A COLORIMETRIC METHOD FOR THE ESTIMATION OF BLOOD CALCIUM.

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Principle.

The calcium is precipitated as calcium phosphate from an alkalinized trichloroacetic acid serum filtrate and estimated as phosphate by the Benedict and Theis (1) molybdic oxide colorimetric method, slightly modified.

Procedure.

Place 2 cc. of blood serum in a small flask and add 4 cc. of distilled water and 4 cc. of 20 per cent trichloroacetic acid. Mix thoroughly, allow to stand 10 minutes, and filter through a double acid-washed calcium-free filter paper. Transfer 5 cc. of the trichloroacetic acid filtrate to a 15 cc. conical centrifuge tube which has been thoroughly cleaned by immersion in bichromate sulfuric acid "cleaning solution" for several hours. Place 1 drop of 1 per cent phenolphthalein in the tube and add, drop at a time, 20 per cent calcium-free sodium hydroxide until a definite pink color is obtained. Add 1 cc. of 1 per cent trisodium phosphate, twirl the tube until thoroughly mixed, cork, and set aside for 1 hour.

After 1 hour's standing, centrifuge for 3 minutes. Decant carefully the supernatant fluid from the calcium phosphate precipitate. Place the inverted tube upon a pad of filter paper to drain for 2 or 3 minutes, then wipe away adherent solution from the mouth of the tube with a clean cloth or paper. Wash twice with 5 cc. portions of 50 per cent alcohol made faintly alkaline to phenolphthalein with a few drops of calcium-free alkali. In washing, the mat of calcium phosphate in the bottom of the tube must be
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thoroughly broken up with a glass stirring rod, and the process of centrifuging, decanting, and draining the tube should be carried out as described above. Dissolve the washed precipitate in 5 cc. of 5 per cent sulfuric acid by volume (5 cc. concentrated H₂SO₄ per 100 cc. of water), and decant into a Rothberg-Evans sugar tube, or a graduated test-tube; wash the centrifuge tube twice with approximately 3 cc. and 2 cc. portions of the 5 per cent sulfuric acid, adding the washings to the graduated tube.

In a similarly graduated tube place 10 cc. of standard phosphate solution containing 0.05 mg. of phosphorus, and add 0.5 cc. of concentrated sulfuric acid. Now add to each tube 1 cc. of 5 per cent sodium molybdate and 1 cc. of hydroquinone bisulfite reagent. Place the tubes in a boiling water bath for 10 minutes. Remove, cool, dilute the standard to 15 cc. and the unknown to a volume giving a color that will approximately match the standard (15 cc. in normal bloods), and compare in a colorimeter in the usual manner.

Calculation.

\[
\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.05 \times \frac{\text{dilution of unknown}}{\text{dilution of standard}} \times \frac{60}{31} \times 100 = \text{mg. Ca per 100 cc. of serum.}
\]

Reagents.

1. Standard Phosphate Solution.—For convenience we use the Bell-Doisy phosphate standard solutions, since these are practically always in use in the laboratory for determining inorganic phosphorus.

(a) Stock Solution.—Dissolve 4.394 gm. of pure dry monopotassium phosphate in 1 liter of phosphate-free water. 1 cc. of this reagent contains 1 mg. of phosphorus. Preserve with chloroform.

(b) Phosphate Solution for Calcium Estimation.—Pipette accurately 5 cc. of the stock phosphate solution into a liter flask, and make up to the mark with phosphate-free water. 10 cc. of this solution contain 0.05 mg. of phosphorus, equivalent to 0.097 mg. of calcium as Ca₅(PO₄)₂.

2. Molybdate Solution.—A 5 per cent solution of pure sodium molybdate (Na₆MoO₄·2H₂O) is used. Pure ammonium molybdate of the same concentration may be used.

3. Hydroquinone Bisulfite Reagent.—Dissolve 30 gm. of pure sodium bisulfite and 1 gm. of highest purity hydroquinone in 200 cc. of phosphate-free water.
The authors at first checked the above procedure against the Kramer-Tisdall (2) and the Fiske (3) methods for estimating blood calcium, and obtained results that agreed satisfactorily. Since these two methods are titration methods which, necessarily, involve personal error in judging end-points, and with which it is difficult to obtain close checks, we discontinued testing against these methods and tried the colorimetric procedure upon known calcium solutions.

**TABLE I.**

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Ca per 100 cc.</th>
<th>Serum plus 4 mg. calcium.</th>
<th>Serum plus 10 mg. calcium.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>1</td>
<td>11.8</td>
<td>15.8</td>
<td>16.1</td>
</tr>
<tr>
<td>2</td>
<td>10.3</td>
<td>14.3</td>
<td>14.2</td>
</tr>
<tr>
<td>3</td>
<td>9.2</td>
<td>13.2</td>
<td>13.0</td>
</tr>
<tr>
<td>4</td>
<td>10.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II.**

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Ca per 100 cc. serum.</th>
<th>Ca per 100 cc. serum diluted 1:2:5 (calculated).</th>
<th>Ca per 100 cc. serum diluted 1:1 (calculated).</th>
<th>Ca per 100 cc. obtained by analysis.</th>
<th>Recovered.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>per cent</td>
</tr>
<tr>
<td>1</td>
<td>10.1</td>
<td>4.04</td>
<td>4.1</td>
<td>101.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10.7</td>
<td>4.28</td>
<td>4.2</td>
<td>98.1</td>
<td></td>
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<tr>
<td>3</td>
<td>10.3</td>
<td>4.12</td>
<td>4.1</td>
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<tr>
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<td>5.75</td>
<td>5.79</td>
<td>100.6</td>
<td></td>
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<tr>
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<td>4.95</td>
<td>4.98</td>
<td>100.6</td>
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<td>6</td>
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<td>5.90</td>
<td>5.70</td>
<td>96.6</td>
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<tr>
<td>7</td>
<td>11.8</td>
<td></td>
<td></td>
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</table>

With a calcium solution containing 10 mg. per 100 cc. we obtained 98 to 100 per cent recoveries. The results were equally as satisfactory when varying amounts of calcium were added to blood serum. We also obtained quantitative recoveries with sera diluted to contain one-half and two-fifths the normal blood calcium, thus demonstrating the applicability of the method to hypocalcemic conditions of 4 to 6 mg. per 100 cc. of serum. Typical protocol are shown in Tables I and II.
Estimation of Calcium

Since the method is dependent upon the fact that calcium phosphate has a low solubility in an alkaline medium, experiments were carried out to determine the conditions that will give the least solubility. The influence of concentrating the alkali was first investigated, and it was found that the lowest solubility exists in hydrogen ion concentrations ranging from pH 7 to pH 12. In this range Ca$_3$(PO$_4$)$_2$ is soluble to the extent of 1.2 to 1.8 mg. per 100 cc. of solution, as shown by the curve in Fig. 1. Above pH 12 the curve rises sharply, showing an increase in solubility to 12.9 mg. per 100 cc. of solution at pH 14. Hence the trichloroacetic acid filtrate is alkalinized until it reaches a hydrogen ion concentration just basic to phenolphthalein in order to obtain maximum precipitation.

We examined various substances that could be used for washing the Ca$_3$(PO$_4$)$_2$ free from the excess soluble phosphate used as a precipitant. The lowest solubility was found with mixtures of alcohol and water. In 50 and 85 per cent alcohol the solubilities determined by the colorimetric method were 0.7 mg. and 0.5 mg. of Ca$_3$(PO$_4$)$_2$ per 100 cc. of solution. These mixtures also proved to be satisfactory washes and were therefore adopted for this purpose.

![Fig. 1. Curve showing solubility of Ca$_3$(PO$_4$)$_2$ at varying hydrogen ion concentrations.](http://www.jbc.org/)

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Analyses carried out upon phosphate precipitates from known calcium solutions to determine the comparative losses from solubility in alcohol and water gave the following results: When washed with 50 per cent alcohol, precipitates representing 10 mg. of calcium per 100 cc. never gave less than 9.8 mg. after two washings, and 9.7 mg. after four washings. Similar experiments, using distilled water, gave 9.0 mg. after two washings, and 8.3 mg. after four washings. We do not feel that the small losses experienced when using 50 per cent alcohol for washing justify the introduction of a correction factor.

The magnesium of the blood does not interfere for either of two reasons: (1) The trichloroacetic acid filtrate is first alkalinized, and, as the solubility of magnesium hydroxide is 0.9 mg. per 100 cc., this step would tend to remove magnesium as the hydroxide, if present to that extent; (2) magnesium phosphate has a solubility of 20.5 mg. per 100 cc., and as this amount is very much in excess of the amount of magnesium found in the blood, there is no phosphate precipitated as magnesium phosphate. We demonstrated that no interference occurs by an experiment in which analyses were carried out upon four samples of pure calcium solution and upon four similar quantities of the same solution to each of which was added 2 mg. of magnesium per 100 cc. The values obtained for calcium were the same in the magnesium additions as in the controls.

Duplicate determinations by this method agree excellently. The technique of precipitating and washing the Ca₃(PO₄)₂ gives practically constant results. Inspection of the calculation formula shows that a variation of 1 mg. in the result will require a difference in colorimeter readings of 2 mm. This is a very liberal margin for errors in technique as compared with conditions that will change results obtained by the titration methods.

Since the proposed method is colorimetric, it conforms to the requirements of a micro method. The equivalent of 1 cc. of serum is used in the regular procedure. This amount can be reduced to the equivalent of 1/4 or 3/8 of a cc. without loss of accuracy, as was shown in the experiments with diluted serum. In the Kramer-Tisdall (2) method, 2 cc. of serum is the amount regularly used for the analysis. This amount will give a burette reading around 1 cc. of 0.01 N KMnO₄ with normal bloods. If 1 cc. of
normal serum, or 2 cc. of a hypocalcemic serum containing 5 mg. of calcium per 100 cc. are being analyzed, the titration with 0.01 N KMnO₄ will be 0.5 cc. If \( \frac{3}{8} \) of a cc. is analyzed, the burette reading will be 0.2 cc. It is obvious that accurate results cannot be obtained when such small quantities of the titrating reagent are used, and that the colorimetric method is capable of being adapted to the analysis of much smaller amounts of serum than the titration methods.

This procedure requires no more skill on the part of the operator than the methods in use at present, and is equally as rapid. It does not necessitate frequent checking of reagents like the permanganate or the alkalimetric titration methods. It is applied to the same trichloroacetic acid filtrate that is used in the determination of inorganic phosphorus, and, as blood calcium and phosphorus are usually studied together, this is a convenient adaptation. It is, therefore, a desirable method for clinical investigation.

The important requirements of this method are pure reagents and the ability to determine inorganic phosphorus accurately. All reagents and apparatus must be calcium-free previous to the precipitation of the calcium phosphate, and, after this, they must be phosphate-free. Most grades of filter paper, even acid-washed, contain calcium, and will require considerable washing before they can be used in preparing the trichloroacetic acid serum filtrate. It is obviously very important to separate the precipitated proteins either by centrifuging, or by using a filter paper of a grade whose washings give negative tests for calcium (Whatman's No. 42). Hydroquinone and sodium molybdate of the highest purity should be used. Many C.P. sodium hydroxide preparations on the market contain considerable calcium. No alkali should be used unless it does not give a precipitate of calcium phosphate when brought to a hydrogen ion concentration slightly alkaline to phenolphthalein (approximately pH 8.5) and soluble phosphate is added in excess. It is important to note that the solubility of calcium phosphate increases with alkalinity, a sharp rise in the solubility curve occurring around pH 13 to 14. A negative result when soluble phosphate is added to a strong solution of alkali therefore does not necessarily indicate that the solution is free from calcium.
SUMMARY.

1. A colorimetric method for estimating blood calcium has been developed which is based upon the precipitation of calcium as phosphate, and the determination of the latter by the molybdic oxide colorimetric procedure.

2. The method is very accurate and is a successful micro procedure, being adaptable to much smaller quantities of serum than other methods in use at the present time.

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