Iron Metabolism in Rats with Phenylhydrazine-Induced Hemolytic Disease

By Marcel E. Conrad, Lewis R. Weintraub and William H. Crosby

The quantity of iron present in the body is regulated by a balance between iron absorption and loss. Iron-deficient and iron-loaded animals and humans attempt to re-establish a normal body store of iron by regulating absorption and a limited but selective loss of body iron. The hemolytic states are unusual in that increased absorption continues in the face of an iron-replete body store without accumulating iron in massive quantities.

The purpose of this study is to examine the mechanisms regulating iron absorption and loss in the hemolytic state produced by administration of acetylphenylhydrazine (AcPh).

Methods and Materials

Male albino rats of the Walter Reed Carworth Farms strain, weighing 225 to 275 Gm. were used. The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed. The rats were raised in a pathogen-free environment and housed in galvanized wire cages or plastic metabolic cages. They were fed a standard rat and mouse diet and the average ingestion of iron was estimated to be 2 mg. daily. During prolonged collections of feces for chemical analyses rats were fed a measured, unrestricted liquid milk diet containing 0.5 μg. of iron per ml.

Solutions of acetylphenylhydrazine were prepared to contain 10 mg. per ml. of distilled water. Rats were either given a single 10 mg. dose intramuscularly or 10 mg. injections of AcPh three times weekly. Certain rats were iron-loaded by intramuscular injection of 5 mg. of iron as iron dextran (Imferon®). Two weeks were allowed for the dextran-iron to equilibrate with body-iron before studies were initiated.

Blood letting was performed by insertion of a heparinized capillary tube into the retroorbital venous plexus. Intravenous injections were administered through the dorsal vein of the penis. The packed red cell volume was determined by the microhematocrit technic. The plasma iron concentration was measured by the Ramsay method.

The segments of gut used in this study were excised from freshly killed animals washed in tap water and opened lengthwise. The intestinal lumen was washed free of contents in running tap water and then rinsed in iron-free distilled water. Intestinal segments used for chemical analysis were obtained from rats after a 16-hour fast. The first quarter of the small intestine was prepared for analysis in a tissue homogenizer (Virtis®) and the nonheme iron content was measured by a modification of the method of Brickman and Zondek. Tubidity was cleared from chemical reaction mixtures by ultracentrifugation for 30 minutes at 25,000 rpm.

Bile was obtained from rats under pentobarbital anesthesia by catheterization of the common bile duct with PE 10 polyethylene tubing. The iron in 1 hour collections was measured by a modification of the method of Bothwell and Mallett.

Plasma iron turnover studies were performed following intravenous injection of 2 to 3 μc. of iron (0.1 μg. of iron) that had been incubated for 30 minutes with 0.2 ml. of normal rat plasma. Blood specimens (0.2 ml.) were collected from the tail vein of the
rat in standard hemoglobin pipettes at 3, 6, 10, 20, 30 and 60 minutes after the intravenous injection. Each specimen was hemolyzed in 2 ml. of distilled water and the radioactivity was assayed in a well-type crystal scintillation detector (Packard Auto Gamma Spectrometer, Model 410A). The plasma iron$^{59}$ T$_{1/2}$ was determined from the first exponential function of a three pool model system.$^{14,15}$

Red blood cell incorporation of iron$^{59}$ was measured in 1 ml. of whole blood obtained from rats 20 hours after the intravenous injection of 1 $\mu$g. of ferrous$^{59}$ citrate. The radioactivity contained in the specimen was quantified in a well-type crystal scintillation detector. The total blood volume of the rate was presumed to be 5 per cent of the body weight and the quantity of iron$^{59}$ incorporated into red blood cells was calculated by the following formula:$^{16}$

\[
\text{Red blood cell uptake (per cent) } = \frac{0.05 \times \text{body weight} \times \text{cpm ml. whole blood}}{\text{Total cpm injected}}
\]

Body retention of test doses of iron$^{59}$ was quantified by measurement of total body radioactivity (0.8 MeV-00) in a small animal whole-body liquid scintillation detector (Packard ARMAC®). The radioactivity in cumulative stool collections and in the entire small intestine was measured in this instrument after the specimens had been acid digested and diluted to 250 ml. in a plastic bottle. The test dose for iron absorption studies was 0.5 $\mu$g. of ferrous$^{59}$ citrate in a carrier of 0.5 mg. of ferrous sulfate dissolved in 0.5 ml. of distilled water. The test dose was administered through an olive-tipped 17 gauge endoesophageal tube to rats fasted for 16 hours. In each experiment, iron absorption was measured in normal control rats. This permitted statistical comparison of observations in normal and treated groups of animals and compensated for variation in the quantity of iron absorbed by different groups of normal rats. The test dose for radioactive excretion studies was 2 $\mu$g. of ferrous$^{59}$ citrate containing 0.2 $\mu$g. of iron in 0.25 ml. of saline. The reliability of these techniques has been reported previously.$^{17}$

RESULTS

Iron Absorption (Table 1, Fig. 1)

The hemolytic state caused by administration of AcPh was associated with increased absorption of iron from the gut$^2$ which persisted so long as the administration of AcPh was continued. Cessation of AcPh therapy was followed by a mild polycythemia and decreased gastrointestinal absorption of iron. Similar to experiments using blood letting, increased absorption of iron was delayed for about 4 days after the injection of AcPh$^1,2$ and occurred even in iron-loaded animals.$^5,15$

Iron Loss (Tables 2 and 3, Fig. 2)

To ascertain if there was increased loss of body iron from AcPh-treated animals, total body radioactivity was measured at weekly intervals for 140 days in normal and AcPH-treated rats that had received a single intravenous injection of iron$^{59}$. Normal rats lost 25 per cent of the test dose whereas rats receiving 10 mg. of AcPh three times weekly lost 42 per cent of the radioiron. To determine if the actual loss in feces of body iron paralleled the loss of an isotopic tracer dose, a metabolic balance study was performed in which accumulating fecal collections from untreated and AcPh-dosed rats were assayed for iron content. The animals were housed in plastic metabolic cages and fed a measured, unrestricted milk diet. To prevent the development of iron deficiency during the period of study each rat was injected with 5 mg,
Table 1

<table>
<thead>
<tr>
<th>Period Following Injection of Acetophenylhydrinazone</th>
<th>Plasma Iron Concentration (μg./100 ml.)</th>
<th>Plasma Iron59 Clearance %</th>
<th>Incorporation of Iron59 into Red Blood Cells %</th>
<th>Incorporation of Iron59 into Small Intestine %</th>
<th>Iron Content of Small Intestine μg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>47.7% (0.8)</td>
<td>108% (2.3)</td>
<td>9.9% (0.8)</td>
<td>5.45% (2.0)</td>
<td>20.8% (2.0)</td>
</tr>
<tr>
<td>Day 1</td>
<td>40.5% (1.0)</td>
<td>385% (22.5)</td>
<td>12.2% (1.4)</td>
<td>9.0% (1.4)</td>
<td>5.16% (2.0)</td>
</tr>
<tr>
<td>Day 2</td>
<td>37.5% (0.9)</td>
<td>228% (7.9)</td>
<td>42.9% (2.4)</td>
<td>9.8% (1.8)</td>
<td>4.18% (0.10)</td>
</tr>
<tr>
<td>Day 3</td>
<td>35.2% (0.3)</td>
<td>169% (8.0)</td>
<td>60.5% (2.8)</td>
<td>11.0% (1.8)</td>
<td>3.57% (0.06)</td>
</tr>
<tr>
<td>Day 4</td>
<td>34.6% (1.2)</td>
<td>132% (4.5)</td>
<td>53.5% (1.9)</td>
<td>20.6% (2.8)</td>
<td>3.23% (0.05)</td>
</tr>
<tr>
<td>Day 5</td>
<td>37.7% (1.3)</td>
<td>230% (34.0)</td>
<td>18.6% (4.9)</td>
<td>25.0% (2.4)</td>
<td>3.05% (0.11)</td>
</tr>
<tr>
<td>Day 14</td>
<td>41.3% (1.2)</td>
<td>192% (16.1)</td>
<td>55.2% (2.9)</td>
<td>30.6% (4.8)</td>
<td>1.34% (0.13)</td>
</tr>
<tr>
<td>Day 30</td>
<td>41.7% (0.7)</td>
<td>174% (12.6)</td>
<td>55.5% (1.5)</td>
<td>3.8% (1.2)</td>
<td>6.98% (0.30)</td>
</tr>
<tr>
<td>Treated 14 days then stopped for 14 days</td>
<td>52.1% (1.2)</td>
<td>174% (12.6)</td>
<td>20.5% (1.5)</td>
<td>5.8% (1.2)</td>
<td>6.98% (0.30)</td>
</tr>
</tbody>
</table>

Nonitalized numbers are the mean of observations at various intervals after a single dose of AcPh. Italicized numbers are the mean of determinations after multiple injections of AcPh. SE is shown within parentheses.

of iron as dextran-iron 2 weeks before the study was begun. One week before the metabolic study was initiated each animal received 2 μc. of iron59 intravenously. During the period of study each rat consumed an average of 80 ml. of milk daily, containing about 40 μg. of iron. Six consecutive weekly collections of feces were obtained from each group of animals and analyzed for iron content. The stool from normal rats contained 404 μg. of iron weekly and 2.1 per cent of the dose of radioiron. Weekly collections from AcPh-treated animals contained an average of 598 μg. of iron and 3.5 per cent of the injected iron59. Calculation of chemical determinations revealed a net mean daily fecal loss of 16 μg. of iron from normal animals and 46 μg. from AcPh-treated rats, whereas, the same fecal collections contained an average daily loss of 0.3 and 0.5 per cent of the parenteral dose of radioiron, respectively. Both methods of measurement indicated that there was a greater fecal loss of iron from AcPh-dosed animals than from normal rats. This difference, between the chemical and radioisotopic methods of measurement, results from failure of the radioiron to mix equally in the entire pool of body iron. To ascertain if biliary loss of iron was enhanced in AcPh-treated rats, the iron content was measured in collections of bile; it was found to be twice the concentration of iron observed in collections from normal animals.
AcPh

Iron Metabolism in Rats

Iron-loaded animals absorbed less of a test dose of iron\(^{59}\) than normal rats. The injection of AcPh into iron-loaded animals increased absorption of iron\(^{59}\) to normal levels.

Iron Kinetics in Blood (Table 1)

Following the administration of AcPh there was accelerated destruction of red blood cells producing a fall in the hematocrit. If the doses of AcPh are not too large, increased production of red blood cells keeps pace with destruction and the anemia improves. Accelerated red blood cell production was detected 2 to 3 days following an injection of AcPh and was sustained until cessation of therapy when a decreased incorporation of iron\(^{59}\) into these cells was associated with a mild polycythemia. Subsequent to the administration of AcPh the plasma iron concentration increased during release of iron from prematurely destroyed red blood cells. Then daily measurements showed that the iron concentration decreased towards normal levels as the bone marrow hypertrophied and the utilization of iron increased. Coincident with these changes in the plasma iron concentration there were alterations in the plasma iron\(^{59}\) clearance (T\(\frac{1}{2}\)). The T\(\frac{1}{2}\) was prolonged for 3 days following the injection of AcPh. Subsequently there was a rapid disappearance of iron\(^{59}\) from plasma which continued despite an increase in the iron content of plasma 5 days after injection of AcPh. It was this rapid rate of clearance of iron\(^{59}\) from the plasma which seemed to parallel the increase in iron absorption.\(^{15}\)

Small Intestinal Kinetics (Table 1, Fig. 3)

Following injection of AcPh into rats, there was decreased incorporation of an intravenous dose of iron\(^{59}\) into the small intestine. In animals that received repeated doses of AcPh the small intestine contained about half the amount of iron\(^{59}\) detected in the gut of normal animals. Radioiron injected at daily intervals following a single dose of AcPh was deposited in the small in-
Table 2.—Iron Balance. Mean Daily Loss of Iron

<table>
<thead>
<tr>
<th>Diet</th>
<th>Intake</th>
<th>Output</th>
<th>Net Loss</th>
<th>Stool Recovery of IV Dose of Iron (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk ml</td>
<td>Iron μg</td>
<td>Fecal Iron μg</td>
<td>Iron μg</td>
</tr>
<tr>
<td>Control</td>
<td>82.5</td>
<td>41.3</td>
<td>57.7</td>
<td>16.4</td>
</tr>
<tr>
<td>AcPh</td>
<td>78.6</td>
<td>39.3</td>
<td>85.5</td>
<td>46.2</td>
</tr>
</tbody>
</table>

Table 3.—Concentration of Iron in Bile (μg./ml.)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>AcPh Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.26</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>2.53</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td>2.35</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>2.87</td>
<td>2.99</td>
</tr>
<tr>
<td></td>
<td>2.07</td>
<td>4.87</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>9.26</td>
</tr>
<tr>
<td></td>
<td>3.15</td>
<td>3.09</td>
</tr>
<tr>
<td></td>
<td>2.04</td>
<td>6.50</td>
</tr>
<tr>
<td>Mean</td>
<td>2.37</td>
<td>4.72</td>
</tr>
<tr>
<td>SE</td>
<td>0.36</td>
<td>0.89</td>
</tr>
</tbody>
</table>

testine in gradually decreasing quantities. That the change in the radioiron content of the small intestine was not caused solely by changes in the plasma iron content was tested by chemical assay of the quantity of non-heme iron in intestinal segments. The upper small intestine contained less iron 4 days after a dose of AcPh than specimens excised from untreated rats and animals 1 day after injection of AcPh.

Discussion

In 1943, Hahn proposed that the iron content of the small intestinal mucosa controlled the quantity of iron absorbed by the body.1 Although this concept was challenged by a number of investigators,15 recent work suggests that the amount of iron in the intestinal epithelium acts to regulate the absorption of iron and serves as a mechanism for selective loss of body iron.5,6 The small intestinal mucosa seems to act as a labile iron store closely controlled by the plasma iron turnover.15 Thus, a decreased body requirement is accompanied by a prolonged plasma iron clearance (T½) and increased deposition of iron in intestinal cells. During the time of increased intestinal iron content, the absorption of dietary iron is reduced and any iron incorporated in the intestinal epithelial cells would be lost into the lumen of the gut when these cells were shed from the intestinal villus.5,15 To ascertain if this hypothesis was valid in animals with simultaneously increased absorption and excretion of iron, we examined certain aspects of iron metabolism in AcPh-treated rats.

Injection of AcPh into rats produced a hemolytic anemia and serial changes in the tests of iron metabolism. Initially, the increased quantities of iron from destroyed red blood cells caused hyperferremia which diluted the doses
Fig. 2.—Retention of parenteral dose of iron$^{59}$. Normal versus phenylhydrazine-treated rats. Total body radioactivity was measured in 6 normal rats (shaded) and 6 AcPh-treated rats (brackets) with a small animal liquid scintillation detector at weekly intervals after an intravenous injection of iron$^{59}$. The AcPh-dosed rats lost significantly more of the radioiron than normal animals. The range of observed values is depicted by the shaded area and the brackets.

The loss of tracer iron, decreasing incorporation of iron$^{59}$ into red cells and prolonging the plasma iron$^{59}$ clearance ($T_1/2$). As the rate of erythropoiesis accelerated there was increased incorporation of iron$^{59}$ into newly formed erythrocytes with an associated decrease in plasma iron concentration and plasma iron clearance ($T_1/2$). Four days after injection of AcPh the $T_1/2$ of iron$^{59}$ in the plasma was less than normal and the iron content of the small intestine was decreased. Simultaneously, absorption of iron from the gut increased. As in experiments with phlebotomized rats, enhanced absorption of iron seemed related to the intestinal iron content and the plasma iron clearance.$^5,15$

If the gastrointestinal absorption of iron is consistently increased the excess must be lost or siderosis develops. In humans with life-long hemolytic disease severe siderosis is rare unless the patient receives multiple blood transfusions.$^{10}$ Thus, an equilibrium must be established between the iron absorbed and lost lest a massive store of iron accumulates.

In AcPh-treated rats the rate of loss of a parenteral dose of iron$^{59}$ exceeded
Fig. 3.—The quantity of iron$^{59}$ sequestered by the small intestine was measured at periodic intervals after injection of an intravenous dose. In normal animals the radioactivity in the excised small intestine increased for 20 hours after administration of the test dose. During the subsequent 48 hours there was a rapid decline in the quantity of iron$^{59}$ detected in the intestinal segments of normal animals. The quantity of iron$^{59}$ sequestered by the small intestine of AcPh-treated rats was approximately half that observed in the normal animals at intervals after administration of the test dose.

that observed in normal animals. Chemical analysis of accumulating fecal collections showed a threefold increase in iron loss from AcPh-treated rats as compared to normal animals. To explain the paradox of increased fecal loss of iron from animals with increased absorption of iron and a decreased iron content in the intestinal mucosa the loss must occur from sources other than the sloughed intestinal epithelium or there must be an increased turnover of these mucosal cells or both. Hahn showed increased quantities of iron in the bile of AcPh-treated animals.$^{19}$ We found a rapid transit of intestinal epithelial cells from the crypts of Lieberkühn to the villous tip.$^{20}$ This shortened lifespan of intestinal mucosal cells in AcPh-treated animals would permit normal or increased loss of iron from the intestinal epithelium with a decreased quantity of iron in each cell.

**SUMMARY**

Increased absorption and excretion of iron were observed in AcPh-treated animals. Enhanced absorption was related to a decrease in the iron content of intestinal epithelial cells and an accelerated plasma iron turnover. Increased excretion of iron from AcPh-treated rats was attributed to an enhanced biliary loss of iron and an increased rate of turnover of the small intestinal epithelium. The balance established between absorption and loss of iron
would prevent the accumulation of a massive iron store in this hemolytic disorder.

**Summario in Interlingua**

Augmentos del absorption e del excretion de ferro esseva observate in animales tractate con acetylphenylhydrazina. Le augmentate absorption esseva relationate con un declino in le contento de ferro del cellulas intestino-epithelial e un accelerate metabolismo del ferro in le plasma. Le augmentate excretion de ferro in rattos tractate con acetylphenylhydrazina esseva attribuite a un augmentate perdita biliari de ferro e un accelerate metabolismo in le epithelio del intestino tenue. Il pare plausibile que establimento de un balancia inter le absorption e le perdita de ferro debe prevenir le accumulation de massive reservas de ferro in le presentia de un morbo hemolytic del typo hic studiate.

**REFERENCES**


17. Forrester, R. H., Conrad, M. E., and Crosby, W. H.: Measurement of total body iron in animals using whole-body liquid scintillation de-
L. R., and Herbert, V.: Rapid char-
18. Moore, C. V.: Iron metabolism and nu-
trition. The Harvey Lectures, series 55, 67. New York and London, Aca-
19. Hawkins, W. B., and Hahn, P. F.: The
biliary excretion of radioactive iron
and total iron as influenced by red
20. Conrad, M. E., Weintraub, L. R., Mer-
rill, B., and Crosby, W. H.: The
effect of acetylphenylhydrazine upon
epithelial turnover in the small in-

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