Estimation of Calcium and Magnesium in Blood Serum by the Cathode-Ray Polarograph

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In our hands precipitation (Clark & Collip, 1925) and visual complexometric (Buckley, Gibson & Bortolotti, 1951) methods of determining calcium and magnesium in blood serum have proved difficult for routine diagnostic purposes. The ammonium oxalate-ammonium phosphate method tended to give difficulty with the precipitates. The end point of the complexometric method varied with operators and even from day to day with any single operator.

Pribil & Roubal (1954) reported that polarographic estimation of calcium in serum and urine was possible with a recording polarograph. This would be of value in routine estimations as it would replace much of the human element with an electronic device. Their method is based on the polarographic determination of Zn^{2+} ions quantitatively displaced from an ammoniacal zinc-ethylenediaminetetra-acetic acid complex by Ca^{2+} ions present in biological material. The authors stated that although a similar quantitative displacement of Zn^{2+} ions by Mg^{2+} ions does not occur, there is some displacement and consequently the presence of magnesium affects the result of the calcium determination. They therefore added saturated ammonium phosphate solution to precipitate magnesium before estimating the calcium.

We studied their method but have not been able to confirm its value for the determination of calcium in blood sera. We did not, however, use the same type of polarograph. We used one in which the pen-recording device is replaced with a cathode-ray tube (K 1000, Southern Instruments, Crawley, England). This gives much greater sensitivity and records the rate of change of current and not the actual diffusion current. This rate of change is greatest at the half-wave potential, a constant for any given element in any particular electrolyte. Maximum suppressors are rarely necessary and, apart from serum itself, were not used in this work. One advantage of such instruments is that the entire polarogram is reproduced every 7 sec., as the whole record is made during the life of a single mercury drop. Furthermore, since the instrument is very sensitive it is possible to restrict the amount of material for analysis.

The differences between the two types of instruments should not have affected the results, but

we found that under our conditions magnesium in serum was not precipitated quantitatively by ammonium phosphate. If the magnesium was less than 3 m-equiv./l., then little or no magnesium was precipitated by the ammonium phosphate. When, however, this amount was exceeded, partial precipitation occurred. It was with such high concentrations of magnesium that Pribil & Roubal were working. Their method will, however, yield approximately correct results for calcium in normal sera since a depressive effect in the peak current due to the presence of serum is offset by the additive effect of the magnesium. This would not be true for serum in cases of hypocalcaemia.

We were able, however, to elaborate a method which we find simple, rapid and accurate.

MATERIALS

Glass-distilled water was used in the preparation of all solutions.

Ammoniacal zinc-ethylenediaminetetra-acetic acid. This consisted of 6.6 mm-disodium ethylenediaminetetra-acetate (EDTA) and 6.6 mm-zinc sulphate dissolved in aqueous 8N-ammonia solution. This was the concentration used by Pribil & Roubal (1954) in their work. As they state, a zinc wave is obtained even in the blank and must be subtracted from subsequent readings. It varies between 2.0 and 2.5 μA according to the solution used, as very slight inaccuracies in the amount of either zinc sulphate or EDTA will affect the reading.

Stock 0.01M-calcium chloride. Calcium carbonate (A.R. grade) was dried at 110° and dissolved in the minimum amount of hydrochloric acid. Carbon dioxide was removed by boiling and the solution diluted to the required volume.

Stock 0.01M-magnesium chloride. This was prepared from magnesium oxide ignited and cooled in a desiccator and dissolved in the minimum amount of hydrochloric acid and diluted to the required volume.

Blank serum. Since proteins of the type present in serum tend to decrease the diffusion current, it is necessary to include serum in the standard solutions. Ox and sheep sera were therefore passed through an ion-exchange column (25 cm. $\times$ 1 cm.³) consisting of the sodium form of Amberlite resin IR-120 in order to remove all calcium and magnesium.

RESULTS

In the preparation of their standard solutions, Pribil & Roubal (1954) added measured amounts of calcium chloride to the ammoniacal zinc-EDTA reagent and included saturated ammonium phos-

phate. We found that the addition of ammonium phosphate lowered the peak-current reading. A similar effect was given by serum. This depressive effect is shown in Fig. 1. The same effect is shown when zinc sulphate is added to the ammoniacal zinc-EDTA reagent in place of calcium.

We therefore prepared a series of standard solutions of calcium and magnesium with treated serum added. These solutions are detailed in Table 1, which also gives the peak-current readings. These show that calcium and magnesium react as a single substance present to the amount of the combined total. The curve constructed from the figures in the second and third columns is therefore a standard curve for total calcium and magnesium in the test solution.

A similar standard curve was prepared for magnesium alone. To do this, measured amounts of magnesium chloride were added to a solution containing treated serum and calcium. A constant volume of ammonium oxalate was added to precipitate the calcium and to produce conditions similar to those in the unknown sera to be tested. The details are given in Table 2, and show that again the peak-current readings bear a linear relationship to the amount of magnesium added.

In order to determine whether or not these peak-current readings represent a stoichiometric displacement of Zn^{2+} ions from the ammoniacal zinc-EDTA reagent, they were compared with the readings obtained by substituting equivalent

amounts of zinc. Fig. 2 shows that, in solutions without serum, equivalent amounts of zinc and of calcium and magnesium produce the same peak current. However, when treated serum is included in the solutions the results for Ca^{2+} and Mg^{2+} ions are not identical with those for Zn^{2+} ions.

Application of the results to the assay of blood sera

To 5 ml. of ammoniacal zinc-EDTA reagent in a stoppered cylinder, 1 ml. of serum and 4 ml. of water were added and the mixture was well shaken.

Table 1. *Standard solutions of calcium and magnesium*

Solutions containing various amounts of 1 mM-calcium chloride and either 0.5 mM- or 1 mM-magnesium chloride were added to 12.5 ml. of ammoniacal zinc-EDTA reagent and 2.5 ml. of treated serum to give the ratios in the first column. The total volume was adjusted to 25 ml. by adding water. Amounts shown in the second column give the concentrations in m-equiv./l. of calcium and magnesium in the solutions assayed. A drop of octanol was added to the solution in the polarograph cell to prevent frothing.

Ratio Ca : Mg	Total Ca + Mg	Peak current (μA)
2 : 1	0.12	1.30
1 : 1	0.16	1.70
2 : 3	0.20	2.10
4 : 1	0.20	2.10
2 : 1	0.24	2.50
4 : 3	0.28	2.95
1 : 1	0.32	3.40
2 : 3	0.40	4.15
4 : 1	0.40	4.15
2 : 1	0.48	5.35
4 : 3	0.56	6.00
6 : 1	0.56	6.00
3 : 1	0.64	7.00
2 : 1	0.72	7.75
8 : 1	0.72	7.75
4 : 1	0.80	8.75
8 : 3	0.88	9.50

Table 2. *Standard solutions of magnesium*

Ammonium oxalate solution (4%; 0.5 ml.) and 0.25 ml. of 1 mM-calcium chloride solution were added to the volumes given in columns 1, 2 and 3 to give a final volume of 2.5 ml. in each case. These solutions were prepared in centrifuge tubes and allowed to stand for at least 4 hr. before centrifuging at 3000 rev./min. The supernatants were poured into cylinders containing 5 ml. of ammoniacal zinc-EDTA reagent and 2.5 ml. of water. After shaking, a portion was placed in the cells, octanol was added, and the peak current determined. Figures in column 4 give the concentration of magnesium in the solution assayed.

Vol. of treated serum (ml.)	Vol. of water (ml.)	Vol. of MgCl_2 (ml.)	Mg (m-equiv./ l.)	Peak current (μA)
1.0	0.25	0.50 (0.5 mM)	0.05	0.46
1.0	0.50	0.25 (2 mM)	0.10	0.96
1.0	0.25	0.50 (2 mM)	0.20	1.96
1.0	0	0.75 (2 mM)	0.30	2.76
0.75	0	1.00 (2 mM)	0.40	3.81

Fig. 1. Depression of peak-current readings by serum and ammonium phosphate. ●, 1 mM-Calcium chloride added to 12.5 ml. of ammoniacal zinc-EDTA reagent and diluted to 25 ml. with water; ○, 1 ml. of saturated ammonium phosphate added; ▲, 2.5 ml. of serum (from which calcium and magnesium had been removed) added; △, 1 ml. of saturated ammonium phosphate and 2.5 ml. of treated serum added. The value of the current produced by the supporting electrolyte in each case has been subtracted.

A drop of octanol was added and the peak current determined. From this was subtracted the reading of a blank solution (5 ml. of zinc-EDTA reagent, 1 ml. of treated serum and 4 ml. of water) and the total calcium and magnesium in the serum was then obtained from the standard curve.

To obtain the amount of magnesium, 1 ml. of serum was added to 1 ml. of water in a centrifuge tube, followed by 0.5 ml. of 4% ammonium oxalate. The mixture was allowed to stand for at least 4 hr. and, after centrifuging for 10 min. at 3000 rev./min., the supernatant was poured into 5 ml. of ammoniacal zinc-EDTA reagent with 2.5 ml. of

water and a drop of octanol. The solution was shaken and the peak current determined. After subtraction of the value of the current produced by the blank (1 ml. of treated serum and 2.5 μ moles of calcium chloride substituted for the unknown serum) the concentration of magnesium in the serum was obtained from the standard graph. The amount of calcium can then readily be obtained by difference.

The accuracy of the method was checked by the estimation of 20 replicates of one ox and of one sheep serum. Each of these replicates was prepared from a single serum, starting afresh for each. At the same time a chemical estimation was made by the method of Clark & Collip (1925). The figures given in Table 3 compare the reproducibility of the two methods.

In addition the method was applied to the routine estimation of calcium and magnesium in sera. Fifty individual samples, both of ox and sheep sera, were estimated in duplicate by polarographic and chemical methods. Table 4 gives the results obtained.

DISCUSSION

Tables 3 and 4 show that the accuracy and reproducibility of the polarographic method compare favourably with those of the precipitation method. The main difficulty with this latter method is a compromise between inadequate washing of the precipitate and possible loss of the precipitate during the removal of excess of oxalate and phosphate. This does not occur in the polarographic method and the estimations of calcium in the

Table 3. *Duplicate polarographic and chemical analyses of 20 replicates each of one ox and one sheep serum*

Results are given in m-equiv./l. and refer to means $\pm$ s.d.

Serum	Method of estimation	Mg	Ca
Ox	Polarographic	1.6 $\pm$ 0.06	4.7 $\pm$ 0.11
	Chemical	1.6 $\pm$ 0.13	4.6 $\pm$ 0.09
Sheep	Polarographic	2.0 $\pm$ 0.12	5.1 $\pm$ 0.17
	Chemical	2.0 $\pm$ 0.06	5.2 $\pm$ 0.12

Table 4. *Comparison of polarographic and chemical analyses of 50 individual samples of ox and of sheep sera*

The mean differences were obtained by subtracting the results of the chemical analyses from those obtained by the polarographic method, with subsequent division of the sum of the differences by the total number of sera. The standard deviations of these means were calculated and probabilities P obtained from a table for distribution of t .

		Range (m-equiv./l.)	Mean difference $\bar{x}$	S.D. of mean $S_{\bar{x}}$	t ($\bar{x}/S_{\bar{x}}$)	P	
Ox	Mg	1.6-3.3	0.01	0.033	0.423	0.6	$< P < 0.7$
	Ca	2.75-6.65	0.07	0.039	1.794	0.05	$< P < 0.1$
Sheep	Mg	1.4-3.4	0.05	0.030	1.666	0.1	$< P < 0.2$
	Ca	2.95-8.6	0.03	0.036	0.833	0.4	$< P < 0.5$

Fig. 2. Effect of serum on the relationship between Zn^{2+} ions added and Zn^{2+} ions displaced by added Ca^{2+} and Mg^{2+} ions. ●, Various amounts of 1 mM-zinc sulphate were added to 12.5 ml. of ammoniacal zinc-EDTA reagent and the volume was adjusted to 25 ml. with water; ○, equivalent amounts of calcium chloride and magnesium chloride (ratio varied) in place of zinc sulphate; ▲, zinc sulphate together with 2.5 ml. of treated serum; △, calcium and magnesium chloride together with 2.5 ml. of treated serum.

different samples of sheep and ox sera tended to be slightly higher than those obtained from the permanganate titration.

The concentration of calcium and magnesium varied little in the majority of samples tested, therefore the differences shown by the two methods are unlikely to depend on the relative concentrations of calcium and magnesium. In the greater proportion of samples these differences, whether positive or negative, were within 5% of the total amounts of calcium or magnesium. A few, however, showed differences up to a maximum of 0.85 m-equiv./l. for calcium and 0.6 m-equiv./l. for magnesium. Table 4 shows that the difference between the two methods is not statistically significant.

The displacement of zinc from its complex with EDTA by calcium is possible in the presence of the high concentrations of ammonia which were used (Schwarzenbach, 1957). This was demonstrated experimentally by Pribil & Roubal (1954). The relation between added calcium and magnesium and zinc displaced is stoichiometric, since the same peak current is obtained whether equivalent amounts of calcium and magnesium or zinc are added (Fig. 2). Since we obtained a linear relationship between calcium plus magnesium added and the peak-current readings (Table 1), whatever the proportions of calcium and magnesium, it is apparent that at the concentrations we used, both calcium and magnesium displace zinc from the complex to an equal extent. In the preparation of the standard magnesium graph, however, the figures obtained for magnesium alone are slightly lower than those for equivalent amounts of calcium plus magnesium. This is due to incomplete decantation of the comparatively viscous supernatant, since similar graphs prepared without serum were identical.

Certain buffers, including phosphate, hinder the displacement of Zn^{2+} ions from ammoniacal zinc-EDTA by calcium or magnesium. Thus ammonium phosphate would be expected to depress the peak current of zinc displaced by calcium from zinc-EDTA. This is so, but when zinc itself is added to the solutions instead of calcium, an equal depression occurs at all concentrations investigated. Thus the depression of the current cannot be due to any effect of PO_4^{3-} ions on the dissociation of zinc-EDTA by calcium since, if it were, the curve for added zinc itself would not be equally depressed. Nor is it due to the formation of complex zinc phosphate ions, since the half-wave potential is the same whether ammonium phosphate is present or not. A new equilibrium must be set up with some slight precipitation of zinc phosphate. Despite the depressive effect of added ammonium phosphate, however, the relationship between zinc and cal-

cium remains stoichiometric, confirming this observation of Pribil & Roubal.

When serum is present, however, the relation between calcium plus magnesium added and zinc displaced ceases to be stoichiometric: the curves diverge as the concentration increases (Fig. 2). In addition, certain complex zinc ions are formed, since the half-wave potential is raised from -0.126 to -0.12 .

In view of the complexity of serum, similar solutions to those in Table 1 and Fig. 2 were prepared with 5% gelatin (passed through an ion-exchange column) instead of serum. Unlike serum, gelatin depressed the peak current by a constant amount, regardless of the concentration of zinc sulphate added, and had no effect on the half-wave potential of the solutions. Nevertheless, the presence of gelatin did affect the displacement of zinc by calcium and magnesium. Hence the effect of serum is, first, the protein effect of lowering the peak current for any given concentration of zinc and the effect of partial inhibition of the displacement of zinc by calcium and magnesium; secondly, a more specific effect of serum is to cause greater inhibition of the peak current in zinc solutions as the concentration rises.

The importance of incorporating serum into the standard solution is therefore obvious.

SUMMARY

1. A polarographic method for the estimation of calcium and magnesium in sera is described in which the total calcium and magnesium is determined and then the magnesium alone. The calcium concentration is found by difference. This method is as accurate as, and faster than, available chemical procedures.

2. The method was checked by the determination of 20 replicates each of one sheep and one ox serum. It was then applied to the analysis of 100 individual sera (50 ox and 50 sheep). None of the results differed significantly from those obtained by chemical methods, though the polarographic estimations of calcium tended to be slightly higher.

3. The presence of serum in the supporting electrolyte affects the stoichiometry of the reaction. This effect is more complex than that of the simple protein gelatin.

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