CVI. THE ESTIMATION OF CALCIUM IN BLOOD.

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(Received June 15th, 1925.)

FOR the estimation of the small amounts (of the order of 10 cmgm.¹ per cc.) of calcium in blood numerous methods and modifications have been described. Of these, the most convenient are those in which the calcium is precipitated as oxalate, and estimated, after solution in sulphuric acid, with permanganate. We propose to detail a short procedure which enables calcium estimations on blood or blood products to be carried out in this way with a probable error not exceeding 5 %.

Preparation of material. Precipitation with trichloroacetic acid removes the proteins without removing appreciable quantities of calcium. When the proteins are not precipitated, the calcium oxalate precipitate contains considerable quantities of organic matter, which use up permanganate.

Precipitation of calcium oxalate. The conditions in which the calcium should be precipitated have been repeatedly studied, and it has been shown that certain limits of p_H must be observed. Precipitation with oxalic acid in the presence of a definite amount of hydrochloric acid and sodium acetate has the advantage that at the concentrations concerned magnesium is not likely to be precipitated as well. The necessary conditions for the oxalic acid precipitation, originally suggested for use on blood by McCrudden [1912], are stated in a paper by Widdows [1923].

Calcium oxalate is appreciably soluble in water. This point has been noticed by Cameron and Moorhouse [1925] in the case of their precipitation in ammoniacal solution, for they calculate the amount lost in washing, but it has escaped the attention of Widdows and others who have used the direct precipitation with oxalic acid and have washed the precipitate with water.

Using the procedure of Widdows (which is in other respects entirely satisfactory) the solution at the time the calcium is precipitated contains a large excess of oxalic acid and has a p_H calculated to prevent any calcium oxalate from remaining in solution. After centrifugalisation a certain (but unstated) amount of this solution remains, and this is then diluted by the water of the first washing; what the solubility of oxalate in this mixture may be it is impossible to calculate. The same applies to the second washing, but as we are then dealing with nearly pure water the solubility may very likely be that of calcium oxalate in water as determined by Richards, McCaffrey and Bisbee [1901].

¹ The abbreviation "cmgm." stands for centimilligram, the hundredth part of a milligram.

Another point which Widdows and others, who have washed the calcium oxalate precipitate by centrifugalisation, have not referred to is the proportionate amount of the liquid remaining in the centrifuge tube after each decantation. Relative to the minute oxalate precipitate the supernatant liquid in the first stage contains an enormous quantity of oxalic acid, and measurement of the undecanted residue is necessary before it can be decided how many washings are enough. The amount of residual liquid will depend on the shape of the centrifuge tube and on the method of decantation. Our own preliminary experiments using Widdows' method gave apparently correct results (4 % to 7 % too high) after two washings, but results 18 % to 21 % too low after three. This was because in those particular centrifuge tubes the amount of residual liquid was comparatively large, and so the free oxalic acid remaining after the second washing was enough to balance the loss due to solution of the oxalate precipitate, and so mask the effect, which, however, became apparent after the next washing. If those centrifuge tubes had been so designed as to permit of the removal of all but a fraction of a cc. of the supernatant liquid, that error would certainly have appeared at the second washing.

The remedy is to wash the precipitate of calcium oxalate not with water but with a saturated solution of calcium oxalate. This is prepared by shaking precipitated and carefully washed calcium oxalate with water and filtering through two or three thicknesses of No. 30 Whatman paper. The saturated solution so obtained must be titrated with $N/100$ permanganate to make sure that no solid particles of oxalate have passed through the filter. Richards [Richards *et al.* 1901] refers to the difficulty of filtering this solution. For this purpose it is sufficient if 15 cc. of the solution require no more than 0.20 cc. to 0.24 cc. of $N/100$ permanganate. The correct figure at 18° is 0.17 cc.

Method of estimation.

(1) *$N/100$ potassium permanganate.* The instability of very dilute solutions of permanganate is well known, and we would therefore draw attention to the method of preparation given by Halverson and Bergeim [1918]. Made in this way we have found $N/100$ permanganate not to change its titre for weeks.

(2) *Centrifuge tubes.* The amount of residual liquid after each centrifugalisation is reduced to a minimum by conducting the precipitation and washings in centrifuge tubes which are about 85 mm. long and 30 mm. in diameter, and taper evenly from about the middle to a blunt point of about 8 mm. internal diameter. The supernatant liquid is removed by means of a small syphon made of capillary tubing, and having its tip drawn out and bent upwards, so that the direction of flow is downwards. With these tubes and this syphon the amount of residual liquid is less than half a cc. so that, as may be proved by calculation, no measurable quantity of soluble oxalate can be left after the initial decantation plus two washings.

(3) *Method.* 5 cc. of plasma are precipitated with 25 cc. of trichloroacetic acid (6.5 %). Of the filtrate 20 cc. are placed in a centrifuge tube. The tube

is supported on a small wire tripod and the small stirrer previously described in connection with the dilution colorimeter [Stanford, 1923] is introduced into the liquid. With the stirrer rotating there are added firstly a drop of methyl red, then concentrated ammonia in drops until the liquid is just alkaline, then $N/2$ hydrochloric acid in drops until the liquid is just acid again. The solution so prepared is treated successively with 1 cc. $N/2$ hydrochloric acid, 1 cc. 4 % oxalic acid and 1 cc. 20 % sodium acetate. The mixture is allowed to stand overnight, and is then centrifugalised and the precipitate washed twice by centrifugalisation with the saturated calcium oxalate solution already described, about 25 cc. being used each time. The oxalate precipitate is finally dissolved in 4 cc. of N sulphuric acid, and titrated with $N/100$ permanganate.

For accurate titrations it is necessary to use a burette having a very fine tip. Ours is of 5 cc. capacity, graduated in fiftieths, and the size of drop is about 0.025 cc. As the titration figures are usually from 1.5 cc. to 2.0 cc. the titration may be regarded as being in itself accurate to about 1 % if the observer has good colour perception.

After centrifugalisation it will always be found that, whilst practically all the precipitate is compactly at the bottom, yet there are minute traces floating on the surface of the liquid. During the syphoning these traces tend to adhere to the limb of the syphon as the liquid descends. For this reason the syphon should be turned off just before the liquid meniscus reaches its intake, and the syphon limb carefully washed down with a jet of the saturated calcium oxalate solution. In trial experiments we found that after the complete set of three decantations traces of oxalate could be dissolved off the syphon limb and on titration were found to be equivalent to 0.06 cc. of permanganate. On the other hand, it is practically impossible to get reagents absolutely free from substances that attack permanganate, and we found in blank experiments on our reagents that such impurities represented, in the quantities used in an estimation, about the same quantity of permanganate (actually 0.05 cc.). We therefore allow these two errors to cancel one another by disregarding both of them.

Purity of calcium oxalate precipitate. Hirth and Klotz [1923] have stated that the calcium oxalate precipitate may be contaminated with organic matter derived from the blood, which uses up permanganate and gives too high results. To satisfy ourselves on this point, we estimated the calcium in several plasma filtrates firstly by the permanganate method above described and secondly by converting the oxalate precipitate into oxide and estimating that acidimetrically. The latter figures were certainly in all cases slightly lower (4 %) than the permanganate results, but this difference is probably due to losses taking place in the additional transferences of material from vessel to vessel necessitated by the acidimetric procedure. This is confirmed by the fact that in two experiments on the estimation of known amounts of calcium in pure CaCl_2 solutions, taking quantities similar to those found in blood, the acidimetric method gave results which were from 3 % to 7 % too low.

Estimation of known calcium solutions. Four consecutive estimations on standard CaCl_2 solutions containing about the same amount of Ca as that present in plasma gave the following errors: -2% , $+1\%$, $+2\%$, $+2\%$.

Recovery of added calcium. Known amounts of calcium chloride added to plasma¹ before the precipitation with trichloroacetic acid are quantitatively recovered with a probable error of about 5% . The errors in seven consecutive experiments were 3% , 1% , -2% , -5% , 2% , 2% and 6% .

DISTRIBUTION OF CALCIUM COMPOUNDS IN THE BLOOD.

As after the experiments above described we were in possession of a method of a proved degree of accuracy of about 5% we thought it well, for purposes of orientation, to examine a few human bloods from this point of view. In each case an estimation was made on the whole blood, on the plasma, and on the corpuscles. In addition the percentage of corpuscles was determined (by weighing) so that the value for the whole blood calculated from those found for the plasma and corpuscles could be compared with the value actually found for the whole blood. The results in the five cases we examined are shown in the following table, and we think that, considering the necessary limitations of micro-quantitative methods, the agreement between the observed figures for whole blood in the first line and the calculated ones in the fourth is a further indication of the reliability of the method.

It will be observed that the quantities of calcium in the corpuscles are very much smaller than those in the plasma. Kramer and Tisdall [1922] found no calcium in the corpuscles, but we consider the amounts recorded below are definitely outside our limits of experimental error. They are doubtless high from adherent plasma, but it would require a 20% plasma contamination to account for the whole of the apparent corpuscle calcium in this way.

Table I. *Distribution of calcium compounds in blood.*

(All figures are cmgm. of Ca per cc.)

	Case 1	Case 2	Case 3	Case 4	Case 5
Blood	6.7	6.3	6.4	5.6	7.1
Plasma	11.0	9.6	10.6	10.0	10.0
Corpuscles	2.0	3.1	1.4	3.2	2.2
Blood (calculated from P + C)	6.4	6.7	6.0	6.3	6.5

The actual estimations were performed as follows:

Blood. 6 cc. blood are pipetted into a small flask, the pipette is washed out with 6 cc. of cold water, the flask is corked and placed in a warm water-bath (40°) for about 20 minutes, and shaken from time to time. After laking is complete 10 cc. are run drop by drop into 25 cc. of 6.5% trichloroacetic acid with constant mixing. The contents of the flask are shaken well and after standing about 30 minutes are filtered through a No. 30 Whatman paper.

¹ Anticoagulant: 3 mg. lithium citrate per cc. of blood [compare Briggs, 1923].

Plasma. 5 cc. plasma are added to 25 cc. of 6.5 % trichloroacetic acid and then treated as for blood above.

Corpuscles. 6 cc. corpuscles are laked as in the case of the blood. 10 cc. of the laked corpuscles are added to 25 cc. of 6.5 % trichloroacetic acid and then treated as for blood above.

From each filtrate 20 cc. are taken for the precipitation of the calcium.

SUMMARY.

Calcium in blood or blood products may be estimated with a probable error of $\pm 5\%$ by precipitating the proteins with trichloroacetic acid, precipitating the calcium as calcium oxalate in certain conditions as to p_H and then estimating the calcium by dissolving in acid and titrating with permanganate. The precipitate must be washed with a saturated solution of calcium oxalate, and not with water in which it is appreciably soluble.

Using this method a series of estimations on the calcium content of blood, plasma and corpuscles has been made with the results set out in Table I above.

We are indebted to our colleagues, Dr J. Walker and Dr S. Grossman, for the collection of the blood samples required for this investigation.

A part of the expenses of the work has been borne by the Medical Research Council, to whom our thanks are due.

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