Titrimetric Analysis of Calcium and Magnesium in Muscle.* (23881)

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Numerous methods of calcium and magnesium analysis for biological tissues, based on the metal chelating property of ethylenediamine tetraacetic acid (EDTA), have utilized the fact that above a pH of 12, EDTA chelates calcium more easily than magnesium. Therefore, in adding EDTA to a solution of magnesium and calcium, (plus any other cations equally well or more easily chelated by EDTA than calcium), a calcium ion indicator changes color at the precise point at which only magnesium remains unchelated, if the pH is greater than 12; subsequently, with the pH reduced to 10, a magnesium ion indicator changes color when further addition of EDTA has chelated the magnesium completely. Magnesium has been measured by EDTA titration using murexide as the calcium indicator and then completing the titration, on the same sample, with Eriochrome Black T as the magnesium indicator(1,2). However, murexide does not give a sharp visual end point. Recently, 2 new indicators, calcein(3) and 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid (Cal-red)(4), have been developed for calcium ion detection. Both give sharp end points in EDTA titrations. This paper deals with the use of these indicators, as well as Eriochrome Black T, in magnesium and calcium EDTA titration methods developed for muscle. In both methods, the cations were separated by precipitation from interfering substances in muscle ash solution. Calcium was precipitated with ammonium oxalate in acetic acid solution(5), subsequently the oxalate ion was destroyed by heating with perchloric acid(6). The calcium, in solution (pH above 12), was then titrated with EDTA, using either calcein or Cal-red as the calcium indicator. Magnesium was precipitated with 8-hydroxyquinoline (oxine) in ammonium hydroxide solution (7). The oxine was destroyed by heating with perchloric acid. EDTA was then added (pH above 12), until the precise end point for the calcium indicator (either calcein or Cal-red) was reached. The pH was then readjusted to 10, and the magnesium titrated with EDTA, using Eriochrome Black T as the magnesium indicator.

Materials. 1. *Oxine* 6% 8-hydroxyquinoline in 95% ethanol; 2. *Ammonium oxalate* saturated sol.; 3. *Methyl red indicator* saturated sol.; 4. *Calcein indicator* 2 mg% calcein (G. Frederick Smith Chemical Co., Columbus, O.) in 0.25 N KOH. Prepare weekly, keep refrigerated; 5. *Cal-red indicator* 40 mg % Cal-red sol. (Scientific Service Lab., Dallas, Texas). Prepare weekly, keep refrigerated; 6. *Eriochrome Black T indicator* 40 mg % Eriochrome Black T sol. (J. T. Baker Chemical Co., Phillipsburg, N. J.). Prepare weekly, keep refrigerated; 7. *Ammonium chloride-ammonium hydroxide buffer.* Add 35.5 g NH_4Cl to 224 ml conc. NH_4OH, dilute to 1 liter, keep refrigerated; 8. *Standard 2 mM calcium solution.* Dissolve 0.2000 g of $CaCO_3$ in min. volume of 1 N HCl, dilute to 1 liter; 9. *Standard 2mM EDTA solution.* Dissolve 0.75 gram (ethylenedinitrilo) tetraacetic acid disodium salt (Distillation Products Industries, Rochester, N. Y.) in water and dilute to 1 liter. Standardize against calcium sol. as follows: To 1 ml of standard 2 mM calcium sol., add 1 ml of 3 N KOH and 1 drop of calcein (or Cal-red) indicator; titrate with the EDTA solution until color changes from yellow-green to brown (If Cal-red is used, color changes from wine red to blue). (1 ml of 2 mM EDTA sol. is equivalent to 1 ml of 2 mM calcium sol.); 10. *Standard 2 mM magnesium solution.* Dissolve 50.83 g $MgCl_2 \cdot 6H_2O$ in water and dilute to 1 liter. The concentration of this stock sol. should be 250 mM. Dilute 8 ml of this stock sol. to 1 liter. Standardize against the EDTA sol. as follows: To 1 ml of the magnesium sol., add 1 ml ammonium chloride-ammonium hydroxide buffer and 1 drop Eriochrome Black T indicator; titrate with the standard 2 mM EDTA solu-

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tion until color changes from wine red to blue. (1 ml of 2 mM magnesium solution is equivalent to 1 ml of 2 mM EDTA solution.); 11. *Hydrochloric acid* 1N and 3N; 12. *Ammonium hydroxide* 1N and conc.; 13. *Potassium hydroxide* 3N; 14. *Acetic acid* 0.07 N; 15. *Perchloric acid* 70%; 16. Platinum crucibles (8 ml) for ashing procedure; 17. Conical centrifuge tubes (12 ml); 18. Metal shields (¾ by 4½ in.) for holding centrifuge tubes in sand bath; 19. Measuring pipettes, 1 ml, graduated to 0.01 ml, tip drawn out to deliver a 0.02 to 0.03 ml drop. The pipettes are coated with Desicote (Beckman Instruments, Fullerton, Calif.) to obtain a flat meniscus and facilitate splitting of drops. A thumbscrew pipette filler is used for filling and ejecting the titrating fluid; 20. Magnetic type stirring rods, about 1 cm long. Prepared by sealing No. 32 galvanized wire within capillary glass tubing (outside diameter 0.85 mm, Arthur H. Thomas, Philadelphia, Penn.). These rods, placed in the centrifuge tubes during the titrations, are agitated to give adequate mixing by moving a small magnet along the outside walls of the tubes; 21. Polyethylene reagent bottles.

Procedures. All volumes are approximate except those for standard solutions. *Calcium determination.* Ash muscle at 500°C (500-1000 mg wet wt) overnight in platinum crucible. Wash ash into centrifuge tube with 0.5 ml of 1 N HCl. Add 2 drops of methyl red indicator and then add dropwise 1 N NH_4OH until the color changes to yellow. Complete washing crucible into centrifuge tube with 3 ml of 0.07 N acetic acid. Add 1 ml of the ammonium oxalate solution, allow precipitate to form overnight. Centrifuge (3000 rpm, 10 minutes) and carefully remove supernatant. Dry precipitate by gentle warming and add one drop of 70% perchloric acid. Lower the tube slowly into metal shield embedded in a sand bath hot enough so that top part of tube is at least 200°C. After the white perchloric acid fumes cease to appear, remove tube from sand bath and dissolve residue in 2 drops of 3 N HCl. Add 1 ml of 3 N KOH and 1 drop of calcein (or Cal-red). Titrate with 2 mM EDTA to the appropriate change. In reading end points, the total volume should be 2 ml or more (add water if necessary). Compare with blank. Back titrations with the 2 mM calcium standard are sometimes helpful. *Magnesium determination.* Ash muscle (about 200 mg wet wt) as above. Wash into centrifuge tube with 0.5 ml of 1 N HCl and 1 ml of water. Add 0.5 ml of oxine and 0.5 ml of concentrated NH_4 OH, and allow precipitate to form overnight. Centrifuge and oxidize with perchloric acid as above. If color is not completely destroyed, repeat perchloric acid treatment. Dissolve residue in 2 drops of 3 N HCl. Add 1 ml of 3 N KOH and 1 drop of calcein (or Cal-red). Add 2 mM EDTA until the color just changes. Add water if necessary to make volume 2 ml. Compare with blank. Adjusting this end point is a critical step. It is sometimes made easier by using the 2 mM calcium solution. Readjust pH to 10 with addition of 1 ml of cold ammonium chloride-ammonium hydroxide buffer followed by 1 ml of 3 N HCl. Add 1 drop of Eriochrome Black T and titrate with 2 mM EDTA. Compare the end point with blank. For back titration, either the 2 mM calcium or magnesium standard may be used.

Results. Tables I and II show calcium and magnesium recoveries from solutions of known composition. With calcium:magnesium ratios of 1 to 10 and 10 to 1, there appeared to be no significant reciprocal interference in either determination. To test the possible interference of other muscle constituents, a pool of several muscles was ashed and dissolved in 1 N HCl. The calcium and magnesium concentrations of this muscle ash solution were determined. Known amounts of calcium and magnesium were also added to the muscle ash solution and were recovered as indicated in Tables I and II.

It is important in completely removing excess perchloric acid that the temperature be at least 200°C, since the constant boiling acid (71.6%) boils at 203°C. If some perchloric acid remains in the sample then insoluble potassium perchlorate will be formed upon KOH addition. However, even in the presence of potassium perchlorate, fairly sharp end points can be obtained.

Cyanide has been recommended to remove interfering cations for EDTA titrations using

Eriochrome Black T(1,2), calcein(3), and Cal-red(4). However, the addition of 1 ml of 0.5 N KCN to some muscle ash solutions just prior to the addition of the calcein (or Cal-red) indicator did not change the calcium and magnesium values.

Duplicate calcium and magnesium determinations were made on individual skeletal muscles from 13 frogs. The calcium was found to be 1.68 ± 0.07 (mean ± standard error of mean) millimoles/kg wet weight and the magnesium, 8.84 ± 0.31 (mean ± standard error of mean) millimoles/kg wet weight. These values yield a magnesium:calcium ratio of 5.26. The correlation coefficient was 0.79 with a probability value of 0.1%. This fairly high coefficient suggests some relationship between muscle calcium and magnesium. These findings are in reasonable agreement with previously reported values for frog muscle calcium(5,8) and magnesium(8).

In conclusion, these chemical methods offer the following advantages: the calcium does not have to be removed from the sample before determining magnesium; the procedures for calcium and magnesium are sufficiently alike so that both cation determinations can be performed concurrently with relative ease; and the actual titrations are relatively easy to perform, and do not require the use of a colorimeter.

TABLE I. Calcium Determinations.

No. of determinations	μmoles in sample Ca	μmoles in sample Mg	Avg μmoles Ca recovered	Stand. dev. of sample*
8	1	10	.96†	.05
8	10	1	9.88†	.14
3	0	2	.00	.00
10	1	1	1.02	.02
3	1	10	1.00	.02
5	2	0	1.99	.00
4	10	1	9.94	.07
4‡	1.3	9.59	1.32	.02
4‡	2.3	8.59	2.22	.10
4‡	3.3	7.59	3.30	.03

* Stand. dev. $= \sqrt{\frac{\Sigma (x-\bar{x})^2}{N}}$, where x is the individual value, $\bar{x}$ is the mean and N is the No. of values.

† These samples were titrated without precipitating the cation.

‡ These recovery experiments were carried out on 0.5 ml aliquots of a muscle ash solution to which known amounts of calcium and/or magnesium had been added. [Estimation of original calcium (1.30 μmoles) and magnesium (7.59 μmoles) of each 0.5 ml aliquot was calculated from the following observed concentrations: 2.60 ± 0.01 mM calcium (mean ± stand. error of mean) in 18 determinations, and 15.18 ± 0.09 mM magnesium (mean ± stand. error of mean) in 16 determinations.] The values given in the Table for calcium and magnesium content of these samples represent the sums of the amount originally estimated to be present plus the added amount, if any.

TABLE II. Magnesium Determinations.

No. of determinations	μmoles in sample Mg	μmoles in sample Ca	Avg μmoles Mg recovered	Stand. dev. of sample*
8	1	10	1.00†	.03
8	10	1	9.94†	.07
2	0	2	.00	.00
11	1	1	1.00	.01
2	1	10	.98	.02
6	2	0	1.98	.01
5	10	1	9.93	.10
4‡	7.59	3.3	7.60	.16
5‡	8.59	2.3	8.62	.24
5‡	9.59	1.3	9.66	.20

*, †, ‡ Same as Table I.

Summary. Relatively simple EDTA titration methods for the determination of muscle calcium and magnesium are given. Calcium is precipitated with ammonium oxalate; the oxalate is destroyed by heating with perchloric acid; and then the calcium is titrated with EDTA using either calcein or Cal-red as the indicator. Magnesium is precipitated with oxine; the oxine is destroyed by heating with perchloric acid; then sufficient EDTA is added in the presence of either calcein or Cal-red until the color of the indicator changes, after which the magnesium is titrated with additional EDTA using Eriochrome Black T as the indicator.

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