A Rapid Method for the Determination of Ultramicro Quantities of Calcium and Magnesium.* (18911)

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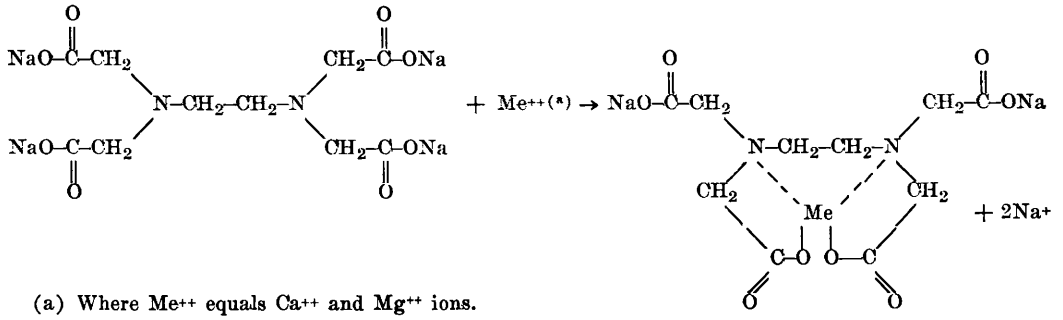
This paper presents a rapid method for the estimation of calcium and magnesium on small volumes of serum (20 to 50 μl) representing 0.05 to 0.175 micromole. Whereas by means of the flame photometer rapid estimation of serum sodium and potassium is possible (and often plays an important part in saving the lives of patients), the hitherto available methods for serum calcium require at least 3 to 4 hours and sometimes longer. This period of time is too long when it becomes necessary to decide whether low serum calcium is a factor in the cause of convulsions. Management of such patients thus usually begins without the benefit of objective evidence.

Since this problem is often encountered in newborns and prematures, a rapid method which requires only small amounts of serum is of greater usefulness. This is especially so when multiple analyses such as pH, CO_2, sugar, K, Na, and Cl are involved.

The present method in principle is an ultramicro modification of the method of Schwarzenbach and Ackerman(1) proposed for the determination of water hardness. Alkaline earth metals (calcium and magnesium) form tightly bound chelate complexes with ethylene-diamine-tetraacetate as shown below:



(a) Where Me^{++} equals Ca^{++} and Mg^{++} ions.

The chelation occurs quantitatively at a pH of 10.0 to 10.5. The dye, eriochrome black T, changes from a wine-red in the presence of free alkaline earth metallic ions to a blue-green in their absence. The proposed buffer of ammonium chloride-ammonium hydroxide(1) was replaced by the more stable ethanolamine which proved to be not only a stable buffer, but under the conditions employed provides essentially the same pH when added to serum as with the pure inorganic solutions of calcium and magnesium. Fortunately the titration can be carried out in serum itself, the color of the end point being blue-green instead of the blue of pure inorganic solutions. This blue-green is due to the coloration of the serum, which is normally yellow and which when combined with the blue of the end-point gives a blue-green. To aid in obtaining the end-point, a match color titration is employed. To a sample of serum, an excess of disodium ethylene-diamine-tetraacetate (SED) is added containing the same proportions of buffer and eriochrome black T as is employed in the method. The titration represents the sum of serum calcium and magnesium. For rapid and approximate calcium values, the titrimetric equivalent of the mean magnesium value found in human serum is subtracted from the total titration, and as seen in Table I one obtains results that will guide the clinician in his treatment. The combined value, how-

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1. Schwartzenbach, G. and Ackerman, H., *Helv. Chim. Acta*, 1947, v30, 1798.

TABLE I. Approximate and Rapid Estimation of Serum Ca (.05 ml serum). All values are expressed as milliequivalents per liter of serum.

Patient	Col. 1 Total Ca + Mg	Col. 2 Approximate Ca	Col. 3 Actual Ca	Col. 4 Difference
1	6.95	5.31	4.63	+.68
2	6.46	4.82	4.83	—.01
3	6.44	4.80	4.77	+.03
4	6.20	4.56	4.63	—.07
5	5.96	4.32	4.58	—.26
6	5.95	4.31	4.66	—.35
7	5.94	4.30	4.19	+.11
8	5.85	4.21	4.38	—.17
9	5.82	4.18	3.99	+.19
10*	5.63	3.99	3.94	+.05
11*	4.80	3.16	3.04	+.12
12*	4.68	3.04	2.98	+.06

* Sera No. 10, 11, 12 were emergency determinations in which the patients were in convulsions. The rapid estimation was followed promptly by calcium salt administration to alleviate the convulsions.

Col. 2—Obtained by subtraction of 1.64 mE/L of magnesium as the mean magnesium content in human sera from total mE/L (Ca + Mg) in Col. 1. Col. 3—Determined by the method of Sobel *et al.*(6,7). Col. 4—Difference between Col. 2 and Col. 3.

TABLE II. Exact Estimation of Serum Calcium and Magnesium. All values expressed as milliequivalents per liter of serum.

	Analytical data			Calculated data			
Patient	Col. 1 New method Ca + Mg	Col. 2 Ca	Col. 3 Mg	Col. 4 Ca	Col. 5 Difference	Col. 6 Mg	Col. 7 Difference
1	6.50	4.78	1.69	4.81	+.03	1.72	+.03
2	6.44	4.77	1.65	4.79	+.02	1.67	+.02
3	6.35	4.57	1.89	4.46	—.11	1.78	—.11
4	6.04	4.48	1.65	4.39	—.09	1.56	—.09
5	5.96	4.77	1.32	4.64	—.13	1.19	—.13
6	5.91	4.57	1.36	4.55	—.02	1.34	—.02
7	5.78	4.57	1.36	4.42	—.15	1.21	—.15
8	5.78	4.08	1.77	4.01	—.07	1.70	—.07
9	5.64	4.24	1.40	4.24	.00	1.40	.00
10	5.54	3.84	1.61	3.93	+.09	1.70	+.09

Col. 2—Determined by method of Sobel *et al.*(6,7). Col. 3—Determined by method of Orange and Rhine(5). Col. 4—Obtained by subtraction of Col. 3 from Col. 1. Col. 5—Difference between Col. 2 and Col. 4. Col. 6—Obtained by subtraction of Col. 2 from Col. 1. Col. 7—Difference between Col. 3 and Col. 6.

ever, may turn out to be more significant in convulsions than the evaluation of serum calcium alone, since convulsions have been observed due to low serum magnesium alone(2-4).

For precise calcium values, magnesium can be determined by the method of Orange and Rhine(5) which in itself requires only half an hour to complete, and as seen in Table II, the calcium values thus obtained compare favorably with those obtained by calcium oxalate precipitation(6,7). For precise magnesium values, the titrimetric equivalent of calcium is determined in the usual way(6,7) on 50 to 100 microliters of serum and the value subtracted from the total titration, and as seen in Table II, magnesium values compare favorably with those obtained by the method of Orange and Rhine(5).

2. Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, v96, 519.

3. Mille, J. F., *Am. J. Dis. Child.*, 1944, v67, 117.

4. Metzge, H. J., *Cornell Veterinarian*, 1936, v26, 353.

5. Orange, M., and Rhine, H. G., *J. Biol. Chem.*, 1951, v189, 379.

6. Sobel, A. E., and Sobel, B. A., *J. Biol. Chem.*, 1939, v129, 721.

7. Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, 1944, v152, 255.

Present experiments in progress indicate that it will be possible by using murexide as an indicator to develop a direct titration for calcium and by using oxalate or fluoride to tie up the calcium and to titrate magnesium directly. Moreover, the precipitated calcium oxalate can be titrated by SED. Serum carbonate (CO_2) can be determined by precipitating as barium carbonate and titrating the barium with SED. Similarly a rapid titrimetric method for sulfates is possible involving precipitation of the sulfate as the barium salt and titration of the barium with SED. Thus it appears that SED titration may become a valuable adjunct to clinical chemistry especially when ultramicro quantities are involved.

Experimental. Reagents and Apparatus—

1. 0.17M Ethanolamine (Approximate) 10.2 ml of ethanolamine is diluted to 1000 ml with doubly distilled water (Eastman-Kodak grade).

2. Disodium ethylene-diamine-tetraacetate (SED)—Dissolve approximately 4.5 g of the salt in 1000 ml of doubly distilled water (Fisher analytical grade). This solution is standardized against a known calcium solution and is stable for at least one month.

3. Stock Calcium Solution—1 ml is equivalent to 0.200mM Ca^{++}. Dissolve 2.0018 g dried $CaCO_3$ in a minimal amount of 6N HCl. Evaporate to dryness on a steam bath and continue to heat until no more fumes of hydrochloric acid are evolved. Dissolve the remaining solid in 100 ml of doubly distilled water.

4. Standard Calcium Solution—1 ml is equivalent to 2.00 micromoles or 4.00 meq/L Ca^{++}. Dilute 1 ml of stock calcium solution to 100 ml with doubly distilled water.

5. Stock Magnesium Solution—1 ml is equivalent to 0.200 mM Mg^{++}. Dissolve 4.9087 g of dried $MgNH_4PO_4 \cdot 6H_2O$ in 100 ml of 0.1 N HCl.

6. Standard Magnesium Solution—1 ml is equivalent to 1.00 micromoles or 2.00 meq/L of Mg^{++}. Dilute 1 ml of stock magnesium solution to 200 ml with doubly distilled water.

7. Eriochrome black T indicator solution—Dissolve 2 g of the solid dye in 500 ml of methyl alcohol and add 20 ml of concentrated ammonium hydroxide.

8. Mixed buffer indicator solution—This solution must be prepared freshly before use. It is stable for approximately 3 hours. To every 5 ml of ethanolamine buffer add 2 drops of the indicator solution. The color of the resulting solution should be blue. If there is any trace of red or purple add trace amounts of SED until color of the solution is blue.

9. Microburette—The Gilmont ultramicro burette was used in this study. The total capacity of this burette is 100 microliters. The total volume can be delivered in steps of 1/1000 of the total so that each scale is 0.1 microliter and 0.01 microliter may be estimated with a fair degree of accuracy. The stirring device of the manufacturer was discarded and in its place a Spinal (Quincke) B-D Yale Luer-Lok, 20 x 3½ inch hypodermic needle was used. The needle point was ground flat and the Luer-Lok end was ground so as to form a round cylinder instead of the normal 4-sided appearance. This modification allows the air stream that is used for agitation during titration to be introduced to the very bottom of the titrating tube.

10. Ultramicro pipettes—Kirk(8,9) type ultramicro pipettes were used in this study. These pipettes are calibrated to contain, and in use the contents are washed into the titration medium and the pipettes are washed by drawing the medium up into and blowing out the pipette. All the washings are added to the titration medium.

11. Small test tubes—75 x 10 mm. These are standard agglutination tubes.

Estimation of total serum calcium and magnesium. With a 20 or 50 microliter pipette transfer a sample of serum to a test tube containing 0.5 ml of the mixed buffer plus indicator solution. The pipette is rinsed by alternately sucking and blowing the contents of the test tube 3 times. Owing to the 10 to 20 fold dilution of the serum with the buffer-indicator mixture, the loss due to the film re-

8. Kirk, P. L., and Craig, R., *J. Lab. and Clin. Med.*, 1933, v18, 81.

9. Kirk, P. L., *Quantitative Ultramicro Analysis*, John Wiley and Sons, Inc., N. Y., 1950, p18.

maining in the pipette is negligible. Titrate the solution with SED contained in the microburette to the blue-green endpoint of a sample of serum plus buffer-indicator mixture that has had an excess of SED added to it. This titration represents the sum of calcium and magnesium. For a normal serum the titration will be approximately 14.0 microliters (which represents 140 full scales on burette) for 50 microliters of serum and 5.6 microliters for 20 microliters of serum.

At the same time, the SED is standardized by transferring 20 or 50 microliters of the standard calcium solution to a test tube containing 0.5 ml of buffer-indicator solution and the solution is titrated with SED to the blue endpoint of the pure buffer-indicator solution. The value obtained is the standardization of the SED. The normality of the SED is then calculated in the usual fashion. For the sake of convenience, millinormality is employed (mE/L) which is obtained by multiplying normality by 1000.

For equal volumes of standards and sera, the total mE/L of calcium plus magnesium in the sera are obtained as follows:

$$\text{mE/L (Ca + Mg)} = \frac{\text{Titration of serum}}{\text{Titration of Ca standard}} \times 4$$

(Table I, Col. 1)

To obtain the approximate calcium content of the serum, a mean value for magnesium of 1.64 mE/L (2.0 mg %) is assumed:

mE/L Ca = mE/L (Ca + Mg)—1.64
(See Table I: Col. 3 = Col. 1—1.64)

Exact determination of serum calcium. Magnesium is determined on a 0.1 ml aliquot of serum using the method of Orange and Rhein(5). For equal volumes of standards and sera the exact calcium content is determined as follows:

$$\text{mE/L Mg} = \frac{\text{mg \% Mg} \times 10}{12.16}$$

mE/L Ca = mE/L (Ca + Mg)—mE/L Mg
(See Table II: Col. 4 = Col. 1—Col. 3

Determination of exact serum magnesium. Serum magnesium may be determined exactly by this method if an aliquot of the serum is analyzed for calcium by the method of Sobel *et al.*(6,7). For equal volumes of standards and sera, the exact magnesium value is determined as follows:

$$\text{mE/L Ca} = \frac{\text{mg \% Ca} \times 10}{20.04}$$

mE/L Mg = mE/L (Ca + Mg)—mE/L Ca
(See Table II: Col. 6 = Col. 1—Col. 2)

Interferences. Iron will interfere with the SED titration resulting in high positive errors. Hemolyzed sera therefore cannot be used with this method. Moreover, since red blood cells contain a higher percent of magnesium than serum, hemolyzed sera will have positive errors due to magnesium when titrated with SED.

Summary. A method is presented for the ultramicro estimation of serum calcium and magnesium with ethylene-diamine-tetraacetate in the presence of eriochrome black T, at a pH of 10.0 to 10.5. The serum (0.02 to 0.05 ml) is titrated directly and the titration is completed in less than one minute; thus the speed of this determination compares favorably with that of the flame photometer. For rapid and approximate calcium value, the mean magnesium concentration of human serum (1.64 mE/L) is subtracted from the total calcium plus magnesium thus determined. For precise calcium values, magnesium is determined on an aliquot of serum and the value subtracted from the calcium plus magnesium values. For precise magnesium values, calcium is determined on an aliquot of serum and the value subtracted from the calcium plus magnesium values.

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