CCXXV. STUDIES OF THE HAEMOGLOBIN AND IRON OF THE BLOOD.

I. THE DETERMINATION OF THE TOTAL IRON OF BLOOD.

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METHODS for the determination of the total iron content of blood have been the subject of very active investigation for many decades and the great advances in knowledge of all forms of anaemia during the past ten years have greatly stimulated these researches.

Gravimetric methods were evolved more than a century ago and gave good results, but they necessitated the use of large volumes of blood of the order of 100–200 ml. Attention was therefore turned to titrimetric methods in which the iron content of ash obtained by incinerating blood was estimated by titration against potassium permanganate, titanous solutions, or thiosulphate after the addition of potassium iodide.

About forty years ago the advantages of colorimetric methods were being realized. It was found that the iron of the ash of blood could be estimated by examination of the Prussian Blue colour developed when ferrocyanides are added, or of the red colour formed on the addition of thiocyanates, under suitable conditions, to the ash. The latter colour reaction has formed the basis of almost all the work of the past thirty or forty years, but many workers have thought that it does not give correct results, especially with regard to tissues rich in phosphorus.

Bogniard & Whipple [1932], Klumpp [1934], Horwitt [1934] and others have therefore revived methods using titanous salts to estimate, by titration, the iron content of ashed material, but the shortcomings of these methods have been pointed out by them and others. Titanous salts react not only with iron salts but also with those of copper, tin, platinum and mercury, so that results in the presence of any of these other elements will give too high a value for the iron content. Moreover, when the end-point is determined colorimetrically, the presence of phosphates tends to obscure this point. The chief difficulty, however, is the necessity of preserving the titanous solution under carbon dioxide, hydrogen, or some other inert gas and of carrying out the titration in an inert atmosphere. In addition, the solutions must be frequently standardized.

Efforts have recently been made to devise, with other reagents, colorimetric methods free from the disadvantages of the thiocyanate reaction. Burmester [1935] and Tompsett [1934] have used thiolacetic acid.

Hill [1931] has shown that dipyridyl, in the presence of small quantities of iron, gives an extremely stable pink colour which is not affected by a wide variation in conditions. This marked specificity for iron, together with other useful properties, has quickly given dipyridyl a definite place in iron analysis and several workers have adapted it for various purposes. Elvehjem *et al.* [1933] have used it for estimating the "available" iron of wheat, yeast etc., as have also Shackleton & McCance [1936]; Engel [1934] has employed it in determining the

iron in the enamel of teeth, and Cooper [1935] for the iron in the sea and in marine plankton.

This communication describes a method of estimating the total iron content of blood by the use of dipyridyl.

Reagents.

1. *Approximately 50% sulphuric acid.* Dilute a convenient quantity of concentrated sulphuric acid to twice its volume with distilled water. It is often not necessary to distil the strong sulphuric acid because so little of the reagent is used for each estimation and most A.R. samples show only the slightest traces of iron. For the purposes of this paper, however, all sulphuric acid was distilled.

2. *Concentrated nitric acid.* As this acid is used in greater quantity it should be distilled.

3. *Approximately 40% solution of sodium acetate.* Sodium acetate is particularly easy to recrystallize and can readily be obtained free from iron. Prepare a 40% solution of CH_3COONa, $3H_2O$ with distilled water.

4. *Approximately 25% solution of glucose.* Most samples of glucose are quite free from iron. Prepare a 25% solution in distilled water.

5. *Dipyridyl reagent.* Dissolve 0·468 g. of dipyridyl in 6 ml. of *N* hydrochloric acid and make up to 100 ml. with distilled water.

6. *Standard iron solution.* Dissolve 0·350 g. of ferrous ammonium sulphate in distilled water containing a few drops of sulphuric acid and make up to 100 ml. in a standard flask. The solution contains 50 mg. of iron per 100 ml.

B.D.H.'s A.R. reagents were used throughout. With glass-distilled water and the above reagents, iron in the blanks should be negligible.

Method.

Measure exactly 0·1 ml. of blood into a pyrex tube which has a mark at the 10 ml. level. The pipette should be washed carefully with the minimum of water. Add 0·2 ml. of 50% sulphuric acid and about 10 drops of concentrated nitric acid. Heat carefully, with constant agitation, over a micro-burner until charring begins. Cool and add more nitric acid, repeating these operations until no further charring occurs. Boil off the excess of nitric acid. The residual drop of sulphuric acid usually contains a white precipitate which is probably anhydrous ferric sulphate. Add about 0·5 ml. of water and warm to dissolve this precipitate. Add 0·3 ml. of dipyridyl reagent, followed by 1 ml. of glucose solution. Mix well by shaking and add 5 ml. of sodium acetate solution. Heat in a boiling water bath for 5 min. for full colour development. Cool and make up to the 10 ml. mark with distilled water.

The standard is prepared in exactly the same way except that 0·1 ml. of the standard iron solution (or 1 ml. of a standard diluted ten times) is used in place of the blood.

Compare in a colorimeter with the standard preferably at 25 mm. If U is the reading for the unknown and S the reading for the standard, then $\frac{S \times 50}{U} = $ mg. of iron per 100 ml. of blood.

Discussion.

Apparatus. Small colorimeter cups are now almost universally used and if these are available the entire analysis should be carried out in pyrex test-tubes 150 mm. × 10 to 12 mm. diameter. These should be accurately marked at 10 ml. If more fluid is required for the colorimeter cups slightly larger tubes should be used and these should be marked at 12 or 15 ml. Tubes should be of as small diameter as convenient in order to obtain as great a length as possible and also to

ensure that the minimum amount of sulphuric acid is required to cover the bottom after incineration is complete. Pipettes should be of the wash-out type and should be accurately calibrated with mercury.

Incineration. Wet ashing is much more convenient than dry ashing. The latter takes a longer time and requires more elaborate apparatus; there is danger of loss of iron by volatilization and the iron and phosphates often tend to be in a more refractory form after ashing. With wet ashing all these difficulties are avoided but some care is necessary to prevent loss by sputtering. With ordinary care, however, this does not occur. Nitric acid was preferred to other oxidizing agents because it can easily be distilled and obtained free from iron [*cf.* Fowweather, 1934]. Excess of the reagent can be removed by boiling.

Buffering action of sodium acetate. The buffer used is ample to maintain the final *p*H well within the limits 5·0–5·5, even when two or three times the stated quantity of sulphuric acid is used.

Reduction of iron by glucose. Glucose was selected as the best reducing agent after many others had been tried. Sodium sulphite and sodium hydrosulphite are difficult to purify and are unstable in solution. Sulphur dioxide is readily available and is not subject to contamination with iron. All three of these materials, however, make the adjustment of *p*H difficult. Glucose on the other hand is easily obtained in a state free from iron and strong solutions keep well. It also has the advantage of not affecting the *p*H. Although glucose reduces ferric iron only slowly at 100° and *p*H 5·0, the presence of dipyridyl hastens the reaction so that it is completed in a very short time.

Colour production. Not less than 0·3 ml. of dipyridyl should be used. This is sufficient to promote colour formation under the experimental conditions, provided that the iron content of the blood does not exceed 75 mg. per 100 ml.—a value which is rarely encountered in practice. The colour attains its maximum intensity very quickly at the temperature of the water-bath and suffers no change if maintained at this temperature for an hour, or if kept at room temperature for as long as 24 hours. The addition of copper salts or of phosphates to the blood to be analysed in no way affects the values obtained for the iron content when estimated by this method.

The colour is not difficult to match using a daylight screen but the definition can be markedly improved by including a bluish-green light filter (which can be bought from any glazier for a few pence) in the lighting system.

Quantity of blood used. Normally, human blood contains approximately 50 mg. of iron per 100 ml., and 0·1 ml. is ample for the estimation. If the blood is markedly anaemic twice this volume should be taken. The method can also be adapted for larger quantities of blood if these are available.

Results.

Some hundreds of analyses have been carried out and the reliability of the method has been checked in several ways, always with good results. Recovery of added iron from blood was excellent and some typical results are given below.

Estimated Fe content of blood mg. per 100 ml.	Estimated Fe content of blood after addition of 25 mg. Fe per 100 ml. mg. per 100 ml.	Difference
37·6	62·5	24·9
39·6	64·8	25·2
53·9	78·3	24·4
49·2	74·8	25·6
50·0	74·7	24·7

SUMMARY.

A micro-method for determining the total iron content of 0·1 ml. of blood, using dipyridyl, is described.

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REFERENCES.

Bogniard & Whipple (1932). *J. exp. Med.* **55**, 653.
Burmester (1935). *J. biol. Chem.* **105**, 189.
Cooper (1935). *Proc. roy. Soc.* B **118**, 419.
Elvehjem, Hart & Sherman (1933). *J. biol. Chem.* **103**, 61.
Engel (1934). *J. dent. Res.* **14**, 273.
Fowweather (1934). *Biochem. J.* **28**, 1160.
Hill (1931). *Proc. roy. Soc.* B **107**, 205.
Horwitt (1934). *J. biol. Chem.* **105**, xli.
Klumpp (1934). *J. biol. Chem.* **107**, 213.
Shackleton & McCance (1936). *Biochem. J.* **30**, 582.
Tompsett (1934). *Biochem. J.* **28**, 1802.