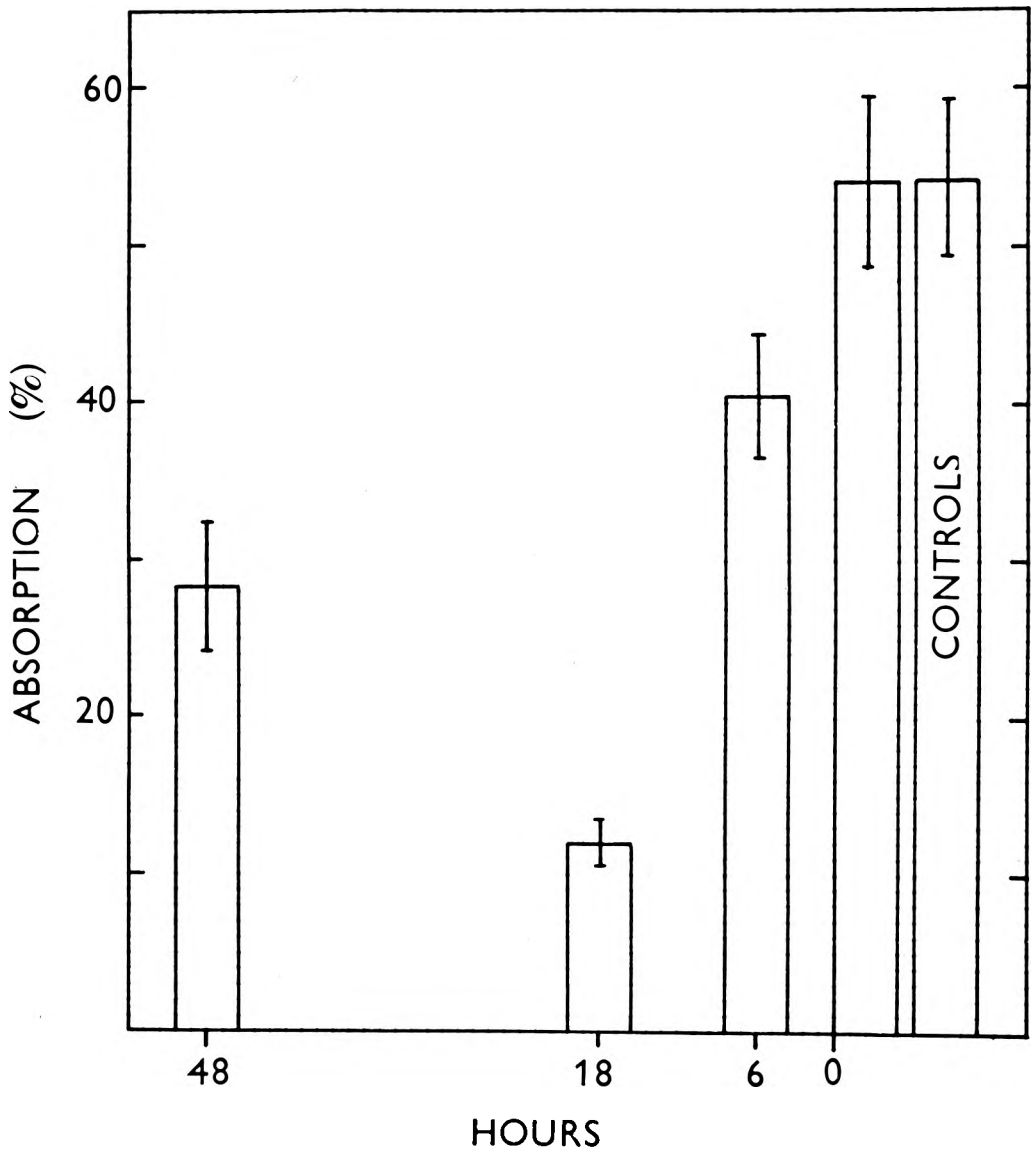


Figure 38.

The effect of the timing of 3 intravenous injections of 50 µg. iron at 5 hour intervals upon the absorption of an oral dose of 10 µg. radioiron. The groups of rats received the first of their three injections 48 hours, 18 hours, 6 hours and immediately before the oral dose. The control group was given a saline injection immediately before the oral dose.

These findings show that raising the serum iron to saturation point has no immediate effect on absorption. Six hours later absorption was depressed and by 18 hours the depression was marked. Forty-eight hours after raising the serum iron the absorption was increasing. The delay between the increased serum iron and the decreased absorption correlate well with the known turnover rate of rat mucosal cells (Messier and Leblond, 1960). It would seem therefore that the delay is due to the time required for cells, produced under the influence of the high serum iron, to grow out to their functional position on the villi.

II. The effect of a change from a high to a low iron diet. In a second experiment an attempt was made to modify the absorption of iron by manipulating the amount of iron taken in the diet, as described by Bannerman, O'Brien and Witts (1962). Forty matched rats receiving their normal diet were given, in addition 1 mg. iron as ferric chloride twice daily for 5 days by oesophageal catheter. They were then put on to an iron-free diet, and randomly subdivided into 4 groups. The absorption of 10 µg. labelled iron was studied 12, 36, 60 and 84 hours respectively after the last 1 mg. dose. Absorption was assessed by whole body counting 7 days after administration. Mean absorptions were 6.3%, 26.1%, 42.8% and 45.1% respectively. This experiment was repeated, but on this occasion absorption was assessed at 12 hours, 24 hours, 36 and 72 hours after the last dose of 1 mg. iron. Mean absorptions at these times were 10.6%,

14.2%, 22.8% and 48.8% (Figure 39). In a parallel experiment, the rats were sacrificed in pairs at various intervals after the last 1 mg. dose, and blood was collected for the estimation of the serum iron. By 24 hours the concentration had returned to normal levels (Figure 39). Since it seemed possible that the low absorption observed at an early stage might be due to dilution of the isotopic label with residual non-radioactive iron, either within the gut lumen or in the absorbing cells, a further experiment was carried out. Ten rats were given a single oral dose of 1 mg. non-radioactive iron 24 hours before, and a further 10 animals 12 hours before, a labelled dose of 10 µg. radioiron. The control group was given water. Mean absorptions in the 3 groups were 32.0% (S.E. 4.9), 33.0% (S.E. 4.1) and 30.2% (S.E. 3.5) respectively. The depressed absorption 12 hours after switching the diet is therefore valid. As in the previous experiment there is a delay between the change in serum iron and the maximal absorption. This delay is again consistent with the time required for newly formed cells to cover the villi.

III. <u>The effect of transferrin saturation on iron absorption</u>.

It has been suggested that the level of saturation of iron-binding protein plays a role in the rate of iron absorption (Laurell, 1952; Sölvell, 1960). However absorption is greatly increased following phenylhydrazine treatment

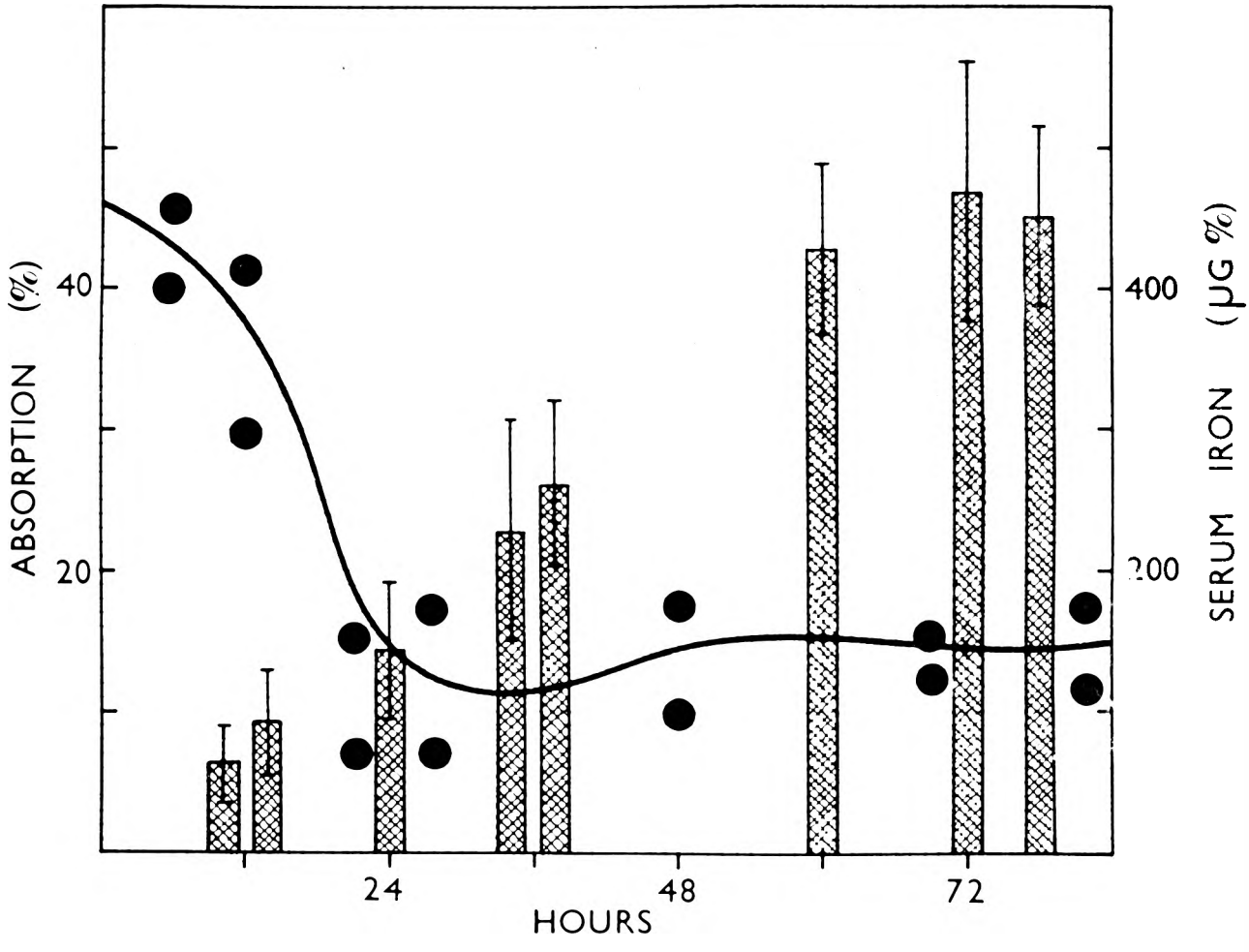


Figure 39.

Absorption of 10 µg. ^{59}Fe at various times after switching from a high iron to a low iron diet (hatched columns). Mean values (±SE) for each group of 10 rats are given. Serum iron concentrations obtained in a parallel experiment are also plotted (black circles). A progressive increase in mean absorptions between 24 and 60 hours is demonstrated.

(which produces haemolysis) in spite of the high saturation of the transferrin. In addition, normal or increased iron absorption has been reported in a child with a congenital absence of transferrin. It therefore seems unlikely that the saturation of the circulating transferrin per se plays a significant role in the control of iron absorption. Nevertheless an experiment was carried out in order to ascertain whether the absorption is depressed when the circulating transferrin is saturated.

Seven rats were given 100 µg. of iron intravenously as ferric ammonium citrate in saline, while 6 control animals received intravenous saline at the same time. An oral dose of 10 µg. radioiron as ferric chloride was administered, and the animals killed after 10 minutes. The gastrointestinal tracts were excised without spilling the contents, and absorption assessed by measuring the radioactivity of the carcasses. In addition, the distribution of the absorbed activity between the liver and the remainder of the degutted carcass was determined. The results of this study are shown in Table 11.

Table 11.

Percentage absorption and distribution of absorbed radioiron in rats with normal and elevated transferrin saturations.

SATURATED		CONTROLS	
Absorption (%)	Liver/rest of animal	Absorption (%)	Liver/rest of animal
5.0	10 : 1	13.3	1 : 4
6.0	10 : 1	16.3	1 : 5
8.5	>20 : 1	7.9	1 : 10
5.3	>20 : 1	11.1	1 : 13
11.6	6 : 1	6.4	1 : 10
7.6	>20 : 1	13.9	1 : 10
10.0	>20 : 1		
7.7(SE2.5)		11.5(SE3.74)	

It can be seen that although no significant change in absorption occurred, there was a profound alteration in the distribution of the absorbed activity. When the transferrin was saturated most of the absorbed activity (90-95%) was present in the liver. This is very different from the controls in which only 10-25% was found in the liver. When plasma saturated with radioiron was injected intravenously, the iron was not preferentially removed by the liver. This indicated that the iron entering the plasma in the previously saturated animals was not bound to iron-binding protein, but was free or bound less tightly

by some other protein, e.g. albumin. In this form the liver rapidly removed the iron from circulation. A similar result was reported by Wheby and Jones (1963) who observed that absorbed iron was removed from circulation by the liver, when the saturation of the transferrin was greater than 60%.

A further experiment was carried out in order to investigate the role of transferrin in iron absorption. The experiment was an attempt to demonstrate the preferential accumulation in the capillaries adjacent to the absorbing mucosa of transferrin molecules having a low iron content. This seemed possible since it has been shown (Jandl et al., 1959) that the iron content of transferrin molecules determines their attachment to reticulocytes for iron transfer.

Rat transferrin isolated by ammonium sulphate precipitation and DEAE cellulose chromatography, was labelled with ^{131}I by the method of MacFarlane (1958). The labelled transferrin was rendered iron-free. The transferrin was injected via the tail vein into a fasted rat which had been given an oral dose of 100 µg. iron as ferric chloride 10 minutes earlier. Five minutes after injection the animal was killed and the proportion of injected activity associated with the small intestine was determined.

Only 0.9% of the injected activity was in the small intestine at this time. A significant number of iron-free transferrin molecules could not have accumulated in the absorbing area since this amount of activity could be accounted for by the

transferrin normally present in the circulating blood of the excised small intestine. This experiment does not completely exclude the possibility that transferrin molecules low in iron do, in fact, accumulate at the absorbing surface, since the experimental conditions may have been incorrectly chosen.

No further experiments were carried out, however, since the evidence given above, as well as that of other authors, makes it unlikely that transferrin saturation per se is important in the control of iron absorption.

IV. The effect of mucosal non-haem iron content.

It is possible that iron absorption is regulated by changes in the non-haem iron content of the mucosal epithelial cells. This was studied after administering acetylphenylhydrazine to produce haemolysis and thus stimulate erythropoiesis. The 50 matched male rats used in this experiment were arbitrarily arranged into 5 groups of 10. Each animal received an intraperitoneal injection of 7 mg. acetylphenylhydrazine dissolved in 0.5 ml. aqueous ethanol. This represents a dose of 35 mg./Kg., which is sufficient to cause the haemolysis of a fairly large proportion of the circulating erythrocytes.

The percentage absorption from a 10 µg. dose of radioiron was determined immediately after injection and on each of the subsequent 4 days. This was done by administering the test dose to animals which has been fasted for 24 hours and killing them 4 hours later by exsanguination. The blood was kept for serum iron and total iron-binding

capacity determinations. The percentage absorption was assessed by removing the entire gastrointestinal tracts intact and counting the carcasses. The small intestines were opened lengthwise and carefully washed in 5 changes of 0.9% saline. After blotting lightly to remove excess washing fluid, the guts were weighed and their radioactivity measured. The non-haem iron contents were then determined by a modification of the method of Brückman and Zondek (1940). The results obtained in this experiment are shown in Figures 40 to 42. It can be seen in Figure 40 that immediately after the administration of the acetylphenylhydrazine hydrochloride the percentage saturation of the transferrin rose rapidly, most probably to 100%, and the total iron-binding capacity (TIBC) was increased. Four hours after treatment the mean serum iron was 483 µg./100 ml. and the unsaturated iron-binding capacity (UIBC) 125 µg./100 ml. giving a TIBC. of 608 µg./100 ml. by the third day. During the same period the UIBC. increased to 383 µg./100 ml. and the TIBC. fell to 462 µg./100 ml.

As expected, the mean percentage absorption showed a steady increase from day 0 to day 4 (Figure 41), reflecting the change in rate of production of new red cells to replace those destroyed by the acetylphenylhydrazine. The iron content of the small intestine gradually decreased over this period. Although it is possible that the stimulation of erythropoeisis caused a decrease in the gut iron which, in turn, was responsible for the increased absorption, it

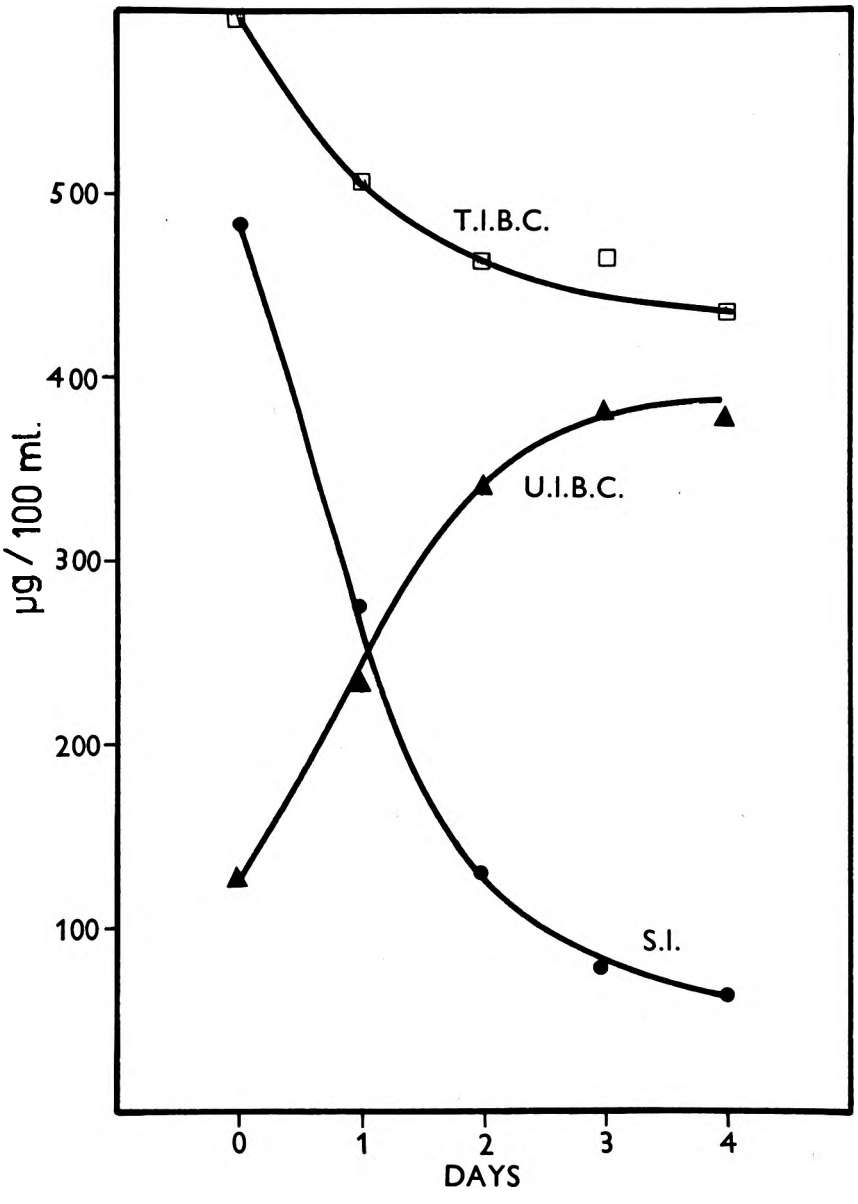


Figure 40.

The changes in serum iron (S.I.), unsaturated iron-binding capacity (U.I.B.C.) and total iron-binding capacity (T.I.B.C.) after administration of acetylphenylhydrazine.

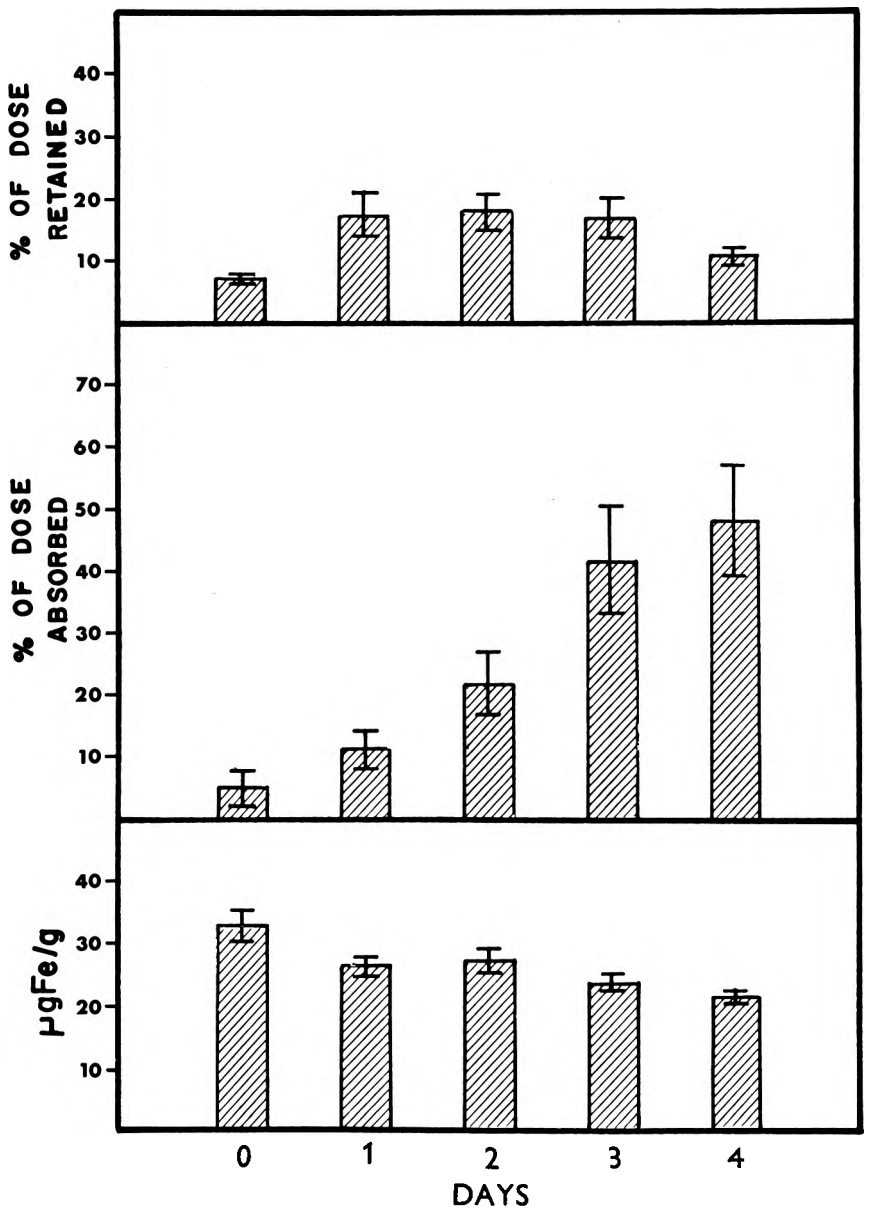


Figure 41.

The effect of intraperitoneal injection of 7 mg. acetylphenylhydrazine on the non-haem iron content of the small intestine (bottom picture); and the percentage absorbed through (middle picture) and retained by (top picture) the small intestine 4 hours after an oral dose of 10 µg. radioiron.

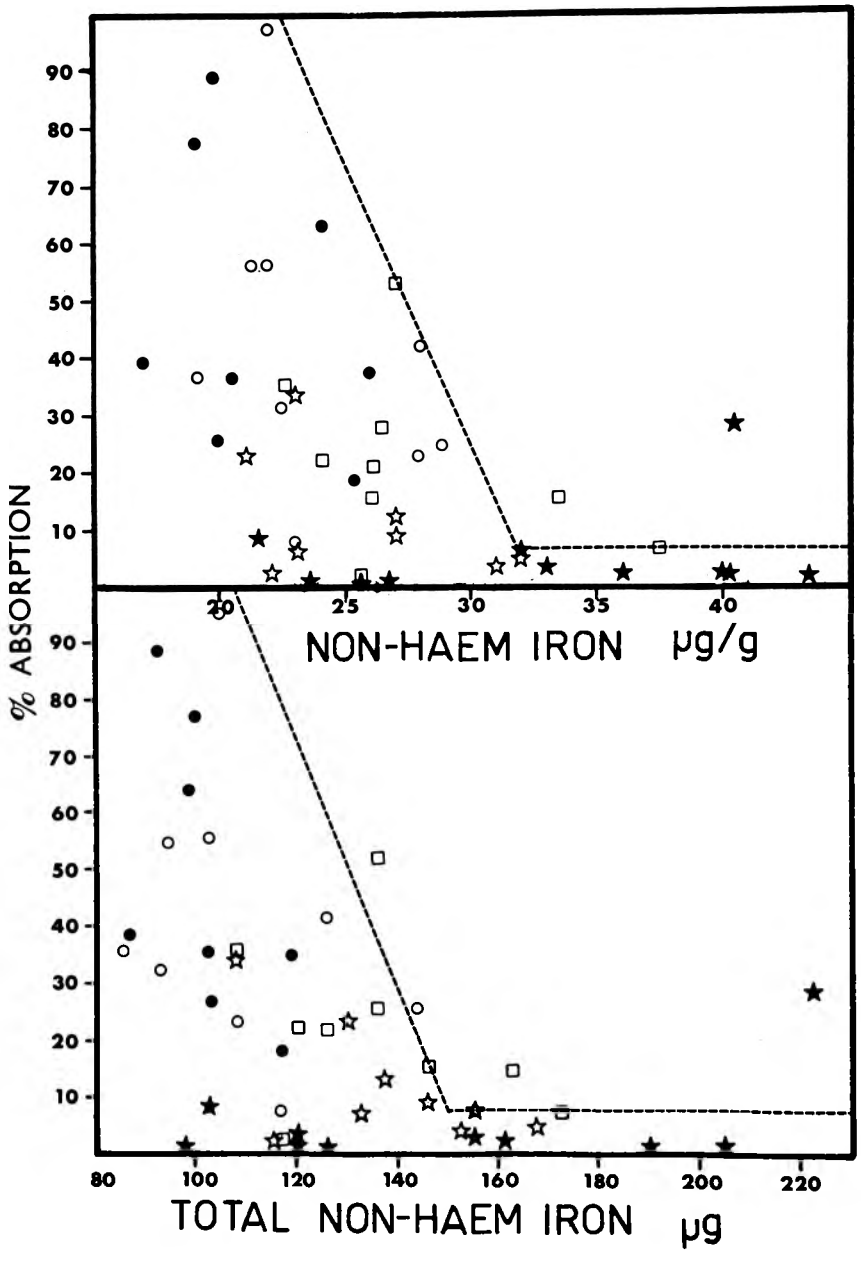


Figure 42.

Comparison of the percentage absorption of a 10 µg. test dose with both the total content and the concentration of non-haem iron in the small intestine after administration of 7 mg. acetylphenylhydrazine.

Closed stars	Day 0	Open circles	Day 3
Open stars	Day 1	Closed circles	Day 4
Open squares	Day 2		

is difficult in this experiment to define cause and effect. It seems quite possible that the increased absorption was directly due to the stimulated erythropoeisis, rather than to the fall in gut iron. Interpretation of these results is also complicated by the demonstration that acetyl-phenylhydrazine increases the rate of exfoliation of intestinal mucosa (Conrad et al., 1965).

These observations were reassessed after plotting each individual result (Figure 42). It can be seen that absorption was low (below 7%) in all except two animals with gut non-haem iron content greater than 150 µg. However the correlation between absorption and intestinal non-haem iron was not good at lower levels. Expressing the results as concentrations rather than as total non-haem iron content did not improve the correlation.

It is important to remember that the non-haem iron in the small intestine measured in this experiment represents not only the iron present in the mucosal cells, but also that present in the muscle tube of the small intestine, which contains iron-containing reticulo-endothelial tissue. It therefore remains possible that if only the iron within the mucosal cells had been measured, a more successful correlation relating iron content and absorptive behaviour could have been obtained.

V. Correlation of mucosal ferritin labelled from the plasma iron and absorption.

The depressed absorption on the day following intravenous

administration of iron suggested that iron incorporated into the developing mucosal cell might govern the cell's absorptive behaviour when it reaches its functioning position 24 hours later. An experiment was performed to establish whether iron incorporated into ferritin in mucosal cells during their development affects their subsequent ability to absorb iron. The experiment was carried out using a twin isotope technique. Rat plasma was incubated with ^{55}Fe and then dialysed against saline to ensure that all the isotope was transferrin-bound. Aliquots of labelled plasma were injected via the tail veins into the 23 rats. In addition to untreated animals, this group contained rats whose avidity for iron had been manipulated either by venesection or parenteral iron loading. The animals were fasted for 24 hours after administration of the ^{55}Fe, after which they received the standard oral dose of 10 µg. iron labelled with ^{59}Fe. Four hours later they were killed in ether and the entire gastrointestinal tract removed. Absorption was assessed by counting the carcasses. The small intestine (unwashed) was homogenised in 0.1M EDTA pH7 in water. The homogenate was heated to 75°C. in a water bath, cooled and centrifuged at 3,000 x g. Ferritin in the supernatant solution was precipitated with antiserum in the presence of carrier. The precipitates were washed with physiological saline, and counted for ^{59}Fe in a well counter. After counting, the precipitates were wet-ashed and prepared for electroplating together with

aliquots of both the intravenous (^{55}Fe) and the oral (^{59}Fe) doses. The electroplated samples were counted for both ^{55}Fe and ^{59}Fe.

The result of this experiment is shown in Figure 43. The percentage absorption has been plotted against the reciprocal of the percentage of the intravenous dose present in ferritin in the small intestine. The figures suggest that the less ^{55}Fe incorporated into ferritin, the greater the absorption of ^{59}Fe on the following day. However, the amount of ^{55}Fe did not reflect the amount of iron incorporated into ferritin, since the plasma iron turnover was markedly different in the different animals. Unfortunately it was not possible to measure plasma iron turnovers directly since it was necessary to use ^{59}Fe for the oral dose so that the whole body counter could be used to measure absorption. This meant that ^{55}Fe (which is not suitable for measuring the plasma iron turnover) had to be used for the intravenous dose. If an approximation of the plasma-iron turnover is made, however, it is apparent that the incorporation of plasma iron into ferritin within mucosal cells was approximately the same in spite of varying absorptions. The rats in which high absorptions were found were those which had been venesected, and it is in these animals that the plasma-iron turnover would be increased to about two to three times normal. It therefore seems that plasma iron incorporated into mucosal ferritin correlates poorly with

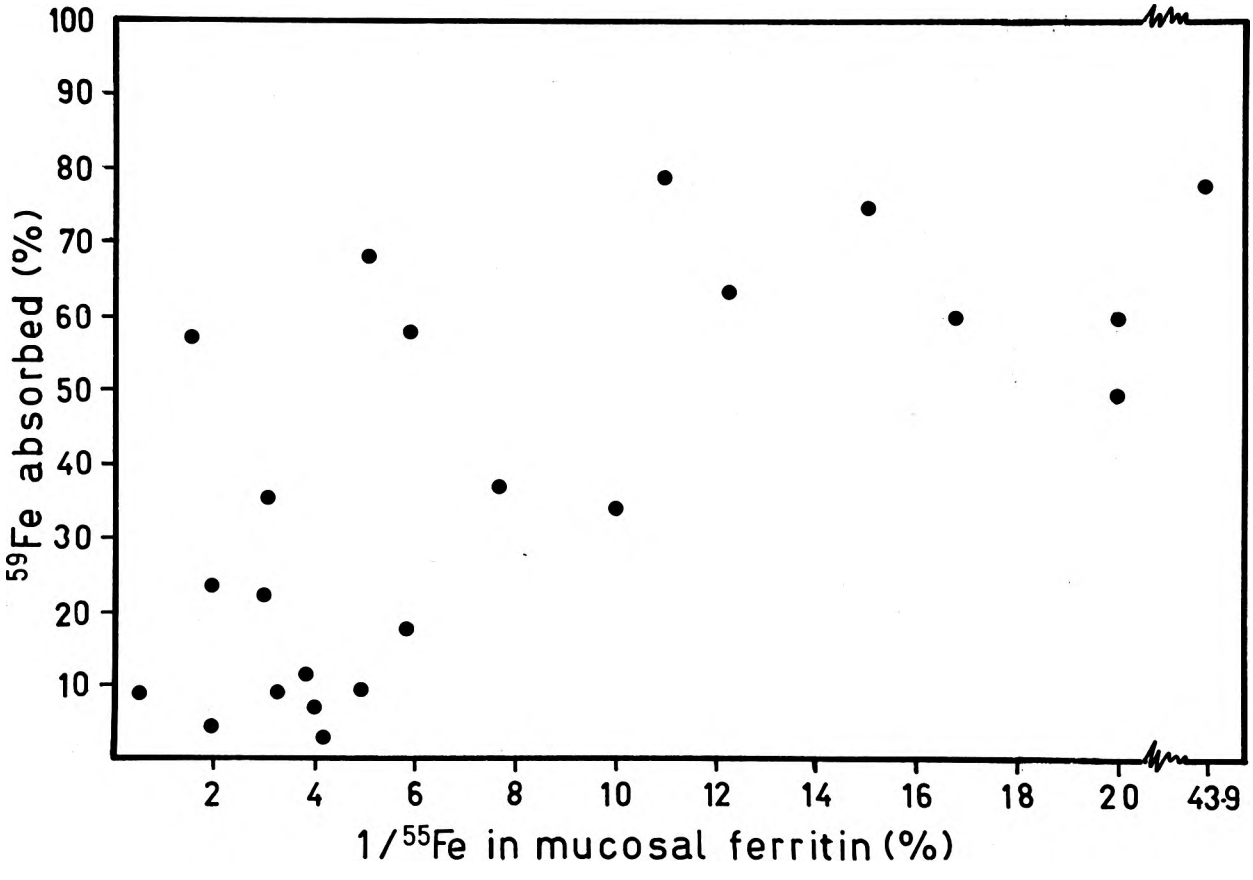


Figure 43.

Correlation of the absorption from an oral dose of 10 µg. ^{59}Fe with the reciprocal of the percentage of an intravenous dose of ^{55}Fe incorporated into ferritin in the small intestine. The ^{55}Fe was bound to plasma and injected 24 hours before giving the oral dose.

absorption. The real correlation revealed in Figure 43 is therefore between plasma iron turnover, a measure of erythroid marrow activity, and iron absorption.

CHAPTER VII.

SUMMARY AND DISCUSSION.

CHAPTER VII.

Although ferritin has previously been shown to be present in the mucosal cells of the small intestine after the oral administration of massive doses of iron, the evidence that it was concerned in the absorption of doses of iron in the normal dietary range was conflicting.

The observations reported in Chapter III establish that not only are small amounts of ferritin present in the intestinal mucosa during absorption of as little as 1 µg. iron, but also that this ferritin plays an integral part in the absorptive process.

An examination of the techniques employed by some of the workers who were unable to identify ferritin in absorbing mucosa reveals the probable explanation for the discrepancies. Brown and Rother carried out Ouchterlony-gel diffusion, but were not able to demonstrate the presence of ferritin in their mucosal extract. Their investigations led them to believe that the mucosal iron-binding protein was more closely related to transferrin than to ferritin. These authors purified their protein-bound iron by several procedures before attempting to identify the protein. Although this appears logical, it may result in a false impression. The starting material was the non-particulate supernatant solution obtained by centrifugation of rat gut homogenate at 105,000 x g for sixty minutes. When we submitted a purified ferritin solution to centrifugation in this manner, 80% of the ferritin iron was present in the dark brown precipitate.

In preparing his non-particulate supernatant solution Brown would therefore be expected to lose a large proportion of the ferritin present in the initial homogenate (figures obtained with gut extracts suggest that about half the radioactivity attached to ferritin is removed by this procedure). The non-particulate supernatant solution was then purified by Brown, using ammonium sulphate precipitation. From precipitation curves carried out using in vitro labelled ferritin and purified transferrin labelled with radioiron (Figure 37) it can be seen that whereas all the transferrin would be precipitated between 45% and 60% saturation, only about 20% of the ferritin would be precipitated due to its heterogeneous nature. The precipitate which Brown obtained by increasing the ammonium sulphate saturation from 45% to 60% was further purified using calcium phosphate gel. The yield from this final step is only 10%. It is apparent from this procedure that if ferritin were initially present most of it would have been eliminated during 'purification' making identification almost impossible on the final product. Beutler was also unable to identify ferritin in rat duodenal mucosa. He tried to identify the protein by the rather crude method originally employed by Granick, i.e. looking for ferritin crystals after mixing mucosal scrapings with cadmium sulphate solution. Although Granick was able to see a few crystals in the duodenal scrapings of untreated guinea pigs, it seems doubtful whether this method is sensitive enough to detect the small

quantities of ferritin involved.

Evidence compatible with our own results was presented recently by Hartman et al. (1963) who demonstrated the presence of ferritin in electron micrographs of the absorbing cells of the jejunum of fasted human iron-replete subjects given 25 mg. of ferrous iron. The ferritin was found chiefly in inclusion bodies in the apical cytoplasm. These ferritin-containing bodies were oval in profile and had a length of 0.5-1.5 μ. They were not bounded by membrane but had a moderately dense background substance which produced a sharp boundary with the cytoplasmic ground substance. However, no evidence that these bodies were concerned with absorption was obtained.

Evidence was also obtained in the present study to suggest that the labelled iron preferentially entered ferritin molecules with a relatively low iron content. Ultra-centrifugation of a solution of ferritin led to the precipitation of 80% of the ferritin iron. However, when the supernatant solution from a mucosal homogenate was subjected to the same procedure only 50% to 60% of the labelled ferritin iron was spun down. Mazur, Litt and Shorr (1950) have shown that centrifugation of a solution of ferritin results in its separation into iron-rich and iron-poor fractions, and the current observations therefore suggest that a greater proportion of mucosal ^{59}Fe is attached to iron-poor ferritin molecules. Similar observations have been reported with regard to hepatic ferritin (Mazur, Green and Carleton, 1960).

When the proportion of mucosal radioactivity attached to ferritin was examined under various circumstances, some insight was gained into its function in the absorptive process. During the first hour after administration there was a progressive rise in the percentage bound to ferritin, with a corresponding fall in the non-ferritin fraction (Figure 14). Both the present study and those of Wheby and Crosby (1963), Stewart and Gambino (1961), Hallberg and Sölvell (1960) and Brown and Rother (1963a) have established that iron moves rapidly across the mucosa at an early stage and much more slowly thereafter. Since most of the mucosal activity is attached to ferritin in the later phases, it seems probable that ferritin is not directly concerned with the transfer across the cell. Results in iron-deficient animals support this interpretation, since a very rapid transfer of a large percentage of the administered dose was observed although little labelled ferritin was present in the mucosa (Figure 16). Brown and Rother (1963b) also noted that most of the radioactivity in the supernatant solution from a mucosal homogenate was not bound to protein when absorption was enhanced. In normal animals they found a build-up of protein-bound iron during the first hour, and, although these workers did not believe that the fraction represented ferritin, it correlated well with the ferritin fraction in the current study.

The ultimate fate of iron sequestered within mucosal cells appears to depend on several factors, Using small doses

of iron, Wheby and Crosby (1963) have produced evidence that some of this iron may feed gradually into the body. Similar findings were obtained in the current study. However, it was not possible to show the late absorption of mucosal activity when larger doses were given, or when small doses were given to animals that had recently received parenteral iron. The results in this experiment were particularly striking. Two hours after the administration of radioiron, a mean of 35% of the administered activity was in the mucosa and 3.3% in the remainder of the carcasses. By 24 hours mucosal radioactivity had dropped to negligible levels, whereas the mean activity in the carcasses was only 4.7%. Mucosal iron must therefore have been lost into the lumen of the gut. Theoretically this could occur either by extrusion of ferritin from the mucosa or via exfoliation of the intestinal cells. Conrad and Crosby (1963) have published observations strongly supporting the second possibility. Using autoradiographs of rat intestinal mucosa at various intervals after oral administration of labelled iron, these workers have demonstrated both the sequestration of ^{59}Fe within the mucosal cells and the progress of the labelled cells towards the tips of the villi. As a result of their studies, these workers envisaged a mucosal mechanism for the control of iron absorption in which iron taken up from the intestinal lumen into the absorbing cells in excess of body requirements is trapped within the mucosa and discarded when these

cells exfoliate. They postulated that the sequestered iron is in the form of ferritin, and the present study provides experimental evidence in support of this suggestion. Although the mechanism appears to function well when small amounts of iron are presented to the mucosa, the percentage of mucosal activity precipitable as ferritin is much lower with large doses.

The nature of the non-ferritin activity in mucosal supernatant solution was also investigated in the present study. High concentrations of activity appeared in this fraction shortly after the administration of labelled iron, and then declined rapidly over the next two hours. These findings indicate that it was probably concerned in the initial rapid phase of iron absorption. Previous reports by Brown and Rother (1963b) have suggested that the iron in this rapid transport fraction is associated with the amino acids serine and glycine. However, results in the present study indicate that the findings obtained by these workers were due to the iron forming a complex with the Chelate Versene-Fe-3 Specific, which had been used in the initial washing of the mucosa. Further investigation was not successful in positively establishing the nature of the bulk of the non-ferritin activity. Although small and variable percentages often appeared to be attached to transferrin, the significance of this finding is open to question, since evidence was obtained suggesting that iron present in some other form might attach to the transferrin of contaminating plasma (Figure 27). The nature of the remaining non-ferritin

fraction was not elucidated, but certain of its characteristics were defined. The iron was in a form that was only dialysable if a chelate such as $\alpha\alpha'$-dipyridly, EDTA, or Versene-Fe-3 Specific had been present during the preparation of the homogenate. It was largely precipitable with ammonium sulphate pH 7.5, but not with perchloric acid. A chelate-free mucosal extract submitted to paper electrophoresis in a barbital system gave rise to bands of activity corresponding to ferritin and transferrin. In addition, activity was present at the origin and between the origin and the transferrin band. This activity disappeared when plasma was added to the mucosal extract. A sharply defined band having a much faster rate of migration than ferritin was also found in autoradiographs in which exposure had been extended for a longer period. This band was not diminished by adding plasma, and after oral administration of radioiron it was more marked at 10 minutes than after 2 hours. This band obviously merits further investigation, since its behaviour suggests that it may be associated with the early active transport system. Although accounting for only a very small proportion of the total mucosal activity it is possible that it may be the chief form of transport iron if it turns over very rapidly.

Finally, an attempt was made to discover how mucosal cell behaviour with regard to the handling of iron is regulated. In general, when body iron stores are depleted or when erythropoeisis is stimulated acutely, more iron passes

through the mucosa into the plasma (Bothwell and Finch, 1962). On the other hand, the present findings indicate that a major portion of the iron taken up from the lumen is trapped in mucosal cells when the body iron content is high, and this is especially so when a recent parenteral injection has been given. This suggests that mucosal cell function may be determined, at least in part, by the iron content of absorbing cells. However, manoeuvres that might be expected to modify the iron content of mucosal cells, such as raising the serum iron (Wheby and Jones, 1963), or administering 'blocking' doses of iron orally (Brown, Dubach and Moore, 1958), have led to equivocal results. In the present study the effects of previous parenteral and oral iron administration were reinvestigated in several ways.

The administration of three intravenous doses of ferric ammonium citrate at five-hour intervals had no immediate effect, but a depression in absorption was apparent six hours after the first injection and was marked at 18 hours (Figure 38). By 48 hours absorption had risen again, although not yet to control levels. These findings are in agreement with those reported by Conrad, Weintraub and Crosby (1964), who noted a depression in absorption in rats 24 hours after the administration of a single large injection of iron. These workers suggested that the behaviour of mucosal cells is conditioned during their developmental period in the crypts of Lieberkühn. On this basis any manipulation of the cellular environment

must be followed by a latent period during which 'conditioned' cells move out into their functional position on the villi. The present results are compatible with this thesis, since the observed changes in absorption correlate well with the turnover rate of intestinal cells (Messier and Leblond, 1960). Parallel studies indicated that the percentage saturation of circulating transferrin was maintained at high levels for the first 12 hours of the study. It is thus possible, though not proven, that the changes in mucosal behaviour were related to this environmental factor.

It is doubtful whether these studies correlating absorption with the amount of intravenously administered iron going to the small intestine are of physiological significance because the amount of iron administered was far in excess of the binding capacity of the serum. In their experiments Conrad et al. injected up to forty times the amount which can be bound by plasma, or eight times the normal daily plasma iron turnover. Increasing the intravenous dose of iron from 10 µg. to 100 µg. produced a negligible drop in absorption. Free iron is not normally present in the body, and the excess iron injected would not be handled by normal physiological mechanisms. Evidence has been obtained to show that unbound iron is rapidly removed from circulation, probably entering the parenchymal cells such as the intestinal mucous membrane. The relevance of these observations to physiological mechanisms is therefore

dubious. However, Weintraub, Conrad and Crosby (1964) obtained further evidence to suggest that the iron content of the mucosa is the controlling factor in iron absorption. They showed that following an acute blood loss there is a four or five day lag before any demonstrable changes in plasma iron kinetics occur. After this delay there is a more rapid clearance of radioiron from the plasma and a transient fall in the serum iron. Associated with these changes there is a decrease in intestinal iron content and iron absorption is increased. In another elegant study (Weintraub et al., 1965) absorption was examined in animals whose erythropoeitic activity had been depressed by returning them to normal atmospheric pressure after a period of seven days at a pressure equivalent to an altitude of 20,000 feet. They found that the reduction in iron absorption exactly paralleled the decrease in erythropoeitic activity, and was associated with increased deposition of iron in the gut. The authors suggested that these experiments indicated that utilisation of iron for erythropoeisis closely regulates the iron content of the absorptive epithelial cells and that this, in turn, determines the ability of these cells to absorb iron from the lumen. It should be pointed out, however, that the interpretation of these results is not strictly in accordance with the hypothesis previously proposed by these workers. According to this hypothesis a change in absorptive behaviour should occur only when mucosal cells which have developed under the altered conditions reach their functional positions.

There should therefore be some delay between the depression of erythropoeitic activity and the fall in mucosal non-haem iron with the concomitant decrease in iron absorption. In the study outlined above, however, these processes occurred exactly in parallel, and no such delay was observed.
The experiment in which mucosal non-haem iron and iron absorption were measured in phenylhydrazine-treated rats provided superficial support in favour of mucosal hon-haem iron governing absorption. The mean percentage absorption increased steadily for four days and was accompanied by a steady decrease in the mean gut non-haem iron content. However, when the results were examined more carefully, and individual absorptions were compared with the gut non-haem iron content, a totally different picture emerged. Whereas it seemed that animals with a high gut non-haem iron absorbed iron poorly, the reverse was not true, since animals with low gut non-haem iron levels had absorptions covering the entire range from 0-100% (Figure 42).
Although there is thus some relation between iron absorption and the gut non-haem iron content, it has not been established that absorption is controlled by the gut non-haem iron content. It seems entirely possible that both variables respond independently to alteration of some third factor.

In a final study, previous observations of Bannerman, O'Brien and Witts (1962) were confirmed. Stock rats were subjected to a high oral intake of iron for several days, and then an abrupt change was made to a diet with a very low iron content. Absorption increased progressively

between 24 and 60 hours after the changeover, and it was shown that the initially low absorption could not be ascribed to dilution of the label with non-radioactive iron still present in the lumen or in functional mucosal cells. Pollack, Kaufman and Crosby (1964b) have reported similar findings in rats switched to a low iron diet from a normal intake. Although the differences were not statistically significant until the animals had been fed the new diet for four days, a steady rise in mean absorption after the changeover was also observed in this study. These workers showed that this increased absorption had not been triggered by the small net loss of iron on the low iron regime, and that neither the half-time of ^{59}Fe in the plasma nor the serum iron level was different from the control group. In the light of these observations, the fall in serum iron concentration in the present study may not be causally related to the subsequent change in absorption, even though the timing of these events is suggestive (Figure 39). Nevertheless, the gradual increase in absorption over several days is again compatible with the steady replacement of the absorbing mucosa by new cells 'conditioned' during their development, in some way not yet established, by the altered iron content of the diet.

The observations reported in the present investigation and those published by other workers, although affording tantalising glimpses of the mechanisms controlling iron absorption, have not fully revealed them. It is apparent that there is still scope for further studies in this area.

A P P E N D I X

THE *IN VITRO* LABELLING OF FERRITIN.

APPENDIX.

I. In vitro labelling.

When a tracer quantity of $^{59}FeCl_3$ was added to a pure solution of ferritin a high percentage of the added radioactivity was found to be associated with the ferritin. This radioiron remained firmly attached to the protein and was not displaced during either starch-gel electrophoresis or passage through Sephadex or ion exchange columns (such as Amberlite CG50 or DEAE cellulose). Ferritin which had become labelled in this way was referred to as in vitro labelled.

II. Prevention of in vitro labelling.

In the present investigation it was necessary to prevent in vitro labelling in order to identify ferritin labelled in vivo. Experiments described in Chapter III established that 0.1M citrate or 0.01M EDTA pH6 or 7 prevented in vitro labelling without removing any in vivo label.

III. Possible mechanisms for in vitro labelling.

The mechanism by which ferritin becomes labelled by tracer radioiron in vitro is unknown. Theoretically there appear to be several ways in which this could occur, and these are discussed below.

A. Direct binding.

Ferritin, even after six crystallisations, is not homogeneous. It contains molecules of varying iron content, ranging from apoferritin, with no iron, to

solely to this mechanism the ^{59}Fe would distribute itself between the ferritin and the solution in the ratio of the concentration of free iron to exchangeable ferritin iron. Such an exchange mechanism would be time dependent, the rate of exchange depending on the strength of the apoferritin-iron bond. Furthermore, if all iron atoms in ferritin are bound to the same extent, the added radioiron should be associated preferentially with the iron-rich fraction.

IV. Investigation of *in vitro* labelling.

Although the mechanism of *in vitro* labelling was not relevant to the present investigation, several experiments were carried out in an attempt to understand the process. These experiments are reported here even though they represent only an initial attack on a problem which still requires further investigation.

A. Iron uptake.

Experiments were carried out to determine how much iron could be taken up by a given ferritin sample. Initial studies failed to provide useful results because of the marked drop in pH caused by addition of large quantities of ferric chloride. This difficulty was overcome by using maleate-sodium hydroxide buffer.

Amounts of non-radioactive iron ranging from 1 to 1000 µg. were added to tubes containing tracer ^{59}Fe and buffer. After readjusting the pH in the tubes to 7.45 they were allowed to stand for one hour. During this time the formation of a precipitate in the tube containing 1000 µg. iron necessitated its exclusion from the study.

After an hour a fixed amount of ferritin was added to each tube. This ferritin was precipitated one hour later by the addition of antiferritin. The proportion of radioactivity present in the washed precipitate was measured and the amount of iron determined chemically. The result of this experiment is shown in Table A1.

Table A1.
Iron uptake by ferritin at pH 7.45.

Fe added µg.	Radioactivity in precipitate (%)	Calculated incorporated Fe (µg)	Observed iron in precipitate Fe (µg.)
0 (tracer only)	60	0	108
1	60	0.6	111
10	23	2.3	111
100	9	9	114
500	52	250	262
1000	PRECIPITATED		

This experiment showed that the percentage binding fell exponentially with increasing iron concentration up to 100 µg. whilst the absolute amount of iron bound increased. However, when 500 µg. iron was added, the amount of iron in the precipitate was far more than anticipated. It seemed possible that the precipitated iron was ferric hydroxide rather than ferritin-bound iron in this tube. Too high a concentration of ferric iron at this pH (7.45) probably leads to precipitation rather than sol formation. In experiments carried out to investigate <u>in vitro</u> labelling it was important to bear this in mind, since radioactivity precipitated in

this way would be measured with the ferritin-bound radioiron and falsely high results would be obtained.

B. Rate of *in vitro* labelling.

An investigation into the rate at which *in vitro* labelling of ferritin occurs was carried out. A tracer quantity of radioiron was added to a ferritin solution. Aliquots were removed after various time intervals, and pipetted directly into rabbit anti-ferritin in order to stop the labelling process and isolate the ferritin. (A preliminary study had indicated that the ferritin-antiferritin complex was unable to bind free tracer radioiron.).
From the result obtained in this experiment (Figure A1 line A) it is apparent that the labelling process, although time dependent, is very rapid. Because of this the early stages have been expanded to show the time-labelling relationship more clearly (Figure A1 line B). When the time is plotted on a logarithmic scale a straight line is obtained (Figure A1 line C).

The formation of the ferritin-antiferritin complex is not instantaneous, so that labelling was not immediately halted in this experiment. A further experiment was performed in which labelling was stopped with EDTA. It is important to remember, however, that although EDTA is immediately able to prevent further labelling, it unfortunately removes approximately 50% of the activity present in freshly prepared *in vitro* labelled

ferritin.

This experiment was carried out in the same way as the previous one, except that the aliquots of ferritin containing tracer radioiron were pipetted into 0.1M EDTA pH7 instead of into the rabbit antiferritin serum. The ferritin was then precipitated with antiserum from the EDTA solution. The precipitate was washed with saline and the activity counted to determine what proportion of the added radioiron was associated with ferritin. A zero time sample was prepared by adding tracer radioiron to an aliquot of ferritin containing EDTA. The result of this experiment (Figure A2) is almost the same as that obtained in the previous experiment, although the time dependence of labelling is more evident. It is possible, however, that this may in part be due to the EDTA removing activity more easily from newly formed in vitro labelled ferritin, progressively less being removed as the labelling matures.

C. The effect of hydrolysis on the uptake of tracer iron.

The effect of hydrolysing the ferric chloride before adding it to the ferritin was assessed by the following experiment.

Tracer iron was added to a freshly prepared solution of ferric chloride containing 10 µg. iron in 1 ml. This solution was heated to boiling and cooled in order to promote hydrolysis of the ferric salt. The radioactive

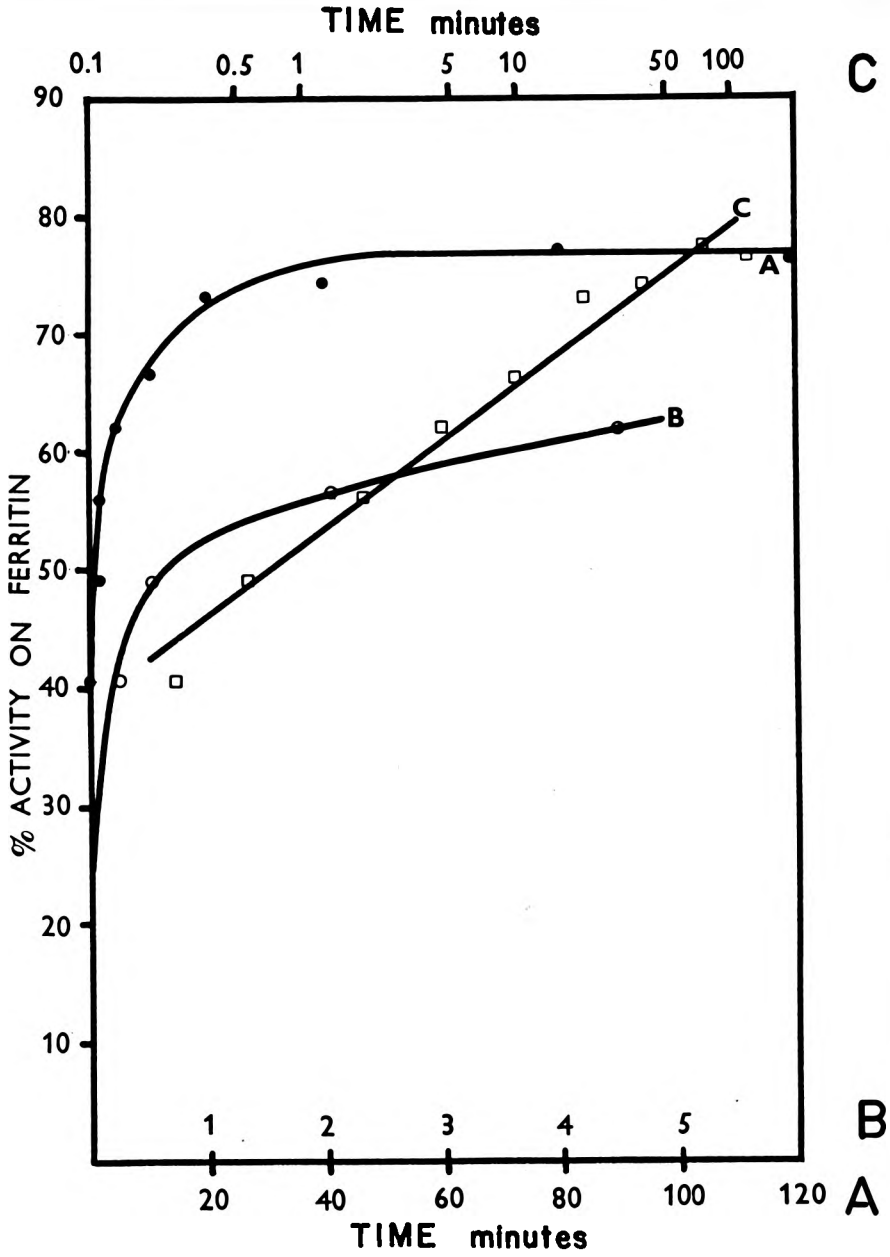


Figure A1.

The rate at which tracer ^{59}Fe added to a ferritin solution becomes protein bound. The rate of labelling up to 120 minutes is shown by closed circles (Scale A). Binding in the initial five minutes is shown by open circles (Scale B). When time is plotted on a logarithmic scale (C) the rate of labelling can be represented by a straight line (open squares).

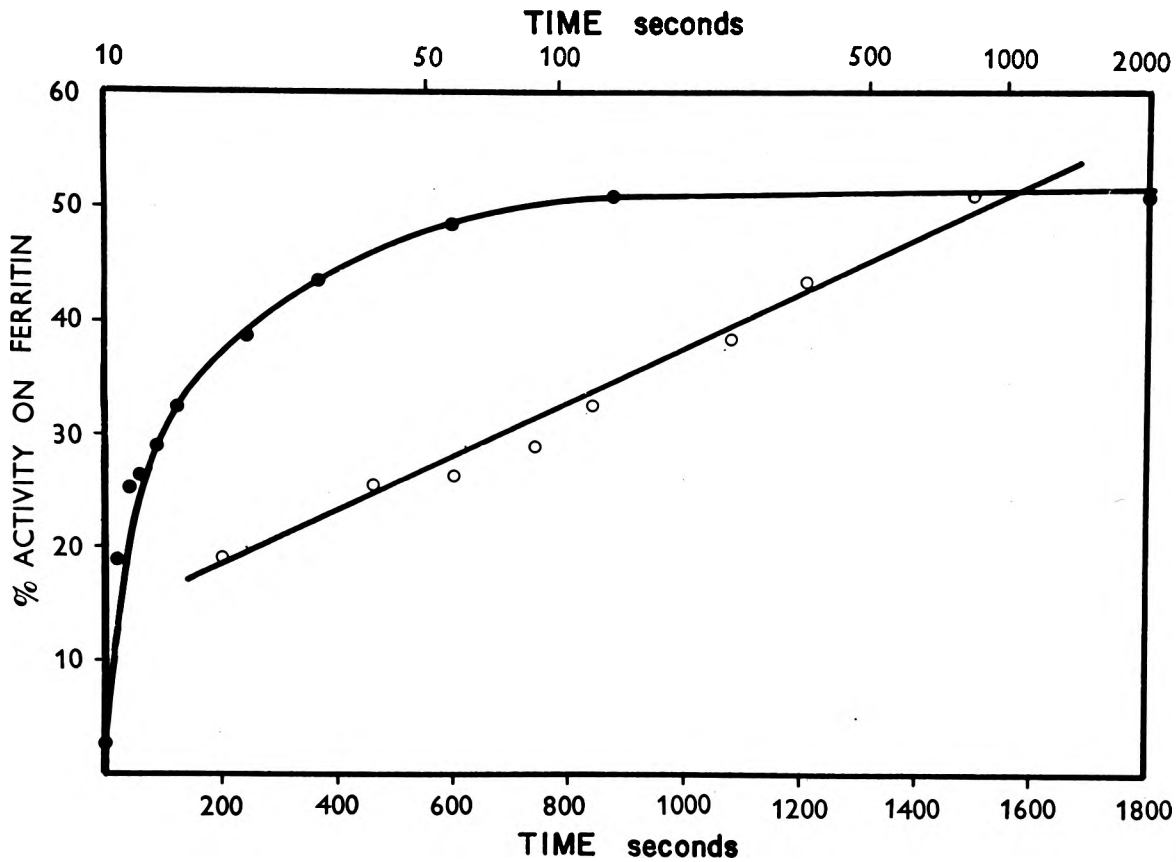


Figure A2.

The curve (black dots) shows the rate at which ferritin appears to take up tracer radioiron when EDTA is used to stop labelling. (EDTA is able to remove up to 50% of *in vitro* label). When time is plotted on a logarithmic scale a straight line is obtained (open circles).

ferric hydroxide sol produced in this way was added to a ferritin solution. Aliquots were removed and precipitated with antiserum after various time intervals as before. A second tube was set up in the same way omitting only the heating step. The percentage of added activity remaining attached to the ferritin-antiferritin precipitate after washing with physiological saline was determined.

It was found that heating a radioiron solution prior to adding it to ferritin enhanced considerably the proportion of label taken up by the protein (Figure A3). Zero time samples were prepared, as previously, by adding heated and unheated radioiron to aliquots of ferritin containing EDTA. Although EDTA prevented completely the uptake of unheated radioiron 44% of the heated sample was taken up by ferritin. It is apparent that EDTA does not inhibit <u>in vitro</u> labelling in the presence of hydrolysed radioiron. These results seem to favour the hypothesis that <u>in vitro</u> labelling is due to the formation of a colloidal solution containing micells having a charge which attracts them to a suitably charged portion of the ferritin molecule.

In another experiment, iron uptake from ferric ammonium citrate and hydrolysed ferric chloride was compared at pH 8.4. One hundred micrograms of labelled ferric chloride in 1 ml. water was heated to boiling. One tenth of this iron was transferred to a tube containing 5 ml. of 0.01M Tris. buffer pH 8.4. The remaining iron

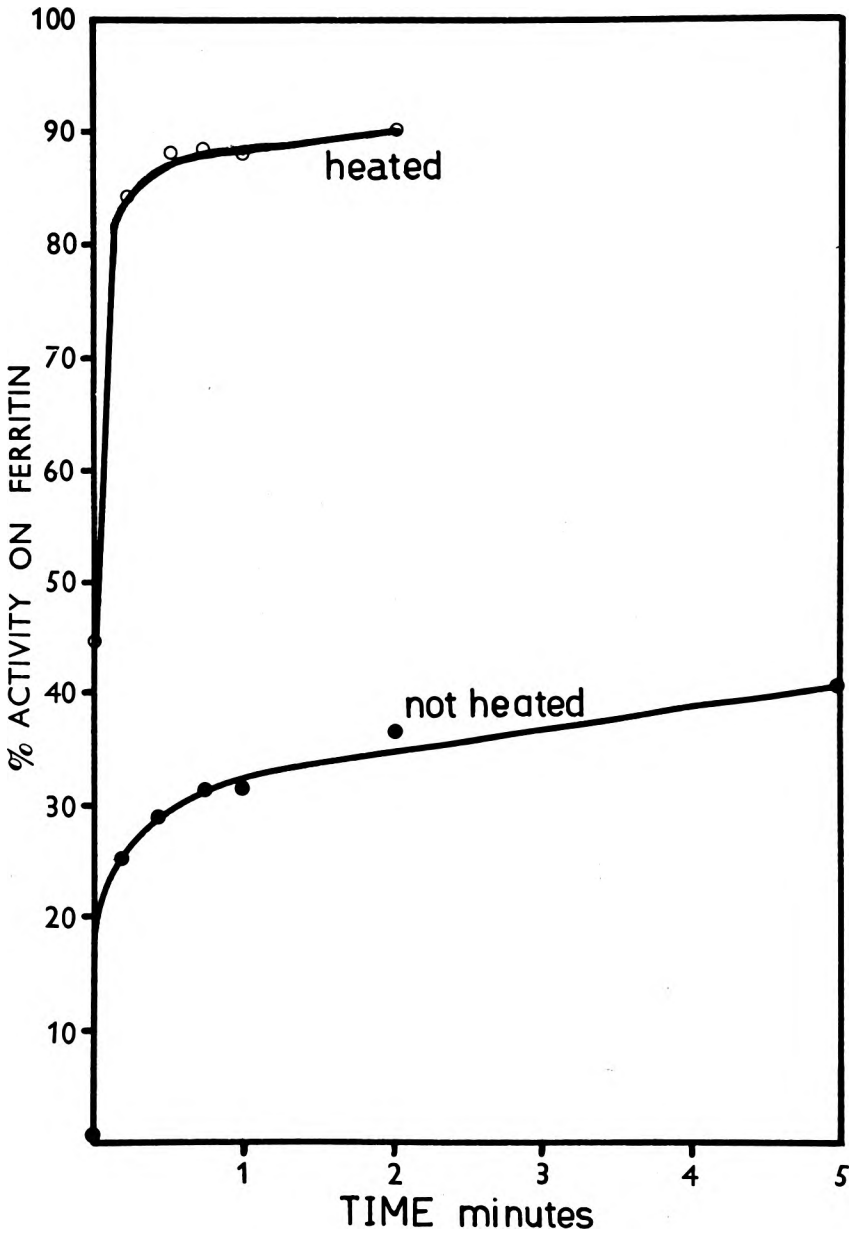


Figure A3.

The effect of heating on the uptake of radioiron by ferritin.

was converted to ferric ammonium citrate and 10 µg. of this iron was also added to 5 ml. of the buffer. One tenth of a ml. of ferritin was added to each tube. One hour later the amount of radioiron associated with ferritin was determined by precipitation with anti-ferritin. In the tube containing ferric ammonium citrate only 4% of the activity precipitated compared with 74% in the other specimen. This suggests that iron in a soluble form is not markedly bound by ferritin *in vitro*.

D. *Exchange*.

An experiment was designed to see whether the addition of non-radioactive iron removed the tracer from *in vitro* labelled ferritin. A sample of ferritin containing 3 mg. iron was labelled by addition of tracer radioiron. The following day 1 mg. ferric chloride freshly dissolved in water was added. The same amount of iron containing tracer $^{59}FeCl_3$ was added to an equivalent sample of unlabelled ferritin. Aliquots were removed from both tubes after various time intervals. The ferritin in these aliquots was precipitated by pipetting them directly into antiferritin serum, and the proportion of activity associated with the washed precipitate was determined. The result obtained is shown in Figure A4.

In this experiment 3000 µg. of ferritin iron and 1000 µg. of free iron were present. If exchange is the sole mechanism by which *in vitro* labelling occurs, the

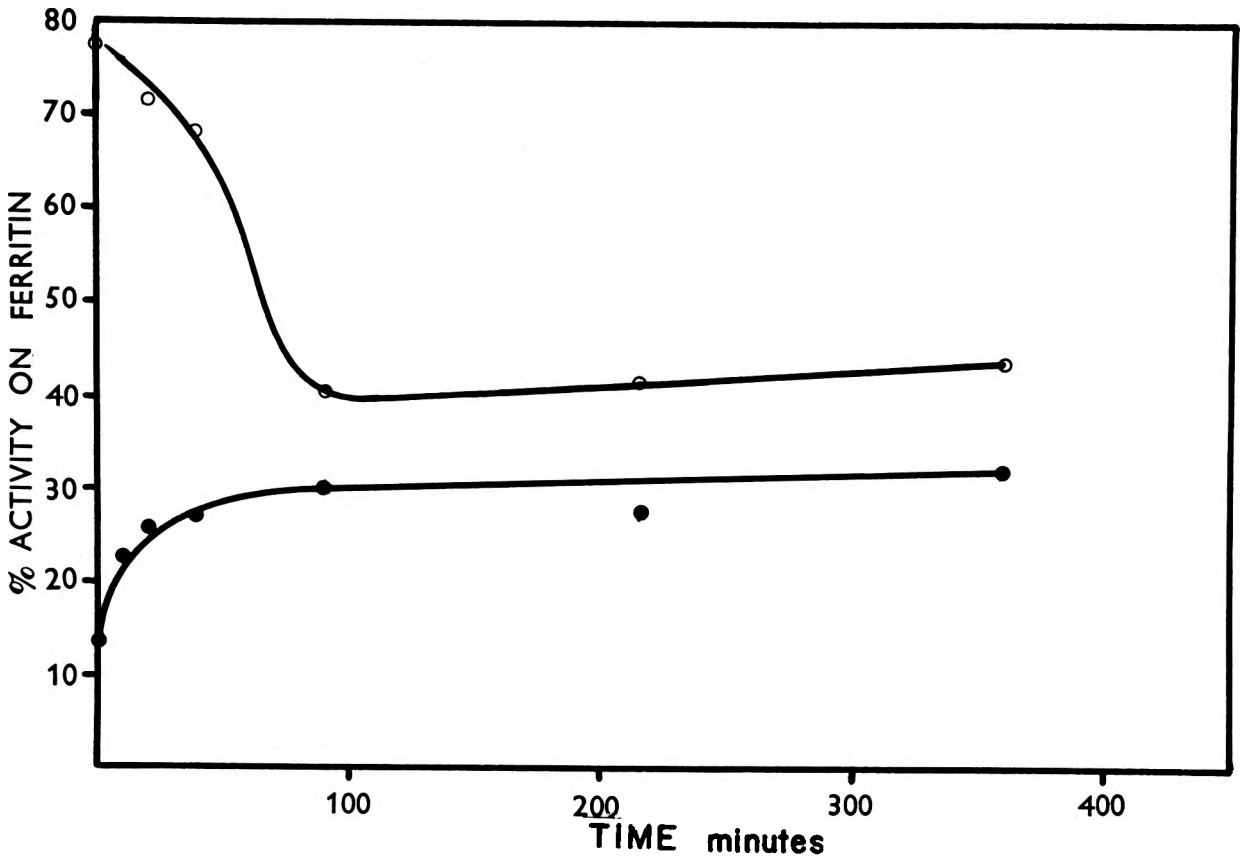


Figure A4.

The removal of <u>in vitro</u> label and the uptake of radioiron in the presence of free iron. A sample of ferritin containing 3 mg. iron labelled by addition of tracer radioiron 24 hours before carrying out the experiment. The percentage of the <u>in vitro</u> label still associated with the ferritin at various times after the addition of 1 mg. of iron as ferric chloride is shown by open circles. The closed circles show the rate at which a similar sample of unlabelled ferritin picked up radioactivity from a solution containing 1 mg. free iron as $^{59}FeCl_3$.

proportion of label attached to ferritin is predictable. The ferritin solution originally labelled by addition of tracer would lose radioactivity, whilst the ferritin solution containing no radioactivity would become labelled. These processes would continue until equilibrium had been established, when 75% of the radioactivity should be associated with ferritin in both samples.

It can be seen from Figure A4 that this prediction was not fulfilled. In both samples approximately 1/3 of the activity was bound to ferritin. This result is compatible with the exchange theory only if it is postulated that not all the ferritin iron is exchangeable. Nevertheless, the fact that radioiron was displaced from ferritin by the addition of unlabelled ferric chloride is difficult to explain on any basis other than exchange.

The removal of <u>in vitro</u> labelling by freshly dissolved non-radioactive ferric chloride was investigated in another experiment. On this occasion <u>in vitro</u> labelled ferritin was incubated at 37°C. with a ferric chloride solution having the same iron content as the ferritin itself. A second sample was incubated with twice as much iron. Aliquots were removed after various intervals and the counts associated with ferritin determined as previously. The result obtained is shown in Table A2.

Table A2.

Removal of *in vitro* label by ferric chloride.

Time (hours)	% of *in vitro* label on precipitate		
	µg. iron added/100 µg. ferritin iron		
	100 µg.	200 µg.	0
0	93	90	93, 91
1/6	90	88	
3/4	87	88	
2	81	80	
18	69	64	
24	62	61	

Once again, the result obtained was unexpected. Theoretically 1/2 and 2/3 of the radioactivity should be removed in the respective samples, if exchange were proceeding according to established principles. However, both the rate of removal and the final amount removed over 24 hours were found to be the same irrespective of the amount of non-radioactive iron added. In a further experiment, various amounts of ferric chloride, labelled with similar amounts of tracer radio-iron, were added to aliquots of ferritin solution. The mixtures were left overnight at 4°C. before precipitating the ferritin with antiserum. The proportion of added activity associated with ferritin was determined by counting the washed precipitates. These figures were compared with those obtained by calculation, assuming that an exchange reaction was responsible for the labelling and that all the ferritin iron was available for such an exchange. Since the observed

values were different from the predicted ones, the proportion of ferritin iron which would have to be exchangeable to give the experimental results was also calculated. These results are shown in Table A3. The observed uptake of label was less than the theoretical amount in each case. Furthermore, the calculated value for the exchangeable pool of ferritin iron was extremely variable.

Table A3.

Comparison of experimental values with the theoretical iron uptake for exchange labelling.

Fe added µg.	Theoretical binding expected (%)	Observed in vitro label precipitated (%)	Calculated pool of Exchangeable Fe to give experimental result (µg.)
10.2	90	61	16
51	65	51	53
102	48	36	57
510	15	4.3	23
1020	8.5	3.2	34

It should be mentioned that this experiment differed from that described on page 184. In the earlier experiment the pH was kept constant at pH 7.45 whilst in this experiment no attempt was made to maintain constant pH. This difference in design probably accounts for the apparent conflict between the results of these experiments. The effect of pH was therefore examined.

E. Effect of pH on *in vitro* labelling.

The following experiments indicated that the labelling was markedly influenced by pH changes. A range of pH from 6.1 to 10 was obtained with 0.1M Tris-HCl buffer. To 1 ml. of buffer was added 0.2 ml. of ferritin solution followed by a tracer quantity of radioiron. One hour was allowed for binding to occur. After this the ferritin was precipitated with antiserum and the proportion of added radioactivity associated with ferritin was determined as before. *In vitro* labelling was found to be greatest at pH 6.1, when 90% of the radioiron was associated with ferritin. As the pH increased the efficiency of labelling decreased rapidly until at pH 10 it was only 12% (Figure A5 Curve A). A similar result was obtained in an experiment in which the method was altered slightly. On this occasion the tracer radioiron was added to the buffer and allowed to stand for one hour in order to allow hydrolysis of the tracer radioiron to occur. Ferritin was then added and the solutions left at 4°C. overnight, before precipitating the ferritin and determining the percentage labelling. The pH range covered was 6.7 - 10.4. Determination of the iron in the precipitates indicated that quantitative precipitation of ferritin by the antiserum had occurred. The diminishing proportion of radioiron precipitated with increasing pH could therefore not be due simply to interference with the antigen-antibody reaction.

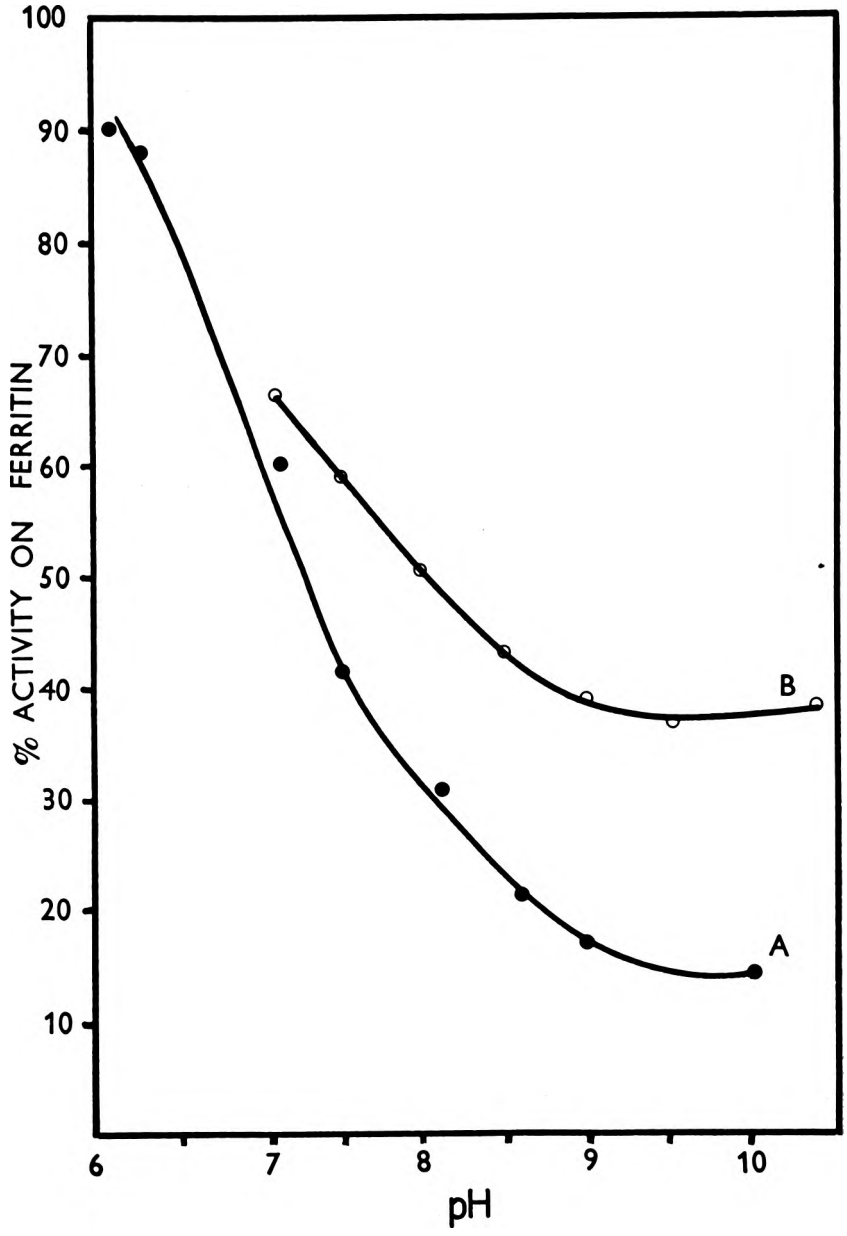


Figure A5.

The influence of pH on the uptake of radioiron by ferritin, Curve A was obtained by adding tracer radioiron to ferritin in Tris. buffer at various pH values. Curve B was the result when tracer radioiron was added to the buffer 1 hour before adding ferritin.

The results of this experiment differed from those of the previous one only in the more efficient labelling observed, particularly at the more alkaline pH values. (Figure A5 curve B). This is compatible with previous observations of the effect of hydrolysing tracer radio-iron by heating (page 186).

An attempt to investigate labelling in a lower pH range using a Tris-maleate buffer was not successful. When the pH was below 5.8 a gel formed immediately on addition of the antiserum, so that estimation of ferritin labelling became impossible.

In each of these experiments the greatest labelling occurred in a specimen to which distilled water (initial pH 5.2) had been added instead of buffer. The significance of this observation was not clear.

The experiments carried out did not enable the optimal pH for labelling to be determined. Nevertheless, labelling in distilled water and in buffer pH 6.1 were so efficient as to suggest that pH 5-6 is the optimal region for labelling.

V. Conclusion.

The demonstration of preferential labelling of iron-poor ferritin molecules (Chapter IV) and the unpredictable results obtained when labelling or removing label in the presence of added iron seem to exclude exchange as the sole labelling mechanism. Although the labelling was time dependent, as would be expected for an exchange reaction, this time lag could equally well be explained

as the rate of hydrolysis of iron under the experimental conditions. This latter mechanism, in which iron is hydrolysed to form a sol which then associates with ferritin by electrostatic forces, received further support from the experiment in which hydrolysis was promoted by heat and from the inability of ferritin to take up a significant proportion of ferric ammonium citrate. The preferential labelling of iron-poor ferritin could be due to these molecules having a more favourable distribution of charge than the iron heavy molecules. This distribution of charge would also be pH dependent which would explain the altered efficiency of labelling at different pH values.

VI. In vitro labelling at various stages in the preparation of ferritin from horse spleen.

In vitro labelling at various stages in the preparation of ferritin was studied by starch-gel electrophoresis.

The stages investigated were:-

(a) The supernatant solution after homogenising.

(b) The supernatant solution after heating to 80°C.

(c) The supernatant solution after adjusting the pH to 4.6 with 50% acetic acid.

(d) The mother liquor after crystallisation of ferritin.

(e) The ferritin after five crystallisations.

(f) The final product after ammonium sulphate precipitation and dialysis.

Tracer radioiron was added to the samples. After standing for one hour the samples were submitted to electrophoresis

at 6 V/cm. for 16 hours using the system of Haut et al. (1962). After electrophoresis the gel was sliced longitudinally through its width. One half was stained for protein whilst the other was set up for autoradiography.

From Figure A6 it can be seen that added radioiron was not confined to the ferritin band. In the crude extract (a) the major portion of the radioactivity is present in a diffuse band migrating rather faster than ferritin. This diffuse band was reduced by both heat treatment and the pH adjustment. After crystallisation with cadmium sulphate all the radioiron was associated with ferritin bands. This pattern was not altered by the final purification with ammonium sulphate. The radioactivity added to the mother liquor on the other hand was distributed between two diffuse bands. One of these corresponded to the previously mentioned band which migrated ahead of ferritin (page 144) whilst the other stretched diffusely from the origin. No radioactivity migrated to the position occupied by ferritin, demonstrating that all the ferritin had been removed from this fraction. This experiment illustrates the fact that ferritin is not the only protein present in the homogenate which is able to bind tracer quantities of radioiron.

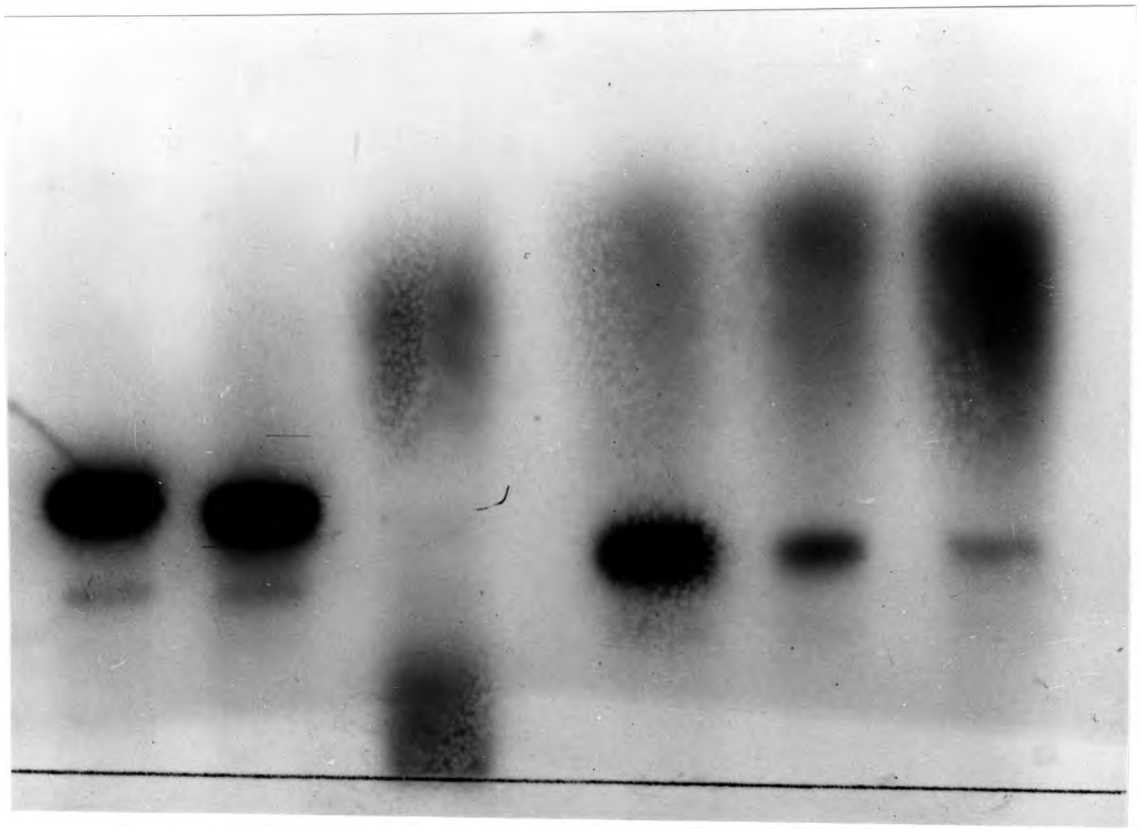


Figure A6.

Autoradiograph showing the starch-gel electrophoretic separation of tracer radioiron added to ferritin solutions at various stages of purification. From right to left. Horse spleen supernatant solution, supernatant solution after heating to 80°C., supernatant solution after adjusting to pH 4.6 with 50% acetic acid, the 'non-crystallisable fraction', the crystallised ferritin before ammonium sulphate precipitation, the final product after five cadmium crystallisations and five ammonium sulphate precipitations. It is apparent that ferritin is not the only compound present which is able to bind tracer quantities of radioiron.

EXPERIMENTAL METHODS.

EXPERIMENTAL METHODS.

I. Experimental animals - care, diet and preparation of iron-loaded and iron-deficient rats.

Throughout this investigation adult rats of the Wistar strain weighing between 150 and 200 g. were used. The rats used in group studies were matched and sorted into random groups. Unless otherwise stated the rats were housed in plastic coated cages and fed rat cubes and distilled water. The rat cubes were prepared by the Delmas Milling Company (Randfontein) and had a composition approximating that shown in Table M1. The daily consumption of diet was between 10 and 15 g. per rat. Since the iron content of the diet is 0.019%, the iron intake of the rats was 2-3 mg. per day. When iron-loaded animals were required these were produced by intramuscular injection of 1 mg. of iron-sorbitol-citrate (Jectofer) on ten successive days. Iron-deficient animals were produced either by raising weanling rats on a semisynthetic diet of low iron content (Valberg et al., 1961) or by venesection of adult rats. The composition of the low iron diet is shown in Table M2. Rats were venesected either by cardiac puncture or by bleeding from the tail vein. When the former technique was used, 2-3 ml. of blood was withdrawn on the fourth and second days before use. In order to bleed rats from their tail veins it was necessary to anaesthetise the animals with ether in order to obtain a suitable flow of blood. After injecting 0.2 ml. of heparinised saline solution into the tail vein the syringe

was removed and the blood allowed to drip from the needle. The rat was kept lightly anaesthetised throughout the procedure. Using this technique up to 5 ml. of blood was removed in a single venesection four or five days before the experiment.

Table M1.

Composition of normal rat diet.

Percentage composition.

Protein	24.27
Fat	4.15
Fibre	4.86
Carbohydrate	56.23
Ash	7.78

Mineral composition %		Vitamin content per 100 g.	
Silica	0.59	Vitamin A	610-680 USP units
Chloride	0.70	Thiamine B_1	228-465 µg.
Sodium	0.41	Riboflavin B_2	436-650 µg.
Potassium	0.72	Pyridoxine B_6	209-275 µg.
Calcium	1.68	Pantothenic Acid	1000-1594 µg.
Phosphorus	0.80		
Sulphur	0.24	Choline equivalent	480-492 mg.
Magnesium	0.33	Inositol	61 mg.
Manganese	0.0084	Vitamin D	148 USP units
Iron	0.019	Vitamin E	1.32 Int. units
Zinc	0.002	Para-amino benzoic acid	78 µg.
Copper	0.0012		
Boron	0.00137	Niacin	6.60 mg.
Cobalt	0.000035	Vitamin C	3.6 mg.
Iodine	0.0012	Carotene	0.42-0.45 mg.
Fluoride	0.0019	Choline	132 mg.

Table M2.

Composition of low iron diet.

L.S. Valberg et al., (1961)

General composition.

Spray-dried skim milk	650 g.
Sucrose	205 g.
Fat mixture (1 part arachis oil, 3 parts lard)	99 g.
Water soluble vitamin mixture	10 g.
Fat soluble vitamin mixture	1 ml.
Choline dihydrogen citrate	5 g.
Salt mixture	30 g.

Salt mixture	g.
Sodium chloride	200
Sodium iodate $NaIO_3H_2O$	22
Manganese sulphate $MnSO_4.4H_2O$	10.81
Copper sulphate $CuSO_4.5H_2O$	2.72
Dextrose	786.45

Water soluble vitamins	g.
Calcium pantothenate	1.200
Pyridoxine	0.500
Riboflavin	0.500
Thiamine hydrochloride	1.0000
Nicotinic acid	1.0000
Vitamin K.	0.1000
Folic acid	0.1000
Para-aminobenzoic acid	1.0000
Biotin	0.020
Inositol	20.00

Water soluble vitamins (Contd.)	g.
Vitamin B_{12}	0.0015
Dextrose	974.5785
Fat soluble vitamins	
Vitamin A palmitate	0.400 g.
Calciferol	0.050 g.
α-Tocopherol (35%)	50 ml.

II. Radioisotopes and counting equipment.

The three isotopes ^{59}Fe, ^{55}Fe and ^{131}I were used in these studies. The two iron isotopes were obtained from Oak Ridge, Tennessee, U.S.A., where they were produced by the following neutron bombardment reactions:

$$^{58}Fe\ (n, \gamma)\ ^{59}Fe$$

$$^{54}Fe\ (n, \gamma)\ ^{55}Fe$$

The ^{131}I on the other hand was produced by fission at Amersham, Herts, England. The isotope was supplied carrier free for protein iodination in N/50 NaOH free of reducing substances. The decay characteristics of these isotopes are summarised in Table M3.

Table M3.

Decay characteristics of isotopes used

Isotope	$T_{\frac{1}{2}}$	Distribution and energy (MeV) of radiations		
		γ	β	X
^{59}Fe	45 days	0.19 2.8% 1.10 57% 1.29 43%	0.27 46% 0.46 54% 1.56 0.3%	
^{55}Fe	2.94 yrs.			Electron capture emits characteristic Mn X-ray 0.006
^{131}I	8 days	0.08 2% 0.28 5% 0.436 80% 0.64 9% 0.72 3%	0.25 3% 0.33 9% 0.61 87% 0.81 1%	

Radioactive measurements.

The γ-emitting isotopes ^{59}Fe and ^{131}I were counted when possible in a well counter consisting of a thallium-activated sodium iodide crystal coupled by a photomultiplier tube to an IDL 1700 scaler, calibrated for the isotope. A whole body counter was used when the volume to be counted was too large (e.g. whole rats) to fit into the well, which could accommodate only test tubes 5/8" in diameter. In early experiments the whole body counter consisted of a ring of twelve Geiger-Muller tubes (type G26PB. 20th Century Electronics Ltd.). The tubes, matched for equal operating voltage, were connected in parallel and used with a Philips

scaling circuit. In later experiments a well counter of plastic phosphor material (Nuclear Enterprises NE 220) was used. This counter was constructed in a similar manner to that of Warner and Oliver (1962). A sleeve of plastic phosphor was connected to a photo-multiplier tube by means of a perspex light guide. This detector was also used in conjunction with Philips high voltage and scaling units.

In experiments requiring only a single isotope ^{59}Fe was used because of the ease with which it could be counted. When both ^{59}Fe and ^{55}Fe were used the iron was electro-plated on to copper discs using the method of Hallberg and Brise (1960), and the radioactivity emitted by the two isotopes measured by the method of Peacock et al. (1946). The tissue containing the isotopes was transferred to a 100 ml. Kjeldahl flask, and 4 ml. concentrated sulphuric and 2 ml. concentrated nitric acid were added. A glass bead was placed in the flask to prevent bumping and the flask was heated until its contents appeared black and oily. The flask was then allowed to cool and approximately 4 ml. of hydrogen peroxide was added. The flask was heated again for 10 minutes. Further additions of peroxide were made until a water clear solution was obtained. Five ml. water was then added and the solution boiled once more to remove traces of peroxide. When cool, sufficient ferric chloride solution was added to give a total iron content of 7.5 mg. The iron was then precipitated by addition of concentrated ammonia liquor. The

precipitate was redissolved by the addition of a few drops of concentrated hydrochloric acid and transferred quantitatively to a 100 ml. centrifuge tube with three washings of 0.6N sulphuric acid. The iron was again precipitated by adding ammonia liquor to the solution while stirring. The mixture was then centrifuged at 2,000 x g for 10 minutes, and the clear supernatant solution discarded. The ferric hydroxide precipitate was dissolved by adding 6 drops of 6N sulphuric acid and washed into the electrolysis cell with 35 ml. of 8% ammonium oxalate solution heated to roughly 60°C.

The electrolysis cell is constructed of acid resistant stainless steel and acrylic plastic. It consists of a heavy base having a depression into which the copper planchette is placed. A rubber O-ring is used as a gasket between the planchette and the plastic upper section of the cell which screws into the metal base. The anode is a platinum spiral with a diameter of 2 cm. The advantages of this design have been elaborated by Hallberg and Brise (1960). The apparatus used in the present study consisted of sets of four cells connected in parallel and screwed to a metal base which acted as a common cathode. Up to four of these units were connected in series, giving a total of twelve cells. The current was supplied by a constant current, constant voltage power pack.

The copper planchettes were cleaned with 30% nitric acid and rinsed with water before assembling the cell. The

iron solution was poured into the cell as soon as the cell had been screwed on to the base. Electrolysis was started immediately and continued for three hours. A current of 0.4 amp. was maintained through each cell. After plating was complete the planchettes were removed, immediately rinsed with distilled water and acetone and dried in a current of warm air. The dry plates were covered with a few drops of 1% mineral oil in benzene in order to prevent oxidation.

The planchettes were differentially counted for the two isotopes. The ^{59}Fe emissions were counted with a Tracerlab Helium-filled TGC 2L end-window Geiger-Muller tube, and the ^{55}Fe activity was measured with a Tracerlab Argon-filled TGC 3 end-window Geiger-Muller tube covered by a beryllium filter. Planchettes on which pure ^{55}Fe and ^{59}Fe standards had been plated were also counted on both tubes enabling an appropriate correction for cross-counting to be made.

When counting both ^{55}Fe and ^{59}Fe it is desirable to have approximately the same count rate for both isotopes in the sample. Since the counting efficiency for ^{55}Fe was much lower than that for ^{59}Fe it was necessary to have more ^{55}Fe activity than ^{59}Fe present in the final sample.

III. Standard procedure for measuring absorption.

Unless otherwise stated the dose of iron used to determine absorption was 10 µg. This dose was prepared by dissolving ferric chloride in distilled water and adding the required amount of $^{59}FeCl_3$. The dose was administered

into the stomach by means of an olive tipped oesophageal cannula. Before receiving the test dose the rats were fasted for 24 hours, but were allowed free access to glucose water. Absorption was usually assessed four or more hours after the oral dose by removing the whole gastrointestinal tract without allowing the luminal contents to escape and determining the radioactivity present in the remainder of the carcass. In some experiments the animal was not killed, the radioactivity retained being measured in the whole body counter seven days after the administration of isotope. By this time all the unabsorbed radioactivity had been excreted. Absorption was calculated using the formula:

$$\text{Percentage absorption} = \frac{\text{counts in carcass}}{\text{counts administered}} \times 100$$

IV. Isolation of horse spleen ferritin.

Ferritin was prepared by a modification of the method of Mazur and Shorr (1948), which was based on Granick's (1943) earlier procedure. Spleens removed from horses at the Johannesburg Municipal Abattoir shortly after slaughter were homogenised with 1.5 times their weight of water. The aqueous suspension was rapidly heated with constant stirring to 80°C. in a water bath. The coagulum of denatured protein was removed by centrifugation. When cool, the supernatant solution was adjusted to pH 4.6 with 50% acetic acid and allowed to stand overnight in the refrigerator. The precipitate which formed was removed by centrifugation. To each 100 ml. of solution was added

with stirring 25 ml. of a 20% solution of cadmium sulphate. After one to two days at room temperature the ferritin crystals were harvested by centrifugation. The mother liquor, which contained non-crystallisable ferritin, was discarded. The ferritin crystals were dissolved in the smallest possible quantity of 2% ammonium sulphate, and recrystallised as before by the addition of cadmium sulphate. After four such crystallisations the ferritin was again redissolved in 2% ammonium sulphate. This solution was then treated with an equal volume of saturated ammonium sulphate and the precipitate recovered by centrifugation. The precipitate was redissolved in water and precipitated again by adding an equal volume of saturated ammonium sulphate. This procedure was repeated three times in order to reduce the cadmium sulphate concentration. Finally the ammonium sulphate was removed by exhaustive dialysis against running tap water. The concentrated ferritin solution was stored in the refrigerator with toluene as preservative.

V. Preparation of antihorse spleen ferritin.

Antihorse spleen ferritin was obtained by bleeding rabbits immunised with horse spleen ferritin. The antigen used for the immunisation of the rabbits was prepared by one of the following methods:

(a) a modification of the method of Proom (1943). In this method the ferritin was prepared for injection as follows: 25 ml. of freshly prepared ferritin was pipetted into 80 ml. of distilled water and mixed well. To this

was added 10% aluminium potassium sulphate with stirring. The pH of the solution was carefully adjusted to 6.5 with 5N NaOH with constant stirring. The precipitate was recovered by centrifugation and the supernatant solution discarded. The deposit was washed twice with 200 ml. physiological saline and resuspended in 100 ml. of physiological saline. Five ml. of the final suspension was injected intramuscularly into each buttock of a rabbit. The procedure was repeated 14 days later. Booster injections of aqueous ferritin were given intraperitoneally 10 days before each bleed. The rabbits were bled periodically from an ear vein, 30-35 ml. being collected in a heparinised tube. The plasma was siphoned off after centrifugation and pooled.

(b) Antigen was also prepared for injection by mixing with Freund's adjuvent (Freund et al. 1948). One volume of adjuvent was mixed well with three volumes of ferritin solution. One ml. of the resulting emulsion was injected into each rear leg. The rabbits received a second injection one month later. Plasma was obtained as before from the ear vein. When Freund's adjuvent was used booster doses were necessary only after an interval of three months.

Aliquots were stored at -20°C. The titre of each pool was determined in the following way.

Increasing quantities of antiserum from 0.1 ml. to 2 ml. were added to a constant volume of ferritin solution of known iron content. The samples were incubated at 37°C.

for one hour and left in the refrigerator overnight. The precipitates were washed twice with saline. The amount of iron in the ferritin-antiferritin complex was then determined. This was done by acid digestion of the precipitate and determination of iron in the digest by the thioglycollic acid method. The minimum volume of antiserum required to precipitate all the ferritin iron (Figure 8) was taken as the titre.

VI. Preparation of *in vivo* labelled ferritin.

In vivo labelled ferritin was prepared in rats. The animals received an intravenous injection of 1 mg. of non-radioactive ferrous ammonium citrate in order to saturate the erythropoeitic demand for iron. The following day the animals received an injection of 250 µg. labelled iron in the form of ferric ammonium citrate. The next day the livers were removed and processed in the same way as horse spleens up to the stage of crystallisation. When this stage was reached carrier horse spleen ferritin was added in order to facilitate the crystallisation and precipitation of the small amount of labelled ferritin.

VII. Isolation of rat transferrin.

Plasma was obtained by centrifugation of blood obtained by bleeding rats from their tail veins. Before isolating the transferrin from the plasma it was labelled with ^{59}Fe and its iron binding capacity was saturated by addition of ferric ammonium citrate. Labelling with radioiron enables the transferrin to be followed through the various

stages of purification. Isolation was carried out with the transferrin saturated with iron because it is most stable in this form (Azari and Feeney, 1958).

A pilot study was carried out on a small quantity of labelled plasma in order to determine the range of ammonium sulphate saturation over which transferrin precipitated. The saturated ammonium sulphate solution was adjusted to pH7 since iron is liberated from transferrin at acid pH. Increasing volumes of the saturated ammonium sulphate were added to aliquots of the plasma and the percentage of labelled transferrin which was precipitated was then determined (Figure 37).

No transferrin was precipitated until the ammonium sulphate saturation reached 35%. As the ammonium sulphate saturation increased above this the transferrin was precipitated. When the saturation reached 55% little or no transferrin remained in solution. Based on this pilot experiment the following procedure was used to isolate transferrin from plasma. Sufficient saturated ammonium sulphate (adjusted to pH7) was slowly added to plasma to produce a final ammonium sulphate saturation of 35%. The plasma was stirred throughout the addition of the ammonium sulphate. The protein precipitate was removed by centrifugation and the transferrin in the supernatant solution was precipitated by addition of sufficient saturated ammonium sulphate to raise the ammonium sulphate saturation to 55%. The same precautions were taken as in the previous addition. The precipitated transferrin was spun down and

the supernatant solution discarded. The transferrin was redissolved in water and prepared for column chromatography by dialysis against 0.01M phosphate buffer pH 7.5. The dialysed transferrin was chromatographed on a DEAE cellulose column 28 x 1.5 cm. The DEAE cellulose was prepared as described in a later section. The column was eluted with a continuous gradient using 0.01M phosphate buffer pH 7.5 as starting buffer and 0.1M phosphate buffer pH7 as limiting buffer.

Five ml. fractions were collected. The activity emerged in fractions 60-100. The fractions 62-80 which contained most of the activity were pooled and concentrated by counter dialysis against polyethylene glycol.

The purity of the final product was checked by paper electrophoresis. Figure M1 shows the electrophoretic separation of the initial plasma and of the purified transferrin. The density tracing was obtained using a Beckman Analytrol density scanner.

VIII. Iodination of transferrin.

The rat transferrin was iodinated before removal of its iron content since the protein is most stable when fully saturated. Iodination was carried out by the method of McFarlane (1958). Ten to fifteen mg. purified transferrin, dissolved in 0.2 ml. 2 M sodium acetate buffer, was prepared for iodination by treating it with 0.8 M iodine solution. After standing, the mixture was passed through a Dowex 1 X 8 column in water and then through a similar column in 0.01M Tris buffer pH8. The required amount of carrier free

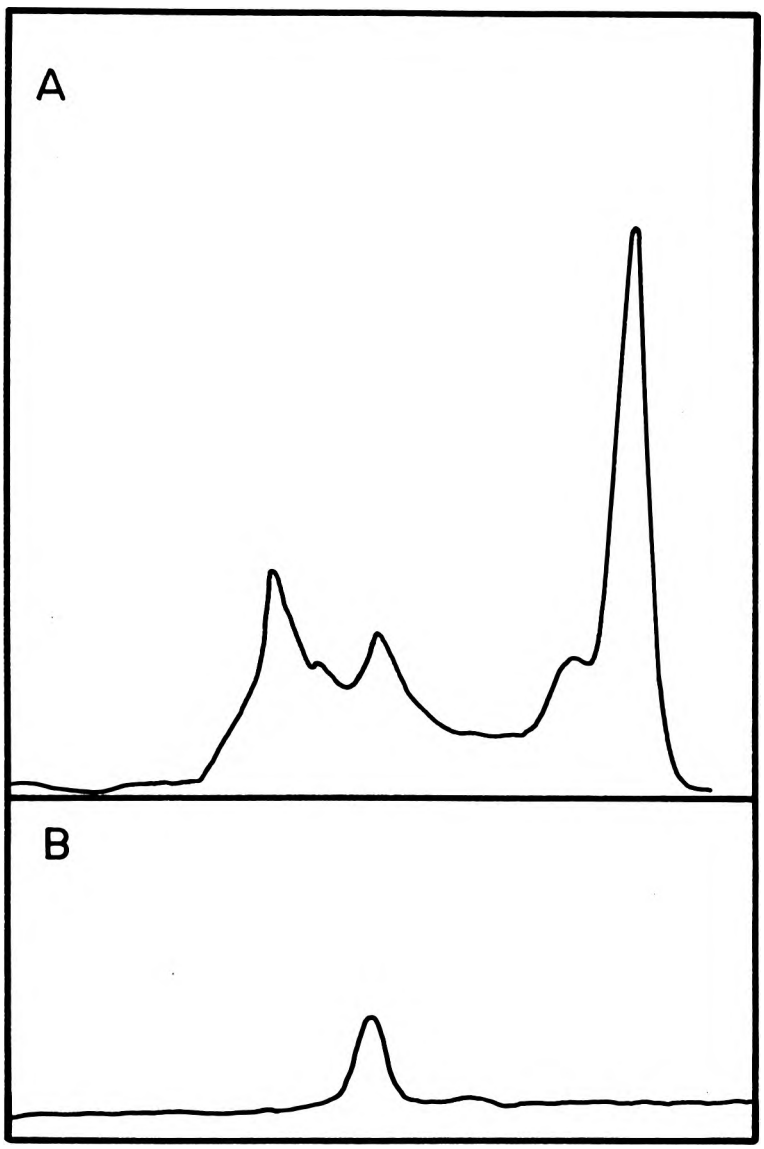


Figure M 1.

Electrophoretic separation of (A) rat plasma and (B) purified rat transferrin.

^{131}I activity was added to 10^{-3}ml.0.4N iodine monochloride, dissolved in N/5 HCl. One ml. of glycine buffer pH 8.8 was injected into the iodine monochloride in order to convert it to hypoiodite. This conversion can be observed by the loss of the characteristic yellow colour of iodine monochloride. The protein solution was added immediately after the glycine buffer and the mixture was well shaken. After standing for 15 minutes the mixture was passed through a Dowex 1^{X} 8 column in order to remove any free iodine. Electrophoresis of the coloured elute showed that the transferrin was adequately iodinated.

IX. Removal of iron from transferrin.

Jandl and Katz (1963) have described a method for the removal of iron from transferrin by lowering the pH to 5.5-5.6 with an acetate-EDTA buffer. At this pH iron bound to transferrin is removed from it and firmly chelated by the EDTA. This latter iron complex is separated from the protein by dialysis against a saline solution containing sodium bicarbonate. This procedure was modified to enable a more rapid separation of the iron from transferrin. It also obviated the exhaustive dialysis necessary to remove all the EDTA. The procedure adopted was basically the same except that separation of the iron-EDTA complex from the transferrin was carried out on a Sephadex column. In order to use a Sephadex column it was necessary to use a concentrated transferrin solution. When the solution to be rendered iron-free was dilute it was concentrated by counter-dialysis against Carbowax M 2,000.

Sufficient solid EDTA was added to produce an 0.1M solution. The addition of EDTA lowered the pH to some extent, but addition of 0.01N HCl was often necessary to attain the required pH of 5.6. Twenty minutes after adjusting the pH, the solution was chromatographed on a Sephadex G50 medium grade column which had been equilibrated with physiological saline. The salmon pink transferrin emerged from the column in roughly twice its original volume. The iron-free transferrin was now in physiological saline ready for injection into animals. The iron-EDTA complex was eluted from the column after the transferrin; it was pale yellow and had been diluted roughly sixfold. The entire procedure took only one and a quarter hours.

X. Determination of the serum iron concentration.

Serum iron concentrations were determined by a modification of the method of Bothwell and Mallett (1955). In this method iron is liberated from transferrin by addition of acid and the proteins are then precipitated with trichloracetic acid. The iron in the clear solution is measured colorimetrically with sulphonated bathophenanthroline. The method is described below.

All apparatus used was soaked overnight in chromic acid and washed in distilled and glass redistilled water. The reagents were made up in glass redistilled water. A standard solution was prepared containing 0.1 mg. ferric iron per ml. in acid solution. This solution was diluted to give a working standard in which 2 ml. contained 2 µg.

iron.

The colour reagent was prepared by adding 0.5 ml. chlorosulphonic acid to 100 mg. 4 : 7 Diphenyl 1 : 10 phenanthroline in a 'pyrex' tube and boiling gently for at least 30 seconds. The tube was then placed in a beaker of nearly boiling water. Cold water was added dropwise until no further reaction occurred. The contents of the tube were kept well stirred throughout the addition. The resulting solution was made up to 100 ml. with distilled water and 1 ml. thioglycollic acid was added.

The determination itself was carried out by acidifying 4 ml. serum by addition of 2 ml. of 2N HCl. After stirring 2 ml. 20% trichloroacetic acid was added and the mixture stirred vigorously for 2-3 minutes. Five ml. of the supernatant solution, obtained by centrifuging the mixture at 2000 x g for 20 minutes, was added to a tube containing 0.5 ml. colour reagent and 2.5 ml. sodium acetate. The optical density of the resulting pink solution was determined on an Evelyn colorimeter using a 540 mμ. filter. The blank was prepared by mixing 2.5 ml. water, 1.25 2N HCl., 1.25 ml. 20% chloroacetic acid, 0.5 ml. colour reagent and 2.5 ml. sodium acetate. The standard was prepared in the same way but substituting 2 ml. of the working standard for 2 ml. of water. The result is obtained from the equation

$$\text{Serum iron } \mu\text{g./100 ml.} = \frac{\text{O.D. of Serum}}{\text{O.D. of Standard}} \cdot \frac{8}{5} \times \frac{100}{4} \times \text{iron in standard}$$

XI. Measurement of unsaturated iron-binding capacity of serum.

A method for the determination of the unsaturated iron-binding capacity has been described by Bothwell et al., (1959).

In this method excess ferric ammonium citrate labelled with ^{59}Fe is added to a 1 ml. aliquot of serum in the presence of a Tris buffer (pH 8.4). The added ferric ammonium citrate not bound by the transferrin is removed with Amberlite IRA-401 resin and an aliquot of the transferrin solution is then counted. From the known specific activity of the added ferric ammonium citrate the amount of iron bound by the transferrin can be calculated.
In the present study it was necessary to measure the iron-binding capacity of serum in which the transferrin was already labelled with radioiron. This could have been carried out by the above method by allowing for the activity already present. This was not done but instead excess ferric ammonium citrate was added and after removing the unbound iron the serum iron concentration of the saturated serum was measured by the method described above. This gave the total iron-binding capacity, and by subtraction of the serum iron the unsaturated iron-binding capacity.
The preparation of the ferric ammonium citrate, the Tris buffer, and the Amberlite resin are described below:
Ferric ammonium citrate was prepared by adding sufficient ammonium hydroxide to a ferric chloride solution to precipitate the iron. If a radioactive preparation was required tracer radioiron was added to the ferric chloride solution before precipitation. The ferric hydroxide is spun down and the supernatant solution discarded. Four ml. iron-free water was added to the precipitate and the tube heated in a boiling water bath. Citric acid was added slowly until the precipitate dissolved. After

cooling, one drop of bromothymol blue was added and ammonium hydroxide added dropwise until the colour changed from yellow to blue. The solution was then diluted to the required concentration with iron-free water. If the solution of ferric ammonium citrate was required for injection, physiological saline was added to the initial ferric hydroxide precipitate in place of water, and saline was again used for the final dilution.

The buffer was prepared by dissolving sufficient Tris (2-amino-2-(Hydroxymethyl)-1 : 3 propanediol) in iron-free water to give an 0.08M solution. The pH was adjusted to pH 8.4 with 2N hydrochloric acid. The Amberlite IRA-401 resin was suspended and mixed in 3N hydrochloric acid for 24 hours to convert it to the Cl^- form. It was then washed repeatedly with distilled water and adjusted to approximately pH 7.5 by suspension in Tris buffer. The resin was dried on a Buchner funnel and then finally at 90°C. in an oven.

The method for determining iron-binding capacity using a resin has recently been modified by Charlton, Hardie and Bothwell (1965). Modification was necessary because water but not protein entered the mesh of the resin causing a slight increase in transferrin concentration. This means that all iron-binding capacities in this study are slightly too high. The difference between results by the initial and modified method is small.

The iron-binding capacity of iron-free transferrin was measured by the method of Bothwell et al. (1959) and by a modification of a method described by Ventura (1952).

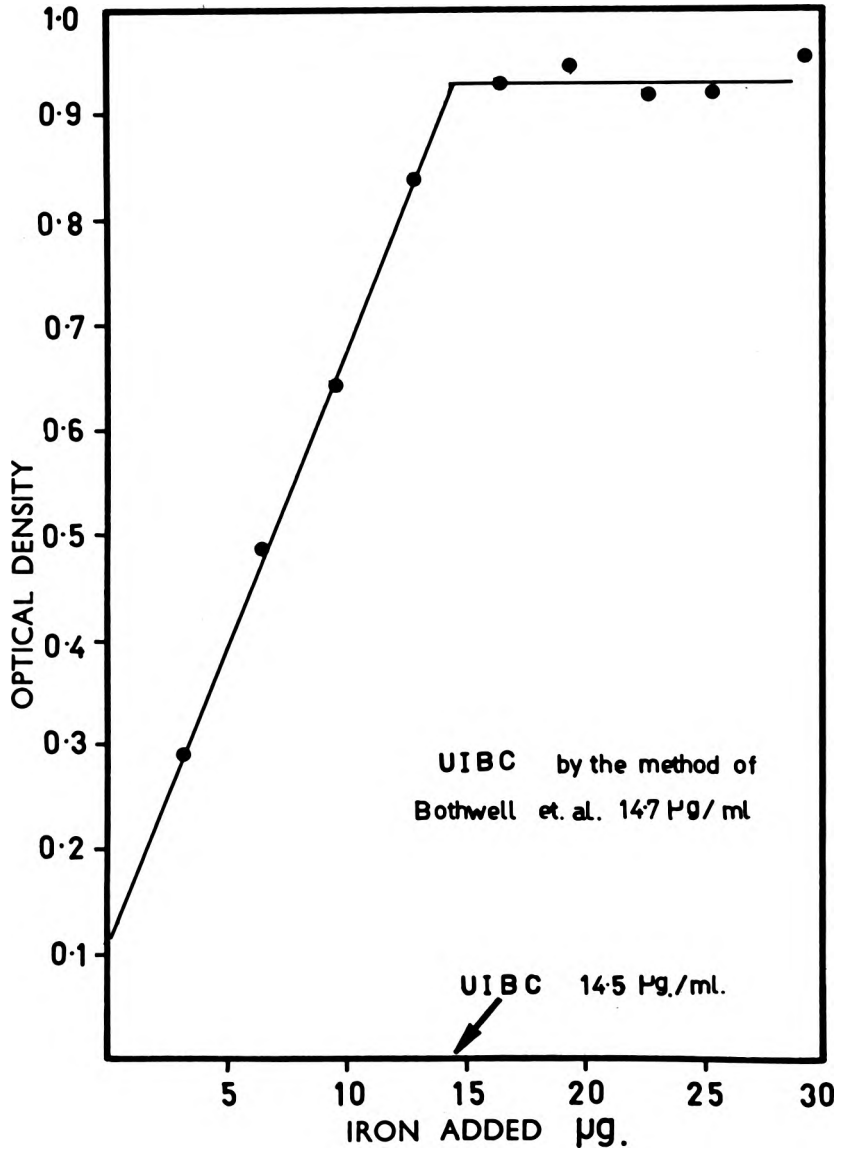


Figure M2.

Determination of the iron-binding capacity of a sample of purified transferrin by the method of Ventura (1952). Increasing quantities of iron are added and the O.D. measured at 540 mµ. The unsaturated iron-binding capacity (UIBC) is indicated by the minimum amount of iron to produce the maximum absorbance.

The latter method was carried out by adding varying known quantities of ferric ammonium citrate to aliquots of the transferrin. Sufficient 0.08M Tris buffer pH 8.4 was added to give a final volume of 6 ml. The tubes were allowed to stand for 5 minutes before determining the optical density on an Evelyn colorimeter using a 540 mμ. filter. Figure M.2 shows a plot of optical density against added iron. Iron added in excess of the iron-binding capacity produces no increase in the optical density. The abscissa at the point where the two lines cross therefore represents the iron-binding capacity of the serum. The sample analysed in Figure M2 had an iron-binding capacity of 14.5 μg./ml. by this method which agreed well with the figure of 14.7 μg./ml. obtained by the method of Bothwell et al., (1959).

XII. Measurement of non-haem iron.

The various methods used to measure non-haem iron in fresh tissue have been reviewed by Gale (1964). These methods depend on the measurement of both total and haem iron or of the total iron after removal of all haem iron either by perfusion (Copp and Greenberg, 1946; Hallgren, 1954) or by extraction (Kooyman, 1949; Labbe and Nishida, 1957). An alternative procedure, the direct extraction of non-haem iron, was selected for this study. This was based on the method of Bruckman and Zondek (1940) which in turn was a modification of Tompsett's (1935) earlier extraction.

The small intestine was opened lengthwise and washed until perfectly clean in five or more changes of physiological

saline. The washed gut was transferred to a measuring cylinder and sufficient saturated sodium pyrophosphate solution was added to give a volume of 30 ml. This was then homogenised and 20 ml. 20% trichloroacetic acid was added before homogenising for a second time. The mixture was then heated in a water bath to 80°C. and maintained at this temperature for 10 minutes with intermittent stirring. After cooling the mixture was filtered through acid washed Whatman No. 5 filter paper. The iron content was determined on an aliquot of the supernatant solution, using thioglycollic acid method. In spite of using a fine filter paper the filtrate was sometimes cloudy; in order to compensate for the cloudiness a blank was prepared by making up a second aliquot omitting only the thioglycollic acid.

XIII. Preparation of the small intestine.

When the uptake of iron into mucosal cells was studied, the intestine was washed before homogenisation. In some experiments a 10 cm. length of intestine from the pylorus distally was perfused for one minute with 0.9% NaCl solution or 0.01M EDTA (pH7.0) in 0.9% NaCl solution, at a pressure of 60 cm. water. In experiments in which uptake into the whole small intestine was determined, the bowel was opened longitudinally and rinsed for half a minute in each of two beakers containing 0.9% NaCl solution with 0.01M EDTA (pH7.0). In some experiments mucosa was freed from the intestine before homogenisation by compression of the intestine with the edge of a glass slide. Pushing

the slide towards the cut end of the bowel resulted in the 'milking' of the mucosa from the muscular layers. The extruded mucosa was then washed by shaking in a test tube with either 0.9% NaCl or 0.01M EDTA (pH7.0) in 0.9% NaCl, centrifuging and discarding the supernatant solution. After washing, the intestine or the mucosa was homogenised either in water or in 0.1M EDTA (pH7.0) in water. Uptake of the labelled iron into intestinal cells was assessed by counting aliquots of the homogenate in a well type scintillation counter, or by counting the whole washed intestine in a whole body counter.

Factors affecting ferritin recoveries: Experiments were carried out to find out to what extent washing of the gut affected the subsequent recovery of ferritin. 10 cm. lengths of duodenum taken from three animals thirty minutes after administration of 10 µg. labelled iron were divided longitudinally in half, the halves being weighed. One half of each duodenum was then washed as before, after which the labelled ferritin content was determined. The remaining halves were not washed, but were homogenised directly into 0.1M EDTA. The labelled ferritin content of the unwashed portions was 20-25% higher than that of equivalent weights of washed intestine, indicating a significant loss of the ferritin-containing cells during the washing procedure. However, no attempt was made in subsequent experiments to correct for this loss.

XIV. Paper chromatography.

Paper chromatography was carried out by the ascending

method in a Smith Universal Chromatography tank (Shandon Scientific Co., London). This consists of a 12 inch cubic tank closed with a heavy glass plate having a ground contact surface. Whatman No. 1 chromatography paper 10 inches square held in a metal frame were developed by placing a frame in a tray 10½ in. x 9 in. x 1 in. containing 200 ml. solvent. Three different solvent systems were used:

(a) n-Butanol: glacial acetic acid: water 120 : 30 : 50.

(b) Phenol: water 160 g. : 4 ml.

(c) 96% Ethanol : H_2O : cyclohexane 70 : 30 : 10. This system which was described by Brown and Rother (1963a), was found to be biphasic.

The top layer, which was very small, was discarded and the lower layer used for chromatography. When two-dimensional chromatography was carried out the butanol-acetic acid system was used first followed by the phenol system. The chromatograms were dried before staining and before the second stage of two-dimensional runs. This was carried out by blowing warm air across the sheet for 4 hours after the phenol system and ½ hour after the other systems. Amino acids were stained by spraying with 0.3% ninhydrin in ethanol using freon as propellant. The sprayed sheets were placed in an oven at 105°C. for 10 minutes.

XV. Electrophoresis.

A. Paper.

Paper electrophoresis was carried out in a perspex tank 17 in. x 17 in. x 4½ in. Whatman No. 3 MM paper strips 2½ in. x 15 in. were used. The strips, the ends of which dipped into the buffer compartments,

were supported on five glass rods. Most separations were carried out using a barbital buffer pH 8.6, prepared by dissolving 10.3 g. sodium barbital and 1.83 g. barbituric acid in 1 Litre of distilled water. An 0.05M sodium acetate buffer pH 4.8, described by Brown and Rother (1963a) was also used. After electrophoresis the strips were dried at 100°C. and stained. Proteins were stained with a 1% solution of bromphenol blue in 95% ethanol saturated with mercuric chloride (30 g./100 ml.). The strips were stained for 5 minutes, after which they were washed in running tap water and finally in pure methanol. Strips were stained for amino acids with ninhydrin in the same way as the paper chromatograms.

B. Starch-gel.

Starch-gel electrophoresis was carried out using the horizontal method described by Smithies (1957). The gel was prepared by using hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada). The amount of starch required varied from batch to batch but was usually about 14 g./100 ml. The starch was suspended in the gel buffer and transferred to a round-bottomed flask. The starch suspension was then heated slowly over a naked bunsen flame with constant swirling. The suspension became viscous and opaque and heating was continued until the consistency changed yielding a homogeneous translucent liquid

containing many small bubbles. The heating was then stopped and negative pressure was applied to the flask. This caused vigorous boiling and eliminated all bubbles. The gel was then poured into perspex trays 25cm. x 9cm. x 6mm. and covered with a glass plate previously coated with liquid paraffin. After cooling for 2-4 hours the plate was removed and the samples applied to 5 mm. strips of Whatman 3MM paper, which were inserted vertically into the gel at the origin with the aid of a razor blade. The gel was then covered with a sheet of parafilm 20 cm. long, leaving 2½ cm. gel exposed at each end. After the tray had been placed in the electrophoresis apparatus, gauze swabs, saturated with the electrode buffer, were used to connect the electrode compartment and the gel itself with the bridge compartment. The buffer system described by Haut et al. (1962) was used. The gel buffer was prepared by adjusting 0.074 M Tris to pH 9.5 with 10% citric acid. The electrode and bridge vessels contained 0.1M boric acid adjusted to pH 9.5 with 2N sodium hydroxide. After electrophoresis the gel was sliced in half through its thickness using a skin grafting blade. The sliced gel was stained for protein with 1% amido-schwarz 10B in methanol : acetic acid : water 50-20-50. The gel was stained for one minute and then washed repeatedly with the methanol : acetic acid : water mixture until the background was clear. The gel was stained for iron in a 3% potassium

ferrocyanide solution containing 1% hydrochloric acid. After the blue bands appeared the gel was washed with methanol : acetic acid : water mixture until the background was white.

XVI. Gel-diffusion.

Sufficient agar was dissolved in water to give an 0.5% solution. This was done by heating the agar and water in a boiling water bath. The hot agar solution was pipetted onto a flat glass to give an 1/8 inch thick layer, held in place by surface tension. After the gel had set, wells were punched into it using a cork borer, the pieces of gel being removed by suction. The pattern of wells can be seen in Figure 13. Antibody was placed in the central well and test solutions were placed in peripheral wells. The plate was kept in a saturated atmosphere until precipitation had occurred. The unprecipitated activity was removed by washing the gel for three days in repeated changes of distilled water. The washed gel was then dried to give a very thin film suitable for autoradiography.

XVII. Autoradiography.

All autoradiographs were made using non-screen X-ray film. Gel-diffusion plates, paper chromatograms and paper electrophoresis strips were firmly attached to the X-ray plate itself which was then returned to its light-tight cover and kept until ready for development. Autoradiographs of starch-gels were produced by the following method: the

gel was sliced through its thickness and one-half used for autoradiography. This was placed, cut surface up, on a glass plate and covered with Parafilm. The covered surface was placed on the surface of a non-screen X-ray plate so that only the Parafilm and the light-tight envelope separated the gel from the film. The gel and plate were left in contact for the required time in a deep freeze. It was essential to freeze the gel to minimise movement of bands.

XVIII. Column chromatography.

Column chromatography was carried out with various grades of Sephadex and DEAE cellulose. The preparation of these media are described below:

A. Preparation of Sephadex columns.

Sephadex columns were prepared according to the method suggested by the manufacturers (Pharmacia). The dry Sephadex powder was suspended in excess 1% sodium chloride and stirred well to prevent clump formation. The Sephadex was allowed to swell for several days before use. Columns were packed under gravity with the tap open. After packing, the columns were equilibrated with the buffer to be used for developing the column.

B. Preparation of DEAE cellulose columns.

DEAE cellulose powder (Whatman) was prepared for use by the following procedure. The DEAE cellulose powder was washed successively with 1N NaOH, distilled water, 1N HCl and distilled water. This washing

procedure was repeated until no further yellow colour was eluted by the NaOH. The excess NaOH was then washed out with distilled water and washing was continued with M/150 phosphate buffer pH 8 until the pH of the washings had dropped to pH 8. Columns were packed under 10 lb/sq. in. pressure. The columns were eluted using either a continuous gradient or stepwise changes in the developing buffer.

1. Gradient elution.

Columns 1.25 x 30 cm. were used. These columns were equilibrated with the starting buffer (M/150 phosphate pH8) by running the buffer through the packed column for 8 to 14 hours. Gradients were produced by siphoning the limiting buffer from a conical flask into the starting buffer in a straight-edged beaker. The buffer from the latter beaker, which was stirred with a magnetic stirrer, was siphoned on to the column. Since it was important to maintain the pH above 7, in order to prevent dissociation of the transferrin-iron complex, a gradient of increasing ionic strength was used. The starting buffer was M/150 phosphate buffer pH8, whilst the limiting buffer was the same buffer made to 0.5 or 1N with respect to sodium chloride. A different gradient was used in the isolation of transferrin from rat plasma. This was achieved in the same apparatus using 0.01M phosphate pH 7.5 as starting buffer and 0.1M phosphate pH7 as limit

buffer.

2. Stepwise elution.

In some experiments stepwise elution was used because satisfactory separations could be obtained quickly. Columns 1 x 10 cm. were used. The starting buffer was again M/150 phosphate pH8. This was changed first to 0.06N NaCl and then to N NaCl dissolved in the starting buffer. Although both transferrin and iron-EDTA were eluted by 0.06N NaCl in the starting buffer, their rates of elution were slightly different, enabling them to be collected in separate fractions. Ferritin was eluted by increasing the NaCl concentration to normal.

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