**THE DETERMINATION OF BLOOD PLASMA IRON**

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Hematopoiesis has become extremely important because of the increased phlebotomy in obtaining blood plasma. Many studies have been made to show the relationship of nutrition to the rate of blood regeneration. During the course of an investigation with experimental anemia and the utilization of iron in foods, a study of the relationship of the plasma iron to the regeneration of hemoglobin was begun. To do this, there was required a rapid and accurate method for the determination of the plasma iron in numerous samples of blood.

Many methods have been published, all of them based on the precipitation or digestion of the plasma proteins and the final colorimetric measurement of the iron. Fowweather (1) found that the precipitation of the proteins with trichloroacetic acid gave lower values than total digestion of the plasma with sulfuric acid and hydrogen peroxide. Because traces of hemoglobin gave high values when the total digestion method was used, he analyzed only samples of plasma that gave a negative test with benzidine. Both Barkan (2, 3) and Tompsett (4) have studied the use of trichloroacetic acid to precipitate the plasma proteins. Barkan found that incubation of the plasma at 37° in the presence of 1.2 per cent hydrochloric acid prevented the coprecipitation of the inorganic iron and gave good recoveries of added iron. Tompsett maintained that ferric iron forms a protein complex more readily than iron in the ferrous state. He found that reduction of the ferric iron with thiolacetic acid or sodium hydrosulfite or the formation of the ferric pyrophosphate complex prevented the coprecipitation of the iron by the proteins. Shorland and Wall (5) and Borgen and Elvehjem (6) found that thiolacetic acid and sodium hydrosulfite reacted with traces of hematin and hemoglobin, releasing iron to give abnormally high values. They found that sodium pyrophosphate gave low values when added to the sample of blood unless a reducing agent was also present. Because of these conflicting reports and because Barkan’s procedure (3) is not convenient, Schaefer and McKibbin of this laboratory investigated the problem and devised a method for the determination of the plasma iron that is simple.

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and seemed to give accurate results. Their method was based on the observation that the inorganic iron was distributed in equal concentration between the filtrate and the precipitate when the proteins were precipitated with trichloroacetic acid. This method has been used in previous investigations and the values obtained agree very well with those reported by others. When this new problem was undertaken, this same method was used but it was found that consistent results were not obtained. A need for a method that would give accurate and reproducible values was apparent.

**Reagents—**

1. Trichloroacetic acid, 25 per cent solution in water. The trichloroacetic acid should be redistilled if it is not free of iron.
2. Hydrochloric acid, 6 N, redistilled.
3. Ammonium hydroxide, approximately 6 N.
4. Buffer solution, pH 4.58, 27.2 ml. of c.p. glacial acetic acid and 33.4 gm. of c.p. sodium acetate (anhydrous) dissolved in water and made to a volume of 250 ml.
5. Thioglycolic acid, Eastman’s practical grade.
6. p-Nitrophenol, 0.1 per cent solution in water.
7. α, α'-Bipyridine, 0.2 per cent solution in 5 per cent acetic acid. Dissolve 0.2 gm. of the reagent in 5 ml. of c.p. glacial acetic acid and dilute to 100 ml. with water.
8. Standard iron solution. Dissolve iron wire (99.8 per cent) in a mixture of nitric acid and hydrochloric acid and dilute with water to give a concentration of 1 mg. per ml. From this stock solution standard solutions of 100 and 1 μg per ml. are made.
9. Iron-free water. Distilled water should be redistilled from glass. All equipment should be thoroughly cleaned and rinsed with iron-free water.

**EXPERIMENTAL**

In order to use the minimum amount of plasma and yet obtain enough iron for the formation of sufficient color intensity, α, α'-bipyridine was selected as the reagent. α, α'-Bipyridine forms an intense pink color with ferrous iron which has an absorption maximum at about 520 mμ and can be used to detect less than 0.05 part per million. Since the iron must be in the ferrous state, thioglycolic acid was used as the reducing agent. It is a very strong reducing agent and only 2 drops of the undiluted reagent are required. Although variations in pH between 3 and 9 do not influence the intensity or hue of the color produced with bipyridine (7), a constant pH was maintained in the standard and test solutions. An acetic acid-acetate buffer was chosen which buffered at pH 4.6. At this pH the color formation is almost instantaneous.

It was found that because of the small amounts of inorganic iron in blood
plasma it would be necessary to measure the color intensity obtained with 1 to 4 γ of iron. The sensitivity of the test for iron with bipyridine was determined by measuring the color obtained with 0.5, 1.0, 2.0, 3.0, and 4.0 γ of iron in 11 ml. of solution. The Evelyn colorimeter was used to measure the intensity of the color. Filter 520 was used, and the transmission of light was read on the galvanometer scale to the nearest 0.1 of a scale division. The L values ($L = 2 - \log G$) were calculated from galvanometer readings ($G$) and plotted against micrograms of iron (Fig. 1). After a series of similar experiments, it was found that 0.5 to 1 γ of iron can be determined with an accuracy of ±6 per cent. The accuracy for 1 to 2 γ is ±3 per cent, and ±2 per cent for 2 to 4 γ.

Various recovery experiments were conducted by the Schaefer-McKibbin method. The procedure is as follows: 5 ml. of blood plasma which had been obtained from a dog by venipuncture, a small quantity of a saturated sodium oxalate solution being used to prevent clotting, were pipetted into a 15 ml. calibrated centrifuge tube. 5 ml. of 10 per cent trichloroacetic acid solution were added and mixed with the plasma. If a recovery experiment were to be made, the iron solution containing a known amount of iron, e.g. 2, 4, 6, 8, and 10 γ, was added before the trichloroacetic acid was mixed with the blood plasma. The tubes were then placed in a water bath (80–90°) for 5 minutes, and then centrifuged for 15 minutes at 2000 r.p.m. The supernatant liquid was poured off into another 15 ml. calibrated centrifuge tube after the volume of the precipitate and of the clear solution had been noted. The iron was then determined in the clear filtrate or an aliquot of

Fig. 1. Standard iron curve
The results are tabulated in Table I. It is seen that the amount of added iron recovered in the filtrate varied from 50 to 78 per cent. If the amount found in the precipitate was also included, the total recovery ranged from 83 to 108 per cent. This was an indication that iron was carried down with the precipitate and that the relationship between the iron in the filtrate and the total iron was variable. An attempt was made to wash the precipitate with a mixture of water and trichloroacetic acid but the recoveries were not appreciably improved. It was necessary to devise a method to eliminate the “carry down” of the iron with the precipitate, or one which would permit the recovery of all the iron by the simple procedure of washing the precipitate.

Since Tompsett (4) found that thioglycolic acid and sodium hydrosulfite improved the recoveries in his method, it was decided to test the effect of reducing agents on the iron content of plasma and on the recovery of added iron. The blood plasma used in this experiment was obtained from dogs. Since the blood had been allowed to remain in the ice chest for 12 hours before centrifugation, there was quite a bit of hemoglobin in the plasma. The Schaefer-McKibbin method\(^1\) was followed and the reducing agent was added to the plasma before the proteins were precipitated. The precipitate was washed with a mixture of the trichloroacetic acid solution and water, and this was added to the filtrate. The results are tabulated in Table II. It will be noted that the plasma iron in Groups II, III, IV, and V is very high when compared to the other groups. It is an indication that iron other than the plasma iron was being measured. This is similar to the results of Shorland and Wall (5), and Borgen and Elvehjem (6). They found that the non-hemoglobin iron content of blood is increased when thioglycolic acid or sodium hydrosulfite is added before the proteins are precipitated.

In order to obviate the situation that was encountered in the preceding experiment, fresh plasma, free of hemoglobin except perhaps for traces

### Table I

Experiments on Recovery of Iron from 6 Ml. of Plasma by Schaefer-McKibbin Method

<table>
<thead>
<tr>
<th>Iron added (mg)</th>
<th>Added iron in filtrate (mg)</th>
<th>Iron recovered (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\gamma)</td>
<td>(\gamma)</td>
</tr>
<tr>
<td>2.00</td>
<td>1.38</td>
<td>69.0</td>
</tr>
<tr>
<td>4.00</td>
<td>2.62</td>
<td>65.5</td>
</tr>
<tr>
<td>6.00</td>
<td>3.12</td>
<td>78.0</td>
</tr>
<tr>
<td>8.00</td>
<td>3.57</td>
<td>59.5</td>
</tr>
<tr>
<td>10.0</td>
<td>5.04</td>
<td>63.0</td>
</tr>
</tbody>
</table>

\(^1\) Schaefer-McKibbin method
which could not be detected, was used. The experiment was repeated, and now the abnormally high values were not obtained with the strong reducing agents, but the recovery of added iron was not good in all cases. The main difficulty seemed to be the coprecipitation of the iron and the incomplete removal of this iron by washing. It is believed that the iron is mechanically carried down by the precipitate. It had been noted that the precipitate formed by the addition of trichloroacetic acid to the plasma was very fine and seemed to be quite hydrated, settling to the bottom of the centrifuge tube to form a thick mass. The problem seemed to be 2-fold: first, to reduce any plasma iron that may be in the ferric state so that protein complexes are not formed, and second, to precipitate the proteins so that very little iron is carried down. The solution to both of these problems came when the properties of the proteins in question were taken into account. Mirsky and Anson (8) maintain that denaturation of most proteins releases sulphydryl groups which have reducing properties. They recognize also the presence in proteins of non-sulphydryl reducing groups which they ascribe to the tyrosine and tryptophane residue. They found that denatured serum globin would reduce the ferricyanide ion. Jaques (9) found that fibrinogen can reduce dilute solutions of iodine and of hydrogen peroxide rapidly at 25° at pH 5 to 7. The reducing power of fibrinogen is not changed by denaturation, but the reducing power toward hydrogen peroxide is increased on conversion to fibrin. He also noted that denaturation of the serum proteins at 100° increased the reduction of iodine by 20 per cent. In

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Additions to 5 ml. plasma</th>
<th>Iron added</th>
<th>Iron in 5 ml. plasma</th>
<th>Iron recovered per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>None</td>
<td>0</td>
<td>13.0</td>
<td>78.0</td>
</tr>
<tr>
<td>II</td>
<td>5 drops thioglycolic acid</td>
<td>8.32</td>
<td>33.0</td>
<td>182</td>
</tr>
<tr>
<td>III</td>
<td>10 &quot;</td>
<td>8.32</td>
<td>46.5</td>
<td>110</td>
</tr>
<tr>
<td>IV</td>
<td>2 ml. 4% Na₂S₂O₃</td>
<td>0</td>
<td>32.0</td>
<td>119</td>
</tr>
<tr>
<td>V</td>
<td>2 &quot; 10% &quot;</td>
<td>8.32</td>
<td>37.0</td>
<td>134</td>
</tr>
<tr>
<td>VI</td>
<td>1 &quot; 10% NH₂OH-HCl</td>
<td>0</td>
<td>12.5</td>
<td>90</td>
</tr>
<tr>
<td>VII</td>
<td>100 mg. vitamin C</td>
<td>8.32</td>
<td>20.0</td>
<td>12.2</td>
</tr>
</tbody>
</table>

TABLE II

Effect of Reducing Agents
following these leads, we found that when the plasma is heated at 100° a gel is formed. Upon addition of trichloroacetic acid a light, curdy precipitate results, the very nature of which facilitates the removal of any residue iron. The following procedure has been perfected so that reproducible and consistent results were obtained with good recoveries of added iron. This method does not require blood plasma free of hemoglobin because any hemoglobin is precipitated by the trichloroacetic acid.

**Proposed Procedure**

3 to 5 ml. of blood plasma containing about 3 to 9 γ of inorganic iron are pipetted into an ungraduated Pyrex 15 ml. conical centrifuge tube. 3 ml. of redistilled water are added and mixed with the plasma. A blank consisting of all the reagents used is also prepared.

Place the tubes in boiling water for 2 to 3 minutes, or until the solution becomes opaque. Cool the tubes in cold water.

Add 2 ml. of 25 per cent trichloroacetic acid solution, and stir thoroughly so that the acid is intimately mixed with the plasma. Use a small blunt end stirring rod so as not to break through the bottom of the tubes.

Place the tubes in a water bath at 90-95° for 3 minutes, stirring the solution once or twice.

Remove from the bath and cool in cold water. Centrifuge at 2000 to 3000 R.P.M. for 5 minutes.

Decant the supernatant liquid into a 15 ml. graduated centrifuge tube.

Add 4 ml. of redistilled water and 1 ml. of trichloroacetic acid to the original tube. Break up the precipitate and stir well. Place in a water bath at 90-95° for 3 minutes, stirring each solution once. Remove and cool the tubes in cold water.

Centrifuge the tubes for 5 minutes at 2000 to 3000 R.P.M. and decant the supernatant liquid into the tube which contains the first filtrate.

Add 1 drop of 0.1 per cent p-nitrophenol indicator solution, and add NH₄OH drop by drop until the solution becomes yellow.

Add 1 ml. of the buffer solution and enough water to make 15 ml. Mix.

Pipette an aliquot, 5 or 10 ml., containing 2 to 3 γ of iron into an Evelyn tube, and add 2 drops of thioglycolic acid and mix. If less than 10 ml. are taken, add water to make 10 ml.

Determine the center setting (100 per cent transmission on the galvanometer scale) for each solution in the colorimeter.

Add 1 ml. of 0.2 per cent α, α'-bipyridine reagent and mix by gentle shaking or tapping of the tube. Read each tube in the colorimeter with the respective center setting previously determined. The galvanometer (G) reading is recorded to the nearest 0.1 of a scale division if the Evelyn colorimeter is used.
Calculate the amount of iron in the solution in each tube by reference to a standard curve or use the following formula after calculating the $L$ values from the $G$ readings.

\[
\frac{\text{Micrograms iron}}{\text{Aliquot}} = 40.6 \times L_{\text{aliquot}} - L_{\text{reagent blank}}
\]

In order to test the validity of the proposed method, recovery experiments were made which are summarized in Table III. The recovery of added iron ranges from 92 to 104 per cent. Considering the sensitivity of

**Table III**

*Recovery of Added Iron*

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Sample</th>
<th>Iron added</th>
<th>Iron found</th>
<th>Iron recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>335</td>
<td>5</td>
<td>3.12</td>
<td>2.94</td>
<td>94.5</td>
</tr>
<tr>
<td>340</td>
<td>3</td>
<td>1.04</td>
<td>0.96</td>
<td>92.3</td>
</tr>
<tr>
<td>338</td>
<td>5</td>
<td>4.16</td>
<td>4.00</td>
<td>96.0</td>
</tr>
<tr>
<td>325</td>
<td>3</td>
<td>1.04</td>
<td>1.05</td>
<td>101</td>
</tr>
<tr>
<td>337</td>
<td>5</td>
<td>2.08</td>
<td>2.16</td>
<td>104</td>
</tr>
</tbody>
</table>

**Table IV**

*Plasma Iron Values of Normal and Anemic Dogs*

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Age</th>
<th>Ration</th>
<th>Iron in 100 ml. plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>333</td>
<td>1</td>
<td>Synthetic ration</td>
<td>90.0</td>
</tr>
<tr>
<td>334</td>
<td>1</td>
<td>Mineralized milk + vitamins</td>
<td>102</td>
</tr>
<tr>
<td>335</td>
<td>1</td>
<td>&quot; + &quot; without added iron</td>
<td>32.0</td>
</tr>
<tr>
<td>325</td>
<td>9</td>
<td>Synthetic ration</td>
<td>200</td>
</tr>
<tr>
<td>300</td>
<td>12</td>
<td>Mineralized milk + vitamins</td>
<td>141</td>
</tr>
</tbody>
</table>

the method and the error incurred by the use of the Evelyn colorimeter with colored solutions of low intensity, these recoveries are good. Recovery of the added constituent does not entirely determine the precision of a method. Reproducibility of results is also important, and we have found that samples of the same blood gave remarkably similar results.

This proposed method was applied by Messrs. Michaud and Ruegamer to their work with nutritional anemia. In numerous analyses of blood plasma, they obtained reproducible values and consistent results. Some typical data as found by them are listed in Table IV.
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SUMMARY

1. A method has been devised to measure the plasma iron. The difficulties encountered because of the precipitation of the plasma proteins by trichloroacetic acid were obviated by a simple procedure. The proteins are denatured by heat before being precipitated by the trichloroacetic acid. One washing of the precipitate seems to recover all the iron.

2. α, α-Bipyridine was used to measure the iron colorimetrically in the range of 1 to 4 γ. When an acetic acid-acetate buffer at pH 4.6 is used, the color formation is instantaneous.

3. The plasma iron may be determined with a maximum error of ±10 per cent for blood plasma containing 30 to 90 γ per cent of iron, and with a maximum error of ±5 per cent for blood plasma containing 100 to 200 γ per cent of iron.

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