Differentiation of Myoglobin and Hemoglobin in Biological Fluids

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ABSTRACT

The use of several methods to differentiate myoglobin from hemoglobin has been investigated. The immunochemical methods, particularly those of hemagglutination inhibition and radioimmunoassay, are the most useful. This report summarizes work in the Ames Research Laboratory over the past 17 years with the several methods.

Introduction

Myoglobin, the oxygen binding pigment of muscle, and hemoglobin, the oxygen binding pigment of erythrocytes, are alike, yet different. They perform similar functions and undergo many of the same reactions. There are some physical and chemical differences.3-11 Despite their many similarities, myoglobin and hemoglobin are immunologically different. This difference, and some of the physical and chemical differences, can be used to differentiate one from the other. With the exception of the immunochemical methods, this usually requires the presence of relatively large quantities of myoglobin and hemoglobin.

Myoglobin is released into the blood plasma as a result of damage to muscle tissue. Because of its small size and lack of binding to haptoglobin, it is rapidly removed from the blood by the kidney and excreted into the urine. Hemoglobin, when released from the red cell by intravascular hemolysis, is rapidly and tightly bound to haptoglobin. The haptoglobin-hemoglobin complex is large, so that it is not excreted in the urine. Thus, no hemoglobin appears in the urine until the blood level of hemoglobin exceeds the haptoglobin binding capacity. Once all the haptoglobin is bound, hemoglobin appears in the plasma, both bound to albumin as methemalbumin and as free hemoglobin.

Myoglobinuria is often inferred from the clinical symptoms of muscle weakness and pain,11,20,21 when there is a peroxidase-like pigment in the urine with few or no erythrocytes seen on the microscopic examination. A rise in serum creatine phosphokinase (CPK) is often used to bolster the suspicion.21 If the laboratory is asked to confirm this presumptive diagnosis, a classical method such as solubility in 80 percent saturated ammonium sulfate might be used. When there is insufficient pigment in the urine to be clearly visible, there generally is no suspicion of hemoglobinuria or myoglobinuria. The conditions would be detected only by routinely running occult...
blood tests. The differentiation would then have to be by the more sensitive immunochemical tests.

Methods

Non-immunological Methods

The more classical methods of differentiation, such as ammonium sulfate solubility, ultrafiltration, gel exclusion chromatography, affinity chromatography, and spectra, are described and discussed in the earlier publication. In some of these, the literature method was modified by using occult blood tests to increase the sensitivity.

Immunochemical Differentiation of Myoglobin and Hemoglobin

The most definitive differentiation of hemoglobin and myoglobin in biological fluids can be achieved by using relatively simple immunochemical methods. These methods, which include immunodiffusion, hemagglutination inhibition, and immunoelectrophoresis, are dependent upon the fact that specific antisera will react only with its homologous antigen. Moreover, the methods will quantitate the hemoglobin or myoglobin present.

Preparation of Antisera. The isolation and purification of the immunogens, the immunization schedules, and the processing of the antisera are essentially the same as previously reported. Myoglobin is isolated from human or rhesus monkey muscle according to the selective precipitation method of Luginbuhl. Hemoglobin and proteins other than myoglobin are precipitated from extracts of muscle at pH 8 with 80 percent ammonium sulfate saturation. Under these conditions, the myoglobin remains in solution. Myoglobin is precipitated at pH 7 with 100 percent ammonium sulfate saturation. The myoglobin is further purified by electrophoresis on acrylamide gel which removes albumin and other serum protein contaminants. To obtain material for immunization, the portion of gel containing the myoglobin bands is electroeluted. Colored eluted fractions are pooled and the absorbance in the Soret band region is measured. After the elution, the myoglobin is in the ferro form; at a concentration of one mg per ml, this has an absorbance of 5.8 at 422 nm. From this value and the volume of elute, the total amount of myoglobin is calculated. The elute, which contains buffer material as well as myoglobin, is lyophilized. For immunization, the myoglobin can be either human or monkey.

The hemoglobin immunogen is prepared from laked erythrocytes as described earlier and purified by electrophoresis.

The earlier immunization scheme has been modified in dose and route. The initial immunization in Freund's complete adjuvant is given into the four footpads of rabbits or into the four dew claws of goats. The boosters in Freund's incomplete adjuvant are given subcutaneously or intramuscularly (roughly equal amounts into the four quarters of the animals). The initial dose is 45 μg per animal and booster doses are 22.5 μg per animal. The schedule of boosting and bleeding remain the same. The antisera are centrifuged and processed as soon as a good clot has formed. On immunodiffusion or immunoelectrophoresis, the antisera to myoglobin should give a precipitin line against a myoglobin solution or a urine containing myoglobin at levels of 40 to 50 μg per ml and no line against human albumin at levels of 1000, 100, and 10 μg per ml or against human serum. The antisera to hemoglobin should give a precipitin line against hemoglobin at levels of 40 to 50 μg per ml and no lines against albumin or other serum proteins at levels of 1000, 100, and 10 μg per ml.

Methods for Detecting Antigen-Antibody Reaction. In this laboratory, the methods employed for the immunochemical reactions of myoglobin and hemoglobin are immunodiffusion, immunoelec-
trophoresis, and hemagglutination inhibition for urine, radioimmunooassay for serum myoglobin, and latex agglutination for special applications. Immunodiffusion and immunoelectrophoresis are the same as described earlier, but with modifications of media and buffer as in a later paper. Precipitin lines develop within four to 16 hours.

**Hemagglutination Inhibition.** Myoglobin is coupled to formalinized sheep red blood cells as previously described with modifications. After dialysis in the carbonate buffer, the crude myoglobin solution is diluted so that it has an absorbance of 0.79 at 410 nm. One half ml of this solution is reacted with 6 mg pyrrole-2-carboxylic acid azide at pH 9.5. The packed cells from 7 ml of the 10 percent formalinized sheep erythrocytes are reacted with 2 ml bis-diazobenzidine at pH 3.5. The myoglobin-pyrrole component is reacted with the diazo-benzidine-cells at pH 7.

Hemoglobin is coupled to formalinized sheep red blood cells. For both myoglobin and hemoglobin conjugates, the stabilized diazonium salt, Fast Black B, can be substituted for the bis-diazobenzidine. A solution of 1.2 mg Fast Black B per ml is equivalent to the prepared bis-diazobenzidine. The preparation of pyrrole-2 carboxylic acid azide has been described.

The antisera are heated at 56° to destroy complement and absorbed with formalinized sheep red blood cells as described. Two fold dilutions of the antisera (starting with 1:20) are made in 0.2 M, pH 8.5 Bicine buffer containing 1 percent nonimmune rabbit serum. The number of dilutions will depend on the titer, but nine dilutions are usually prepared.

Microtiter plates* (the titer may vary slightly depending on the particular brand) are used for the titration. Ten vertical rows on the tray are marked for the nine antisera dilutions and one for protein-buffer without antisera. One horizontal row is marked for a negative urine (titer row) one is marked for each standard urine, and one for each unknown urine. In each well of the first vertical row is placed, with a Pasteur pipet (0.025 ml), one drop of the 1:20 antiserum dilution; in each well of the second vertical row is placed one drop of the 1:40 dilution. This is continued through the ninth vertical row. In the tenth row is placed one drop of the protein-buffer. One drop of negative urine is added to all the wells of the first horizontal row; one drop of standard or unknown urine is added to each well of the other horizontal rows. One drop of the appropriate conjugate is added to all the wells.

The contents of the wells are mixed either by vigorously rotating the whole plate or by stirring each well with an applicator stick or toothpick. The plate is placed either over a mirror or on a white background for one hour. A positive is indicated by a ring or button of cells in a well to the left of the first well with a ring in the titer row. The greater the number of wells with rings, the greater the concentration of myoglobin or hemoglobin.

The best standard for myoglobin is a urine containing a large amount of myoglobin but no hemoglobin. The amount of myoglobin can be determined by a peroxidase activity method and then the urine can be diluted with negative urine to give appropriate standards. A standard for myoglobin can be prepared by chromatography of a muscle extract on Sephadex® G-100. The first peroxidase-like reactive band is catalase, the second one is hemoglobin, and the third is myoglobin. This myoglobin is not as immunoreactive as myoglobin excreted in urine. If treated with 6 M urea, which then is removed on a column of Sephadex G-25, the immunoreactivity is increased to that of excreted myoglobin. The amount of myoglobin is determined by the adsorption in the soret band region (about 410 nm): A (mg per ml) = 10.

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* U bottom Ames Autotray, Cooke, or Linbro.
Hemoglobin standards can be prepared from urines and hemolysates in a similar manner.

**Serum Myoglobin.** Because of the relatively low amounts of myoglobin in serum compared to that in urine (nanograms vs. micrograms) and because of interference by other serum proteins, the hemagglutination inhibition method is generally not suitable for serum myoglobin. If the serum myoglobin concentration is high, as may be caused by renal shutdown so that the serum proteins can be diluted out, the hemagglutination inhibition method may be used. Serum myoglobin may be determined by separating myoglobin from most of the serum proteins by passing it through an Amicon XM 50 filter and then concentrating it on a UM 10 filter before using the hemagglutination inhibition method. Radioimmunoassays are best for serum myoglobin. A column radioimmunoassay was reported previously. A double antibody method can be set up in the following manner.

For the first antibody, goat anti-human myoglobin is diluted in goat gamma globulin solution (1 mg per ml in 0.2 M, pH 8.5 Bicine buffer) so that 0.1 ml will bind 30 to 40 percent of the labelled myoglobin. For the second antibody, the solution used is rabbit anti-goat gamma globulin in 0.2 M, pH 8.5 Bicine buffer, 0.03 M in ethylenediamine tetraacetic acid (EDTA), and containing 2 percent Brij 35. Labelled myoglobin and standards are prepared in the same manner as for the column method.

Disposable tubes (75 × 12 mm) are labelled 100 percent blank, serum blank, each standard, and each unknown serum. Into each tube is placed 0.1 ml standard or unknown serum as labelled; 0.1 ml non-immune serum is used for both the blanks. To each tube, except the serum blank, is added 0.1 ml of the first antibody. To the serum blank is added 0.1 ml Bicine buffer. The tubes are vigorously mixed. After 15 minutes, 0.1 ml of labelled myoglobin is added to each tube. The tubes are vigorously mixed. After 30 minutes, 0.2 ml of the second antibody is added to each tube. The tubes are vigorously mixed. After 30 minutes, the tubes are again mixed and then centrifuged at 3000 rpm for 15 minutes. The supernates are decanted; the lips of the tubes (while in an inverted position) are wiped with a tissue. The tubes are inverted on a towel. The tubes are counted in a well counter. The counts of the serum blank are subtracted from the counts of the other tubes. The net counts of the standards and unknowns are expressed as percentages of the net count of the 100 percent blank. The percentage of the standards is plotted on log logit paper against the ng of myoglobin. From this curve the myoglobin in the unknown sera is calculated. A similar commercial kit is available.*

The use of the hemagglutination method in detecting myoglobin in tissues and tissue culture and a fluorescent antibody technique for detecting myoglobin deposited in the kidney was described in the earlier publication. The use of immunochemical methods to distinguish endogenous hemoglobin from myoglobin and hemoglobin of dietary origin was described at the 1973 seminar.

**Results and Discussion**

Many laboratories are depending on the clinical history or the classical ammonium sulfate precipitation method for differentiating hemoglobinuria from myoglobinuria. The original method used only the appearance of the urine before and after 80 percent saturation with ammonium sulfate. The sensitivity is increased by combining this with an occult blood test. With this technique, many urines which contain myoglobin when analyzed by immunochemical methods, are reported to contain only hemoglobin. Chu et al suggest that the ammonium sulfate method fails many times because the

* Nuclear Medical Systems.
pH is not adjusted above 7.5. This is not the complete answer since pH 8 has been used routinely by us and yet these false positives for hemoglobin are obtained. In the ultrafiltration method, myoglobin sometimes fails to pass the filter.

Both the ammonium sulfate precipitation and the ultrafiltration methods also fail to detect a small amount of one of the pigments in the presence of a large amount of the other. Only the immunochemical methods will do this.

Minimal Detectable Levels

In addition to being more discriminating, the immunochemical tests have the lowest minimal detectable levels of myoglobin or hemoglobin. The radioimmunoassay will measure 5 to 10 ng per ml of serum.\(^5,13,23\) The hemagglutination method will detect and measure from 0.3 to 1 µg of myoglobin or hemoglobin per ml of urine. The immunodiffusion and immunoelectrophoresis systems are generally positive with 5 to 10 µg per ml. The occult blood sticks will detect from 1 to 10 µg of hemoglobin or myoglobin per ml of urine, but this is without differentiation of the two pigments. It would be expected that when combined with the stick test, the ammonium sulfate precipitation and the ultrafiltration methods should be able to detect 10 µg of hemoglobin or myoglobin per ml. Chu\(^9\) however, found a minimal detectable level of 50 µg per ml with the ammonium sulfate precipitation method.

Normal Levels

The normal levels of hemoglobin or myoglobin in urine have never been clearly defined. From our experience with the hemagglutination inhibition methods and the stick test, it was found that the normal amount of the pigments in urine is less than 0.3 µg per ml. With vigorous muscular activity, myoglobin in urine sometimes increases to detectable levels. Saranchak and Bernstein\(^22\) reported using radial immunodiffusion normal levels to be below 5 µg per ml and following myocardial infarction to be above 50 µg per ml. Both of these numbers are 10 times higher than ours. It is believed this is a reflection of an error in standards.

Using the radioimmunoassay, it was found in our laboratory that the levels of myoglobin in the serum of 10 normal persons ranged from 0 to 69 ng per ml with a mean value of 26 and a standard deviation of 26. This is in agreement with values reported by Jutzy\(^13\) and by Stone.\(^23\) There is a wide variation in reported hemoglobin levels in plasma depending on the care with which the blood is collected and the method of assay. Naumann\(^19\) reported values of 0.3 to 2.5 mg per dl with an average of 1.3 mg per dl ± 0.12 S.D.

Originally, our immunochemical methods were developed because the question was asked, “Why are the occult blood tests positive and yet no red blood cells are seen under microscopic examination?” Longfield\(^17\) reported that out of 2,700 routine hospital urines, 145 gave positive occult blood reactions when no red blood cell or only an occasional cell was seen. In 1962,\(^1\) it was reported by us of 2,050 urine specimens, 212 contained occult blood and had sufficient volume for further study. By behavior of the reactive material on ultrafiltration, ion exchange cellulose, coprecipitation with albumin by ammonium sulfate, and precipitation with nanonoic and decanoic acid followed by examination of their spectra, circumstantial evidence of the presence of hemoglobin was obtained.

In retrospect, it is now realized that some of the urines may have contained myoglobin. In the past two years, 6,888 urine specimens were tested for occult blood by a research laboratory at a local hospital. Of these specimens, 1,025 gave positive occult blood tests and 187 of these were shown by the hemagglutination test to contain myoglobin.

Most of the studies have been carried out to show myoglobinuria following
myocardial infarction. In Table I are shown the positives obtained with the various studies. The studies are not directly comparable to each other since the reagents and the form of the test have varied over the period of time. The diagnosis of myocardial infarction was based on clinical symptoms, ECG changes, and enzyme elevations, but ultimately on the willingness of the participating cardiologist to commit himself. When other conditions known to cause myoglobinuria are noted, positive tests in the absence of myocardial infarction are negligible.

In a previous report on the radioimmunoassay for serum myoglobin, there was a scattergram showing the distribution of values from normal persons, hospitalized patients without myocardial infarction, and patients with diagnosed myocardial infarction. Nineteen of the 24 patients diagnosed as having myocardial infarction had at least one elevated serum myoglobin. For two of the five patients without elevated serum myoglobin, the serum enzymes (total CPK, SGOT, LDH) were not elevated, the urine myoglobin was negative, and the ECG was questionable; however, the diagnosis was myocardial infarction. For the other three patients without elevated serum myoglobin, it is likely the critical sera were not available. Values as high as 1120 ng per ml were found with most being in the 300 to 500 ng per ml range. When the time of myocardial infarction could be established by an objective sign such as onset of chest pain, it was shown that the serum myoglobin level rose in the first six hours, peaked in about 12 hours, and fell to normal within 24 hours. In some cases the serum myoglobin level remained elevated, indicating continued damage to the heart. In other cases, the level peaked, fell to normal, and then later rose again,—probably indicating a new attack. Sometimes there were elevated levels of myoglobin in the serum drawn on admission. This suggests that the infarction had occurred some time earlier and was generally confirmed by the clinical history.

The importance of the timing of the collection of the specimen was also seen in all the studies with detection of urinary myoglobin. Usually, the amount of urinary myoglobin in the first 12 hours following the insult was greater than in the second 12 hours. For 15 of the 24 patients with myocardial infarction, it was judged that both urine and serum had been collected at the critical times. The sera was assayed by radioimmunoassay and the urines by hemagglutination inhibition. Fourteen of the patients had myoglobin in both serum and urine. One had myoglobin in serum, but not in the urine. This may reflect the differences in sensitivities of the methods.

A variety of conditions resulting in myoglobinemia and myoglobinuria or in hemoglobinemia and hemoglobinuria were listed in earlier publications. During the various studies we, or our cooperating investigators, have noted myoglobinuria in cases of dermatomyositis, trichinosis, fever and virus infections, arterial occlusion, carbon monoxide intoxication, crush injuries (both myoglobin and hemoglobin), drug overdose, heroin addiction, collagen vascular disease, myositis, alcoholic binges, myasthenia gravis with collagen disease, electrical shock, malignant hypertension, disorder of muscle lipid metabolism, and heart and arterial surgery. Some of these

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<th>Institution</th>
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have been associated with renal failure. In the case of the patient with trichinosis, enough serum myoglobin became available, owing to renal shutdown, that the serum could be diluted to give a positive indication in the hemagglutination test. The urine of this patient was reported to be, owing to renal shutdown, that the

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References