Iron in the Duodenal Mucosa of Normal, Iron-loaded, and Iron-deficient Rats

By Roberto Mattii, C. Harold Mielke, Jr., Peter H. Levine, and William H. Crosby

Iron absorption studies with oral $^{59}$FeSO$_4$ were performed on 13 iron-depleted, 11 iron-loaded, and 10 control rats, and non-heme iron was determined in both isolated epithelial cells and defoliated mucosa obtained from the duodenum. Mean absorption by the control animals was 18.5% of the dose. Both iron-depleted and iron-loaded groups showed significant differences in iron absorption (54.5% and 2.2% respectively). Compared with the normal controls, iron was decreased in the epithelial cells of the iron-deficient group, whereas higher concentrations were observed in the defoliated mucosa of iron-loaded animals. The latter observation was confirmed by the presence of iron-laden macrophages seen in sections of the lamina propria of the iron-loaded rats.

In the absence of an adequate excretory system the balance of iron in mammals is maintained by limiting the absorption of available, unneeded iron. This concept was announced by McCance and Widdowson. It received substantial experimental support from Hahn, who proposed a theory of "mucosa block" and suggested that the newly discovered iron-storage protein, ferritin, might in some way control the intestinal block. Others have suggested some correlation between the presence of mucosal ferritin and intestinal avidity for iron.

Using radioactive iron it has been shown that iron from the plasma is incorporated into intestinal absorptive cells in animals that do not need iron, whereas iron-deficient animals do not incorporate any radioactive plasma iron into these cells. It was suggested that this investment of plasma iron might serve as a "messenger," informing the epithelial cells about the body's requirement.

Attempts to measure the iron in the small intestine of normal and iron-deficient animals have yielded conflicting results. The material subjected to analysis varied in these experiments. Some used epithelium plus lamina

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propria, some used material from the entire length of the small intestine, not limiting observation to that portion involved in the control of iron absorption.

The present observation attempts to relate the absorption of iron to the iron concentration chemically determined in both isolated epithelial cells from the duodenum as well as in the defoliated mucosa.

MATERIALS AND METHODS

Male albino Sprague-Dawley rats weighing 400-500 g were used in the experiment. One group of eight rats was made iron deficient through 8 phlebotomies from the postorbital venous sinuses (about 6-7 ml of blood were removed each time). Five more rats were submitted to a total of 16 phlebotomies, with the intent of obtaining a greater depletion of their body iron. Both groups were maintained on a casein-base iron-poor diet (General Biochemicals, Chagrin Fall, Ohio). The average ingestion of iron was estimated to be 0.1 mg daily.

Another group was given a total dose of 100 mg dextran-iron (Imferon, Lakeside Laboratories, Inc., Milwaukee, Wis.), administered by two intravenous injections with a 1-wk interval. A third group of rats was fed a regular diet and used as control. At least 10 days after either the last phlebotomy or the iron injection, the rats, together with controls, were tested for iron absorption. The test dose consisted of 0.5 μCi of 59FeCl3, 0.1 mg of FeSO4 as carrier, and 1 mg of ascorbic acid in 0.5 ml of distilled water. Oral test doses were administered through gastric intubation with a polyethylene tubing with an outside diameter of 0.075 inches under very light ether anesthesia. The animals were then placed in vented quart-size ice-cream cartons and counted for two 60-sec periods in a small-animal whole-body liquid scintillation detector (Packard ARMAC).

The animals were killed by exsanguination through cardiac puncture under light ether anesthesia. The duodenum of each animal was promptly removed and stripped of mesenteric and pancreatic residues. The proximal and distal ends were cut off with a piece of glass, fixed in 10% buffered formalin, blocked in paraffin, cut in sections 6 μ thick, and submitted to histochemical test for hemosiderin (Perl’s prussian blue). The central portion of each duodenum, measuring about 6-8 cm, was cooled for 10 min in 0.9% NaCl at 4°C. The specimen was then removed, gently everted onto a glass spiral and vibrated to obtain a relatively pure epithelial cell suspension according to the method of Levine and Weintraub. The epithelial cells were collected and transferred into freeze-drying ampules (Johnson & Jorgenson, Ltd., London SE 7, England). The defoliated duodenum was removed from the spiral, placed on blotting paper, and a sample of about 1-1.5 cm was cut with a piece of glass from its middle third. These specimens were put into ampules as described above. All of the specimens were lyophilized in a Speedivac lyophilizer (Edwards High Vacuum, Ltd., Crawley, Sussex, England) either promptly or after storage at -70°C (dry ice). Weights of epithelium recovered from normal, iron-deficient, and iron-loaded animals were similar (see Table 2 below; minor contamination of epithelial suspensions by naked nuclei and leukocytes was similar in the three models.

Iron Determination

Glassware used in the experiment was made iron free by soaking in a solution of potassium dichromate in concentrated sulphuric acid, then rinsed with iron-free distilled water.

The lyophilized specimens were weighed. The duodenal portions were then finely pulverized and placed into 10 x 75-mm test tubes; 0.2 ml of iron-free distilled water was added to each tube, and a fine suspension was thus obtained. The tubes were centrifuged at 1000 rpm for 10 min.

Table 1. Recovery of Iron From Hemoglobin Solution

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme Fe (μg) predicted</td>
<td>12.5</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Heme Fe (μg) recovered</td>
<td>12.2</td>
<td>21.4</td>
<td>43.0</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>97.6</td>
<td>84</td>
<td>86</td>
</tr>
</tbody>
</table>
and a hemoglobin determination was performed on the supernatant. Hemoglobin determination was omitted in the epithelial cell samples. There was too little to measure.

The whole content of each test tube was transferred into 30-ml Kjeldhal digestion flasks using iron-free water to rinse out the tissue suspensions. An approximately equal amount of iron-free water was also added to the flasks with the lyophilized epithelial cells. Two milliliters of concentrated iron-free nitric acid (G. Fredrick Smith) and 1.0 ml of a 12.5% solution of sulfuric acid, double vacuum distilled, were added to each flask and digestions carried out for 90 min on a gas or electrical Kjeldhal digestor (Labconco, Kansas City, Mo.).

At the end of digestion 7-10 ml of iron-free distilled water were added to each flask, which was kept boiling for 5 min, to allow total water evaporation. This step was to ensure complete removal of any residual trace of HNO₃ from the digest. The digested material was then quantitatively transferred into Kolmer graduated tubes (Will catalog No. D-526C) and brought up to 1 ml volume with iron-free distilled water.

A colorimetric iron determination was performed on 0.5 ml of the digested products, upon sequential addition of the various reagents as follows:
1. Hydroxyl ammonium chloride (20%, solution), 0.1 ml.
2. Bathophenanthroline, sulfonated, sodium salt, (0.5%, solution) 0.1 ml.
3. Na acetate (40% solution), 0.8 ml.

The O.D. of the sample was read in a Coleman Jr. spectrophotometer at 535 mÅ against a standard, where the sample was substituted with 0.2 ml of an iron solution (20 μg/ml) (Harleco Reagents), 0.2 ml of a 31.25%, solution of sulfuric acid, double vacuum distilled (G. Fredrick Smith), and 0.1 ml of iron-free distilled water. The total amount of iron in the epithelial-cell samples was then calculated according to the formula

$$Fe(μg) = \frac{O.D. \text{ sample} - O.D. \text{ sample blank}}{O.D. \text{ standard} - O.D. \text{ standard blank}} \times 8$$

The nonheme iron content of the residual gut was likewise obtained from the total iron of the sample after subtraction of the heme iron previously determined. The method proved to be reliable for the measurement of small aliquots of iron. In a recovery test, increasing amounts of heme iron as hemoglobin solution were wet ashed. As shown in Table 1 over 80% of the original amount of iron was recovered from each sample.

The method was further tested for its reproducibility in samples of a relatively wide weight range. Iron was measured in seven samples of increasing weight from the same portion of a dog duodenum. As shown in Table 2 an almost equal concentration of iron was detected in every sample, with only small variations, perhaps due to technique, perhaps due to differences of iron concentration within the same duodenum.

Statistical Evaluation

Results were evaluated for their statistical significance with student’s t test for unpaired observations.

RESULTS

Iron Absorption

Iron absorption was found significantly different in the three groups tested (Table 3). When compared to control animals (° iron absorption 18.5 ± 12.1
Table 3. Iron Absorption Levels of Three Groups Tested

<table>
<thead>
<tr>
<th></th>
<th>Fe Deficient</th>
<th>Normal</th>
<th>Fe Loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{59}$FeSO$_4$ absorption (%)</td>
<td>Mean SD</td>
<td>Mean SD</td>
<td>Mean SD</td>
</tr>
<tr>
<td>Fe deficient vs normal</td>
<td>54.5 ± 20.5</td>
<td>18.5 ± 12.1</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>Significance</td>
<td>$p &lt; 0.001$</td>
<td>$p &lt; 0.001$</td>
<td>$p &lt; 0.001$</td>
</tr>
</tbody>
</table>

SD) the iron loaded animals absorbed only 2.2% ± 0.3% ($p < 0.001$). Iron absorption of iron-deficient animals was found to be 54.5% ± 20.5% significantly different from both the normal ($p < 0.001$) as well as the iron-loaded rats ($p < 0.001$). The severely iron-deficient animals, rats 9–13, showed a mean iron absorption of 85.1% ± 3.0%, quite different from the absorption found in the remaining rats, 1–8, of the less iron-deficient group (absorption 35.4% ± 11.6%; $p < 0.001$).

Epithelial Cells

Iron was measured in the isolated epithelial cells of 11 iron-deficient, 10 normal, and 10 iron-loaded animals. A significant difference ($p < 0.05$) was found in the epithelial iron of the iron-depleted group (mean 0.10 μg/mg of tissue ± 0.06 SD) as compared to both the control (mean 0.16 ± 0.07) and the iron-loaded group (mean 0.16 ± 0.06) (Fig. 1 and Table 4).

In the group of five animals more extensively phlebotomized, rats 9, 11, and 12 showed only traces of iron in their epithelial cells. The mean epithelial iron of this group, however, was not different from that found in the other six iron-deficient animals (Table 4).

Defoliated Intestinal Wall

Nonheme iron was measured in the duodenal wall remaining after epithelial cell stripping in 13 iron-deficient and 11 iron-loaded animals. The same deter-

Table 4. Iron-Deficient (Nos. 1–8) and Severely Iron-Deficient (Nos. 9–13) Rats

<table>
<thead>
<tr>
<th>No.</th>
<th>Weight (g)</th>
<th>Fe (μg/100 ml)</th>
<th>TIBC (μg/100 ml)</th>
<th>$^{59}$FeSO$_4$ Absorption (%)</th>
<th>Serum Fe Suspension</th>
<th>Mucosal Fe Denuded Gut</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>615</td>
<td>171*</td>
<td>770*</td>
<td>51.8</td>
<td>—</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>615</td>
<td>185*</td>
<td>770*</td>
<td>40.3</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>3</td>
<td>615</td>
<td>214*</td>
<td>396*</td>
<td>48.3</td>
<td>0.06</td>
<td>0.01</td>
</tr>
<tr>
<td>4</td>
<td>593</td>
<td>257*</td>
<td>730*</td>
<td>22.4</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>610</td>
<td>229*</td>
<td>730*</td>
<td>39.4</td>
<td>0.17</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>650</td>
<td>237*</td>
<td>710*</td>
<td>19.8</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>7</td>
<td>610</td>
<td>257*</td>
<td>685*</td>
<td>30.4</td>
<td>—</td>
<td>0.06</td>
</tr>
<tr>
<td>8</td>
<td>520</td>
<td>250*</td>
<td>730*</td>
<td>30.7</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>36</td>
<td>708</td>
<td>84.2</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>10</td>
<td>726</td>
<td>80.2</td>
<td>0.22</td>
<td>0.04</td>
</tr>
<tr>
<td>11</td>
<td>—</td>
<td>86</td>
<td>726</td>
<td>87.8</td>
<td>0.03</td>
<td>0.19</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>42</td>
<td>750</td>
<td>86.6</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>13</td>
<td>—</td>
<td>52</td>
<td>—</td>
<td>86.8</td>
<td>0.16</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*Sera and TIBC at time of death (about 90 days after Fe absorption test).

For rats 2–8 the mean value for iron in cell suspensions was 0.1, SD ± 0.04.

For rats 9–13 the mean value for iron in cell suspensions was 0.09, SD ± 0.08 ($p > 0.8$).
Iron in duodenal mucosa was also performed on ten control animals. To minimize the source of error due to the variable amounts of hemoglobin iron in the capillary blood of each sample, hemoglobin was also quantified and heme iron was subtracted from the total iron; the amount of heme iron contained even in the most congested samples, however, was quite small, when compared with the total iron of the same specimen. Nonheme iron was practically the same in the control and the iron-deficient groups (mean 0.08 μg Fe/mg of tissue ± 0.05 SD and 0.07 μg Fe/mg ± 0.04 SD, respectively). A much higher iron content was found in the iron-loaded group (mean 0.28 μg Fe/mg ± 0.06 SD), and this proved to be significantly different ($p < 0.01$) from the amount found in the other two groups of animals (Fig. 2).

**Histology**

The intestinal sections from both the iron-deficient and the control groups showed no visible iron in the duodenal villi. Heavy deposits of iron were observed in the iron-loaded animals. Iron-laden macrophages were found in the lamina propria at the tips of the villi.
DISCUSSION

Iron was measured in the epithelial cells of duodenal villi after separation by vibration from the underlying lamina propria, and also in the remaining intestinal wall after removal of the epithelial layer.

Iron in the epithelial cells from iron-deficient animals was found to be quite low and significantly different from the other two groups. The total body iron of the iron depleted animals was reflected both in the lack of iron in the duodenal epithelium, and in the elevated absorption of orally administered ferrous sulfate.

Among the iron-deficient group five animals were further phlebotomized to produce a more severe deficiency. A significantly higher absorption was obtained in these rats, without a significant decrease in the iron concentration of the epithelium. Three of the five specimens, however, contained only trace amounts of iron, a condition not found in any of the others from the iron-deficient group. The number of samples, however, is definitely too small to attach any significance to this finding.

Different techniques have been employed by other investigators, who failed to find any meaningful correlation between the iron content of the intestinal epithelium and the iron absorbed by the body. In order to achieve a severe
degree of iron depletion our animals had been subjected to a great number of phlebotomies throughout a 2–3 mo period. This was paralleled by elevated absorption rates, higher than previously reported. The iron determinations in our study were limited to the duodenum, the segment most involved with iron absorption, excluding the rest of the small intestine, which tissue might result in a “diluting out” of significant differences. And finally the mechanical vibratory separation of the epithelium provided us with a “clean” suspension, unaltered both by iron-chelate contamination from chemical separation, and by contamination with lamina propria, inevitable with separation by scraping.

Our results indicate that severe iron deficiency results in a reduced iron concentration of the epithelium of the intestinal villi. This in turn is associated with increased iron absorption. The degree of absorption varies with the severity of iron deficiency. We may conjecture that in varying degrees of iron deficiency the absorptive cell can respond to minor differences in iron concentration, differences that are capable of delivering the “message,” yet not detectable by chemical techniques.

Our study was also extended to investigate the lamina propria. Middle sections of each duodenum, after epithelial stripping, were processed for iron analysis like the epithelial counterparts. These tissues from iron-loaded animals were found to contain high concentrations of intestinal iron, significantly different from the controls. The histological sections confirmed the chemical analysis and showed iron to be present in the macrophages of the lamina propria, at the tips of the villi. The presence of iron-loaded macrophages is bound to increase the nonheme iron in tissues. These macrophages are excretory cells found in iron-storage disease carrying iron into the intestinal lumen. They may also help to limit the accession of iron by intercepting some of the absorbed iron and returning it to the lumen.

REFERENCES

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