Myoglobin and hemoglobin are often present in the urine of hospitalized patients. Differentiating between the two is difficult but important, and textbooks still may contain misconceptions regarding these two proteins.

Here we discuss (a) the difficulties of detection, (b) how to differentiate myoglobinuria from hemoglobinuria, (c) the importance of such differentiation, (d) the etiology of acute renal failure in myoglobinuria, (e) the need for further basic research in myoglobinuria and hemoglobinuria, and (f) the newer techniques used to study myoglobin, such as "high-performance" liquid chromatography (HPLC) and isoelectric focusing.

Case 1
A 28-year-old white man with a history of familial colonic polyposis was in good health, working as a receiving clerk. He was admitted for surgery for rectal resection and creation of an ileostomy (incision of the ileum). At admission, results of urinalysis, hematological studies, and routine chemical screening were within normal limits except for a borderline increased uric acid.

During surgery, he was in the lithotomy position for 7 h. Later, in the recovery room, he exhibited the bilateral acute anterior compartment syndrome (a localized compromised circulation in the leg muscles) secondary to prolonged ischemia, and he was rushed back to surgery, where fasciotoomies (excision of fibrous tissues) were performed. He received a blood transfusion.

His urine was dark brown. Urinalysis revealed a trace of protein and a positive Hemastix dip-stick test. Microscopic examination showed one to three leukocytes and zero to one erythrocyte per high-power field.

Serum lactate dehydrogenase (LDH; EC 1.1.1.27) and serum creatine kinase (CK; EC 2.7.3.2) exceeded by severalfold the upper limit of their intervals (Table 1). Figure 1 shows the LDH isoenzyme pattern. The proportion of CK-MB was <2%. Postoperatively measured urine myoglobin was 700 mg/L. Table 1 gives results for routine chemical analyses. Intravenous fluids were administered and the patient was discharged a week later.

Case 2
A 30-year-old woman presented herself to the emergency room complaining of a two-day history of fever, nausea, and vomiting, and a one-day history of dark urine. She had been discharged a week earlier after an evaluation of hematemesis. She had received a 1060-mL transfusion of erythrocytes and was discovered to have gastric antral erosions.

Table 1. Clinical Chemistry Results for Hemoglobinuria (Case 2) and Myoglobinuria (Case 1)

<table>
<thead>
<tr>
<th>Test</th>
<th>Admission</th>
<th>24-h later</th>
<th>Hemoglobinuria*</th>
<th>Units/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>140</td>
<td>142</td>
<td>138</td>
<td>mmol</td>
</tr>
<tr>
<td>K⁺</td>
<td>3.9</td>
<td>3.7</td>
<td>4.9</td>
<td>mmol</td>
</tr>
<tr>
<td>CI⁻</td>
<td>105</td>
<td>107</td>
<td>98</td>
<td>mmol</td>
</tr>
<tr>
<td>CO₂</td>
<td>25</td>
<td>27</td>
<td>2</td>
<td>mmol</td>
</tr>
<tr>
<td>Urea nitrogen</td>
<td>120</td>
<td>130</td>
<td>100</td>
<td>mg/L</td>
</tr>
<tr>
<td>Creatinine</td>
<td>10</td>
<td>10</td>
<td>18</td>
<td>mg</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>88</td>
<td>73</td>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>Uric acid</td>
<td>56</td>
<td>87</td>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1500</td>
<td>820</td>
<td></td>
<td>mg</td>
</tr>
<tr>
<td>Total protein</td>
<td>64</td>
<td>45</td>
<td></td>
<td>g</td>
</tr>
<tr>
<td>Albumin</td>
<td>39</td>
<td>28</td>
<td></td>
<td>g</td>
</tr>
<tr>
<td>Bilirubin, total</td>
<td>27</td>
<td>16</td>
<td>20</td>
<td>mg</td>
</tr>
<tr>
<td>Bilirubin, direct</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>mg</td>
</tr>
<tr>
<td>Alkaline phosphatase⁵</td>
<td>106</td>
<td>92</td>
<td>64</td>
<td>U</td>
</tr>
<tr>
<td>LDH⁶</td>
<td>1380</td>
<td>1441</td>
<td>1137</td>
<td>U</td>
</tr>
<tr>
<td>CK⁷</td>
<td>135</td>
<td>220</td>
<td>50 000</td>
<td>U</td>
</tr>
<tr>
<td>Iron</td>
<td>1500</td>
<td>230</td>
<td></td>
<td>μg</td>
</tr>
<tr>
<td>AST⁸</td>
<td>71</td>
<td>980</td>
<td></td>
<td>U</td>
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<tr>
<td>ALT⁹</td>
<td>14</td>
<td></td>
<td></td>
<td>U</td>
</tr>
<tr>
<td>Urine Hb</td>
<td>+++</td>
<td>+++</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Urine protein¹</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Myoglobin</td>
<td>–</td>
<td></td>
<td>700</td>
<td>mg</td>
</tr>
</tbody>
</table>

⁵ 24-h post-rhabdomyolysis. ⁶ Reference intervals: CK 10–180, LDH 90–250, alkaline phosphatase 30–110, AST 5–35, ALT 5–25, and myoglobin 0–15. ¹ +, positive; –, negative. Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Relevant laboratory results included serum LDH activity of 1380 U/L and a total bilirubin concentration of 27 mg/L (Table 1). The LDH isoenzyme pattern is illustrated in Figure 1. Urinalysis showed a protein of 3000 mg/L, one to three erythrocytes per high-power field, a positive Hemastix test for hemoglobin, and a small amount of bilirubin. Measurement of urinary myoglobin was ordered because of the dark color of the urine.

The patient was admitted with suspected delayed hemolytic transfusion reaction and possible rhabdomyolysis of undetermined etiology. Blood-bank studies showed a neg-
tative result for direct anti-globulin test, but results of her previously negative antibody screen had become positive. Additional workup showed the presence of antibody in the serum, despite the negative direct anti-globulin test, consistent with a delayed hemolytic transfusion reaction. She eventually recovered.

Discussion
Case Discussion
Both of the above cases involved a dark urine with a positive Hemastix test after blood transfusion, both had symptoms suggesting rhabdomyolysis, and both had an above-normal value for serum LDH. Red or brown urine alerts one to the possible presence of hemoglobin or myoglobin, which can be confirmed by the presence of peroxidase-like activity, e.g., a positive reaction with Hemastix.

Urine from the first patient was positive for myoglobin (700 mg/L). The second patient's urine was negative for myoglobin but positive for hemoglobin (3000 mg/L), and had an increase in total bilirubin, owing to the excessive hemolysis, which the liver could not accommodate.

The first case involved rhabdomyolysis as a result of ischemia with a release of myoglobin. The second case involved hemolysis of the erythrocytes secondary to a delayed transfusion reaction. In both cases, cellular contents, enzymes, and electrolytes were released into the blood. The erythrocytes contain no myoglobin or creatine kinase, so results for these two tests were within normal limits in the second patient. However, both were greatly supranormal in the first patient. Figure 2 illustrates that myoglobin in urine was the first abnormality to appear and subside, in the first case, probably because of the small size of the molecule. Creatine kinase also exceeded the reference interval by >200-fold, with <2% of it being CK-MB.

Values for aspartate aminotransferase (EC 2.6.1.1) were also quite supranormal in the first case, but not very much in the second. This also reflects the aspartate aminotransferase content of muscle as compared with erythrocytes. LDH was increased in both cases; however, LDH isoenzyme 5 was the prominent isoenzyme in case 1, whereas LDH-1 and LDH-2 were prominent in the second case, findings that reflect the source of LDH. Potassium and phosphorous tend to increase after myoglobinuria and hemoglobinuria because both muscle cells and erythrocytes are rich in these elements. Note that the serum creatinine concentration tended to increase in the first case, but not in the second.

Mannitol was given to the first patient to induce diuresis and to prevent acute renal failure.

General Discussion
Until early in the 19th century the red color of muscle was thought to reflect the presence of hemoglobin. However, Xavier Bichat (in 1803) and Kolliker (in 1850) discovered a unique pigment, different from hemoglobin, in muscle, named myoglobin by Gunther Leipziz in 1921 (1).

Hemoglobin and whole myoglobin were among the first proteins for which the amino acids sequence was determined and the three-dimensional structure ascertained. Myoglobin thus was used extensively as a model protein in investigations of the basic mechanism for binding of antibodies to antigens. Hemoglobin and myoglobin share similar functions and basic structure. Both deliver oxygen to the tissues and both contain a heme group in addition to amino acid homologies in the globulin part of their molecules. Myoglobin delivers oxygen from the cell membrane to the mitochondria in the striated muscle at low oxygen tension. It is a spheroidal polypeptide chain with 153 amino acid residues and 75% of the chain in an α-helix. Rich in the basic amino acid lysine, it contains no cysteine (2). It is present as a monomer with a molecular mass of 17 000 Da. Hemoglobin is a tetramer with a molecular mass of 65 000 Da. A 24-amino-acid sequence of the peptide is common to myoglobin and hemoglobin. Muscle cells, including myocardial muscle, contain 4 mg of myoglobin per gram of tissue (1).

Causes of Myoglobinuria
A red or dark-brown urine showing positive peroxidase activity by dip-stick screening tests such as Hemastix
(Ames Division, Miles Laboratory, Elkhart, IN) or Chemstrip (Boehringer Mannheim Diagnostics, Indianapolis, IN) indicates that hemoglobin or myoglobin may be present. The latter results from breakdown of muscle cells, the former from breakdown of erythrocytes. Hemoglobinuria occurs in most renal disorders, in hemolytic disorders, and in transfusions of incompatible blood. A very common cause of occult hemoglobinuria is tumors. In females, it may result from contamination with menstrual blood. In either sex, it can arise from hemolysis of erythrocytes in the urine. Myoglobin is seen in the urine less frequently than hemoglobin. It usually is present as a result of trauma but may be present in nontraumatic disorders such as alcohol overdose, toxin ingestion, or certain metabolic disorders (1, 3, 4) (Table 2).

Significance of Myoglobinuria

Muscle tissue, including the myocardium, is rich in both myoglobin and CK. Detectable amounts normally (and harmlessly) leak from the muscle into the serum. However, in excessive concentrations, such as those found in crush injuries, myoglobin becomes toxic to the kidney. Acute renal failure associated with crush injuries has been recognized since the studies of Bywaters et al. (5, 6) during the Battle of Britain in 1941. Because of its low molecular mass (17 000 Da), myoglobin is rapidly cleared through the glomeruli and reabsorbed into the renal tubular cells, where it is metabolized. Hemoglobin, with its greater molecular mass (65 000 Da), is cleared less rapidly. Excessive amounts of myoglobin in serum, such as may occur in crush injuries, are known to be toxic to the kidney tubule, causing acute renal failure (tubular necrosis) (4–6).

The presence of myoglobin in serum and urine indicates dysfunctioning of a major muscle, hypotension, and volume depletion. Hemoglobin is also nephrotoxic, but its nephrotoxicity relative to that of myoglobin is not well established, because several factors play a role in the acute renal failure, as will be discussed. With improvements in blood-collecting techniques, acute renal failure of hemoglobinuria due to mismatched blood is much less common, but some cases of it still occur.

Nontraumatic myoglobinuria with acute renal failure is a relatively common disease in patients with alcohol overdose or a history of heroin addiction, accounting for 5% to 7% of all cases of acute renal failure (4, 7). Many of those patients requiring dialysis exhibit myoglobinuria (8). Thus, differentiating myoglobinuria from hemoglobinuria is important for proper diagnosis and treatment.

Pathogenesis of Acute Renal Failure in Myoglobinuria

It is unclear why heme pigmenturia is associated with acute renal tubular necrosis. The occurrence of spontaneously myoglobinuria without acute renal failure in about half of the patients with muscle phosphorylase deficiency (McArdle’s disease) suggests that coincident factors may enhance the toxicity of hemoproteins to the kidney (7). Also, the difficulty with which acute renal failure is induced with pure myoglobin or hemoglobin solutions in experimental animals indicates the importance of such factors (9–11). Dehydration, hypotension, pigmentation, and aminoglycoside exposure often are significant cofactors that increase the risk of developing clinical acute renal failure (12).

Morphological features of myoglobin acute renal failure include severe degenerative changes in the proximal convoluted tubule and the formation of brown casts in the distal convoluted tubule. Myoglobin deposits have been detected by immunofluorescent antibody techniques (13).

Different studies indicate that multiple factors contribute to the pathogenesis of acute renal failure of myoglobinuria. A toxin-induced vasocostriction may be the initiating event for acute renal failure (14). Morphological studies support the role of tubular obstruction by myoglobin casts in the pathogenesis of acute renal failure; however, micropuncture studies have shown that the increase in intratubular pressure caused by the casts is an early, transient, and variable phenomenon (15). Mesangial changes that alter permeability of glomerular basement membranes (16) may also play a role in the pathogenesis of acute renal failure. Coincident volume depletion and aciduria (17) enhance the development of acute tubular nephrosis. In vivo experiments (18) and metabolic studies of kidney tissue slices (19) show that acid hematin is more toxic than myoglobin or hemoglobin, a finding that has been ascribed to the ease of penetration into tubular cells of the smaller hematin molecule. Prostaglandin-inhibition may enhance the toxic effects by interfering with ischemia-induced vasodilatation (10).

Clinical Symptoms

The symptoms and history of myoglobinuria may be obvious such as in crush injuries or burns. In general, the diagnosis of rhabdomyolysis depends on clinical impressions and on the patient’s symptoms, with laboratory results for confirmation (20). However, in about one-fourth of the cases—e.g., in nontraumatic rhabdomyolysis—the symptoms are vague, and biochemical analysis is necessary for diagnosis (8). Most patients with nontraumatic myoglobinuria have intense myalgia and swollen and tender muscles. Patients developing acute renal failure from hemoglobin experience chills, fever, nausea, and vomiting.

Treatment of Acute Renal Failure

Correction of pre-existing volume deficits with isotonic saline is cardinal in management of acute tubular necrosis. The observations of Ron et al. (21) and Enea et al. (22) suggest that prompt and aggressive volume expansion, alkalization of the urine, and administration of mannitol may prevent the development of the acute tubular necrosis. Hyperkalemia from the release of potassium from the damaged muscle is often life-threatening and requires treatment with ion-exchange resins and (or) hemodialysis. Furosemide has not been efficacious in animal models of myoglobinuria (23, 24). The prognosis in nontraumatic myoglobinuria remains good (4), but when traumatic myoglobinuria is complicated by sepsis and multiple organ failure, the prognosis is poor (25).

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**Table 2. Causes of Myoglobinuria**

| Hereditary: phosphorylase deficiency, phosphofructokinase deficiency, malignant hyperthermia |
| Metabolic (with or without coma): alcoholic myopathy, carbon monoxide, hypokalemia, McArdle’s disease |
| Trauma and ischemia: wars, prolonged surgery, arterial ischemia to the extremities, severe burns |
| Severe exercise |
| Myositis: dermatomyositis, polymyositis |
| Toxins, heroin, quai-eater’s disease, animal venoms |
| Status epilepticus, convulsions |
Abnormal Test Results in Rhabdomyolysis and Hemolysis

In addition to increased myoglobin in the urine, rhabdomyolysis is usually accompanied by greater than 40-fold increases of serum myoglobin and increases of several serum enzymes: CK >40 (4, 8, 20, 26, 27), aspartate aminotransferase >4 (27), and LDH >2 (27) times the upper limit of their reference intervals, with a CK-MB <5% of total CK and an increase in LDH-5 isoenzyme. Usually the CK-MB in rhabdomyolysis is <2%; however, we have occasionally seen CK-MB increases of up to 5% in malignant hyperthermia. Figure 2 illustrates the increase of several enzymes with time after rhabdomyolysis. Urine myoglobin is the first test analyte to increase; it subsides rapidly within the first few days, reflecting its small molecular mass and its half-life in serum of about 2–3 h. CK increases within a few hours after muscle damage but remains increased a few days longer than myoglobin.

An increase in both serum myoglobin and serum CK of up to 40 times the upper reference interval has been detected in healthy military recruits (27) and in Olympics trainees performing strenuous exercises. A fairly high correlation (r = 0.74) between serum myoglobin and CK has been found in patients with myocardial infarction or burns (28, 29). A similar correlation is expected between serum CK and urine myoglobin, but this has not yet been determined.

Hemolysis is usually accompanied by an increased activity of LDH and normal CK; however, the LDH isoenzymes reflect an increase in LD-1 and LD-2, with a low LDH-5 (Figure 1). Thus serum LDH isoenzymes are very helpful in differentiating hemoglobinuria from myoglobinuria. Serum potassium and phosphorus increase very rapidly after both muscle injury and hemolysis, whereas uric acid increases only after muscle injury. The usual 10:1 ratio for blood urea nitrogen/creatinine tends to be lower in myoglobinuria, as is the concentration of serum calcium.

Haptoglobin, which binds hemoglobin, tends to decrease after hemolytic anemia. However, this test is not very useful for detecting hemolytic anemia because some of the haptoglobin variants do not bind very well with hemoglobin. It also lacks specificity because the concentration of haptoglobin is affected by hepatic dysfunction, infection, malignancy, and inflammation. Hemopexin, another β-glycoprotein, also binds hemoglobin and tends to decrease after hemolytic anemias. Albumin binds heme, forming methemalbumin with a long half-life, so that concentrations of this compound are increased for a few days after the hemolytic episode. Reticulocytosis and urine urobilinogen tend to increase after hemolytic anemias.

Reference interval

A well-defined reference interval is important for: (a) identifying the patients at risk of developing acute renal failure, and (b) selecting an analytical method with appropriate sensitivity.

Serum: In normal subjects, the serum myoglobin reference interval is 0.01–0.06 mg/L (29). An increased concentration in serum is associated with several disorders: myocardial infarction, polymyositis, muscular dystrophy, malignant hyperthermia, and renal failure (30). Serum myoglobin is one of the first markers to increase after myocardial infarct, peaking as early as 9.9 h post-infarction (29) and as much as 30-fold normal values (29–31). In rhabdomyolysis, serum myoglobin increases 40– to 400-fold; the urinary concentration increases 40– to 20 000-fold

the top of the reference interval. The mean for eight serum samples we have seen from patients with rhabdomyolysis was 6.1 mg/L (range 2–25 mg/L) (32). Myoglobin is stable in serum but relatively unstable in urine. Thus, all radiimmunoassay kits can detect myoglobin in serum, and a very few kits can detect it in the urine (31, 33).

Urine: Myoglobin is filtered by the glomeruli and reabsorbed by the renal tubules, where it is degraded. Very little, if any, intact myoglobin can be detected in the urine of normal subjects. However, when the kidney is overwhelmed by myoglobin, such as in crush injuries, the tubules cannot metabolize all the myoglobin and thus intact myoglobin can be detected, in addition to its degradation products.

In normal subjects, urinary myoglobin is usually below the detection limits of most methods and is probably <0.4 mg/L (33–35). Unfortunately, the concentration of urinary myoglobin associated with acute renal failure has not been determined. However, because serum myoglobin, during severe exercise, can increase up to 40-fold without acute renal failure (27), similar increases in the concentrations of urinary myoglobin can be anticipated. Thus, one can infer that values <15 mg/L will be tolerated by the kidney without risk of acute renal failure. This value, based on myoglobin concentration in muscle, corresponds to about 5 g of damaged muscle. Urinary myoglobin between 1 and 15 mg/L can be detected occasionally in patients after vigorous exercise (8, 34) or with myocardial infarction. Thus patients with urinary myoglobin >15 mg/L are expected to be at risk of acute renal failure. In the 20 cases of rhabdomyolysis we have seen, the concentration of myoglobin was 20–8000 mg/L (mean 580 mg/L). Values (presumably erroneous) as high as 500 mg/L for serum and 30 mg/L for urine in healthy individuals have been reported in the literature.

Because screening with urine dipsticks (e.g., Hemastix) is sensitive to myoglobin in concentrations of 0.15–0.62 mg/L (average 0.38 mg/L), a 40-fold dilution of the urine is required before using the Hemastix, to decrease the sensitivity of this test to 15 mg/L. Samples that give negative results at this dilution (the majority of those encountered in the laboratory) are not tested further; those giving positive results will require differentiation of hemoglobin and myoglobin by one or a combination of the methods described below.

Hemoglobin/Myoglobin Differentiation

The differentiation of myoglobin from hemoglobin can be based on several physical or chemical properties of the two molecules. In general, two types of assays are available: assays that can detect 1 mg of myoglobin per liter (such as the immunoassays, HPLC, the dipstick) and assays that detect concentrations >100 mg/L (such as electrophoresis). Obviously the difference between these two general types of assays is very wide.

Visual inspection. A red or brown urine with a positive peroxidase activity indicates the presence of a heme-containing protein and eliminates such causes as the presence of homogentisic acid, porphyria, and aicaletonuria. Other causes excluded by the peroxidase activity include drugs such as aminopyrine, pyridium, or food such as beets. In general, the presence of cherry-red color indicates the presence of fresh blood or fresh hemoglobin. The presence of a red sediment after centrifuging the tubes for 30 s in a microfuge supports the presence of erythrocytes and leads
to a positive Hemastix as a result of hemolysis of some cells in vitro. Myoglobin in general will appear light brown. However, some unstable hemoglobins and methemoglobin give brown byproducts. A 50 mg/L solution of hemoglobin or myoglobin in water can be detected by the naked eye. However, in urine, values <300 mg/L cannot easily be detected by eye.

The renal threshold for myoglobin in serum is between 3 and 15 mg/L (8). Hemoglobin is not cleared as rapidly as myoglobin by the glomeruli and binds tightly to haptoglobin, up to 1350 mg/L, giving a red color to the serum before any hemoglobin can be detected in the urine. In myoglobinuria, on the other hand, the serum color does not appear red. However, in vitro hemolysis should be ruled out.

Scanning spectrophotometry. Myoglobin can be differentiated from hemoglobin on the basis of absorption of the oxy derivative at about 542 and 577 nm (36-38) vs about 540 and 576 nm for oxyhemoglobin. These wavelengths are very close to each other, and the rapid conversion of oxymyoglobin to metmyoglobin is accompanied by a change in the spectra (36-38).

Because of the presence of many chromogens in urine, myoglobin values <50 mg/L cannot be detected by scanning spectrophotometry. Realizing that the critical concentration of both myoglobin and hemoglobin in urine is close to 15 mg/L demonstrates that one cannot rely on either visual or spectrophotometric detection of a red color to infer the presence of clinically significant hemoglobinuria or myoglobinuria. In general, the spectra of myoglobin and hemoglobin lack sensitivity and change with time and with denaturation, giving unreliable results (37).

Difference in molecular mass. Separating myoglobin from hemoglobin by their difference in molecular mass by using special dialysis membranes should be easy; such methods have been described (39). However, in our experience, as well as that of others (40), results with membranes are not reproducible: small but variable amounts of hemoglobin leak through the membrane, giving false-positive results when the dialysate is tested with Hemastix. In addition, the membranes are expensive and require long centrifugation times for the separation.

Ammonium sulfate precipitation. Hemoglobin reportedly precipitates at 80% saturation with ammonium sulfate, whereas myoglobin does so only after 100% saturation (41, 42). We added ammonium sulfate to hemoglobin solutions (62–1000 mg/L) to bring the concentrations to 80% saturation (40–42), but observed no visible precipitation at hemoglobin concentrations <300 mg/L (Figure 3). Thus, the method would give false-negative results for urine samples containing <300 mg/L. Precipitation also depends on pH (43), temperature, time, centrifugal force, etc. Furthermore, testing the supernate with sensitive tests such as Hemastix (positive at a hemoglobin concentration of 0.38 mg/L) would give false-positive results for myoglobin on many of the myoglobin-negative samples. Thus, we believe this procedure, reported in most clinical textbooks, should be abandoned.

Electrophoresis. Myoglobin can be separated from other proteins electrophoretically, by using either the conditions for separating hemoglobin or for the high-resolution of serum by electrophoresis shown in Figure 4. Being rich in lysine, myoglobin migrates far from hemoglobin A but close to A2 and C variants on alkaline agarose membranes (37). Myoglobin concentrations <100 mg/L are difficult to make visible after electrophoresis by protein-staining methods. Concentrating the urine is not a successful strategy, because most of the myoglobin will denature on the membrane used for the concentration.

Isoelectric focusing has also been used for separating these two compounds (36, 37), but in general this does not lend itself to emergency work. Recently, with the use of narrow pH gradients, Wu et al. (44) found that muscle myoglobin of the dog differs from that of the canine heart; moreover, these myoglobins produced two different antisera. If such is the case for human myoglobin, we might be able to produce specific antisera for the rapid and specific diagnosis of myocardial infarction. Isoelectric focusing might be useful for studying the presence of different variants of myoglobin in pathological conditions.

Immunoassays. Myoglobin, because of its simple structure, has been used as a model to study the basic mechanism of interaction of antigens and antibodies (45, 46). Radial immunodiffusion (35, 38, 47), nephelometry/immunoturbidimetry (34), and radioimmunoassays (30, 33) have been described for the assay of myoglobin in urine and

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Fig. 3. Ammonium sulfate precipitation of hemoglobin: percentage of hemoglobin in the precipitate or the supernate vs hemoglobin concentration

Fig. 4. Electrophoresis on high-resolution agarose gels (Helena Laboratories, Beaumont, TX) of concentrated urine from (A) patient 1, and (B) patient 2
serum. Radial immunodiffusion and nephelometric methods can detect myoglobin concentrations as low as 2 mg/L in urine. The anti-human myoglobin antibodies cross-react with the monkey and camel myoglobins (47, 48). Many of the commercial antisera form soluble complexes, which are not useful for radial immunodiffusion or for nephelometry; others react only with serum but not with the urine myoglobin (31). These reaction differences may reflect variations in the antigenic sites and the presence of different degradation products of myoglobin in the urine (49–51). In addition, several myoglobin-breakdown peptides in urine can compete with the myoglobin molecule itself, inhibiting the reaction with myoglobin (51). We found that rabbit antisera are better than those of goat origin for immunodiffusion assays.

Myoglobin can easily be detected qualitatively in Ouchterlony plates by applying 10 μL of the rabbit anti-human myoglobin (Organon Teknika-Cappel, West Chester, PA) in the middle well and 10 μL of the urine sample or standards in the outer wells. For the radial immunodiffusion plates, we mixed 300 μL of the rabbit antisera with 10 mL of melted agar (100 mg of agar and 300 mg of polyethylene glycol 6000 in 10 mL of phosphate buffer, 20 mmol/L, pH 7.4, containing 90 g of sodium chloride per liter) at 45 °C. The ratio of myoglobin antisera to agar is about fivefold higher than what we usually use for other similar tests such as that for microalbuminuria. The precipitin rings are weak relative to those observed for other assays. The reaction can be speeded up by incubating the plates at 37 °C. At this temperature, precipitin rings can be detected as early as 3 h in the radial immunodiffusion or 8 h for the Ouchterlony precipitin lines. Urinary myoglobin concentrations between 5 and 100 mg/L can be detected by these two methods. For the described immunoassays, it is important to assay the patients’ samples at several dilutions to avoid the prozone effects encountered with antigen excess.

HPLC. Myoglobin can be separated from hemoglobin by HPLC by using an ion-exchange column, with detection at 405 nm (32). Two peaks (MI and MII), in equilibrium and differing by a single charge, can be detected for myoglobin from muscle tissues or serum of patients with myoglobinuria (Figure 5). These two peaks also have slightly different absorption maxima. This method (used in our laboratory) is rapid and simple and can detect as little as 2 mg/L in urine or serum; however, special instrumentation is required. HPLC methods are potentially useful for detecting myoglobin variants and for preparation of standards and antigens.

Peroxidase-like activity. Either hemoglobin or myoglobin can catalyze a reaction between hydrogen peroxide and several dyes such as tetramethylbenzidine, aniline, and gum guaiac. Unfortunately, most of these dyes are potentially carcinogenic; besides, these reactions do not follow zero-order kinetics, and are subject to interference from reducing agents such as ascorbic acid, uric acid, and cysteine. The Hemastix assay avoids many of the previous problems by using reagents impregnated in a convenient dry form onto filter paper. In general, the Hemastix method is valuable as the first step in urine screening.

Stability of Myoglobin and Hemoglobin in the Urine

Urine is a hostile environment for enzymes and proteins in general. It contains several proteolytic enzymes and various salts that can denature or hydrolyze proteins (46). The activity of most enzymes is lost rapidly in urine. As with other proteins, myoglobin and hemoglobin break down in the urine and also denature upon freezing (32). Less than 3% of the myoglobin injected into volunteers was recovered intact in the urine (52, 53). In general, myoglobin in urine is stable for a few days in the refrigerator; however, the stability differs among urine samples. Myoglobin and hemoglobin added to some urine samples may disappear within a few hours or may persist for several weeks. Because the various methods detect different physical or chemical attributes of hemoglobin and myoglobin molecules (e.g., catalytic activity, charge, size), myoglobin stability will vary depending on the method of analysis. We recommend that urine samples be analyzed promptly, both to avoid denaturation and to permit earlier therapy.

Concluding Remarks

Myoglobinuria and hemoglobinuria are quite common in hospitalized patients and occur in a wide variety of disorders. Differentiation of the two proteins is important for diagnosis and therapy. Very sensitive techniques such as immunoassays and dipsticks can be misleading through detection of clinically insignificant amounts of myoglobin. Because the urinary concentration of myoglobin associated with a risk of acute renal failure is unknown, we recommend using 15 mg/L as a cutoff. Below this range samples can be considered negative for myoglobin, because the danger of acute renal failure at such concentrations is low.

Given the difficulties associated with reliable specific tests for myoglobin determination and unavailability of simple assays for emergency testing, we recommend that myoglobin testing be offered as a part of a “Rhabdomyolysis/Hemolysis Profile,” obtained with both serum and urine. The first step is to analyze the urine at a 40-fold dilution. If the result is negative, both rhabdomyolysis and hemolysis are ruled out, and no further testing is indicated. If the urine is positive, total CK in serum is analyzed: CK <10 times the upper reference interval indicates hemolysis is present, whereas CK >40 times the reference interval indicates that rhabdomyolysis is present. A large number of erythrocytes in the urine is usually accompanied by in vitro hemolysis. Assays of isoenzymes of CK and LDH in serum and (or) myoglobin in urine are reserved mainly for confirmation in questionable cases. On the basis of the
results of all these tests, the laboratory can offer a more reliable interpretation regarding the presence or absence of rhabdomyolysis than is possible through performing a single chemical analysis for the presence of myoglobin. Assaying serum for CK activity and screening 40-fold-diluted urine with Hemastix probably are the two most ignored but most reliable, simple, and quickly performed tests for detecting rhabdomyolysis.

Recent evidence indicates that the activity concentration of CK, in addition to clinical signs of dehydration and asiasis, is an important risk factor for predicting acute renal failure (64). At present, it is not clear whether quantitative tests for myoglobin (in serum or urine) will be helpful in predicting acute renal failure. Because both serum CK (64) and pigmenturia (12) are both important risk factors for acute renal failure, we infer that myoglobin content (serum or urine) is another one of the multi-risk factors reflecting the degree of muscle damage. However, the relative importance of this factor in the development of acute renal failure and in predicting a patient’s survival remains to be determined.

Myoglobin in the urine is not very stable, necessitating a rapid assay. Reliable methods for quantifying both myoglobin and hemoglobin, as well as determining the reference ranges for these two compounds, remain a challenge for the clinical chemist. Additional areas that remain open for research include the cause of myoglobin nephrotoxicity, the presence of other nephrotoxic factors in myoglobinuria, the critical concentrations (serum and urine) of myoglobin that cause nephrotoxicity, the concentrations of urinary myoglobin after exercise, the correlation between serum CK and urinary myoglobin, the detection of different variants of myoglobin, and the preparation of stable controls and standards for myoglobin assay.

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References


