Radioautographic Observations on Iron Absorption by the Duodenum of Mice With Iron Overload, Iron Deficiency, and X-linked Anemia

By Yvan C. Bédard, Peter H. Pinkerton, and Gérard T. Simon

The uptake of iron by the absorptive cells of the duodenum, and its subsequent transfer to the lamina propria, has been studied using $^{55}$Fe and high-resolution radioautography in mice rendered iron deficient by diet, in mice with dietary iron overload, and in mice with hereditary malabsorption of iron (s/a). In all, as in normally iron replete mice, two phases of iron absorption can be distinguished. There is an early active phase of uptake and transfer, followed by a second "storage" phase. The pathway of iron absorption is qualitatively similar to the normal with rapid uptake by the brush border, passage through the terminal web to the rough endoplasmic reticulum, and areas rich in free ribosomes. Iron passes through the lateral cell membrane, intercellular spaces and epithelial basement membrane to the vessels of the lamina propria. The rough endoplasmic reticulum and areas rich in free ribosomes form the major localization of iron at all stages of absorption. Only a relatively small amount of radioactive iron is found over ferritin in iron-loaded mice and mice with s/a; morphologically recognizable ferritin was not observed at any stage in iron-deficient animals. Significant numbers of grains have not been seen over mitochondria in any group of animals studied. In iron-deficient mice, the rate of uptake and of transfer of iron is increased. With the 10 µg dose of iron used, all iron taken up by the absorptive cell is transferred to the lamina propria within 3 hr, with none remaining in the cell during the second, storage phase. In mice with iron overload, transfer of iron is decreased, resulting in considerable storage of iron in the cell. In s/a mice, uptake does not appear to be unduly reduced, but transfer is reduced, thereby resulting in increased storage of iron in the absorptive cell. The rough endoplasmic reticulum and areas rich in free ribosomes appear to play an important role in the uptake, transfer, and storage of iron by the absorptive cell regardless of the state of the body iron stores. Ferritin would seem to have a less important part in iron absorption, possibly acting as a storage or detoxifying mechanism.

HIGH-RESOLUTION RADIOAUTOGRAPHY with $^{55}$Fe has been used to study sequentially the subcellular localization of iron in the duodenal mucosa of the normal mouse during iron absorption.1 Iron absorption occurs in two phases: an early phase lasting about 3 hr during which there is rapid transfer of iron through the absorptive cell, involving brush border, terminal web, areas rich in rough endoplasmic reticulum and free ribosomes (RER), and...
the lateral cell membrane, with the RER the main localization of tracer iron. In a later phase, during which there is little transfer of iron to the lamina propria, the RER remains the main localization of iron, with a small amount occurring in association with ferritin.

Using the same technique, we have studied mice with enhanced iron absorption due to iron deficiency and mice with impaired iron absorption due to oral iron overload and to the sla gene.2

MATERIALS AND METHODS

Three groups of mice were used: iron-deficient mice, iron-overloaded mice, and sla mice.

Iron-deficient Mice

Sixteen male C57 Bl/6J mice were fed on an iron-deficient diet3 for 4.5–6 mo. Tap water was given ad libitum. Body weight was 22.7 ± 0.8 g; age was 230 ± 5 days.

Iron-overloaded Mice

Sixteen male C57 Bl/6J mice were given iron supplements in their drinking water for a period of at least 10 wk; ferrous ammonium sulfate in weak citrate buffer (pH 6.5) was added to provide elemental iron, 10 mg/100 ml. Rockland rat and mouse diet was provided ad libitum. Body weight was 25.4 ± 0.6 g; age was 190 ± 5 days.

sla Mice

Sixteen hemizygous (sla/—) male mice were studied. All were sixth backcross generation descendants of the original mixed stock4 bred to C57 Bl/6J mice. These mice were maintained on Rockland rat and mouse diet, and tap water was given ad libitum. Body weight was 22.5 ± 0.6 g; age was 201 ± 17.4 days.

The mean iron absorption of an oral dose of 1 µg of FeSO₄ labeled with 1 µCi ⁵⁹FeSO₄ was found to be 85.3% in mice on the iron-deficient diet and 14.6% in mice receiving iron supplements, as compared to a mean of 33.2% in normal mice on a regular diet. The difference from normal mice is significant for both iron-deficient (p < 0.001) and iron-supplemented animals (p < 0.02). The sla mice have a mean absorption of 7.7% of similar test dose of iron.2

Each mouse, after fasting for 8–12 hr, was given radioiron infrastructurally (⁵⁵FeCl₃) to which had been added anhydrous unlabeled FeSO₄ to provide an arbitrary dose of 10 µCi and 10 µg of elemental iron in a volume of 0.1 ml. (⁵⁵FeCl₃, specific activity 2.78 Ci/g; ⁵⁵FeCl₂, specific activity 3.87 Ci/g, and ⁵⁵FeCl₁, specific activity 3.56 Ci/g were obtained from Atomic Energy of Canada Ltd., Ottawa, Canada. Contamination with ⁵⁹Fe is less than 2 × 10⁻⁹ µCi/Ci of ⁵⁵Fe.) The time intervals after administration of the dose, the sampling of duodenal tissue, and the preparation and examination of electron microscopic radioautographs have been previously described.¹ The assessment of grain localization was done according to the method of Williams.⁵ For the purposes of comparison, results on normal mice have been reassessed by the same method.

With Caro’s method,⁸ the resolution of radioautography with ⁵⁵Fe has been estimated to be 1000 Å. The number of background grains and the loss of label during preparation of tissues for microscopy were negligible, as previously reported.¹

The detailed distribution of grains over the duodenal mucosa was analyzed in the same way as for the normal mice.¹ Since the variations in the grain counts between the two mice studied at each time interval were less than 5%, the results for each member of the pair were combined. Furthermore, for comparative purposes, the results were converted for a standard surface area of 10,000 sq µ of duodenal absorptive epithelium.

Circle and point analyses⁵ were applied to measure the relative surface occupied by each organelle in the absorptive cells of the three experimental groups, as well as of normal mice. No significant variation was found within or between these groups of mice (χ²: p = 0.9).
RESULTS

Localization of $^{55}\text{Fe}$

In the iron-deficient, iron-loaded, and $sla$ mice, $^{55}\text{Fe}$ is found in the duodenal mucosa in the same sites as in normal mice\(^1\) (Fig. 1). Radioiron is found almost exclusively in absorptive cells and in the lamina propria. The number of grains over goblet, Paneth's, argentaffine, and undifferentiated cells is insignificant and is in the range of the number of background grains.

Uptake of radioiron by the duodenum is rapid, as is shown by the presence of grains over the brush border and terminal web of the absorptive cells within 5 min of dosing with $^{55}\text{Fe}$. Radioiron then becomes disseminated in the cytoplasm of the absorptive cells, and numerous grains are seen over the rough endoplasmic reticulum and adjacent cytoplasm and over areas rich in free ribosomes, both of which remain the predominant localization of $^{55}\text{Fe}$ throughout the experiment (Fig. 1). Another important localization is over the folds of the lateral cell membrane (Fig. 1). Some grains also overlie the extracellular spaces. At the same time, there is evidence of transfer of iron to the lamina propria, where the majority of grains are found over the epithelial and vascular basement membranes, the intercellular spaces, and the endothelium and lamina of capillaries and occasionally of lymphatics.

From 1½ hr onward, in iron-overloaded and $sla$ mice, grains also appear over lysosomes in the absorptive epithelial cells usually in association with ferritin granules. In the iron-deficient mice, grains have not been seen over lysosomes or ferritin.

Throughout the experiment, few grains are present over the mitochondria, nucleus, or Golgi zone of the absorptive cells of iron-overloaded and $sla$ mice. In iron-deficient animals, in the first 20 min, several grains are observed over the nucleus.

Differential Distribution of Grains

The three groups of mice show differences in the distribution and number of grains in the duodenal epithelium at the various time intervals studied (Table 1).

The results of grain counts over the brush border and terminal web, rough endoplasmic reticulum and adjacent cytoplasm, and lysosomes and ferritin are graphically illustrated in Fig. 2 for the iron-deficient, $sla$, and iron-overloaded mice; results for normal mice are also included for comparison. The results are plotted for the first 8 hr after dosing, since afterward all the counts show a sharp decline (Table 1) that may be attributed to the duodenal epithelial cell turnover. The lifespan of these cells is about 48 hr in the mouse.\(^7\) In the iron-deficient mice, grains were seen over the duodenal mucosa only in the first 3 hr after administration of $^{55}\text{Fe}$. The grain counts over the brush border and terminal web are maximal at 5 min and decline abruptly until 30 min. Over the rough endoplasmic reticulum and adjacent cytoplasm the grain counts are also maximal at 5 min and decline abruptly until 30 min to rise slightly at 1 hr and decrease progressively until 3 hr. Grains have not been seen over ferritin granules or lysosomes at any of the time intervals studied.
Fig. 1. Shows s/a mouse; 20 min. Grains are disseminated in cytoplasm of absorptive cells, mainly over rough endoplasmic reticulum and free ribosomes. Note that some grains are overlapping rough endoplasmic reticulum and adjacent mitochondria (arrows). Few grains are over ferritin-containing lysosomes (Ly) and lateral cell membrane (LCM). $\times$ 14,000.
**Iron Absorption by Duodenum**

### Table 1. Distribution of Iron Throughout Absorptive Epithelium

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<tr>
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**sla**

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**Iron-overloaded**

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**BB,** brush border; **TW,** terminal web; **RER,** rough endoplasmic reticulum and adjacent cytoplasm; **LCM,** lateral cell membrane; **ExS,** extracellular spaces; **M,** mitochondria; **N,** nucleus; **G,** Golgi zone; **L,** lysosomes; **Total,** total number of grains per 10,000 sq μ.

In *sla* mice, the counts over the brush border and terminal web reach a first peak at 30 min and a second smaller peak at 1 1/2 hr; thereafter, the counts become progressively less. The counts over the RER and adjacent cytoplasm also show two peaks, the first at 20 min and the second, smaller one at 1 1/2 hr. Thereafter, the number of grains over ferritin and lysosomes increases progressively from 1 hr to 8 hr but represents only 12% of the total grain counts at 8 hr.

In the iron-overloaded mice, the counts over the brush border and terminal web rise to a peak at 30 min, decline to a low value at 3 hr, and rise very slightly at 8 hr. The rough endoplasmic reticulum and adjacent cytoplasm counts show a first peak at 1 hr, decline until 1 1/2 hr, and rise to a second greater peak at 8 hr. The ferritin and lysosome counts rise from 1 1/2 hr to 8 hr when they reach a maximum. Here again, grains over lysosomes and recognizable ferritin represent only a minor portion (about 5%) of the total cellular iron at 8 hr.
DISCUSSION

Localization of $^{55}\text{Fe}$ During Iron Absorption

The present study shows that in states with altered iron stores, including sla, duodenal iron absorption proceeds through the same subcellular pathways as in states of normal iron repletion. Iron is taken up from the lumen by the brush border and terminal web of the absorptive cells; it then becomes disseminated over the areas rich in rough endoplasmic reticulum and free ribosomes. Three possibilities now arise: some of this iron is transferred to the lamina propria via the lateral cell membrane and the extracellular spaces and/or via the basal cell membrane; some remains in the rough endoplasmic reticulum and adjacent cytoplasm; and some is transformed into ferritin.

The Golgi zone and mitochondria are not part of the absorptive pathways, since the analysis of the number of both “junctional” and “exclusive” grains over these organelles excludes them as a possible source of radioactivity in the cell. The organelles involved in iron absorption, as shown here by high-resolution radioautography, differ from those identified by subcellular fractionation. Brown and Rother, in normal iron-replete rats, observed most of the radioactivity in the “nuclear” fraction in the later times of the absorption. Worwood and Jacobs, in normal, iron-loaded and iron-deficient rats, stressed a role for the mitochondria in the absorptive pathway. Unfortunately, the purity of their fractions has not been verified by electron microscopy.

In a light microscopy radioautographic study, Conrad and Crosby observed very little radioactivity in the small intestinal mucosa of iron-depleted and iron-loaded rats from 2 to 48 hr after gastric administration of $^{59}\text{Fe}$. However, their first radioautographs were taken 2 hr after dosing, by which time iron absorption in iron-deficient animals may have been complete. In iron-overloaded animals, the reason for discrepancy is not obvious but could be explained by different methods of iron overloading or by differences in the iron “test” dose, or both.

Phases of Iron Absorption

Analysis of the distribution and frequency of grains over the various organelles of absorptive cells at different time intervals after dosing permits comparison of the “absorptive pattern” in mice with altered iron stores, including sla, with that in normal iron-replete mice. However, for detailed kinetic analysis, the nature of the technique imposes limitations on the use of the data derived from this study. The limitations include restriction in sample size due to the time-consuming nature of high-resolution radioautography, the inconstant rate of gastric emptying, and the absence of direct measurement of iron uptake and transfer. Furthermore, the grain counts observed at any time after dosing are the resultant of both uptake and transfer of iron. However, since it has been demonstrated that the iron taken up by the mucosa does not return to the gut lumen during the lifespan of the absorptive cells, the difference between the peak amount of iron in the cell in the early times and the value reached in the later times as iron is retained in the absorptive cells...
may be regarded as an indirect estimate of the amount of iron transferred to the plasma.

The mice rendered iron deficient by diet show a decline to zero of the curve of the RER and adjacent cytoplasm from 5 min to 3 hr (Fig. 2). This indicates an accentuation of the first “normal” phase of iron uptake and transfer,¹ during which all the iron taken up by the mucosa is transferred to the plasma. Hence, there is no late phase of retention of iron either in the rough endoplasmic reticulum and free ribosomes or as ferritin. This absorptive pattern is in keeping with the previous demonstrations that absorption in iron deficiency has a very rapid onset¹⁴ and is enhanced,¹⁵ with the increase greater in the serosal transfer step than in the mucosal uptake step.¹⁴ The observations of others,¹⁶,¹⁷ however, indicate that a second late phase of iron retention may be present in iron deficiency, depending on the size of the iron test dose and on the severity of the iron deficiency.

In sla mice (Fig. 2), the curve of the RER and adjacent cytoplasm between 5 min and 3 hr shows uptake of iron that is as rapid as normal. Transfer occurs during the same period and is less than normal. The grain counts in the RER show two peaks at 20 min and 1½ hr. The observed pattern probably reflects fluctuations in gastric emptying. This suggestion receives support from the coincident changes in the grain counts over the RER and over the brush border and terminal web. After 3 hr, counts over the RER and adjacent cytoplasm remain relatively high as compared with normal mice and decline only slightly, implying increased retention of iron during the second phase of absorption. The radioautographic findings are in keeping with everted gut sac studies that have shown normal mucosal uptake with defective serosal transfer.¹⁸-²⁰ Thus, sla mice, despite their iron deficiency,²¹,²² exhibit an absorptive pattern that differs strikingly from that of normal mice rendered iron deficient by diet and is in keeping with an impaired iron transfer mechanism in sla. However, high-resolution radioautography does not show any qualitative alteration in the subcellular pathway of iron absorption in sla mice, and the precise nature of the defect in the iron transfer mechanism remains to be determined.

In the iron-overloaded mice (Fig. 2), the curve of the RER and adjacent cytoplasm shows uptake and transfer of iron from 5 min to 1½ hr. For similar reasons as in sla, the small decline in the curve from 20 to 30 min may be attributed to changes in relative rates of iron uptake and transfer influenced by gastric emptying. In this first phase of absorption, iron transfer is reduced in comparison with normal mice,¹ as indicated by the relatively small difference in the peak number of grains at 1 hr and the low level at 1½ hr. The second phase that starts at 3 hr shows a concurrent rise in the curves for the brush border and terminal web, RER and adjacent cytoplasm, and lysosomes, suggesting some renewed iron uptake, probably from desquamated cells. In the second phase, there is reduced transfer and marked retention of iron in the cell. This pattern of iron absorption is consistent with the results of kinetic studies which demonstrated that iron overloading decreases both the mucosal uptake and the serosal transfer of iron, with the latter being reduced to a greater extent.¹⁴,¹⁶,²³,²⁴

In mice with decreased iron absorption as well as in normal mice,¹ there are
Fig. 2. Graphs showing results of grain counts (distribution of grains per 10,000 sq μ of absorptive epithelium) on electron microscope radioautographs of duodenal mucosa at various time intervals after administration of 59Fe. Results are presented for the brush border and terminal web, rough endoplasmic reticulum and adjacent cytoplasm, and lysosomes. No grains were observed over lysosomes or ferritin in iron-deficient mice.

two morphologic pools of iron in the cell: one is associated with the rough endoplasmic reticulum and free ribosomes, and the other is associated with ferritin. The pool associated with morphologically recognizable ferritin appears as a minor and late component in the absorptive process and may represent a pool for storing12 and excreting unneeded15 and possibly toxic iron.26 The pool associated with rough endoplasmic reticulum and free ribosomes is involved in both transfer and storage of iron. In the transfer of iron, it may be related
to the existence of a nonferritin iron carrier for which there is increasing evidence in the literature.\textsuperscript{9-17,27-31} The fact that the low molecular weight label employed, \textsuperscript{55}FeCl\(_3\), is not removed by the fixing and dehydrating fluids in the processing of tissues suggests that the iron in the RER pool is not "free" but is bound to a macromolecule or onto a supramolecular element.\textsuperscript{5}

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**REFERENCES**

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