Urinary Iron Excretion and Renal Metabolism of Hemoglobin in Hemolytic Diseases

By DAVID A. SEARS, PEARL R. ANDERSON, ARTHUR L. FOY, HAROLD L. WILLIAMS AND WILLIAM H. CROSBY

It has been known for many years that hemosiderin can be identified in the urinary sediment of patients with intravascular hemolysis, and significant urinary losses of nonhemoglobin iron have been shown to occur in cases of paroxysmal nocturnal hemoglobinuria and sickle cell anemia. Since nonhemoglobin iron compounds appearing in the urine of patients with intravascular hemolysis presumably are products of renal tubular metabolism of filtered hemoglobin, quantitative and qualitative studies of the urinary iron in such patients should provide information about the nature and capacity of tubular hemoglobin metabolism and its significance in hemolytic disease.

The present study was undertaken to quantify urinary iron in patients with various hemolytic diseases, particularly those in which intravascular hemolysis is prominent, to ascertain the location of iron in different fractions of the urine, to identify ferritin in the urine as one of the products of renal tubular metabolism of hemoglobin, to compare diurnal patterns of hemolysis and iron excretion, to relate the findings to current concepts of renal clearance and metabolism of hemoglobin, and to assess the importance of this avenue of iron loss in body iron balance. In addition to the study of patients, urinary iron and hemoglobin excretion were evaluated in a normal subject given an infusion of hemoglobin.

Patients Studied, Methods and Materials

Description of Patients. In Table 1 are the lists of patients studied, pertinent hematologic data, and the ranges of values for total urinary iron. Patients 1–4 developed mechanical hemolysis following open heart surgery and implantation of intracardiac prosthetic devices. In Patient 1 an ostium primum septal defect was closed with a teflon patch. After surgery, mitral insufficiency and persistent or recurrent left-to-right shunting of blood produced turbulence of blood flow and mechanical intravascular hemolysis. Studies were performed while he was anemic and again after his hematocrit had returned almost to normal. In

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Patients 2-4 intravascular hemolysis followed replacement of their aortic valves with caged ball-valve (Starr-Edwards) prostheses. All three of these patients developed aortic diastolic murmurs postoperatively, suggesting that their artificial valves were not functioning optimally. All four patients had typical findings of intravascular hemolysis, including schistocytosis, reticulocytosis, elevated plasma heme pigment levels, anhaptoglobinemia, methemalbuminemia and hemosiderinuria; hemolysis was considered to be on a mechanical basis. Patient 4 had compensated hemolysis without anemia. Of all the patients studied, only Patient 1 had significant hemoglobinuria. Patients 1 and 3 have been described in detail in a previous report.5

Patients 5 and 6 had paroxysmal nocturnal hemoglobinuria with positive acid hemolysis and thrombin tests and typical findings of intravascular hemolysis. At the time that Patient 5 was studied, he had a normal hematocrit and had been clinically well for several years without treatment. Studies were carried out in Patient 6 at three different times during a 12-month period. Her clinical condition did not change during that interval; she was active and attended school regularly despite her low hematocrit.

Patient 7 had sickle cell anemia; Patient 8 had a variant of sickle cell disease, probably sickle cell-thalassemia; and Patient 9 had thalassemia major. The latter patient, a 17-year-old boy, was studied on two occasions 3 weeks apart. The first urinary iron determination was performed before treatment, the second after he had received folic acid for a week because of megaloblastic changes in his bone marrow. He responded to folic acid therapy with a rise in hematocrit, marked reticulocytosis, and an increase in plasma heme pigment levels.

Patient 10 had pernicious anemia. Patients 11 and 12, who had typical hereditary spherocytosis, were studied prior to splenectomy.

**Hematologic Studies.** Routine hematologic studies were performed by standard methods.6 Serum iron and total iron-binding capacity were determined by the methods of Ramsay.7-8 The acid hemolysis test was performed according to Ham9 and the thrombin test for paroxysmal nocturnal hemoglobinuria by the method of Crosby.10 Hemosiderin in urinary sediments was detected by the Prussian blue reaction11 and was graded from 0 to 4+.

**Plasma Heme Pigment Studies.** Heparinized blood was obtained by atraumatic venipuncture, and the plasma was separated immediately by centrifugation. Total plasma heme pigment was determined by the modified benzidine method of Crosby and Furth.12 Dilutions, when necessary, were made with 0.1 M sodium chloride as recommended by Hanks et al.13 The individual plasma heme pigments were separated by paper electrophoresis in 0.05 M phosphate buffer at pH 7.0, stained with benzidine-hydrogen peroxide, and quantified by photometric scanning.* as described by Lathem and Worley.14 Plasma haptoglobin levels were determined by the same electrophoretic technic after incubation of plasma with known amounts of hemoglobin for 1 hour at 37 C. The amounts of all plasma pigments were expressed in terms of oxyhemoglobin standards and reflected, therefore, the relative amounts of heme bound to globin and albumin. The method assumed that heme bound to albumin exhibited the same peroxidase activity toward benzidine as that bound to globin. In studies of the reactivity of various heme pigments in the benzidine test, heme bound to albumin had 90 to 95 per cent as much activity as heme bound to globin. The hemoglobin concentrations of the standards were measured by the cyanmethemoglobin method.15 Plasma samples were preserved in the frozen state when necessary.

**Urine Collection and Hemoglobin Determination.** Timed collections of urine were placed in refrigerated iron-free plastic containers. Urinary hemoglobin was measured soon after collection, and frozen or refrigerated aliquots were saved for later iron analysis. Urinary hemoglobin was determined by a slight modification5 of the benzidine method of Crosby and Furth.12

**Urine Fractionation.** The fractionation procedure is outlined in Figure 1. Urine was divided into sediment and supernatant fractions by centrifugation for 15 minutes at 900 g.

*Spinco Analytrol, Spinco Division, Beckman Instruments, Inc., Belmont, California.*

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Table 1.—Patients Studied, Representative Hematologic Data, and Values for Urinary Iron Excretion

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Date of Study (Month-Year)</th>
<th>Hema- notrit (Vol. %)</th>
<th>Reticu- locyte Count (%)</th>
<th>Urinary Sediment Stain for Hemosiderin</th>
<th>Plasma Heme Pigments (mg./100 ml.)*</th>
<th>Serum Fe/TIBC† (µg./100 ml.)</th>
<th>Marrow Iron Stores</th>
<th>Urinary Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. B.</td>
<td>46 M</td>
<td>Mechanical hemolysis (atrial septal defect repair)</td>
<td>6-64</td>
<td>29</td>
<td>9.7</td>
<td>++++</td>
<td>68</td>
<td>14</td>
<td>54</td>
<td>65/300</td>
</tr>
<tr>
<td>B. H.</td>
<td>44 M</td>
<td>Mechanical hemolysis (prosthetic aortic valve)</td>
<td>2-65</td>
<td>31</td>
<td>7.9</td>
<td>++++</td>
<td>29</td>
<td>5</td>
<td>15</td>
<td>42/450</td>
</tr>
<tr>
<td>E. S.</td>
<td>53 M</td>
<td>Mechanical hemolysis (prosthetic aortic valve)</td>
<td>7-64</td>
<td>36</td>
<td>9.2</td>
<td>++++</td>
<td>42</td>
<td>14</td>
<td>28</td>
<td>54/389</td>
</tr>
<tr>
<td>H. F.</td>
<td>36 M</td>
<td>Mechanical hemolysis (prosthetic aortic valve)</td>
<td>2-65</td>
<td>49</td>
<td>2.5</td>
<td>++</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>67/243</td>
</tr>
<tr>
<td>R. B.</td>
<td>54 M</td>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td>8-64</td>
<td>45</td>
<td>2.5</td>
<td>++++</td>
<td>20</td>
<td>1</td>
<td>19</td>
<td>32/3172</td>
</tr>
<tr>
<td>K. C.</td>
<td>13 F</td>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td>6-64</td>
<td>23</td>
<td>12.4</td>
<td>++++</td>
<td>17</td>
<td>3</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>W. Y.</td>
<td>27 M</td>
<td>Sickle cell anemia</td>
<td>1-65</td>
<td>27</td>
<td>5.6</td>
<td>++++</td>
<td>34</td>
<td>17</td>
<td>17</td>
<td>27/377</td>
</tr>
<tr>
<td>F. D.</td>
<td>25 M</td>
<td>Probable sickle cell-thalassemia</td>
<td>8-64</td>
<td>28</td>
<td>9.9</td>
<td>±</td>
<td>27</td>
<td>12</td>
<td>15</td>
<td>11/260</td>
</tr>
<tr>
<td>B. M.</td>
<td>17 M</td>
<td>Thalassemia major</td>
<td>7-65</td>
<td>17</td>
<td>0.9</td>
<td>±</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>280/204</td>
</tr>
<tr>
<td>W. A.</td>
<td>23 M</td>
<td>Pernicious anemia</td>
<td>10-65</td>
<td>19</td>
<td>0.7</td>
<td>0</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td>170/240</td>
</tr>
<tr>
<td>T. D.</td>
<td>27 M</td>
<td>Hereditary spherocytosis</td>
<td>2-65</td>
<td>39</td>
<td>7.6</td>
<td>0</td>
<td>2 — —</td>
<td>77/261</td>
<td>normal</td>
<td>2</td>
</tr>
<tr>
<td>L. J.</td>
<td>23 M</td>
<td>Hereditary spherocytosis</td>
<td>8-65</td>
<td>35</td>
<td>5.6</td>
<td>0</td>
<td>3 — —</td>
<td>2</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

*None of the patients had any demonstrable haptoglobin.
†Normal serum iron in this laboratory is 70–140 µg./100 ml.; normal TIBC (total iron binding capacity) is 250–350 µg./100 ml.
‡The patient had taken 0.3 Gm. ferrous sulfate daily for years but stopped in September 1965. Serum for this iron determination was drawn 2 months later.
§Evaluated from Prussian blue stain of marrow smear.
Whole Urine

Centrifugation

Sediment
("Insoluble Iron")

Supernate

50% Saturation with \((\text{NH}_4)_2\text{SO}_4\)

Precipitate
("Protein-bound Iron")

Supernate
("Soluble Iron")

Fig. 1.—Outline of the procedure for fractionating urine.

The sediment was washed once with water before iron analysis. Iron in the sediment is referred to as "insoluble iron" or hemosiderin. Further fractionation of the supernatant portion was achieved by mixing an aliquot with an equal volume of an iron-free aqueous saturated solution of ammonium sulfate. The protein was allowed to precipitate overnight at 4°C and was separated by centrifugation and then washed once with a 75% saturated aqueous solution of ammonium sulfate. Ferritin and hemoglobin are precipitated under these conditions. The iron in this ammonium sulfate precipitate is referred to as "protein-bound iron," and hemoglobin iron, as calculated from the results of the benzidine test, is subtracted and tabulated separately. (For all calculations iron is assumed to comprise 0.338% of hemoglobin by weight.) Iron in the supernatant fraction after ammonium sulfate treatment is referred to as "soluble iron." The state of the iron in this fraction has not been characterized, but it obviously contains ionic iron and iron bound to proteins which were not precipitated by the ammonium sulfate. Most or all transferrin iron remains in the supernatant fraction after half-saturation with ammonium sulfate.

Urinary Iron Analyses. All glassware used for iron studies was made iron-free by acid-washing. Reagents were freed of iron contamination by addition of 8-hydroxyquinoline* to bind the iron, with subsequent removal of the iron complex by repeated extraction with chloroform. Redistilled acids were used in the digestion procedures. Duplicate urine samples were acid-digested. To 5–20 ml of urine in pyrex tubes were added 0.5–1.0 ml of concentrated nitric acid and 0.5 ml of 50% sulfuric acid. Planks containing only acids were included with each set of urine samples. The samples were heated overnight at 90–110°C to reduce the volumes to 1–2 ml. S-shaped air condensers were then fitted to the tops of the tubes, and the samples were boiled on an oscillating hot plate until digestion was complete; that is, the solutions were clear and colorless, and nitric fumes were no longer liberated. In most cases a few drops of 30% hydrogen peroxide were added to complete the digestion. At high temperatures phosphates may be converted to pyrophosphates which interfere with iron determination. Therefore, digested samples were refluxed for 1–2 hours or heated overnight at 90–100°C to hydrolyze any pyrophosphates formed. To an aliquot of the final digest was added 40% sodium acetate to raise the pH to 4–5 and hydroxylammonium chloride† to reduce the iron to the ferrous state. Iron was measured colorimetrically by its reactions with \(\alpha,\alpha'\)-dipyridyl. Optical densities were read at 520 nm in a Coleman Jr. spectrophotometer.§ corrected for reagent blank.

†Iron-free, 10% solution, G. Frederick Smith Chemical Co., Columbus, Ohio.
‡Fisher Scientific Co., Fair Lawn, N. J.
values, and concentrations calculated by comparison with standards included with each set of determinations.

Iron determinations on the urinary sediment, supernatant fraction, and the ammonium sulfate precipitate were carried out in similar fashion after acid digestion. Iron in the ammonium sulfate supernatant fraction ("soluble iron") was determined by heating an aliquot overnight at 37 C. with hydroxylammonium chloride and \( \alpha, \alpha' \) dipyridyl. For the latter fraction, urine blanks containing water instead of dipyridyl were run with each sample, and the optical densities of samples at 520 nu. were appropriately corrected and compared to standards to determine iron concentration.

During the course of these studies, it was discovered that all the urinary iron was released for complexing with \( \alpha, \alpha' \) dipyridyl by the initial overnight concentration step. When this partial digestion procedure was utilized, sodium hydrosulfite was employed as the reducing agent instead of hydroxylammonium chloride, and an aliquot of the partially digested material was used as a sample blank. After addition of the reducing agent and \( \alpha, \alpha' \) dipyridyl, color was allowed to develop at 37 C. for 16 hours in the case of whole urine and for 2 hours in the case of urine fractions. In all cases, results obtained after partial digestion were the same as those obtained with the total digestion procedure. Using either total or partial digestion methods, the amount of iron in a specimen of whole urine was generally within 10 per cent of the sum of the amounts present in its fractions.

Studies of Urinary Ferritin. Human liver ferritin was prepared by the method of Granick. To obtain antihuman ferritin, rabbits were immunized by subcutaneous injection of ferritin homogenized with Freund's adjuvant followed by a series of intravenous ferritin injections. The rabbit antiserum was diluted 1:10 with saline for use in qualitative and quantitative tests.

Qualitative precipitin reactions were carried out on glass slides by double diffusion in 0.5 per cent agar gel prepared in buffered saline at pH 8.4. The precipitate resulting from half-saturation of the supernatant fraction of urine with ammonium sulfate was dissolved in saline and dialyzed thoroughly against saline at 4 C. Rabbit antihuman ferritin and the redissolved dialyzed urinary protein precipitate were in surrounding wells. The slides were kept in an atmosphere saturated with water vapor for 24-48 hours to allow precipitin lines to develop. The slides were then washed for 3 days in saline and 1 day in 1 per cent acetic acid, air-dried, and stained for iron by the Prussian blue reaction. Quantitative precipitin reactions using the urinary supernatant fraction and rabbit antihuman ferritin were performed as described by Mazur and Shorr, using 8 ml. of urinary supernate and 0.5 ml. of 1:10 antiserum. The saline-washed precipitate was acid digested and its iron content determined as described above.

Electrophoresis of human liver ferritin and the urinary supernatant fraction from Patient 1 was performed in acrylamide gel. Some segments of the gel were stained for protein with amido black, others for iron with Prussian blue.

Hemoglobin Infusion Experiment. From a normal 41-year-old male volunteer, 150 ml. of blood were taken into a 250 ml. plastic transfusion bag containing 37.5 ml. of acidified citrate dextrose. The red cells were hemolyzed by freezing and thawing in the bag, and the hemolysate was administered to the subject intravenously through a transfusion set with filter over a 20-minute period. He received a total of 19 grams of hemoglobin or 210 mg./Kg. body weight. Plasma samples were obtained at intervals after the infusion, and urine was collected for 48 hours.

RESULTS

Urinary Iron in Normal Subjects. Urine specimens from four normal subjects contained amounts of iron ranging from 0.0 to 0.14 mg./24 hours with a mean of 0.05 mg./24 hours.

†Purified, low in iron, Fisher Scientific Co., Fair Lawn, N. J.
Table 2.—Iron in Urinary Fractions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Days Studied</th>
<th>Total Urinary Iron (mg./24 hrs.)</th>
<th>Per Cent of Total Iron in Urinary Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&quot;Insoluble Iron&quot;</td>
<td>&quot;Protein-Bound Iron&quot;</td>
</tr>
<tr>
<td>E. B.</td>
<td>7</td>
<td>7.21 (5.41-8.58)</td>
<td>61 (46-72)</td>
</tr>
<tr>
<td>B. B.</td>
<td>6</td>
<td>9.37 (7.76-10.75)</td>
<td>69 (63-76)</td>
</tr>
<tr>
<td>E. S.</td>
<td>1</td>
<td>3.15</td>
<td>68</td>
</tr>
<tr>
<td>H. F.</td>
<td>4</td>
<td>0.60 (0.54-0.65)</td>
<td>62 (56-70)</td>
</tr>
<tr>
<td>H. H.</td>
<td>2</td>
<td>3.54 (5.50-5.58)</td>
<td>59 (50-60)</td>
</tr>
<tr>
<td>K. C.</td>
<td>4</td>
<td>3.43 (3.04-3.57)</td>
<td>76 (64-82)</td>
</tr>
<tr>
<td>W. Y.</td>
<td>1</td>
<td>3.03</td>
<td>60</td>
</tr>
<tr>
<td>F. D.</td>
<td>2</td>
<td>0.72 (0.60-0.85)</td>
<td>35 (32-38)</td>
</tr>
</tbody>
</table>

The numbers shown are mean values with the ranges in parentheses. The urinary supernatant fraction was not fractionated in Patient 4.

**Urinary Iron in Patients.** The results are summarized in Table 1. Urinary iron excretion was definitely increased in the four patients with mechanical hemolysis, the two with paroxysmal nocturnal hemoglobinuria, and the single patients with sickle cell anemia, sickle cell-thalassemia, and thalassemia major; excretion was normal or only slightly increased in the patient with pernicious anemia and in the two patients with hereditary spherocytosis.

**Iron in Urinary Fractions.** In Table 2 are the results of determinations of the fractions of urinary iron from patients with total losses greater than 0.5 mg. per day (Patients 1–8). In all but the subject with sickle cell-thalassemia (Patient 8), over half the total iron was in the urinary sediment as "insoluble iron" or hemosiderin. Hemoglobin accounted for as much as 8 per cent of the urinary iron in the patient with mechanical hemolysis due to atrial septal defect repair (Patient 1) but was absent from the urine of the other patients or accounted for less than 1 per cent of the total urinary iron. The percentage of the total iron in the "protein-bound iron" fraction varied considerably among the different patients and in individual patients on different days.

**Day-Night Patterns of Iron Excretion:** Patient 1 had a striking diurnal pattern of hemoglobinuria. Hemoglobin excretion was invariably greater during the day than at night, and this increased excretion, reflecting increased hemolysis, was shown to be correlated with physical activity.5 Urinary iron excretion showed diurnal fluctuations parallel to those of hemoglobin, although the differences were not so marked (Fig. 2). In the patients with mechanical hemolysis caused by aortic valve prostheses (Patients 2–4), who had negligible hemoglobinuria, it was not possible to demonstrate conclusively that hemolysis was accentuated during the day. In Patient 2 total plasma heme pigment levels determined on 4 consecutive days were lower in the morning before arising when he was at rest (mean 29.1 mg./100 ml., range 28.3 to 29.7 mg./100 ml.) than later in the day when he was active (mean 34.9 mg./100 ml., range 32.1 to 39.7 mg./100 ml.), but the differences, though consistent, were not statistically significant. Similar studies in Patient 3 showed no significant differences in total plasma heme pigment levels during rest or activity, although free hemoglobin levels in the plasma were higher when he was active.5 The diurnal-nocturnal rates of urinary iron excretion for Patients 2–4 are shown in Figure 3. In Patients 2 and 4 there was daytime accentuation of iron excretion, while in Patient 3 there was no difference.
Fig. 2.—Diurnal variations in the rate of urinary excretion of hemoglobin and iron in a patient with mechanical hemolysis after repair of an atrial septal defect with a teflon patch (Patient 1).

Fig. 3.—Diurnal variations in the rate of urinary excretion of iron in three patients with mechanical hemolysis caused by prosthetic aortic valves (Patients 2–4). White columns = day; lined columns = night.
It was assumed that individuals with paroxysmal nocturnal hemoglobinuria (Patients 5 and 6) would have maximal hemolysis at night. We were unable to test this assumption, however, since they had no hemoglobinuria and were not available for frequent plasma pigment studies. The time pattern of urinary iron excretion in these two patients is shown in Figure 4. Patient 5 showed a moderate nocturnal increase in 1 of 2 studies. Patient 6, on the other hand, consistently excreted more iron during the day than at night.

No consistent relationship was observed between total urinary iron and the percentage of the total present in any fraction, nor was a relationship apparent between the time of urine collection and the relative amounts of iron in certain fractions.

Urinary Iron in Normal Subject after Hemoglobin Infusion. The urinary excretion of iron and hemoglobin by a normal subject after hemoglobin infusion is shown in Table 3. Essentially all the urinary iron was accounted for by hemoglobin itself, although small amounts of nonhemoglobin iron appeared after 8 hours. No hemosiderin was detected in the urinary sediment for 48 hours after the infusion. Thus the pattern of urinary iron excretion in a normal subject with acute hemoglobinemia and hemoglobinuria was different from that in the patients with chronic intravascular hemolysis in whom hemoglobin accounted for a negligible fraction of the urinary iron excreted.

Studies of Ferritin. The "protein-bound iron" fraction of urine from two subjects with mechanical hemolysis (Patients 1 and 2) and two with paroxysmal nocturnal hemoglobinuria (Patients 5 and 6) formed clear precipitin lines with rabbit anti-human liver ferritin after double diffusion in agar gel (Fig. 5), and these precipitin bands were stained by the Prussian blue reaction. To ascertain how much of the "protein-bound iron" fraction was accounted for by
Table 3.—Urinary Excretion of Hemoglobin and Iron after Infusion of 19 Grams of Hemoglobin in a Normal Subject

<table>
<thead>
<tr>
<th>Time after Infusion</th>
<th>Hours</th>
<th>Hemoglobin (mg.)</th>
<th>Iron (mg.)</th>
<th>Total Hemoglobin (g.)</th>
<th>Total Iron (g./hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.5</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
<td>8.4</td>
</tr>
<tr>
<td>0–4 Hours</td>
<td>4</td>
<td>1292</td>
<td>4.37</td>
<td>4.12</td>
<td>1092</td>
</tr>
<tr>
<td>4–8 Hours</td>
<td>4</td>
<td>407</td>
<td>1.38</td>
<td>1.24</td>
<td>344</td>
</tr>
<tr>
<td>8–12 Hours</td>
<td>4</td>
<td>113</td>
<td>0.38</td>
<td>0.45</td>
<td>96</td>
</tr>
<tr>
<td>12–24 Hours</td>
<td>12</td>
<td>9.6</td>
<td>0.03</td>
<td>0.19</td>
<td>2.7</td>
</tr>
<tr>
<td>24–48 Hours</td>
<td>24</td>
<td>5.0</td>
<td>0.02</td>
<td>0.29</td>
<td>0.7</td>
</tr>
</tbody>
</table>

ferritin, quantitative precipitin studies were carried out on the urinary supernatant fractions from two patients with mechanical hemolysis (Patients 1 and 2) and one with paroxysmal nocturnal hemoglobinuria (Patient 5). In Table 4 the amounts of iron precipitated by the rabbit antihuman ferritin are compared to the amounts precipitated from the same samples after half-saturation with ammonium sulfate. Ferritin accounted for all the "protein-bound iron" in Patients 1 and 5 and most of that fraction in Patient 2.

Additional evidence for the presence of ferritin in the urine of patients with intravascular hemolysis was obtained by acrylamide gel electrophoresis. After electrophoresis of the urinary supernatant fraction of a patient with mechanical hemolysis (Patient 1), a Prussian blue-positive band was found, and its mobility was identical to that of human liver ferritin. Attempts to isolate and crystallize ferritin from the urine by Granick's method for tissue ferritin have been unsuccessful.

DISCUSSION

Urinary Iron in Normal Subjects and Patients with Nonhemolytic Diseases. Although reported values for normal urinary iron excretion have varied widely, most recent studies have cited amounts less than 0.1 mg. per 24 hours. Our values in four normal subjects were similar.

Hemosiderinuria and increased urinary iron excretion have been reported in idiopathic hemochromatosis. In the nephrotic syndrome, transferrin-bound iron may be lost through damaged glomeruli. Administration of chelating agents induces urinary iron loss. Renal iron may be increased in patients with iron-loading anemia and in transfusional siderosis, but urinary iron studies have not been reported.

Urinary Iron in Hemolytic Diseases. Sigler et al. reported increased concentrations of urinary iron in three patients who developed intravascular hemolysis after teflon-patch repair of atrial septal defects, and in one patient the renal proximal tubular epithelium was laden with iron at autopsy. Stohlman and co-workers found heavy deposits of hemosiderin in the kidneys of dogs with valvular prostheses and intravascular hemolysis. Numerous studies have shown renal siderosis and increased urinary iron in patients with paroxysmal nocturnal hemoglobinuria. Dacie and Lewis described a patient with
URINARY IRON EXCRETION AND RENAL METABOLISM

Fig. 5.—Precipitin lines formed by agar gel immunodiffusion of rabbit antihuman ferritin and urinary fractions. The antiserum was placed in the center well and human ferritin in the wells marked F. In the other wells was placed the redissolved, dialyzed urinary protein precipitated from the urinary supernatant fraction by half-saturation with ammonium sulfate. The numbers refer to the patients studied, two with mechanical hemolysis (Patients 1 and 2) and two with paroxysmal nocturnal hemoglobinuria (Patients 5 and 6). The lines demonstrate identity of the urinary protein from these four patients with human liver ferritin.

daily urinary iron loss of 10 mg. without overt hemoglobinuria. Our four patients with mechanical hemolysis and two with paroxysmal nocturnal hemoglobinuria excreted large amounts of iron in the urine. Substantial iron loss occurred even in patients with compensated hemolysis and no anemia.

Renal tubular siderosis has been described in patients with sickle cell anemia. Children with that disease may have hemoglobinemia, hemosiderinuria, and increased urinary iron excretion. Intravascular hemolysis occurs in thalassemia major, and deposition of iron in the kidneys and other viscera is characteristic of that iron-loading disease. Our patient with thalassemia excreted relatively small amounts of iron in his urine even after treatment with folic acid, which was accompanied by increased intravascular hemolysis (judged by the marked rise in plasma heme pigment). Thus renal loss did not provide a pathway for “unloading” his presumably excessive iron stores.

Pernicious anemia is associated with an increase of stainable iron in body stores, and prior to the discovery of vitamin B₁₂ renal hemosiderosis was described in patients said to have pernicious anemia. Intravascular hemolysis is uncommon in hereditary spherocytosis, but hemosiderinuria and renal
Table 4.—Amount of Iron Precipitated from the Urinary Supernatant Fraction by Rabbit Antihuman Ferritin and by Half-Saturation with Ammonium Sulfate

| Patient | Antibody Precipitate | Ammonium Sulfate Precipitate | A/B (%)
|---------|----------------------|------------------------------|----------
| 1. E. B. | 58.3 | 57.0 | 102 |
| 2. B. B. | 45.5 | 80.5 | 57 |
| 3. R. B. | 68.9 | 76.7 | 90 |

The results are expressed as µg. of iron per 100 ml. of supernatant fraction.

siderosis have been described. In the present study one patient with pernicious anemia and two with hereditary spherocytosis demonstrated essentially normal urinary iron excretion.

Renal Clearance of Hemoglobin. For many years the renal threshold for hemoglobin was attributed to tubular reabsorption of filtered hemoglobin. In 1957 observations in man related the threshold to the hemoglobin-binding capacity of plasma since the haptoglobin-hemoglobin complex is not filtered through the glomerulus. However, when plasma unbound hemoglobin levels were plotted against urinary excretion in 10 normal subjects infused with hemoglobin, Lathem demonstrated a small renal threshold unrelated to haptoglobin (20 to 60 mg./100 ml. of plasma free hemoglobin) and a tubular transport maximum for hemoglobin (1.3 ± 0.9 mg./min./1.72 m²). In similar studies of 59 normal subjects, Lowenstein and co-workers reported tubular transport maximum values for hemoglobin of 1.43 ± 0.96 mg./min. In our subject given an infusion of hemoglobin, the tubular transport maximum for hemoglobin, based on three urine collection periods, was calculated to be 0.27 mg./min., a value below the means but within the ranges observed in the series of Lathem and Lowenstein et al. Uptake of hemoglobin by proximal tubular cells has also been demonstrated in animals by stop-flow analysis and by histochemical and electron microscopic technics. Whether actual transfer of hemoglobin between tubular cells and plasma occurs is not known.

In animals a considerable fraction of free hemoglobin is removed from the plasma by the reticuloendothelial system. In our normal subject, after infusion had produced a peak plasma heme pigment level of 574 mg./100 ml., only 10 per cent of the infused hemoglobin was excreted in the urine. If appreciable amounts of hemoglobin were not retained in the tubules beyond 48 hours, it can be estimated that less than 20 per cent of the plasma free hemoglobin was cleared by the kidneys. In the patients with hemolytic disease, the plasma heme pigment levels correlated poorly with the amount of iron excreted in the urine (Table 1). This lack of correlation may be due to differences in renal clearance of hemoglobin or differences in the amounts of heme pigment in the plasma as methemalbumin. The amount of hemoglobin presented to the renal tubules may be only a small fraction of that released into the plasma during chronic intravascular hemolysis.

Renal Metabolism of Hemoglobin. The processes of renal clearance and metabolism of hemoglobin are summarized in Figure 6. Hemoglobin removed from the glomerular filtrate by tubular epithelium is converted to hemosid-
Fig. 6.—Schematic outline of the renal clearance and metabolism of hemoglobin.

In the urinary sediments of the patients in the present study, Prussian blue-positive material (hemosiderin) was seen both within and outside cells. Thus, cellular sloughing was the mode of excretion of at least some of the hemosiderin formed in tubular cells. It is possible that tubular cells also regurgitate iron compounds into the lumen.

Hemosiderinuria is characteristic of patients with intravascular hemolysis, and ferritinuria was demonstrated in our patients. To our knowledge, urinary excretion of ferritin has not been demonstrated previously.

The percentage of the total urinary iron found in the sediment was fairly consistent (Table 2). It is possible that the “insoluble iron” fraction contained some ferritin within the hemosiderin structure or that other soluble forms of iron were trapped within intact cells. Ferritin in the supernatant fraction constituted a significant percentage of the total urinary iron and accounted for most or all of the “protein-bound iron” fraction.

Since the nonhemoglobin iron in the urine of our patients was derived from renal catabolism of hemoglobin, calculations may be made to ascertain the quantitative significance of this pathway. The largest 24-hour urinary iron loss we observed was 10.75 mg., representing the iron from 3.2 grams of hemoglobin (Patient 2). Thus hemoglobin was removed by the tubular epithelium at the rate of 2.2 mg. per minute (assuming no transfer of hemoglobin or iron compounds between cells and plasma). This figure for the rate of tubular transport of hemoglobin is above the mean but within the range of values found in acute studies of normal subjects. Thus, the siderotic kidneys of patients with chronic intravascular hemolysis retain a normal capacity to absorb and metabolize hemoglobin, as much as 3 grams per day.

In our normal subject infused with a red cell hemolysate, the large, acute hemoglobin load greatly exceeded the renal tubular transport maximum. Nevertheless, from the transport maximum for hemoglobin it can be estimated that the tubules removed 194 mg. of hemoglobin from the glomerular filtrate.
during the first 12 hours after infusion. The 656 μg. of iron in that amount of
hemoglobin would have been detectable as nonhemoglobin iron in the urine.
The absence of nonhemoglobin iron in the urine during the 48 hours after
infusion suggests two possibilities: either hemoglobin or iron-containing meta-
bulic products were actually reabsorbed into plasma, or the nonhemoglobin
iron compounds were retained by the tubular cells beyond the period of
observation. The first possibility is consistent with electron microscopic observa-
tions of ferritin in the peritubular capillaries of kidneys of patients with
paroxysmal nocturnal hemoglobinuria. Contrariwise, the excretion of hemos-
derin and ferritin may be delayed if it depends on sloughing of tubular cells
or accumulation of a certain amount of intracellular iron before iron com-
ounds are regurgitated into the tubular lumen. It has been demonstrated that
ferritin may be retained in tubular cells for prolonged periods of time follow-
ing intraperitoneal administration of hemoglobin to rats and mice.

Vicissitudinous Patterns of Iron Excretion. Three of the four patients with
mechanical hemolysis excreted more iron during the day than at night (Figs. 2
and 3). Increased daytime hemolysis was clearly shown in Patient 1 and may
have occurred in the others as well. One of the two patients with paroxysmal
nocturnal hemoglobinuria showed a slight nocturnal accentuation of iron ex-
cretion (Fig. 4), as did a patient reported by Myhre and Flatmark. This
apparent relationship between the rates of hemolysis and excretion of iron in
the urine suggests that the renal tubules catabolize hemoglobin and excrete the
products rapidly or that filtered hemoglobin stimulates the excretion of pre-
formed iron compounds by the tubules. The increased daytime excretion of
iron by the other patient with paroxysmal nocturnal hemoglobinuria (Fig. 4)
remains unexplained. Patients with that disease have been reported in whom
the usual nocturnal increase in hemolysis did not occur.

Role of Renal Loss in Total Iron Balance. In hemolytic states with pre-
dominantly extravascular destruction of red cells, the net result of increased
absorption and excretion of iron is generally a normal or slightly increased
store of body iron. In the presence of intravascular hemolysis, however,
large quantities of iron may be lost in the urine. Three of our patients with
mechanical hemolysis and both patients with paroxysmal nocturnal hemo-
globinuria developed iron deficiency with low serum iron levels or depleted
marrow stores of iron (Table 1). These patients were unable to absorb
sufficient quantities of dietary iron to compensate for their massive “iron
diuresis.” From pathological studies in similar patients, it is reasonable to
assume that the kidneys of our patients contained substantial amounts of iron.
At the same time other body stores were depleted. Thus the iron deposited in
the renal tubular epithelium was relatively unavailable for mobilization and
incorporation into red cells. An analogous situation occurs in idiopathic pul-
monary hemosiderosis in which iron deficiency of the red cells may occur while
there is excessive deposition of iron in the lungs.

Kidney Function in Renal Siderosis. Occasional reports have suggested a
deleterious effect of chronic hemoglobinuria and renal siderosis on the kid-
ney, but most patients with chronic intravascular hemolysis and siderotic
URINARY IRON EXCRETION AND RENAL METABOLISM

Kidneys do not have significant impairment of renal structure or function.\textsuperscript{47,48,84} The capability of the kidney to shed and replace its iron-laden tubular cells may protect it against injury by siderosis.\textsuperscript{50} Gross abnormalities of renal function were not observed in the patients reported here.

Summary

Quantitative and qualitative studies of urinary iron excretion were performed in 12 patients with hemolytic disease and in one normal subject given an intravenous infusion of hemoglobin. In 9 patients with significant intravascular hemolysis, increased urinary excretion of nonhemoglobin iron was observed with amounts as high as 10.75 mg per 24 hours. In 7 of 8 patients in whom fractions of the urinary iron were studied, the majority of the iron was in the sediment (hemosiderin). Ferritin was demonstrated in the urine by immunologic and electrophoretic technics and accounted for a significant percentage of iron excreted. In several patients, day-night variations in hemolysis were associated with parallel fluctuations in iron excretion.

The results were analyzed in relation to current concepts of glomerular clearance and renal tubular metabolism of hemoglobin. The significance to body iron balance of the massive “iron diuresis” occurring in some of these patients was discussed.

Summary in Interlingua

Studios quantitative e qualitative del excretion uninani de fenro esseva executate in 12 patientes con morbo hemolytic e in un subjecto normal qui habeva recipite un infusion intravenose de hemoglobina. In 9 patientes con grados significative de hemolyse intravascular, un augmentate excretion uninani de ferro non-hemoglobinic esseva observate con valores de usque a 10,75 mg per 24 horas. In 7 de 8 patientes in qui fractiones del ferro uninani esseva studiate, le plus grande parte del ferro esseva in le sedimento (hemosiderina). Ferritina esseva demonstrate in le urina per technicas immunologic e electrophoretic e representava un procentage significative del ferro excernite. In plure patientes, variationes in le hemolyse inter die e nocte esseva associate con fluctuationes parallel in le excretion de ferro.

Le resultatos esseva analysate in relation a conceptiones curnente del clearance glomerular e del metabolismo renno-tubular de hemoglobina, es commentate le signification, con respecto al balancia de ferro in le corpore, del massive "diurese de ferro" que occurre in certes de iste patientes.

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Urinary Iron Excretion and Renal Metabolism of Hemoglobin in Hemolytic Diseases

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