UNIVERSITY of OREGON.

ESTIMATES of CALCIUM in BLOOD.

A Thesis Submitted for Master of Science Degree

by

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ESTIMATION OF CALCIUM IN BLOOD.

Since variation in the amount of calcium present in the blood has recently been thought to have clinical significance,it has been made the subject of much study by physiologists and biochemists. A number of prevalent pathological conditions have been found to be characterized by a calcium content of the blood distinctly different from normal. It is evident that there is need for a good method of estimation. The method must be accurate, and must be capable of being carried out with ease and without too great expenditure of time. The time element is especially important if the estimation of calcium is to become part of the routine of blood analysis.

The first purpose, then, of this work was to study the methods which have been used and to try them out. Our aim was to discover improvements which would make one of the methods completely satisfactory for accurate estimations and at the same time suitable for clinical use.

The second purpose of this study was to determine whether calcium estimations should be made on the serum or on the plasma of the blood. According to the Mills' theory of the clotting of blood,variable amounts of calcium may enter into the fibrin during the process of clotting.Mills uses as a starting point,tissue fibrinogen.This tissue fibrinogen is a protein that is capable of taking up or combining with one to thirteen parts of a phospho-lipin called cephalin. Each cephalin molecule in turn can take up one calcium ion, and finally each calcium ion takes up one part of blood fibrinogen.If this theory is correct,estimations of calcium should be somewhat higher in plasma than in serum. Also the difference between the two estimations would not always be the same,due to the fact that different amounts of calcium might be used each time in the formation of the fibrin.

REVIEW OF METHODS.

There are three ways of treating blood for the estimation of calcium:

1. By oxidizing or ashing the proteins before precipitating the calcium.
2. By precipitating the proteins with picric, tungstic or trichloracetic acid and using aliquot parts of the filtrate for calcium precipitation.
3. By direct precipitation of calcium from serum or plasma.

The first method takes considerable time, and is much too troublesome for routine work. The second method calls for the use of a larger amount of blood than do the other two methods and also involves the use of an additional reagent. The method of direct precipitation saves time and reagents while giving equally good results.

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1. The nephelometric method by which the calcium is changed into a calcium soap, which remains suspended as fine particles. The degree of cloudines or turbidity is determined by a special apparatus called a nephelometer
2. The gravimetric method, by which the calcium is precipitated as calcium oxalate, this precipitate ignited and weighed as calcium oxide.
3. The volumetric method, by which the calcium is precipitated as calcium oxalate, the precipitate is dissolved in sulphuric acid and titrated with a solution of potassium permenganate.

The first of these methods was one of the earliest used for blood. Lyman proposed it in 1917(5). This method involves considerable difficulty and expenditure of time, because of having to remove the protein, and because of the danger of introducing calcium from the filter paper. There is also needed a greater number of reagents than are essential for the other methods. Some claim, however that it gives satisfactory results(12).

The second method obviously can not be used because of the small amount of blood on which the estimation must be made. Although the third method has its drawbacks, it seemed the most promising of all methods, so that we have tried to make as many improvements in it as possible.

The Kramer and Tisdall method(13,14,15), a modification of which was used by us (See Technique of Estimation), calls for the precipitation of the calcium with ammonium oxalate, washing the precipitate three times, centrifuging after each washing, and siphoning off the supernatant liquid. The precipitate is then dissolved in sulphuric acid and titrated with N/100 potassium permanganate. The modification by E.P.Clark and J.B.Collip(18) saves much time and is equally accurate. One washing with 3 cc. of dilute ammonia water is substituted for three washings with larger quantities of dilute ammonia water. The tubes are then inverted and allowed to drain for five minutes, after centrifuging long enough to thoroughly pack the precipitate in the bottom of the tube. These workers found that by draining the tube the amount of mother liquor left in the tube was only 0.02 cc. as compared with 0.1 cc. without draining. The error due to the trace of oxidizable materials other than the calcium oxalate left in the tube is balanced by the slight loss of calcium due to the solubility of calcium oxalate in the wash solution. By this method the calcium can be estimated with an error of not over two per cent as compared with the possibility of an error of five per cent with the original Kramer and Tisdall method. The tubes must be thoroughly cleaned with a mixture of potassium dichromate and sulphuric acid to insure uniform drainage.

PROCEDURE FOR OBTAINING PLASMA.

Since one of the purposes of this study was to determine whether the plasma contains more calcium than the serum, one of the first problems was to secure the plasma. This necessitated making a choice of an anti-coagulant. Potassium or sodium oxalate is suitable when estimations of organic constituents are to be made, but calcium is precipitated by both of these. Fluorides have been used but they also precipitate calcium. Several investigators claim to have secured proper calcium estimations on sodium citrate plasma, although they recommend the use of serum. The directions for using 1 cc. of saturated sodium citrate solution(over 60 %) for 10 cc. of blood are manifestly absurd. This method involves making a determination

of the plasma volume of the blood so as to be able to calculate the dilution factor. We have used this proportion of citrate and secured only a trace of calcium oxalate precipitate. If sodium or lithium citrate is used, a very small amount should be taken(e.g. 0.02 to 0.05 cc. for 5 cc. of blood),the citrate being reduced to the minimal quantity that will prevent clotting.

We decided to try the substance recently placed on the market, called heparin.This substance, a fine grayish white powder,is produced in the liver in small amounts and is extracted therefrom. It is easy to handle and presents no problem of dilution as does a solution of a citrate. The cost of the heparin is $10.00 per gram, but since the extremely small amount of 1.1 mg. is sufficient to prevent the clotting of 5 cc. of blood, the cost for each estimation is very small(1.1 cents).

Before the use of hepsrin could be adopted it was necessary to determine whether the presence of the heparin affected in any way the determination of the calcium. Several estimations were made on ox serum, 2 cc. of serum being used,to which 1.0 mg. of heparin was added.Controls were run on the same serum without heparin.No appreciable change could be detected in the final titration figure,so it was decided that heparin was a very suitable anticoagulant.

TECHNIQUE OF PREPARING SAMPLES.

Two estimations were made on each blood at the same time,one on the serum and one on the plasma, in order to determine any difference in the calcium content of the two. About ten cubic centimeters of blood was drawn each time. Five cc. of each sample was transferred quickly to a conical centrifuge tube containing the weighed amount(1.1 mg.) of heparin.The tube was shaken vigorously to insure the uniform distribution of the anticoagulant.This was centrifuged immediately for 5 minutes or until there is a good separation of the plasma from the cells.The time depends somewhat on the rate of speed of the centrifuge.

We found that the plasma could best be removed by means of a capillary pipette to which a small rubber bulb was attached.By this means the plasma can be almost completely removed without disturbing the cells, which is a difficult thing for one person to do with an ordinary pipette. The plasma was collected in this way in a clean dry tube.

The other 5 cc. of blood was placed in a centrifuge tube and allowed to clot.It was then centrifuged and the serum was removed from the clot in the same manner as the plasma was removed from the cells.

TECHNIQUE OF ESTIMATION.

Measure 2 cc. of serum or plasma ino a centrifuge tube,add an equal volume of redistilled water(See Preparation of Reagents) and 1 cc. of 4%ammonium oxalate solution.Mix with a stirring rod,and then rinse the rod with two or three drops of the oxalate solution.Allow the tube to stand at least thirty minutes to insure complete precipitation Centrifuge for about 5 minutes or until the precipitate of calcium oxalate is well packed in the bottom of the tube.The supernatant liquid is carefully poured off and the tubes inverted,resting on a pad of filter paper and allowed to drain 5 minutes.The mouth of the tube is wiped dry. The precipitate is stirred up and washed with 3 cc. of dilute ammonia ~~wat~~ water(2 cc. of concentrated ammonium hydroxide in 98 cc. of redistilled water),directed in a fine stream from a wash bottle. Centrifuge again and drain as before.The precipitate is then dissolved in 2 cc. of approximately normal sulphuric acid(28 cc. of pure concentrated acid to 970 cc. of redistilled water).The acid is blown on to the precipitate from a pipette in order to stir up the calcium oxalate and cause the mat to dissolve more quickly.If the mat of precipitate is not broken up by blowing the acid upon it,it must be broken up with a stirring rod.It is undesirable to have the rod in the tube during the main titration,and it must be rinsed carefully before removal with a few drops of dilute sulphuric acid or redistilled water.

The tube is then placed in a water bath(a large Pyrex beaker serves this purpose nicely) kept at a temperature between 70 and 75°C., and titrated while hot with N/100 potassium permanganate solution to a faint pink color which persists 1 minute after the last drop is added.When the end point is almost reached,that is, when the pink color disappears rather slowly,the solution should be stirred with a rod so that the part in the tip of the tube may come in contact with the potassium permanganate. It usually requires from one to three drops additional to reach the end point The potassium permanganate solution is used in a micro burette of 5 cc. capacity,each mark representing 0.02 cc. It is fitted with a glass stop-cock and capillary tip.The tip is oiled frequently to make the drops which are delivered as small as possible.The drops can be made smaller than 0.02 cc.

In case of shortage of serum or plasma,1 cc. or even less may be used for the estimation,although 2 cc. is desirable.

CALCULATION.

One cc. of N/100 $KMnO_4$ is equivalent to 0.2 mg. of calcium

$$\text{Titration in cc.} \times 0.2 \times \frac{100}{\text{cc. of serum or plasma used}} = \text{mg. of Ca per 100 cc. of serum or plasma}$$

If 2 cc. of plasma or serum is used multiply the cc. of $KMnO_4$ by 10.If 1 cc. is used,multiply the titration figure by 20.

RESULTS.

Part of the work was done on human blood and part of it on dog's blood.The average calcium content of the blood of normal persons is 10.0 mg. per 100 cc. of serum and 13.1 mg. per 100 cc. of plasma. Our estimations that show very low calcium figures were made on dogs which were being used for experimental purposes in physiology research. The data obtained is as follows:

TABLE SHOWING RESULTS OF ESTIMATIONS OF SERUM AND PLASMA.

Source	Plasma	Serum	Difference	Per Cent of increase of Ca. in Plasma over that in Serum
Human	10.32 mg.	8.49 mg.	1.83 mg.	21.5
Human	10.32 mg.	9.5 mg.	0.82 mg.	8.6
Human	14.00 mg.	11.2 mg.	2.80 mg	25.0
Human	14.00 mg	10.2 mg	3.80 mg.	37.0
Human	14.06 mg.	11.97 mg	2009 mg.	17.4
Human	13.91 mg.	11.65 mg.	2.26 mg.	11.6
Human	13.77 mg.	1159 mg	2.18 mg	18.8
Human	14.80 mg	14.00 mg.	0.80 mg	5.7
Human	14.60 mg.	13.97 mg.	0.63 mg.	4.5
Human	13.50 mg.	10.38 mg	3.12 mg	30.00
Human	12.70 mg.	9.50 mg	3.20 mg.	33.6
Human	11.90 mg.	9.56 mg.	2.34 mg.	24.4
Dog	12.00 mg	11.70 mg.	0.30 mg	2.5
Dog	12.90 mg	10.80 mg	2.10 mg	19.4
Dog	5.32 mg.	3.86 mg	1.46 mg	37.5
Dog	11.70 mg	9.60 mg	2.10 mg	21.8
Dog	8.51 mg	6.90 mg.	1.61 mg.	23.3
Dog	10.60 mg	7.80 mg	2.80 mg.	35.9
Dog	13.30 mg	12.40 mg.	0.90 mg.	7.2
Dog	6.90 mg	4.70 mg	2.20 mg	46.8
Dog	6.60 mg	5.50 mg.	1.10 mg	20.0
Dog	7.33 mg	5.52 mg	0.81 mg	32.7
Dog	7.47 mg	6.60 mg	0.87 mg	1.31
Dog	7.30 mg	5.80 mg	1.50 mg	25.8

TABLE SHOWING RESULTS OF ESTIMATIONS OF SERUM AND PLASMA (Cont'd)

Source	Plasma	Serum	Differences	Per Cent of increase of Ca. in Plasma over that in Serum.
Dog	7.30 mg	5.50 mg	1.80 mg	32.7
Dog	7.90 mg	5.60 mg	2.30 mg	40.0
Dog	9.44 mg	9.19 mg	0.25 mg	2.7
Dog	9.18 mg	7.05 mg	2.13 mg	30.2

In these twenty eight estimations, the variation in the percentage increase of calcium in the plasma over that of the serum ranges from 1.31 to 46.8. Seven estimations showed a percentage increase between 1.31 and 8.6; ten showed a percentage increase between 11.6 and 25.0; and eleven showed a percentage increase between 25.8 and 46.8.

DISCUSSION OF RESULTS.

It is to be noted that there is a decided increase of calcium in the plasma over that of thebserum,but that the difference is variable which indicates that the Mills' theory of the variation in the amounts of calcium used in fibrin formation may be correct,and that to get the true calcium content of the blood,estimations should be made on the plasma rather than on the serum. We secured no evidence of the adsorption of organic substance to the calcium oxalate precipitate in the case of plasma that did not occur in the case of serum, so that we are convinced that the difference in titration is due to actual difference in the calcium content.

G.W.Clark(2) checked the accuracy of the direct method of precipitation and titration by adding known amounts of calcium to serum. Ninety-eight per cent of the added calcium was recovered.

Controls.

1. The 2 cc. of approximately normal sulphuric acid gave a titration value of 0.04 cc. of N/100 $KMnO_4$. This was deducted from the titration figure in each estimation.

2. A test was made to determine how much soakage there might be of the reagents in the calcium oxalate precipitate.A quantity of fine pumice about equal to the amount of a calcium oxalate precipitate was treated with the precipitating reagent,centrifuged,drained,washed,centrifuged again and drained,treated with sulphuric acid and titrated just like a calcium precipitate.The result was that the titration figure was increase by only 0.005 cc. above that for the control(See 1. above).This is negligible.

PREPARATION OF REAGENTS. KEEPING QUALITY OF REAGENTS.

Considerable difficulty was met with in the matter of reagents,especially the keeping quality of N/10 and N/100 potassium permanganate solutions, and also of solutions of recrystallized oxalic acid and

sodium oxalate.We recrystallized the oxalic acid ourselves from C.P. crystals, in order to be sure of the amount of water of crystallization. At first ordinary once distilled water was used in the preparation of all solutions.A stock solution of potassium permanganate ,made a little stronger than N/10 was prepared,using distilled water.This was allowed to stand two weeks. It was titrated against a freshly prepared solution of N/10 oxalic acid. The potassium permanganate was then diluted with the required amount of distilled water to make it exactly N/10,and checked again against N/10 oxalic acid. A fresh solution of the N/100 potassium permanganatewas made each day by dilution of the stock N/10 solution. This dilution had to be made by the trial and error method because exact measurements did mot produce a solution which was exactly N/100. Furhtermore the N/10 stock solution changed perceptibly within a m month,

ILLUSTRATION OF THE CHANGE IN N/100 KMnO AND N/100 $KMnO_4$

January 12 2 cc. N/10 oxalic acid(fresh)- 2 cc. N/10 $KMnO_4$

January 26 2 cc.N/10 oxalic acid(fresh)- 2.07 cc. N/10 $KMnO_4$

February 2 2 cc. N/10 oxalic acid(fresh)- 2.1 cc. KMnO (N/10)

This shows a weakening of the N/10 potassium permanganate solution.

Illustration of the change in N/100 $KMnO_4$

January 15 2 cc. N/100 oxalic acid(fresh)- 1.86 cc. $KMnO_4$

January 19 2 cc. N/100 oxalic acid(fresh)- 1.88 cc. $KMnO_4$

March 25 2 cc. N/100 oxalic acid(fresh)- 2.00 cc. $KMnO_4$

This shows a weakening of the N/100 potassium permanganate solution

The poor keeping quality of the reagents caused the loss of a great deal of time in the preparation and standardization of materials each day when there were estimations to be made.We decided that this constant change might be due to oxidizable substances in the distilled water. Titrations were made on the water as in the calcium method and 1cc. was found to reduce 0.005 cc. of N/100 $KMnO_4$(freshly prepared).Then to 500 cc. of distilled water 2.5 cc. of N/100 $KMnO_4$ was added and the mixture allowed

to stand a week. It was then redistilled into a special non-sol flask and tightly corked.

This redistilled water was then used in the preparation of all reage gents, and for dilutions.It was found that an exact N/10 solution of potassium permanganate could be diluted by calculation and an exact N/100 solution obtained.One sample of N/100 solution of potassium permanganate was found unchanged after two weeks time. The N/10 solution was exact a after six we ks time.This was also true of the N/10 oxalic acid.

ILLUSTRATION TO SHOW THE KEEPING QUALITY OF N/10 AND N/100 $KMnO_4$

April 7 5 cc. N/10 oxalic acid(fresh)- 5 cc.N/10 $KMnO_4$

May 21 5 cc. N/10 oxalic acid(fresh)- 5 cc.N/10 $KMnO_4$

May 21 5cc. N/10 Oxalic acid(made April 7)- 5 cc. N/10 $KMnO_4$

April 7 2 cc. N/100 oxalic acid(fresh)- 2 cc. N/100 $KMnO_4$

April 21 2 cc. N/100 oxalic acid(fresh)- 2 cc.N/100 $KMnO_4$

April 21 2cc. N/100 oxalic acid(made April 7)- 1.9 cc. $KMnO_4$

The N/10 oxalic acid was also checked against N/10 NaOH and the results agreed.

April 6 5 cc. N/10 oxalic acid- 5 cc. N/10 NaOH

April 14 2 cc. N/10 oxalic acid- 2 cc. N/10 NaOH

April222 5 cc. N/10 oxalic acid- 5 cc. N/10 NaOH

May 21 5 cc. N/10 oxalic acid- 5 cc. N/10 NaOH

The solution of N/100 oxalic acid,however did not retain its s strength more than one day.

IMPROVEMENTS IN THE METHOD.

1.The use of heparin as an anticoagulant in securing plasma for the estimations is a great advantage.

2. Heparin plasma gives more nearly the true calcium content of than the blood than does serum.

3. Distilled water which has been allowed to stand for a week with a small amount of N/100 $KMnO_4$ and then redistilled is used in making the

normal sulphuric acid, the precipitating reagent (ammonium oxalate) the potassium permanganate and oxalic acid solutions and the dilute ammonia water for washing. This greatly adds to the keeping quality of the reagents. The redistilled water is also used for the dilution of plasma and serum and for all dilutions. This removes the possibility of introducing organic matter which might reduce part of the potassium permanganate used in the titration. This redistilled water can be made up in large quantities and kept in special non-sol flasks. tightly corked, and much time is saved in the preparation of reagents.

CONCLUSIONS.

1. Special technique must be used in the preparation of reagents. Many of the problems associated with the method are solved if this type of distilled water is used in the preparation of all solutions.

2. The method of direct precipitation of the calcium as Oxalate and titrating with potassium permanganate is satisfactory, both as a research and as a clinical method of prodedure if good technique is used.

3. The estimation of calcium in blood should be made on the plasma rather than on the serum, as the calcium content of the plasma ishigher than that of the serum, and approximates more closely the true calcium content of the fluid portion of the blood

4. Heparin has proved to be an excellent substance for preventing coagulation. It is indispensable for this method.

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The other 5 cc. of blood was placed in a centrifuge tube and allowed to clot.It was then centrifuged and the serum was removed from the clot in the same manner as the plasma was removed from the cells.

TECHNIQUE OF ESTIMATION.

Measure 2 cc. of serum or plasma ino a centrifuge tube,add an equal volume of redistilled water(See Preparation of Reagents) and 1 cc. of 4% ammonium oxalate solution.Mix with a stirring rod,and then rinse the rod with two or three drops of the oxalate solution.Allow the tube to stand at least thirty minutes to insure complete precipitation Centrifuge for about 5 minutes or until the precipitate of calcium oxalate is well packed in the bottom of the tube.The supernatant liquid is carefully poured off and the tubes inverted,resting on a pad of filter paper and allowed to drain 5 minutes.The mouth of the tube is wiped dry. The precipitate is stirred up and washed with 3 cc. of dilute ammonia ~~wat~~ water(2 cc. of concentrated ammonium hydroxide in 98 cc. of redistilled water),directed in a fine stream from a wash bottle. Centrifuge again and drain as before.The precipitate is then dissolved in 2 cc. of approximately normal sulphuric acid(28 cc. of pure concentrated acid to 970 cc. of redistilled water).The acid is blown on to the precipitate from a pipette in order to stir up the calcium oxalate and cause the mat to dissolve more quickly.If the mat of precipitate is not broken up by blowing the acid upon it,it must be broken up with a stirring rod.It is undesirable to have the rod in the tube during the main titration,and it must be rinsed carefully before removal with a few drops of dilute sulphuric acid or redistilled water.

The tube is then placed in a water bath(a large Pyrex beaker serves this purpose nicely) kept at a temperature between 70 and 75, and titrated while hot with N/100 potassium permanganate solution to a faint pink color which persists 1 minute after the last drop is added.When the end point is almost reached,that is, when the pink color disappears rather slowly,the solution should be stirred with a rod so that the part in the tip of the tube may come in contact with the potassium permanganate. It usually requires from one to three drops additional to reach the end point The potassium permanganate solution is used in a micro burette of 5 cc. capacity,each mark representing 0.02 cc. It is fitted with a glass stop-cock and capillary tip.The tip is oiled frequently to make the drops which are delivered as small as possible.The drops can be made smaller th than 0.02 cc.

In case of shortage of serum or plasma,1 cc. or even less may be used for the estimation,although 2 cc. is desirable.

CALCULATION.

One cc. of N/100 $KMnO_4$ is equivalent to 0.2 mg. of calcium

$$\text{Titration in cc.} \times 0.2 \times \frac{100}{\text{cc. of serum or plasma used}} = \text{mg. of Ca per 100 cc. of serum or plasma}$$

If 2 cc. of plasma or serum is used multiply the cc. of $KMnO_4$ by 10.If 1 cc. is used,multiply the titration figure by 20.

RESULTS.

Part of the work was done on human blood and part of it on dog's blood.The average calcium content of the blood of normal persons is 10.0 mg. per 100 cc. of serum and 13.1 mg. per 100 cc. of plasma. Our estimations that show very low calcium figures were made on dogs which were being used for experimental purposes in physiology research. The data obtaines is as follows:

TABLE SHOWING RESULTS OF ESTIMATIONS ON SERUM AND PLASMA.

Source	Plasma	Serum	Difference	% Increase of calcium in Plasma over that in Serum
Human	10.32 mg.	8.49 mg.	1.83 mg.	21.5
Human	10.32 mg.	9.5 mg.	0.82 mg.	8.6
Human	14.00 mg.	11.2 mg.	2.80 mg.	25.0
Human	14.00 mg.	10.2 mg.	3.80 mg.	37.0
Human	14.06 mg.	11.97 mg.	2.09 mg.	17.4
Human	13.91 mg.	11.65 mg.	2.26 mg.	11.6
Human	13.77 mg.	11.59 mg.	2.18 mg.	18.8
Human	14.80 mg.	14.00 mg.	0.80 mg.	5.7
Human	14.60 mg.	13.97 mg.	0.63 mg.	4.5
Human	13.50 mg.	10.38 mg.	3.12 mg.	30.00
Human	12.70 mg.	9.50 mg.	3.20 mg.	33.6
Human	11.90 mg.	9.56 mg.	2.34 mg.	24.4
Dog	12.00 mg.	11.70 mg.	0.30 mg.	2.5
Dog	12.90 mg.	10.80 mg.	2.10 mg.	19.4
Dog	5.32 mg.	3.86 mg.	1.46 mg.	37.5
Dog	11.70 mg.	9.60 mg.	2.10 mg.	21.8
Dog	8.51 mg.	6.90 mg.	1.61 mg.	23.3
Dog	10.60 mg.	7.80 mg.	2.80 mg.	35.9
Dog	13.30 mg.	12.40 mg.	0.90 mg.	7.2
Dog	6.90 mg.	4.70 mg.	2.20 mg.	46.8
Dog	6.60 mg.	5.50 mg.	1.10 mg.	20.0
Dog	7.33 mg.	5.52 mg.	0.81 mg.	33.7
Dog	7.47 mg.	6.60 mg.	0.87 mg.	1.31
Dog	7.30 mg.	5.80 mg.	1.50 mg.	25.8

TABLE SHOWING RESULTS OF ESTIMATIONS OF SERUM AND PLASMA(cont'd)

Source	Plasma	Serum	Difference	% Difference(increase of calcium in plasma over that in serum
Dog	7.30 mg.	5.50 mg.	1.80 mg.	32.7
Dog	7.90 mg.	5.60 mg.	2.30 mg.	40.0
Dog	9.44 mg.	9.19 mg.	0.25 mg.	2.7
Dog	9.18 mg.	7.05 mg.	2.13 mg.	30.2

In these twenty eight estimations, the variation in the percentage increase of calcium in the plasma over that of the serum ranges from 1.31 to 46.8 .Seven estimations showed a percentage increase between 1.31 and 8.6; ten showed a percentage increase between 11.6 and 25.0; and eleven showed a percentage increase between 25.8 and 46.8.

DISCUSSION OF RESULTS.

It is to be noted that there is a decided increase of calcium in the plasma over that of thebserum,but that the difference is variable which indicates that the Mills' theory of the variation in the amounts of calcium used in fibrin formation may be correct,and that to get the true calcium content of the blood,estimations should be made on the plasma rather than on the serum. We secured no evidence of the adsorption of organic substance to the calcium oxalate precipitate in the case of plasma that did not occur in the case of serum, so that we are convinced that the difference in titration is due to actual difference in the calcium content.

G.W.Clark(2) checked the accuracy of the direct method of precipitation and titration by adding known amounts of calcium to serum. Ninety-eight per cent of the added calcium was recovered.

Controls.

1. The 2 cc. of approximately normal sulphuric acid gave a titration value of 0.04 cc. of N/100 KMnO. This was deducted from the titration figure in each estimation.

2. A test was made to determine how much soakage there might be of the reagents in the calcium oxalate precipitate.A quantity of fine pumice about equal to the amount of a calcium oxalate precipitate was treated with the precipitating reagent,centrifuged,drained,washed,centrifuged again and drained,treated with sulphuric acid and titrated just like a calcium precipitate.The result was that the titration figure was increased by only 0.005 cc. above that for the control(See 1. above).This is negligible.

PREPARATION OF REAGENTS. KEEPING QUALITY OF REAGENTS.

Considerable difficulty was met with in the matter of reagents,especially the keeping quality of N/10 and N/100 potassium permanganate solutions, and also of solutions of recrystallized oxalic acid and

sodium oxalate.We recrystallized the oxalic acid ourselves from C.P. crystals, in order to be sure of the amount of water of crystallization. At first ordinary once distilled water was used in the preparation of all solutions.A stock solution of potassium permanganate ,made a little stronger than N/10 was prepared,using distilled water.This was allowed to stand two weeks. It was titrated against a freshly prepared solution of N/10 oxalic acid. The potassium permanganate was then diluted with the required amount of distilled water to make it exactly N/10,and checked again against N/10 oxalic acid. A fresh solution of the N/100 potassium permanganatewas made each day by dilution of the stock N/10 solution. This dilution had to be made by the trial and error method because exact measurements did mot produce a solution which was exactly N/100. Furhtermore the N/10 stock solution changed perceptibly within a month.

ILLUSTRATION OF THE CHANGE IN N/100 $KMnO_4$ AND N/100 $KMnO_4$

January 12 2 cc. N/10 oxalic acid(fresh)- 2 cc. N/10 KMnO

January 26 2 cc.N/10 oxalic acid(fresh)- 2.07 cc. N/10 KMnO

February 2 2 cc. N/10 oxalic acid(fresh)- 2.1 cc. KMnO (N/10)

This shows a weakening of the N/10 potassium permanganate solution.

Illustration of the change in N/100 $KMnO_4$

January 15 2 cc. N/100 oxalic acid(fresh)- 1.86 cc. $KMnO_4$

January 19 2 cc. N/100 oxalic acid(fresh)- 1.88 cc. $KMnO_4$

March 25 2 cc. N/100 oxalic acid(fresh)- 2.00 cc. $KMnO_4$

This shows a weakening of the N/100 potassium permanganate solution

The poor keeping quality of the reagents caused the loss of a great deal of time in the preparation and standardization of materials each day when there were estimations to be made.We decided that this constant change might be due to oxidizable substances in the distilled water. Titrations were made on the water as in the calcium method and 1cc. was found to reduce 0.005 cc. of N/100 $KMnO_4$(freshly prepared).Then to 500 cc. of distilled water 2.5 cc. of N/100 $KMnO_4$ was added and the mixture allowed

to stand a week. It was then redistilled into a special non-sol flask and tightly corked.

This redistilled water was then used in the preparation of all reage gents, and for dilutions.It was found that an exact N/10 solution of potassium permanganate could be diluted by calculation and an exact N/100 solution obtained.One sample of N/100 solution of potassium permanganate was found unchanged after two weeks time. The N/10 solution was exact a after six weeks time.This was also true of the N/10 oxalic acid.

ILLUSTRATION TO SHOW THE KEEPING QUALITY OF N/10 AND N/100 $KMnO_4$

April 7 5 cc. N/10 oxalic acid(fresh)= 5 cc.N/10 $KMnO_4$

May 21 5 cc. N/10 oxalic acid(fresh)= 5 cc.N/10 $KMnO_4$

May 21 5cc. N/10 Oxalic acid(made April 7)= 5 cc. N/10 $KMnO_4$

April 7 2 cc. N/100 oxalic acid(fresh)= 2 cc. N/100 $KMnO_4$

April 21 2 cc. N/100 oxalic acid(fresh)= 2 cc.N/100 $KMnO_4$

April 21 2cc. N/100 oxalic acid(made April 7)= 1.9 cc. $KMnO_4$

The N/10 oxalic acid was also checked against N/10 NaOH and the results agreed.

April 6 5 cc. N/10 oxalic acid= 5 cc. N/10 NaOH

April 14 2 cc. N/10 oxalic acid= 2 cc. N/10 NaOH

April222 5 cc. N/10 oxalic acid= 5 cc. N/10 NaOH

May 21 5 cc. N/10 oxalic acid= 5 cc. N/10 NaOH

The solution of N/100 oxalic acid,however did not retain its s strength more than one day.

IMPROVEMENTS IN THE METHOD.

1.The use of heparin as an anticoagulant in securing plasma for the estimations is a great advantage.

2. Heparin plasma gives more nearly the true calcium content of the blood than does serum.

3. Distilled water which has been allowed to stand for a week with a small amount of N/100 $KMnO_4$ and then redistilled is used in making the

normal sulphuric acid, the precipitating reagent (ammonium oxalate), the potassium permanganate and oxalic acid solutions and the dilute ammonia water for washing. This greatly adds to the keeping quality of the reagents. The redistilled water is also used for the dilution of plasma and serum and for all dilutions.. This removes the possibility of introducing organic matter which might reduce part of the potassium permanganate used in the titration. This redistilled water can be made up in large quantities and kept in special non-sol flasks, tightly corked, and much time is saved in the preparation of reagents.

CONCLUSIONS.

1. Special technique must be used in the preparation of reagents. Many of the problems associated with the method are solved if this type of redistilled water, described above, is used in their preparation.

2. The method of direct precipitation of the calcium as oxalate and titrating with potassium permanganate is satisfactory, both as a research and as a clinical method of procedure if good technique is used.

3. The estimations of calcium in blood should be made on the plasma rather than on the serum. as the calcium content of the plasma is higher than that of the serum, and approximates much more closely the true calcium content of the fluid portion of the blood.

4. Heparin has proved to be an excellent substance for preventing coagulation of blood. It is indispensable for this method.

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