

THE IRON-BINDING PROTEINS OF
IRON-ABSORBING RAT INTESTINAL
MUCOSA

by

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2. SUMMARY.

Iron deficiency anaemia is perhaps the most widespread nutritional deficiency disease; as result, the topic of iron absorption has received intensive investigation over a relatively long period of time. Most of the investigative thrust has come from clinical medicine and allied fields, with some associated biochemical investigation. Evidence from the latter has pointed towards the involvement of iron-binding proteins especially ferritin and transferrin in the absorptive process. While the biochemical literature on these two proteins, particularly transferrin, is vast, their roles in iron absorption are obscure. This study was undertaken, therefore, as an investigation into these proteins, their quantitation and role in iron absorption. The physiology of absorption was studied by injection of radiolabelled ferrous ascorbate into isolated intestinal loops and the determination of mucosal, blood and carcass uptake. Uptake by the mucosa was found to be uniform throughout the intestine whereas absorption (carcass uptake) showed a steep gradient from duodenum to ileum. Absorption was shown to increase in states of iron deficiency and stimulated erythropoiesis and to decrease in iron loading, in all cases ceasing after 30-60 minutes, the total amount increasing only slightly thereafter. Prolonged exposure of distal jejunal and ileal mucosa to iron did not result in an increase in absorption. Absorption increased linearly with increasing iron dose, was partially inhibited by cyanide and was totally inhibited by the cytoskeletal poisons colchicine, vincristine and cytochalasin-B. Isolated cells in vitro showed a similar uptake pattern, which was found to be temperature-dependent, non-saturable and only partially inhibited by cyanide. These results are consistent with a time-dependent mechanism of pinocytic uptake.

Fractionation of the mucosa by isopycnic density gradient centrifugation showed the presence of two iron-binding proteins, identified by gel filtration, electrophoresis and radioimmunoassay as transferrin and ferritin. The presence of a low molecular weight iron compound less than 10000 daltons in the cytosol was noted. Further investigation by gel filtration and immunoabsorption showed that there were no other iron-binding components. Ferritin was found in three-fold greater amounts in the iron-absorbing regions of the intestine and iron loading by the protein correlated with absorption; iron loading was absent in distal jejunum and ileum and also after pretreatment of the mucosa with the cytoskeletal poisons colchicine, vincristine and cytochalasin-B. Absolute amounts of the protein increased in iron-loaded states and decreased in iron-deficient states and in all cases ferritin loading increased over time. Prolonged exposure of ileal mucosa did not result in ferritin loading except in the case of iron loaded animals. Homogenisation of cells in the presence of excess iron showed ferritin to be accumulating the metal: the radiolabelled ferritin density gradient peak shifted to maximal ferritin density without displacement of the radiolabel. Transferrin was found in equal quantities and showed similar iron-loading behaviour throughout the intestine. Density gradient fractionation showed the protein to be present in both free and membrane-associated forms; the concentration of the latter, approximately 10% of the total transferrin in normal iron absorbing cells, was found to correlate with absorption, suggesting that it may play some role in the absorptive process. Increased amounts of transferrin were found in iron deficiency; parenteral iron loading had no effect on transferrin levels. Cytoskeletal inhibitors had no effect on free transferrin loading, although the membrane-associated transferrin was significantly reduced. Administration of a macromolecular iron source, iron

polymaltose, in the loop and subsequent fractionation of the mucosa, resulted in minimal uptake of iron by transferrin, in spite of ferritin loading. These results suggest that iron-binding by free transferrin might be an artefact of the homogenisation process. Investigation of the contribution of serum transferrin to the mucosal transferrin concentration by injection of iodinated biologically screened transferrin indicated that approximately 24% of the transferrin in the mucosa could be accounted for as serum-derived; the remainder was inaccessible to proteolytic digestion of whole cells.

During the course of the study, it was noticed that transferrin from mucosal homogenates showed a highly significant immunological difference to that of the serum. This difference could not be attributed to proteolysis or technical artefacts. "Mucosal" transferrin was therefore purified by rivanol extraction, ammonium sulphate precipitation and ion-exchange chromatography. The last-named procedure separated two transferrins. Electrophoresis in 6-8% polyacrylamide gels with 1% sodium dodecyl sulphate showed that one of these species corresponded to serum transferrin, while the other was of greater molecular size. Electrophoresis of serum transferrin showed no evidence of this larger species. The difference was not related to iron-binding, shown by addition of iron to transferrin in vitro and subsequent analysis and also by the parallel migration of the two transferrins on isoelectrofocusing. It is thus possible that this larger transferrin species represents a structurally different protein.

This study, therefore, has succeeded in determining and quantitating the iron-binding proteins of the mucosa. However, principally due to problems such as the cross-contamination of fractions and the failure to identify a high-flux iron compartment, a definite conclusion as to the exact nature of the

transport and regulation of iron absorption cannot be reached. The results are consistent with an hypothesis of pinocytic means of uptake and transport in which transferrin may or may not be involved. The free diffusion of low molecular weight chelates thus seems to be ruled out since this should be independent of cytoskeletal activity. Ferritin does not appear to play a direct role in the absorptive process, although iron loading by this protein, wherever this might occur, would appear to be sited near the crucial point in absorption. The role of transferrin, and in particular the role of the "mucosal" transferrin, in absorption is uncertain. Further work into the structure and function of this mucosal species will have to be conducted.

3. INTRODUCTION.

Iron is required by all living organisms, and it has been said (Neilands 1972) that "life in any form without iron is, in all likelihood, impossible". Iron is capable of existing in a variety of oxidation states, of which the FeII and the FeIII are the most common, and the only forms in which the aqueous ions are stable. FeII salts when dissolved in water give rise to a pale green weakly acidic aquo-ion, with $K_a = -9.5$ and a maximum solubility at pH 7 of $10^{-1}M$. Similar salts of FeIII are, however, extensively dissociated, with a K_a of -3.0 and a maximum solubility at pH 7 of $10^{-18}M$. The hydrated FeIII cation is only able to exist in the presence of added acid at $pH < 1$. As the pH rises, protons are removed, the FeIII salts undergo further hydrolysis and form polynuclear aggregates in which the iron atoms are linked by hydroxo bridges. If further base is added, polymerisation may occur, with the formation of a colloidal gel of "ferric hydroxide" (Phipps 1976).

At the time of the evolution of the earliest biomolecules, the earth's atmosphere was strongly reducing, with the result that iron was readily available in the soluble FeII form. Iron was, as it is today, extremely abundant, being the fourth most abundant element in the earth's crust, and possesses very useful chemical properties, being able to alter oxidation state rapidly and reversibly. A combination of these factors led to the metal's incorporation into the biomolecules. However, with the advent of photosynthetic plants, the partial pressure of oxygen in the atmosphere rose. Environmental iron was thus oxidised to the highly insoluble ferric form, where it is present largely as insoluble hydroxides and sulphides. This presented a dual problem to living organisms: the acquisition, necessitating solubilisation of iron from the environment, followed by the necessity for

maintaining the iron, once acquired, in soluble form. Different groups of organisms have solved the problems in different ways.

Microorganisms have evolved iron scavenging molecules, the siderophores, which are secreted into the environment. These molecules are found in a wide variety of microorganisms, including bacteria and certain species of blue-green algae (Neilands 1972). The siderophores, or siderochromes as they are sometimes known, are among the strongest chelators of iron known; they are hexadentate ligands containing hydroxamate or phenolic binding groups. They act by wrapping themselves tightly around FeIII, leaving no room for hydrolysis and are taken up again by the organism by a mechanism involving binding to cellular receptors and internalisation of the complex, and the subsequent release of the iron. Whether microorganisms have the capacity for iron storage is not certain, but recently iron-protein complexes with properties very similar to ferritin have been found in *Escherichia coli* and *Azotobacter vinelandii* (Harrison et al 1981). Whether these organisms have the capacity to excrete iron is not known; however, they do increase secretion of siderophores into the environment in the case of iron deficiency (Morgan 1981).

The majority of multicellular organisms have a degree of structural complexity restricting the free exchange of nutrients, including iron, with the environment, and therefore necessitating the development of a circulatory system. Because of the insolubility of iron at physiological pH, a carrier is required. In plants, this function is served by small organic chelates. Plants require a continuous supply of iron as much of the metal is lost with the leaves. The only source is from the soil where it is present, again, in insoluble form. Plants have coped with the problem in a number of ways, principally by the secretion of organic acids into the soil to solubilise the iron which can then

be taken up in low molecular weight form through the roots (Morgan 1981). Some plant root nodules may in fact secrete siderophore-like compounds that deny iron to pathogenic root fungi thereby increasing the plant's yield of iron. The iron storage protein, ferritin, has been demonstrated in higher plants (Hyde et al 1962), where it is called phytoferritin. The literature of iron metabolism in certain species of mammals, particularly *Rattus* and *Homo*, is vast; literature on other species and groups of animals is relatively scanty. Furthermore, because of the variation in experimental aims and techniques used, it is difficult to compare results, and very few studies have concerned themselves with comparative iron metabolism. Animals, because of the high degree of structural complexity, have evolved a class of very high affinity iron-binding proteins, the transferrins, that serve as carriers of iron and also for the denial of iron to pathogenic organisms. Transferrins have been found in all vertebrate species in which their presence has been investigated, and transferrins are now being discovered in invertebrates as well (Huebers et al 1982). Animals acquire iron in foodstuffs of either plant or animal origin. The latter are generally better absorbed, as compounds in vegetable material, principally phosphate and phytate, bind the iron and reduce its bioavailability, whereas in food of animal origin, most of the metal is present in iron-porphyrin complexes which are absorbed as such (see below). The iron is solubilised by digestive enzymes, and in mammals, by the hydrochloric acid of the stomach. Relatively little is known of iron balance in invertebrates, and what studies there are are not quantitative. Mammals have been the most extensively studied, and consideration of iron balance has tended to depend on the observation of McCance and Widdowson (1937) that the excretion of iron, in man, is very limited. The necessary corollary to this is that body iron balance depends on a

mechanism of regulated absorption. Iron loss in man occurs largely through sloughing off of intestinal mucosal cells which, like all cells, contain iron, principally in the iron storage forms, ferritin and haemosiderin. However, the actual amount of iron lost does seem to vary from species to species, being apparently higher in rodents (Conrad et al 1964).

The amount of iron in the human body ranges from 2-6g, with an average of approximately 4g (Moore 1960). Most (approximately 65%) is present in the haemoglobin of circulating red cells, the remainder is present largely in the non-erythroid tissues as the storage forms, ferritin and haemosiderin, and a small, though essential, 1% is present in the transferrins, the cytochromes and iron sulphur proteins, and myoglobin (Crichton 1973). There is continual turnover of red cells and tissue proteins so that the iron in the body is in a continuous state of flux. The carrier involved in this system is transferrin. Approximately half of the body's transferrin is in the plasma and the remainder circulates through the extravascular fluid (Morgan 1974). Iron can leave the plasma by two routes: one leads to the erythroid tissue, principally the erythroid marrow, where the iron is taken up by maturing reticulocytes for haemoglobin synthesis. All of this iron will eventually return to the plasma either as result of destruction of red cells at the end of their lifespan, or following intramedullary catabolism of erythroid cytoplasm (Cavill and Ricketts 1981). The former is accomplished by the reticuloendothelial system, responsible for breaking down of red cells and the catabolism of their haemoglobin. This iron is then passed to transferrin and recycled, although some may be stored in the reticuloendothelial storage sites (Cavill and Ricketts 1981). The second pathway, less defined than the first, is passage of iron to non-erythroid tissue, which appears to be mediated through the extravascular circulation (ibid). Iron is present in all

non-erythroid tissues as storage compounds and enzymes. The metal is acquired by the body from absorption through the intestinal mucosa, and there is limited means of excretion, a fact recognised by McCance and Widdowson (1937) who stated: "There are indeed indications that in man and certain animals the bowel excretes practically no iron. If this is the case, the amount of iron in the body must be regulated by controlled absorption". It follows, therefore, that the intestine receives information concerning deficiency or surfeit of iron and by means of sensory or effector mechanisms it acts in response to this information (Moore 1960).

3.1. Iron in the Gastrointestinal Tract

Iron enters the gastrointestinal tract bound to various foodstuffs of either animal or vegetable origin, from which it is released by the enzymes and hydrochloric acid of the stomach. This highly acidic environment also serves to maintain the iron in solution, a function that may be assisted by the presence of a high molecular weight iron-binding mucopolysaccharide which has been reported in gastric juice (Turnbull 1974). Once out of the stomach and passing into the intestine, however, the pH of the food material rises steadily. It is very well documented that FeII is better absorbed than FeIII (Brise and Hallberg 1960). This was at first thought to be due to an intrinsic advantage of ferrous over ferric iron (Bothwell and Finch 1962); however, the importance of iron solution chemistry has increasingly been realised, and that the greater solubility of the FeII form may simply be due to the greater solubility of this ion at pH's approaching neutrality. It has in fact been demonstrated that FeIII iron, provided it is maintained in solution, is as well absorbed as its FeII counterpart (Spiro and Saltman 1969). In this more alkaline environment, the iron may either be hydrolysed, forming ferric

hydroxide polymers which are not available for absorption or combine with any of a variety of ligands present. These complexes may or may not be available for absorption. It is known that inorganic iron is more efficiently absorbed than iron of animal origin which is in turn better absorbed than iron of vegetable origin. The last-named is due largely to the presence of compounds such as oxalate, phosphate and particularly phytate which combine with the iron and reduce its solubility (Forth and Rummel 1973; Peters et al 1971). On the other hand, certain compounds enhance absorption. These are generally chelates that serve to keep the iron in solution: ascorbic acid, citric acid, fructose and other sugars (Davis and Deller 1967) (Conrad and Schade 1968). The effect of ascorbic acid is thought to be due to the reduction of the iron and the formation of the soluble ferrous ascorbate chelate (Conrad and Schade 1968). Certain amino acids, e.g. histidine, cysteine and lysine, also enhance absorption (Van Campen 1973). This enhancement is abolished by decarboxylation, further evidence that efficient absorption depends on the presence of soluble chelates (Van Campen 1973). Synthetic chelates such as EDTA and desferrioxamine, however, form stable, charged iron complexes that are not available for absorption, if present in excess.

None of these compounds affects the absorption of haem, however. It has been shown (Conrad et al 1966) that the haem complex is not split but is absorbed as such. A specific haem receptor has been identified and characterised on brush border microvillous membrane (Grasbeck et al 1982), the haem-receptor complex is presumably then absorbed by endocytosis and the iron split off only inside the cell by a microsomal haem oxygenase. An interesting point is that the absorption of haem is low in comparison with that of haemoglobin; this has been attributed to the reduced solubility of the complex in the absence of globin (Conrad et al 1966a).

3.1.1. The Mucosal Epithelium.

The control of iron absorption and hence of iron homeostasis in mammals rests entirely with the mucosal cell of the small intestine. The small intestine of the rat may be divided into three sections: the duodenum, forming the upper 10 cm or so of the rat intestine just distal to the pylorus becoming continuous with the jejunum, the next two fifths, which is in turn followed by the ileum, the remaining three fifths. Intestinal villi project from the membrane into the lumen; the villi of the duodenum are broader and longer, decreasing gradually in size to a minimum in the ileum just before the ileocaecal junction. The villi have cores of lamina propria, but no connection to the muscularis mucosa or submucosa (Figure 1). Coating the villi are the epithelial or mucosal cells of the intestine. These are tall columnar epithelial cells having a thick "striated border" shown by electron microscopy to be composed of packed microvilli or projections of the luminal membrane. The luminal plasma membrane may therefore be described as a "brush border". The membrane itself is a typical trilaminar structure (Kenny and Booth 1976), 8- 10 nm in thickness. Thin sections of the brush border frequently show a polysaccharide coat, the glycocalyx or "fuzz" particularly marked at the tips of the microvilli.

The mucosal cells have abundant cytoplasm, are enclosed on the sides by highly convoluted cell membranes and have abundant mitochondria, rough endoplasmic reticulum and Golgi apparatus but few free ribosomes (Altmann and Enesco 1967). The epithelium in the crypts between the villi -- the crypts of Lieberkuhn-- is composed of large numbers of glandular cells with small columnar epithelial cells. Electron microscopy shows that these cells are poor in cytoplasm, have smooth lateral membranes, few mitochondria and endoplasmic reticulum cisternae and small Golgi apparatus, but

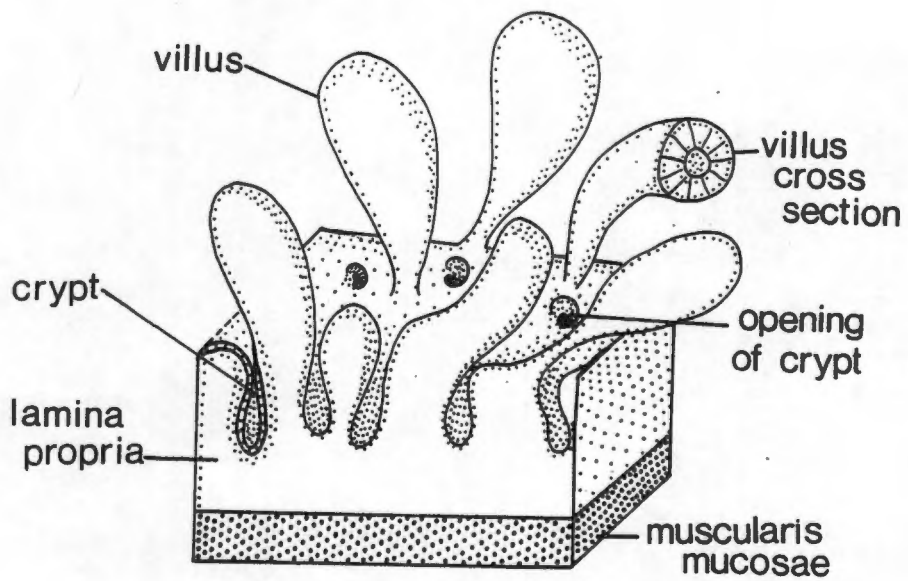


Figure 1. Three-dimensional drawing of the lining of the small intestine, showing the villi and crypts of Lieberkuhn as well as the submucosal layers. After Ham (1965).

are packed with free ribosomes (Altmann and Enesco 1967). This would indicate that these cells are in a relatively undifferentiated state and show a high rate of division. Epithelial cells on the villi, on the contrary, do not divide. Radioautography shows that columnar cells in the crypts migrate up the villus, appearing on the surface within about 1 day and after about 3 days have reached the villus tip where they are extruded (Altmann and Enesco 1967). The mucosal epithelium of the rat is thus renewed approximately every 3 days.

The cores of the villi are composed of lamina propria, consisting of connective tissue having many of the attributes of lymphatic tissue. A single arterial twig from the submucosa penetrates the muscularis mucosa beneath each villus and ascends the latter for some distance before breaking down into capillaries. These capillaries approach the mucosal cells very closely. Separate arterial twigs break up into capillaries surrounding the crypts of Lieberkuhn. Smooth muscle fibres extending from the muscularis mucosa have their long diameter parallel with those of the villus and are located around the lacteal, the single large lymphatic capillary of the villus.

Two phases of iron absorption have been recognised: an initial rapid phase, starting within seconds of the presentation of iron to the brush border membrane and reaching a maximum within 30-60 minutes, and a subsequent slow phase lasting up to 24 hours (Hallberg and Söllvell 1960; Wheby and Crosby 1963). The absorptive process can also be divided, according to the barriers presented to the iron, into 3 components: uptake across the brush border membrane, transfer across the cell and finally release to the blood across the serosal surface. The mechanism or mechanisms by which these processes occur are not known.

Effective absorption is dependent in the first instance on the

ease with which the compound traverses the membrane (Albert 1973). The lipid layers of membrane are a major factor in restricting their permeability, and it is well known that lipophilic substances tend to pass cell membranes more readily than those which are water soluble. Water soluble substances may, however, pass through pores in the lipid barrier or by being transported by mechanisms that form part of the membrane structure. The mechanism by which iron enters the cells may be either active or passive (Table 1): characteristics of active transport are, first and foremost, a dependence on metabolic energy, inhibition by metabolic poisons, partial or complete substrate specificity, transport against either a concentration or potential gradient and finally saturation of the mechanism at higher substrate levels. Passive diffusion on the other hand shows no reliance on metabolic energy, moves molecules down gradients only and will only permit the passage of lipophilic molecules through the membrane. Charged molecules would be unable to penetrate the membrane due to the high energy required to move them from an aqueous to a lipid phase. Three other cases that do not fall into these two categories may be recognised: firstly, the phenomenon of facilitated diffusion, a saturable process that can move charged species. This does not rely on oxidative metabolism nor is it capable of working against a concentration gradient. Secondly, when pores exist in the membrane; these allow the passage of molecules smaller than albumin (molecular weight 70000 daltons). Thirdly, pinocytosis and phagocytosis, cell "drinking" and "eating" respectively (Silverstein et al 1977), the former concerned with the vesiculation of small particles and the latter with large particles such as viruses.

3.1.2. Transport across the Brush Border Membrane.

Most semipermeable membranes do not appear to possess active transport mechanisms specific for transition metal ions (May et al 1978). The alternative, passive diffusion, requires a low molecular weight lipophilic complex, as neither high molecular weight proteins nor free aquated ions could enter the cell in this manner.

The requirement for both facilitated diffusion and pinocytosis is a specific receptor within the membrane. There is a great deal of evidence (see below) concerning the uptake of iron by mucosal cells, much of it conflicting. May et al (1978) have stressed that the interpretation of results in a case such as this may be very difficult, as all the criteria used to identify the kind of transport processes involved may be ambiguous. For instance, it has been found (Yeh and Shils 1966) that inhibitors of protein synthesis inhibit iron absorption: this may be related to ferritin or storage iron synthesis within the cell, inhibited synthesis of a protein carrier or inhibition of some form of an active transport system. A further complication may be that of complex formation of the iron: a phenomenon that may cause movement of an ion against a concentration gradient and may show typical effects of saturation. It is well documented that at low iron doses (less than 50 μM) uptake is saturable, whereas at higher doses uptake shows first order kinetics (Gitlin and Cruchoad 1962; Thomson and Valberg 1972; Wheby et al 1964). Further, changes in species distribution between media on either side of the membrane, due to differences in composition of pH, may tend to produce a flux for reasons that are not easy to discern. A further complication is that of changes in oxidation state. The evidence, as mentioned above, is conflicting. Early research by Hahn et al (1943) showed that the transfer of iron across the intestinal epithelium is

Table 1. A brief summary of the major transport processes through membranes. Information was obtained principally from Allison and Davies (1974) and Silverstein et al (1977).

Transport process	Metabolic energy	Intact cytoskeleton	Saturable	Substances
Active	Yes	No	Yes	Variety
Passive diffusion	No	No	No	Low MW; lipophilic
Diffusion through pores	No	No	No	Water-soluble; MW < 70000
Facilitated diffusion	No	No	Yes	Variety
Pinocytosis	?Yes	Yes	Yes	Variety

influenced by cell metabolism. Since then, a dependence on metabolic energy has been shown repeatedly (Dowdle et al 1960; Manis and Schachter 1962; Ruliffson and Hopping 1963; Jacobs et al 1966; Linder et al 1975) and a number of authors (Dowdle et al 1960; Manis and Schachter 1962) proposed an active transport mechanism specific for FeII, consisting of two steps: rapid uptake across the brush border membrane, followed by transport across the cell and slower release at the serosal membrane. There is some disagreement about the saturation kinetics of the system (Manis and Schachter 1962; Jacobs et al 1966). It is possible that this could be due to loss of cellular function which can occur when gut sacs are incubated over several hours (Smith et al 1969). Other workers, have, however, failed to confirm the active transport hypothesis (Brown and Justus 1958; Pearson and Reich 1965). The active transport studies are difficult to evaluate, as most of them were done on everted gut segments or whole cells in situ, which do not separate the processes of uptake and transport or events occurring within the cell, such as protein synthesis or mitochondrial uptake, which is known to be energy-dependent (Romslo and Flatmark 1973). Studies using isolated brush borders (Kimber et al 1973; Savin and Cook 1978) have suggested that uptake by the brush border membrane is not energy-dependent. Passive transport, either by simple or facilitated diffusion, has tended to be the more favoured theory especially in recent years. Workers in the mid-sixties (e.g. Saltman 1965; Forth et al 1965; Hopping and Ruliffson 1966) stressed the importance of equilibrium binding and chelation phenomena: if both FeII and FeIII species are available for absorption provided suitable chelates are present, the need for metabolic energy is removed. The importance of soluble chelates has been observed (Spiro and Saltman 1969); Kimber et al (1973) found uptake of iron by brush borders was

greater from ferric citrate or ferric ascorbate than from ferrous sulphate; similarly Cox and O'Donnell (1981) observed similar behaviour. Whether the function of the chelates is simply to maintain the iron in soluble form in the gut lumen or whether the complex is absorbed as such is not certain. Terato et al (1973) found that absorption of iron from chelates was dependent on the molecular weight of the compound, which suggested absorption. It has been found that the fat soluble and therefore membrane permeable chelate ferrocene is almost quantitatively absorbed following oral administration (May et al 1978). Sex-linked anaemic (sla) mice, normally unable to absorb inorganic iron, when fed on a lipophilic iron compound showed effective transfer of iron from lumen to serum (Edwards et al 1974). Absorption of iron from 8-hydroxyquinoline or its sulphonic acid analogue was increased by a factor of four (Forth and Rummell 1973); this was attributed to the charge on the compound formed. In this respect, iron absorption from the synthetic chelate EDTA is interesting: iron in EDTA is effectively unavailable for absorption (Jandl et al 1959). The complex formed is charged and hydrophilic.

It would appear, therefore, as though both active and passive transport processes have a role to play. The relative importance of each is difficult to evaluate. Dowdle et al (1960) suggested that the active transport mechanism may come into play if passive diffusion is restricted. This would fit the observed differences in kinetics at different iron concentrations (Forth and Rummell 1973). Active transport mechanisms do have role to play in many instances, but the principle of energy conservation applies here as it applies elsewhere in nature: if such expenditure can be avoided, it will be.

3.1.3. Intracellular Transport of Iron.

The second step in absorption is that of transport through the mucosal cell. A wealth of information has accumulated on this topic, some confusing and conflicting, but a number of general trends are apparent. It has been shown that absorption is diminished by inhibitors of protein synthesis (Yeh and Shils 1966); phenobarbitone, a stimulator of protein synthesis (Rosenzweig et al 1969) increases absorption (Thomas et al 1972). It has been assumed on this evidence that a protein carrier is involved, however, as has been described above, the effect of the inhibitors may not be direct, affecting perhaps ferritin synthesis or that of some other species not necessarily on the direct path of absorption. Iron-binding proteins have however been found by almost all investigators: the general trend is that of ferritin and transferrin or a transferrin-like protein. A summary of these investigations is presented in Table 2.

Using differential centrifugation, Pearson and Reich (1969) found, in the rat, evidence of ferritin and a "high molecular weight protein other than ferritin".

Worwood and Jacobs (1971a and b) found, in the rat, most of the absorbed iron in the first early phase of absorption to be in the cytosol. After 12-18 hours, the mitochondrial fraction was the most active however. As far as proteins were concerned, ferritin, a protein of MW 80000 and an apparent high molecular weight non-ferritin protein were found. Evidence of the latter was also found on polyacrylamide gel electrophoresis.

Halliday and Powell (1973) and Halliday et al (1976), in the rat, found ferritin, transferrin and a protein similar in molecular weight to transferrin but immunologically distinct. Iron was also associated with intracellular membrane particles; chaser studies

Table 2. The association of radioiron with components of the intestinal mucosa during absorption. Table (a) shows studies based on subcellular fractionation. Tf = transferrin; Fe = iron; G-pig = guinea pig. Table (b) shows studies based on autoradiography.

ER = endoplasmic reticulum;
Mitoch. = mitochondria.

(a) Studies based on subcellular fractionation.

Tf	Ferritin	Other	Species	Reference
Yes	Yes	Tf-like	Rat	Halliday and Powell (1973)
Yes	Yes	Membrane	Rat	Halliday et al (1976)
Yes	Yes	Low MW	Rat	Batey and Gallagher (1977)
Yes	Yes	-	Rat	Kaufman et al (1977)
Yes	Yes	Tf-like	Rat	Huebers et al (1971,1976)
No	Yes	Fe-salts	Rat	Sheehan and Frenkel (1972)
Yes	Yes	-	Rat	Savin and Cook (1980)
?	Yes	Tf-like	Rat	Osterloh and Forth (1981)
No	Yes	Tf-like	G-pig	Pollack and Lasky (1976)
Yes	Yes	Low MW	Pig	Furoguori (1977)
Yes	Yes	-	Rat	Charlton et al (1965)

(b) Studies based on autoradiography.

ER	Mitoch.	Ferritin	Species	Reference
-	Yes	-	Rat	Humphrys et al (1977)
-	Yes	-	Rat	Hopkins and Peters (1979)
Yes	-	No	Mouse	Bedard et al (1971)

indicated that these particles and the unidentified protein were involved in absorption.

Batey and Gallagher (1977), in the rat, found ferritin, transferrin and another protein of lower molecular weight than transferrin and immunologically different, that increased in iron deficiency.

Kaufman et al (1977), using differential centrifugation and polyacrylamide gel electrophoresis on rat intestinal homogenates, found proteins of molecular weight 400000 and 80000 daltons both of which were present in the ribosomal-enriched subfraction obtained during purification of the microsomal membrane but were not identified among the purified membrane proteins.

Huebers et al (1971, 1976) in the rat, observed ferritin and a protein of similar molecular weight but differing in amino acid composition and isoelectric behaviour to transferrin. Similar results were reported by Pollack and Lasky (1976) who found that, in the guinea pig, this transferrin-like protein was not precipitated by serum transferrin antiserum.

Humphrys et al (1977) using both differential centrifugation and radioautography, found a concentration of activity in both the soluble fraction and the mitochondria. As regards the latter, they concluded that although these organelles would seem to be quantitatively important in iron metabolism, they appeared to have no direct involvement in iron transport.

Hopkins and Peters (1979) using density gradient centrifugation of rat intestinal homogenates, similarly reported an association of iron with the mitochondria.

Sheehan and Frenkel (1972) found ferritin which accounted for approximately 90% of the observed radioactivity, and apparently

free iron salts, the amount of which increased in iron deficiency.

Savin and Cook (1980) identified and quantitated the amounts of ferritin and transferrin in rat mucosa. No evidence of other species was found.

Osterloh and Forth (1981) reported the presence of a transferrin-like protein in the rat, similar in size and structure but differing in immunological reactivity.

The possibility of the involvement of a low molecular weight component or components in iron absorption has been suggested in a number of models particularly Jacobs (1977) and May et al (1978). Such a component would have ease of transfer through membranes and the cell and could form a "pool" or reservoir within the cell from which the various cellular structures and components could draw. These models are attractive and it is known that low molecular weight chelators play a role, certainly in vitro, in the transfer of iron from transferrin to ferritin (Miller and Perkins 1969). The evidence for such a species in iron absorption is scanty however: the only investigator to report such evidence is Furoguori (1977) in the piglet.

Autoradiographic studies on the other hand have shown a different pattern (Bedard et al 1973): rapid uptake of iron at the brush border and passage through the terminal web to rough endoplasmic reticulum and areas rich in free ribosomes. The iron then passed through the lateral cell membrane, the intercellular spaces and the epithelial basement membrane to the vessels of the lamina propria. Regardless of iron status, the rough endoplasmic reticulum and ribosomes formed the major localisation of iron at all stages of absorption. Ferritin, visible by electron microscopy, did not appear to play an important role in absorption.

The respective roles, if any, of the iron-binding proteins in absorption remains controversial. There is general agreement that ferritin is not directly involved in the absorptive process although it might play a role in the regulation of absorption (see the Regulation of Absorption, below). There is evidence and suggestions (Halliday et al 1976; Huebers et al 1976) that the transferrin or transferrin-like protein is closely involved with absorption. The possibility of low molecular weight compounds being involved in absorption has been discussed above. Pinocytic transport remains another possibility. There is evidence that haem absorption involves binding to a specific brush-border receptor (Grasbeck et al 1982) followed by pinocytosis and splitting of the haem complex within the cell by a mucosal haem oxygenase, whereupon the released iron follows the same path as for iron salt, subject to the same regulatory factors (Wheby et al 1970). The possibility of vesicles being involved in transport of iron salts across the brush border membrane and through the cell has been suggested (Evans and Grace 1974; Cox and O'Donnell 1981; Marx and Aisen 1981).

3.1.4. Transport across the Serosal Membrane.

The last step is that of transfer of iron across the serosal membrane to the transferrin in the blood. Very little evidence on this point exists. Results of in vitro experiments using suspensions of free mucosal cells (Levine et al 1972) suggested that transferrin potentiates this process. Other studies however, do not support such a conclusion: Wheby and Jones (1963) found that absorption continued after the iron-binding capacity of the serum had been saturated and Jacobs et al (1966) found that a gut segment with an artificial circulation transferred iron rapidly,

while Schade et al (1969) found that increasing the amount of available transferrin had no effect on absorption. Morgan (1980) presented evidence that transferrin probably acts primarily to supply iron to the cells and that iron passes from the cells to the portal plasma in low molecular weight form. Clinical evidence is provided by the disease of atransferrinaemia characterised by virtual absence of transferrin; Heilmeyer (1961) describes the case of a 7 year old girl with this disorder whose absorption of iron was not impaired in comparison with normal children although it was low for anaemia.

3.1.5. The Control of Iron Absorption.

It is very well documented that absorption of iron rises in situations of need such as reduced stores and increased erythropoietic activity and diminishes in states of iron repletion (Bothwell and Finch 1962). The means by which this occurs remains one of the perennial problems of iron metabolism. Bothwell et al (1979). The theories that have been proposed may be divided into 3 groups:

- (i) those involving regulation by the mucosal cell;
- (ii) those involving regulation by transferrin and
- (iii) a mechanism of humoral control.

(i) Firstly, control by the mucosal cell. Hahn et al (1943) presented evidence that absorption of iron increased in iron deficiency in comparison to normal subjects. Similarly, feeding dogs a large dose of iron resulted in depressed absorption of a subsequent dose. Hahn et al (1943) proposed the existence of a "mucosa block" and the existence of a "mucosal acceptor" for iron, "physiological saturation" of which prevented further entry of iron through the mucosa into the body. It was suggested that this

acceptor might be apoferritin. These ideas were confirmed by Granick (1946) who observed that the mucosa of guinea pigs became "saturated" a few hours after the administration of an oral dose of iron and further absorption was prevented for several days. This led him to propose the "mucosal block" theory of iron regulation as follows: iron is absorbed in the ferrous form, oxidised to the ferric and stored as such in ferritin. An equilibrium between ferrous and ferric iron stored in ferritin is postulated in the cell, with the cell being in a state of "physiological saturation" with respect to ferrous iron. Lowering the concentration of serum iron in the blood stream would lead to removal of iron, reduced to the ferrous form, from ferritin and from the mucosal cell, resulting in a decrease of ferritin in the mucosa. Only when the ferritin iron was diminished to a point where the cell was no longer "physiologically saturated" with respect to ferrous iron, would more iron be absorbed by the cell. It was later shown, however, (Wohler et al 1957) that absorption continues in spite of maximal ferritin levels and Moore (1960) discounted the theory altogether for this reason and for others including the observation that the large iron dose administered, resulting in the "block" observed by Hahn et al (1943) and Granick (1946) was highly artificial, the amount of iron present in the food being too small to produce such an effect; also, as the size of the test dose increases, the total amount of iron absorbed increases, and also, that absorption is greater than normal not only in states of iron deficiency but also in states of accelerated erythropoiesis when tissue stores are high. Further evidence is that after mucosal ferritin synthesis was stimulated by iron in both normal and iron deficient rats, normal ferritin concentrations resulted in the latter, but absorption was still greater (Brittin and Raval 1970)^{and} that ferritin synthesis itself, while increased in the iron loaded mucosa, is equal in both normal

and iron deficient cells (Cumming et al 1970) and that, in the case of animals with turpentine abscesses which reduce absorption, incorporation into ferritin was the same as in normal animals, indicating that the reduction of absorption was not due to diversion of iron into ferritin (Cumming et al 1970).

Modifications to the theory were proposed by Crosby (1963) and Conrad et al (1964): it was proposed that the iron incorporated into mucosal ferritin is there not as a stage of absorption but is permanently detained there to prevent its absorption, being incorporated from the plasma during the cells' development in the crypts of Lieberkuhn. This "loading from the rear" is essentially a mechanism for the excretion of excess iron. By saturating ferritin and iron-carrier mechanisms of absorption, the cells may induce a "refractory state" thus inhibiting the acceptance of unneeded iron. Crosby (1963) suggested that the disorder of haemochromatosis, in which large quantities of iron are absorbed resulting in pathological iron overload, may represent a genetically-determined lack of the ability to form ferritin in cells, so that the passage of iron is relatively unimpeded through the cells.

The iron status of the mucosal cell is dependent on iron both from the plasma and from the lumen. Parenterally administered iron enters the cells from the serosal pole at the time of their emergence from the crypts of Lieberkuhn. The actual iron content of the mucosa is not increased with loading but is reduced in iron deficiency (Richmond et al 1972; Mattli et al 1973). These authors found the greatest concentration of iron in the mitochondrial fraction. Similar results were reported by Worwood and Jacobs (1971, 1972), who showed that plasma iron is taken up largely by the mitochondria and that, in iron deficiency, mitochondrial iron is reduced, as is iron-enzyme activity. In iron loading, mitochondria are normal.

A second possibility of mucosal cell control rests on the assumption of receptors in the brush border membrane. There have been various studies showing that brush borders and microvillous membranes play an important role in determining the rate and specificity of intestinal transport processes, other than iron (e.g. Miller and Crane 1961). Both Greenberger et al (1969) and Kimber et al (1973) found increased attachment of iron to isolated brush borders in iron deficiency; Cox and O'Donnell (1981) have isolated a glycoprotein membrane receptor for iron that also responds to changes in iron status.

(ii) It has also been suggested that transferrin may play a role in iron absorption regulation, presumably acting at the serosal surface of the cell. Clinical data however does not support the hypothesis: in humans, increased absorption is found in patients in whom haemoglobin, plasma iron binding capacity and transferrin saturation were normal (Pirzio-Birolli and Finch 1960); absorption was also found to be decreased by increased stores although the plasma iron was normal.

An explanation was suggested by Fletcher and Huehns (1968) based on the hypothesis of functional heterogeneity of the binding sites of transferrin. The hypothesis suggests that one site of transferrin is more directed to the uptake of iron from the mucosa and stores and delivery to the marrow, while the other site is oriented to delivery of iron to the stores. The mucosal cell could thus be informed of the need for iron without there being any abnormalities in transferrin saturation or plasma iron. Recent evidence (see "Transferrin" below) suggests that, for uptake of iron by reticulocytes at least, this hypothesis is not valid, that the binding sites are in fact functionally homogeneous. No evidence exists for transferrin site differences or otherwise in uptake of iron from the intestine.

Levine et al (1972) presented data suggesting that the amount of iron-free transferrin available at binding sites at the serosal membrane may play a role in regulation, but no increase in absorption could be demonstrated after acute intravenous administration of unsaturated transferrin. Similarly Morgan (1980) presented data suggesting that transferrin in intestinal fluid and binding to the mucosa probably functions primarily in supplying iron to the cells rather than as a carrier.

there are
(iii) Lastly, [^]humoral factors, external to the cell itself. The search for a hormone promoting absorption has thus far proven unsuccessful (Krantz et al 1959; Beutler and Buttenweiser 1960). Administration of erythropoietin increases absorption (Mendel 1961) but presumably through its effect on erythroid marrow rather than the intestine; similarly hypoxia has been shown to increase absorption (Brittin et al 1968), but also presumably because of erythropoietic stimulation (Hathorn 1971). An inhibitor of absorption in gastric juice, "gastroferrin", was reported by Davis and Deller (1966) but is not confirmed (Jacobs and Miles 1968). Confusingly, absorption was apparently increased in rats (Turnberg 1968) and humans (Murray and Stein 1968) by administration of gastric juice from iron deficient subjects. Pancreatic secretions have also been proposed (Davis and Badenoch 1962) but not confirmed.

On the strength of this evidence, it appears as though control of absorption rests with some sort of mucosal cell regulation. The influence of humoral factors can probably be discounted; the evidence is minimal and, at best, contradictory. Similarly, it appears as though on the strength of recent demonstrations of the functional homogeneity of the binding sites of transferrin and the invalidation of the Fletcher-Huehns hypothesis, that this, too, can be discounted as a means of control. As far as regulation by

the mucosal cell itself is concerned, it is apparent that, while ferritin may play a part in the absorption and regulation of iron, it is not the only factor involved. The hypothesis of brush border receptors thus appears, on present evidence, to be the most acceptable. However, subcellular fractionation studies have indicated the presence of transferrin or a transferrin-like protein, apparently involved in iron absorption. It is therefore possible that the process consists of uptake by the brush border receptor, followed by handover to transferrin, perhaps with an intervening regulatory step. Present evidence suggests that the iron-binding components of the mucosa represent known moieties, such as transferrin and ferritin, which would argue for the involvement of these in the process. The alternative is a completely novel mechanism, a possibility that cannot be discounted.

3.2. Transferrin.

The transferrins are a group of high affinity iron-binding glycoproteins, of molecular weight 80000 - 90000 daltons and each binding 2 atoms of ferric iron whose functions include the transport of iron between sites of absorption, utilisation and storage, and the denial of iron to pathogenic organisms (Aisen and Listowsky 1980). The transferrins have traditionally been thought to exist only in vertebrates and to consist of the three related proteins, serum transferrin, lactoferrin and ovotransferrin. However, a number of publications in the last few years attest that the traditional classification is far too limited: transferrins do exist in animal groups outside the vertebrate subphylum, as shown by the recent discovery of a transferrin in the crab (Huebers et al 1982), fish transferrins lacking glycans have been found (cited Williams 1982) and a number of "new"

proteins similar in structure and properties to transferrin have been found (e.g. Brown et al 1982; Antanaitis and Aisen 1982). Structural variation within transferrin itself is well-known, particularly the genetic polymorphism found in many species (Aisen and Leibman 1972), as well as the fact that different tissues of a single individual may produce "different" transferrins e.g. cerebrospinal fluid (Parker and Bearn 1962) and the Sertoli cells of the testis (Skinner and Griswold 1980). The classification of the group thus appears considerably more complex than at first thought.

The transferrins consist of a single polypeptide chain of molecular weight about 80000 daltons (Greene and Feeney 1968) that folds up into 2 compact globular structures or domains (Gorinsky et al 1979). Each domain has a specific binding site for one ferric atom and are generally thought to be independent although Aisen et al (1973) have suggested that there may be some interaction. The domains are formed from the N and C terminal halves of the protein and have been isolated after partial proteolysis as single chain iron-binding fragments of molecular weight about 40000 daltons each (Williams 1974). The two halves of the polypeptide chain are homologous: it has been shown (MacGillivray et al 1977; Jeltsch and Chambon 1982; Williams et al 1982) that about 40% of the residues are identical. The domains also have similar iron-binding properties, with the same ligands involved in binding in each case (see later). There are however spectroscopic differences which may be caused by different geometries at each site and the distribution of iron between the two is not random (Lestas 1976; Princiotto and Zapolski 1976). The observed homology may be confirmation of an hypothesis suggested by Greene and Feeney (1968) that the two domains are descendants of a common ancestral protein and that gene duplication and fusion during evolution led to the present-day

structure. After fusion, the two domains diverged both in structure and iron-binding properties. Although the two domains do show some physical differences (see below), present evidence indicates that there is no difference in the *in vivo* behaviour of the sites with respect to iron uptake and delivery to cells. This hypothetical ancestral protein is supposed to have had a molecular weight of around 400000 and suggestions that this protein was itself derived from an even smaller protein have also been made (MacGillivray et al 1977; Jeltsch and Chambon 1982). A search for the 400000 dalton ancestral transferrin led to the finding in the hagfish *Eptatretus stoutii*, a primitive vertebrate (Palmour and Sutton 1971) of a 400000 molecular weight transferrin; however, Aisen et al (1972) were unable to confirm this, and indeed wrote in a later paper (Aisen and Listowsky 1980) "Why a two-sited protein should possess biological advantages enabling it to endure to the exclusion of its single-sited ancestor remains a mystery to transferrin biochemistry". The answer to this question lies in the problem of renal excretion of serum proteins: proteins of molecular weight less than about 700000 (the molecular weight of albumin) are able to pass through the glomerular membrane and thus are excreted (Brenner and Rector 1981). Williams et al (1982) have in fact demonstrated the excretion of isolated domains (molecular weight ca. 400000 daltons) of serum transferrin from the kidney tubule. If indeed, the ancestral protein was as small as this, it seems unlikely to have been retained in the blood; there would be strong selective pressure to enlarge it while retaining its highly useful functional characteristics. A low molecular weight membrane-bound glycoprotein which appears to be homologous to a single domain of immunoglobulin has recently been discovered (Cohen et al 1981). By analogy, Williams et al (1982) have postulated that the ancestral transferrin may have been a membrane-bound metal receptor protein. Ancestral transferrin may

have followed a similar evolutionary pathway. In this respect, it is interesting that p97, MW 97000, a membrane tumour antigen found in melanomas and foetal intestine, is homologous to transferrin and binds iron (Brown et al 1982). P97 could represent an intermediate stage, a "missing link", in the evolution of transferrin.

3.2.1. Structure of Transferrins.

For a comparison of the different transferrins isolated to date, see Table 3.

The molecular weight of transferrins, and in particular that of serum transferrin, has been variously reported as ranging from 70000 to 90000 daltons, with more recent estimates (e.g. MacGillivray 1977) inclining to a figure between 80000 and 81000. However, MacGillivray et al (1982) in a complete amino acid sequence analysis, has set the figure at 79550, containing 678 amino acid residues. The single polypeptide chain is divided into 2 domains, as described above, corresponding to residues 1-336 and 337-678, each bearing an iron-binding site and are apparently homologous. Transferrin contains approximately 6% carbohydrate in the form of two nearly symmetrical branched heterosaccharide chains of molecular weight ca. 2200 daltons each (Dorland et al 1977). These two chains were previously thought to be identical, but recent investigation (Spik and Mazurier 1977) has shown that they differ. Both chains are located on the C-terminal region of the protein, and are joined by β -1 glycosidic linkage to asparagine residues 415 and 608 (Jamieson et al 1971; MacGillivray et al 1977). It is interesting that this pattern is conserved in other transferrins: in chicken ovotransferrin, both glycosylation sites are also present in the C-domain (Kingston and Williams 1975). While the amino acid sequence of the polypeptide chain is genetically coded and determined by the usual template mechanism,

Table 3. Members of the transferrin class that have been discovered to date. *MW: molecular weight.

Transferrin	Source	MW*	Sites for	Reference
iron				
Mammalia				
Transferrin	Human serum	79550	2xFeIII	MacGillivray et al (1982)
Lactoferrin	Milk; secretions	80000	2xFeIII	Leibman and Aisen (1972)
Transferrin	Human CSF	79000	2xFeIII	Parker and Bearn (1962)
Transferrin	Rat testis	80000	2xFeIII	Skinner and Griswold (1980)
p97	Melanoma	97000	2xFeIII	Brown et al (1982)
Uteroferrin	Pig uterus	?	2xFeIII; plus phosphatase	Ananaitis and Aisen (1982)
Aves				
OvoTf	Egg white	81000	2xFeIII	Greene and Feeney (1968)
Reptilia				
Transferrin	Turtle	96000	?	Palmour and Sutton (1971)
Amphibia				
Transferrin	Frog	72000	?	Palmour and Sutton (1971)
Osteichthyes				
Transferrin	Tench	81000	2xFeIII	Van Eijk et al (1972)
Chondrichthyes				
Transferrin	Shark	78000	?	Got et al (1967)
Agnatha				
Transferrin	Hagfish	76000	2xFeIII	Aisen et al (1972)
Arthropoda				
Transferrin	Crab	150000	2xFeIII	Huebers et al (1982)

the carbohydrate residues are added at post-translational level only, directed by transferases (Gottschalk 1969) which may result in heterogeneity of the carbohydrate groups. Variations in the primary structure, as seen in human transferrin polymorphism, are caused by mutations at a single autosomal locus (Aisen and Leibman 1972). Thus two transferrin species in different tissues of a single individual may show variation in carbohydrate, while the amino acid backbone is identical; this is seen in the hen with serum transferrin and ovotransferrin or conalbumin (Williams 1962) and in the human, with serum transferrin and cerebrospinal fluid transferrin where the same situation prevails. The function of the carbohydrate chains are unknown, although it has been suggested (Van Bockxmeer and Morgan 1977) that acylation of the carbohydrate groups may be involved in the binding of the protein to its cell surface receptors (see later).

Lactoferrin, also known as lactotransferrin, the "red protein" of milk and other secretions, has so far been found only in mammals. Lactoferrin, although clearly homologous to transferrin, differs both in polypeptide and carbohydrate structure (Aisen and Leibman 1972), does not cross-react immunologically and thus may be thought of as a separate gene product. It is present in normal human serum at concentrations of about 3 mg/l (Bennett and Mohla 1976) but whether it plays any role in the transport of iron is not known; it has been suggested (Morgan 1981) that the protein may be involved in iron transport from plasma to reticuloendothelial cells and may thus contribute to the reduction in plasma iron concentration that occurs in acute inflammatory states. A further function of lactoferrin is that of defence against bacterial infection, by "mopping up" surplus iron that might otherwise be utilised for the proliferation of microorganisms, a function also attributed to transferrin itself. A third possibility is that of control of iron absorption: Cox et al

(1979) found specific lactoferrin receptors in human duodenal brush-border membrane. Recently, two further proteins have been discovered that appear to have characteristics of transferrin and may thus be tentatively included in the discussion of the class. p97, a protein of molecular weight 97000 daltons, is a membrane protein found only in trace amounts in normal tissue. It has recently been purified and characterised from human melanoma cells (Brown et al 1982) and its N-terminal sequence shown to be homologous to the respective sequences of transferrin and lactoferrin. Denatured p97 was shown to cross-react immunologically with both denatured transferrin and lactoferrin. Its function appears to be that of transmembrane iron transport (ibid).

Uteroferrin, a purple progesterone-induced glycoprotein with phosphatase activity, is secreted by the pregnant porcine uterus. Placentation in the pig is of the epitheliochorial type, where there is no invasion of maternal tissue by the developing trophoblast, so that the latter is dependent on the secretion of nutrients by the mother. Uteroferrin forms a major constituent of these secretions, binds two atoms of iron (Antanaitis and Aisen 1982) and is capable of transferring iron to transferrin in utero (Buhl et al 1982). It has thus been postulated that the major function of this little-known protein is that of transplacental iron transport (ibid), the other being its known phosphatase activity.

The transferrin membrane receptor is a topic that has received much attention in recent years. Receptors have been found in a variety of cells, but are particularly abundant in cells requiring large amounts of iron such as cells of the erythroid marrow and the placenta. Active reticulocytes can incorporate over 1×10^6 atoms of iron per minute. These cells have approximately 300000

transferrin receptors (Williams 1982), and the transferrin binding, internalisation, release of the iron and return of the apotransferrin to the medium takes place within 15 - 30 seconds (Newman et al 1982). Other types of tissue in which transferrin receptors have been found include the placental trophoblast (Galbraith et al 1980a and b), fibroblast, lymphocytes, kidney tissue, epithelia, various tumour cells (Larrick and Cresswell 1979) and haemopoietic progenitor cells (Sieff et al, cited Newman et al 1982). The literature on the reticulocyte receptor and receptor-transferrin interaction is vast and will be discussed in detail under functional aspects of binding sites (see below). The receptor has been purified and characterised: there is one ubiquitous receptor throughout all tissues; it is a glycoprotein, a dimer of molecular weight 150000 (Van Bockxmeer et al 1975), 180000 (Ecarot-Charrier et al 1980) or 213000 (Enns and Sussman 1981) with subunits each 90000 - 94000 daltons (Seligman et al 1979). The latter authors reported a molecular weight of 364000 for the transferrin-receptor complex; the receptor, therefore, binds two molecules of transferrin. Much of the work on the receptor has been done with the aid of monoclonal antibodies which have been used to isolate and identify the receptor. Trowbridge and Omary (1981) reported that monoclonal antibody B3/25 raised against a human erythroid leukemia cell line was directed against the transferrin receptor; Sutherland et al (1981) found that monoclonal anti-T cell reagent OKT9 was also directed against the receptor. The receptor possesses N-asparagine-linked carbohydrate chains, and it has been reported (ibid) that it contains high-mannose chains and one complex chain, an unusual configuration. The receptor also possesses phosphoryl serine residues and covalently bound fatty acid (Newman et al 1982). The receptor is solubilised with detergents and is readily labelled with lipophilic nitrene reagents, suggesting that it is

an integral membrane protein, and since phosphoryl serine residues are not present in the trypsin-cleaved fragment, it may also be a trans-membrane protein. Owen et al (1980) used a different method, that of protease digestion of microsomes, and were able to demonstrate that the receptor is in fact a trans-membrane protein, having a trans-membrane tail of about 5000 daltons. An interesting point is that the genes for transferrin itself and its receptor, both molecules of very similar size, are located on the same chromosome, chromosome 3 (Williams 1982).

3.2.2. Iron Binding by Transferrin.

Transferrin binds two Fe^{3+} atoms tightly but reversibly; the equilibrium constant for the two binding sites at physiological pH is of the order of 10^{30} (Aasa et al 1963). Apart from iron, the protein also binds a variety of divalent and trivalent metal and rare earth cations (Perkins 1966; Ford-Hutchinson and Perkins 1971), although the highest affinity is for the FeIII ion (Warner and Weber 1953). Transferrin does not, however, bind FeII or at least only very weakly (Bates and Schlabach 1975). A characteristic of the iron-binding process is the requirement for an anion; at physiological pH this is usually carbonate or possibly bicarbonate (Bates and Schlabach 1975; Price and Gibson 1972); others such as oxalate, nitrilotriacetate and thioglycolate may also bind; inorganic anions do not. Both the anion and the metal bind cooperatively in a 1:1 ratio with the protein, neither binding appreciably without the other. Metal binding itself involves two or three tyrosyl (Tan and Woodworth 1969) two histidyl (Line et al 1967) and an equal number of tryptophanyl (Tan and Woodworth 1970) residues. Electron paramagnetic resonance evidence indicates that oxygen and four nitrogen nuclei interact with the metal (Aasa and Aisen 1968). The anion is itself bound to

the protein, a phenomenon resulting in the characteristic salmon-pink colour of the iron-complexed protein (Jamieson et al 1971). EPR studies have found the minimum possible distance between the binding sites to be 9\AA (Aasa et al 1963); a similar study using fluorescence (Luk 1971) found this to be 43\AA . Conformational changes occur with the binding of iron: diferric transferrin is a prolate ellipsoid with a major axis of 55.2\AA , a minor axis of 27.6\AA and a hydration volume of $16.9 \times 10^{-20} \text{cm}^3$ (Rosseneu-Motreff et al 1971), in contrast to the apoprotein's major axis of 62.0\AA , minor axis of 24.6\AA and hydration volume of $15.4 \times 10^{-20} \text{cm}^3$ (Rosseneu-Motreff et al 1971). Furthermore, iron-binding reduces hydrogen-tritium exchange, indicating a more folded state, and nuclear magnetic resonance changes are apparent (Woodworth et al 1970). Kornfeld (1969) was able to show differences in elution on gel filtration between the diferric and apotransferrin; while Charlwood (1971) demonstrated a 0.7% decrease in the Stokes radius of the molecule per iron atom bound. It is interesting that the N-terminal domain of transferrin contains fewer disulphide bonds (MacGillivray et al 1982) suggesting less conformational stability; Price and Gibson (1972) showed that the two sites varied in their susceptibility to conformational changes induced by perchlorate. The importance of conformation in iron-binding is further attested to by Baldwin (1980) who observed that denaturation with detergents caused loss of iron from the protein.

Transferrin may bind none, one (on either binding site) or two iron atoms. The currently accepted nomenclature (Figure 2) is as follows (Frieden and Aisen 1980): Tf for apotransferrin containing no iron; Fe_NTf for monoferric transferrin with Fe^{III} attached to the site closer to the N-terminal (previously designated the B site); TfFe_C for monoferric transferrin with

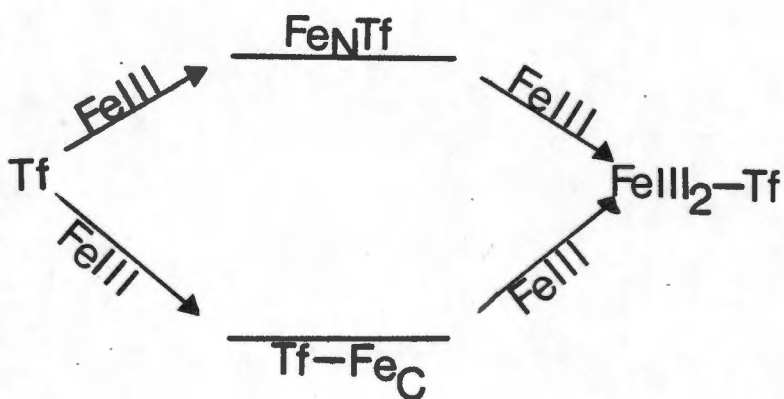


Figure 2. Diagram illustrating the formation of the species of Fe(III)-transferrin. Tf = apotransferrin; FeN-Tf represents transferrin with iron attached to the N-terminal site (formerly designated the B site), and TfC-Fe represents transferrin with iron attached to the C-terminal binding site (formerly designated the A site), and Fe(III)₂-Tf represents diferric transferrin. After Frieden and Aisen (1980).

FeIII bound to the site closer to the C terminus (previously designated the A site); Fe_2Tf or $\text{Fe}_\text{N}\text{TfFe}_\text{C}$ for diferric transferrin with FeIII on both sites.

The metal-free and complexed transferrins differ in their solubilities in 40% ethanol, resistance towards proteolysis, alkali, thermal and urea denaturations, iodination and treatment with disulphide-breaking reagents (Warner 1954; Azari and Feeney 1958; Glazer and McKenzie 1963). In all cases, the iron-transferrin complex is found to be more stable than the apoprotein. Zschocke et al (1972) found 6-7 protected free amino acid groups that were less reactive in the iron-complexed than the metal-free protein. Although these groups were apparently not involved in the binding process, the further fact that modification of these residues led to denaturation of the protein suggests that these residues may be involved in determining the tertiary structure vis-a-vis iron chelation. Much earlier, Fraenkel-Conrat and Feeney (1950) had suggested that metal chelation may not arise from a simple interaction of iron with some group on the protein, but may be dependent on a specific configuration assumed by a number of residues to form a site of proper geometry for the iron atom. Evidence was provided by titration studies (Wistinia et al 1961) that a large number of tyrosine residues, including those involved in the binding of iron, are hydrogen-bonded, thus contributing to both the secondary and tertiary structures. Further supportive evidence is provided by Bezkorovainy and Grollich (1971) who showed that the tyrosyl residues that bind iron are distributed over a relatively large section of the polypeptide chain. In recent years the technique of separating transferrin according to its iron content by polyacrylamide electrophoresis in the presence of high concentrations of urea has been developed (Makey and Seal 1976). Urea denatures by affecting the tertiary structure of the protein: the hydrophilic outer residues "prefer" the urea environment to

that of the hydrophobic inner residues, resulting in unfolding of the protein. The urea electrophoretic technique is dependent on the greater resistance of the iron-complexed protein to unfolding. The two monoferric forms are distinguishable due to the different configurations of the two domains.

Metal-binding also affects the isoelectric behaviour of transferrin: the synergistic binding of one iron atom together with one bicarbonate molecule results in an increase in negative charge, by -1, on the protein. Thus monoferric transferrin will have a lower pI than apotransferrin and diferric transferrin will be even lower. Thus the three transferrin species - the two monoferric forms cannot of course be separated - may be separated (Wenn and Williams 1968) at pH's 5.2, 5.6 and 5.9 respectively. It has also been shown (Kourilsky and Burtin 1968) that the three transferrin species react differently on immunoelectrophoresis in agarose, probably due to charge differences between them.

3.2.3. Functional Differences in the Binding Sites.

The Fletcher-Huehns hypothesis

There are clearly physical differences between the binding sites, as shown by electron paramagnetic resonance studies (Aisen et al 1969, 1973; Cannon and Chasteen 1975). The two sites also show different pH dependencies (Lestas 1976; Princiotto and Zapolski 1975), with the N-terminal site being more susceptible to proton attack. This point is, incidentally, interesting, in view of the fewer disulphide bonds contained in this domain, presumably leading to a less stable conformational state. Whether the two sites are functionally different or not is still a matter of controversy. This hypothesis, (Figure 3) originally proposed by Fletcher and Huehns (1968) states that iron from one binding site,

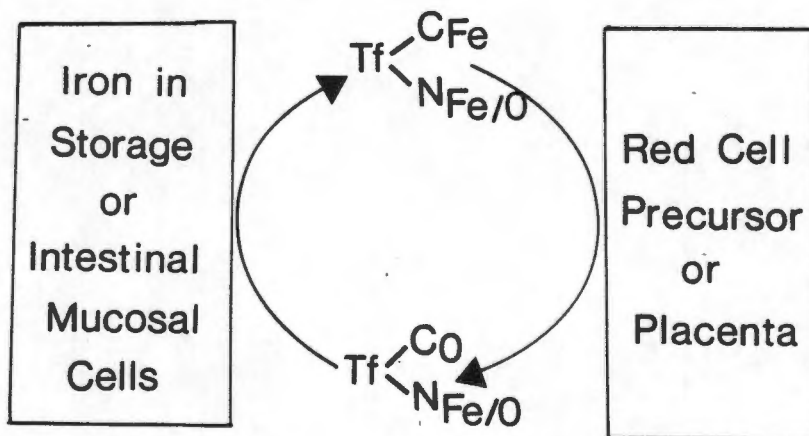


Figure 3. Diagrammatic representation of the Fletcher-Huehns hypothesis. Regulation of absorption could occur if there is a means of controlling amounts of iron transferred from the mucosa to the plasma. It is postulated that this could be if the ironbinding site (the C-terminal site) on transferrin preferentially cleared by red cell precursors and placenta is particularly avid for iron from the intestinal mucosal cells. After Fletcher and Huehns (1968).

which labels first, is delivered preferentially to metabolically active sites such as maturing erythrocytes, while that from the second, subsequently labelled site, is delivered primarily to storage sites. Thus the saturation of the binding sites may act as a "messenger" to the body or the tissues' requirement for iron. This hypothesis was further extended to include the control of iron absorption: the amount of iron on plasma transferrin's binding sites could act as the "messenger iron" of Conrad et al (1964), although these authors used the term in the context of ferritin. The saturation of transferrin and in particular the amount of diferric transferrin present in the plasma, the fraction that delivers iron preferentially to storage sites, is dependent on both erythropoietic activity and body iron stores, both of which control the rate of absorption (see above). Thus the saturation of transferrin would determine the amount of iron incorporated into developing mucosal cells, as any other storage site, and, superimposed on this, would be the effect of the binding sites available to pick up absorbed iron. States of iron need such as iron deficiency or pregnancy would increase the transferrin concentration in the plasma as well as reducing its iron saturation, thus less iron would be deposited in newly formed cells and more sites would be available for picking up iron, hence the observed increase in absorption. It has been found that FeNTf is the major transferrin species in the serum of normal subjects, a phenomenon that has been ascribed to the ionic composition of the serum (Leibman and Aisen 1979). Dialysis of serum leads to the migration of iron to the C-terminal site. Baldwin (1980) was able to manipulate the sites by using various salts and detergents: the sites could be made kinetically either homo- or heterogeneous; this was ascribed to conformational changes.

A great deal of work has been done on the roles of the two binding sites in the delivery of iron to reticulocytes, both in vivo and

in vitro, in attempts to confirm or refute the Fletcher-Huehns hypothesis. According to the hypothesis, these red cell precursors should take up iron preferentially from diferric transferrin. In summary, studies using heterologous systems, such as human transferrin and rabbit reticulocytes (e.g. Awai et al 1975; Okada et al 1978, 1979) support the hypothesis, while those using homologous systems, human-human or rabbit-rabbit (e.g. Huebers et al 1981) do not. A recent paper (Van der Heul et al 1981), using sophisticated techniques of producing accurately labelled monoferric transferrin in a homogeneous human system, shows categorically that there is no functional difference between the two sites. The mechanism of uptake of transferrin by reticulocytes and other cells and the receptors involved have also received much attention. That transferrin may actually be taken up by reticulocytes was first suggested by Jandl et al (1959). Later, evidence was provided that transferrin binds to a receptor (Baker and Morgan 1969). It has been suggested (Fielding and Speyer 1974; Workman and Bates 1975) that the iron is removed at this stage, while the transferrin is attached to the receptor on the cell surface; how the iron enters and is transported through the cell was not postulated. There is however morphological evidence (Sullivan et al 1976; Hemmaplardh and Morgan 1977; Karin and Mintz 1981) that transferrin is actually taken up in an energy-dependent vesicular process, releases its iron within the cell and is returned to the circulation intact. Details of the steps occurring within the cell are not known.

3.3. Ferritin.

The iron-storage molecule ferritin was first described by Schmiedeberg in 1894 who called it "ferratin". The protein was isolated from horse spleen in 1937 by Laufberger who described it

as an iron depot and gave it the name of "ferritin". Ferritin is found widely throughout the animal kingdom; so-called "phytoferritins" have been found in plants and recently a related species in fungi (Aisen and Listowsky 1980). This widespread distribution is an indication of its early evolution and physiological importance. Ferritin is found in most tissues especially in iron storage sites such as liver, spleen and bone marrow. The related form, haemosiderin, an insoluble complex of inorganic iron associated with protein and other substances in variable proportions, is thought to be a degradation product of ferritin (Richter 1959; Fischbach et al 1971) and will not be discussed further.

Ferritin is a hollow nearly spherical protein, with an internal radius of 37\AA and an external radius of 61\AA , as measured by X-ray and electron microscopic studies (Fischbach and Anderegg 1965). It has a molecular weight of 450000 and a sedimentation constant of 17-18S (Crichton 1973). The iron is deposited within the shell and single molecules may accommodate little or no iron to a maximum of approximately 4500 atoms per molecule: the fact that the iron is encapsulated within means that it has no direct effect on electrophoretic mobility, immunological and other properties (Harrison 1964) which depend only on the protein shell. Ferritin consists of 24 subunits of molecular weight approximately 18500 daltons each; these are arranged in 4 3 2 symmetry to form a spherical molecule (Treffrey et al 1979). These subunits may be dissociated by acetic acid at 0°C (Harrison and Gregory 1968) or by sodium dodecyl sulphate at $60-80^{\circ}\text{C}$ (Bryce and Crichton 1971).

Ferritins from a wide variety of species show very constant amino acid compositions: all contain about 40% non-polar residues and a nearly constant 13-15 residues per subunit of alanine (Crichton et al 1973). However, ferritins from different tissues of the same

species or individual show significant differences (Arosio et al 1978). These differences show up on polyacrylamide gel electrophoresis, where a monomer band containing most of the ferritin and more slowly moving oligomer bands are found (Harrison et al 1974) and on isoelectrofocusing where the protein may focus in a series of small peaks (Wagstaff et al 1978). It has been suggested that the latter may be due to differences in iron content (Ishitani et al 1975), although neuraminidase treatment of the sialic acid residues simplifies the isoelectrofocusing profiles (Cynkin and Knowlton 1977), suggesting that part of the heterogeneity may be due to differences in carbohydrate composition.

It has been suggested that the microheterogeneity of so-called "isoferritins" from different tissues of the same individual may arise from the presence of two subunits of different primary structure (Drysdale et al 1977; Arosio et al 1978): the so-called "L" or "light" subunit which predominates in liver ferritins and the "H" or "heavy" subunit, predominating in heart ferritins. These two subunits may be present in varying proportions ranging from the homopolymer $L_{24}H_0$ to the other homopolymer L_0H_{24} . Six channels exist through the protein shell into the interior of the molecule (Banyard et al 1978); these are believed to be large enough to allow the passage of small ions or molecules e.g. Fe^{2+} aq., $Fe(OH)_2$ aq., O_2 ; IO_3 etc. which can diffuse into the inner cavity. Large polymeric Fe^{III} species would not however be able to penetrate. The iron core consists of an inorganic iron polymer or micelle, predominantly $FeO-OH$, but also with some 1-1.5% phosphate, with the formula $(Fe_0\theta\theta H)_8 (FeO:OPO_3H_2)$ and a radius of approximately 35A. The structure of the iron core is suggested (Towe and Bradley 1967) to consist of Fe^{III} atoms arranged in octahedral positions between hexagonally packed oxygen layers. This model has received support from electronic absorption spectra

and extended X-ray absorption fine structure, EXAFS (Webb and Gray 1974).

It has long been known that deposition of iron into ferritin requires the metal in the FeII form and that, once deposited, this iron exerts a catalytic effect, inducing more and more deposition. A number of theories have been proposed to account for the deposition of iron into ferritin: firstly, a model based on the chemistry of ferric chelates and the tendency of iron to polymerise giving water and soluble micellar cores (Spiro and Saltman 1969). The theory (Pape et al 1968) proposed that the ferric oxyhydroxide core is first formed with the protein subunits subsequently forming a shell around it. This theory has received little support, particularly in view of the demonstration (Niederer 1970) that iron is taken up gradually, as shown by the increasing density of ferritin samples over time, demonstrated by isopycnic centrifugation of ferritin samples over time. The more accepted theory involves the penetration of FeII through the channels in the shell, followed by oxidation on the inner surface of the protein. It is presumed that some coordination sites are first occupied by water, protons are lost and linkage of further FeIII occurs through oxo and hydroxy bridges. This is the stage where a catalytic effect occurs: it has been observed (Macara et al 1972, 1973) that iron taken up by ferritin exerts a "catalytic" effect, resulting in the deposition of more iron. The polymer formed soon becomes too large to escape the shell (Clegg et al 1980). A modification of this theory involves the presence of active sites on the apoferritin shell which catalyse the oxidation of FeII (Crichton et al 1977) although other authors have thought this unlikely (Macara et al 1972, 1973).

The kinetics of formation of the iron core are consistent with those of crystal growth, following a sigmoidal curve. This has led

to the proposal of the "crystal growth model" (Macara et al 1972) in which the initial nucleation step is followed by a more rapid growth stage, increasing as the surface area available for crystallisation increases, which would account for the catalytic effect.

It has long been known that administration of iron leads to an increase in the ferritin concentration of the tissue concerned. It was noted by Granick (1946) for instance, that oral doses of iron lead to increased amounts of ferritin present in the duodenal mucosa of guinea pigs; he put forward the hypothesis that the iron causes an apparent induction of ferritin by stabilising a previously unstable precursor (apoferritin). There is evidence that iron-rich ferritin is more resistant to enzymic and lysosomal (Crichton 1971) degradation than the iron-free form. Fineberg and Greenberg (1955) however, showed that iron stimulates de novo synthesis of ferritin, with apoferritin as the initial product. This was confirmed by Drysdale and Munro (1966) who, using density gradient ultracentrifugation, found that the apoferritin fraction was more active soon after administration of ^{14}C -glycine. The iron-induction of ferritin synthesis has been confirmed in numerous tissues, e.g. rat liver (Coleman and Matrone 1969), spleen, kidney, duodenum and testis (Yoshino et al 1968).

Actinomycin-D does not affect the stimulation of ferritin synthesis (Drysdale and Munro 1966; Zahringer et al 1976). Iron administration does however increase the amount of ferritin mRNA engaged in protein synthesis (Zahringer et al 1975). Pretreatment of animals with cordycepin, an inhibitor of polyadenylic acid 3'-tail formation on addition to mRNA before transport from the nucleus to cytoplasm, failed to reduce the increase in ferritin mRNA induced by iron administration (Zahringer et al 1976), implying a cytoplasmic source for the latter. It was suggested

that in the tissues of animals not receiving excess iron, a ferritin subunit is attached to the initiation site of ferritin mRNA preventing the latter from binding to the ribosomes. The iron entering the cell would then shift the equilibrium towards assembly of completed ferritin shells, so that the inhibiting subunits are mobilised and more mRNA becomes available.

Ferritin has been demonstrated in the intestinal mucosa (e.g. Granick 1946; Smith et al 1968) and has been postulated as the factor regulating iron absorption: the so-called "mucosal block" theory (see above).

There is thus a vast literature on iron metabolism, of which this review has attempted to give only a brief outline, and only in relation to the topic under investigation, iron absorption. Much of the literature on absorption is itself confusing: there are data supporting both active and passive transport as the mechanism involved. It would appear from consideration of the available evidence that both may have a role to play, perhaps depending on the dose of iron presented to the cells. Iron-binding proteins have been identified in almost all the cellular fractionation studies: consensus of opinion is that these are transferrin, or a transferrin-like protein, and ferritin. Other proteins of both higher and lower molecular weight have also been found.

Absorption has been shown to be dependent on protein synthesis, suggesting a protein carrier, although there is the possibility that this could represent an indirect event not on the actual transport pathway. Iron has been found in association with the endoplasmic reticulum and the mitochondria: the role of these in absorption is unknown.

This study, therefore, is an investigation of the iron absorption process, into the possible mechanism involved, and, in particular, into the iron-binding proteins of the mucosa. There is little

quantitative evidence on the last-named topic and this study will attempt to quantitate the proteins and explore their role in iron absorption.

4. METHODS.

4.1. Animals.

Adult (>300g) male Long-Evans rats were used throughout. Animals were fed on a standard rat diet (Epol, Johannesburg) containing 1µg/g of iron as ferrous sulphate. Iron stores were reduced and erythropoiesis stimulated by venesection of 20 ml of blood over a two week period, or increased by intramuscular injections of 100mg iron as iron dextran (Imferon, Fisons Pharmaceuticals) two weeks prior to study. Haemoglobin was measured by the cyanmethaemoglobin method (International Committee for Standardisation in Haematology, 1971), reticulocytes by the method of Hillman and Finch (1969) and splenic non-haem storage iron by the method of Torrance and Bothwell (1968).

4.2. Absorption in Vivo from Isolated Loops.

Rats were anaesthetised with pentobarbital sodium (Sagatal, Maybaker, S.A.), the abdomen opened and loops, 5 or 10 cm in length, ligated between ligatures in the small intestine. Care was taken not to damage the blood supply. 0.3 ml of a radioiron solution containing 0.15M NaCl, 50µM (3µg)^{ml} iron + ⁵⁹Fe (0.75 µCi; Amersham International Limited) and 0.4mM ascorbic acid, pH 4.2, was injected into the loop and absorption allowed to proceed for one half or one hour. Data on the viability of such loops and reversibility to normal function after removing ligatures, is given in Appendix 1. Blood samples were taken from the tail vein at 5 minute intervals and aliquots of these counted for ⁵⁹Fe activity. Thereafter, the loop was removed, washed with 40 ml cold saline and counted for ⁵⁹Fe in a gamma counter (Beckman

Instruments). The remaining carcass was counted in a small animal whole body counter, both against standards contained in a similar volume (see Counting of Radioactivity).

To determine the effects of increasing iron dose on absorption, iron doses of 50 μ M (3 μ g), 500 μ M (30 μ g) and 5mM (300 μ g) iron as ferrous ascorbate were used.

The kinetics of iron loading by iron-binding components of the mucosa were investigated by pulse-chase studies using either "hot" or "cold" chaser solutions. The iron solution labelled with ^{59}Fe , was incubated in the loop for 5 minutes, followed by either "cold" chase of 50 μ M cold ferrous ascorbate or "hot" chase of 50 μ M iron + ^{55}Fe (1 μ Ci ^{55}Fe ; Amersham International Limited) which was left for 5 - 30 seconds before removal and washing as above.

4.3. Cell Isolation.

Cells from 5 cm segments were removed by one of two techniques: firstly by vibration of everted loops (Savin and Cook 1978) on a glass rod at 50Hz for 10 minutes; the cells, resuspended to a final volume of 1 ml, gave, on average, cell counts of 5.5×10^6 cells/ml measured in a haemocytometer, and 5.81 ± 0.90 mg protein as determined by the biuret method (n=5). Secondly, the loop was opened, the cells gently scraped off the underlying lamina propria with a glass slide and resuspended to a final volume of 1 ml. This yielded an average count of 12.3×10^6 cells/ml and 15.51 ± 4.42 mg protein (n=20). For both techniques, Trypan Blue exclusion confirmed >95% viability.

4.4. Iron Uptake by Isolated Cells.

Based on the method of Savin and Cook (1978), the radioiron

solution was added to 200 μl of a cell suspension containing 3×10^3 cells/ μl and incubated at 37°C with shaking. Aliquots were removed at 5 minute intervals, pipetted onto a filter (pore size: 0.45μ), washed with 40 ml cold saline and the activity on the filters counted. In control experiments, cells were incubated at 4°C or preincubated with 1 mM potassium cyanide or 1 mM sodium arsenate for 10 minutes before washing and being added to the iron-containing solution. The effects of ethylene diamino-tetra-acetic acid (EDTA) on iron uptake were tested firstly by vibration of cells into buffer containing 5 mM EDTA, followed by washing in two changes of buffer, and secondly, after incubation with iron-containing solution, cells were washed with saline containing the same concentration of the chelate. To investigate the effect of variations in iron concentration and incubation time on uptake, three experiments were performed: firstly, the concentration of iron in the uptake medium was increased from the standard $50\mu\text{M}$ ($3\ \mu\text{g}$)^{1.5} to $100\mu\text{M}$ ($6\ \mu\text{g}$)^{1.5} and $150\mu\text{M}$ ($9\ \mu\text{g}$)^{1.5} iron. Secondly, cells were incubated with $50\ \mu\text{M}$ iron for 10 minutes after which the iron concentration was increased to $75\ \mu\text{M}$ ($4.5\ \mu\text{g}$) with cold ferrous ascorbate; thirdly, cells were incubated with $25\ \mu\text{M}$ ($1.5\ \mu\text{g}$) iron as cold ferrous ascorbate for 10 minutes, followed by addition of the further $1.5\ \mu\text{g}$ (to $50\ \mu\text{M}$ iron) in the form of ^{59}Fe ferrous ascorbate.

4.5. Density Gradient Fractionation of Cell Homogenate

Washed cells were suspended to final volume of 1 ml in 0.15M saline containing proteolytic inhibitors (5 mM ϵ -amino caproic acid, $20\ \mu\text{M}$ phenylmethylsulphonylfluoride (PMSF) and 100 i.u. Trasylol) and homogenised with 30 strokes of a Dounce homogeniser. 10-50% sucrose density gradients of 17.5 ml in MES buffer were used (0.1M 2-(N-morpholino) ethane sulphonic acid (MES); 1mM

ethyleneglycol-bis (B-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA, Sigma), 0.02% NaN₃, pH 6.5) (Pearse 1975). 0.8 ml cell homogenate was applied to each and centrifuged for 16 hours at 52000g in a swinging bucket rotor (SW 27.1, Beckman L2-65B ultracentrifuge). Fractionation was by upward displacement of the gradient on a density gradient fractionator (Instrumentation Specialties Company, Nebraska). Data on the calibration of the gradients is shown in Figure 4. The collected fractions were counted for ⁵⁹Fe and assayed for transferrin and ferritin. 10 mM ferrous sulphate was added to cells during homogenisation when required. The gradient densities established after centrifugation were measured by densitometry and ranged from 1.04 to 1.20 g/ml in the first and last of 30 fractions. Mitochondria have reported densities of 1.29 g/ml (Beaufray et al 1964) and 1.15-1.20 g/ml (Hopkins and Peters 1979). Assay for the marker enzyme creatine kinase found on the mitochondrial inner membrane on a Centrifuchem System 400 centrifugal analyser (Roche) indicated the presence of mitochondria at 1.18 g/ml density (fractions 27-28).

To determine whether handover of iron occurred between the different iron-binding components separated on the density gradient, 0.2 ml from one radiolabelled peak was added to the same volume from under another, unlabelled, peak, incubated at 37°C for 1 hour with shaking, the density equilibrated with buffer to a final volume of 0.8 ml and applied to the gradient before centrifugation as above.

4.6. Immunoabsorption.

Antisera produced in rabbits against transferrin and ferritin were dialysed overnight at 4°C against 0.05M Tris/HCl, pH 7.4, and the IgG separated by DE-52 (Whatman) chromatography. The IgG was covalently coupled to cyanogen bromide-activated

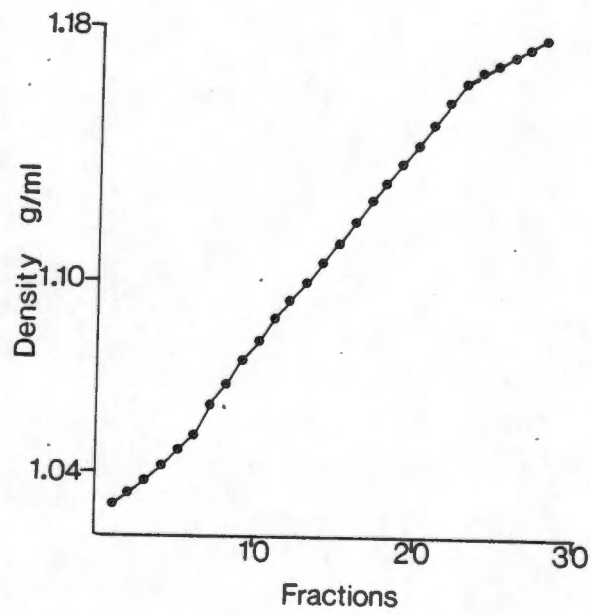


Figure 4. Calibration of sucrose density gradients after centrifugation. Measurement was determined by direct densitometry of each fraction. Each fraction is equal to 0.6ml; the total volume of the gradient is approximately 17.5 ml.

Sepharose 4B (Pharmacia). The transferrin and ferritin antigen-binding capacity was estimated for each column using varying concentrations of labelled transferrin and ferritin with unconjugated Sepharose 4B as a control. The capacity of the anti-transferrin column was 16 μ g transferrin/ml and that of the anti-ferritin column 1.5 μ g ferritin/ml. Cell homogenates, clarified by centrifugation at 1000g for 10 minutes, were incubated with 1 ml immunoabsorbent with constant mixing at room temperature for 4 hours and eluted through a further 1 ml of immunoabsorbent with 0.15M NaCl. The eluates were fractionated on sucrose density gradients and the fractions assayed for transferrin and ferritin.

4.7. Gel Filtration.

A 100 x 1cm column of Ultrogel AcA-34 (LKB) was calibrated with gel filtration molecular weight markers (Pharmacia). 0.3 ml Triton-treated sample was applied and eluted with MES buffer. 2 ml fractions were collected and counted for ^{59}Fe and assayed for transferrin and ferritin.

4.8. SDS-Polyacrylamide Gel Electrophoresis.

1x150x150mm polyacrylamide gels containing 1% sodium dodecyl sulphate (SDS) were run at 50V for 16 hours in a Hoechst Model SP1000 electrophoresis apparatus. The acrylamide gradient ranged from either 5-20% or 6-8%. Gels were stained with Coomassie Blue (Laemmli 1970).

4.9. Metabolic Inhibitors.

A solution of 5mM cytochalasin-B (Sigma), initially dissolved in dimethylsulphoxide (B.D.H.) was incubated in the intestinal loop

for 15 minutes (Carter 1967) before washing with 10ml saline and injection of the iron- ascorbate solution. To investigate the dose-response effects behaviour of the substance, concentrations of 100µM, 1mM and 10mM were used. Incubation of the iron-ascorbate solution was allowed to proceed for 5 minutes; in order to test the recovery time, incubation times of 5, 10, 15 and 20 minutes were used. Blood samples were taken during this time, and ⁵⁹Fe activity in 100µl aliquots of these counted to determine whether absorption was occurring.

Similarly, 10mM colchicine (Sigma) was incubated in the loop for 2 hours (Taylor 1965); doses tested were 100µM, 1mM and 10mM. The effects of vincristine (Petersen Ltd., South Africa) at normal dose of 0.1mg/ml (Hemmaplardh et al 1974) and test doses of 1µg/ml, 10µg/ml and 100µg/ml were also investigated. Incubation time in this case was 15 minutes. Similarly, 1mM KCN was incubated in the loop for 15 minutes.

4.10. Radioimmunoassay of Transferrin.

Rat serum transferrin was prepared by the method of Sutton and Karp (1968) with the additional step of passage through a 1x5 cm column of Blue Sepharose (Pharmacia) to remove contaminating albumin. Purity was tested by homogeneity on polyacrylamide gel electrophoresis.

The antisera used were either commercially produced rabbit antirat transferrin (Cappel Laboratories) or antisera raised in rabbits with initial injection of 0.1ml solution containing 1µg protein in Freund's Complete Adjuvant into the retrocruar lymph node followed by weekly intramuscular injections of the same. The rabbits were bled after 4 weeks and the presence of antibodies tested by immunoelectrophoresis (Graber and Burtin 1964) and

precipitation on Ouchterlony plates (Ouchterlony 1962) in 1% agar gel with Beckman (Beckman Instruments) buffer. The immunoprecipitates were stained with amido black and destained in 7% acetic acid. Radioautographs (X-Omat, Kodak) were taken to determine the position of radiolabelled transferrin, if such was used. Iodination was performed by the Chloramine-T method of Greenwood et al (1963). The optimum concentrations of both Chloramine-T and transferrin were established in advance and the latter was standardised as 10 μ g transferrin. The excess unreacted ¹²⁵I was separated from the labelled protein by Sephadex G-25 chromatography using a 25 x 1 cm column eluted with phosphate buffer (5mM NaH₂PO₄; 0.01M Na₂HPO₄, pH 6.8).

Samples were assayed in duplicate together with a doubling dilution series of 14 standards of purified serum transferrin and tubes (4 of each) containing the total amount of radiolabelled protein added to each sample, the radiolabel with addition of antiserum, to give the uninhibited upper limit of the assay and radiolabel with no antibody added, for the non-specific binding. All these tubes were incubated in the same manner as the standards and samples. Samples were incubated in plastic test tubes (Falcon, Oxnard, U.S.A.) in a reaction volume of 400 μ l; bovine serum albumin buffer (0.04M NaH₂PO₄; 0.15M NaCl; 0.1% NaN₃; 0.01M EDTA and 0.5% BSA pH 7.4) was used as a carrier. The antibody titre was determined so as to give 35% binding of tracer to antiserum in the absence of unlabelled antigen. This was established by the incubation of serial dilutions of antiserum with constant amounts of ¹²⁵I-transferrin according to the standard assay protocol. The optimum titre for the antiserum from rabbit number 311 was found to be 1/10000 and that for the commercially produced antiserum 1/200000 (Figure 5). Tubes were incubated at room temperature overnight. Separation of

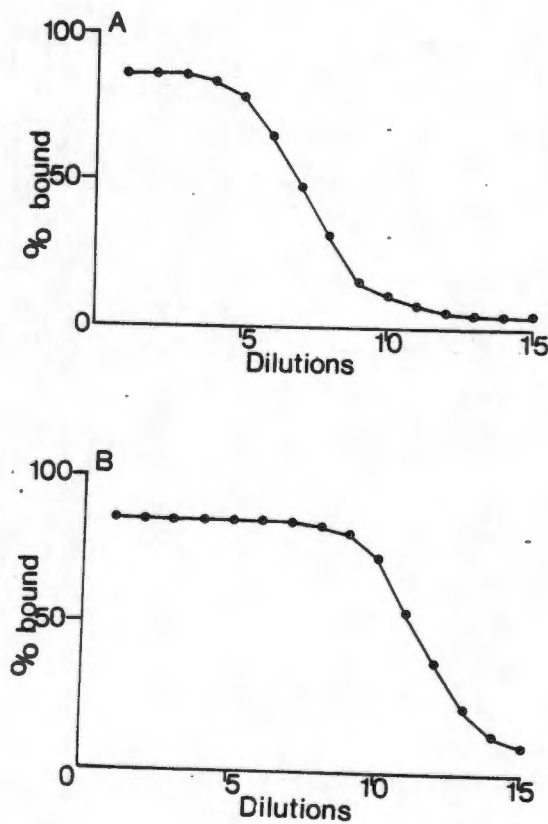


Figure 5. Antibody dilution curves of transferrin antiserum 311 and commercially-produced antiserum (Cappel Laboratories). 15 doubling dilutions of antisera were incubated with radiolabelled transferrin and BSA buffer according to the standard assay procedure. The antibody dilution giving approximately 35 % precipitation was used in subsequent assays. A, Antiserum 311. The starting dilution for this antiserum was 1/100 and the antiserum dilution used for subsequent assays was 1/1000000; B, Commercially-produced antiserum. The starting dilution was 1/100 and the dilution used in subsequent assays was 1/2000000.

the bound and free tracer was achieved by addition of second antibody system: 100µl of normal rabbit serum followed by 100µl of donkey anti-rabbit serum, on the second day. Precipitates were washed the following day with 1ml BSA buffer and counted for ¹²⁵I activity.

Results were calculated by an adaptation of the Fortran programme of Rodbard and Lewald (1970) run on a Univac 1106 computer. This programme fits the standard curve of the assay to a linear function from which the values or "potency estimates" of unknowns can be calculated. This linearisation of the standard curve is performed using the logit-log relationship:

$$\text{logit } (Y) = a + b \log_e (X)$$

where: Y is B1/B0, the ratio of bound counts to counts bound in the absence of unlabelled antigen, with both values corrected for non-specific binding counts;

$$\text{logit } (Y) \text{ is } \log_e (Y/(1 - Y));$$

X is the dose of unlabelled antigen;

and a and b are the intercept and the slope, respectively, of the linearised curve.

The derivation of antiserum affinity data by means of the Scatchard plot analysis, the derivation of part of the data relevant to proficiency testing and quality control of the assay were also performed by the programme.

To investigate immunological differences between rat transferrins from different tissues, 5 duplicate doubling dilutions of purified serum transferrin, rat serum, rat mucosa, including whole homogenate, as well as Peaks 1 and 3 from density gradient fractionation were assayed in the presence of proteolytic inhibitors (see above), 2% Triton-X100 and BSA buffer. The effects of other buffers, notably MES and saline, as well as the presence

of sucrose from the density gradients, were also investigated. No effects of these variables were found. To investigate immunological differences between diferric, monoferric and apotransferrin, these species were assayed, in 5 duplicate doubling dilutions, in BSA buffer without EDTA. $0.02M$ FeIII-nitrilotriacetic acid (FeIII-NTA) was prepared by the method of Woodworth (1966). It was calculated that 1ml of FeIII-NTA contained $110\mu g$ of iron. 1mg in 1ml of serum transferrin was used. Apotransferrin was prepared by dialysis of transferrin for 20 hours at $4^{\circ}C$ against 4 litres of $0.05M$ EDTA, $0.1M$ NaH_2PO_4 , pH 5.5, followed by dialysis for 24 hours at $4^{\circ}C$ against 4 litres of distilled water, followed by dialysis for 24 hours against 4 changes of $10mM$ Tris, $150mM$ NaCl, pH 8.5. FeIII-NTA was added, 1ul at a time, to 1ml of 1mg/ml transferrin solution. Optical densities 200-500nm were scanned in a Unicam SP1800 Ultraviolet Spectrophotometer. Aliquots of each solution after addition were retained for parallel line radioimmunoassay.

The computer programme used to analyse this data was a parallel line affinity analysis adaptation of the Rodbard and Lewald (1970) programme.

4.11. Radioimmunoassay of Ferritin.

Rat liver ferritin was purified by the method of Penders et al (1971). Purity was confirmed by homogeneity on polyacrylamide gels.

Antiserum was raised in rabbits in the same manner as for transferrin. The presence of antiserum was tested by immunoelectrophoresis and precipitation on Ouchterlony plates. 5 μg ferritin were used for iodination, otherwise the assay and computer calculations were performed in exactly the same manner as

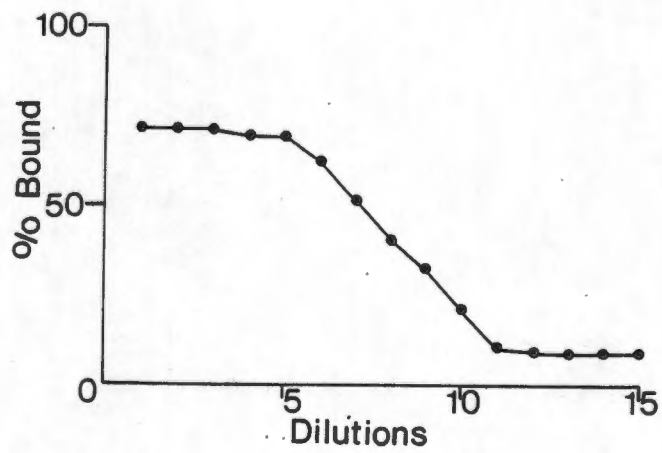


Figure 6. Antibody dilution curve for ferritin antiserum. The method was the same as that used for transferrin antibody dilution. The starting dilution for this antiserum was 1/50 and the dilution used in subsequent assays, 1/1500.

for transferrin. The antibody dilution curve of the ferritin antiserum is shown in Figure 6.

4.12. Protein Concentrations.

Protein concentrations were determined by the biuret method or by the Bio-Rad Protein Estimation Kit (Bio-Rad Laboratories).

4.13. Counting of Radioactivity.

^{125}I and ^{59}Fe were counted in a Beckman Gamma 310 system. The efficiency for both isotopes was about 75%. The window setting used for ^{125}I was 50-250 and that for ^{59}Fe , 400-600. Samples were counted for 1 minute or a preset error of 5%. The counter was equipped with a teletype and paper tape puncher; the paper tape was used for input of data to the computer. ^{55}Fe was counted in a Beckman liquid scintillation system LS233 where the efficiency of counting was established at 34%. The window used was 50-125. It is thus apparent that ^{59}Fe and ^{55}Fe can be counted simultaneously. An efficiency of 68% was calculated for ^{59}Fe on the liquid scintillation system. Samples, in 5ml Instagel (Packard) were counted for 10 minutes or to a preset error of 5%. The cross-counting between the two isotopes was $21.25 \pm 1.63\%$ for ^{59}Fe in the ^{55}Fe setting and 0% in the reverse situation.

4.14. Absorption from Iron Polymaltose.

Iron polymaltose, an iron-carbohydrate complex, molecular weight 100000, was radiolabelled with ^{59}Fe by the method of Mueller et al (1973). It has been shown previously (Jacobs et al 1979) that absorption of iron from this complex is comparable to that from iron salts. This was confirmed by comparing absorption from

differently labelled salt and polymaltose (see Appendix 2)

Iron polymaltose, containing 3µg of iron as ^{59}Fe , was administered in isolated loops in the same manner as for ferrous ascorbate.

Data on iron polymaltose, including its handover of iron to transferrin, ferritin and iron chelates, is given in Appendix 2.

4.15. Serum Contamination of Mucosal Extracts.

Following the iodination procedure, rat serum transferrin was biologically screened to remove free ^{125}I and damaged molecules by injection into a rat and harvesting the serum after 24 hours.

This whole serum was intravenously injected into test animals. Blood samples were taken to follow the protein equilibration and distribution (Figure 7). After 4 hours when the slow clearance phase, denoting equilibration with the tissues had been reached, the rat was sacrificed and cells harvested from a series of 5cm intestinal segments, counted for ^{125}I and assayed for transferrin by radioimmunoassay. By assuming that equilibration has been attained, the amount of transferrin in the mucosa may be expressed as equivalent to a volume of serum (the "serum-equivalent volume"). As control, ^{125}I -alpha-fetoprotein was used in the same manner.

To determine whether transferrin adhering to the outer surface of cells can be removed, cells were digested with 0.25% trypsin in Hanks Balanced Salt Solution for 1 hour at 37°C. The action of the enzyme was stopped with 200 i.u. Trasylol. The transferrin concentration in cells before and after digestion was measured by radioimmunoassay. As controls, 50µg pure transferrin or 50µg transferrin added to cells from a single segment were digested under similar conditions.

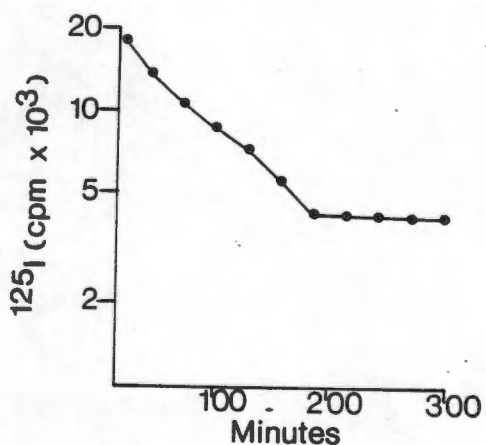


Figure 7. Biological screening of transferrin. Iodinated transferrin was injected intravenously into a rat. After 24 hours the rat was bled by cardiac puncture and 1ml of this serum injected intravenously into a second rat. Blood samples were taken at half-hourly intervals from the tail vein and 100ul aliquots of these were counted for ^{125}I activity.

4.16. Isoelectrofocusing.

A preparative isoelectrofocusing column of 220ml volume was filled with a 5-50% sucrose (w/v) in water density gradient containing 2% ampholytes (Ampholine, Pharmacia), pH range 3.5-9.5. The density gradient rested on a cushion of 55% sucrose in 1M phosphoric acid, and a 1M ethanolamine solution was layered on the top of the gradient. A small portion of the gradient at the estimated density of the sample was removed and the sample inserted. The column was run at a constant power of 1000 watts (Hoeffer Model PS1200 power supply; Hoeffer Scientific Instruments, San Francisco), at 4°C for 20 hours. The column was eluted under gravity; 2ml fractions collected and their pH, ⁵⁹Fe activity and transferrin and ferritin concentrations measured.

4.17. Purification of Mucosal Transferrin.

The small intestines from rats were removed, washed with 0.15M NaCl and 5mM PMSF, pH 7.4 and quick frozen in liquid nitrogen. Intestines were homogenised in a Waring Blender without thawing and extracted with approximately 2l of the above buffer. The first extract was centrifuged at 13000rpm (26000g) for 2 hours (JA-20 refrigerated centrifuge; Beckman Instruments) and the precipitate treated with 1% Triton-X100. The second extract, — obtained from washing the precipitate prior to Triton extraction, was spun at 13000g for 2 hours. Rivanol (6,9 diamino-2-ethoxyacridine lactate; Sigma) was added to the supernatants and Triton-extracted precipitate to a final concentration of 0.31% (w/v), with constant stirring at 4°C. After standing for 2 hours, the solution was filtered through Whatman No 1 filter paper and recentrifuged. The precipitate was discarded. The supernatant was treated with hydrolysed starch and washed in a sintered glass funnel to remove

the rivanol. Any excess rivanol was removed by passage through Sephadex G25 (Pharmacia). To the salmon-pink eluate was added 33g/100ml of ammonium sulphate, allowed to stand overnight then centrifuged at 26000g for 2 hours. To the supernatant a further 11g/100ml ammonium sulphate was added and the precipitate collected. The precipitate was washed twice with 44% ammonium sulphate. The transferrin in the final solution was stabilised by addition of 0.2mM FeCl₃, together with 0.5mM NaHCO₃ and 0.5mM sodium citrate.

The transferrin-enriched extracts were further purified by gel filtration on Biogel P100 (Bio-Rad Laboratories) column (100x1cm) eluted with 5mM Tris pH 7.4; column isoelectricfocusing, as described above, and ion-exchange chromatography on DE-52 (Whatman) resin eluted with a linear gradient of 0-100mM NaCl in 5mM Tris, pH 8.0 buffer. The transferrin-containing fractions were concentrated in polyethylene glycol and dialysed against the starting buffer prior to application to the ion-exchanger.

5. RESULTS.

5.1. Absorption in Vivo from Isolated Loops.

Washed loops from all regions of the small intestine showed almost identical uptake of iron at the 3 μ g dose (21.06 \pm 1.68%; n=22) for 10cm loops. Absorption, whether measured by the appearance of radioiron in venous blood or in the carcass, showed a steep gradient over the upper 10cm of the intestine: at the 50 μ M (3 μ g) dose of iron carcass uptake ranged from 27.40 \pm 3.29% (0.822 μ g) in the duodenum to 0.48 \pm 0.18% (0.014 μ g) in the ileum (Figure 8 and Table 4). In iron deficient animals, duodenal absorption increased to 45.30 \pm 13.19% (1.359 μ g, n=10) and in iron loaded animals, decreased to ^{2.2}13.20 \pm 0.89% (0.066 μ g, n=10) (Table 4). Similarly, when the amount of iron presented to the lumen increased, percentage absorption decreased, but the absolute amount of iron transferred to the carcass rose: 0.82 \pm 0.20 μ g (27.4%) iron for a 50 μ M (3 μ g) dose of iron, n=10; 9.75 μ g (32.5%) for a 500 μ M (830 μ g) dose of iron, n=5; and 61.35 μ g (20.4%) for a 5mM (3000 μ g) dose of iron, n=3. Exposure of the gut segment to iron for longer periods of time did not result in significantly increased absorption: absorption of 31.59 \pm 2.57% (0.948 μ g; n=2) was found after 4 hours for a duodenal segment, and 1.03 \pm 0.25% (0.031 μ g, n=3) for an ileal segment. Based on the amount of iron consumed by each rat in a 24 hour period, all subsequent studies were carried out in the physiological dose range of 50 μ M (3 μ g) of iron as radiolabelled ferrous ascorbate.

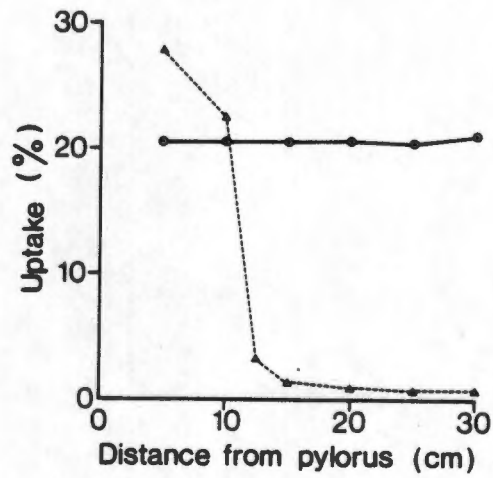


Figure 8. Percentage uptake of iron ($3\mu\text{g}$, $50\mu\text{M}$ dose) by washed loops (●—●) and carcass (▲---▲) from isolated 10cm intestinal loops at different distances from the pylorus.

Table 4. Carcass absorption of 50 μ M (3 μ g) dose of iron by normal, iron deficient and iron loaded rats, showing the haematological measurements of haemoglobin (column 3), reticulocyte count (column 4) and splenic non-haem storage iron (column 5), expressed as μ g iron per gram wet weight of tissue.

Animals	Number	Hb (g/dl)	Retic. (%)	Storage Fe (μ g/g)	Absorption (%)
Normal	17	13.26 \pm 1.32	3.30 \pm 0.43	2442.0 \pm 103.7	27.4 0.822 \pm 3.29
Iron deficient	20	8.05 \pm 1.55	21.10 \pm 4.36	1050.1 \pm 211.3	45.3 1.359 \pm 13.19
Iron loaded	20	13.73 \pm 1.38	2.80 \pm 0.31	3430.4 \pm 422.1	2.2 0.066 \pm 0.89

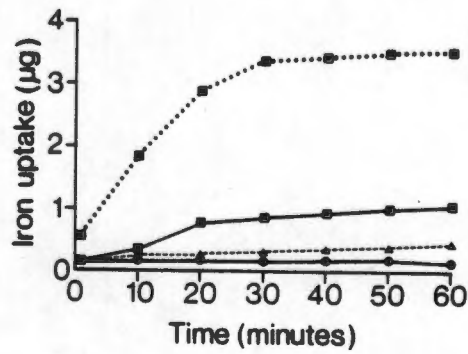


Figure 9. Uptake of iron by isolated duodenal mucosal cells in vitro. A dose of 3µg (50µM) iron was used in all cases except for (■.....■) in which 9µg (150µM) was used. Cells were preincubated with 1mM KCN for 10 minutes before washing and incubation with ferrous ascorbate (▲---▲); cells were incubated at 37°C (■—■) except for (●—●) in which the temperature was 4°C.

5.2. Iron Uptake by Isolated Cells.

There was an instantaneous uptake of approximately 0.3 μ g (10%) iron in all cases in the 3 μ g dose range (Figure 9). It is possible that this may represent non-specific binding to basolateral and plasma cell membranes. The isolated cells in suspension initially took up iron rapidly but ceased absorbing after 30 minutes. That this uptake represented specific binding to or uptake into the cell was shown by the inability to remove any radiolabel by washing with 1mM EDTA. Furthermore, the control experiments of applying the radioiron solution alone, without the presence of cells, to the filters, followed by washing, did not result in adherence of iron to the filters.

Uptake beyond the 10% point was inhibited at 4°C and partially by prior exposure of the cells to cyanide and arsenate (Figure 9). If the poisons were added to the incubation medium, uptake of iron ceased. Very similar results were obtained by either preincubation with or addition of the iron chelator EDTA. Increased concentrations of iron (100 and 150 μ M) in the incubation medium resulted in increased uptake of iron in linear fashion (Figure 9). Similarly, addition of iron to cells during incubation resulted in increased uptake of the metal. Evidence that uptake *in vitro* by isolated cells was not an artefact was provided by the fact that, on fractionation of the cells, uptake of iron by mucosal ferritin was observed to have occurred. This indicates that uptake into cells was occurring (see below) and that the uptake observed was not a non-physiological artefact.

5.3. Fractionation of Cell Homogenates.

Three iron-containing peaks were found (Figure 10):

1. at density 1.050-1.068g/ml a peak was present equally throughout the small intestine;
2. at density 1.080-1.110g/ml there was a peak maximal in the duodenum, diminishing in size in the jejunum and absent in the distal ileum; this peak was less prominent in *in vitro* labelled cells;
3. at density 1.155-1.180g/ml there was a peak present throughout the intestine.

Recentrifugation of fractions from peaks 1 and 2 indicated that they retain their position; peak 3 did likewise except on addition of Triton X-100, when the activity moved quantitatively to the peak 1 position. Mixtures of fractions under the various peaks indicated that peak 1 hands over iron to peak 2, but not to peak 3, peak 2 appeared to hand over a small amount of label to peak 1, but again none to peak 3, while the latter was capable of yielding small quantities of iron to both peaks 1 and 2 (Figure 11).

Addition of 10 mM cold ferrous ascorbate to the homogenate did not affect the positions of peaks 1 and 3, but shifted the radiolabelled peak 2 at density 1.080-1.110g/ml to density of approximately 1.095-1.110g/ml without displacing the radiolabel (Figure 12). The ferritin antigen shifted together with the radiolabel.

Time studies, using varying lengths of exposure time (30 seconds to 1 hour) of the gut to the radioiron solution, showed maximal activity retained by peaks 1 and 2 throughout the time periods studied; the activity of peak 2, however, increased over time (Figure 13).

Pulse/chase experiments using differently labelled radioiron solutions, gave no conclusive results. No high-flux component was identified.

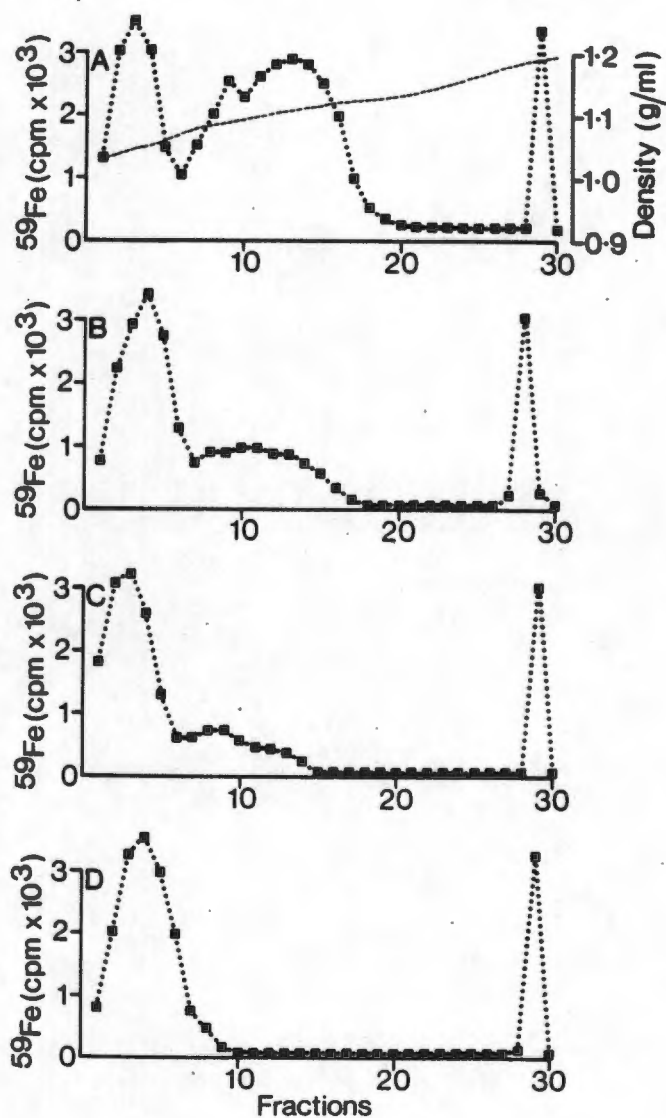


Figure 10. Density gradient fractionation of mucosal cell homogenates from A, duodenum; B, proximal jejunum, 15cm from the pylorus; C, jejunum 15cm from the pylorus; and D, ileum. The sucrose density gradient was determined by direct densitometry on each fraction (----). Similar gradients were used in all the other Figures. Counts per minute ^{59}Fe (■.....■).

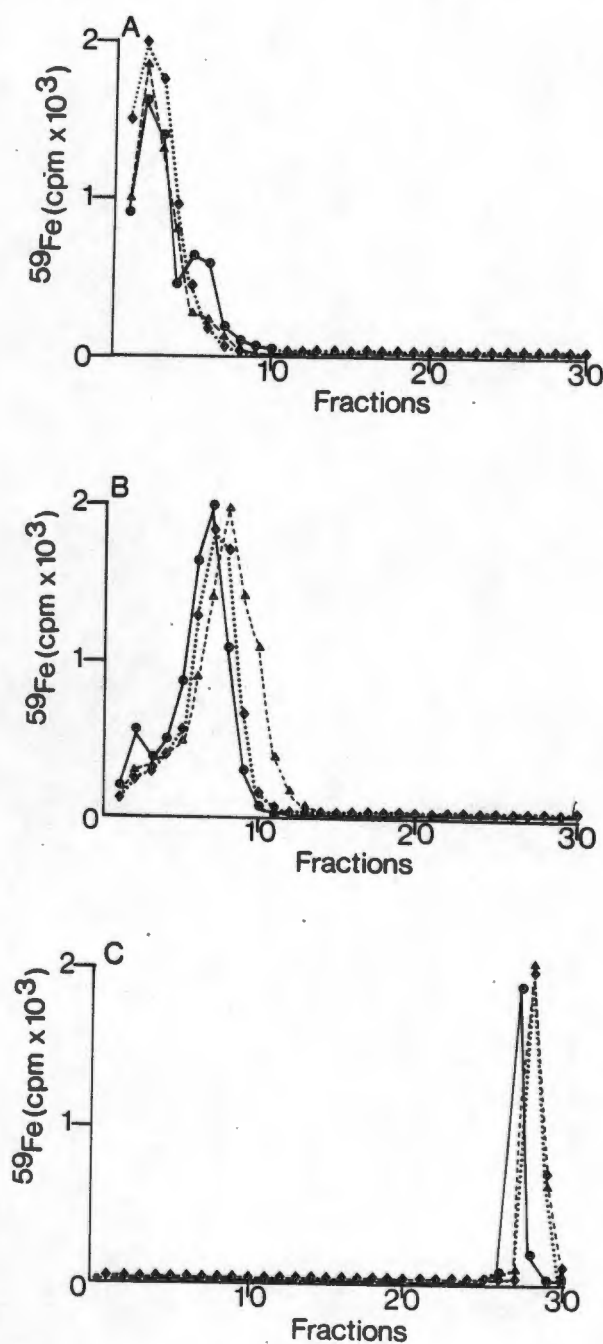


Figure 11. Density gradient re-centrifugation of density gradient peaks. Both *in vivo*-labelled and unlabelled mucosal cells were fractionated on 10-50% sucrose gradients; fractions from under the peaks were mixed, as shown in the graphs, incubated at 37°C for 30 minutes and re-centrifuged on 10-50% gradients.

- A, Peak 1 plus: Peak 1 only (\blacklozenge \blacklozenge); Peak 1, labelled and unlabelled Peak 2 (\bullet — \bullet); Peak 1, labelled and unlabelled Peak 3 (\blacktriangle --- \blacktriangle).
- B, Peak 2 plus: Peak 2 only (\blacklozenge \blacklozenge); Peak 2, labelled and unlabelled Peak 1 (\bullet — \bullet); Peak 2, labelled, and unlabelled Peak 3 (\blacktriangle --- \blacktriangle).
- C, Peak 3 plus: Peak 3 only (\blacklozenge \blacklozenge); Peak 3, labelled and unlabelled Peak 1 (\bullet — \bullet); Peak 3, labelled and unlabelled Peak 2 (\blacktriangle --- \blacktriangle). The density gradients are the same as in Figure 10.

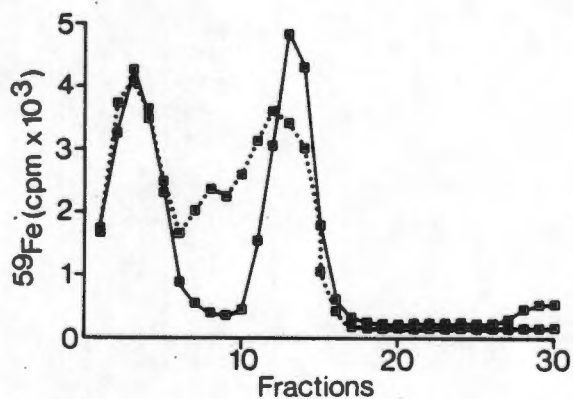


Figure 12. Density gradient fractionation of ^{59}Fe loaded duodenal cells homogenised in saline (■.....■) and homogenised with 10mM ferrous ascorbate (■——■). The density gradients are the same as in Figure 10.

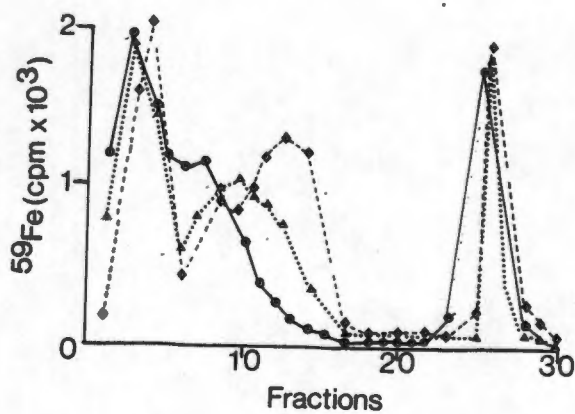


Figure 13. Labelling of mucosal components with ^{59}Fe as a function of time. Radiolabelled ferrous ascorbate ($3\mu\text{g}$ or $50\mu\text{M}$ iron) was incubated in isolated loops for 2 (●—●), 10 (▲...▲) and 120 (◆--◆) seconds. The gradient is the same as in Figure 10.

5.4. Identification of Iron-binding Fractions.

5.4.1. Rats on a Normal Iron Diet.

Transferrin was found in peaks 1 and 3 (Figure 14). The transferrin content of peak 3 was $9.91 \pm 2.01\%$ ($n = 6$) that of peak 1 in the duodenum while in the ileum the transferrin content of peak 3 in relation to peak 1 was $2.88 \pm 0.69\%$ ($n = 3$) (Table 6). This difference is significant ($p < 0.05$). Transferrin levels in duodenum and ileum were very similar: $4.25 \pm 1.16 \mu\text{g}$ transferrin/mg protein ($n = 19$) in the duodenum and $4.32 \pm 0.09 \mu\text{g}$ transferrin/mg protein ($n = 19$) in the ileum (Table 5). The specific activity of transferrin was not able to be calculated due to the presence of other iron-binding components in the fractions (see Gel Filtration and Immunoabsorption).

Ferritin was present predominantly in peak 2, with a density between 1.080 and 1.110g/ml. Small amounts were present in peak 1 (density 1.050-1.070g/ml) and in the high density region (density 1.150-1.170 g/ml). Ferritin levels, expressed in μg ferritin per mg total protein, were greater in the iron-absorbing areas: 0.22 ± 0.08 in the duodenum ($n = 19$) and 0.08 ± 0.02 in the ileum ($n=19$) (Table 6). Most of the ferritin in the latter case is present in the low density, iron depleted forms (Figure 14). The specific activity of ferritin was calculated as $56500 \text{ } ^{59}\text{Fe}$ cpm per μg ferritin.

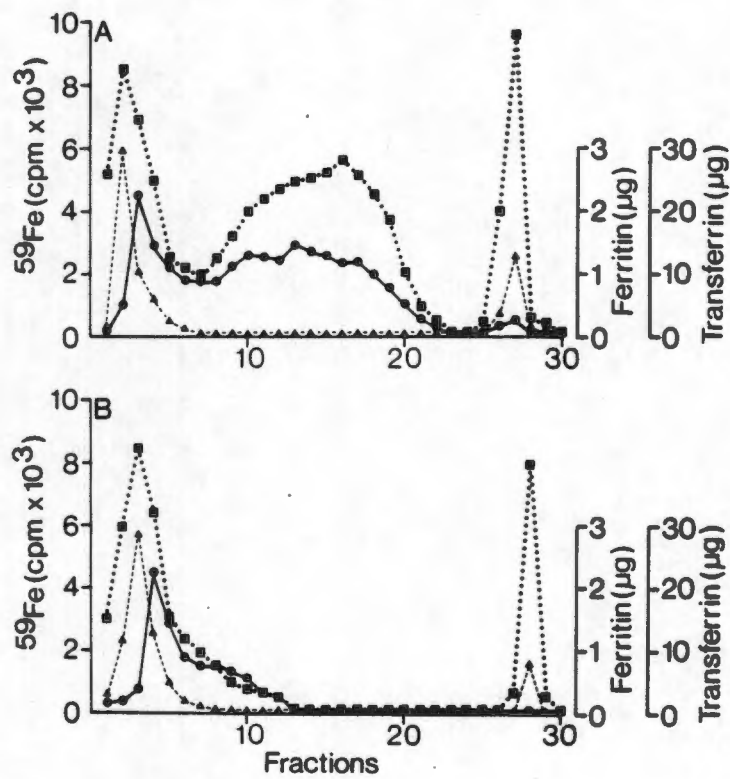


Figure 14. Density gradient fractionation of normal iron load mucosal homogenate.

A, Duodenum;

B, Ileum.

Counts per minute ^{59}Fe (■.....■), transferrin (▲---▲) and ferritin (●—●). The density gradients are the same as in Figure 10.

Table 5. The transferrin and ferritin content of the mucosa. The total protein concentration of each 5cm duodenal segment is shown in column 2; transferrin and ferritin values in columns 3 and 5 are expressed as $\mu\text{g}/\text{mg}$ total protein in columns 4 and 6 respectively. Only the latter values are given for the ileal segments (columns 7 and 8); the mean total protein for the 19 segments was found to be $12.58 \pm 3.37\text{mg}$, which is not significantly different ($p > 0.05$) from that of the duodenum.

Rat	Duodenum				Ileum		
	Protein (mg)	Tf (μg)	Tf/mg ($\mu\text{g}/\text{mg}$)	F (μg)	F/mg ($\mu\text{g}/\text{mg}$)	Tf/mg ($\mu\text{g}/\text{mg}$)	F/mg ($\mu\text{g}/\text{mg}$)
1	19.29	48.70	2.52	7.51	0.38	4.35	0.09
2	16.36	54.08	3.31	4.18	0.25	3.42	0.12
3	23.77	33.20	1.40	3.67	0.15	4.68	0.07
4	33.55	103.35	3.08	5.44	0.16	5.45	0.05
5	14.64	52.87	3.61	1.74	0.12	4.61	0.09
6	10.34	58.94	5.70	1.36	0.13	5.36	0.08
7	16.36	50.51	3.09	4.63	0.28	3.51	0.09
8	9.99	28.65	2.86	2.36	0.23	5.57	0.07
9	10.16	39.88	3.93	2.44	0.24	4.55	0.08
10	10.16	66.91	6.58	2.62	0.25	3.24	0.08
11	11.20	53.46	4.77	7.17	0.06	4.31	0.07
12	18.95	51.71	2.73	1.32	0.06	3.97	0.08
13	9.48	54.14	7.82	2.83	0.29	3.93	0.05
14	14.64	59.54	4.06	1.36	0.29	4.35	0.10
15	16.54	54.64	4.69	5.61	0.52	4.42	0.06
16	9.99	59.55	5.96	2.58	0.26	4.25	0.09
17	10.34	59.29	6.73	2.12	2.04	2.90	0.12
18	24.97	51.93	3.79	7.63	0.30	4.68	0.05
19	12.58	69.64	4.13	2.82	0.22	4.39	0.05
Mean	15.43	55.32	4.25	3.39	0.22	4.32	0.08

Table 6. The transferrin content of density gradient Peak 3 in relation to Peak 1. The total transferrin protein is not necessarily derived from 5cm segments, hence the variation.

Duodenum			
Expt.	Peak 1	Peak 3	Peak 3/Peak 1
	(μg)	(μg)	(%)

1	65.71	8.50	11.93
2	16.15	2.00	12.38
3	6.49	0.60	9.23
4	21.80	2.25	10.32
5	49.97	5.72	8.74
6	47.34	3.21	6.97
		Mean:	9.91

Ileum			
1	39.71	0.93	2.33
2	29.48	1.09	3.71
3	40.25	0.84	2.09
		Mean:	2.71

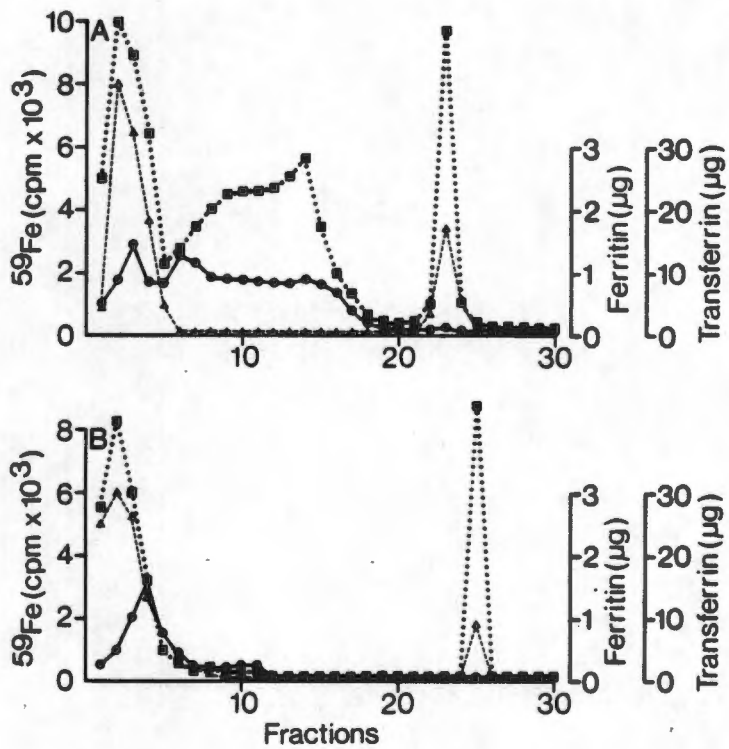


Figure 15. Density gradient fractionation of venesected (iron deficient) mucosal homogenate.

A, Duodenum;

B, Ileum.

Counts per minute ^{59}Fe (■.....■), transferrin (▲---▲) and ferritin (●—●). The density gradients are the same as in Figure 10.

Table 7. Transferrin and ferritin in the duodenum and ileum of iron deficient rats. The values are expressed as $\mu\text{g}/\text{mg}$ total protein. Total protein values were non-significantly different from those shown in Table 5.

Duodenum		Ileum	
Transferrin ($\mu\text{g}/\text{mg}$)	Ferritin ($\mu\text{g}/\text{mg}$)	Transferrin ($\mu\text{g}/\text{mg}$)	Ferritin ($\mu\text{g}/\text{mg}$)
8.72	0.05	9.73	0.02
9.95	0.01	8.52	0.01
10.30	0.03	7.64	0.02
Mean			
9.66	0.03	8.63	0.02

Table 8. The transferrin content of Peak 3 in relation to Peak 1 in iron deficient duodenum and ileum.

Duodenum			
Expt.	Peak 1 (μg)	Peak 3 (μg)	Peak3/Peak 1 (%)
1	89.62	19.03	21.24
2	52.89	12.54	23.71
3	37.90	9.25	24.40
		Mean:	23.11
Ileum			
1	9.09	0.62	6.82
2	14.09	2.01	14.58
3	47.34	3.30	6.97
		Mean:	9.50

5.4.2. The Effects of Iron Deficiency.

The venesection programme alters the need for iron absorption both by reducing body iron stores and stimulating erythropoiesis (Table 4). In iron deficient animals, carcass absorption was increased from $27.4 \pm 3.29\%$ in the normal animal to $45.3 \pm 13.19\%$ ($n=10$) from a 10 cm duodenal segment.

Considering the iron-binding profiles, peaks 1 and 3 were increased and peak 2 was reduced in mucosal cell homogenates of the iron-deficient animals (Figure 15) compared to normal (Figure 14). Transferrin levels were increased to $9.66 \pm 1.22 \mu\text{g}$ transferrin/mg protein in the duodenum and $8.65 \pm 1.09 \mu\text{g}$ transferrin/mg protein in the ileum ($n = 4$) (Table 7). Ferritin levels decreased in iron deficiency to $0.031 \pm 0.01 \mu\text{g}$ ferritin/mg protein in the duodenum and $0.019 \pm 0.005 \mu\text{g}$ ferritin/mg protein in the ileum ($n = 3$) (Table 7). The transferrin content of peak 3 in relation to peak 1 was also increased to $23.11 \pm 1.35\%$ ($n = 3$) in the duodenum and $9.50 \pm 2.22\%$ ($n = 3$) in the ileum (Table 8). The specific activity of ferritin, however, was found to increase: $84900 \text{ } ^{59}\text{Fe cpm}/\mu\text{g}$ ferritin.

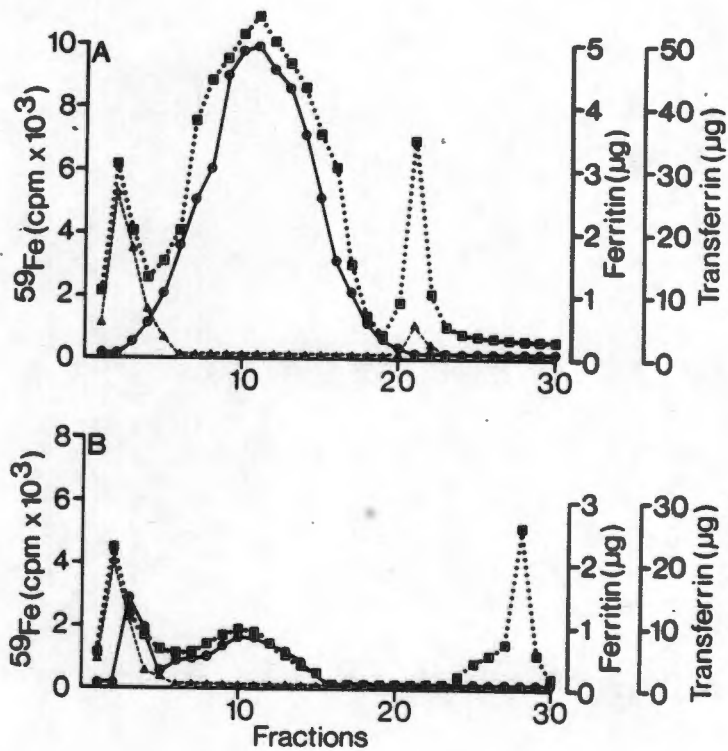


Figure 16. Density gradient fractionation of iron-loaded mucosal homogenate.

A, Duodenum;

B, Ileum.

Counts per minute ^{59}Fe (■.....■), transferrin (▲---▲) and ferritin (●—●). The density gradients are the same as in Figure 10.

Table 9. Transferrin and ferritin in the duodenum and ileum of iron loaded rats. The values are expressed as $\mu\text{g}/\text{mg}$ total protein. Total protein values were non-significantly different from those shown in Table 5.

Duodenum		Ileum	
Transferrin/mg ($\mu\text{g}/\text{mg}$)	Ferritin/mg ($\mu\text{g}/\text{mg}$)	Transferrin/mg ($\mu\text{g}/\text{mg}$)	Ferritin/mg ($\mu\text{g}/\text{mg}$)
3.09	2.83	2.57	0.39
3.93	2.39	3.55	0.27
4.13	2.58	3.24	0.47
4.77	6.50	4.31	0.21
2.73	9.29	3.19	0.43
4.06	2.04	2.97	0.34
4.39	2.23	2.61	0.23
Mean	3.86	3.51	0.33

Table 10. The transferrin content of Peak 3 in relation to Peak 1 in iron loaded rats.

Expt.	Peak 1 (ug)	Peak 3 (ug)	Peak 3/Peak 1 (%)
Duodenum			
1	36.66	1.68	4.99
2	21.19	0.89	4.20
3	25.30	1.04	4.10
		Mean:	4.42
Ileum			
1	57.41	0.00	0.00
2	29.22	0.78	1.47
3	25.62	0.01	0.05
		Mean:	0.80

5.4.3. The Effects of Iron Loading.

In iron-loaded animals carcass absorption was decreased to $2.20 \pm 0.80\%$ ($n = 10$) from duodenal segments. Transferrin levels were found to be similar to those in the normal animal: 3.86 ± 0.74 $\mu\text{g}/\text{mg}$ total protein ($n = 4$) in the duodenum and 3.51 ± 1.02 μg transferrin/mg protein in the ileum (Figure 16 and Table 9). Transferrin content of Peak 3 was $4.42 \pm 0.51\%$ ($n = 3$) that of peak 1 in the duodenum and $0.80 \pm 0.25\%$ ($n = 3$) in the ileum (Table 10). Ferritin levels were increased to 3.98 ± 2.59 μg ferritin/mg protein ($n = 7$) in the duodenum and 0.33 ± 0.09 μg ferritin/mg protein in the ileum ($n = 3$) (Table 9). The specific activity of ferritin was however reduced to 7016 ^{59}Fe cpm/ μg ferritin.

5.5. Gel Filtration.

The membrane-free homogenate yielded 3 peaks (Figure 17). The first peak had a molecular weight of approximately 400000 daltons, ran in the position of ferritin on polyacrylamide gel electrophoresis and was confirmed as this protein by radioimmunoassay and immunoabsorption. The second peak had a molecular weight of approximately 90000 daltons, ran in the position of transferrin on polyacrylamide gel electrophoresis and its identity was similarly confirmed by radioimmunoassay and immunoabsorption. A third, low molecular weight peak, less than 10000 daltons molecular weight, did not stain for protein on polyacrylamide gel electrophoresis and did not react in radioimmunoassay or immunoabsorption against either transferrin or ferritin antiserum. Gel filtration of density gradient peak 1 showed a transferrin peak and a large low molecular weight peak. Peak 2 yielded a ferritin peak and a smaller low molecular weight peak. Gel filtration of the Triton-treated peak 3 (not shown) yielded ferritin, transferrin and low molecular weight/free iron.

5.6. Immunoabsorption.

Transferrin-specific immunoabsorption of the membrane-free homogenate removed $56.84 \pm 7.28\%$ of peak 1 ($n = 4$). Ferritin-specific immunoabsorption removed $89.25 \pm 0.21\%$ of peak 2 ($n = 4$). The results were confirmed by radioimmunoassay (Figure 18).

5.7. Metabolic Inhibitors.

The microtubular poisons colchicine and vincristine abolished the transfer of iron from the gut lumen to the blood. Cytochalasin-B,

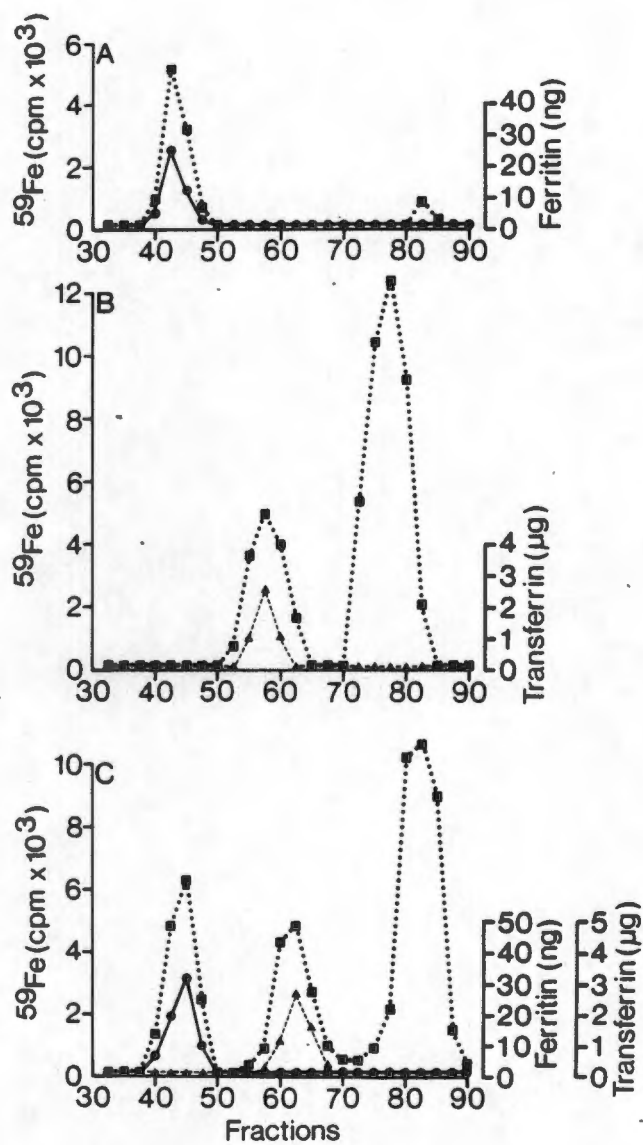


Figure 17. Gel filtration of the membrane-free mucosal homogenate.

A, Gel filtration of density gradient Peak 2;

B, Gel filtration of density gradient Peak 1;

C, Gel filtration of the membrane-free homogenate.

Counts per minute ^{59}Fe (■.....■), transferrin (▲---▲) and ferritin (●—●).

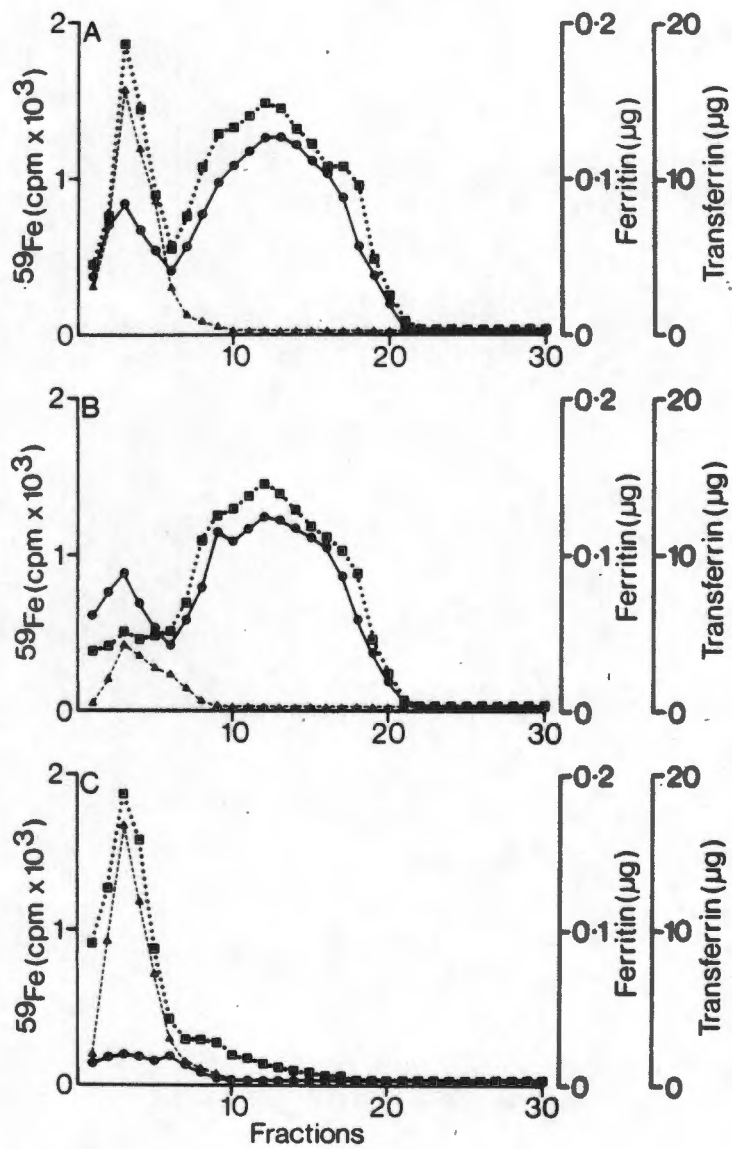


Figure 18. Density gradient fractionation of eluates after ferritin and transferrin-specific immunoabsorption.
 A, Starting material;
 B, Anti-transferrin immunoabsorbent and
 C, Anti-ferritin immunoabsorbent.
 Counts per minute ^{59}Fe (■.....■), transferrin (▲---▲) and ferritin (●—●). The density gradients are the same as in Figure 10.

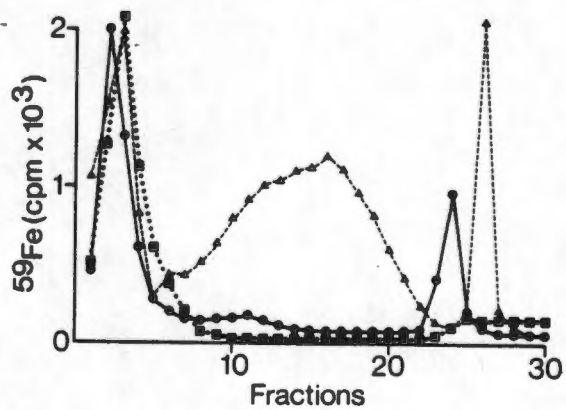


Figure 19. Density gradient fractionation of cells from mucosa pretreated with 10mM colchicine for 2 hours (■---■); 5mM cytochalasin-B for 15 minutes (●—●) and control (▲---▲). The density gradient is the same as in Figure 10.

Table 11. The transferrin content of Peak 3 in relation to Peak 1 in cells treated with metabolic inhibitors.

Expt.	Peak 1 (ug)	Peak 3 (ug)	Peak 3/Peak 1 (%)
Cytochalasin-B			
1	37.93	0.29	0.76
2	17.73	0.50	2.80
Colchicine			
1	22.11	0.21	0.95
2	17.73	0.31	1.74
3	20.93	0.25	1.20
		Mean:	1.49

a microfilament poison, did likewise, although in this case the effect was reversible. The uptake of iron by Peak 2 was also abolished (Figure 19) while the transferrin content of Peak 3 was significantly reduced to $1.49 \pm 0.74\%$ of Peak 1 ($n=5$) in the duodenum ($p < 0.01$) (Table 11). Dose and time-response curves for the three substances are shown in Figures 20, 21 and 22. Removal of cytochalasin-B led to a progressive increase in iron absorbed and incorporated into ferritin (Figure 23).

The respiratory poison cyanide, reduced, but did not abolish either carcass uptake or uptake into ferritin (Figure 24).

5.8. Absorption from Iron Polymaltose.

Absorption of iron from the macromolecular iron source, iron polymaltose, an iron-carbohydrate complex of molecular weight 100000 daltons, measured as radioiron incorporation into circulating haemoglobin of red cells two weeks after administration of iron salt and iron polymaltose labelled with different isotopes is non-significantly different from that of ^{ferrous ascorbate} ($p > 0.05$) for normal, iron deficient and iron loaded rats (Appendix 2). However, fractionation of the mucosa after exposure to the iron polymaltose complex showed minimal incorporation of iron into Peak 1 for normal, iron deficient and iron loaded animals (Figure 25). It can be seen from the gel filtration studies on Peak 1, after exposure of the mucosa to ferrous ascorbate, that the low molecular weight component of Peak 1 comprises approximately 1.5 times the activity of the transferrin fraction; exclusion of the low molecular weight moiety from the total activity of the peak, thus nevertheless results in a high-specific activity of the transferrin fraction. Assuming no contribution of low molecular weight moiety to the Peak 1 activity in iron-polymaltose-treated cells, the specific activity of

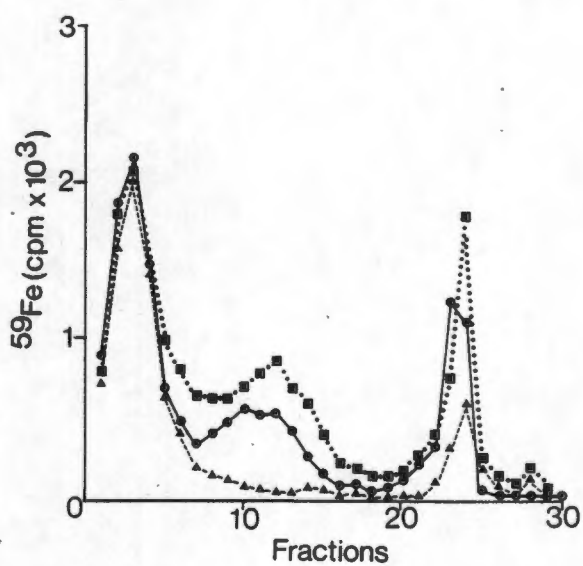


Figure 20. Density gradient fractionation of cells from mucosa pretreated with 100 μM (■...■), 1 mM (●—●) and 10 mM (▲---▲) colchicine. The colchicine was incubated in the loop for 2 hours, before washing and injection of the radiolabelled ferrous ascorbate, containing 3 μg of iron. The density gradients are the same as in Figure 10.

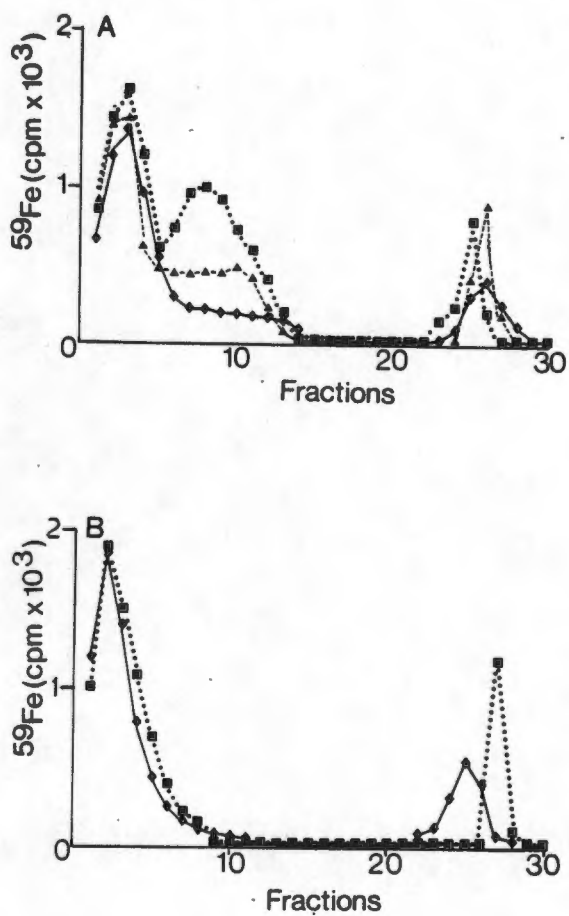


Figure 21. Density gradient fractionation of cell homogenates pretreated with vincristine.

A, Cells were pretreated with 0.0025mg/ml (■····■), 0.005mg/ml (▲---▲) and 0.01mg/ml (◆——◆) vincristine before washing the loop and injection of the radiolabelled ferrous ascorbate solution.

B, The mucosa was pretreated with 0.1mg/ml vincristine for 10 minutes (■····■) and 30 minutes (◆——◆) before washing and injection of the radiolabelled ferrous ascorbate solution. Density gradients are the same as in Figure 10.

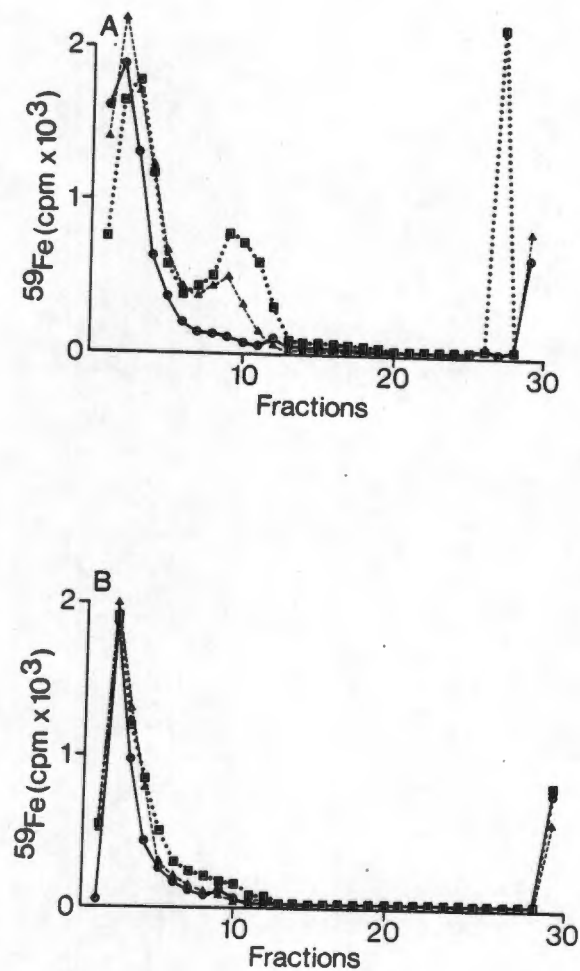


Figure 22. Density gradient fractionation of cell homogenates pretreated with cytochalasin-B. A, Mucosa was pretreated with 100 μM (■.....■), 1 mM (▲----▲) and 10 mM (●——●) cytochalasin. The radiolabelled ferrous ascorbate was allowed to incubate for 5 minutes. B, Mucosa was pretreated with 5 mM cytochalasin-B for 5 minutes (■.....■), 10 minutes (▲----▲) and 20 minutes (●——●) before washing and injection of the radiolabelled ferrous ascorbate solution. Density gradients are the same as in Figure 10.

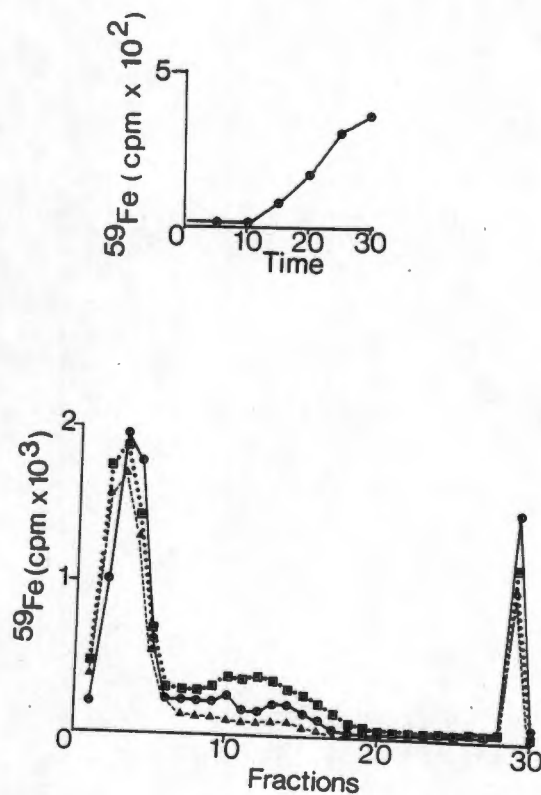


Figure 23. Density gradient fractionation of cell homogenates after incubation with 5mM cytochalasin-B for 15 minutes, washing the mucosa, injection of the radiolabelled ferrous ascorbate solution which was allowed to incubate for 10 minutes (\triangle --- \triangle), 15 minutes (\bullet — \bullet) and 20 minutes (\blacksquare \blacksquare). Density gradients are the same as in Figure 10. Inset, ^{59}Fe activity in 100 μl aliquots of blood as a function of time after removal of the cytochalasin and injection of the radiolabelled ferrous ascorbate.

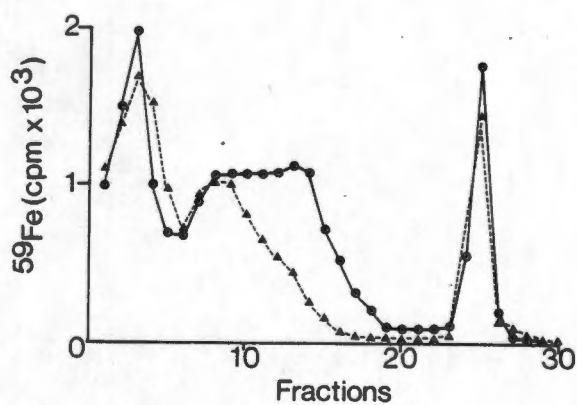


Figure 24. Density gradient fractionation of cells pretreated with 5mM KCN (▲---▲) for 10 minutes before washing and injection of the radiolabelled ferrous ascorbate solution, and control (●—●). Density gradients are the same as in Figure 10.

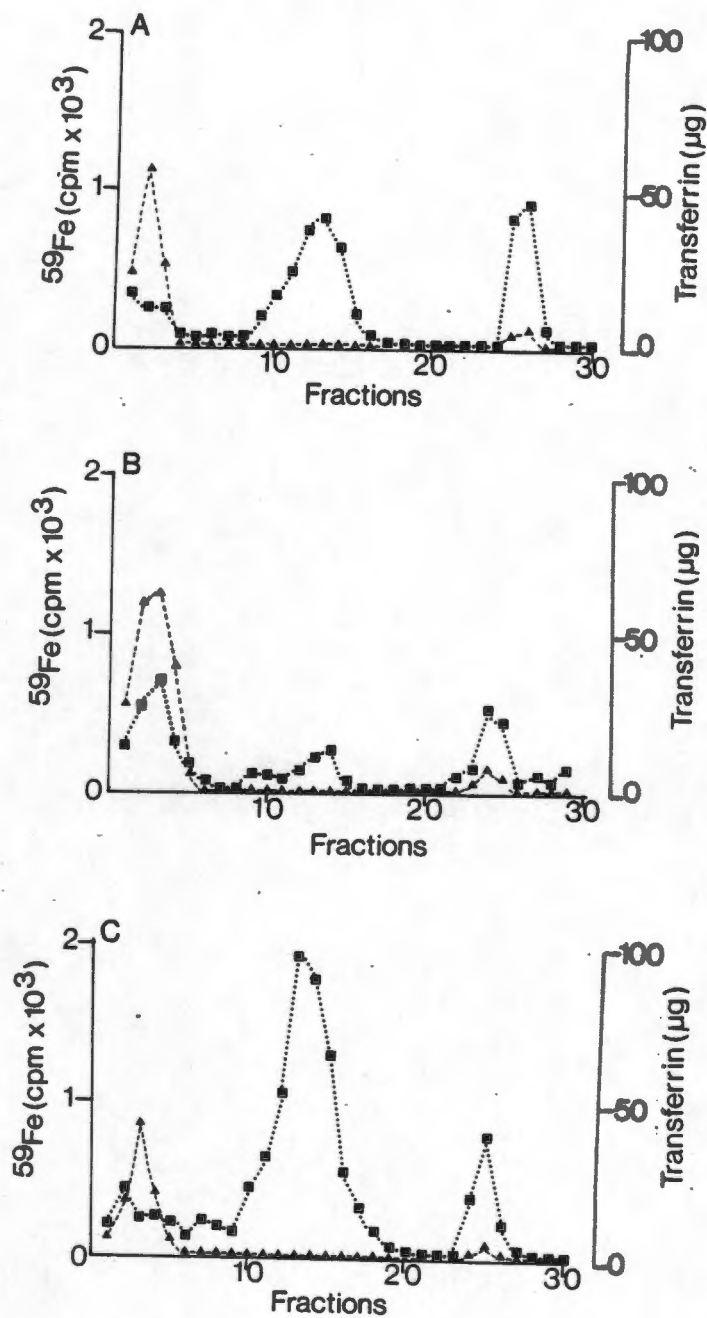


Figure 25. Density gradient fractionation of mucosal cells incubated with iron polymaltose solution, containing $3\mu\text{g}$ of iron. A, Mucosal homogenate from normal iron load animal; B, homogenate from a venesected (iron deficient) animal; and C, mucosal homogenate from iron loaded animal. Counts per minute ^{59}Fe (■····■) and transferrin (▲---▲).

It would thus appear that transferrin in the mucosa of polymaltose-treated cells is binding only small amounts of iron. Little is known of the mechanism of absorption of iron from the polymaltose complex, and while it is possible that the iron from this source is traversing the cell by a different absorptive pathway to that from ferrous ascorbate, it is possible that the absence of free or low molecular weight-complexed iron has resulted in the absence of iron uptake by transferrin.

Transferrin does not accept iron from the polymaltose complex (see Appendix 2). The possibility therefore remains that iron binding by transferrin, as shown by other experiments, in the mucosa is an artefact.

5.9. Serum "Contamination" of Mucosal Extracts.

Using the method of intravenous injection of biologically screened proteins, the mean contaminating serum volume per 5cm segment, as estimated by the ^{125}I -transferrin method, in 12 segments in two rats, was found to be $8.64 \pm 1.41 \mu\text{l}$ ($8.73 \pm 1.07 \mu\text{l}$ in rat 1 and $8.56 \pm 1.47 \mu\text{l}$ in rat 2). The ^{125}I alpha-fetoprotein method yielded a mean contaminating serum volume of $9.68 \pm 2.04 \mu\text{l}$ per 5cm segment (no significant difference; $p > 0.05$). Iodinated biologically-screened serum added to cells from a 5cm segment in vitro yielded a contaminating volume of $7.99 \pm 2.13 \mu\text{l}$ (no significant difference, $p > 0.05$).

Serum transferrin levels ranged from 1.04 - 2.10mg/ml with a mean of 1.56mg/ml ($n=15$). Using the mean volume of 1.56mg/ml , a contaminating serum volume of $8.64 \mu\text{l}$ would imply that $13.48 \mu\text{g}$ of transferrin would be accounted for as serum contaminant. The mucosal cell homogenates contained, on average, $55.4 \pm 12.2 \mu\text{g}$ of transferrin/5cm segment ($n=39$), ranging from 28.7 to $74.1 \mu\text{g}$ of transferrin/5cm segment. Since this is well in excess of the

estimated serum contaminant, it is apparent that an intrinsic mucosal cell transferrin is present. The contaminating serum volume is a calculated parameter, and does not preclude the possibility that serum transferrin can become intrinsic to the mucosal cell during the course of the experiment.

5.10. Trypsin Digestion of Mucosal Cells.

Whole cells digested with trypsin showed a small reduction in transferrin content measured by radioimmunoassay (Table 12), equivalent to a mean contaminating serum volume of $6.55 \pm 1.70 \mu\text{l}$. Transferrin itself was undetectable by radioimmunoassay after trypsin digestion and there was only a slight retention of additional transferrin after digestion of cells to which transferrin had been added in vitro.

5.11. Immunological Comparison of Transferrins.

The transferrin radioimmunoassay, using labelled serum transferrin, was standardised with rat serum transferrin and potency estimates were obtained from five serial dilutions of serum and mucosal homogenates. Using the parallel line method of Rodbard and Lewald (1973), the transferrin in serum was shown to have an identical slope to purified serum transferrin, but there was a highly significant difference ($p < 0.001$) in the slopes of serum and mucosal transferrin (Figure 26 and Table 13). Mixtures of serum and mucosal extracts yielded an intermediate slope predictable for non-interacting components. Constant conditions for both samples were maintained in terms of proteolytic inhibitors, Triton X-100 and buffer used. As a control,

Table 12. Trypsin digestion of mucosal cells. Whole mucosal cells were digested with 0.25% trypsin for 1 hour at 37°C and aliquots were homogenised and assayed for transferrin (column 2). Aliquots retained before digestion were also homogenised and assayed (column 1). The differences between these values (column 3) is the trypsin-accessible transferrin. This is also expressed as a serum equivalent volume (column 4) using a mean serum transferrin concentration of 1.50mg/ml. As controls, approximately 50ug transferrin was also digested under the same conditions. The added transferrin is completely accessible to trypsin.

	µg Tf before digestion	µg Tf after digestion	Difference	Serum equivalent volume (µl)
Cells alone	124.54	112.34	12.20	8.13
	62.24	51.14	11.10	7.40
	45.99	39.79	6.20	
Cells plus	107.39	51.52	55.87	
Tf protein	118.75	58.29	60.46	
Tf protein	52.50	0.00	52.50	
alone	56.7	0.00	56.7	

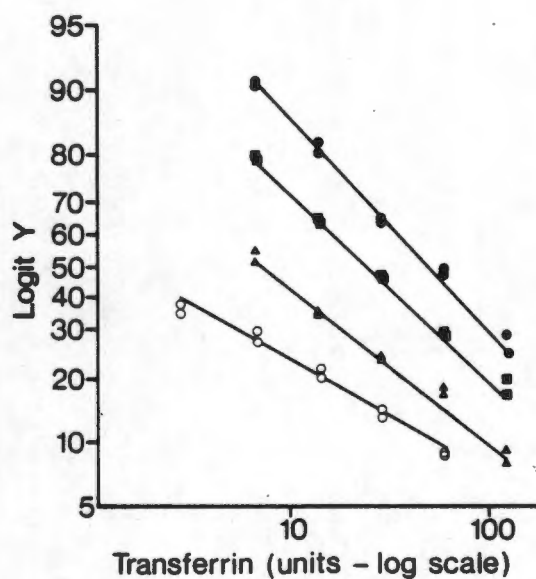


Figure 26. Parallel line assays of serial dilutions of rat serum (○—○); duodenal cell homogenate (●—●); ileal cell homogenate (■—■); and mixture of serum and duodenal cell homogenate (▲—▲). The F test for homogeneity of variance and the Student's t test showed a significant difference ($p < 0.001$) in the slopes of serum and intestinal transferrins (method of Rodbard and Lewald (1970)). The transferrin is in arbitrary units. The ordinate is logit Y ($\ln Y/1-Y$) where Y is bound count/uninhibited count rate.

Table 13. Immunological comparison of serum and mucosa. The slopes shown are the result of 5 duplicate doubling dilutions, calculated by the parallel line adaptation of the Rodbard and Lewald (1970) programme. Serum, duodenal and ileal samples were obtained from each rat. The "mix" (column 5) is a 1:1 mixture of serum and duodenal mucosa in appropriate dilutions.

Rat	Serum	Duodenum	Ileum	Mix
1	-0.8168	-1.2575	-1.8410	-1.1189
2	-0.8290	-1.4015	-1.3256	-1.1454
3	-0.8586	-1.5287	-1.7198	-1.1754
4	-0.8726	-1.2740	-1.3053	-1.1889
5	-0.8126	-1.5888	-1.4633	-1.2449
6	-0.9172	-1.3759	-1.9367	-1.2253
7	-0.9446	-1.4808	-1.3963	-1.2424
8	-0.9838	-1.5872	-1.8070	-1.2754
9	-0.9746	-1.3362	-1.3790	-1.2931
10	-0.9357	-1.6741	-1.5357	-1.3201

transferrin protein was assayed in the presence and absence of the above conditions and no difference was found. No difference was found between the transferrins of duodenal and ileal mucosal extracts nor between transferrin from Peaks 1 and 3 (Table 14). These differences between serum and mucosal transferrin would be greater if part of the mucosal transferrin were not due to serum contamination.

When increasing amounts of iron, as FeIII-NTA, were added to serum transferrin, and aliquots after each addition analysed by the parallel line assay, no significant difference ($p > 0.05$) was found between any of the samples (Table 15). Iron-binding was monitored by changes in the absorption at 465nm. Furthermore, there was no significant difference ($p > 0.05$) in the affinity slopes of antiserum for sera obtained from normal, iron-deficient and iron-loaded animals.

5.12. Isoelectrofocusing.

Column isoelectrofocusing, pH range 3.5-9.5, of density gradient Peak 1 (Figure 27) yielded three transferrin fractions, as determined by optical density, polyacrylamide gel electrophoresis and radioimmunoassay, at pH's 5.2, 5.5 and 5.6; a small peak at pH 4.98-5.00, confirmed as ferritin by optical density, polyacrylamide gel electrophoresis and radioimmunoassay, and a large free iron or low molecular weight peak at pH 1-3.5, which does not correspond to an OD 280 peak nor stain for protein on polyacrylamide gel electrophoresis, and would thus appear to correspond to the low molecular weight compound observed in gel filtration studies. Separation of transferrin according to its iron content -- increasing negative charge is associated with the binding of further iron and bicarbonate atoms -- is well described (Wenn and Williams 1968). A similar pattern was obtained on

Table 14. Immunological comparison of density gradient Peaks 1 and 3. Serum samples were obtained from the same rats as the mucosal samples. The slopes shown ^{below} ~~above~~ were derived from 5 duplicate doubling dilutions of each sample and calculated by an adaptation of the Rodbard and Lewald (1970) programme.

Rat	Serum	Peak 1	Peak 3
1	-0.7214	-1.0080	-1.0724
2	-0.7504	-1.1052	-0.9164
3	-0.7467	-1.0184	-1.1156
4	-0.8218	-1.4637	-1.5120
5	-0.8582	-1.8255	-1.6592
6	-0.9151	-1.4763	-1.6756
7	-0.8179	-1.5200	-1.8242

Table 15. Immunological comparison of normal, iron deficient and iron loaded sera, and the effect of addition of iron to serum transferrin in vitro. Iron deficient serum was obtained from iron deficient rats (see Table 4) and iron loaded serum from iron loaded rats (Table 4).

Normal Serum	Iron deficient serum	Iron loaded serum	Tf+ Iron
-0.8856	-0.7519	-0.8790	ApoTf -1.0342
-0.9937	-0.8210	-0.8961	1µl FeNTA -0.9413
-0.8790			2µl FeNTA -1.1400
			3µl FeNTA -0.9377
			5µl FeNTA -0.9605
			10µl FeNTA -0.9116
			20µl FeNTA -0.8854

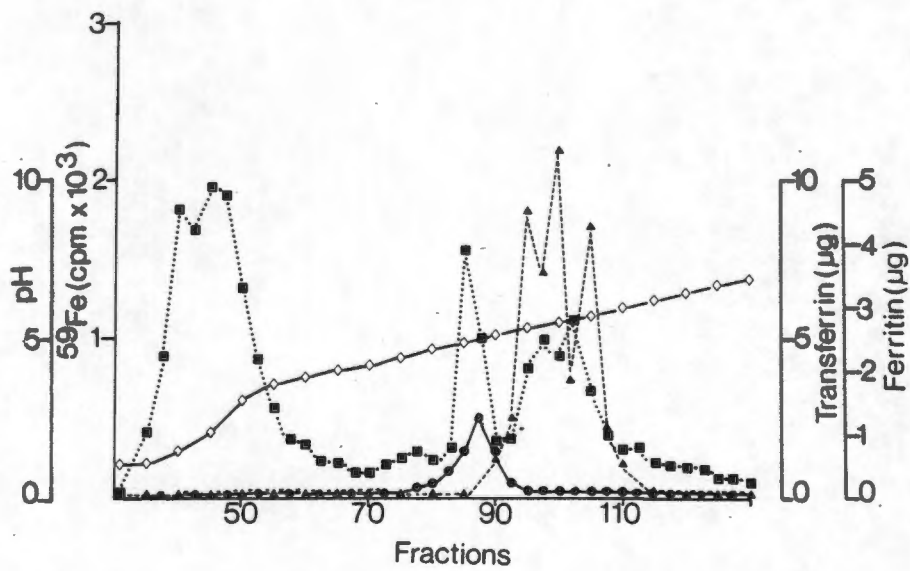


Figure 27. Column isoelectrofocusing of density gradient Peak 1. The pH gradient used was 3.5-9.5. Counts per minute ⁵⁹Fe (■.....■); pH (◇—◇); transferrin (▲---▲) and ferritin (●—●).

isoelectrofocusing of Peak 3: in this case, the transferrin content of the sample was lower and only two antigen peaks were obtained, corresponding to diferric and monoferric transferrin (Figure 28). A large ferritin peak was found at pH 4.98 and, similar to the Peak 1 profiles, a low molecular weight/free iron peak was observed at pH 1-3.5. Isoelectrofocusing of ^{55}Fe -labelled rat serum together with ^{59}Fe -labelled mucosal homogenate, yielded an identical ^{59}Fe and ^{55}Fe distribution in the transferrin fractions (Figure 29).

5.13. Mucosal Transferrin.

Mucosal transferrin preparation, analysed by the Rodbard and Lewald (1973) parallel line method, showed no significant difference to previously assayed mucosal homogenates ($p > 0.05$). The difference in immunological reactivity between it and serum was, however, highly significant ($p < 0.001$).

5.13.1. Ion Exchange Chromatography.

The eluted fractions from the DE-52 ion-exchanger, when analysed by optical density at 280nm and radioimmunoassay, showed two major transferrin peaks (Figure 30). When these fractions were electrophoresed on 6-8% polyacrylamide gels it was found that these peaks corresponded to transferrins of different electrophoretic mobility (Figure 31). The faster-moving of the two bands corresponded to serum transferrin; electrophoresis of the latter (Figure 31) showed no evidence of the slower-moving band.

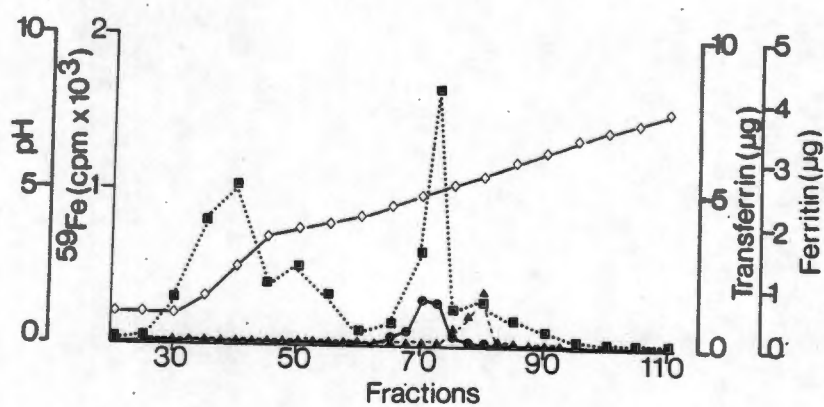


Figure 28. Column isoelectrofocusing of density gradient Peak 3. The pH gradient used was 3.5-9.5. Counts per minute ^{59}Fe (■.....■); pH (◇—◇); transferrin (▲---▲) and ferritin (●—●).

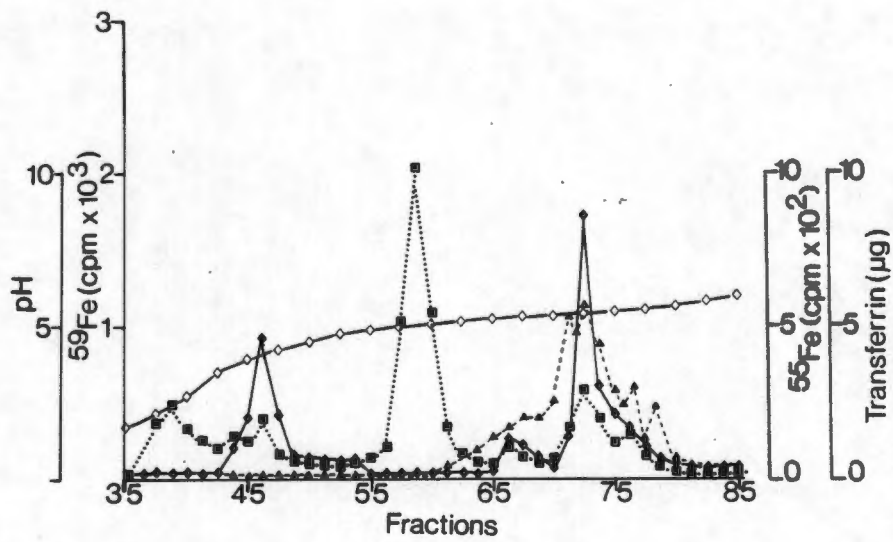


Figure 29. Column isoelectrofocusing of serum and mucosa. The serum transferrin was labelled with ^{55}Fe and the mucosal with ^{59}Fe . The pH gradient used was 3.5-9.5. Counts per minute ^{55}Fe (\blacklozenge — \blacklozenge); counts per minute ^{59}Fe (\blacksquare — \blacksquare); pH (\diamond — \diamond) and transferrin (\blacktriangle — \blacktriangle).

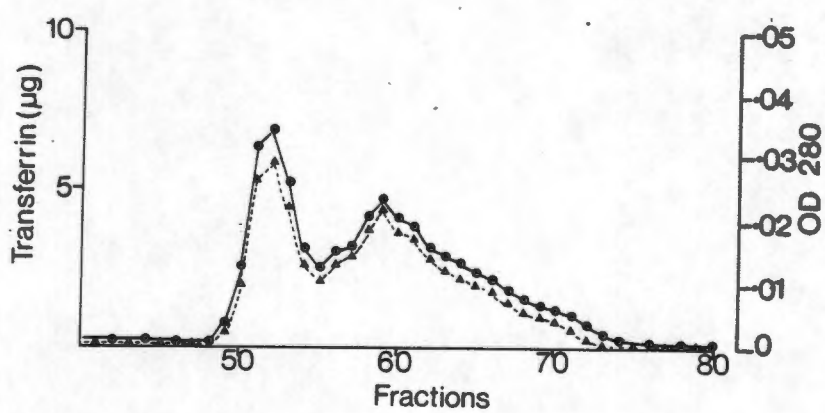


Figure 30. DE-52 chromatography of mucosal transferrin. Optical density (●—●) and transferrin (▲---▲).

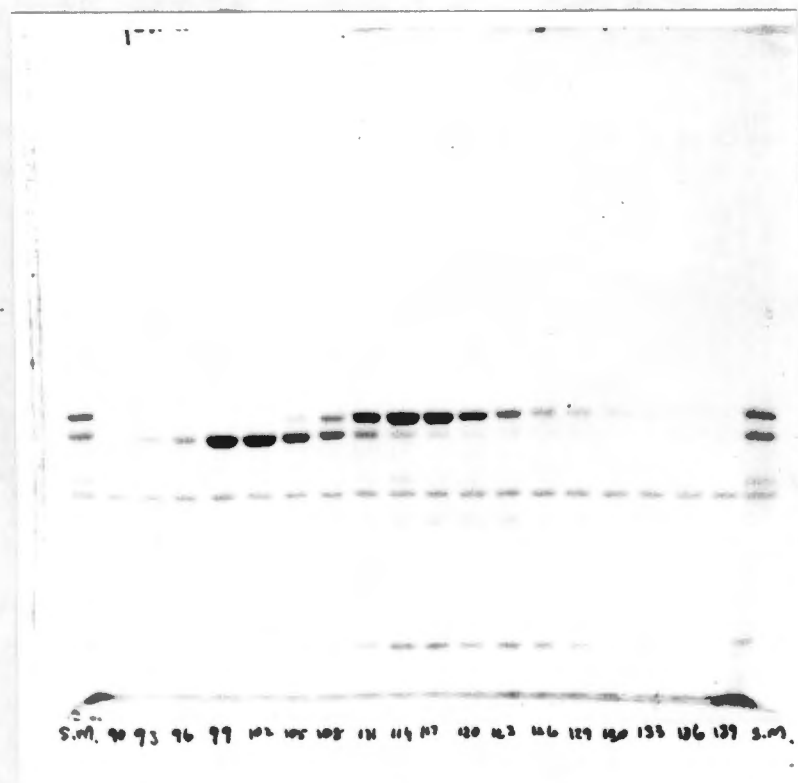


Figure 31. 6-8% sodium dodecyl sulphate polyacrylamide gel electrophoresis of fractions from DE-52 ion-exchanger. The two outer lanes contain the starting material, and show the presence of the two forms of mucosal transferrin (marked with arrow), with the DE-52 fractions in between, in which the separation of the two transferrins can be seen. The material is contaminated primarily with albumin (MW 67000) and another unidentified component of lower molecular weight.

It is known (Peterson 1970) that DE-52 and other DEAE-cellulose media fractionate according to molecular size as well as charge, on the principle that a larger molecule will have a greater distribution of surface charge than a smaller species.

Electrophoresis in the presence of SDS separates wholly according to molecular size (Reynolds and Tanford 1970), as expressed by the Stokes radius of the molecule, the charge differences being effectively screened by the presence of the detergent. It thus appears that the mucosa contains two transferrin species of unequal size. The fact that the "odd" transferrin has a larger molecular weight or size than the "normal" variety would suggest that it is not a proteolytic degradation product. It is perhaps significant that neither purified serum transferrin nor whole serum show this separation either on electrophoresis or on ion-exchange chromatography, where the protein elutes as a single homogeneous peak (e.g. Sutton and Karp 1968).

5.13.2. Isoelectrofocusing.

Preparative column isoelectrofocusing of mucosal transferrin in the pH range of 5-7 yielded three transferrin peaks, as determined by optical density, polyacrylamide gel electrophoresis and radioimmunoassay, at pH 5.2, 5.5 and 5.6 (Figure 32). This pattern corresponds to diferric, monoferric and apotransferrin (see above) respectively. Electrophoresis in the presence of SDS, however, showed no separation of the two transferrin fractions which showed parallel migration in all fractions (Figure 33). It appears therefore that charge and iron-binding do not account for the differences in the transferrin species. Electrophoresis of clarified mucosal homogenates labelled in vivo with ^{59}Fe , with or without a "cold" iron chase, followed by autoradiography, yielded no substantive results. No ^{59}Fe activity could be detected on

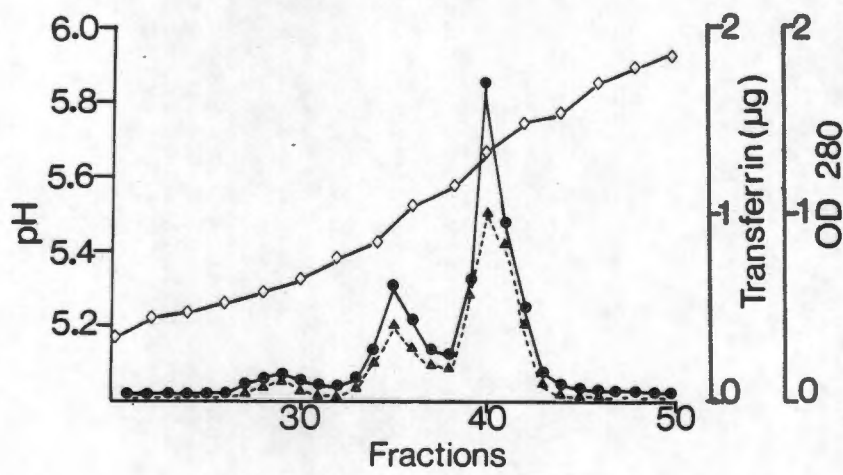


Figure 32. Column isoelectrofocusing of mucosal transferrin. The pH gradient used was 5-7. Optical density at 280nm (●—●); pH (◇—◇) and transferrin (▲—▲).

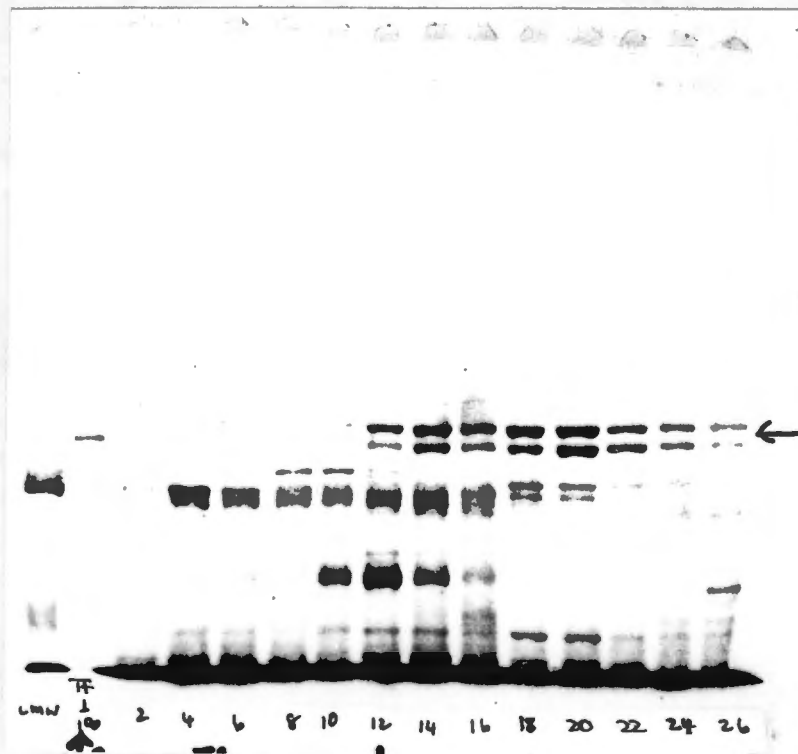


Figure 33. 6-8% sodium dodecyl sulphate polyacrylamide gel electrophoresis of isoelectrofocusing fractions of mucosal transferrin. The molecular weight markers, phosphorylase b (MW 94000), albumin (MW 67000) and ovalbumin (MW 43000) are in lane 1; purified rat serum transferrin in lane 2, followed by the isoelectrofocusing fractions. Although the fractions are contaminated by the presence of other proteins, the two forms of mucosal transferrin (marked with arrow) can be clearly seen.

mucosal transferrins, although the iron was present as free iron or on ferritin, not on the serum transferrin marker. It is thus supposed that the running conditions adversely affect iron-binding by the protein. To sum up, the presence of the two transferrin species appears to be a unique property of the mucosa and that the fractionation of "mucosal transferrin" in this way may be due to structural differences in the protein. Isoelectrofocusing, which fractionates according to iron content, indicated that iron binding was not responsible. The nature of the difference or differences remains to be determined. Possibilities are a difference in the carbohydrate moieties, although the immunological distinction would suggest a difference in primary or perhaps tertiary structure, both of which would account for the observed molecular size difference.

6. DISCUSSION.

The demonstration of the gradient of absorption, with maximal absorption occurring in the proximal 10-15cm of intestine, confirms previous reports (e.g. Duthie 1964; Wheby et al 1964) and validates the isolated loop method used in this study. Identical pH conditions were maintained in all incubation media and the iron was present as the soluble ferrous ascorbate complex, refuting the suggestion (Kavin et al 1967) that the low intraluminal pH of the duodenum, contributing to the maintenance of iron salts in solution, is responsible for the gradient of absorption and substantiating the results of Manis and Schachter (1966) and Greenberger et al (1967) with everted intestinal sacs. It has also been suggested (Booth 1967) that a longer transit time in the ileum may increase absorption; it was found here, however, that prolonging the incubation time to 4 hours had little effect. These factors suggest that the enhanced absorptive capacity of the duodenum is a property of the mucosa, and that a transport mechanism present in the duodenum is lacking in the ileum (see below). In contrast to the absorption gradient, measured by blood and carcass activity, uniform adsorption or non-specific binding of iron occurred in all regions of the intestine. Iron uptake by isolated cells showed a rapid initial adsorption that may be related to the uniform mucosal uptake of washed loops of bowel (Savin and Cook 1978). This may represent tight binding on the membrane or deeper structure or even internalisation of the iron. A clear distinction may therefore be made between, firstly, uptake into or adhesion onto the cell of iron and, secondly, absorption or the transfer of iron across the mucosa to the bloodstream (Turnbull 1974).

Similar kinetics of absorption with maximal uptake occurring between 30 and 60 minutes was shown by both blood and carcass as

well as isolated cells, and confirms the results of Savin and Cook (1978) on isolated cells and Kimber et al (1973) on brush borders. The finding that uptake by cells was partially inhibited by cyanide and low temperature concurs with the observation of Greenberger et al (1969) and Savin and Cook (1978) and suggests that the initial uptake step after binding to the membrane is at least partially energy-dependent. The similarity of uptake by cyanide- and EDTA-treated cells as well as the effects obtained after addition of cyanide during incubation i.e. an immediate reduction of uptake, might, however, suggest that the iron is being removed from the system and would support Savin and Cook's (1978) hypothesis that the former is acting as a chelator of iron rendering the metal unavailable for absorption. The initial rapid uptake of iron would presumably correspond to the "rapid absorption phase" described by Bothwell and Finch (1962) which is followed by a slow phase lasting up to 24 hours. The essential cessation of absorption after 60 minutes with minimal further uptake would suggest saturation of the mechanism. Increasing the amount of iron in the incubation medium, both in vivo and in vitro, resulted in linear increase in the amount taken up. This confirms the results of, for example, Wheby et al (1964) and Thomson and Valberg (1972). For all the iron doses, saturation time was the same, similarly in iron deficient and loaded animals. These results suggest that a time-related mechanism may be operative. An interesting observation from the literature is that competition exists between iron and other transition metal ions, such as cobalt (Thomson et al 1971). The evidence, thus, is as follows: the uptake mechanism is apparently time-dependent and relies on adsorption to the membrane; it is only partially inhibited by respiratory poisons such as cyanide and arsenate, but wholly by cytoskeletal poisons such as colchicine, vincristine and cytochalasin-B. Uptake is diminished by the presence of a charged

iron chelator, EDTA, in the lumen, but, as is very well documented in the literature, enhanced by the more lipophilic neutral complexes such as ascorbate, certain sugars and amino acids (Davis and Deller 1967; Bates et al 1972). It should be mentioned here that ferritin loading was found to correlate exactly with absorption in terms of the specific activity of the protein, and thus may be used as an index of absorption. Investigation of the iron transport across membranes has shown that the more lipophilic the iron complex the better it is absorbed (Princiotta et al 1964). Similar results have been found in intestinal absorption (Princiotta et al 1970; Edwards et al 1974). The importance of the maintenance of luminal iron in soluble form must also not be forgotten nor the fact that absorption occurs in the duodenum only, in spite of the satisfaction of this requirement. The results are not compatible with mechanisms of either active transport or simple diffusion, as has been suggested (e.g. Manis and Schachter 1962; May et al 1978). Most semipermeable membranes, including those surrounding cells, are not endowed with active transport mechanisms for transition metal ions (May et al 1978), this theory has been discounted for various other reasons described in the Introduction, and is not compatible with the observation that absorption continues in spite of treatment with cyanide and arsenate. The mechanism of simple diffusion for iron absorption has in recent years attained some popularity (e.g. Savin and Cook 1978). However, although the partial inhibition by cyanide can be explained on the basis of chelation, as did these authors, the temperature- and hence energy-dependence of uptake would argue against this; similarly the linear increase in uptake with increasing iron dose would suggest simple diffusion, but does not explain the saturation effect after 30 minutes. The reliance of effective absorption on membrane-compatible and lipophilic iron complexes, as well as the necessity for soluble low molecular

weight iron would suggest diffusion, but can be explained in terms of other mechanisms. Lastly, and most important, the dependence of absorption on an intact cytoskeleton effectively rules out simple diffusion as a mechanism since this should be independent of cytoskeletal activity. The evidence is thus consistent with a mechanism of pinocytosis. During pinocytosis substances may be captured in two ways: they may be taken up in solution, so-called fluid-phase pinocytosis, or alternatively, they may be carried into the cell attached to the invaginating plasma membrane (Jacques 1969) or by a combination of the two, with the possible participation of specific receptors. Sugars and amino acids are taken up by facilitated diffusion (Pratten et al 1981). There is controversy as to whether all pinocytic processes are or are not energy-dependent; a classification depending on vesicle size was presented by Allison and Davies (1974) but does not seem definitive. Certainly the evidence presented here, such as partial energy-dependence -- the invagination of the membrane is energy-dependent-- and complete cytoskeletal dependence, would argue in favour of a pinocytic mechanism. Colchicine and vincristine are microtubule inhibitors, while the cytochalasins are inhibitors of microfilament function: both are implicated in pinocytic processes (Allison and Davies 1974) and indeed both have been implicated in the pinocytosis of transferrin (Galbraith and Galbraith 1980b). Pinocytosis is dependent on membrane function and movement, which would account for the dependence of effective absorption on the membrane-compatibility of complexes. The rate-limiting step in all pinocytic phenomena is the number of pinosomes formed per unit time (Pratten et al 1981); little is known as to how this is controlled, but this might account for the cessation of absorption after 30 minutes, especially if a non-recycled ligand or receptor is involved. Furthermore, the presence of absorption in the duodenum only, in spite of identical

pH and solubility conditions maintained in the ileum, argues against simple diffusion and in favour of the presence of a specific mechanism.

Fractionation of the labelled mucosal homogenates indicated the presence of two iron-binding proteins only: ferritin and transferrin. This confirms previous findings (Batey and Gallagher 1977; Halliday et al 1976; Huebers et al 1972; Savin and Cook 1980). No evidence was found of the high molecular weight non-ferritin protein reported by Pearson and Reich (1969) and Worwood and Jacobs (1971), nor was evidence found of association of iron with the mitochondria (Hopkins and Peters 1979). In all cases and under all conditions, transferrin was found to label with iron, whereas ferritin labelled only in cells through which absorption was occurring. In *in vitro*-labelled cells, only a small amount of iron accumulated in ferritin: this may be result of the polar nature of mucosal cells and the need for correct orientation to the iron, and thus may only in this respect be non-physiological. Fractionation of cells treated with cytoskeletal poisons showed labelling of transferrin only. Such indiscriminate uptake of iron by transferrin suggests that this labelling may be an artefact. As the mucosal iron-binding proteins showed different immunological reactivities compared with those with which the radioimmunoassays were standardised, the protein concentrations obtained are not quantitative. The values obtained, however, fall within the same range as those reported by Savin and Cook (1980) using an immunoradiometric assay and Osterloh and Forth (1981) using radial immunodiffusion. The problem of the quantitation of mucosal-specific transferrin was partially overcome by estimating the possible serum contribution to the mucosal preparation. In spite of the differences in reactivities of serum and mucosal transferrin, a certain amount of transferrin was present in excess of any possible serum

contamination and an equivalent amount was shown to be inaccessible to proteolysis. While the "free" transferrin was present in equal quantities and showed identical iron-binding behaviour throughout the intestine, the membrane-associated protein showed a close correspondence to absorption, whether under normal conditions or modified by position in intestine, iron status or cytoskeletal inhibitors. Colchicine and vincristine disrupt the microtubules of the cell, the former binding non-covalently to the tubulin subunits preventing assembly and creating conditions favouring dissociation (Taylor 1965) while vincristine precipitates and hence disrupts microtubules (Wilson et al 1970). The cytochalasins, including cytochalasin-B, are inhibitors of microfilament function: their effect is immediate and reversible even after long periods of incubation (Carter 1967) in contrast to the progressive effects of the microtubular inhibitors (Taylor 1965). Both the immediacy and reversibility of effects of cytochalasin-B and the progressive effects of colchicine and vincristine were observed in this study. Dose ranges of both colchicine and vincristine are similar to those reported by Hemmaphysal et al (1974) and the former are similar to those required for the binding of colchicine to tubulin from porcine brain (Owellen et al 1972). Microtubules are implicated in vesicle movement through cells (Dumont and Wallace 1972; Wessells et al 1971) and microfilaments have been implicated in receptor mobility in the membrane (Galbraith and Galbraith 1980b). At low temperatures, receptors are randomly distributed through the membrane; warming results in aggregation of the receptors to form a "cap" (Raff and De Petris 1973), the fate of which is either to be shed from the cell or internalised (Unanue and Karnovsky 1973). Galbraith and Galbraith (1980b) showed that cytochalasin-B significantly inhibited the "capping" process of transferrin receptors in human mitogen-activated lymphocytes and hence

transferrin uptake. Hemmaplardh et al (1974) showed iron uptake by reticulocytes to be inhibited by microtubule poisons, including colchicine and vincristine. Both receptors (Cox et al 1979; O'Donnell and Cox 1982) and vesicles (Evans and Grace 1974; Eastham et al 1977; Marx and Aisen 1981) associated with iron transport have been observed. It thus seems probable that iron transport in the mucosa is dependent on vesicle movement and in this respect the membrane-associated transferrin fraction may be significant. The role of the "free" transferrin is difficult to explain. This was found in all regions of the intestine, where it showed the same iron-binding behaviour during iron absorption and the same immunological reactivity. The latter finding, together with the demonstration that only approximately a quarter of this transferrin was serum-derived, indicate that it is a mucosal protein. Whether iron-binding by this species is an artefact or not is uncertain. Iron-binding occurs equally in all situations whether or not absorption is occurring; furthermore, there is the observation that during absorption from the macromolecular iron source, iron polymaltose, which does not, unlike ferrous ascorbate, donate iron to transferrin (see Appendix 2), almost no binding occurred. The latter should not be taken as conclusive evidence; little is known about the mechanism of absorption from the polymaltose complex, although the preliminary studies reported here as well as previously published work (Jacobs et al 1979) have shown that absorption occurs with the same efficiency as from ferrous ascorbate suggesting that at least part of the transport and regulatory pathway is common. The role in absorption of this transferrin is thus at present uncertain.

Mucosal ferritin, like mucosal transferrin, is also immunologically different from the ferritins of other tissues. Unlike transferrin, this is not in itself significant: electrophoretic and electrofocusing differences between the

isoferritins of different tissues are well known (Drysdale 1974; Linder-Horowitz et al 1970; Powell et al 1974) and Hazard et al (1977) have shown immunological differences between human isoferritins from different tissues. The reason for the differences lies in subunit heterogeneity: the genome apparently codes for several species of ferritin protein. Ferritin, in contrast to transferrin, is present in greater quantities in the iron-absorbing areas and only takes up iron in these areas. The observation that iron-binding by ferritin, unlike that of transferrin, is inhibited by the microtubule and microfilament poisons suggests that it is not an artefact. On this evidence alone, one might conclude that it is involved in absorption. However, the protein is present in greater quantities in iron loaded animals and decreases in iron deficiency, factors which would argue against its involvement. Further evidence was given by the homogenisation with cold iron studies: ferritin is accumulating, not transporting, iron. The protein would therefore appear to lie on a sidepath of iron absorption, serving as a sink for excess iron. The greater concentration in iron-absorbing areas is probably the effect rather than the cause: exposure of cells to iron results in ferritin synthesis (Drysdale and Munro 1966) and exposure of intestinal cells to iron in the lumen likewise results in ferritin synthesis within a matter of hours (Bernier et al 1970). Most ferritin synthesis was found to occur in the duodenum. Iron also enters the cells from the serosal pole, resulting in ferritin synthesis, although in this case there was no preference for the duodenum (Bernier et al 1970). Results from parenteral iron loading experiments and fractionation of the mucosa at various time intervals suggested that the cells were incorporating their "ferritin apparatus" during their development in the crypts of Lieberkuhn as suggested by Crosby et al (1963). This, it was hypothesised, acted as the regulator of absorption;

Brittin and Raval (1970), however, found in iron-deficient rats, despite maximal intestinal ferritin levels induced by luminal iron administration, absorption continued at a greater rate than normal, suggesting that ferritin is not the regulator. The results presented here suggest that the crucial point in absorption, and the point at which regulation occurs, is at the site before iron incorporation into ferritin.

The immunological difference between mucosal and serum transferrin is highly significant. The presence of a "different" mucosal transferrin has been suggested by a number of workers: Halliday et al (1976) who suggested that there might be an immunological difference, Huebers et al (1976) who found a transferrin different in pI -- this was not confirmed here-- and amino acid composition in the mucosa of the rat, and Pollack and Lasky (1976) who found a physically and immunologically distinct transferrin in the mucosa of the guinea pig. The demonstrated immunological difference might be due to several factors; firstly, proteolysis. This was extensively controlled and tested for: cells were washed and homogenised in the presence of proteolytic inhibitors as soon as removed from the gut and the radioimmunoassay was run on mucosal homogenates in the presence and absence of various factors including proteolytic inhibitors, detergent and buffers. No differences at all were found. Further, the polyacrylamide gel electrophoresis of the mucosal transferrin, showing a slower-moving, hence larger, species than the serum, would argue against proteolytic degradation. One is therefore forced, in the absence of extraneous factors, to conclude that the mucosal protein is a structurally different transferrin. The separation of mucosal transferrin into two major electrophoretic bands is apparently unique. Fractionation of transferrin into two or more fractions has been reported: Jeppsson and Sjöquist (1963) found separation of human diferric transferrin into two fractions on

CM-cellulose, Lane (1971) reported separation into three peaks on anion-exchange chromatography, according to iron content and Huebers et al (1976) reported the electrophoretic fractionation of serum transferrin into two species, transferrin slow and transferrin fast. These results are not comparable to those obtained in this study however; with the exception of the last-named report, none of the fractions were electrophoretically distinguishable; with respect to the last-named report, no fractionation of serum transferrin was found in the present study. The reported results do show that heterogeneity within a single transferrin species may exist. This has also been reported under other circumstances e.g. Van Eijk et al (1982). Variations in carbohydrate composition among members of the transferrin class are fairly well-known (e.g. Parker and Bearn 1962; Wenn and Williams 1968); it is unlikely however that such differences would account for an immunological difference: antigenic determinants exist on the polypeptide backbone, not on the carbohydrate moieties. This however remains to be investigated.

The remaining possibilities are those of differences in primary and tertiary structure. The former would be due to an intrinsic difference in the polypeptide backbone in terms of the number of amino acid residues or the composition of the sequence, and would imply a separate gene product. Purification and sequencing of mucosal transferrin is at present being carried out in this laboratory. A difference in tertiary structure, or the conformation of the protein, could produce an immunological difference by shielding of the antigenic determinants during folding, and the molecular size difference by the compactness of the folded structure. If this is so, and the mucosal species represents a different conformational state, the major or one of the major, differences between serum and mucosal transferrin is the ability of the latter to exist in a different stable

conformational state. This would be in itself a very interesting phenomenon, and one that is apparently rare in proteins (Creighton 1979), although such stable conformational intermediates have been reported for cytochrome c (Myer 1968), bovine α -lactalbumin (Kuwajima et al 1976), bovine carbonic anhydrase B (Wong and Tanford 1973) and *Staphylococcus aureus* penicillinase (Carrey and Pain 1978). It is known that iron binding to transferrin induces conformational changes (Kornfeld 1969; Charlwood 1971). Iron binding, as described in the Introduction, involves a number of ligands not all in the same position on the molecule, hence binding of an iron atom presupposes the assumption of a more folded, spherical and compact configuration to accommodate the metal. This could very well result in the shielding of antigenic sites, hence the difference in immunoreactivity. However, the evidence is against this: in vitro addition of iron, as FeIII-NTA to serum transferrin resulted in no change in immunoreactivity, nor could the sera of iron deficient and loaded rats be found to show an immunological difference. Isoelectrofocusing of mucosal transferrin preparations, both whole homogenates and partially purified protein, resulted in no immunological difference between the fractions; it is concluded therefore that the immunological difference is not a result of differing iron content. It is interesting to speculate on why such a structural or conformational difference between serum and mucosal transferrin might exist. The problem of iron uptake by reticulocytes and other cells is one that has puzzled investigators for many years: it is known that transferrin binds to the membrane and is apparently internalised (e.g. Sullivan et al 1976) before releasing the iron and returning to the serum. Most of the early studies e.g. Jandl et al (1959), Kornfeld (1968), Aisen et al (1966) were performed with human transferrin and rabbit reticulocytes, and a significant difference was found between

uptake from diferric and monoferric transferrin. Later studies using homologous systems of rabbit-rabbit and human-human transferrin and reticulocytes respectively e.g. Esparza and Brock (1980) and Huebers et al (1981) could not repeat these findings, and on this basis the Fletcher-Huehns hypothesis was discounted (Van der Heul et al 1982). It is known that the binding sites of transferrin show spectral and iron affinity differences as well as differences in their susceptibility to modification (Kornfeld 1968) and the protein undergoes conformational changes induced by iron binding. It is thus suggested that in the case of the heterologous human-rabbit situation, enhanced binding of diferric transferrin to the membrane, resulting in enhanced uptake of iron by the cell -- binding to the membrane is inevitably followed by endocytosis of the transferrin regardless of its iron content (Baker et al 1982) -- is a result not of its iron content but of its conformational state. It is known that, generally, alteration in tertiary structure causes modification of molecule-receptor interaction (Pratten et al 1981) but little is currently known about such interactions. In other words, the receptor in this non-physiological situation is recognising some facet of the transferrin's conformation; that this conformation is also associated with iron binding is coincidental. In this respect, Kornfeld (1968) using various reagents to modify the biological properties of transferrin, found that, regardless of reagent used, interaction of transferrin with reticulocytes was more sensitive to chemical modification than the ability to bind iron, suggesting that these are two separate events. Mucosal transferrin is clearly very closely related to serum transferrin and there is no reason to believe that its behaviour should be radically different. The Fletcher-Huehns hypothesis proposed that different affinity of binding sites of transferrin for different tissues was responsible for body iron regulation. The hypothesis has, for reasons

described previously, fallen into disfavour. However, it is possible that, as far as iron absorption is concerned, the hypothesis may not be too far wrong, that transferrin may act as the regulator of iron absorption, but that regulation may occur not as result of differential iron binding, as proposed by Fletcher and Huehns, or by the conformational states thus induced, but by an alteration in conformational state. This is purely hypothetical.

The question of iron absorption has been intensively studied for close on twenty years, both physiologically and biochemically, but little concrete evidence as to the mechanism of absorption has emerged. This is very probably due to intrinsic problems associated with the fractionation of the mucosa, particularly cross-contamination of fractions (Hopkins and Peters 1979). This problem was encountered here also, and renders conclusions as to the mechanism of absorption difficult to draw. No small high-flux compartment that might be associated with a carrier pool could be identified; furthermore, it is uncertain whether the low molecular weight compound or even iron-binding by transferrin itself, are artefacts, the results of cross-contamination. It does appear, however, that a cytoskeletal-dependent transport mechanism is operative; the possibility of brush border vesicles in iron uptake has been suggested (Evans and Grace 1974; Eastham et al 1977) but this study is apparently the first in which absorption has been shown to be dependent on the function of microtubules and microfilaments.

Transferrin and ferritin were quantitated, as far as possible allowing for the differences in immunological reactivity between the mucosal proteins and those with which the assays were standardised. The role of ferritin can be explained with reasonable certainty, the role of transferrin less so. The

finding of an immunologically and electrophoretically distinct mucosal transferrin species is significant and further investigation into the structure and function of this protein is under way. It is tempting to postulate that this transferrin represents the carrier and/or regulator of absorption, but the possibility remains that its iron-binding activity under the conditions of absorption represents an artefact. It is also tempting to postulate, in view of both its demonstrated partial membrane-association and larger molecular size that it may be homologous to p97 that has recently been shown to be a member of the transferrin class (Brown et al 1982). It has recently been proposed (Williams 1982) that p97 may represent an evolutionary intermediate in the development of transferrin: it is almost certainly true, in view of the close sequence homology between the two domains (MacGillivray et al 1982) that gene duplication occurred during transferrin's evolution, and that the ancestral protein had a molecular weight of approximately 40000. The small size would have resulted in excretion through the kidney as has been demonstrated for 40000 molecular weight fragments of transferrin (Williams et al 1982) necessitating anchorage of some sort, hence the proposal of a membrane-associated form. Mucosal transferrin is found partially in association with membrane and furthermore is located within the mucosal cells, rather than in the serum and thus subject to renal excretion. It is thus possible that mucosal transferrin may represent an intermediate stage in the evolution of the transferrin class.

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8. APPENDICES

8.1. Appendix 1.

8.1.1. Viability of intestinal loops.

The rat was anaesthetised with Sagatal, the abdomen opened and a 5cm loop ligated in the duodenum. Ligatures were removed after 30 minutes and the abdomen was sutured. After 5 hours, the loop was again ligated and the radiolabelled ferrous ascorbate solution injected. After 30 minutes the animal was sacrificed and the mucosal cells from the loop harvested and fractionated on a 10-50% sucrose density gradient . The iron-binding profile of this cell homogenate is shown in the Figure.

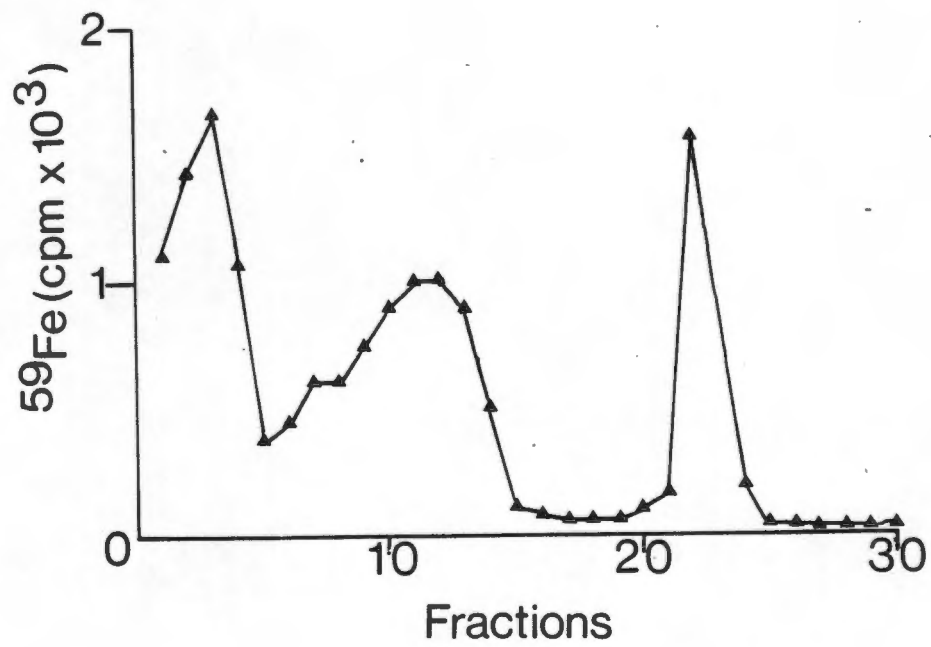


Figure. Density gradient fractionation of cell homogenate from an intestinal loop, ligatured for 30 minutes, allowed to recover for 5 hours, re-ligatured and the ferrous ascorbate solution injected. It can be seen that uptake of iron into ferritin has occurred, indicating that normal absorption is taking place.

8.2. Appendix 2.

8.2.1. Iron Polymaltose.

8.2.1.1. Iron release from iron polymaltose

	Total Cpm	60 min.	120 min.	180 min.
Control	43900	13	14	28
+ Amylase	43814	15	8	9
Gut - inhib	43204	25	25	27
Gut + inhib	43367	4	4	8
+ Succus	43204	19	15	11
Asc.*	43663	39	38	42
EDTA*	42436	138	215	308
Tf*	42851	19	21	54
Ferritin*	42333	16	39	53

Table. Iron release from iron polymaltose in the presence of various agents. Amylase (200µl saliva), cells from 5cm intestinal segments, homogenised with and without proteolytic inhibitors (see **Methods** for details), and succus entericus (200µl washing from the duodenum) were incubated together with 3µg iron as iron polymaltose, in 10ml 5mM Tris/HCl, pH 7.4 buffer, in a dialysis bag. This was dialysed against 20ml of the same buffer with shaking at room temperature. At specified time intervals, 10ml In a second experiment (*), 0.4mM ascorbic acid, 5mM EDTA, 10µg rat serum transferrin and 10µg rat liver ferritin were present in the 20ml of buffer, The dialysis bags contained 3µg iron as iron polymaltose in 10ml buffer only. At specified time intervals, 10ml aliquots of buffer were removed, counted for ⁵⁹Fe activity and replaced.

8.2.1.2. Comparison of absorption

The absorption from salt and from polymaltose was investigated by comparing absorption from differently labelled salt and polymaltose as follows: after overnight fast, animals received an oral dose by nasogastric tube of 3µg iron as either ferrous ascorbate or iron polymaltose. Each form of iron was labelled with a different isotope. Thereafter no food was allowed for the following 4 hours. The following day, under comparable conditions of fasting, the second compound, labelled with the other isotope, was given. After 2 weeks, when it was estimated that optimal incorporation of iron into haemoglobin had occurred, the rats were venesected by cardiac puncture under ether anaesthesia and the blood collected in anticoagulant. 2ml aliquots of blood with addition of 5mg iron as carrier were digested by the method of Katz et al (1964). Aliquots of the orally administered iron doses were retained, diluted to approximately expected blood concentrations and treated in the same manner as the blood samples. ^{55}Fe and ^{59}Fe activities were determined simultaneously as described in **Methods**. The amount of iron absorbed was calculated on the assumption that 100% of the retained radioactivity was present in the haemoglobin of circulating red cells and computed using an average blood volume of 60ml/kg body mass.

Group	Number	% Salt	% Polymaltose absorption
Normal	10	9.42±1.29	10.69±0.98
Iron deficient	10	32.37±2.09	26.33±2.70
Iron loaded	10	2.21±0.52	1.72±1.61

Table. A comparison of absorption from ferrous ascorbate (column 3) and iron polymaltose (column 4). There is no significant difference between the respective percentage absorptions for salt and polymaltose in each group ($p > 0.05$).