LXIII. THE ACCURATE ESTIMATION OF CALCIUM IN WHOLE BLOOD.

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THE study of calcium in the blood, in relation to its metabolism in health and disease, of which very little is known, is based upon its accurate quantitative estimation.

In regard to methods, it is not considered necessary to recall the well-known work of Wright [1912], Vines [1921], Howland and Kramer [1920], Kramer and Tisdall [1921], and Laidlaw and Payne [1922] but reference may be made to the recent paper by Ling and Bushill [1922]. The method they adopt is based on the work of McCrudden [1909], Halverson and Bergeim [1917], and de Waard [1919]. 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 by Halverson and Bergeim's modification of McCrudden's method. The calcium oxalate is washed twice, dissolved in dilute sulphuric acid and the solution titrated with $N/100$ potassium permanganate.

The calcium content of whole blood should be estimated. Although a number of investigators perform quantitative estimations of calcium on serum only, the data afforded is hardly sufficient for an accurate study of calcium metabolism. The corpuscular elements are known to contain calcium. Cowie and Colhoun [1919] found that red corpuscles contained calcium in somewhat smaller concentration than the serum.

The technique of the method I employ in the estimation of calcium in whole blood is both accurate and simple and is as follows.

About 8 cc. of blood are withdrawn from a vein and transferred either direct into a platinum dish, or first into a test-tube containing 0.1 g. of potassium oxalate and later into the platinum dish. The blood in the dish quickly loses weight by evaporation and should be weighed at once on a balance. The actual weight of the blood $\times \frac{1000}{1060}$ enables one to express the amount of blood in cc. sufficiently accurately. It is then dried on a boiling water-bath. When dry it is carefully incinerated over a low flame on an Argand burner, the temperature being gradually raised. Finally it is transferred to the more intense flame of a powerful Bunsen burner until incineration

is completed. It should be noted that calcium may be lost by spluttering or by "creeping over" the edge of the dish if the temperature is increased too quickly. Another method, which should be employed with care, is the addition of a little redistilled methylated spirit to the blood and the application of a light, repeat this three or four times and then incinerate. By this means blood can be completely ashed in about an hour.

The ash, which owing to the temperature of incineration has fused leaving a vitreous residue, consists chiefly of phosphate of iron. If this is treated with water and acetic acid only, the phosphate of iron does not dissolve and consequently a considerable proportion of the calcium is left behind. It is therefore necessary to treat the ash with hydrochloric acid which dissolves the vitreous residue completely. By evaporation to dryness on the water-bath, a granular residue is obtained from which the calcium is readily extracted with acetic acid, sodium acetate and water, leaving the iron phosphate in an insoluble form.

In practice, I take up the ash with about 5 cc. of water and five drops of strong HCl and after evaporation to dryness on the water-bath, the residue is treated with six drops of glacial acetic acid, 0.5 g. of sodium or ammonium acetate and about 8 cc. of water. A good excess of the acetate is required to ensure the removal of any free HCl which may be present or produced by chemical action. The liquid is reduced to about 4 cc. by evaporation on the water-bath, cooled and filtered through a filter paper (7 cm. in diameter) into a 10 cc. glass centrifuge tube of an ordinary water turbine pattern centrifuge. The lixiviation of the residue is repeated with another 2 cc. of liquid acidified with two drops of acetic acid and this passed through the filter.

To remove every vestige of calcium, it is necessary to wash the filter paper with at least another 15–20 cc. of water. But the whole procedure must be completed in the one centrifuge tube and there is only space available for another 3 cc. To overcome this difficulty, I remove the centrifuge tube, wash out the platinum dish with 4 cc. of water acidified with a few drops of glacial acetic acid, and pass this liquid through the filter paper into a glass evaporating dish. This is repeated three or four times, following which the liquid is evaporated on the water-bath to about 2 cc. and transferred to the filtrate in the centrifuge tube. The glass dish is then finally washed out with 1 cc. of water, which is added to the rest of the liquid in the centrifuge tube.

This procedure is essential, as I have on a number of occasions incinerated the paper after ordinary filtering and found that about 20–25 % of the total calcium had been retained in the filter paper. Six drops of ammonium oxalate solution are now added and the liquid heated. If the cloudy precipitate of calcium oxalate which appears is not satisfactory, add a few more crystals of sodium acetate to make certain of the complete replacement by acetic acid of the HCl which was first used. The precipitation of the calcium oxalate must be done in the presence of acetic acid, as in ammoniacal solution the oxalates of both calcium and magnesium are precipitated.

The next step is to render the precipitate of calcium oxalate sufficiently granular to enable it to be readily centrifuged. I tried adding six drops of a saturated solution of an electrolyte, either aluminium acetate or sodium chloride, and heating the centrifuge tube over a boiling water-bath for 20 minutes. This acted perfectly when estimating solutions containing known quantities of calcium, but on applying the principle to blood estimations, I found a considerable amount of the calcium retained in the supernatant fluid pipetted off after the first centrifuging. The same thing happened, when, following the method recommended in ordinary quantitative estimations of calcium in solutions, I made the solution alkaline with ammonium hydroxide and let it stand for 20 minutes with the intention of subsequently rendering it acid with acetic acid to redissolve the magnesium oxalate. Thus it is necessary, after adding the oxalate, to heat the tube over a water-bath for at least three hours to insure the precipitation of the total amount of calcium present. The oxalate is then washed three times by centrifuging and removing the supernatant fluid. The latter is done by means of a glass pipette with an upturned point to prevent sucking up any of the precipitate. I do not recommend decanting off the supernatant fluid. Test the last wash in a small test-tube, by adding a drop each of NH_4OH and CaCl_2 and bring to the boil. If cloudiness appears add a few drops of glacial acetic acid which should make the solution perfectly clear. If cloudiness persists, there is still soluble oxalate present and the calcium precipitate must be rewashed and the washings re-tested. Excess of soluble oxalate must be removed, otherwise it will react with the permanganate and give an artificially high calcium value.

Dissolve the washed calcium oxalate in 3 cc. of distilled water containing four drops of dilute H_2SO_4 (1 in 10), heat to 60° and titrate with $N/100$ potassium permanganate solution. The end point is the faintest pink and the solution should again be heated to 60° when this appears—the addition of the permanganate solution having reduced the temperature to below that figure. The thermometer should be used for stirring the solution and washing off any calcium which may adhere to the upper part of the tube during the centrifuging process. It is important as a control to titrate four drops of the H_2SO_4 in distilled water with permanganate to ascertain whether there is any organic matter present in these reagents. The recognition of the pink end point is dependent upon the personal factor in the estimation and is thus liable to error. To insure accuracy I have made use of the reaction of acid permanganate solution and KI and the subsequent titration of the iodine liberated with thiosulphate, using starch as indicator. This reduces the error to a minimum if the $N/100$ solutions employed are accurate. After obtaining the end point which should persist for at least a minute, run in an excess of KMnO_4 (1 cc.) from the burette. Transfer to a beaker and wash the outside of the mouth as well as the inside of the tube with distilled water, to make certain that all the solution has passed to the beaker. Add two large crystals of a sample of potassium iodide which has been tested and found to

contain no free iodine—there must be an excess of KI—and titrate the yellow coloured iodine solution which results with *N*/100 sodium thiosulphate, using starch as indicator. The end point disappearance of the blue coloured compound of starch and iodine is one of the most delicate indicators known. Subtract the amount of sodium thiosulphate used from the total potassium permanganate figure and you have the actual end point. This should coincide with the first reading of the burette and represents the exact amount of KMnO_4 required to oxidise the oxalic acid in the centrifuge tube after the decomposition of the calcium oxalate precipitate by sulphuric acid.

The burettes (10 cc.), the pipettes and standard flasks used for making up *N*/100 solutions, should be of the best Charlottenburg or National Physical Laboratory standard pattern and should be carefully cleaned and dried after use. The *N*/10 solutions must be made up with the best analytical reagents, weighed out on an accurate balance by the investigator himself, and the solutions freshly prepared at frequent intervals. The *N*/10 potassium permanganate should be tested every fortnight against an accurately prepared stable solution such as *N*/10 oxalic acid, and the thiosulphate against the KMnO_4 solution. The thiosulphate solution is particularly liable to deterioration and must be kept in a dark bottle in a dark place. This applies to the KMnO_4 solution as well. The *N*/100 solutions, prepared from *N*/10 solutions, should be made up during the last washing of the calcium oxalate and used immediately. All reagents used must be free from calcium. The more concentrated the solutions are, the more liability there is to contamination by calcium salts.

The accuracy of the method is illustrated below:

Case 1. 20 cc. of blood were taken from a student's arm and divided into two parts.

Part I.

Weight of blood = 8.84 g.

Volume ,, = $8.84 \times \frac{1000}{1060} = 8.3$ cc.

Titration figure = 2.25 (cc. of KMnO_4) $\times 0.2^1 = .45$.

$.45 \times \frac{100}{8.3} = 5.42$ mg. of Ca per 100 cc. of blood.

Part II.

Weight of blood = 11.699 g.

Volume ,, = 11.03 cc.

To the blood, before incineration, were added 2 cc. of an accurate solution of calcium chloride containing 0.2 mg. Ca in each cc.

Titration figure = 5.05 (cc. of KMnO_4) $\times .2 = 1.01$ mg. Ca.

1.01 – 0.4 mg. Ca added = .61 mg. Ca present in the blood.

$.61 \times \frac{100}{11.03} = 5.53$ mg. Ca per 100 cc. of blood.

There is thus a difference of only .11 mg. Ca per 100 cc. of blood between the two estimations, which is well within the limits of experimental error.

¹ 1 cc. of *N*/100 solution of a calcium salt contains 0.2 mg. Ca.

Further results obtained in a similar manner are as follows:

	Volume of blood in cc.	Titration figure cc. of N/100 KMnO_4	Mg. of Ca per 100 cc. of blood
Case 2	11.0	3.0	5.45
„ 3	7.8	2.3	5.90
„ 4	10.5	3.0	5.71
„ 5	8.49	2.4	5.65
„ 6	10.94	3.15	5.76
„ 7	8.72	2.8	6.42
„ 8	11.20	3.85	6.87

The blood from Case 6 was incinerated in about an hour by the addition of methylated spirit in the manner described above. All the investigations were done on normal individuals. The findings range from 5.42 to 6.87, the average being 5.8 mg. Ca per 100 cc. of blood. It should be noted that the results are given in milligrams of calcium and not lime.

Kramer and Tisdall [1921] found that the calcium content of whole blood in normal persons varied from 5.3 to 6.8. An average of 5.8 mg. Ca per 100 cc.

All these figures vary within comparatively wide limits and in using calcium estimations for clinical purposes, it is obviously futile to attempt to draw conclusions from very slight variations. It is possible that in some cases sufficient attention has not been given to technique and consequently I have thought it worth while to set forth step by step a technique which, if followed exactly, should give accurate results.

Cowie and Colhoun [1919] state "that the limits of variation of blood calcium in normal individuals are marked. Lyman [1917] found the same variations in normal individuals which he was unable to explain. Determinations of the number of corpuscles and of the specific gravity of the blood might aid in clearing up this point by giving an idea of the concentration." Their figures range from 6.64 to 9.08 with an average of 8.45 mg. of Ca per 100 cc. of blood.

CONCLUSIONS.

(1) The titration of calcium oxalate by N/100 potassium permanganate is an extremely accurate and comparatively simple method of estimating calcium in the blood, if due attention is paid to technique.

(2) Using the method described above I found that normal values in human blood range from 5.42 to 6.87 with an average value of 5.8 mg. of Ca per 100 cc.

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