XV. THE DETERMINATION OF IRON IN BLOOD, TISSUES AND URINE.

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INTRODUCTION.

OF all colorimetric methods used for the determination of small quantities of iron the thiocyanate method is the best known, and is one which, provided its limitations are well understood, gives very good results. An extremely useful modification was introduced by Marriott and Wolf [1906] which consists in the development of the thiocyanate colour in the presence of acetone. By this means not only is the colour intensified and stabilised, but in our experience, the effect of interfering substances is considerably minimised and in some cases entirely prevented. Walker [1925], who in other respects has published a useful study of the thiocyanate method, appears to have overlooked this important modification. Wong [1923] omits its use in the method he describes on account of possible changes in the volume of the final solution due to the volatility of the acetone. This objection is of no practical importance provided the final solution is made up in a stoppered graduated flask, for, since the final solution contains 50 % of water with considerable quantities of ammonium thiocyanate in solution, the vapour pressure will be considerably lower than that of pure acetone; and as the solution has a free surface exposed to the air only during the actual comparison in the colorimeter, any change in the concentration of the solution due to evaporation of acetone can have no appreciable effect on the final result. The advantages to be derived from the use of acetone considerably outweigh any objections to its use, particularly in the case of tissues and urine, where the intensification of the colour is highly desirable, and where interfering substances, notably phosphates, are present in relatively large quantities.

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Lapicque [1889] appears to have been the first to use the thiocyanate method for this purpose. Berman [1918] used concentrated hydrobromic acid to split off the iron held in combination in blood and oxidised it to the ferric condition, at the same time destroying organic matter, with potassium permanganate. Wong carried out oxidations by means of sodium or potassium chlorate.

In the present method we have largely followed Wong's procedure, but have introduced the use of "perhydrol" (100-volume hydrogen peroxide) in place of chlorate and utilised the acetone modification as described by Berman. "Perhydrol" has two advantages over chlorate, namely, that it leaves no residue, and that the steady evolution of oxygen which results from its addition ensures steady ebullition, thus avoiding the possibility of loss by spurting which we have found to be a real danger in Wong's method.

Method.

1 cc. blood is measured into a test tube containing 4 cc. of distilled water. After thorough mixing, 1 cc. of this diluted blood is transferred to a Pyrex test tube (200 × 25 mm.) followed by 1 cc. of concentrated sulphuric acid. The tube is clamped and held at an angle of 40° to the horizontal. The contents are boiled rather vigorously until practically all the water has been driven off and white fumes begin to be evolved. Heating is discontinued for about half a minute, after which time 0.5 cc. of "perhydrol" is added to the tube, drop by drop, from a teat pipette. Boiling is then repeated. A brisk evolution of oxygen occurs and the solution in the tube assumes an amber colour. When white fumes are again evolved heating is again discontinued and after cooling for half to one minute a further 0.5 cc. of "perhydrol" is added as before. Heating is resumed and the solution usually becomes colourless. If all colour has not disappeared a further 0.3 cc. of "perhydrol" is added. Heating is continued for one minute after the solution has become colourless. The solution is now allowed to cool completely, then it is diluted with about 5 cc. of distilled water and transferred to a 50 cc. stoppered graduated flask. Into another similar flask are placed 1 cc. of a standard iron solution containing 0.1 mg. iron per cc. and 1 cc. concentrated sulphuric acid. Water is added to both flasks up to a volume of about 18 cc. after which 25 cc. acetone are added. The contents of the flasks are thoroughly mixed and allowed to cool to room temperature. To each flask are then added 5 cc. of 3 *M* ammonium thiocyanate, the contents mixed and made up to the mark. The two solutions are then compared in a colorimeter.

If the standard is set at 20 mm., then

$$\frac{20}{R} \times 50 = \text{mg. iron per 100 cc. blood,}$$

or $\frac{20 \times 50}{R \times 3.35} = \text{haemoglobin in blood (haemoglobin contains 0.335 \% of iron),}$
where *R* is the colorimeter reading of the solution tested.

Preparation of standard iron solution.

Dissolve 0.7 g. pure ferrous ammonium sulphate in about 50 cc. of distilled water. Add to the solution 20 cc. 10 % iron-free sulphuric acid, warm slightly and then add 0.1 *N* (approx.) potassium permanganate solution to

oxidise the ferrous salt completely. Dilute with distilled water to 1 l. Each cc. contains 0.1 mg. iron.

The accuracy of the method was tested by adding to a specimen of blood, diluted to five times its original volume with distilled water, various quantities of the standard iron solution and then determining the total amount of iron present in each case. The concentration of the standard was varied so as to obtain colorimeter readings for the solutions tested lying between 15 and 25 when the standard was set at 20. The blood used was found to contain 47.5 mg. iron per 100 cc.

The following results were obtained:

Amount of iron added mg.	Total amount present mg.	Total amount recovered mg.
0.02	0.115	0.113
0.04	0.135	0.132
0.06	0.155	0.154
0.08	0.175	0.177
0.10	0.195	0.194

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For this purpose most methods require complete destruction of organic matter either by ignition or by oxidation with sulphuric and nitric acids.

The present method involves the partial destruction of organic matter with sulphuric acid followed by complete oxidation of remaining organic matter in an aliquot portion of the resulting fluid by "perhydrol." The iron is then determined by the thiocyanate method described above.

The presence of acetone has been found necessary in order to obtain satisfactory results as otherwise the presence of phosphates introduces an appreciable error.

Method.

The organ or tissue to be examined is minced and washed with water to remove blood. It is then dried in a steam oven. The dried material is then ground in a mortar until it passes through a 30-mesh sieve.

1 g. of this material is weighed into a 300 cc. Kjeldahl flask to which are added 10 cc. concentrated H_2SO_4 . The flask is heated in a fume chamber, gently for 15 minutes, and then strongly for 30 minutes. At the end of this time the walls of the flask are free from charred material and its contents consist of a brown homogeneous fluid. This is allowed to cool and then diluted with an equal volume of water and transferred to a 50 cc. stoppered graduated flask. A rise of temperature occurs on diluting and washing the fluid into the graduated flask. When room temperature is again reached the contents of the flask are made up to the mark. A certain amount of light, flocculent precipitate forms on adding water to the acid digestion fluid. The contents of the flask are therefore thoroughly shaken and 5 cc. are withdrawn immediately and placed in a Pyrex tube, to which 1 cc. of "perhydrol" is

added. The tube is inclined as in the previous method and the oxidation with "perhydrol" there outlined is followed. If much iron is present the final solution has a slight yellow colour after oxidation which almost entirely disappears on cooling.

After cooling, the solution is transferred to a 50 cc. graduated flask and acetone and thiocyanate solution added as before, a standard for comparison being also prepared as already described.

In the case of organs containing more than the normal amount of iron (*e.g.* where siderosis is present) 0.5 g. should be taken instead of 1 g., and it may be found necessary to prepare a stronger standard solution for colorimetric comparison.

The following table indicates the accuracy with which known amounts of iron added to various tissues before the sulphuric acid digestion have been recovered by this method.

Tissue	Iron in tissue mg.	Iron added mg.	Total iron present mg.	Total iron recovered mg.
Kidney A, 0.25 g.	2.90	0.20	3.10	3.09
" B, 1.0 g.	0.98	0.30	1.28	1.26
Liver B, 0.5 g.	1.30	0.40	1.70	1.68
" A, 0.5 g.	0.60	0.50	1.10	1.105
Spleen A, 1.0 g.	0.66	0.60	1.26	1.30
" B, 0.5 g.	0.63	0.70	1.33	1.29

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The concentration of iron in urine is extremely small, amounting to about 1 mg. in the whole 24-hour specimen in the case of normal persons. Further, the presence of phosphates in considerable amounts introduces difficulties whatever method of analysis be employed.

In the present method partial destruction of organic matter is carried out as in the previous method. After the final complete oxidation by "perhydrol," strong heating is continued for at least 3 minutes until practically all sulphuric acid is driven off. Under these conditions orthophosphoric acid is changed to metaphosphoric acid, which appears to cause less interference with the colour development. The sulphuric acid driven off is replaced before the colour is developed as it is essential to have a sufficient quantity of sulphuric acid in order to get correct results. Marriott and Wolf showed that the interference of phosphates can be prevented by having a sufficient concentration of mineral acid.

Acetone is essential in the case of urine for under the conditions employed in the following method the colour produced by the thiocyanate is barely perceptible in its absence. Its presence however introduces a complication since it precipitates some of the inorganic salts which are present. The precipitation amounts to little more than a cloudiness which however is quite sufficient to make colorimetric comparison impossible. The precipitate is, however, readily removed by centrifugalisation.

The colour produced in this method fades much more rapidly than in the previous methods, hence it is necessary to lose no time in making the colorimetric comparison.

Still, though the method is more difficult than the one described for blood and tissues, it is simpler than the methods at present in use for urine, and provided careful attention is paid to all the details no fault will be found with the accuracy of the results obtained.

Method.

To 100 cc. of the 24-hour specimen of urine placed in a 300 cc. Kjeldahl flask, 10 cc. of concentrated sulphuric acid are added. The contents of the flask are vigorously boiled until frothing begins to take place. Heating is then carried on with caution until frothing ceases. A full flame is then used again until the walls of the flask are free from charred material and a homogeneous fluid results. After cooling, this fluid is diluted and transferred to a 50 cc. graduated flask whose contents are made up to the mark when they have cooled again to room temperature. 10 cc. of the liquid are transferred to a Pyrex tube and concentrated by boiling. Until the volume has been considerably reduced the flame of the burner must not be directed to the bottom of the tube, but to the wall of the tube just below the surface of the liquid. When nearly all the water has been driven off and white fumes begin to be evolved, 0.5 cc. perhydrol is added as before, followed by further quantities of 0.3 cc. and 0.2 cc. After the last addition the resulting liquid is boiled vigorously for 3 minutes, when practically no more fumes are evolved. The liquid is then allowed to cool, diluted with about 2 cc. distilled water and transferred to a 25 cc. stoppered graduated flask. 1 cc. concentrated sulphuric acid is added and 12.5 cc. acetone. The contents of the flask are made up to about 20 cc. and allowed to cool to room temperature. 3 cc. of the thiocyanate solution are then added and the contents of the flask well mixed and made up to the mark. The standard for comparison is prepared at the same time in a similar flask using 1 cc. of a solution containing 0.01 mg. iron per cc., 1 cc. concentrated sulphuric acid, and 12.5 cc. acetone. The unknown solution as thus prepared is cloudy. A portion of it is therefore immediately centrifuged in a closed tube for 5 minutes, when the clear supernatant liquid which results is compared with the standard in the colorimeter.

The iron solution used for preparing the standard for comparison is one-tenth the concentration of the standard previously used and is prepared from the latter by suitable dilution with distilled water.

It is essential in this method, where very small quantities of iron are estimated, to prevent dust from falling into the solutions, as it has been found that the atmospheric dust, at any rate in an industrial centre, contains sufficient iron to cause errors in the result if precautions are not taken to exclude it.

The following table shows the agreement obtained between the results of two separate determinations on the same specimen, and also shows the accuracy with which known quantities of iron added to urine, before the acid digestion, may be recovered by this method.

Iron in urine mg. %	Iron added mg.	Total iron present mg.	Total iron recovered mg.
0.043	—	—	—
0.044	—	—	—
0.0435	0.05	0.0935	0.092
"	0.10	0.1435	0.145
"	0.15	0.1935	0.186
"	0.20	0.2435	0.250

SUMMARY.

Methods are described for determining the amount of iron present in blood, tissues and urine using the colorimetric method dependent on the formation of ferric thiocyanate in presence of acetone. It is shown that these methods are capable of a high degree of accuracy. They are simpler and less time-consuming than the methods at present in use.

These methods are being employed in a quantitative study of certain aspects of iron metabolism.

REFERENCES.

- Berman (1918). *J. Biol. Chem.* **35**, 231.
 Lapique (1889). *Compt. Rend. Soc. Biol.* **41**, 167.
 Marriott and Wolf (1906). *J. Biol. Chem.* **1**, 456.
 Walker (1925). *Analyst*, **50**, 279.
 Wong (1923). *J. Biol. Chem.* **55**, 421.