CLVII. THE DETERMINATION OF IRON IN BLOOD-PLASMA.

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A NUMBER of investigators have made determinations of iron in blood-serum, chiefly with the object of ascertaining what changes, if any, occur in the non-haemoglobinous iron of the blood in the anaemias. Most of the published methods involve the precipitation of the serum-proteins and the determination of iron in the protein-free filtrate. As a method for determining the iron present in solution in the blood (apart from that present in the cells) the use of protein-free filtrates of serum is open to two objections. Firstly there is the possibility of the removal of some iron in the clot when serum is formed, and secondly iron may be removed in the protein precipitate. Whether such removal does or does not occur, can only be ascertained by experiment, for as yet we have no knowledge of the chemical nature of the iron in question and so cannot infer from the known constitution and properties of the compounds we are concerned with the extent to which these objections are valid. Hence determinations of the iron in plasma are preferable to determinations made on protein-free filtrates of serum, unless it can be demonstrated that the objections just mentioned are in practice of no account; and determinations on plasma are necessary in order to investigate the practical value of these objections.

Some time ago I published a method for the determination of iron in blood, tissues and urine [1926] and made preliminary experiments to see if this method could be modified so as to be applied to blood-plasma. These experiments indicated that such a modification was possible, but the matter was not then pursued further. The interest recently directed to the subject, however, and the advisability of developing this method caused me to take up the matter again.

The first difficulty in any such method is to obtain plasma free from haemoglobin. This difficulty does not arise when protein-free filtrates of serum are used, for the small amounts of haemoglobin, which are practically always present in the serum after blood has been allowed to stand long enough to clot, are removed along with the other proteins. The use of anticoagulants is best avoided since they can only exercise their action if properly mixed with the blood, and such mixing causes rupture of some red cells and consequent contamination of the plasma with haemoglobin. By taking blood with a perfectly dry syringe and needle and transferring at once to a centrifuge-tube lined with paraffin wax, clotting can be delayed long enough to obtain satisfactory separation of the plasma from the cells without the use of any anticoagulant. The test I have used for determining the freedom of the plasma from haemoglobin is the benzidine test, and plasma obtained with proper precautions in the way I have mentioned, generally gives a negative result to this test. As will be seen later, the amount of iron in plasma is in the neighbourhood of 1γ per ml. or 1 part per million. The benzidine test is capable of detecting blood when present in a

dilution considerably greater than 1 in 1,000,000 and blood contains approximately 47 mg. iron per 100 ml., *i.e.* roughly 1 part in 2000. It will be seen therefore that the amount of haemoglobin which might be present in plasma giving a negative benzidine reaction is much too small to have any effect on the iron content of such plasma; in other words a negative benzidine reaction is a sufficiently good criterion of freedom from haemoglobin. Marlow and Taylor [1934], who also describe a method for determining iron in plasma, use spectroscopic examination for the presence of haemoglobin. It is generally agreed that the benzidine test is more sensitive than spectroscopic examination for this purpose, and the fact that their values are over four times those found by the present method would seem to support this. The cause of these increased values appears to be mechanical rupture of some red cells and the consequent presence of some haemoglobin, since the blood is stirred with a glass rod to bring it into contact with potassium oxalate for the purpose of preventing coagulation.

Plasma obtained in the way I have mentioned is completely oxidised by sulphuric acid and 100 volume hydrogen peroxide and the resultant solution is treated with ammonium thiocyanate. Acetone is added so that the final solution (made up to a fixed volume) contains 50 % by volume of acetone and the resulting pink solution is compared in a colorimeter with that obtained from a standard solution of iron, acidified with sulphuric acid, similarly treated with ammonium thiocyanate and acetone and made up to the same fixed volume.

During the oxidation of the plasma approximately one-third of the sulphuric acid is lost; hence the amount of acid used in the standard is two-thirds of that originally added to the plasma.

Special precautions must be taken to exclude dust from the solutions and apparatus used, and reagents as free as possible from iron must be selected. In any case blank determinations must be made on all reagents and appropriate corrections made in calculating the final result.

Since the standard itself includes reagents which may contain iron, *viz.* ammonium thiocyanate, sulphuric acid and acetone, the iron content of the standard solution may require correction. Actually, using B.D.H. acetone and ammonium thiocyanate, each of A.R. quality, and redistilled iron-free sulphuric acid supplied by the same firm, the amount of iron in the quantities used for this estimation was found to be negligible, but this fact should never be assumed without actual test. The 100 volume hydrogen peroxide, also supplied by B.D.H., does contain appreciable amounts of iron, for which a correction is necessary.

Experimental.

10–12 ml. of blood are taken by venipuncture in the usual way, using a 20 ml. Record syringe and iridio-platinum needle. The syringe and needle are dried by drawing in and expelling first methylated spirit, then ether and finally air. The needle must be a well-fitting one, since air leaking in at the joint between syringe and needle, and passing as small bubbles through the blood, tends to cause rupture of red cells. The blood must be drawn into the syringe in a slow, steady stream. The needle is then detached and the syringe is held vertically to allow any air in the blood to rise to the surface. The blood is then expelled, again in a slow, steady stream, into an ordinary conical centrifuge-tube, previously lined with paraffin wax, until only 2 ml. remain in the syringe. This quantity, which includes the upper surface of the blood in the syringe, may contain a few bubbles or a little froth and is rejected. The remainder is centrifuged for at least 10 minutes; 4–5 ml. of plasma will then be found to have separated. Of this, about 2·5 ml. are carefully withdrawn with a teat-

pipette, always keeping the tip of the pipette just below the surface of the plasma. On account of the quantity of blood dealt with, it should never be necessary for the tip of the pipette to approach very closely to the boundary between plasma and cells. The plasma drawn off is placed in a test-tube, and 2 ml. are at once measured out and transferred to a pyrex test-tube (200 × 25 mm.). The remainder is submitted to the benzidine test, which, if the above instructions have been carefully followed, will generally be found to give a negative result. If positive, the plasma is of course rejected. To the plasma in the pyrex tube are added 1·5 ml. of sulphuric acid, 0·5 ml. of the 100 volume hydrogen peroxide and a small piece of platinum wire to facilitate smooth boiling. The tube is clamped at an angle of 40° to the horizontal and the contents are heated with a micro-burner until boiling occurs. After a few moments of cautious heating, boiling may be allowed to become vigorous, while water is being driven off, but as soon as charring begins, heating must again be very cautious, as at this stage spitting is liable to occur. During the next few moments charring increases and some carbon is deposited on the sides of the tube. White fumes then begin to be evolved and become copious and the liquid begins to froth but not dangerously. In fact, at this stage, heating may again become vigorous as any danger from spitting is now over. After this stage the white fumes get much less and sulphuric acid is seen to condense just above the liquid and run back, removing much of the carbon deposited on the tube. The liquid is now allowed to cool for about 1 minute. 0·5 ml. of the hydrogen peroxide is now added, drop by drop, and the liquid again heated. A brisk evolution of oxygen occurs. Heating is continued until sulphuric acid is again seen to be condensing on the walls of the tube and running back. At this stage all carbon deposit should be removed, and the liquid be transparent and reddish brown in colour. After cooling again for a short time, 0·5 ml. of hydrogen peroxide is again added and heating resumed. The liquid now becomes pale yellow. On the further addition of 0·5 ml. hydrogen peroxide and heating again, the liquid becomes entirely colourless. Heating is continued for a few moments after the loss of all colour, to decompose the hydrogen peroxide thoroughly. The liquid is then allowed to cool completely, after which it is diluted with about 2 ml. of water and washed into a 20 ml. stoppered graduated flask. The total volume of the liquid and washings must not exceed 8 ml. Meanwhile, in a similar flask are placed 1 ml. of sulphuric acid, 0·5 ml. of a standard iron solution containing 0·01 mg. of iron per ml. and 5–6 ml. of water. To both flasks are added 10 ml. of acetone and the contents thoroughly mixed. Some rise of temperature occurs, and the flasks are set aside until the contents have cooled to room temperature. To each are then added 2 ml. of 3*M* ammonium thiocyanate solution and the contents mixed and made up to the mark. The two solutions are then compared in a colorimeter.

I have found illumination of the colorimeter by artificial light unsatisfactory for this comparison and have therefore adhered to daylight illumination throughout.

Preparation of standard iron solution.

A stock solution containing 0·1 mg. of iron per ml. is first prepared. 0·7 g. pure ferrous ammonium sulphate is dissolved in about 50 ml. of water. 20 ml. of 10 % iron-free sulphuric acid are added, the solution is slightly warmed and 0·1*N* potassium permanganate solution added to oxidise the ferrous salt completely. The solution is then diluted to 1 l.

The required standard is prepared from this solution by making a 1 in 10 dilution with water.

Determination of blanks.

(*a*) *For standard solutions.* Two different quantities of the standard iron solution, *e.g.* 0·5 ml. and 0·7 ml., are each treated with sulphuric acid, acetone and ammonium thiocyanate as above described and compared in the colorimeter.

If A and B are the amounts of iron present in the standard solution taken, a and b the colorimeter readings of the solutions prepared from them, and x the amount of iron in the other reagents used, then

$$\frac{A+x}{B+x}=\frac{b}{a}$$

from which x is calculated.

(*b*) *For unknown solutions.* 1 ml. of the standard iron solution is transferred to a pyrex tube and treated by the above method exactly as if it were plasma. It is then compared with a standard made up from the same volume of iron solution, and the difference between the iron content of the two solutions is calculated.

The accuracy of the method was tested by adding to various quantities of serum whose iron content had previously been determined different quantities of the standard iron solution and then determining the total amount of iron present in the mixtures so obtained.

The following results were obtained:

Iron in serum mg.	Iron added mg.	Total amount present mg.	Total amount recovered mg.
0·00265 (2 ml.)	0·0010 (0·1 ml.)	0·00365	0·00353
0·00199 (1·5 ml.)	0·0025 (0·25 ml.)	0·00449	0·00444
0·00211 (1 ml.)	0·0050 (0·5 ml.)	0·00711	0·00687
0·00076 (0·5 ml.)	0·0100 (1 ml.)	0·01076	0·01050

The iron content of serum, plasma and protein-free filtrates of serum (prepared by adding an equal volume of 20 % trichloroacetic acid to the serum) were determined by this method in the case of three healthy individuals, with the following results:

Plasma (γ iron per ml.)	Serum (γ iron per ml.)	Protein-free filtrate of serum (γ iron per ml. serum)
1·8	2·02	1·36
1·02	1·60	0·68
1·14	1·58	0·72

The higher values found for serum are explained by the fact that in all cases the serum was benzidine-positive while the plasma was benzidine-negative. Hence it is not possible to say if any iron is removed by the clot. The lower values for protein-free filtrates of serum than for plasma indicate removal of an appreciable proportion of iron either in the clot or the protein precipitate or both. It is possible that these differences may be greater in certain anaemic conditions than in health.

Values for iron in plasma by the present method.

The plasma of 10 male students and 10 female students, all presumably healthy, has been examined by this method. In all cases blood was taken between 10 a.m. and 10.30 a.m., breakfast having been allowed. The iron found in the males varied from 0·95γ per ml. to 1·80γ, the mean being 1·25γ. In the females the values were found to be between 0·60γ and 1·56γ, the mean being 1·05γ.

Locke *et al.* [1932], using protein-free filtrates of serum, found an average of 1·00γ per ml. of serum for males, and 0·77γ for females. In this case blood was taken before breakfast after a 12 hours' fast. Allowing for the difference which I have found between plasma and protein-free filtrates of serum, these figures agree well with those I have just given.

Warburg and Krebs [1927], using a totally different method, report a few values which are in the neighbourhood of 0·7γ per ml. of serum. Many of the reported values however are considerably higher. Thus Marlow and Taylor, whose method has already been referred to, found values varying between 4 and 7γ per ml. of plasma; and Riecker [1930] found an average of 11γ per ml. of serum.

SUMMARY.

A method is described for the determination of iron in blood-plasma. The precautions necessary for obtaining plasma free from haemoglobin are indicated, together with the test which is used to determine if the required freedom from haemoglobin has been obtained. It is shown that the method is capable of a very satisfactory degree of accuracy.

Results are given which show that the iron in plasma is higher than that found in serum when protein-free filtrates of serum are used for analysis. The difference appears to be due to removal of iron in the blood-clot or the protein precipitate.

The results of iron determinations in plasma in the case of 10 males and 10 females are given.

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