SIMPLIFIED ESTIMATION OF CALCIUM IN BIOLOGICAL MATERIALS BY FLAME PHOTOMETRY

BY

J. G. LLAURADO

From the Postgraduate Medical School, London

(RECEIVED FOR PUBLICATION SEPTEMBER 3, 1953)

Flame photometry has introduced rapidity, accuracy, and simplicity into the estimation of sodium and potassium in biological materials. When it is desired to prepare for the estimation by flame photometry of Ca^{++} in serum containing Na^+ and K^+ merely by diluting with distilled water, it must be kept in mind that for every 10 mg./100 ml. of Ca^{++} there is 325 mg./100 ml. of Na^+ . Furthermore, the sodium spectrum lines are very near to, and more intense than, those of Ca^{++} so that Ca^{++} values obtained in this way are greater than actual values.

Following the wide use of flame photometry since 1945 (Barnes, Richardson, Berry, and Hood, 1945) many attempts have been made to estimate calcium; unsuccessful data remain unpublished, and hitherto reported methods are inaccurate or tedious.

In one of the earliest reports in this field Belke and Dierkesmann (1948) attempted the estimation using a flame photometer equipped with acetylene gas and a green filter reputedly not absorbing completely Na^+ and K^+ lights. Attempts were made to allow for Na^+ and K^+ interference by the addition of comparable amounts of these elements to the standard Ca^{++} solutions. The accuracy of this procedure is impaired by relative variability of Na^+ and K^+ levels in biological fluids and also by the fact that Ca^{++} *per se* is not measured.

Herrmann (1952) has described a method for the estimation of calcium, in which the main features are the recording of the green line in the Ca^{++} spectrum at 558 $\text{m}\mu$, the use of acetylene gas for combustion, and a simple serum dilution of 1 in 5. Neither normal values for Ca^{++} in serum nor objective data are reported, but this method has been used by Bohnstedt, Herrmann, Baumann, and Füller (1953) to estimate the $\text{K}^+/\text{Ca}^{++}$ serum ratio in normal subjects and eczematous patients. Isolated values for K^+ and Ca^{++} are not given in this paper, and a remark is made that "bei unserer Methode die K/Ca -Quotienten stets relativ niedrige Werte ergaben." The value

for Ca^{++} can, however, be determined theoretically from their data: for normal people the ratio is given as 1.54, and, if the accepted normal K^+ serum value of 20 mg./100 is considered, the "normal" for Ca^{++} serum by this method is 13 mg./100. This value is high and agrees with values found by us when Ca^{++} was estimated merely by dilution of serum.

It has been realized for some time that this problem could be solved by precipitating Ca^{++} , thus removing interfering cations and carrying out the estimations on the redissolved and suitably diluted Ca^{++} sample.

The manufacturers of the Beckman spectrophotometer with flame attachment recognize (Application Data, DU-9-B) the necessity of precipitating Ca^{++} in Na^+ and K^+ mixtures in spite of all the advantages of this apparatus. Precipitated calcium oxalate is washed, centrifuged, and taken up in perchloric acid and isopropylalcohol. Explanations for the use of these two substances are not given. Acetylene gas is also used in this method.

While our investigations were being carried out Schlütz (1953) described a method based on the same principle of precipitation as calcium oxalate. No advantage is really gained with this method over the usual chemical methods, since three centrifugations and two washings are required. Furthermore, no details of the filter used are given and no objective data showing sensitivity and accuracy are reported. Acetylene gas was also used in this case.

Since the start of these investigations, the aim has been to establish a method to estimate the true amount of Ca^{++} in biological materials under the following conditions: (1) without Na^+ and K^+ interference; (2) saving time on the ordinary chemical methods, especially the two or more washings, re-centrifugations, and tedious titration; (3) adapted to a simple flame photometer, using coal-gas and compressed air instead of acetylene gas and additional oxygen.

An Evans "electroselenium" Ltd. flame photometer supplied with coal-gas and compressed air from a cylinder was used. Air pressure was adjusted at 10 lb./sq. in. (0.7 kg./cm.²). The gas was regulated to produce 10 small cone-shaped flames at the top of the burner. Working with this empirical procedure, flame conditions usually change each time the burner is lit. This is obviated by interpolating with the unknown samples immediately higher and lower standards. Nevertheless, an absolute relationship, as shown below, between deviations in galvanometer scale-readings and concentrations of Ca^{++} exists. An interference filter with a peak at 620 m μ and additional special didymium glass to cut out interference by Na^+ was used.

With these conditions a whole deflexion of the galvanometer scale is reached with 30 p.p.m. of Ca^{++} when the potentiometer is adjusted to 1. Sometimes, however, due to variations in the supply or quality of gas, a complete deflexion is not reached with 30 p.p.m. For this reason, a 40 p.p.m. Ca^{++} solution is used as the strongest, and the potentiometer is adjusted between 1 and 2 until the whole deflexion is recorded in the scale of the galvanometer.

Preliminary Experiments

The first objective was to establish a linear relationship between deviations of the galvanometer scale-reading and the concentrations of Ca^{++} in a mixture containing no other cations: this simple relationship in a range from 0 to 40 p.p.m. is obvious from Fig. 1. Therefore, estimations of solutions containing only Ca^{++} do not represent any problem with this type of flame photometer.

The second objective was to measure the Ca^{++} content in samples of normal serum by simple dilution with distilled water. Values of about 13 mg./100 ml. were obtained by this procedure.

Finally it was necessary to determine the efficiency of the Ca^{++} filter in cutting out light due to Na^+ and K^+ . Standard samples of Na^+ and K^+ were sprayed in the flame photometer. A Na^+ content of 8 p.p.m. (0.8 mg./100 ml.) and a K^+ content of 7.5 p.p.m. (0.75 mg./100 ml.) do not produce any deviation when the filter for Ca^{++} is tested. As, on the other hand, other trials showed (see below) that the Na^+ contained as impurity or residuum in the precipitated samples of Ca^{++} does not exceed this amount, this experiment assured that below that range all light due to Na^+ and K^+ was cut off.

Preparations of Standards

Calcium chloride prepared in the following way was used: AnalaR CaCO_3 is put in the oven overnight at 40° C. to dry it. Next day 12.5 g., which represents exactly 5,000 mg. of Ca, is weighed out. This is

suspended with a small amount of distilled water in a volumetric flask and Na-free *N*-HCl is added (about 250 ml.) until it is completely dissolved. This is made

FIG. 1.—Linear relationship between galvanometer scale-readings and other cation-free Ca^{++} solutions.

up to 1.1 with distilled water. This solution is called the stock solution, and when kept in the refrigerator can be used indefinitely. From the stock solution the following standard solutions are prepared:

Solution	Stock Solution (ml.) Made up to 1,000 ml. H ₂ O	Ca ⁺⁺ Content in p.p.m.
A	2	10
B	4	20
C	6	30
D	8	40

Solutions used for recovery were either the same standard solutions or solutions prepared from stock solution.

Precipitation of Ca^{++} Samples

Samples containing 5 mg., 7.5 mg., 10 mg., 12.5 mg., and 15 mg./100 ml. of Ca^{++} were used. Two lots were made: (1) containing only Ca^{++} , and (2) Ca^{++} dissolved in a solution of NaCl, containing 305 mg./100 ml. of Na^+ , a concentration similar to that of serum.

Two millilitres of each sample was taken, precipitated with ammonium oxalate, and allowed to stand for 30 minutes. Four millilitres distilled water was then added and the solution was centrifuged. The supernatant fluid was discarded, the precipitate dried and redissolved with 10 ml. 0.2 *N*-HCl.

Samples of the solutions containing only Ca^{++} were also estimated directly, i.e., without any manipulation. As a rule, values found after precipitation were lower than those found by direct estimation. Results are shown in Table I. From this experiment

TABLE I
RESULTS OF PRECIPITATING CALCIUM SAMPLES

Theoretical Values (mg./100 ml.)	Samples Estimated Directly by Flame Photometer	Samples Precipitated			Samples Precipitated Containing 305 mg./100 ml. Na ⁺				
		Ca ⁺⁺ Content	Na ⁺ Content (mg./100 ml.) as Impurity	Ca ⁺⁺ Content of Decanted Water	Ca ⁺⁺	Na ⁺	Duplicate		Mean Ca ⁺⁺
							Ca ⁺⁺	Na ⁺	
5	4.9	4.6	0.5	0	5.6	2.25	5.1	1.2	5.3
7.5	7.5	5.8	0.5	0	7.4	2.0	7.1	1.2	7.2
10.0	10.0	9.1	0.49	0	10.0	2.6	8.8	1.2	9.4
12.5	12.5	11.06	0.62	0	10.8	1.5	10.8	1.1	10.8
15.0	15.0	12.5	0.42	0	12.7	2.4	12.3	1.1	12.5

Experiments with 2 ml. of the sample.

two points became clear: (1) The "loss" of Ca⁺⁺ after oxalate precipitation does not follow any linear relationship (Fig. 2) but is a curve. As the Ca⁺⁺ content in the decanted water of samples containing pure Ca⁺⁺ (in samples to which Na⁺ was added this experiment cannot be made because of the high Na⁺ content) was zero, the apparent loss of Ca⁺⁺ can only be due to interference. (2) Na⁺ in a concentration similar to that of human serum does not produce any additional interference when Ca⁺⁺ samples are simply precipitated without any washing.

Oxalic Acid Interference

When HCl is added to redissolve the precipitate, CaCl₂ is formed and oxalic acid is liberated. Moreover, some ammonium oxalate is still present as impurity, which is now converted to oxalic acid. As this substance remains in the solution analysed by flame photometry, we thought that oxalic acid was responsible for the interference. To prove this a simple experiment was done: 50 p.p.m. of oxalic acid was added to each standard and measured by flame photometry, these samples having been checked by oxalic acid-free standards. Results are expressed in Fig. 3 and confirmed our suspicion. The similarity of this curve with that shown in Fig. 2 is obvious. That oxalic acid produced a depressant effect on the Ca⁺⁺ flame has not hitherto been reported, in spite of others (Application Data, DU-9-B; Schlütz, 1953) having tried to estimate Ca⁺⁺ by flame photometry after similar precipitation. This effect was quite unexpected but not surprising, since Bills, McDonald, Niedermeier, and Schwartz (1949) have described a similar action of other organic compounds on Na⁺ estimations. Oxalic acid can be destroyed by several oxidizing agents, but either some of these contain K⁺ as a cation (it is obviously ridiculous to mix the precipitate with Na⁺ or K⁺, after it has been precipitated specifically to remove these cations) or else the added substance itself might produce a new interference effect. On the other hand, there is a very simple procedure to destroy the calcium oxalate molecule, i.e., to heat it at any temperature between 300° and 400° C. At this

temperature it is converted to CaCO₃. This step has another advantage: the unknown sample is prepared in the same way as the standards, i.e., HCl is added to CaCO₃ to dissolve it.

Results of Precipitating Samples and Destroying Oxalic Acid

Samples containing 2.5 mg., 5 mg., 7.5 mg., and 10 mg./100 ml. of pure Ca⁺⁺ were chosen to carry out this experiment. Different samples containing the same amount of Ca⁺⁺ were estimated as above, and others after being left overnight in the furnace at 300°-400° C. All samples were carried out in duplicate. Results are shown in Table II. Complete agreement between expected and found results was obtained in the samples put in the furnace.

FIG. 2.—Relationship between theoretical values and those found by flame photometry. ● Non-precipitated samples. ▲ Na⁺-free precipitated samples. ■ Samples precipitated containing 305 mg./100 ml. of Na⁺.

SIMPLIFIED ESTIMATION OF CALCIUM

113

TABLE II
RECOVERY OF CALCIUM IN DISTILLED WATER

Theoretical Values (mg./100 ml.)	Samples Estimated Directly by Flame Photometer	Samples Precipitated						
		Ca ⁺⁺ Content		Ca ⁺⁺ Mean	Na ⁺ as Impurity (Mean)	Ca ⁺⁺ After 400° C. in Furnace		
			Duplicate				Duplicate	Mean
2.5	2.6	2.8	2.9	2.85	1.8	2.7	2.7	2.7
5.0	5.0	4.4	4.2	4.3	1.1	5.0	5.0	5.0
7.5	7.5	6.6	6.4	6.5	1.1	7.5	7.4	7.45
10.0	10.0	8.2	8.2	8.2	1.2	9.8	9.8	9.8

Experiments with 2 ml. of sample.

FIG. 3.—Interference effect of oxalic acid on Ca⁺⁺ flame excitation. ● Samples diluted with distilled water only. ▲ Samples diluted with distilled water containing 50 p.p.m. of oxalic acid.

Experiments on Recovery of Ca⁺⁺ in Serum

Since the method was working perfectly, it was decided to apply the technique to human serum. Serum was obtained from the same subject throughout and was diluted 3:2 (v/v) with distilled water. This dilution was used to economize in serum and to bring the Ca⁺⁺ values to about 9 mg./100 ml., when different amounts of Ca⁺⁺ were added. One mg., 2 mg., 3 mg., and 4 mg./100 ml. of Ca⁺⁺ were added to different samples of this serum. All were done in duplicate. In this experiment only 1 ml. of the sample was taken in each tube, instead of 2 ml. used in the above experiments. The results are shown in Table III. Recovery of Ca⁺⁺ was complete.

TABLE III
RECOVERY OF CALCIUM IN SERUM

Samples	Ca ⁺⁺ Added (mg./100 ml.)	Theoretical Values (mg./100 ml.)	Ca ⁺⁺ Content			Na ⁺ Content (Mean) (mg./100)	K ⁺ Content
				Duplicate	Mean		
Diluted serum	—	—	7.0	7.0	7.0	4.0	0
" "	1	8.0	8.0	8.0	8.0	4.9	0
" "	2	9.0	9.0	9.0	9.0	4.0	0
" "	3	10.0	10.0	10.0	10.0	3.9	0
" "	4	11.0	10.9	10.9	10.9	4.5	0

Experiments with 1 ml. of sample. Initial Na⁺ content of diluted serum, 233.0 ml./100 ml. Initial K⁺ content of diluted serum, 10.15 mg./100 ml.

Several estimations in normal sera have since been made, and results ranging from 9.6 to 10.5 mg./100 ml. found. Values found in urine change with the metabolic status of the person as shown by chemical methods. It has also been applied to the estimations of Ca⁺⁺ in cerebrospinal fluid, tap water, and milk.

Detailed Technique of Calcium Estimation by Flame Photometry

One ml. of sample is put in a 15 ml. conical "pyrex" centrifuge tube, perfectly clean and dry. One ml. of distilled water and 1 ml. of saturated (about 4 g./100 ml.) ammonium oxalate are added. They are mixed by rolling the tube between the palms of the hands and are left standing for 30 minutes. Then 4 ml. of distilled H₂O is added to "wash" free of Na⁺ and the sample mixed again. Samples are centrifuged at 2,500 r.p.m. for 10 to 15 minutes. Supernatant fluid is discarded by very careful decantation and tubes are left inverted on a sheet of filter paper for five minutes. Thus the last drops of liquid containing Na⁺ are absorbed. Tubes are placed overnight in a furnace adjusted at any temperature between 300° and 400° C. The precipitate is then dissolved in 5 ml. 0.2 N-HCl (Na⁺-free). Dissolution is favoured by putting the tubes for two minutes in a boiling water-bath. Samples are diluted 1:1 (this is the proper dilution for serum; greater dilutions may be required by urine or other biological samples), transferred to small beakers, and the actual Ca⁺⁺ content measured by flame photometry, interpolating the reading of the unknown sample between immediately lower and higher standards.

Ca⁺⁺ solutions containing 10, 20, 30, and 40 p.p.m. are used as standards. The method of prepar-

ation has been described above. As a routine we make up beforehand the dilutions of all the samples to be analysed in one session.

The procedure to make the Ca^{++} estimations of samples is the following:

Once the air pressure has been adjusted at 10 lb./sq. in. and the gas supply has been regulated according to the empirical procedure described above, the 0 and the 100 are checked in the galvanometer scale-reading. Distilled water is sprayed in the flame photometer and the galvanometer is adjusted at 0. Then the standard solution D (40 p.p.m. of Ca^{++}) is sprayed in the flame photometer and the potentiometer is adjusted between divisions 1 and 2 until a whole deflexion (that is, 100 divisions) is recorded in the galvanometer. The other standard solutions A, B, and C (10, 20, and 30 p.p.m. of Ca^{++} respectively) are now sprayed and the galvanometer deviations are recorded. Thus, when an unknown solution is estimated, it is possible to know between which two standards it lies. The unknown sample is now sprayed and the galvanometer reading recorded. The standard solution which produced deviations immediately lower and higher than this sample are checked again and the result is calculated by interpolation.

An example illustrates this procedure: a serum sample diluted finally at 1 in 10 for flame photometry estimation gives a deviation of 26 in the galvanometer dial. This lies between standards A and B, which on re-checking now give deviations of 25 and 52 respectively. The unknown sample contains obviously all the Ca^{++} of standard A plus an extra amount. This latter amount is calculated by means of the following

formula: $\frac{D}{C} = \frac{d}{x}$ where D= difference of galvanometer

deviations between the two standard solutions, C= difference of Ca^{++} content between these standard solutions, d= difference of galvanometer deviations between the unknown sample and standard solution immediately below it, x= Ca^{++} content of difference d. In the present instance, the equation would be

$$\frac{52-25}{10} = \frac{26-25}{x} \text{ and } x = \frac{10}{27} = 0.3$$

Thus, 0.3 is the extra amount which must be added to 10 p.p.m. (Ca^{++} content of standard A). Therefore, the Ca^{++} content of the diluted analysed sample will be $10+0.3=10.3$ p.p.m., and as it is a 1 in 10 dilution the Ca^{++} value of the serum will be $10.3 \times 10 = 103$ p.p.m., or converting p.p.m. to mg./100 ml., it will be 10.3 mg./100 ml.

When a great number of estimations have to be carried out, it is convenient to construct a table, from which the calculated values may be obtained directly.

Occasionally when analysing urine, milk, or other biological materials with high Ca^{++} content, a solution of 1 in 10 contains still too much Ca^{++} and the galvanometer deviation reaches more than a whole deflexion. In this case it is necessary to dilute again the sample until the figure recorded in the dial is less than 100. This dilution is a factor by which the Ca^{++} content of the sample sprayed in the flame

photometer must be multiplied in order to obtain the real Ca^{++} content of the original sample.

When calculating the results in the case of Ca^{++} serum estimations attention must be drawn to the fact that the initial volume of the serum sample was 1 ml. the volume when redissolved in HCl was 5 ml. This solution was then diluted by a factor of 2 to make it suitable for flame photometer estimation. Therefore, in the case of serum, the value obtained by analysing the diluted sample must be multiplied by 10.

In Table III values of Na^{+} apply to non-diluted samples, i.e., those solutions analysed by flame photometry contained 1 in 10 of the values given; therefore, all values of Na^{+} are below 0.8 mg./100 ml., that is completely cut off by the Ca^{++} filter.

Although the step of converting oxalate to carbonate by leaving the tubes in the furnace is very simple, it requires a thermostat, which may not be readily available in all laboratories. To avoid this possible hindrance it was decided to make further experiments using a simple sand-bath. Fine sand was placed in a tin tray and heated by one or more Bunsen burners, a thermometer being placed in the sand to record temperatures. It was observed that once the required temperature (about 360°C .) was reached, it could be readily controlled within 10°C by slightly adjusting the burner(s). When half the length of the tubes containing the calcium oxalate precipitate was buried in the sand and left for 30 minutes at 360°C . the complete conversion of oxalate to carbonate was achieved and the values of Ca^{++} recovery were the same as those obtained by putting the samples in the furnace.

Certain precautions, however, must be taken. (1) The precipitate at the bottom of the tube must be dry before placing it in the sand. (2) When several series of estimations have to be carried out time can be saved by placing the tubes in the sand-bath at 200°C . When the temperature reaches 360° , the conversion of oxalate is completed in 30 minutes. (3) Care must be taken that the sand-bath is uniformly heated.

Conclusion

This is a combined chemico-physical method that makes feasible and easy the estimation of the true amounts of Ca^{++} in biological samples even when mixed with Na^{+} , K^{+} , and other biological substances, such as proteins, etc. The advantages over usual chemical methods can be summarized as follows:

It avoids the repeated washings and the repeated centrifugations.

It suppresses the tedious step of titration, substituting an estimation on the flame photometer that takes 10 seconds. For this reason it is an extremely useful method when a large number of Ca^{++} estimations must be made.

Complete accuracy is reflected in the objective data given. The slightly high results that are often

obtained by manganometry, due to the presence of reducing substances, are obviated by this method.

Flame photometry for Na^+ and K^+ estimations is becoming a common method in this country and the same apparatus can be used for Ca^{++} estimations with the simple addition of the filter for Ca^{++} .

Summary

A method is described in detail for estimating Ca^{++} when mixed with other cations in biological materials, using a commonly available type of flame photometer, burning simple coal-gas and equipped with an interference filter.

An absolutely linear relationship between galvanometer deviations and Ca^{++} concentrations ranging from 0 to 40 p.p.m. is shown.

Na^+ and K^+ concentrations which can be admitted as "impurities" without affecting Ca^{++} estimation are discussed.

An interference effect of oxalic acid on Ca^{++} flame excitation is described for the first time.

This is overcome by heating the samples at 300° – 400° C., which destroys oxalic anion.

The complete recovery of Ca^{++} in distilled water and in human serum is shown in tables.

Advantages of the present method over routine chemical methods are that it avoids the repeated washings and subsequent recentrifugations and the tedious titration, and gives greater accuracy.

I am indebted to the British Council for the tenure of a grant during the performance of this work; to Professor E. J. King for suggesting this work; to Professor J. McMichael, in whose Department this research was carried out; to Dr. I. D. P. Wootton for his helpful advice; to Dr. C. L. Cope and Dr. C. E. Dalglish for their criticism and help when preparing the manuscript.

REFERENCES

- Application Data for Beckman Instruments, DU-9-B.
Barnes, R. B., Richardson, D., Berry, J. W., and Hood, R. L. (1945). *Industr. Engng Chem., Anal. Ed.*, **17**, 605.
Belke, J., and Dierkesmann, A. (1948). *Arch. exp. Path. Pharmac.*, **205**, 629.
Bills, C. E., McDonald, F. G., Niedermeier, W., and Schwartz, M. C. (1949). *Anal. Chem.*, **21**, 1076.
Bohnstedt, R. M., Herrmann, R., Baumann, R., and Füller, H. (1953). *Arzil. Forsch.*, **7**, 82.
Herrmann, R. (1952). *Z. ges. exp. Med.*, **118**, 187.
Schlütz, G. O. (1953). *Schweiz. med. Wschr.*, **83**, 383.