Iron Binding Proteins of Iron-absorbing Rat Intestinal Mucosa

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ABSTRACT The distribution and quantitation of the iron-binding proteins of rat small intestinal mucosa was studied, in iron-deficient and replete animals, to explore their role in the absorption of iron. Adsorption (mucosal uptake) of iron in in situ ligated loops of small intestinal mucosa was found to be uniform throughout the length of the small intestine whereas absorption (carcass uptake) showed a steep decreasing gradient from the duodenum to the ileum. The disrupted, in vivo labeled mucosal cells were fractionated by isopycnic centrifugation and transferrin and ferritin were quantitated by radioimmunoassay. Transferrin derived from mucosal cells was shown to have a higher affinity for the antibody than transferrin in serum. Of the transferrin present in the mucosal extract, only a portion could be accounted for by contamination from the serum; the proteolysis resistant and intrinsic transferrin may be mucosal cell specific. Transferrin was found in similar amounts in all regions of the small intestine, was not affected by iron loading but doubled in response to iron deficiency. Mucosal ferritin was found in greater amounts in the iron-absorbing areas of the intestine, increased in the duodenum of iron-loaded animals, and decreased in iron-deficient animals. The incorporation of newly absorbed radioiron into ferritin was only found in iron absorbing regions and was completely inhibited by colchicine and cytochalasin-B, suggesting that ferritin was loaded with iron at the point of iron absorption and that the process is associated with vesicle movement and not simple diffusion. Transferrin and ferritin-specific immunoabsorption and also gel filtration established that no other soluble iron binding proteins were involved in absorption.

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Received for publication 7 July 1982 and in revised form 19 January 1983.

INTRODUCTION

In mammals the limited availability of soluble dietary iron is reflected in the lack of a true excretory system and the use of high affinity binding proteins for iron transport and storage that also function to deny iron to pathogenic organisms. This unique set of circumstances results in body iron balance being controlled predominantly by regulated absorption, and under physiological circumstances both body iron stores and erythropoietic activity exert a major influence at the level of the intestinal cell (1). Reduction in storage iron and accelerated erythropoiesis are associated with increased absorption of dietary iron, whereas iron overload and erythroid hypoplasia decrease absorption.

In the interests of clarity a clear distinction must be made between uptake of iron into or adsorption onto mucosal cells and absorption that is defined as transfer of iron across the mucosa into the blood stream and carcass.

It has been shown that iron absorption occurs mainly from the proximal small intestine (2, 3) and depends on maintenance of the iron in a soluble form (4). Iron passing through the mucosal cells and appearing in the blood bound to the serum glycoprotein transferrin is transported to various sites. The major turnover of this iron is to erythrocyte precursors in the marrow, for hemoglobin synthesis: a process associated with the reversible binding, internalization, and release again of the protein from specific membrane receptors (5, 6). Another major iron binding protein, ferritin, is present in most tissues, and although it is currently considered to provide a flexible capacity for iron storage, it has, in the past, had attributed to it or the apoferritin moiety, a role in the regulation of iron absorption (7).

Both transferrin and ferritin have been found in the intestinal mucosa (8–10) and other mucosal iron binding proteins, similar in size and properties to trans-

ferrin, have also been reported (11, 12). In one species a claim has been made that the transferrin-like protein is unrelated immunologically (12). Low molecular weight iron-binders (13) and mitochondria (14) have also been suggested as being involved in absorption. The function and interrelationships of these intracellular iron-containing components and their participation in iron absorption remains controversial.

The aims of this study were to quantitate the iron-binding components of rat intestinal mucosa in various states of iron load or erythropoietic activity and to explore the role of transferrin and ferritin in the absorptive process.

METHODS

Animals. Adult (300 g) male Long Evans rats were used throughout. Rats were fed on a standard rat diet (Epol (Pty) Ltd., Johannesburg) containing 100 parts per million iron as ferrous sulphate. Iron stores were reduced by venesection of 20 ml blood over a 2-wk period, or increased by intramuscular injections of 100 mg iron as iron-dextran 2 wk before study. The hematology and iron status of the animals is shown in Table I.

Absorption of iron from isolated loops. Rats were anesthetized with pentobarbital sodium (Sagatal, May Baker S.A. (Pty) Ltd. Port Elizabeth, South Africa) the abdomen opened and either 5 or 10-cm long loops isolated between ligatures at different levels from pylorus to ileum. Care was taken not to damage the blood supply. A radioiron solution (0.3 ml) containing 0.15 M saline, 50 μM iron (0.75 μCi ^{59}Fe; Radiochemical Centre, Amersham, England) and 0.4 mM ascorbic acid, pH 4.2 was injected into the loop and absorption allowed to proceed for either 30 min or 1 h. Thereafter the loop was removed, washed clean with 40 ml cold saline, and counted for ^{59}Fe activity in a gamma counter (Beckman Instruments Inc., Fullerton, CA) as a measure of uptake (i.e., adsorption to the mucosa), and the remaining carcass counted (as a measure of absorption into the system) in a small mammal whole body counter against standards contained in a similar volume.

Cell isolation. Cells from 5-cm segments were removed by one of two techniques: firstly, by vibration of loops (18) on a glass rod at 50 Hz for 10 min. The cells, resuspended to final volume of 1 ml gave on average, cell counts of 5.5 $\times 10^6$ cells/ml measured in a hemocytometer, and 5.81±0.90 mg protein as determined by the biuret method (n = 5).

TABLE I
Hematology of Normal, Iron-deficient by Venesection, and Iron-loaded Rats

Group	No.	Hemoglobin	Reticulocytes	Storage iron
		g/dl	*%*	*μg/g tissue*
Normal	17	13.36±1.32	3.30±0.43	2,442.0±103.7
Iron deficient	20	8.05±1.55	21.10±4.36	1,050.1±211.9
Iron loaded	20	13.73±1.38	2.80±0.31	3,230.5±422.1

Hemoglobin was measured by the cyanmethemoglobin method (15); the reticulocytes measured by the method of Hillman and Finch (16); and storage iron (spleen) was measured by the method of Torrance and Bothwell (17).

Secondly, the loop was opened and the cells 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 $\times 10^6$ cells/ml and 15.51±4.42 mg protein (n = 20).

Iron uptake by isolated cells. Based on the method of Savin and Cook (18), the radioiron solution was added to 200 μl of a cell suspension containing 3 $\times 10^6$ cells/μl and incubated at 37°C with shaking. Aliquots were removed at 5-min intervals, pipetted onto a filter (pore size: 0.45 μm) (Millipore Corp., Bedford, MA) and washed with 40 ml cold saline, and the ^{59}Fe activity on the filter counted. In control experiments, the cells were incubated at 4°C, or preincubated with 1 mM potssium cyanide before washing and being added to the iron-containing solution.

Density gradient fractionation of cell homogenates. Washed cells were suspended to final volume of 1 ml in 0.15 M saline containing proteolytic inhibitors (5 mM ϵ-amino caproic acid (Sigma Chemical Co., St. Louis, MO), 20 μM phenylmethylsulfonylfluoride (Sigma) and 1,000 I.U. Trasylol (The Bayer Co., New York) and homogenized with 30 strokes of a Dounce homogenizer. Linear 10–50% sucrose density gradients (17.5 ml) in MES buffer were used: 0.1 M 2-(*N*-morpholino) ethane sulfonic acid (MES, Sigma Chemical Co.), 1 mM ethyleneglycol *bis*-(B-aminoethyl-ether) *N,N'*-tetra-acetic acid (EGTA, Sigma Chemical Co.) 0.02% sodium azide, pH 6.5 (19). Cell homogenate (0.8 ml) was applied to each and centrifuged for 16 h at 52,000 *g* in a swinging bucket rotor (SW27.1, Beckman L2-65B ultracentrifuge). Fractionation was by upward displacement of the gradient on a density gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebraska). The collected fractions were counted for ^{59}Fe and assayed for transferrin and ferritin. Ferrous sulfate (10 mM) (BDH Chemicals, Poole, England) was added to cells during homogenization when required. The gradient densities established after centrifugation were measured in each fraction by densitometry and ranged from 1.040 to 1.200 g/ml in the first and last of 30 fractions. Mitochondria have reported densities of 1.19 g/ml (20) and 1.15–1.2 g/ml (14). Assay for the marker enzyme creatine kinase found on the mitochondrial inner membrane on a Centrifuchem System 400 centrifugal analyzer (Roche Laboratories, Nutley, NJ) indicated the presence of mitochondria at 1.18 g/ml *d* (fractions 27–28).

Purification of proteins and radioimmunoassay. Rat serum transferrin was prepared by the method of Sutton and Karp (21). Rat liver ferritin was prepared by the method of Penders et al. (22). Purity of both was confirmed by homogeneity on polyacrylamide gel electrophoresis. Antibodies were raised in rabbits with an initial injection of 0.1 ml solution, containing 1 μg protein in Freund's complete adjuvant, into the retrocrural lymph node. The specificity of the antibodies were tested by immunoelectrophoresis and precipitation on Ouchterlony plates. Ferritin (5 μg) and transferrin (10 μg) were iodinated by the method of Greenwood et al. (23). Bovine serum albumin buffer [0.04 M NaH_2PO_4, 0.15 M NaCl, 0.1% sodium azide, 0.01 M ethylenediamine tetraacetic acid (EDTA, BDH Chemicals, Poole, England), 0.5% bovine serum albumin (Sigma Chemical Co.), pH 7.4] was used as a carrier and sufficient antiserum for 35% precipitation was used. The second antibody system was normal rabbit serum and donkey anti-rabbit gamma globulin serum (Wellcome Research Laboratories, Kent, England). Precipitates were washed and counted for residual ^{125}I activity. Results were calculated by the logit-log parallel line method of Rodbard and Lewald (24), where potency estimates were calculated from at least five duplicate doubling dilutions of each sample. The ferritin assay was stan-

dardized with the whole ferritin molecule; the relative reactivities of the monomeric and oligomeric species are not known. The transferrin assay was standardized with the purified serum transferrin. Density gradient fractions and cell homogenates were treated with 2% (final concentration) Triton X-100 before assay.

Biological screening of transferrin and determination of serum contamination of mucosal preparations. Following the iodination procedure, rat serum transferrin was biologically screened to remove free ^{125}I and damaged molecules by harvesting the serum from a rat that had been given an intravenous injection of labeled transferrin 24 h previously. This biologically screened whole serum was intravenously injected into test animals. Blood samples were taken to follow the protein equilibration and distribution. After 4 h when the slow clearance phase (denoting equilibrium with the tissues) had been reached, the rat was killed and cells were harvested from a series of 5-cm intestinal segments, counted for ^{125}I, and also assayed for transferrin by radioimmunoassay. By assuming that equilibration had been attained, the amount of transferrin in the mucosa could be expressed as equivalent to a volume of serum. 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 h at 37°C. The action of the enzyme was stopped with 200 I.U Trasylol. Transferrin concentration in cells before and after digestion was measured by radioimmunoassay. As controls 50 μg pure transferrin alone or 50 μg transferrin, added to cells from a single 5-cm segment, were digested under similar conditions.

Immunoabsorption. Transferrin and ferritin antisera were dialyzed overnight at 4°C against 0.05 M Tris/HCl pH 7.4 and the IgG separated with DE-52 (Whatman Inc., Clifton, NJ) chromatography. IgG was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, NJ). The transferrin and ferritin antigen-binding capacity was estimated for each column using varying concentrations of labeled transferrin and ferritin, with unconjugated Sepharose 4B as a control. The capacity of the antitransferrin column was 15 μg transferrin/ml and that of the antiferritin column 1.5 μg ferritin/ml. Cell homogenates, clarified by centrifugation at 1,000 *g* for 10 min were incubated with 1 ml immunoabsorbant with constant mixing at room temperature for 4 h and eluted through a further 1 ml of immunoabsorbant, with 0.15 M NaCl. The eluates were fractionated on sucrose density gradients.

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

Metabolic inhibitors. A solution of 5 mM cytochalasin-B (Sigma Chemical Co.), initially dissolved in dimethylsulfoxide (BDH Chemicals) was incubated in the intestinal loop for 15 min before washing with 10 ml saline and injection of the iron-ascorbate solution. Similarly, 10 mM colchicine (Sigma Chemical Co.) was incubated in the loop for 2 h and 1 mM KCN was incubated in the loop for 15 min.

RESULTS

Absorption of iron from isolated loops. Washed loops from all regions of the small intestine show almost identical uptake of iron (21.06±1.68%, n = 22) for 10-cm loops. Absorption, whether measured by the appearance of radioiron in venous blood or in the carcass, shows a steep gradient over the upper 10 cm of small intestine; at the 50-μM (3 μg) dose of iron carcass uptake ranges from 27.40±3.29% (0.822 μg) in the duodenum to 0.48±0.19% (0.014 μg) in the ileum (Fig. 1). In animals made iron deficient by venesection, duodenal absorption increases to 45.30±13.19% (1.359 μg, n = 10) and in iron-loaded animals decreases to 13.20±0.89% (0.066 μg, n = 10). Similarly, when the amount of iron presented to the lumen is increased, percentage absorption decreases but the absolute amount of iron transferred to the carcass rises: 0.82±0.20 μg iron for a 50-μm (3 μg) dose of iron (n = 10); 9.75 μg for a 500-μM (30 μg) dose of iron (n = 5); and 61.35 μg for a 5-mM (300 μg) dose of iron (n = 3). Based on the amount of iron consumed by each rat in a 24-h period, all subsequent studies were carried out in the physiological dose range of 0.05 mM (3 μg) of iron as radiolabeled ferrous ascorbate.

Iron uptake by isolated cells. There is a constant, instantaneous uptake of ~0.3 μg iron (10% of total dose) in all cases in the 3-μg dose range (Fig. 2). The isolated cells in suspension initially take up iron rapidly but cease absorbing iron after 30 min. Uptake is completely inhibited at 4°C and is partially inhibited by prior exposure of the cells to cyanide but the initial increment is not affected.

Fractionation of cell homogenates on sucrose density gradient centrifugation. Three iron-containing peaks are found (Fig. 3): (*a*) at d = 1.050–1.068 g/ml, a peak is present equally throughout the small intestine. (*b*) At d = 1.080–1.110 g/ml there is a peak maximal in the duodenum, diminishing in jejunum and absent in distal jejunum and ileum. This peak is less prominent in the in vitro-labeled duodenal cells. (*c*) At d = 1.155–1.180 g/ml there is a peak present throughout the intestine. Recentrifugation of fractions

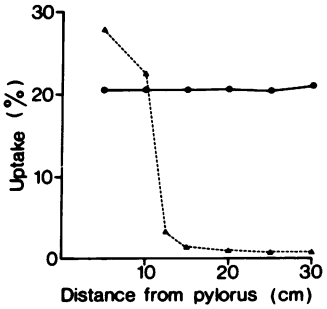

FIGURE 1 Percentage uptake of iron (3 μg, 50 μM dose) by washed loops (● —— ●) and carcass (▲ - - - ▲) from isolated 10-cm intestinal loops at different distances from the pylorus.

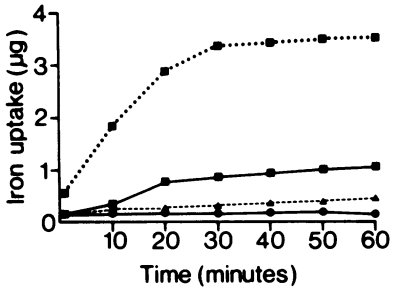

FIGURE 2 Uptake of iron by isolated duodenal mucosal cells in vitro. A dose of 3 μg (50 μM) iron was used in all cases except for (■ ----- ■) in which 9 μg (150 μM) was used. Cells preincubated with 1 mM KCN for 10 min before washing and incubation with iron-ascorbate (▲ - - - ▲); cells were incubated at 37°C (■ —— ■) except for (● —— ●) in which the temperature was 4°C.

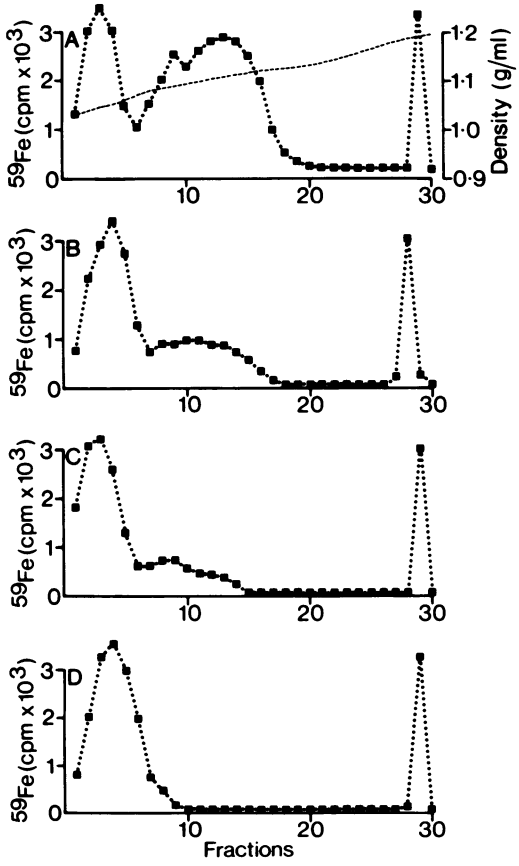

FIGURE 3 Density gradient fractionation of mucosal cell homogenates from A, duodenum; B, proximal jejunum, 15 cm from the pylorus; C, jejunum 25 cm from pylorus; and D, ileum. The sucrose density gradient was determined by direct densitometry on each fraction (- - -). Similar gradients were used in all the other figures. Counts per minute ^{59}Fe (■ ----- ■).

from peaks 1 and 2 indicates that they retain their position, while the activity of peak 3, on addition of Triton X-100, moves quantitatively to the peak 1 position. Addition of 10 mM cold acidic ferrous sulfate to the homogenate does not affect the position of peaks 1 and 3 but shifts the radiolabeled peak 2 at d = 1.080–1.110 g/ml to d ~1.095–1.110 g/ml without displacing the radiolabel (Fig. 4). The ferritin antigen shifts together with the radiolabel (not shown).

Identification of iron-binding fractions by radioimmunoassay in rats on a normal iron diet (Fig. 5). Transferrin is found in peaks 1 and 3. The transferrin content of peak 3 is 9.91±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 is 2.88±0.69% (n = 3). This difference is significant ($P < 0.05$). Transferrin levels in duodenum and ileum are very similar: 4.25±1.16 μg transferrin/mg protein (n = 19) in the duodenum and 4.32±0.09 μg transferrin/mg protein (n = 19) in the ileum.

Ferritin is present predominantly in peak 2, with a density between 1.080 and 1.110 g/ml. Small amounts are present in peak 1 (d = 1.050–1.070 g/ml) and in the high density region (d = 1.150–1.170 g/ml. Ferritin levels, expressed in micrograms of antigen per milligram total protein, are 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). The specific radioactivity of ferritin in the duodenum, when measured in the basal state is 56,500 cpm ^{59}Fe/μg ferritin.

The effects of iron deficiency induced by venesection (Fig. 6). The venesection program alters the need for iron absorption by reducing body iron stores and stimulating erythropoiesis (Table I). In these animals carcass absorption from a 10-cm segment is increased

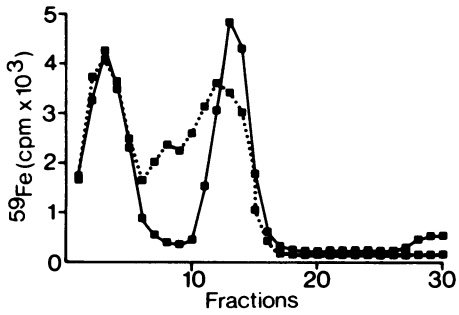

FIGURE 4 Density gradient fractionation of ^{59}Fe-loaded duodenal cells homogenized in saline (■ ----- ■) and homogenized with 10 mM iron ascorbate (■ —— ■). The density gradients are the same as in Fig. 3. Note that peak 3 is missing as a result of filtration of the large particles during passage of the homogenate through Sephadex G-25 to remove free iron and low molecular weight compounds.

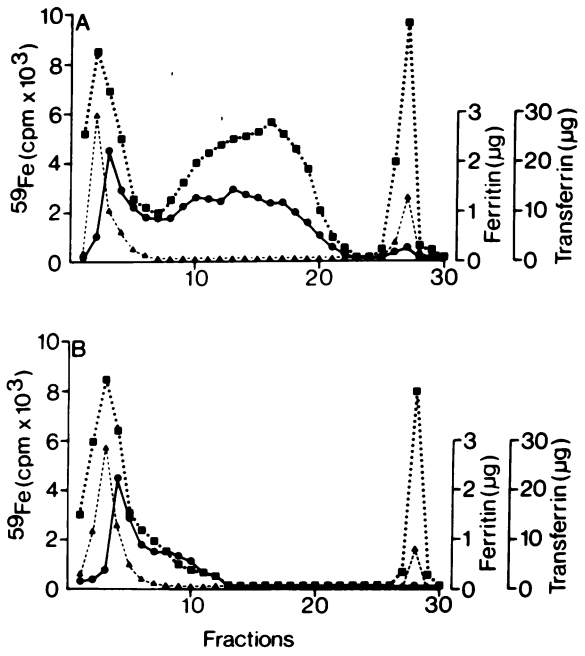

FIGURE 5 Density gradient fractionation of mucosal homogenate from normal iron-load animals. A, Duodenum; B, Ileum. Counts per minute ^{59}Fe (■ ----- ■), transferrin (▲ - - - ▲), and ferritin (● —— ●). The density gradients are the same as in Fig. 3.

from 27.4±3.29% in the normal animal to 45.3±13.19% (n = 10), from a 10-cm duodenal segment.

Considering the iron-binding profiles, peaks 1 and 3 are increased and peak 2 reduced in mucosal cell homogenates of the iron-deficient animals (Fig. 6) compared to normal (Fig. 5). Transferrin levels are increased to 9.66±1.22 μg transferrin/mg protein in the duodenum and 8.65±1.09 μg transferrin/mg protein in the ileum (n = 4). Ferritin levels decrease in iron deficiency to 0.031±0.01 μg ferritin/mg protein (n = 3) in the duodenum and 0.019±0.005 μg ferritin/mg protein in the ileum. The transferrin content of peak 3 in relation to peak 1 is also increased to 23.11±1.35% (n = 3) in the duodenum and 9.50±2.22% (n = 3) in the ileum. The specific radioactivity of ferritin, however, is found to increase to 84,900 ^{59}Fe cpm/μg ferritin.

The effects of iron loading (Fig. 7). In iron-loaded animals carcass absorption is decreased to 2.20±0.8% (n = 10) from duodenal segments. Transferrin levels are found to be similar to those in the normal animal: 3.86±0.74 μg/mg total protein (n = 4) in the duodenum and 3.51±1.02 μg transferrin/mg total protein (n = 4) in the ileum. The transferrin content of peak 3 is 4.42±0.51% (n = 3) that of peak 1 in the duodenum and 0.89±0.25% (n = 3) in the ileum. Ferritin levels are increased to 3.98±2.59 μg ferritin/mg protein

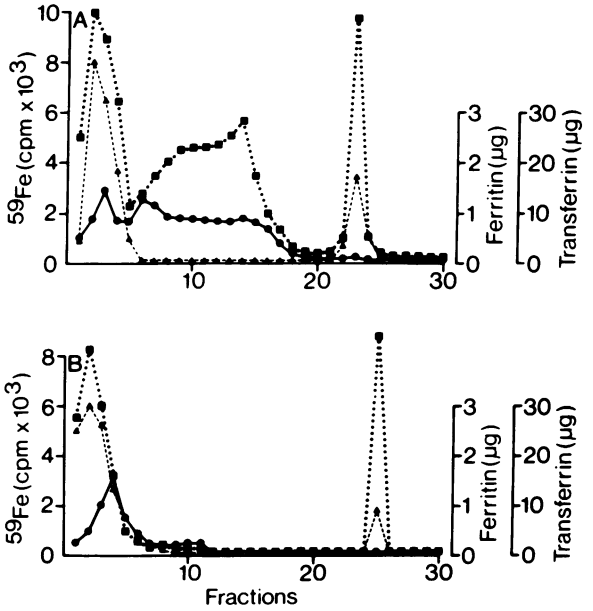

FIGURE 6 Density gradient fractionation of mucosal homogenate from animals made iron deficient by venesection. Counts per minute ^{59}Fe (■ ----- ■), transferrin (▲ - - - ▲) and ferritin (● —— ●). The density gradients are the same as in Fig. 3.

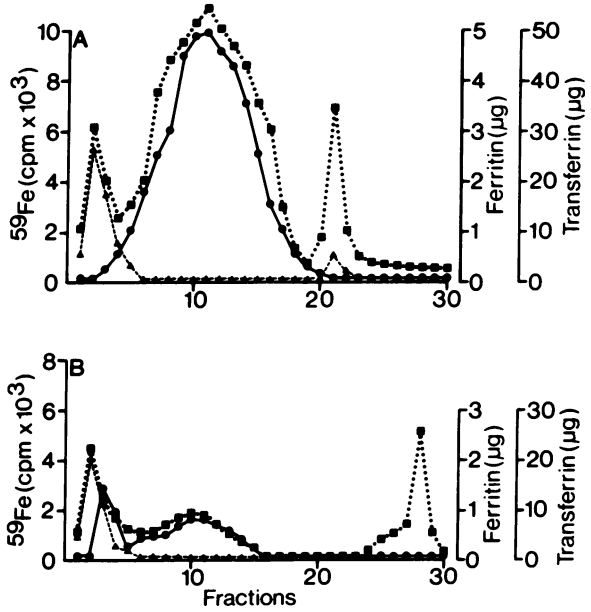

FIGURE 7 Density gradient fractionation of mucosal homogenate from animals loaded with iron. A, Duodenum; B, Ileum. Counts per minute ^{59}Fe (■ ----- ■), transferrin (▲ - - - ▲), and ferritin (● —— ●). The density gradients are the same as in Fig. 3.

(n = 4) in the duodenum and 0.33±0.09 μg ferritin/mg protein in the ileum (n = 3). The specific radioactivity of ferritin is, however, reduced to 7,016 ^{59}Fe cpm/μg ferritin.

Gel filtration. Gel filtration of the membrane-free mucosal homogenate yields three peaks (Fig. 8C). The first peak has a molecular weight of ~400,000, runs in the position of ferritin on polyacrylamide gel electrophoresis and was confirmed as this protein by radioimmunoassay and immunoabsorption. The second peak has a molecular weight of ~90,000, runs 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, <10,000, does not stain for protein on polyacrylamide gel electrophoresis and does not react in radioimmunoassay or immunoabsorption against either transferrin or ferritin antiserum. Gel filtration of density gradient peak 1 shows a transferrin peak and a large low molecular weight peak (Fig. 8B). Peak 2 yields a ferritin peak and a smaller low molecular weight peak (Fig. 8A), while peak 3 (not shown) yields a small amount of ferritin, transferrin and low molecular weight compounds.

Immunoabsorption. Transferrin-specific immunoabsorption of the membrane-free homogenate removes 56.84±7.28% of peak 1 (n = 4). Ferritin-specific immunoabsorption removes 89.25±0.21% of peak 2 (n = 4). The results were confirmed by radioimmunoassay (Fig. 9).

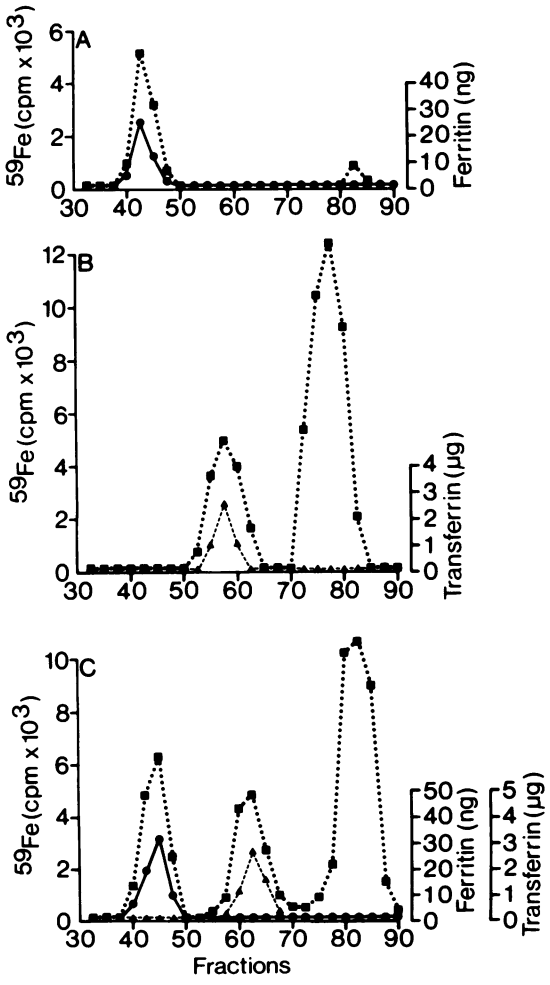

FIGURE 8 Gel filtration of the membrane-free mucosal homogenate. A, gel filtration of density gradient peak 2; B, gel filtration of density gradient peak 1 and C, gel filtration of membrane-free homogenate. Counts per minute ^{59}Fe (■ ----- ■), transferrin (▲ – – – ▲), and ferritin (● —— ●).

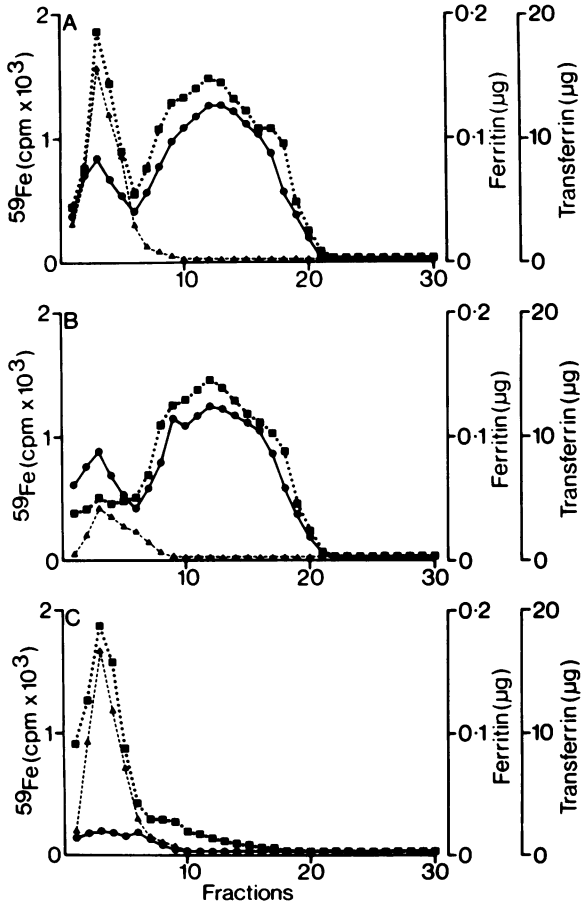

FIGURE 9 Density gradient fractionation of eluates after ferritin and transferrin-specific immunoabsorption. A, Starting material; B, anti-transferrin immunoabsorbent; and C, anti-ferritin immunoabsorbent. Counts per minute ^{59}Fe (■ ----- ■), transferrin (▲ – – – ▲), and ferritin (● —— ●). The density gradients are the same as in Fig. 3.

Immunological comparison of transferrins. The transferrin radioimmunoassay, using labeled serum transferrin, was standardized with rat serum transferrin and potency estimates were obtained from five doubling dilutions of serum and mucosal homogenates. Using the parallel line method of Rodbard and Lewald (24) the serum transferrin was shown to have an identical slope to purified serum transferrin but there was a highly significant difference ($P < 0.001$) between the slopes of serum and mucosal transferrin (Fig. 10). Mixtures of serum and mucosal extracts yield an intermediate slope predictable for unmodified components. Constant conditions for both samples were maintained in terms of proteolytic inhibitors, Triton X-100 and buffer used. No difference is found between the transferrins of duodenal and ileal mucosal extracts nor between transferrin from peaks 1 and 3. The differences between serum and mucosal transferrin would be greater if part of the mucosal transferrin were not due to serum contamination.

Serum contamination of mucosal extracts. Using the method of intravenous injection of biologically screened proteins the mean contaminating serum volume per 5-cm segment, as estimated by the ^{125}I-transferrin method, in 12 segments in two rats, is found to be 8.64±1.41 μl (8.73±1.07 μl in rat 1 and 8.56±1.47 μl in rat 2). The ^{125}I-alpha-fetoprotein method yields a mean contaminating serum volume of 9.68±2.04 μl per 5-cm segment (no significant difference, $P < 0.05$). Iodinated biologically screened serum added to cells in vitro yields a contaminating volume of 7.99±2.13 μl/5-cm segment. Serum transferrin levels ranged from 1.04 to 2.10 mg/ml, with a mean of 1.56 mg/ml ($n = 15$). Using the mean level a contaminating serum volume of 8.64 μl would imply that 13.48 μg of transferrin would be accounted for as serum contamination. The mucosal cell homogenates contained on average 55.4±12.2 μg transferrin/5-cm segment ($n = 39$), ranging from 28.7 to 74.1 μg transferrin/5-cm 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 but this does not preclude the possibility that serum transferrin can become intrinsic to the mucosal cell in the course of the experiment.

Trypsin digestion of mucosal cells. Whole cells digested with trypsin show a small reduction in transferrin content measured by radioimmunoassay (Table II), mean 9.83 μg transferrin per cells from a 5-cm segment. This is equivalent to a volume of serum of 6.55±1.7 μl. Transferrin itself is undetectable by radioimmunoassay after trypsin digestion and transferrin added to cells is also completely accessible to trypsin and is digested.

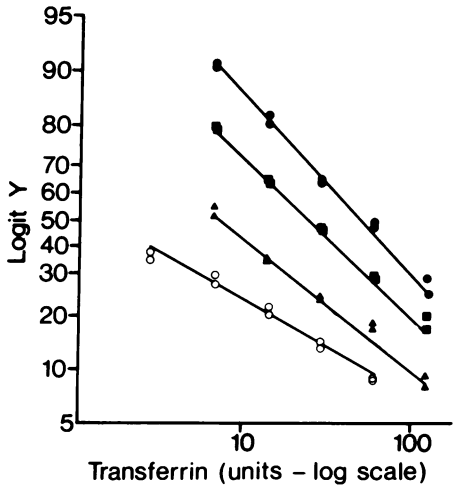

FIGURE 10 Parallel line assays of serial dilutions of rat serum (○ —— ○); duodenal cell homogenate (● —— ●); ileal cell homogenate (■ —— ■); and mixture of serum and duodenal cell homogenate (▲ —— ▲). The F test for homogeneity of variance and the Student's *t* test showed a significant difference ($P < 0.001$) in the slopes of the serum and intestinal transferrins (method of Rodbard and Lewald [24]). The transferrin is in arbitrary units. The ordinate is logit Y (ln Y/1-Y) where Y is bound count/uninhibited count rate).

TABLE II
Trypsin Digestion of Mucosal Cells

	Tf before digestion	Tf after digestion	Difference	Serum equivalent volume
	μg	μg		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	4.13
Cells plus Tf protein	107.39	51.52	55.87	
	118.75	58.29	60.46	
Tf protein alone	52.5	0.00	52.5	
	56.7	0.00	56.7	

Whole mucosal cells were digested with 0.25% trypsin for 1 h at 37°C and aliquots were homogenized and assayed for transferrin (column 2). Aliquots retained before digestion were also homogenized and assayed (column 1). The difference 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 1.50 mg/ml. As controls ~50 μg transferrin was added to cells and the same amount of pure protein was also digested under the same conditions. The added transferrin is completely accessible to trypsin.

Metabolic inhibitors. Both cytochalasin-B and colchicine abolish the transfer of iron from the gut lumen to the blood. The uptake of iron by peak 2 is also abolished (Fig. 11), while the transferrin content of peak 3 is significantly reduced to 1.49±0.74% of peak 1 ($n = 5$) in the duodenum ($P < 0.01$). Cyanide reduces but does not abolish uptake into ferritin.

DISCUSSION

The demonstration of the intestinal gradient of absorption, with maximal absorption occurring in the proximal 10–15 cm, as shown by blood and carcass activity, confirms previous reports (2, 3) and validates the isolated loop method used in this study. In contrast, uniform adsorption or nonspecific binding of iron occurs in all regions of the gut. Iron uptake by isolated cells shows a rapid initial adsorption that may be related to the uniform mucosal uptake by washed loops of bowel (18). This may represent tight binding on the membrane, deeper structures or even internalization of the iron. The finding that the uptake by cells was only partially inhibited by cyanide and low temperature concurs with the observations of Greenberger et al. (25) and Savin and Cook (18) and supports their conclusion that iron uptake by brush borders is probably not energy dependent. We may thus distinguish quite clearly between (*a*) adsorption, i.e., uptake into or adhesion onto the cell of iron, and (*b*) absorption, i.e., the transfer of iron across the mucosa to the bloodstream (26). Cells from various levels of the intestine show different iron-binding profiles on fractionation but loading of ferritin with radioiron is found only in iron-absorbing areas. Since the techniques used to study absorption of iron appear to be physiologically valid, the dispersion of iron in the various components is likely to have significance for the mechanism of absorption.

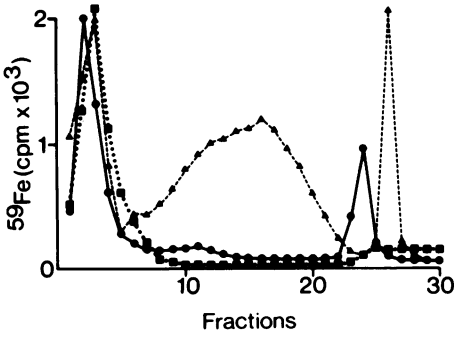

FIGURE 11 Density gradient fractionation of cells from mucosa pretreated with 10 mM colchicine for 2 h (■ ----- ■); 5 mM cytochalasin-B for 15 min (● —— ●); and control (▲ – – – ▲). The density gradients are the same as in Fig. 3.

The mucosal proteins show different immunological reactivities compared with those with which the radioimmunoassays were standardized; the results therefore are not quantitative. The values obtained, however, fall within the same range as those reported by Savin and Cook (10), using an immunoradiometric assay, and Osterloh and Forth (27), using radial immunodiffusion. The problem of the quantitation of the 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 contribution and an equivalent amount was shown to be inaccessible to proteolysis. The presence of a mucosal transferrin, differing in amino acid composition and isoelectric behavior (12) in the rat, and not precipitated by serum transferrin antiserum (11) in the guinea pig, has been described. Although unable to confirm the isoelectric difference (unpublished), we found a very significant difference in immunological reactivity between serum and mucosal transferrin, the latter having at least a twofold higher affinity for the antiserum. This difference was maintained in mixtures of serum and mucosa and appears to be a stable property of the protein. It was not due to aggregation, organellar binding, proteolysis, or other interference with the assay. The structure and properties of mucosal transferrin are not known at this stage. Transferrin levels were found to increase in the mucosa of iron-deficient animals, as reported (12, 27, 28), in conjunction with an increase in the amount of iron absorbed. It thus appears possible that the mucosal-specific transferrin may be involved in the absorption process. The increase is however not restricted to cells of the iron-absorbing region of the intestine.

Ferritin levels in cells reflect the iron status of the animal: ferritin, however, is present in even greater amounts in the iron-absorbing regions. Iron-loading of ferritin does not occur in non-iron-absorbing areas, with the exception of the iron-loaded ileum, suggesting perhaps a regulatory role for ferritin in iron absorption. Treatment of cells with microtubular poisons inhibits ferritin loading completely. Microtubules are implicated in vesicle movement in cells (29, 30) and have been shown to be involved in the uptake of transferrin by reticulocytes (31). The presence of high density transferrin, i.e., membrane or organelle associated, in amounts correlated with the absorptive capacity of the cell, suggests that vesicular movement may be important in iron absorption. The free diffusion of small molecular weight iron chelates to the site of absorption

within the cell seems to be ruled out since this should be independent of cytoskeletal activity.

In order to rationalize our data we consider that the findings of this study could be consistent with an hypothesis that iron absorption is primarily regulated by the degree of saturation of the transferrin that is brought into the cells in vesicles from the plasma at some stage, perhaps bound to specific membrane receptors. This partially saturated transferrin is loaded with newly adsorbed iron that had been tightly bound to pinocytosed villous membrane. This would require fusion of vesicles from the opposite poles of the cell and subsequent selective release of the iron-loaded transferrin into the blood stream. However, although part of the mucosal transferrin may be intrinsic, its role is unclear and we have also not been able to identify the small high-specific activity and/or high-flux transferrin pool that this hypothesis would require. The determining factors for the site specific localization of iron absorption in the intestinal mucosa would then have to depend on an anatomically localized vesicle fusion process since the transferrin uptake into the mucosal cell and membrane iron binding occur everywhere in the small intestine. Considerably more information is therefore needed to substantiate an hypothesis of this type.

In conclusion, it appears that transferrin and ferritin are the only major iron-binding proteins involved in absorption. A role for a low molecular weight substance and possible uptake by nonprotein components of the mitochondria seems unlikely but cannot be discounted. We have demonstrated the presence of a mucosal cell-specific transferrin, the nature of which is as yet unknown, and suggest a possible role for transferrin, in association with specific receptors on vesicles, in iron absorption. Ferritin loading is exactly correlated with absorption and may therefore have a direct role in the regulation of absorption or on the other hand at least define the site of ferritin loading as occurring at the precise point of iron absorption wherever that is.

ACKNOWLEDGMENTS

The assistance received from the South African Medical Research Council, the Atomic Energy Board, the University of Cape Town Leukemia Centre, the University Staff Research Fund and the Hausmann Laboratory (Switzerland) are gratefully acknowledged. The technical assistance of N. Linton was indispensable to the project. We thank Professor M. C. Berman for access to facilities in the Department of Chemical Pathology.

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