Alterations in the mucosal processing of iron in response to very-short-term dietary iron depletion and repletion

Richard W. TOPHAM,* Cynthia E. EADS and Bonnie F. BUTLER

Department of Chemistry, University of Richmond, Richmond, VA 23173, U.S.A.

The transfer of control rats to a low-iron diet for only 24 h resulted in a 2-fold increase in iron uptake by brush-border membrane vesicles. Extension of the low-iron feeding period to 72 h or 2 weeks resulted in only small additional increases in iron uptake by vesicle preparations. In contrast, the transfer of iron-deficient rats to a control diet resulted in a progressive decrease in iron uptake by vesicles that reached a level equivalent to that of control rats in 2 weeks. ^{59}Fe labelling of detergent extracts of these vesicle preparations provided evidence for the presence of an iron-binding protein composed of subunits of 52000 Da. The changes in the ^{59}Fe labelling of this protein component were consistent with the changes observed in iron uptake by intact brush-border membrane vesicles. The ^{59}Fe -labelling profiles of mucosal ferritin and transferrin from a test dose also were changed substantially in response to very-short-term alterations in dietary iron. Even though changes in dietary iron rapidly altered iron uptake by brush-border membrane vesicles and the incorporation of ^{59}Fe from the test dose into mucosal transferrin, changes in the incorporation of ^{59}Fe into mucosal ferritin best reflected the actual changes in the transfer of iron from dose to plasma.

INTRODUCTION

In contrast with many other trace elements, no regulated excretory mechanism exists for iron in mammals. Consequently, iron homeostasis in mammals is controlled by the regulation of the absorption of dietary iron and not by excretion (McCance & Widdowson, 1937). It has been known for many years that intestinal mucosal cells rapidly adjust to decreases in body iron stores to facilitate the absorption of a greater fraction of the dietary iron (Bannerman *et al.*, 1962). Although extensively studied, the molecular events that lead to the increased iron absorption are still unclear (Flanagan, 1989).

It has been demonstrated that intestinal iron absorption occurs in distinct phases that can be separated from one another (Manis & Schachter, 1962); however, controversy exists concerning the relative importance of these in the regulation of iron absorption. The first phase entails the transport of iron into the mucosal cell via the brush-border membrane. On the basis of studies with brush-border membrane vesicles prepared from normal and severely iron-deficient mice, it has been proposed that increases in this step of iron absorption could be solely responsible for the increased iron absorption known to occur in iron deficiency (Muir *et al.*, 1984; Muir & Hopfer, 1985). Evidence has been reported for the possible involvement of protein and lipid components as mediators of iron transport across the brush-border membrane (Huebers *et al.*, 1983; Idzerda *et al.*, 1986; Simpson & Peters, 1986, 1987; Stremmel *et al.*, 1987; Simpson *et al.*, 1988, 1989).

Additional steps in iron absorption involve the processing of iron within the mucosal cell, with the eventual transfer of a fraction of the mucosal iron to plasma. Wheby & Crosby (1963) first postulated that the transfer of iron from within the cell to plasma was the primary regulatory step in iron absorption, and experiments with normal and iron-deficient rats have provided support for this proposal (Linder & Munro, 1977). Once inside the mucosal cell, a number of studies indicate that most of the iron becomes bound to one of two proteins, mucosal ferritin and a modified form of plasma transferrin (Worwood & Jacobs, 1972; Huebers *et al.*, 1976; Johnson *et al.*, 1983; Purves *et al.*,

1988). Studies monitoring the labelling pattern of these proteins with ^{59}Fe from a test dose in control and iron-deficient animals have suggested that these proteins could be involved in the regulation of dietary iron absorption (El-Shobaki & Rummel, 1977; Savin & Cook, 1980; Topham *et al.*, 1985). On the basis of these studies, mucosal ferritin was postulated to bind the iron not rapidly transported to plasma and to serve as a reservoir of iron for the mucosal cell. Mucosal transferrin was proposed as a possible vehicle for the transport of iron from within the mucosal cell to plasma. Transferrin receptors have been detected in the basal-lateral membrane of the mucosal cell, and iron deficiency is accompanied by an increase in these receptors in duodenal absorptive cells (Banerjee *et al.*, 1986). More recently, evidence has been presented for an iron-binding protein in the duodenal mucosa of rats in addition to mucosal ferritin and transferrin, but its function is not yet known (Conrad *et al.*, 1990).

In the studies described in the present paper, rats raised on a control diet with normal tissue iron stores were fed on a low-iron diet for 24 h, 72 h, and 2 weeks, and rats raised on a low-iron diet with greatly decreased tissue iron stores were fed on a control diet for identical time intervals to determine the speed and degree of co-ordination of the changes that occur in the uptake of iron by brush-border membrane vesicles and in the distribution of iron between iron-binding proteins of the mucosal cytosol, and to explore the molecular bases for these changes.

MATERIALS AND METHODS

Animals and diets

Weanling male Fischer rats (CDF strain; Charles River Laboratories, Wilmington, MA, U.S.A.) were raised for 6 weeks on a control or low-iron diet. After 6 weeks, rats raised on the control diet were transferred to the low-iron diet for 24 h, 72 h or 2 weeks. Similarly, rats raised on the low-iron diet for 6 weeks were transferred to the control diet for 24 h, 72 h, and 2 weeks. The control and low-iron diets had identical compositions (American Institute of Nutrition-76 rat diet; ICN Biomedicals, Cleveland, OH, U.S.A.), except that the low-iron diet had no

* To whom correspondence should be addressed.

iron salt in the mineral mixture. For each diet and time interval, at least eight animals were analysed.

Preparation of brush-border membrane vesicles, and iron-uptake studies

Brush-border membrane vesicles were prepared with minor modifications to the procedure previously described (Simpson & Peters, 1984). Specifically, mucosal tissue scrapings from rats from each diet group were suspended in 50 mM-mannitol/2 mM-Hepes buffer, pH 7.1 (28 ml/g of tissue) and homogenized for 2 min at high speed in a Waring blender. Sufficient solid MgCl_2 was added to give a concentration of 10 mM. After standing at 4 °C for 20 min, the homogenate was centrifuged for 10 min at 4 °C and 3000 *g*. The supernatant was carefully decanted and centrifuged for 30 min at 4 °C and 27000 *g*. The pelleted vesicles were resuspended uniformly in cold 0.1 M-mannitol/0.1 M-NaCl/0.1 mM- MgSO_4 /20 mM-Hepes/NaOH, pH 7.4 (20 ml/g of original tissue weight) by passage of the suspension through a 25-gauge needle three times. Buffers were always filtered through a 0.2 μm Millipore filter before use. After resuspension, the vesicle preparation was centrifuged for 15 min at 4 °C and 6000 *g*. The supernatant was carefully removed and centrifuged for 30 min at 4 °C and 27000 *g* to collect the vesicles. The vesicle pellet was resuspended in 3 ml of the resuspension buffer by passage through a 25-gauge needle twice. The protein concentration of the final suspension of vesicles was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with bovine γ -globulin as standard. The volume of the vesicle preparation was adjusted with the resuspension buffer to yield a protein concentration of 4–5 mg/ml. Freshly prepared vesicles were used for all iron-uptake studies.

Iron-uptake studies with these brush-border membrane vesicle preparations were conducted with minor modifications to the protocol previously reported (Simpson & Peters, 1987). The stock ^{59}Fe -labelled solution had a final Fe^{2+} concentration of 1200 μM , with 3 μCi of $^{59}\text{FeSO}_4$ added per 10 ml and a 20-fold molar excess of ascorbic acid to maintain the iron in the Fe^{2+} state. This stock solution was prepared fresh daily, and the pH of this solution was adjusted to 7.4 immediately before use. In all iron-uptake studies, the final incubation volume was 140 μl and incubations were conducted at 37 °C. After each incubation, duplicate 50 μl portions of the incubation mixture were filtered through 0.2 μm membrane filters (Supor-200; Gelman Sciences, Ann Arbor, MI, U.S.A.). The isolated vesicles were immediately rinsed with an ice-cold stop solution consisting of 0.1 mM- FeCl_3 and 100 mM-citrate, pH 7.0. The filters were immersed in 10 ml of scintillation cocktail and counted for ^{59}Fe radioactivity. To compensate for any non-vesicle binding of ^{59}Fe to the membrane filters, controls were run for each assay by substituting buffer for the vesicle preparation.

Extraction of iron-binding components from brush-border membrane vesicles

Sufficient Triton X-100 (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to 5 ml samples of brush-border membrane vesicles (5 mg of protein/ml) to yield a final concentration of 1%. The samples were gently incubated at 4 °C for 1 h and then centrifuged for 1 h at 100000 *g*. To the supernatant was added 1 ml of a ^{59}Fe -labelled stock iron solution (3.48 ml of 3.44 mM- FeSO_4 , 4 ml of 60 mM-ascorbic acid, 3 μCi of $^{59}\text{FeSO}_4$, adjusted to pH 7.4, and made to a final volume of 10 ml with distilled water). These ^{59}Fe -labelled samples were incubated at 37 °C for 1 h, centrifuged at 25000 *g* for 20 min, and dialysed overnight at 4 °C to remove unbound iron. To the dialysed sample, 1 g of SM-2 Bio-Beads (Bio-Rad) was added to remove the Triton X-100. This sample was left overnight at 4 °C, and then the liquid was

carefully removed from the beads. A 0.5 ml portion of this sample was placed on a column of Sepharose CL-6B (1 cm $\times$ 47 cm) pre-calibrated with proteins of known molecular mass (MW-GF-1000 kit; Sigma). The eluent for this column was the same buffer used in the original resuspension of the brush-border membrane vesicles. Fractions of 30 drops were collected, and 0.1 ml of each fraction was counted for ^{59}Fe content.

Iron status

The non-haem iron content of a liver sample from each rat was determined by the method of Torrance & Bothwell (1968). Total blood haemoglobin and total serum iron were analysed with diagnostic kits 525 and 565 from Sigma.

Radioiron dosing, preparation of mucosal cytosol, and separation of mucosal ferritin and transferrin

Rats from each diet group were given 4 μCi of $^{59}\text{FeSO}_4$ in 2 ml of 0.005 M-HCl per 200 g body wt. by intragastric tube. After 30 min exposure to the dose, a blood sample was obtained from each rat and the entire small intestine was excised. Duplicate 0.5 ml portions of the blood plasma samples were counted for ^{59}Fe content. The mucosal tissue was immediately removed from the entire length of the small intestine, and a 20% (w/v) homogenate of this tissue was prepared in 0.05 M-Hepes buffer, pH 7.4. These homogenates were initially centrifuged for 20 min at 15000 *g* and 4 °C. The resulting supernatants were filtered through glass-wool to remove any floating lipid material and then centrifuged for 1 h at 125000 *g* and 4 °C to obtain cytosol. Duplicate 0.5 ml portions of the mucosal cytosol preparations were counted for radioactivity to determine the ^{59}Fe content.

In previous studies, mucosal ferritin and transferrin have been effectively separated by gel filtration of mucosal cytosol (Huebers *et al.*, 1976; El-Shobaki & Rummel, 1977; Topham *et al.*, 1985). In the present studies, the mucosal ferritin and transferrin in the cytosol of rats given ^{59}Fe were separated by gel filtration of a 5 ml sample of cytosol on a column (2.5 cm $\times$ 63 cm) of Sepharose CL-6B. The elution buffer was 0.05 M-Hepes, pH 7.4. Protein elution from the column was continuously monitored at 280 nm, and 6.3 ml fractions were collected. A portion (0.3 ml) of each fraction was counted for radioactivity to determine its ^{59}Fe content. The elution positions of ferritin and transferrin were established by passage of authentic samples of these proteins through the column.

Polyacrylamide-gel electrophoresis

In a previous study, an electrophoretic technique was used to estimate the amounts of mucosal ferritin and transferrin in mucosal cytosol samples (Topham *et al.*, 1982). In the present study, cytosol samples containing the same total amount of protein (280 μg) from each rat group were subjected to electrophoresis in 8.5%-polyacrylamide gel slabs. Samples of purified horse spleen ferritin (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.) and purified rat plasma transferrin (Cappel Research Products, West Chester, PA, U.S.A.) were also loaded on these gels to aid in identifying the bands corresponding to these proteins. After electrophoresis, these gels were cut into two sections. One section was stained with Coomassie Blue, and the other section was treated with a specific stain for iron-containing proteins that is sensitive to nanogram levels of iron (Kuo & Fridovich, 1988).

RESULTS

Comparison of iron status of dietary animal groups

As shown in Table 1, the control rats transferred to the low-iron diet for 24 and 72 h had normal blood haemoglobin

Table 1. Iron status of test animals

Each animal group consisted of eight male Fischer rats (CDF strain, Charles River Laboratories).

Animal group	Liver non-haem iron ($\mu\text{g/g}$ of tissue)	Total blood haemoglobin (g/dl)	Total serum iron ($\mu\text{g/dl}$)
Raised on control diet	73.8 $\pm$ 10.6	16.3 $\pm$ 0.557	96.4 $\pm$ 6.18
24 h on low-iron diet	75.9 $\pm$ 9.25	16.4 $\pm$ 1.54	103 $\pm$ 4.27
72 h on low-iron diet	70.6 $\pm$ 7.35	17.0 $\pm$ 0.515	99.2 $\pm$ 6.20
2 weeks on low-iron diet	51.7 $\pm$ 8.60	15.9 $\pm$ 0.784	75.8 $\pm$ 6.82
Raised on low-iron diet	20.2 $\pm$ 0.949	10.3 $\pm$ 0.691	79.9 $\pm$ 5.29
24 h on control-iron diet	23.3 $\pm$ 1.76	11.3 $\pm$ 1.38	84.2 $\pm$ 2.49
72 h on control-iron diet	21.6 $\pm$ 1.58	12.1 $\pm$ 0.956	87.1 $\pm$ 3.04
2 weeks on control-iron diet	45.3 $\pm$ 3.30	16.1 $\pm$ 0.374	116 $\pm$ 10.9

concentrations, total serum iron concentrations and liver non-haem iron stores. After 2 weeks on the low-iron diet, the blood haemoglobin concentration was still virtually normal, but the total serum iron concentration and the liver non-haem iron stores had decreased to 75% and 70% of the controls respectively. Rats raised on the low-iron diet that were transferred to the control diet for 24 h and 72 h showed virtually no increase in liver non-haem iron stores and only small increases in blood haemoglobin and total serum iron concentrations. However, after 2 weeks on the control diet, these rats had blood haemoglobin and total serum iron concentrations equal to the control rats, but the liver non-haem iron content had increased only to 60% of the controls.

Comparison of mucosa and plasma contents of an intragastric dose of ^{59}Fe

As shown in Fig. 1(a), 30 min after intragastric administration of ^{59}Fe , the rats transferred to the low-iron diet for various time intervals showed markedly increased ^{59}Fe in plasma and slightly decreased retention of ^{59}Fe in mucosal cytosol compared with controls. Most of the change in both of these factors occurred within 72 h after transfer to the low-iron diet.

When the rats raised on the low-iron diet were transferred to the control diet (Fig. 1b), some decrease in the transfer of ^{59}Fe from dose to plasma was observed. However, even after 2 weeks on the control diet, rats raised on the low-iron diet still exhibited a greatly elevated transfer of ^{59}Fe from the intragastric dose to plasma compared with rats raised on the control diet, and only a small increase in the ^{59}Fe retained in the mucosal cytosol.

Comparison of iron uptake by brush-border membrane vesicles

Fig. 2 illustrates the iron uptake with respect to incubation time and iron concentration by comparable amounts of brush-border membrane vesicles from control rats and control rats fed on the low-iron diet for various time intervals. The iron uptake by vesicles from rats fed on the low-iron diet for only 24 h was approximately twice that of vesicles from control rats. Extending the feeding period of the low-iron diet to 72 h and 2 weeks led to increases in iron uptake that were only slightly greater than the increase observed with vesicles from rats transferred to the low-iron diet for 24 h. These results suggest that the adjustment that

Fig. 1. Transfer of ^{59}Fe from the test dose to plasma

Four rats from each diet group were given 4 μCi of $^{59}\text{FeSO}_4$ in 2 ml of 0.005 M-HCl per 200 g body wt. by intragastric tube. After 30 min exposure to the dose, blood samples were obtained and mucosal cytosol was prepared as described in the Materials and methods section. Duplicate 0.5 ml portions of blood plasma (●) and mucosal cytosol (○) were counted for radioactivity to determine ^{59}Fe content. (a) Rats raised for 6 weeks on the control diet and then transferred to the low-iron diet for 24 h, 72 h and 336 h. (b) Rats raised for 6 weeks on the low-iron diet and then transferred to the control diet for 24 h, 72 h and 336 h.

results in increased iron uptake by the brush-border membrane in response to decreased dietary iron is extremely rapid and essentially complete after only 24 h of exposure to dietary iron depletion.

As shown in Fig. 3, when rats raised on the low-iron diet were transferred to the control diet, the iron uptake of brush-border membrane vesicle preparations progressively decreased with respect to the length of time for which the animals were given the control diet. The iron uptake by vesicles after 2 weeks of feeding of the control diet was decreased to a level equal to that in the vesicles from the rats raised on the control diet.

Saturation kinetics were observed for iron uptake with respect to the Fe^{2+} concentration for all diet groups.

Extraction of iron-binding components from brush-border membrane vesicles

Brush-border membrane vesicles from rats in all diet groups were extracted with 1% Triton X-100. After removal of unsolubilized material by centrifugation, the extracts were labelled with ^{59}Fe . The Triton was removed from the preparations, and the Triton-free samples were subjected to gel filtration on a Sepharose CL-6B column that had been calibrated with protein molecular-

Fig. 2. Iron-uptake studies with brush-border membrane vesicles from control rats transferred to the low-iron diet

Brush-border membrane vesicles were prepared and iron uptake analysed as described in the Materials and methods section. (a) In the time-course study of iron uptake, each assay contained 22 μg of vesicle protein and 430 μM-Fe(II). (b) In the study of the effect of Fe(II) concentration on iron uptake, each assay contained 22 μg of vesicle protein and a Fe(II) concentration of 54, 107, 215 or 430 μM. All data points in (a) and (b) represent means of three separate measurements. Key: □, 2 weeks on low-Fe diet; ■, 72 h on low-Fe diet; ○, 24 h on low-Fe diet; ●, rats raised on control-Fe diet.

mass standards. As illustrated in Fig. 4(a), samples from vesicles of rats raised on the control diet showed only one major ^{59}Fe -labelled peak, which was eluted at the void volume for the column. In contrast, samples from vesicles of similar rats transferred to the low-iron diet for 24 or 72 h had a second ^{59}Fe -labelled peak. The ^{59}Fe labelling and the A_{280} (results not shown) associated with this peak increased progressively with respect to the time interval of low-iron feeding.

As demonstrated in Fig. 4(b), rats raised on the low-iron diet also exhibited the second ^{59}Fe -labelled peak. The ^{59}Fe labelling and the A_{280} (results not shown) of this peak decreased progressively in the low-iron rats switched to the control diet for 24 and 72 h, and virtually disappeared from the vesicle extracts from rats returned to the control diet for 2 weeks.

The slight difference in the fraction number for the elution of the ^{59}Fe -labelled peaks between Figs. 4(a) and 4(b) was the result of a small difference in the height of the Sepharose CL-6B column used in the two experiments and not the result of any

Fig. 3. Iron-uptake studies with brush-border membrane vesicles from iron-deficient rats transferred to the control diet

The experimental protocol was identical with that described for Fig. 2. (a) Time-course study of Fe(II) uptake by brush-border membrane vesicles. (b) Effect of Fe(II) concentration on iron uptake by brush-border membrane vesicles. All data points in (a) and (b) represent means of three separate measurements. Key: □, rats raised on low-Fe diet; ■, 24 h on control-Fe diet; ○, 72 h on control-Fe diet; ●, 2 weeks on control-Fe diet.

inherent difference in the size of the ^{59}Fe -labelled peaks. Calibration of the column used in each experiment with molecular-mass standards indicated that this second ^{59}Fe -labelled component had a molecular mass of approx. 216 000 Da. In each experiment, the fractions composing this second ^{59}Fe -labelled peak were combined and concentrated, and samples of the concentrated material were subjected to SDS/PAGE. A single protein band with an apparent molecular mass of 52 000 Da was detected when stained with Coomassie Blue. This finding suggests that this iron-binding protein is most likely a tetramer composed of four identical subunits.

Comparison of the intracellular distribution of radioiron between mucosal ferritin and transferrin

Rats in each diet group were administered an intragastric test dose of ^{59}Fe for 30 min, and cytosol was prepared from mucosal cell homogenates of each group. The mucosal ferritin and transferrin in these cytosol preparations were separated by gel filtration, and the amount of ^{59}Fe associated with each protein

Fig. 4. Iron-binding components in detergent extracts of brush-border membrane vesicles

^{59}Fe -labelled detergent extracts of brush-border membrane vesicles were prepared as described in the Materials and methods section. After removal of the Triton X-100 with SM-2 Bio-Beads, 0.5 ml of each sample was subjected to gel filtration on a column of Sepharose CL-6B (1 cm $\times$ 47 cm) as described in the Materials and methods section. Protein elution from the column was monitored continuously at 280 nm; 1 ml fractions were collected, and 0.1 ml of each fraction was counted for ^{59}Fe content. (a) Gel filtration of ^{59}Fe -labelled Triton extracts of brush-border membrane vesicles from rats raised on the control diet (●) and rats transferred from the control diet to the low-iron diet for 24 h (○) and 72 h (■). (b) Gel filtration of ^{59}Fe -labelled Triton extracts of brush-border membrane vesicles from rats raised on the low-iron diet (□) and rats transferred from the low-iron diet to the control diet for 24 h (■), 72 h (○) and 2 weeks (●).

was determined. As shown in Fig. 5, the cytosol of control rats had a much greater fraction of the ^{59}Fe from the test dose associated with mucosal ferritin than transferrin. After only 24 h on the low-iron diet, the amount of ^{59}Fe from the test dose incorporated into mucosal ferritin had decreased significantly, and there was an increase in the amount of ^{59}Fe incorporated into transferrin. A large additional decrease in the incorporation of ^{59}Fe into mucosal ferritin, but only a small additional increase in the incorporation of ^{59}Fe into mucosal transferrin, was observed when the low-iron feeding period was extended to 72 h. After 2 weeks of feeding of the low-iron diet, the total fractional decrease (~ 8 -fold) in the ^{59}Fe incorporation into mucosal ferritin

Fig. 5. Intracellular distribution of ^{59}Fe in the cytosol of control rats transferred to the low-iron diet

Mucosal cytosol samples (5 ml) from rats receiving an intragastric dose containing ^{59}Fe (as described in Fig. 1) were subjected to gel filtration (as described in the Materials and methods section) to separate mucosal ferritin (F) and transferrin (T). Protein elution from the column was continuously monitored at 280 nm; 6.3 ml fractions were collected, and 0.3 ml from each fraction was counted for ^{59}Fe content.

was much greater than the fractional increase (~ 2 -fold) in the ^{59}Fe incorporation into mucosal transferrin.

As illustrated in Fig. 6, the rats raised on the low-iron diet also showed very little incorporation of the ^{59}Fe dose into mucosal ferritin and a 2-fold increase in the incorporation of ^{59}Fe into mucosal transferrin compared with the rats raised on the control diet (Fig. 5). The ^{59}Fe incorporation into mucosal ferritin increased progressively with the length of time for which these rats were given the control diet; however, after 2 weeks of feeding of the control diet, the ^{59}Fe incorporation into mucosal ferritin still did not approach that of the rats that had been raised from weanlings on the control diet. The incorporation of ^{59}Fe into mucosal transferrin from the dose remained elevated even after the rats raised on the low-iron diet were fed on the control diet for 2 weeks.

PAGE of mucosal cytosol samples

Duplicate samples of the mucosal cytosol preparations from

Fig. 6. Intracellular distribution of ^{59}Fe in the cytosol of iron-deficient rats transferred to the control diet

Apart from the rat diets, the experimental protocol was identical with that described for Fig. 5.

each group of rats, containing equivalent amounts of protein, were subjected to PAGE. After electrophoresis, the gels were cut into two sections. One section was stained with Coomassie Blue, a general protein stain, and the other section was treated with a specific stain for iron-containing proteins that is sensitive to nanogram amounts of iron (Kuo & Fridovich, 1988). As illustrated in Fig. 7(a), the staining intensity with Coomassie Blue of the two bands corresponding to the ferritin standard was not greatly diminished in the cytosol samples of the rats transferred to the low-iron diet for 24 h or 72 h. In contrast, the staining intensity with the iron stain was diminished in the slower-moving ferritin band in these same samples. These results suggest that the dramatic decrease in the amount of iron entering the ferritin pool in the mucosal cytosol of the rats exposed to the low-iron diet for 24 h and 72 h was not simply the result of a large decrease in the total amount of ferritin, but could have resulted from an impairment in the incorporation process.

As illustrated in Fig. 7(b), cytosol samples from rats raised on the low-iron diet and similar rats transferred to the control diet for only 24 h showed decreased staining intensity with Coomassie Blue for the slower-moving ferritin band compared with controls. The iron staining of the slower moving ferritin band, although virtually absent in the cytosol samples of rats raised on the low-

Fig. 7. PAGE of mucosal cytosol samples

Duplicate samples of the mucosal cytosol preparations from each group of rats, containing equivalent amounts of protein, were subjected to PAGE as described in the Materials and methods section. The left half of the gel was stained with Coomassie Blue, and the right half was treated with the stain specific for iron (Kuo & Fridovich, 1988). Key: F, ferritin standard; T, transferrin standard; C, cytosol from control rats; 0, 24, 72, 336, time (h) for which rats had been transferred to low-Fe or control diet. (a) Cytosol from rats raised on the control diet and then transferred to the low-Fe diet for various time intervals. (b) Cytosol from rats raised on the low-Fe diet and then transferred to the control diet for various time intervals.

iron diet, re-appeared in the cytosol samples of rats fed on the control diet for only 24 h. Thus, on return of iron-deficient rats to a diet of normal iron content, rapid incorporation of iron into the ferritin of slower electrophoretic mobility must occur.

DISCUSSION

Two of the most conspicuous changes in the mucosal processing of iron that accompany the depletion of body iron stores are: (1) an increased uptake of iron into the mucosal cell, which has been demonstrated with isolated brush-border membrane vesicles and gut loops (Muir *et al.*, 1984; Muir & Hopfer, 1985; Nathanson *et al.*, 1985; Huebers *et al.*, 1990); and (2) a radically decreased incorporation of iron into mucosal ferritin (Worwood & Jacobs, 1972; El-Shobaki & Rummel, 1977; Savin & Cook,

1980; Johnson *et al.*, 1983; Topham *et al.*, 1985). Both of these changes should increase the exchangeable pool of iron within the mucosal cell and facilitate the transfer of a greater fraction of dietary iron to plasma; however, the molecular basis, relative importance and degree of co-ordination of these changes are not yet clear.

In studies of the effect of iron deficiency on iron absorption, iron deficiency is most frequently induced by a combination of iron-deficient feeding and repetitive bleeding. This procedure results in much decreased body iron stores and increased iron absorption. In recent studies, where this combined procedure was compared with the effect of simple 8-day feeding of an iron-deficient diet to rats, no significant differences were found in the stimulating effect on the overall process of intestinal iron absorption, even though the simple 8-day iron-deficient feeding resulted in little depletion of body iron stores (Schumann *et al.*, 1989, 1990). In other recent studies (Topham & Eads, 1990, 1991), it was demonstrated that feeding of a low-iron diet for 2 weeks is sufficient to initiate an increase in the uptake of iron by brush-border membrane vesicles and a decrease in iron incorporation into mucosal ferritin equivalent to the changes observed in more severely iron-deficient rats.

The present studies showed that most of the increase in the transfer of iron from dose to plasma that accompanied low-iron feeding occurred within 72 h after transferring control rats to a low-iron diet. It has been suggested that increases in the uptake of dietary iron via the brush-border membrane of the mucosal cell could be solely responsible for the increased iron absorption in iron deficiency (Muir & Hopfer, 1985). In the present studies, only 24 h of feeding of the low-iron diet resulted in a 2-fold increase in iron uptake by brush-border membrane vesicles. Extension of the low-iron feeding period from 24 h to 72 h resulted in only a small additional increase in iron uptake, but a large incremental increase in the transfer of ^{59}Fe in 30 min from a test dose to plasma. Furthermore, on transfer to a diet of normal iron content, iron-deficient rats showed a progressive decrease in iron uptake by brush-border membrane vesicles that reached a level equal to that in vesicles from control rats in 2 weeks; however, the transfer of ^{59}Fe in 30 min from the test dose to plasma was still elevated to 2.4 times that observed with control rats. Even though increases in the uptake of iron by the brush-border membrane are rapid and may contribute to the increased iron absorption in iron deficiency, these results suggest that it is unlikely that this increased uptake is solely responsible for the increased iron absorption. After 2 weeks of feeding of the control diet, blood haemoglobin levels and total serum iron levels in the originally iron-deficient rats had returned to normal levels, but liver iron stores were still only 60% of those in control rats. Thus the continued elevated iron absorption in these rats most probably reflects an attempt to restore these iron reserves to normal levels.

It has been proposed that iron entry into the mucosal cell via the brush-border membrane is a carrier-mediated process regulated by the iron status of the animal (Muir *et al.*, 1984; Muir & Hopfer, 1985). In the present studies, the saturation kinetics of iron uptake observed with respect to the Fe^{2+} concentration with all brush-border membrane vesicle preparations would be consistent with a carrier-mediated transport mechanism. The detergent treatment of these vesicle preparations provided evidence for the presence of an iron-binding component, the ^{59}Fe labelling of which was increased in control rats transferred to the low-iron diet and was decreased in iron-deficient rats transferred to the control diet. In respect to the length of feeding of the diet of altered iron content, the changes in ^{59}Fe labelling of this component were consistent with the changes observed for iron uptake by the intact brush-border membrane vesicles. These

findings would suggest that alterations in the amount or iron-binding ability of this membrane component could be involved in the adjustments made in vesicle iron uptake in response to fluctuations in dietary iron. This iron-binding component appeared to be an oligomeric protein composed of subunits with a molecular mass of 52 000 Da. The subunit molecular mass of this protein is similar to that of iron-binding proteins identified by others in microvillous membrane vesicle preparations from the upper small intestine of Sprague-Dawley rats and humans (Stremmel *et al.*, 1987; Teichmann & Stremmel, 1990).

It has been demonstrated consistently that iron deficiency results in a marked decrease in the incorporation of ^{59}Fe from a test dose into mucosal ferritin, and a smaller but significant increase in the incorporation of ^{59}Fe into mucosal transferrin (El-Shobaki & Rummel, 1977; Savin & Cook, 1980; Topham *et al.*, 1985; Topham & Eads, 1990, 1991). The present studies showed that 24 h of feeding of the low-iron diet was sufficient to result in a large incremental decrease, and 72 h was sufficient to diminish almost entirely the incorporation of iron from the test dose into mucosal ferritin. These changes in iron incorporation into ferritin correlated well with the large incremental increases observed in the transfer of iron from dose to plasma after 24 h and 72 h of low-iron feeding. In these short time intervals of low-iron feeding, decreases in the total amount of mucosal ferritin did not appear to account for the decreased iron incorporation. The decrease in ^{59}Fe incorporation into mucosal ferritin could result from an impairment in the incorporation mechanism, or from a rapid throughput mechanism that leaves little iron in a form accessible to mucosal ferritin. Earlier studies (Worwood & Jacobs, 1972; Richmond *et al.*, 1972) showed that mitochondria of mucosal cells of iron-deficient rats have increased avidity for iron, and suggested that increased mitochondrial iron uptake also could be a factor in decreasing the availability of mucosal iron to ferritin.

The incremental increases in ^{59}Fe incorporation in mucosal transferrin after various periods of low-iron feeding correlated less well with the incremental changes in the transfer of ^{59}Fe from the dose to plasma. A much larger incremental increase in the incorporation of ^{59}Fe into mucosal transferrin was observed after 24 h of low-iron feeding than when the low-iron feeding period was extended from 24 h to 72 h. This is in contrast with the larger incremental increase in the actual transfer of ^{59}Fe from dose to plasma that was observed on extension of the low-iron feeding period from 24 h to 72 h.

In the iron-deficient rats transferred to the control diet, changes in the transfer of ^{59}Fe from dose to plasma also correlated better with the changes in ^{59}Fe labelling of mucosal ferritin than of mucosal transferrin. Even after 2 weeks of feeding the control diet, the ^{59}Fe labelling of mucosal ferritin from the dose had increased to only 30% of that observed in control rats, and this correlated well with the small decrease in the elevated transport of ^{59}Fe from dose to plasma. In contrast, the ^{59}Fe labelling of mucosal transferrin from the dose had decreased by 70%, and was only 30% greater than that observed in the control rats. Although mucosal transferrin may facilitate transfer of iron from the mucosal cell to plasma, these findings suggest that it is unlikely that it is the sole means by which this is accomplished.

In summary, these results are consistent with the proposal that short-term decreases in dietary iron produce a stimulating effect on the mucosal processing of iron equivalent to that observed in more severely iron-deficient animals. These studies demonstrate that adjustments in iron uptake by the brush-border membrane occur extremely rapidly in response to a change in the iron content of the diet. They also support the proposal that iron entry into the mucosal cell is largely a carrier-mediated transport process and provide evidence for the presence of an iron-binding

protein in the brush-border membrane that is responsive to short-term changes in the dietary iron content. Although roles in the processing of iron within the mucosal cell have been proposed for mucosal ferritin and mucosal transferrin, considerable controversy exists about whether these proteins are active participants in the regulation of iron absorption (Flanagan, 1989). These studies suggest that the substantial changes in the incorporation of iron into mucosal ferritin that accompany short-term fluctuations in dietary iron more closely reflect changes in the transfer of iron from dose to plasma than either altered uptake by brush-border membrane vesicles or changes in the incorporation of iron into mucosal transferrin.

This work was supported by Grant DK 38313 from the National Institutes of Health.

REFERENCES

- Banerjee, D., Flanagan, P. R., Cluett, J. & Valberg, L. S. (1986) *Gastroenterology* **91**, 861–869
- Bannerman, R. M., O'Brien, J. R. P. & Witts, L. J. (1962) *Blood* **20**, 532–546
- Conrad, M. E., Umbreit, J. N., Moore, E. G., Peterson, R. D. A. & Jones, M. B. (1990) *J. Biol. Chem.* **265**, 5273–5279
- El-Shobaki, F. A. & Rummel, W. (1977) *Res. Exp. Med.* **171**, 243–253
- Flanagan, P. R. (1989) *Acta Paediatr Scand. Suppl.* **361**, 21–30
- Huebers, H., Huebers, E., Rummel, W. & Crichton, R. R. (1976) *Eur. J. Biochem.* **66**, 447–455
- Huebers, H. A., Huebers, E., Csiba, E., Rummel, W. & Finch, C. A. (1983) *Blood* **61**, 283–290
- Huebers, H. A., Csiba, E., Josephson, B. & Finch, C. A. (1990) *Blut* **60**, 345–351
- Idzerda, R. L., Huebers, H., Finch, C. A. & McKnight, G. S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3723–3727
- Johnson, G., Jacobs, P. & Purves, L. R. (1983) *J. Clin. Invest.* **71**, 1467–1476
- Kuo, C. F. & Fridovich, I. (1988) *Anal. Biochem.* **170**, 183–185
- Linder, M. C. & Munro, H. N. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 2017–2023
- Manis, J. G. & Schachter D. (1962) *Am. J. Physiol.* **203**, 73–80
- McCance, R. A. & Widdowson, E. M. (1937) *Lancet* **i**, 680–684
- Muir, W. A. & Hopfer, U. (1985) *Am. J. Physiol.* **248**, G376–G379
- Muir, W. A., Hopfer, U. & King, M. (1984) *J. Biol. Chem.* **259**, 4896–4903
- Nathanson, M. H., Muir, A. & McLaren, G. D. (1985) *Am. J. Physiol.* **249**, G439–G448
- Purves, L. R., Purves, M., Linton, N., Brandt, W., Johnson, G. & Jacobs, P. (1988) *Biochim. Biophys. Acta* **966**, 318–327
- Richmond, V. S., Worwood, M. & Jacobs, A. (1972) *Br. J. Haematol.* **23**, 605–614
- Savin, M. A. S. & Cook, J. D. (1980) *Blood* **56**, 1029–1035
- Schumann, K., Elsenhans, B., Hunder, G., Strugala, G. & Forth, W. (1989) *Z. Versuchstierkd.* **32**, 261–267
- Schumann, K., Elsenhans, B., Ehtechami, C. & Forth, W. (1990) *Biochim. Biophys. Acta* **1033**, 277–281
- Simpson, R. J. & Peters, T. J. (1984) *Biochim. Biophys. Acta* **772**, 220–226
- Simpson, R. J. & Peters, T. J. (1986) *Biochim. Biophys. Acta* **859**, 227–236
- Simpson, R. J. & Peters, T. J. (1987) *Biochim. Biophys. Acta* **898**, 181–186
- Simpson, R. J., Moore, R. & Peters, T. J. (1988) *Biochim. Biophys. Acta* **941**, 39–47
- Simpson, R. J., Venkatesan, S. & Peters, T. J. (1989) *Cell Biochem. Funct.* **7**, 165–171
- Stremmel, W., Lotz, G., Niederau, C., Teschke, R. & Strohmeyer, G. (1987) *Eur. J. Clin. Invest.* **17**, 136–145
- Teichmann, R. & Stremmel, W. (1990) *J. Clin. Invest.* **86**, 2145–2153
- Topham, R. W. & Eads, C. E. (1990) in *Iron Biochemicals* (Frankel, R. B. & Blakemore, R. P., eds.), pp. 417–425, Plenum Publishing Corp., New York
- Topham, R. W. & Eads, C. E. (1991) *Biochem. Int.* **23**, 759–768
- Topham, R. W., Walker, M. C., Calisch, M. P. & Williams, R. W. (1982) *Biochemistry* **21**, 4529–4535
- Topham, R. W., Joslin, S. A. & Prince, J. S. (1985) *Biochem. Biophys. Res. Commun.* **133**, 1092–1097
- Torrance, J. D. & Bothwell, T. H. (1968) *S. Afr. J. Med. Sci.* **33**, 9–11
- Wheby, M. S. & Crosby, W. H. (1963) *Blood* **22**, 416–428
- Worwood, M. & Jacobs, A. (1972) *Br. J. Haematol.* **22**, 265–272

Received 22 October 1991/2 January 1992; accepted 20 January 1992