XXXII. THE ESTIMATION OF CALCIUM IN BLOOD¹.

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INTRODUCTORY.

THE estimation of calcium in blood, serum etc., is a matter of extreme delicacy seeing that, in the case of human blood at least, it is not often easy to obtain more than 1 to 5 cc. of the sample. Working with such small quantities therefore it is necessary to employ a method capable of measuring calcium to a limit of accuracy of at least 0.006 mgm. It is obvious also that in these circumstances the reagents employed must be of the highest purity, especially so far as their freedom from calcium is concerned.

Among published methods are some based on the use of the haemocytometer (counting the calcium oxalate crystals) and on principles, such as (1) the determination of the concentration of calcium ions necessary to produce clotting of the blood, (2) mixing the blood with ammonium oxalate solutions made isotonic with sodium chloride, and calculating the calcium from that concentration of oxalate which just prevents clotting, (3) mixing the blood with sufficient ammonium oxalate to prevent coagulation and subsequently determining the quantity of calcium chloride necessary to produce clotting. We agree with de Waard [1919] that these give comparative rather than absolute results. Recourse must therefore be had in our opinion to chemical methods.

McCrudden [1909] suggests a gravimetric method. The calcium is precipitated as oxalate in a solution in which the hydrogen ion concentration is so adjusted that the iron and phosphates remain in solution.

Halverson and Bergeim [1917] propose to estimate calcium in blood which has not been incinerated, but from which the proteins have been precipitated by picric acid. The precipitation of the calcium is carried out under McCrudden's conditions, the hydrogen ion concentration being adjusted to the point at which alizarin gives a violet coloration. The calcium oxalate is washed by centrifuging and after dissolving it in dilute sulphuric acid the solution is titrated with *N*/100 potassium permanganate. This method, according to the examples shown in the paper, gives results agreeing closely,

¹ This investigation was undertaken at the request of my colleague, Prof. Carlier. (A. R. L.)

i.e. within the limit of experimental error, with similar estimations made on the ash of the blood, etc.

Marriott and Howland [1917] have devised an ingenious method for the estimation of calcium and magnesium in blood serum. It depends on the fact that solutions of ferric thiocyanate are decolorised by oxalates and by phosphates. Calcium is precipitated as oxalate and magnesium as ammonium magnesium phosphate. The precipitates are dissolved in acid, and added to solutions of ferric thiocyanate, the degree of decolorisation being determined by comparison in small Nessler tubes. It is said to be possible by this method to work on 2 cc. samples of serum with a maximum error of less than 5 %.

Jansen [1918] describes a gravimetric method of estimating calcium. He incinerates the blood; and he considers the removal of iron and phosphoric acid necessary before precipitating the calcium as oxalate.

De Waard [1919] has devised a method for the estimation of calcium in organic substances for which he claims an accuracy of 4 %. The substance is incinerated, the ash dissolved in hydrochloric acid in a special tube, drawn out to a capillary end, which is subsequently used for centrifuging the liquid when the calcium has been precipitated. The tube containing the hydrochloric acid solution of the ash is placed in a bath of boiling water, 0.5 cc. of saturated ammonium oxalate solution added, then a slight excess of ammonia, and finally a slight excess of acetic acid. In these circumstances he finds that the iron, magnesium and phosphates remain dissolved, whilst the calcium is precipitated quantitatively.

EXPERIMENTAL.

The method we have adopted is based on the methods of McCrudden, Halverson and Bergeim, and de Waard.

The blood is incinerated in a platinum dish, the ash dissolved in concentrated hydrochloric acid, and the solution washed into a centrifuge tube of special shape. In this tube the calcium is precipitated as oxalate as described later. When the liquid is centrifuged, the precipitate collects in the narrow tube leaving the supernatant liquid clear. This liquid can then be removed by a tube drawn out to a capillary without disturbing the precipitate. The calcium oxalate is washed twice in this way, dissolved in dilute sulphuric acid and the solution titrated with *N*/100 potassium permanganate.

The shape of the centrifuge tube is shown in Fig. 1. It has the following dimensions: diameter 1.5 cm., total length 12 cm., length of narrow tube 1.3 cm., bore of capillary tube 0.5 cm. The tube is graduated for 2 cc. and for 25 cc. A glass rod capable of entering the narrow tube (as shown) is used for stirring the precipitate after the addition of sulphuric acid before titrating. The advantage of this centrifuge tube is that it is of such dimensions that all the operations can be performed in it. It is large enough for

the precipitation to be carried out, the narrow tube at the base enables the precipitate to be washed easily without being disturbed, and finally the end point can be seen easily when titrating as the tube does not taper gradually to a point. The solutions may be titrated to 0.01 cc.

The procedure is as follows:

Incineration. About 2–5 cc. of blood is measured into a platinum dish. This is heated on a piece of wire gauze with a coating of asbestos, by a very small flame until all the water has evaporated and a spongy mass of carbonaceous matter is left, when it is heated more strongly. During the final heating the dish is placed on a pipe-clay triangle and brought to redness. To prevent extraneous matter from gaining access to the dish during the first part of the incineration, an inverted funnel is fixed over it by a clamp. The residue is treated with 1.5 cc. concentrated hydrochloric acid 0.5 cc. at a time—each time the solution is gently warmed and washed into the centrifuge tube. The dish is then washed three times with water. Care must be taken in the operation to keep the volume to a minimum.

Precipitation. This was carried out by McCrudden's method as modified by Halverson and Bergeim. The solution in the centrifuge tube is neutralised with 0.880 ammonia using alizarin as indicator. The neutral point is overshot and then titrated back with $N/2$ HCl until the colour just commences to change. Then 2.5 cc. of $N/2$ HCl and 2.5 cc. of 2.5 % oxalic acid solution are added and water to make to the 25 cc. mark. The tube is then placed in a water-bath and brought to the boiling point. To the hot solution 5 cc. of 3 % ammonium oxalate is added and the tube kept at the boiling point for 15 minutes when it is removed, placed in ice water and 5 cc. of 20 % sodium acetate solution added (or until the colour commences to change). When the calcium oxalate has precipitated which takes about 6–8 hours (it is usually allowed to remain over night), the solution is centrifuged. The clear supernatant liquid is removed by means of a tube (drawn out to a capillary) leading to a bottle connected with a filter pump. By this means the liquid is removed to the 2 cc. mark on the tube. The outside of the capillary tube is washed with water to prevent the loss of any precipitate which may adhere to it.

Fig. 1. Diagram of centrifuge tube used in the estimation of calcium in blood.

Water is then added to the 25 cc. mark, the centrifuging repeated, and the supernatant liquid removed as before to the 2 cc. mark, etc. It is washed twice in this way, when 4 cc. of 5 % sulphuric acid are added and the precipitate stirred with the glass rod. The tube is then placed in a water-bath at 65° C. for 1–2 minutes, when the oxalic acid is titrated with *N*/100 potassium permanganate by means of a burette capable of reading to 0.01 cc. 1 cc. of *N*/100 potassium permanganate is equivalent to 0.020 mgm. of calcium.

A "blank" experiment must be performed on the materials. Coagulation of the blood was prevented by the addition of sodium citrate to the extent of 2.5 %.

Solutions used. All materials used were recrystallised twice from water which had been redistilled twice from a Jena glass flask, and such water was used throughout the operations. It was found necessary to have the end of the burette drawn out to a fine point and vaseline was placed on the outside so that it was possible to reduce the volume of one drop to 0.01 cc.

The permanganate solution. About 0.5 g. of potassium permanganate crystals is dissolved in 1 litre of redistilled water in a litre flask. The flask is placed on the water-bath for 2–3 days, a funnel and watch glass being placed in the neck to prevent extraneous matter from entering. The solution is then carefully filtered through an ashless filter paper on a Buchner funnel to remove the deposit of manganese dioxide. The solution should be kept in the dark. It is standardised against an oxalic acid solution prepared by dissolving about 0.25 g. of recrystallised oxalic acid in 250 cc. of water. When standardising allowance must be made for the titre of the sulphuric acid used. It is as well to standardise the permanganate about once every fortnight.

Alizarin solution. An aqueous solution of 0.2 % strength was used.

The following are some of the results obtained:

								Calcium per 100 cc. g.
1.	Standard calcium solution (containing 0.0076 g. per 100 cc.)							0.0077
2.	"	"	"	"	"	"	"	0.0077
3.	"	"	"	"	"	"	"	0.0077
4.	"	"	"	"	"	"	"	0.0077
1.	Human blood	...	...	...	...	...	...	0.0082
2.	"	"	...	...	...	...	...	0.0080
1.	Ox blood	...	...	...	...	...	...	0.0070
2.	"	"	...	...	...	...	...	0.0070
1.	"	"	(clotted)	...	...	...	...	0.0055
2.	"	"	"	...	...	...	...	0.0055
3.	"	"	"	...	...	...	...	0.0056

In all cases 5 cc. were used for the determination with the exception of the human blood when 3 cc. and 2 cc. respectively were used.

Duplicate experiments show an agreement equal to two drops of *N*/100 permanganate or in other words to 0.006 mgm. of calcium.

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