THE DETERMINATION OF IRON IN SMALL VOLUMES OF BLOOD SERUM*

By HELEN B. BURCH,‡ OLIVER H. LOWRY,‡ OTTO A. BESSEY,§ AND BELLA Z. BERSON

(From the Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., New York)

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In an effort to find practical means of distinguishing iron deficiency anemias from other types in large population groups, an investigation was made of the possibility of measuring iron in an amount of blood serum obtainable from the finger. Iron deficiency anemias are characterized by low serum iron, while other states of diminished hemoglobin formation show normal or even high values for serum iron (1-4). The prevalence of iron deficiency anemias in large population groups has not been widely investigated, owing partly to the difficulty of obtaining necessary quantities of blood for analysis and partly to the troublesomeness of existing methods. Present methods require 1 to 10 ml. of blood serum and time-consuming techniques.

Most widely used of the methods for iron in blood serum are those involving color reactions of ferrous iron with α,α'-bipyridyl (5, 6) or o-phenanthroline (4, 7) and of ferric iron with thiocyanate ion (1,8-10). Of these three reagents, thiocyanate ion appeared to offer advantages for a simple microprocedure. Both bipyridyl and o-phenanthroline require several steps for their use (pH adjustment and reduction of ferric to ferrous iron prior to color formation) which it is possible to avoid by using a thiocyanate procedure. It has been found possible, with thiocyanate ion, to measure iron with satisfactory precision in 20 c.mm. of serum. In this method, which is described below, acid and thiocyanate ion are added as one reagent to the serum and the iron thiocyanate is extracted directly from the mixture with isoamyl alcohol. This avoids the preparation of serum filtrates and improves the extraction of iron from the serum proteins.

In addition to a description of the procedure, illustrative data are presented; viz., the daily and hourly variation in serum iron in several normal persons.

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‡ Present address, Department of Chemistry, Columbia University, New York.
§ Present address, Department of Pharmacology, Washington University School of Medicine, St. Louis.
§§ Present address, Department of Biological Chemistry, University of Illinois College of Medicine, Chicago.
DETERMINATION OF IRON IN BLOOD SERUM

EXPERIMENTAL

Reagents—All water used is carefully redistilled from glass and stored in Pyrex glass-stoppered bottles.

1. NH₄SCN, 27 per cent. Traces of iron are removed by adding 0.05 ml. of concentrated HCl per 100 ml. and extracting with a 3:1 mixture of isoamyl alcohol and ethyl ether until no pink color is visible. Stored at 4° this reagent is satisfactory for several months.

2. Hydrochloric acid, 1.35 N, prepared from redistilled 6 N (constant boiling) acid.

3. Isoamyl alcohol, redistilled, saturated with water, and stored at 4°. On the day used, to 10 ml. is added 0.1 ml. of 0.2 per cent HzO₂ prepared from 30 per cent HzO₂ (superoxol).

4. Complete reagent. Equal volumes of 27 per cent NH₄SCN and 1.35 N HCl are mixed for each series of determinations. This reagent keeps not more than 2 hours.

5. Standard iron solution. A stock, 100 mg. per cent, iron solution is prepared from iron wire (Bureau of Standards) by means of redistilled HCl and a few drops of redistilled nitric acid. A working standard of 200 γ per cent is prepared in 0.01 N HCl.

6. Ascorbic acid, 30 per cent in ethylene glycol. Warming to 60° is necessary to dissolve completely; higher temperatures result in discoloration. Preparation of fresh solution daily is recommended.

Apparatus for 20 C.mm. of Serum—

1. Constriction pipettes 2, 20, 60, and 90 c.mm. (11, 12) and two fine tipped constriction pipettes of about 80 c.mm. capacity for transferring samples in and out of the spectrophotometer cuvettes.

2. Blood-collecting tubes. Melting point capillaries, 1.5 to 2.0 mm. outside diameter, 7 to 10 cm. long, e.g., No. 34500, Kimble Glass Company, Vineland, New Jersey. These and the serological tubes listed below are cleaned by boiling in half concentrated nitric acid, rinsing in distilled water, boiling in distilled water, rinsing finally with redistilled water, and dried in an oven at 45-50°.

3. Serological tubes 6 X 50 mm.; e.g., Kimble, No. 45060.

4. Wooden or plastic racks for tubes.

5. Glass-stoppered flasks for reagents. These are cleaned in acid and rinsed thoroughly with distilled and glass-redistilled water.

6. Centrifuge fitted with cups suitable for small tubes.

7. Beckman spectrophotometer adapted to small volumes (13). Micro cells of 2 to 2.4 mm. inner width are appropriate.

8. High speed hand drill with a flattened nail for violently agitating the samples to effect the extraction of the aqueous phase with isoamyl alcohol (14).
Apparatus for 0.160 Ml. of Serum—
1. Constriction pipettes, 0.015, 0.150, 0.45, and 0.6 ml., and two transfer pipettes of about 0.5 ml. capacity.
2. Blood-collecting tubes. Pyrex tubing 4 mm. outside diameter, 10 cm. long; cleaned as described above.
3. Sleeve stoppers (vaccine caps) for stoppering the blood-collecting tubes. The outside sleeve is cut off, and the inner core is used. No. 1A-66F, West Company, Phoenixville, Pennsylvania.
4. Pyrex test-tubes, 5 ml.
5. Other equipment as described above. A Coleman junior (model 6-A) spectrophotometer with a special adapter may be used for this volume (12).

Procedure

Collection of Samples—Special care must be taken to have the surface of the finger clean. It is then wiped with 80 per cent alcohol and incised with a new, clean, Bard-Parker scalpel blade (No. 11). The first drop of blood is discarded by wiping with a clean towel. The small samples, e.g., for 20 c.mm. of serum, are collected and sealed in the small capillary as previously described (15). Volumes up to 150 c.mm. of serum (300 c.mm. of blood) are more conveniently collected by the use of the larger tubes. By squeezing the finger this amount of blood is easily obtainable. If necessary, the hand should be warmed. The first few mm. of the dry end of the tube are coated with vacuum wax or vaseline and a vaccine cap firmly pressed into place after filling. The unwaxed end of the tube is closed by another cap. Before centrifuging, the cap on the waxed end of the tube is taped on with adhesive tape. After sealing, the samples are allowed to clot, are centrifuged, and the serum is collected at once and stored in iron-free tubes, preferably frozen, until analyzed.

Analysis—It is, of course, necessary to work in rooms as free as possible from dust and to take every precaution to avoid contamination of samples and clean vessels by covering them whenever possible with dust-proof materials. To 20 c.mm. of serum in a 6 X 50 mm. tube are added 60 c.mm. of the “complete reagent.” The sample is well shaken at once by tapping to obtain a finely divided precipitate from which iron can be extracted. 90 c.mm. of isoamyl alcohol containing H_2O_2 are added. The tube is covered with Parafilm, and mixed violently by vibration with the high speed motor and nail (“Apparatus,” item (8)).

Blanks and standards are prepared at the same time by substituting either water or standard iron solution for the serum.

The tubes are chilled in centrifuge cups to 4-10° and then centrifuged at 3000 R.P.M. for 10 minutes. Approximately 80 c.mm. of the upper
isoamyl alcohol layer are pipetted carefully into the Beckman cell with care to avoid contamination from the precipitate interface or from the water layer. (To clean the narrow Beckman cells, allow them to stand for 10 or 15 minutes filled with 1 N HCl, rinse well with redistilled water, and dry with alcohol and ether. Finally, one of the reagent blanks is used for rinsing the cell.) With isoamyl alcohol in the reference cell, the optical density of the sample is read at 520 mμ (R₁). The entire sample is removed from the cell with a fine pipette and delivered into a clean tube. 2 c.mm. of the 30 per cent ascorbic acid reagent are added and the solution is mixed. After 10 to 60 minutes, the optical density of the solution is again measured (R₂).

In practice, a series of samples is read, removed from the cells, and reduced with ascorbic acid. The tubes are covered with Parafilm and allowed to stand until the completion of the original readings on a series. Then the optical density of the reduced solutions is determined.

Calculation—Since the same pipettes are used for serum and for iron standard solutions, the volume of the pipettes does not enter the calculations.

\[ R₂ - R₁ = R₁ - R₃ \]

\[ \frac{(R₂ - R_{\text{blank}}) \times 200}{R_{\text{standard}} - R_{\text{blank}}} \times \frac{100}{90} = \% \text{Fe} \]

The factor 100/90 is introduced to correct for the incomplete recovery (90 per cent) of iron from serum.

DISCUSSION

Extraction of Iron from Serum—For the conversion of iron into a determinable form, two types of methods have been employed previously: (1) ashing and (2) preparation of protein-free filtrates. The various ashing methods give total iron values for serum without correction for the possible contribution of iron due to hemolysis. Since hemolysis is likely to occur to a slight degree even under very favorable conditions, ashing methods for large numbers of samples were considered unsatisfactory.

Methods depending on precipitation of the protein and removal of iron in the filtrate were found to be unsatisfactory on the micro scale. The recovery of added iron was low and irregular. It was found possible to obtain better recovery of iron and with fewer steps by adding acid and NH₄SCN as one reagent to the serum and extracting the Fe(SCN)₃ directly with isoamyl alcohol. Since irregularities in results were found when trichloroacetic acid was used to precipitate the proteins, it was omitted from the reaction mixture. The thiocyanic acid itself causes a considerable precipitation of the proteins.
The concentrations of HCl and NH$_4$SCN and peroxide were chosen to
give maximal extraction of iron and color development and minimal blank
values. Increasing any of these three reagents beyond the given concen-
tration tends to cause an increase in the blank.

Correction for Acid Hematin—In addition to Fe(SCN)$_3$, acid hematin is

![Absorption curves of isoamyl alcohol extracts of aqueous solutions: acid
hematin, acid hematin plus ascorbic acid, ferric thiocyanate, ferric thiocyanate plus
ascorbic acid.](image)

...unfortunately also extracted into isoamyl alcohol together with other un-
known materials present in serum which absorb light at the wave-length
used for measurement (520 m$_\mu$). A means was, therefore, sought to
eliminate, correct for, or reduce to a minimum this interference.

Since acid hematin has an absorption 12 times greater at 400 m$_\mu$ than at
520 m$_\mu$ (Fig. 1), it is possible to achieve an approximate correction for acid
hematin by measuring the absorption of the isoamyl alcohol extracts at 400 m\(\mu\) and calculating the contribution of hematin to the absorption at 520 m\(\mu\). However, other materials extracted from serum make the proper correction factor somewhat uncertain and, therefore, this procedure unsatisfactory.

The selective destruction of the color of Fe(\(\text{SCN}\))\(_2\) without changing the absorption of the interfering materials was another possibility. The addition of ammonia decolorized Fe(\(\text{SCN}\))\(_3\) but also caused a change in the color of acid hematin. Reagents such as stannous chloride and sodium arsenite reduced the Fe(\(\text{SCN}\))\(_3\) but proved unsatisfactory for other reasons. However, it was found that minute quantities of ascorbic acid rapidly reduced Fe(\(\text{SCN}\))\(_3\) in isoamyl alcohol solution and that acid hematin was not affected, particularly in the 500 m\(\mu\) region (Fig. 1). A 30 per cent solution of ascorbic acid in ethylene glycol proved to be a satisfactory reducing agent because of the low volatility of the solvent and the relatively small volume of solution required for the reaction.

In addition to acid hematin there may be a slight contribution to the blank from other substances present in serum. These substances show minimal absorption at 520 m\(\mu\) (Fig. 2). Although the absorption of Fe(\(\text{SCN}\))\(_3\) is somewhat less than its maximum at this wave-length, absorption at 520 m\(\mu\) was chosen as best for the serum iron measurements in order to keep the blank values as low as possible.

The difference curves (Fig. 2) furnish further evidence that only Fe(\(\text{SCN}\))\(_3\) is changed by reduction with ascorbic acid. The absorption curves for Fe(\(\text{SCN}\))\(_3\) extracted from serum and for the same extract after addition of ascorbic acid were measured between 420 and 560 m\(\mu\). The difference curve represents the change in absorption on reduction. This difference curve is compared with a difference curve calculated from the absorption curve of Fe(\(\text{SCN}\))\(_3\). It can be seen that the actual and calculated difference curves nearly coincide.

Standards and Recoveries—Proportionality of the Fe(\(\text{SCN}\))\(_3\) color produced at low concentrations of iron in the micromethod is satisfactory (Fig. 3). Iron standards containing 95, 184, and 328 \(\gamma\) per cent of Fe were used in 20 c.mm. quantities (19, 37, and 66 m\(\gamma\) of Fe per sample) for these measurements.

Similar concentrations of iron standards were added to serum for recovery experiments (Table I). Whether or not the reagent stands in contact with the serum before extraction with isoamyl alcohol seems to make no significant difference in the recovery, which averaged 89 per cent (Table I). Additional tests have shown that recovery was not increased by allowing the serum to stand with one-fourth its volume of 6 N HCl for 15 minutes prior to the addition of NH\(_4\)\(\text{SCN}\) and isoamyl alcohol. The peroxide is added to convert and maintain iron in the ferrie (III) state.


Reproducibility—Separate samples of blood from each of four fingers of the same hand taken in consecutive order were analyzed in triplicate (Table II). The standard deviation among the twelve analyses was 3.6 \( \gamma \) per cent, which agrees with other replicate analyses. The agreement between the averages for each finger is about that expected from the standard deviation, which suggests that the method of obtaining blood from the finger need not introduce additional errors. This is borne out by comparison of analysis on blood serum from the finger and from the vein (Table III). It is indicated in Table III that simple cleansing of the finger with alcohol is more effective in preventing contamination of the sample than attempting to coat the finger with vaseline, etc.

Daily and Hourly Fluctuations—Fig. 4 records the hourly variations observed in the serum levels of three subjects during three morning periods. In each instance the subjects had been without food for about 15 hours.

![Absorption curve](http://www.jbc.org/)

**Fig. 2.** Absorption curves (Curve A) of \( \text{Fe(SCN)}_3 \) extracted from serum with isoamyl alcohol and (Curve B) of this extract reduced with ascorbic acid and difference curves obtained by subtracting the values for Curve B from Curve A and by calculating the difference curve from the absorption curve of \( \text{Fe(SCN)}_3 \).
prior to the test to avoid possible alimentary effects. Rather marked fluctuations were observed without any definite trend. The greatest

![Graph showing proportionality between iron concentration and optical density.](image)

**FIG. 3.** Curve showing proportionality between the concentration of iron in standard solutions and the optical density of the reaction product (Fe(SCN)\(_3\)) as measured in the isoamyl alcohol layer.

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery of Iron Added to Serum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum Fe, initial</th>
<th>Added Fe</th>
<th>Total Fe, found</th>
<th>Recovery per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standing with reagent 15 min. before addition of isoamyl alcohol</td>
<td>54 (\gamma) per 100 ml. serum</td>
<td>100 (\gamma) per 100 ml. serum</td>
<td>141 (\gamma) per 100 ml. serum</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>202</td>
<td>234</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>396</td>
<td>400</td>
<td>89</td>
</tr>
<tr>
<td>Reagent followed immediately by isoamyl alcohol extraction</td>
<td>48</td>
<td>100</td>
<td>134</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>202</td>
<td>234</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>396</td>
<td>400</td>
<td>90</td>
</tr>
</tbody>
</table>

change in any one 3 hour period was 40 \(\gamma\) per cent, the smallest was 6, and the average was 20 \(\gamma\) per cent. In no case, however, did any sample fall below 65 or rise above 122 \(\gamma\) per cent. Heilmeyer and Plötner (4)
have reported a decrease in serum iron after 6 hours fasting, with a subsequent rise of 25 per cent after 24 hours. Changes in the serum iron levels also occur on different days, as noted by Skouge (2) and more recently by Hyger (16). The latter describes a fall in serum iron from morning to evening of an average of 42 γ per cent, and daily and weekly average varia-

Table II

<table>
<thead>
<tr>
<th>Serum Fe in Consecutive Samples of Finger Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finger 1</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Fe, γ %</td>
</tr>
<tr>
<td>94</td>
</tr>
<tr>
<td>98</td>
</tr>
<tr>
<td>96</td>
</tr>
<tr>
<td>Average</td>
</tr>
<tr>
<td>&quot; 12 analyses &quot;</td>
</tr>
<tr>
<td>Standard deviation</td>
</tr>
</tbody>
</table>

Table III

Comparison of Iron in Blood Serum from Vein and Finger

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>Fe, γ per 100 ml.</th>
<th>Source of Serum</th>
<th>Fe, γ per 100 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein</td>
<td></td>
<td>Finger coated with mineral oil</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>107</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>107</td>
<td>112</td>
<td>141</td>
</tr>
<tr>
<td>Average</td>
<td>106</td>
<td></td>
<td>138</td>
</tr>
<tr>
<td>Finger cleaned with alcohol</td>
<td>113</td>
<td>113</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>120</td>
<td>127</td>
</tr>
<tr>
<td>Average</td>
<td>115</td>
<td></td>
<td>128</td>
</tr>
</tbody>
</table>

Periodically during a period of 1 month, at 11.00 a.m., blood serum was obtained from each of four adult women and analyzed for iron by the proposed micromethod. The individual averages were 92, 94, 100, and 109 γ per cent. The standard deviations were, respectively, 24, 23, 23, and 21 γ per cent (Table IV). The lowest value observed was 60 and the highest 155 γ per cent. No correlation of the iron values with the menstrual cycle
**FIG. 4. Hourly serum iron levels in fasting subjects**

**TABLE IV**

<table>
<thead>
<tr>
<th>Time interval days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject A γ per 100 ml.</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>19</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>26</td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td>31</td>
</tr>
</tbody>
</table>

Average: 92, 94, 100, 110

s.d.: ±24, ±23, ±23, ±21
was observed. These averages and fluctuations concur with the data in the literature obtained with macro serum iron methods.

It is apparent from these measurements and those in the literature that the serum iron levels may undergo considerable hourly and daily variation. This must surely be taken into account in interpreting data designed to reveal nutritional status with respect to iron. On the basis of knowledge now available on the subject, the following conclusions seem reasonable. Serum iron values less than 60 \( \gamma \) per cent are rarely found in well nourished persons and, therefore, in the absence of infection or other pathology (17) such low values can probably be interpreted as indicating an iron deficiency. Conversely, values above 60 \( \gamma \) per cent indicate an adequate iron supply. Since factors other than iron supply lead to rather wide fluctuations in serum values, such analyses cannot be safely used to estimate iron intake except on a rather rough scale. This limitation notwithstanding, serum iron analysis, if a suitable method were available, would seem to provide a useful and practical means of determining the occurrence of iron deficiency in large population groups. It is felt possible that the proposed micromethod may serve this function.

SUMMARY

1. A simple method is described for the determination of iron in 20 c.mm. of blood serum with ferric thiocyanate as the colored compound measured. Iron added to serum is consistently recovered to the extent of about 90 per cent. The standard deviation of replicates is 3 to 5 \( \gamma \) per cent. Since blood serum from the finger can be used and since one analyst can perform twenty-five to fifty analyses in a day, the method appears suitable for nutritional surveys.

2. Hourly variations in serum iron values averaging 20 \( \gamma \) per cent were observed during 3 hour periods for three well nourished persons. The variations over a period of a month for four adult women were greater but seemed to be restricted within the limits of 60 to 155 \( \gamma \) per cent. Even with this variation it is felt that, since serum iron of less than 60 \( \gamma \) per cent is rare among well nourished persons, serum iron analysis by the method described provides a useful and reliable means for determining the occurrence of iron deficiency in large population groups.

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