CXXXIII. THE DETERMINATION OF THE TOTAL CALCIUM CONTENT OF BLOOD-SERUM.

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THE values given for the normal calcium content of blood-serum vary a good deal. It is therefore advisable, when giving such figures, to mention the method used in their determination.

Various investigators have compared their results with determinations made in the ash of serum.

The idea of the following investigation was as follows.

1. To make determinations according to the method of Trevan and Bainbridge [1926], both in serum and ash, and at the same time in a known standard solution which corresponds as closely as possible with a solution of serum-ash.

2. To ascertain whether the magnesium present in the serum appreciably affects the calcium values.

3. To ascertain in what standard solution comparative tests can be made.

4. To compare the figures found by the Trevan-Bainbridge method with those of some of the permanganate methods.

When ammonium oxalate is added to serum, a precipitate of calcium oxalate is formed, and many methods of determination are based on this reaction.

The objection to precipitating calcium as oxalate is the difficulty of separating it from magnesium. The separation is based on the difference in solubility of the two oxalates. But from solutions in which the limit of solubility of the magnesium oxalate present is far from being reached, magnesium is still occluded by the calcium oxalate that is precipitated [Lemarchand, 1925]. When, therefore, a large amount of magnesium is present, the calcium oxalate may be precipitated twice; a more favourable proportion of the two metals is obtained the second time.

Under certain circumstances, however, even with only one precipitation, a separation to a certain degree may be obtained. An investigation of this has been carried out by Richards, McCaffrey and Bisbee [1901], who show that the quantity of magnesium oxalate occluded by calcium oxalate depends on the concentration of undissociated magnesium oxalate. This quantity is decreased by the addition of ammonium chloride, which forms complex compounds with magnesium. Ammonium chloride has a similar solvent effect, but to a much smaller extent, on calcium oxalate.

An excess of ammonium oxalate would increase the quantity of undissociated magnesium oxalate and this is avoided by adding the reagent slowly, an excess of ammonium oxalate being added only after precipitation has taken place.

The most favourable conditions are obtained when an acid solution is slowly neutralised by ammonia, and an excess of ammonium oxalate is added afterwards.

In the following determinations, in which due regard was paid to these conditions, which were established by Richards and his associates, the following method was adopted for determination in serum-ash and in stock solution.

0.02 cc. tropaeolin 000 No. 1 is added to 1 cc. solution in a glass tube such as is described in the paper of Trevan and Bainbridge, and the liquid is stirred with a small glass rod. 0.01 cc. 2.5 % ammonium chloride, 0.8 cc. $N/100$ oxalic acid, and 0.12 cc. $N/10$ hydrochloric acid are then added successively. The liquid is then neutralised by the addition of 0.2 N ammonia, 0.01 cc. being added at a time, with a 5 minutes' pause after each addition. The neutralisation is continued until the colour indicates a p_H of 8.0, a buffer solution being used for comparison. A further 0.6 cc. of 3.5 % ammonium oxalate is then added, and the mixture is put aside for four hours to precipitate, the further treatment being as indicated by Trevan and Bainbridge. This method of neutralising differs somewhat from that of Richards who used methyl orange as an indicator, heating the liquid to boiling point, this being necessary when the calcium oxalate is to be filtered off. The precipitate is collected in this case by centrifuging, and may be formed without heating. There was no difficulty about the addition of alkali to p_H 8.0, and the colour changed back again towards the acid shade when the excess of ammonium oxalate was added.

Richards advises the addition, when calcium has to be determined, of an amount of ammonium chloride ten times as large as the amount of magnesium present. In the analyses of which the results are here published, the quantity of ammonium chloride added was only roughly equivalent to the magnesium, a considerable quantity of ammonium chloride being formed during neutralisation, and the metals being present in a very dilute solution.

By taking all these precautions, Richards, McCaffrey and Bisbee obtained results which were approximately equal to the right values. These were supposed to be due to the fact that two slight errors neutralised each other, viz. a small loss of calcium, and the occlusion of some magnesium in the precipitate. Phosphates are not mentioned in their paper.

The method of Kramer and Tisdall [1921], which was afterwards simplified by Tisdall [1923], has furnished some data on this point with regard to serum. The original method of Kramer and Tisdall was based on the fact that in the presence of phosphates calcium oxalate can be precipitated in acetic acid solution.

The method of McCrudden [1909-10] for a quantitative separation of calcium, magnesium, and phosphates in food, urine, and faeces was modified for

that purpose. Kramer and Tisdall precipitated the calcium oxalate in serum or plasma after adding acetic acid. But Tisdall found later that when nothing was added to serum but ammonium oxalate, the precipitate of calcium oxalate was sufficiently pure, and his simplified method of precipitation is about the same as that already applied by de Waard [1919]. In mixtures dissolved in water, which were approximately neutral, McCrudden obtained a precipitate which contained calcium phosphate. This, however, took place in rather different conditions from those of the method here employed for solutions of serum-ash and analogous stock solutions.

Tests were made with the precipitate obtained by the latter method for phosphates. A liquid was used as a stock solution, the composition of which was about the same as one of the ash of serum, containing per 100 cc.:

- 21.8 mg. K_2SO_4 .
- 22.9 mg. Na_2HPO_4 .
- 39.1 mg. KCl.
- 800.0 mg. NaCl.
- 25.0 mg. $CaCO_3$ (dissolved in HCl).
- 21.0 mg. $MgCO_3$ (dissolved in HCl).

As much hydrochloric acid was added as was required to dissolve the whole of these substances.

The calcium oxalate was slowly precipitated in 10 cc. of this stock solution. Calcium oxalate was also obtained from 10 cc. of serum by simply following the method of Trevan and Bainbridge. To prevent the chlorides from upsetting the test for phosphates the precipitate was washed as usual, and then dried after adding nitric acid. The solution was allowed to stand for 4 hours at 50° mixed with ammonium molybdate reagent. The same was done with 1 cc. 0.0045 % $Na_2HPO_4 \cdot 2H_2O$. The amount of phosphate present in 10 cc. of the stock solution was thus 60 times as great as in 1 cc. of the phosphate solution. This latter gave a distinctly positive reaction. Both the others, on the contrary, yielded a practically inappreciable amount of yellow precipitate.

The object of the next experiments was to find out what calcium values are found by the Trevan-Bainbridge method.

In a stock solution containing nothing but 8 mg. of calcium and 6 mg. of magnesium per 100 cc. of the liquid, the calcium was precipitated by that method, that is, without neutralising slowly. The values obtained (mg.) in three series were:

- I. 8.4, 8.4, 8.0, 8.4, 8.2, 9.0.
- II. 8.6, 8.6, 9.0.
- III. 8.4, 8.6, 8.2, 8.8.

When slow precipitation was applied, four series showed:

- I. 8.2, 8.0, 8.0.
- II. 8.3, 8.1, 8.4.
- III. 7.6, 7.6, 7.8, 7.6.
- IV. 8.2, 8.0, 7.8, 8.0.

A stock solution containing 10 mg. of calcium and 6 mg. of magnesium in 100 cc., direct precipitation being applied, yielded:

11.0, 10.7, 10.8, 10.7, 10.4.

Slowly precipitated:

9.6, 9.8, 9.7, 9.6, 9.7, 10.5.

When the average values of these series are considered, it is evident that a small percentage of error in either sense was not avoided. The higher values obtained by direct precipitation are demonstrated by these tests.

It was also found possible to perform a slow neutralisation in serum. But no difference in the values was found between the results and those of the direct precipitation by the Trevan-Bainbridge method. The values given are the average of several determinations:

	Direct precipitation	Slow precipitation
First serum	9.3	9.3
Second serum	10.4	10.4
Third serum	10.2	10.4

The higher values, attributed to the occlusion of magnesium, do not appear in this case. A test for magnesium was made in the precipitates by the reaction of Hahn, Wolf and Jäger [1924]. This showed that, when the calcium oxalate was precipitated directly, the test for magnesium was distinctly positive, but when it was precipitated slowly, the reaction was less intense, but not quite negative. In serum the calcium oxalate seems to be precipitated more slowly, and the occlusion of magnesium is apparently less.

The question is now whether more calcium can be obtained by allowing the mixture to stand for a longer time, or by adding more highly concentrated ammonium oxalate.

Calcium was now precipitated from ox-serum, but in this case by the addition of 2 cc. of 7 % ammonium oxalate, which was kept in solution by means of gentle warmth. For the rest, the method of Trevan and Bainbridge was followed, and in addition to this the usual precipitation was performed with 3.5 % ammonium oxalate. The former determinations gave 12.0 mg. of calcium, the latter 10.1 mg. in 100 cc.

The experiment was repeated with human serum; 7 % ammonium oxalate gave a value of 10.6 mg. of calcium, and 3.5 % ammonium oxalate gave 9.9 mg. The tests for magnesium again differed, being more positive when 7 % ammonium oxalate was used, but not quite negative with 3.5 % ammonium oxalate.

The method of Trevan and Bainbridge was then again employed, with a different time for precipitation. The test was made with sheep-serum. After standing 1 hour for precipitation 9.3 mg. Ca were found, after 4 hours 9.3 mg. Ca, and after 24 hours 9.2 mg. Ca per 100 cc. The test for magnesium was slightly positive in all three cases, but no difference in quantity was visible. A small amount of magnesium seems always to be occluded, but when the method of Trevan and Bainbridge is followed, the amount remains within the limits of the error which, with the tests here made, was unavoidable.

The following data may serve as an example of the degree of accuracy attained:

Solution used	mg. Ca per 100 cc. found	Average
Stock containing 8 mg. Ca per 100 cc.	8.4, 8.3, 7.6, 8.2, 7.7	8.0
Stock containing 10 mg. Ca per 100 cc.	10.1, 10.4, 10.1, 10.1, 10.3	10.2
Stock resembling serum-ash and containing 10 mg. Ca per 100 cc. Neutralised slowly	(9.8, 10.2, 10.2, 10.6 9.6, 10.1, 10.1, 10.2, 10.1, 10.1 9.7, 10.1, 9.7, 9.7	10.2 10.0 9.8
Normal serum I	10.5, 10.2, 9.9, 10.2, 10.3	10.2
Normal serum II	10.5, 10.5, 10.4, 10.2	10.4
Normal serum III	10.0, 10.2, 9.8, 10.2	10.05

Tests in ash and serum were now made side by side—in the ash solution by slow neutralisation.

The ashing was done by the method of Stolte [1911]. 5 cc. of serum were ashed in a platinum crucible, which was placed in a quartz crucible and covered with a quartz cover. The ash obtained was practically white, and most of it could be transferred to a centrifuge tube marked at 5 cc. The rest of the ash was dissolved in 1 cc. of *N* HCl, and this liquid was introduced into the tube, the volume being brought up to 5 cc. by washing with water. After centrifuging, a clear liquid was obtained, of which 1 cc. at a time was transferred to tubes for Trevan and Bainbridge's calcium test. The acid solutions, on being heated to dryness, left a crystalline residue, in which, after it had been dissolved in 1 cc. of water, the calcium oxalate was precipitated by slow neutralisation. Most of the tests were done in ox-sera, some of which were haemolytic.

Serum	Ash
10.9	10.7
11.4	11.1
9.2	9.4
10.7	10.7
10.9	11.3
9.9	10.0

Occasionally a test was made in a stock solution by way of a check. Tests were also made on the sera of ten persons in whom a normal calcium value might be expected. The averages of these determinations were:

10.3	10.3	9.8	10.4	10.2
10.0	10.2	9.9	10.0	10.3

Finally, some determinations by the method of Trevan and Bainbridge were compared with the values given by two permanganate methods. It may be remarked that by the latter methods, which require to be carried out with the utmost regularity, more constant values would probably be found in the long run.

Most of the values simultaneously obtained for one serum did, however, correspond fairly well. For instance:

	Method of Clark and Collip [1925]	Method of de Waard [1919]
Serum IV	10.8	9.8
	10.5	9.8
	10.8	10.2
	10.5	10.2
	10.7	
Serum VI	9.9	10.0
	9.8	10.0
	10.0	

The average results of the three methods were:

	Trevan and Bainbridge	Clark and Collip	de Waard	Ash
I	11.4	11.6	11.1	11.1
II	11.3	11.3	11.0	—
III	9.2	9.6	8.8	9.4
IV	10.7	10.7	10.0	10.7
V	10.9	11.8	11.3	11.3
VI	10.2	9.9	10.0	—
VII	9.8	10.2	10.5	—
VIII	10.4	10.5	10.2	—

A check seems also advisable with permanganate methods, if the tests are not carried out by the same person. The method of Trevan and Bainbridge occasionally gave distinctly wrong results, as shown by the considerable divergence in one series of values. They were evidently caused by heating too much or too little.

SUMMARY.

When calcium oxalate is slowly precipitated, so as to avoid the occlusion of magnesium, values which agree fairly well are obtained by the determination of calcium in serum and ash according to the Trevan-Bainbridge method. The degree of accuracy obtained by Trevan and Bainbridge in their tests was, however, not attained here. The conditions for the precipitation of calcium are, so far as the influence of magnesium is concerned, evidently favourable in serum.

As a standard solution for comparative tests a solution may very well be taken which contains calcium only, in which case the method of Trevan and Bainbridge can be followed in its entirety.

The results given by the Trevan-Bainbridge method were, in the case of a limited number of determinations, compared with those of two permanganate methods. The method of Clark and Collip generally gave somewhat higher, that of de Waard generally somewhat lower values than the Trevan-Bainbridge method.

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