solution was effected by adding the least possible amount of sodium carbonate. The amount of alkali introduced in this way is too small to affect appreciably the pH of the strongly buffered Czapek–Dox agar.

The melted agar in the tubes was poured into the corresponding number of Petri dishes and allowed to set. Each dish was inoculated centrally with mycelium from a young culture of the *Pythium*. The results were recorded after 3 days' incubation, which is the time taken for a control plate to be completely covered. There was complete inhibition at a dilution of 1:25 000 and partial inhibition at 1:50 000. Thus two fungi belonging to widely different orders are inhibited completely by the same concentration.

SUMMARY

1. The inhibition of fungal growth by quadrilineatin was tested against two species of fungi. Complete inhibition of germination of *Botrytis allii* conidia and of mycelial growth of *Pythium debaryanum* occurred at a dilution of 1:25 000 (40 μg./ml.).

2. Quadrilineatin is a less potent inhibitor of *Botrytis allii* than the other known phthalaldehydes of fungal origin.

REFERENCE

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The Flame-Spectrophotometric Determination of Calcium in Biological Fluids and an Isotopic Analysis of the Errors in the Kramer–Tisdall Procedure

By I. MacINTYRE*
Postgraduate Medical School of London, Ducane Road, London, W. 12

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Although chemical methods have been superseded by the flame photometer in the determination of sodium and potassium in biological fluids, calcium is still usually determined chemically. The method in most widespread use is the procedure of Kramer & Tisdall (1921) or one of its modifications (Tisdall, 1923; Clark & Collip, 1925). This method involves the direct precipitation of calcium from serum with ammonium oxalate, isolation by centrifuging, and titration with permanganate after washing with dilute ammonia. This procedure is tedious and has other disadvantages.

Kirk (1954) regards the procedure as one of known unreliability. The same conclusion may be reached from a consideration of the conditions of precipitation which are far from optimum (Kolthoff & Sandell, 1952). Smith *et al.* (1950) showed that magnesium may be co-precipitated to the extent of 10% of the normal calcium content (expressed in equivalents) when serum is analysed. That such accuracy as the method possesses must depend on compensation of errors was well known to Clark & Collip (1925).

That flame photometry has not been widely used for calcium determination is due to two main difficulties. The first is the proximity of the relatively weak calcium oxide bands at 554 and 620mμ to the intense sodium line at 590 mμ. This, together with the preponderance of sodium, makes marked spectral interference inevitable with the relatively simple instruments now in wide use (Powell, 1953). Even when instruments incorporating a monochromator (such as the Beckman flame photometer) are used, this difficulty is not entirely removed (Severinghaus & Ferrebee, 1950). Incorporation of sodium in the standard solutions (Baker, 1955) may allow correction to be made when serum calcium is being estimated, but the variability of sodium concentrations makes this procedure inaccurate when applied to urine.

The second difficulty is the fact that calcium is much more susceptible to interference from anions, in particular phosphate (Brealey, Garratt & Proctor, 1952; Chen & Toribara, 1953; Denson, 1954; Leyton, 1954; Baker & Johnson, 1954), than are sodium and potassium (Shapiro & Hoagland, 1948; Collins & Polkinhorne, 1952; Domingo & Klyne, 1949). As will be shown, neglect of this effect, as by Severinghaus & Ferrebee (1950), may cause serious error. Prior precipitation of calcium as oxalate (Powell, 1953; Llaurado, 1954) should overcome both these difficulties; however, it leads only to combining some of the error of the oxalate procedure with little of the convenience of the flame photometer.

* Drummond Fellow.

In view of these difficulties, a suitable flame spectrophometer was constructed (MacIntyre, 1954, 1955) and a study made of those interfering effects of importance in biological fluids. As a result a method was developed for the determination of calcium in plasma and urine, deproteinization being the only prior step necessary.

In view of the claims of Smith *et al.* (1950) and Lehmann (1953) that the traditional figures for normal serum calcium are too high, a series of normal sera were examined, parallel estimations being carried out by the flame photometer and by the Kramer–Tisdall method as described by King (1951), for which an adequate series of normal figures is available (Wootton, King & Smith, 1951). There was only fair agreement between these two methods. In the author's hands the ethylenediaminetetra-acetic acid procedure of Fales (1953), which employs murexide and a spectrophotometric end-point, was too imprecise to cast light on the source of the discrepancy. A similar imprecision was found with a modification of the procedure of Sweetser & Bricker (1954) in which the end point of the ethylenediaminetetra-acetic acid titration is detected by the sudden change in extinction in the ultraviolet.

It was necessary, therefore, to test the flame-photometric method exhaustively, and to attempt to analyse in detail the sources of error in the oxalate procedure. As the use of isotopes provides a reliable tool for investigating analytical errors, ^{45}Ca was employed to measure the losses of calcium in the Kramer–Tisdall procedure during precipitation and washing. Analyses of precipitates for magnesium and phosphorus were also carried out. The results presented are believed to offer further evidence on the unreliability of the oxalate procedure of Kramer & Tisdall.

EXPERIMENTAL

Description of the flame spectrophotometer

The component parts are an atomizer, spray chamber, burner, monochromator and photomultiplier (Fig. 1).

Gas supply. Air and acetylene are supplied from cylinders fitted with two-stage regulators (British Oxygen Co. Ltd., Wembley, Middlesex).

Atomizer. The atomizer is constructed of Perspex, stainless steel and platinum–iridium alloy. It is concentric in type (Fig. 3).

Spray chamber. This consists of a simple glass cylinder with suitable baffles (Fig. 2).

Burner. The burner is that of Baker (1955). It is held in an adjustable holder allowing both vertical and lateral adjustment. Precise vertical adjustment is important to ensure maximum sensitivity.

Monochromator. This is the Hilger Barfit Wavelength Spectrometer D. 186, which uses a constant-deviation glass prism (Hilger and Watts Ltd., London, N.W. 1).

Photocell. This an RCA-931 A multiplier phototube (RCA Photophone Ltd., Sunbury-on-Thames, Middlesex). It is placed in the compartment in place of the phototube

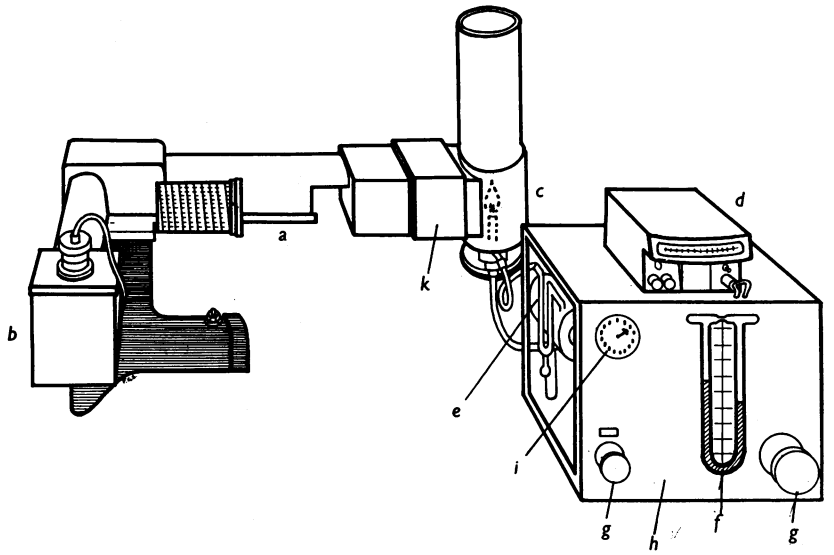

Fig. 1. Flame spectrophotometer (semi-diagrammatic). The main components (monochromator *a*, burner and chimney *c*, and metal box *h*) are screwed to a wooden base not shown in the figure. The metal box houses the spray chamber *e*, flowmeter *f*, rubber-tubing connexions and air-pressure gauge *i*. The galvanometer *d* rests on top of *h*, needle valves *g*, controlling air and acetylene, are set in the front panel. There is a light-tight metal connexion *k* between *c* and *a* which serves to exclude extraneous light from the entrance slit. The phototube housing *b*, contains the photomultiplier.

supplied with the spectrometer. During the course of this work it was operated at 1080 v supplied from batteries. The photomultiplier cathode is connected to the battery cathode. The last dynode is connected to the battery anode and to earth. 120 000 Ω resistors are connected between each dynode. The galvanometer measures the current flowing between the last dynode and the photomultiplier anode. Since the work was completed the phototube has been operated at 1200 v, with a consequent increase in sensitivity.

Galvanometer. The instrument used for this work was a Scalamp galvanometer (sensitivity 90 mm./μA) (W. G. Pye and Co. Ltd., Cambridge). This has now been replaced with a Scalamp galvanometer of double this sensitivity.

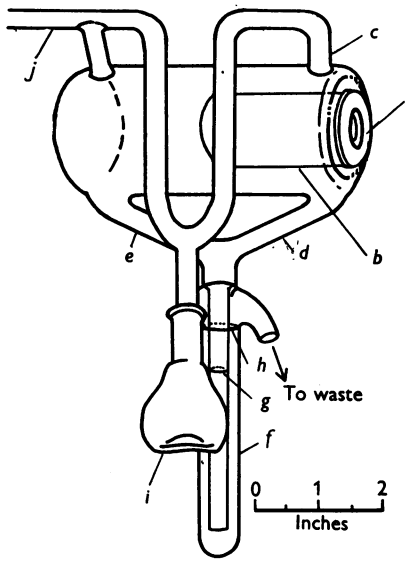

Fig. 2. Spray chamber. This is constructed of glass except for the brass plate *a*, into which is screwed the atomizer. A cylinder *b* prevents large droplets reaching *c*, the exit tube from the chamber. Two limbs *d* and *e* conduct condensed liquid to the constant-pressure drain *f*. The two fluid levels *g* and *h* are shown. Overflow from *h* is led to waste. The small detachable bottle *i* collects condensed fluid from *c*. Tube *j* leads to the burner.

Operation of the flame spectrophotometer

The air pressure is adjusted to 16 lb./sq.in. and the aspiration rate adjusted to 4–6 ml./min. The acetylene pressure is adjusted to 4 lb./sq.in. at the cylinder and the flowmeter adjusted so that the rate is about 500 ml./min.

The flame is now lighted and the monochromator entrance and exit slits are adjusted. Slit widths of 0·2 mm. were used for calcium estimations. The greater sensitivity since obtained now allows the use of 0·06 mm. slit widths.

The wavelength control is set at the desired figure (423 mμ for Ca). The photomultiplier circuit is completed and the galvanometer switched to full sensitivity. A suitable solution, e.g. the 0·25 mM-calcium standard, is sprayed and the wavelength setting checked to ensure that maximum sensitivity is achieved. The acetylene adjustment is slowly altered to a setting which gives maximum sensitivity. This setting is also the one least affected by any possible variations in the acetylene flow rate.

Distilled water is sprayed for about 10 min. At the end of this period steady reproducible deflexions are constantly obtained and the instrument is ready for use. Distilled water is repeatedly sprayed between the sprayings of test and standard solutions.

Determination of interference effects

The solutions used were all made from AnalaR chemicals. The distilled water used was passed through a column of mixed anion- and cation-exchange resins (Amberlite MB-1).

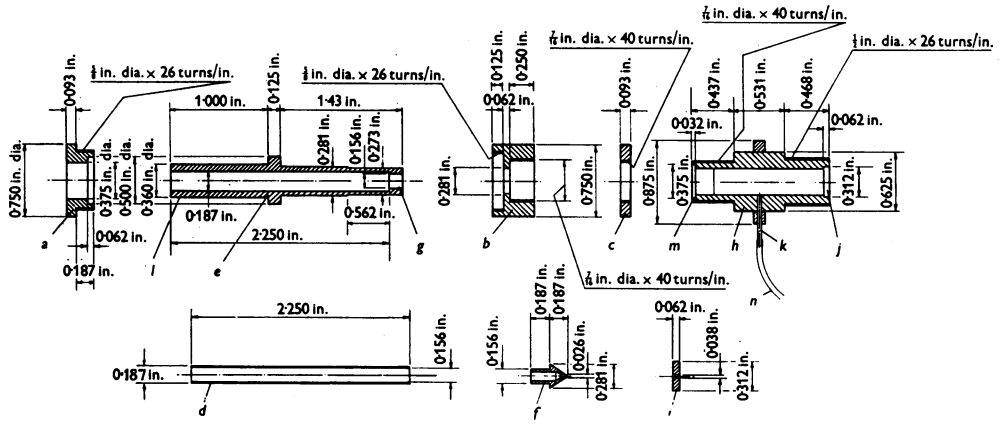

Fig. 3. Atomizer. The sectional drawing shows the retaining ring *a*, adjusting ring *b* and locking ring *c*. These components are made of brass. The stainless-steel tube *d* fits tightly inside the Perspex inner tube *e*. The platinum–20% iridium tip *f* fits into *g*. The platinum–20% iridium disk *i* fits into the body of the atomizer *h* at *j*. A rubber O-ring seal fits into *m*. The test solution enters the body of the atomizer via the polythene tube *n* and Perspex inlet-tube *k*. The Perspex inner tube fits inside the body. A rubber connexion from the air cylinder fits over *e* at *l*. The rate of aspiration is determined by the distance between *f* and *i*. This is controlled by *b*, which, together with *a*, advances *e* into *h*. A satisfactory position is maintained by locking ring *c*.

The standard calcium solution was made from AnalaR $CaCO_3$ (British Drug Houses Ltd., Poole, Dorset), dried at 350° and dissolved in the minimum amount of hydrochloric acid. As with the standard solutions used for blood and urine analyses, polythene containers were used for all solutions kept more than a few hours.

All glassware used was washed in dichromate–sulphuric acid cleaning mixture, and then rinsed thoroughly in several changes of distilled water.

All solutions were kept from contact with rubber bungs or filter paper.

The procedure for determining interference effects was to record the galvanometer deflexions produced by various solutions of the same calcium content, containing increasing amounts of the constituent being examined. 'Blank' readings without calcium were recorded in every case, and in addition a standard solution was sprayed between each pair of test solutions. This enabled any slight change in the sensitivity of the instrument to be detected. Where this occurred the readings were corrected.

Determination of calcium in biological fluids

All solutions were made with deionized distilled water.

Stock solutions. These were as follows. Calcium solution: 25 mM-$CaCl_2$; mixed salt solution: 30 mM-KCl, 5 mM-K_2SO_4, 1·4 M-NaCl, 50 mM-KH_2PO_4; magnesium solution: 8 mM-$MgCl_2$; perchloric acid: $HClO_4$ 60% (w/w); phosphate solution: 44·4 mM-KH_2PO_4. Working standards were prepared by adding 10 ml. of mixed salt solution, 10 ml. of magnesium solution and an appropriate volume of calcium solution to 700 ml. of water in a litre flask. Perchloric acid (50 ml.) was now added, and after mixing the volume was made to a litre. A range of standards at intervals of 0·025 mM was prepared from 0·05 mM to 0·5 mM with respect to calcium concentration. The constituents of the working standards were chosen to approximate to the mean plasma filtrate concentrations after deproteinization of 1 vol. of plasma with 9 vol. of a phosphate–perchloric acid solution. This deproteinizing solution contained sufficient phosphate to eliminate the interfering effect of varying plasma concentrations. The 0·05 mM-sulphate in the working standard solutions is somewhat higher than that suggested by the normal figures of Letonoff & Reinhold (1936), namely 0·03 mM. However, these authors consider that acid deproteinizing reagents liberate an increased amount of inorganic sulphate from protein.

In general, the amounts of the various salts added were adjusted to minimize the interference effects. They were such that the variation of these constituents which occurs in biological fluids would not affect the results.

Combined diluting and deproteinizing reagent. Phosphate solution (10 ml.) was diluted to 700 ml., mixed with 55·5 ml. of perchloric acid and made up to 1 l. with water.

Plasma or serum was prepared for analysis by adding 1 vol. to 9 vol. of deproteinizing reagent and centrifuging, the clear supernatant being used. Urine (1 vol.) was added to 18 vol. of deproteinizing reagent and 1 vol. of water. The diluted urine was centrifuged if necessary before analysis. It is advisable to collect 24 hr. samples of urine in Winchester containers, to which 10 ml. of concentrated HCl has been added. This prevents precipitation of calcium as phosphate before analysis.

It was found that the galvanometer deflexion was related linearly to calcium concentration. However, a small deflexion is produced at 423 mμ by the other constituents of the standard, owing to a small extent to the NaCl (spectral interference is of the order of 2% for a normal serum calcium) and to a larger extent to the perchloric acid, which gives a low-intensity continuous spectrum. Therefore at least two standards were sprayed for each test solution, and the final answer was arrived at by interpolation. Two readings of test and each of two standards were taken.

The direct oxalate precipitation procedure of Kramer & Tisdall (1921) as described by King (1951) was compared with the flame-spectrophotometric method in a series of twenty-two normal and nine pathological sera. Half an hour was allowed for precipitation.

Investigation of the Kramer–Tisdall procedure

The losses of Ca in precipitation and washing were investigated with ^{45}Ca. Calcium acetate (1 μc) of specific activity of 1 c/g. was added to each of ten 2 ml. serum samples. The sera were then set aside for 12 hr. The calcium was precipitated and washed by the same technique as in the comparison series. Samples of the dissolved precipitate, 'decalcified' serum, and washings were now counted after drying on planchets. The addition of the calcium acetate involved only a negligible change in the total calcium content of the sera (about 0·5%).

In eight of these sera, samples of the calcium oxalate precipitate were analysed for Mg content after dissolution in nitric acid and evaporation to dryness. The method of Orange & Rhein (1951) was used. In the author's hands this method had a precision of 5%. A standard was prepared containing an amount of calcium equivalent to that in the test sample. The mean phosphate content of these precipitates was found by pooling and analysing by the method described by King (1951).

In addition oxalate precipitates were obtained from a further eight sera and analysed individually for phosphate content.

RESULTS

Interference effects

Inorganic constituents. Interference effects were studied in the greatest detail with 0·25 mM-$CaCl_2$ but other concentrations within the range 0·05–0·50 mM showed corresponding effects. The emission spectrum of a solution containing 14 mM-NaCl and 0·25 mM-$CaCl_2$ was studied; these are the proportions which exist in human plasma. The proportions of the total emission due to Ca, Na and the flame were: 71·5, 1·5 and 27% respectively at 423 mμ; 80, 8 and 12% respectively at 554 mμ; and 82, 12·5 and 5·4% respectively at 624 mμ.

Both Na and K caused an increase in emission of Ca. In neither case was spectral interference responsible for more than a minor part of this effect. This increase remained constant at 5% for concentrations of Na and K from 3·0 to 20·0 mM. Magnesium chloride was without effect in the concentrations examined from 0·05 to 0·20 mM.

In Fig. 4 the effect of phosphate is shown. There is a marked depression of calcium emission, reaching a maximum when the P:Ca atomic ratio is about

1:1·25. Further increase in the phosphate concentration is without effect.

With 0·25 mM-$CaCl_2$, containing also 14 mM-NaCl, 0·4 mM-KCl and 0·4 mM-KH_2PO_4, K_2SO_4 produced no detectable depression of calcium emission between concentrations of 0·02 and 1·0 mM. Although sulphate is usually reported to depress flame emission, Baker & Johnson (1954) showed that in the presence of excess of phosphate the depressing effect of sulphate was not additive.

The effect of $HClO_4$ and HCl are shown in Fig. 5.

Organic constituents. Neither 0·75% bovine albumin nor 0·75% glycine produced any effect on the emission of 0·25 mM-$CaCl_2$. However, in the presence of 0·4 mM-KH_2PO_4 the albumin, but not the glycine, produced a greater calcium emission than was found with 0·25 mM-$CaCl_2$ and 0·4 mM-KH_2PO_4 alone. A similar effect was obtained by Chen & Toribara (1953).

The adequacy of the plasma-deproteinization procedure in recovering the calcium is shown in Table 1. It is evident from columns (*c*) and (*d*) that the combined effect of non-protein organic constituents and the slight concentration of calcium due to the volume displacement of plasma proteins is not appreciably more than 1%.

The interference of organic substances in urine is shown to be small in Table 2.

Recovery experiments

The adequacy of recovery of calcium added to serum and urine is shown in Tables 3, 4*a* and 4*b*. [The Kramer–Tisdall procedure gave unsatisfactory recoveries when applied to urine (Table 4*a*).]

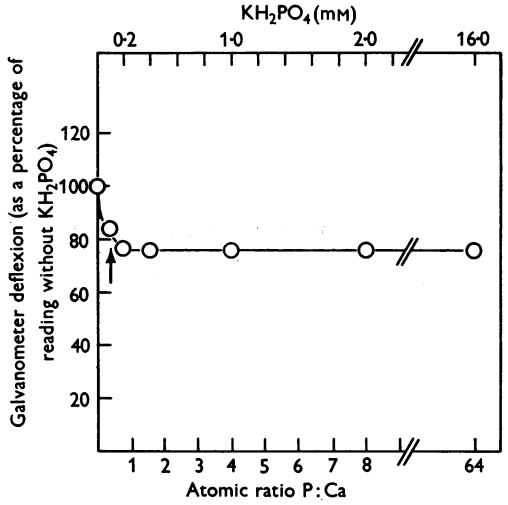

Fig. 4. Effect of phosphate on the emission of calcium. λ 423 mμ; 0·25 mM-$CaCl_2$ used. 16·0 mM-KH_2PO_4 alone produced an emission of 5% of that due to 0·25 mM-$CaCl_2$. The lesser concentrations of KH_2PO_4 tested produced no detectable emission. The arrow indicates mean normal serum concentration.

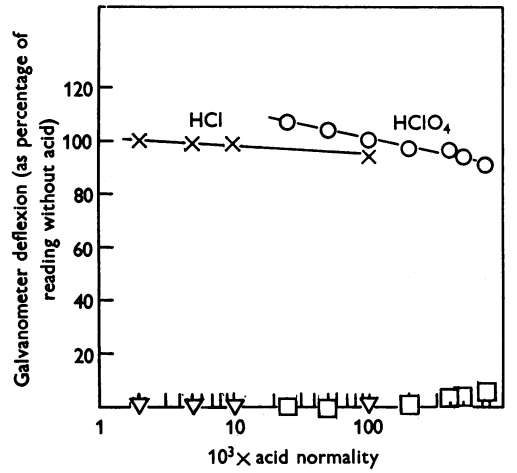

Fig. 5. Effect of hydrochloric and perchloric acids on calcium emission. λ 423 mμ; 0·25 mM-$CaCl_2$ containing also 14·0 mM-NaCl, 0·40 mM-KCl and 0·40 mM-KH_2PO_4 was used. ×, $CaCl_2$ + HCl; ▽, HCl alone; ○, $CaCl_2$ + $HClO_4$; □, $HClO_4$ alone.

Table 1. *Effect of ashing, direct dilution with water and deproteinization on the apparent calcium content of serum*

(*a*) 1 ml. of serum was ashed, dissolved in 2 ml. of 0·1 N-HCl, 1 ml. of 4 mM-KH_2PO_4 and 7 ml. of water.
(*b*) 1 ml. of serum was added to 2 ml. of 0·1 N-HCl, 1 ml. of 4 mM-KH_2PO_4 and 6 ml. of water.
(*c*) 5 ml. of diluted ashed sample as in (*a*) to which 0·3 ml. of 60% (w/w) $HClO_4$ was added.
(*d*) 5 ml. of serum diluted as in (*b*), to which was added 0·3 ml. of 60% $HClO_4$ before centrifuging to remove the protein.

Sample no.	Galvanometer deflexion (mm.) Ashed (*a*)	Direct dilution (*b*)	Ashed + $HClO_4$ (*c*)	Deproteinization with $HClO_4$ (*d*)
1	46·5	54·0	43·0	43·0
2	44·5	53·0	41·0	41·5
3	44·5	52·5	41·5	42·0
4	47·5	54·5	44·0	44·5

Table 2. *Effect of ashing on the apparent calcium content of urine*

The samples were ashed in platinum crucibles at a temperature of 420° before analysis by flame spectrophotometry.

Description of urine sample	Ashed (m-equiv./l.)	Direct estimation (m-equiv./l.)
Normal	12·90	12·95
Normal	4·00	4·00
Proteinuria (0·5 g./100 ml.)	2·55	2·57
Bile + urobilin	2·55	2·40
Glycosuria (6 g./100 ml.)	21·40	22·80

Table 3. *Recovery of calcium added to serum*

Varying quantities of calcium were added to serum no. 1. The analyses were carried out in duplicate by the flame spectrophotometer. The serum used was a pooled specimen of five normal sera.

Sample no.	Obtained (m-equiv./l.) *a*	Expected (m-equiv./l.) *b*	$a-b$ (m-equiv./l.)	$(100a/b)$
1	4·45	—	—	—
2	5·25	5·34	−0·09	98·3
3	5·85	5·78	+0·07	101·2
4	6·25	6·21	+0·04	100·7
5	6·42	6·36	+0·06	101·0
6	6·91	7·03	−0·12	98·3
7	7·36	7·43	−0·07	99·0
Mean (±S.E.M.)			−0·018±0·034	99·8±0·55

Table 4*a*. *Recovery of calcium added to urine*

The analyses were carried out by the flame-spectrophotometer (f.s.) and by the Kramer–Tisdall (K.T.) procedures.

Sample no.	*a* Obtained by K.T. (m-equiv./l.)	*b* Obtained by f.s. (m-equiv./l.)	*c* Expected (m-equiv./l.)	$100a/c$ (K.T.)	$100b/c$ (f.s.)
1	1·18	1·18	—	—	—
2	3·15	3·40	3·50	90·0	97·2
3	4·10	4·42	4·43	92·5	99·8
4	5·00	5·57	5·60	89·3	99·5
5	6·05	6·95	6·55	92·4	106·2
6	7·20	8·13	8·13	88·6	100·0
7	8·95	9·47	9·30	96·2	101·9
8	12·10	12·13	12·45	97·2	97·4
Mean (±S.E.M.)				92·3 (±1·35)	100·3 (±1·05)

Table 4*b*. *Recovery of calcium added to urine*

Calcium solution to increase the concentration by 2·50 m-equiv./l. was added to 12 urines. The samples were analysed by the flame spectrophotometer.

No. of expts.	$\frac{\text{Result obtained}}{\text{Result expected}} \times 100$ Mean	Range	S.D.	S.E.M.
12	100·6	95·0–103·0	2·07	0·62

The figure in the second column does not differ significantly from 100·0 (*t* test).

The precision of the flame-spectrophotometric method is 0·06 m-equiv. of Ca/l. This was calculated from duplicates of the seven serum analyses shown in Table 3, and eight urine analyses in Table 4*a* from the formula $\sqrt{(\Sigma d^2/2n)}$, where d is the difference between duplicates and n is the number of pairs.

Comparison with the Kramer–Tisdall method

Table 5 shows the comparison with the Kramer–Tisdall procedure.

In the twenty-two analyses on normal sera, the flame-spectrophotometric procedure gave a mean deviation of $+0{\cdot}19 \pm 0{\cdot}049$ m-equiv./l. from the oxalate procedure. When the remaining analyses from the 'pathological' sera are included, the mean deviation is $+0{\cdot}28 \pm 0{\cdot}052$ m-equiv./l. Both these deviations are highly significant ($P < 0{\cdot}001$, t test).

Investigation of the Kramer–Tisdall method

In Table 6 are presented the results of studies on the composition of the precipitates from ten sera, by using the isotopic method of analysis described above (p. 167). This shows that 0·8–2·0 % of the Ca originally present was not precipitated, and a further 2·0–8·9 % was lost in washing. The total loss in this series varied between 2·8 and 10·9 %. The results of analyses for magnesium on eight of the ten precipitates are also presented. The extent of magnesium precipitation was extremely variable and sometimes considerable, and the mean coprecipitation of phosphorus was almost negligible. Coprecipitation of phosphate may on occasion be of more significance, since in another series of eight precipitates the mean phosphorus content was 1·1 μg. (range 0·4–3·5 μg.). This would result in a mean negative error of 0·65 % (range 0·2–2·1 %).

When the negative errors due to Ca^{2+} loss are contrasted with the positive errors due to coprecipitation of Mg, the Ca loss outweighs the Mg loss by 3·3 % (range −7·9 to +7·2 %). The exact magnitude of this figure may not be representative, in a small series, since the coprecipitation of Mg was so variable, but this result is in good agreement with the series of comparisons between the flame-photometric and Kramer–Tisdall methods shown in Table 5.

DISCUSSION

Interference effects

The calcium line in the blue is much more suitable for use in the flame photometry of biological fluids than the bands in the green and red spectral regions, if a suitable photocell is available. When combined with a monochromator, use of this line effectively eliminates the spectral interference of sodium.

Table 5. *Comparison of the flame-spectrophotometric and Kramer–Tisdall procedures*

Thirty-one sera were analysed—twenty-two from normal subjects and nine from those with diseases affecting calcium metabolism.

	No. analysed	Flame spectro-photometer (m-equiv./l.)	Kramer–Tisdall (m-equiv./l.)	Mean diff.	S.E.M. diff.	*P* (*t* test)
Normal sera	22	5·39	5·20	0·19	0·049	<0·001
Including abnormal sera	31	5·48	5·20	0·28	0·052	<0·001

Table 6. *Analysis of errors in the Kramer–Tisdall procedure*

The precipitates from ten sera were analysed. The figures for percentage coprecipitation of Mg and P were calculated in equivalents, assuming the calcium to be the mean normal (5·39 m-equiv./l.). The Mg was assumed to be precipitated as oxalate and the phosphate as $CaHPO_4$. The net error was calculated as total Ca loss minus Mg coprecipitation.

Sample no.	Ca loss: Precipitation (%)	Ca loss: Washing (%)	Ca loss: Total (%)	Mg coprecipitation (%)	Net error (%)
1	1·6	6·2	7·8	—	—
2	1·2	6·7	7·9	0·0	−7·9
3	1·5	4·7	6·2	0·7	−5·5
4	1·3	4·9	6·2	0·0	−6·2
5	2·0	8·9	10·9	11·0	+0·1
6	1·3	5·3	6·6	0·0	−6·6
7	0·8	2·0	2·8	10·0	+7·2
8	1·5	6·9	8·4	—	—
9	1·0	3·9	4·9	2·3	−2·6
10	1·2	5·1	6·3	2·3	−4·0
Mean	1·3	5·5	6·8	3·3	3·2

The mean phosphate content of precipitates 2–10 (pooled) was such as to cause a negative error of 0·22 %.

The enhancement effects due to sodium and potassium are small and constant over the biological ranges.

The most marked interference found was that due to phosphate. That it becomes maximal at a P:Ca atomic ratio of about 1:1·25, remaining constant at higher ratios, is fortunate, since it allows elimination of phosphate interference by diluting all samples with phosphate solution. Brealey *et al.* (1952), Leyton (1954), Denson (1954) and Baker & Johnson (1954) have reported a similar effect. The amount of phosphate in plasma is only about half of the minimum amount required to ensure calcium results that are independent of further phosphate increase. This means that incorporation of the mean plasma–phosphate content in the standard solution is not an effective method of eliminating error from phosphate since the emission of calcium in deproteinized plasma will vary inversely with the plasma phosphate. Failure to add any phosphate to standard solutions, however, especially when deproteinized plasma is analysed, inevitably gives results which are too low. Severinghaus & Ferrebee (1950) used such a procedure, and reported excellent recoveries, as might be expected from the results of Leyton (1954). His systematic studies of phosphate interference show that good recoveries would be expected in the analysis of deproteinized plasma by comparison with phosphate-free standards; although the original calcium content would be underestimated.

The use of the internal-standard procedure with lithium (Baker, 1955) is almost certainly an unsound method of dealing with phosphate interference. Even if the lithium emission is subject to an identical degree of depression by phosphate, it is improbable that lithium will be bound by protein to exactly the same extent as calcium. The presence of protein would therefore affect the lithium and calcium emissions to a different degree.

Magnesium and sulphate were without detectable influence on calcium emission in the concentrations tested, but were nevertheless incorporated in the standard solutions. This was done to compensate for any combined interference effect of the plasma salts which had not been detected when their effects were studied individually.

Comparison with the Kramer–Tisdall method

The mean difference of 0·28 ± 0·052 m-equiv./l. in the serum-comparison series cannot be attributed to the effect of the concentration of calcium consequent upon deproteinization. From consideration of the specific volume of plasma proteins (Van Slyke *et al.* 1950), together with the amount of non-solvent water associated with similar proteins (Höber, 1945), it would appear that the volume displacement of proteins should not cause a positive error greater than 0·6%. However, even if the calcium values obtained by flame photometry are reduced by 1% the differences remain highly significant.

The Kramer–Tisdall procedure

The original Kramer–Tisdall method published in 1921 advocated three washings with dilute ammonia, each wash being removed by aspiration. The modification of Tisdall (1923), which is substantially the method used in the comparison series, substituted decantation for aspiration, and two washings with ammonia were considered sufficient. In the modification of Clark & Collip (1925), one washing with ammonia was employed after 5 min. drainage of the centrifuge tube in an inverted position. Clark & Collip were not able to recover calcium added to serum by using the modification of Tisdall (1923). Their own method gave 100% recovery, but it did not agree with the Tisdall modification.

The absence of any generally accepted conclusion about which, if any, of these methods is accurate may have been due to the lack of a sound method of detecting and measuring analytical errors. The use of radioactive tracers does provide such a method—one which is certain to be increasingly used in biochemical analysis (e.g. Bothwell & Mallett, 1955). The results (obtained with ^{45}Ca) presented here show that such errors in coprecipitation of magnesium as may occur, even assuming all the magnesium to be present as oxalate, are less than the errors due to loss of calcium in precipitation and washing. Further, as pointed out by Kolthoff & Sandell (1952), addition of ammonium oxalate to a neutral calcium solution produces a precipitate of uncertain composition, contaminated with basic calcium oxalate, or calcium hydroxide, and tends to give low results. These authors also point out that in the presence of ammonia and phosphate some of the magnesium may be precipitated as magnesium ammonium phosphate. The errors due to precipitation of calcium as phosphate or to inclusion of non-oxalate reducing substances in the precipitate seem to be of the same magnitude (0·5–1·0%) as judged from the analyses of phosphate reported here and from Sendroy's (1944) results. Since the errors tend to cancel each other out, they are not considered in the discussion above.

Rothlin & Bidder (1945) were unable to obtain complete precipitation of calcium directly from serum, and consistently found large mechanical losses in decantation and washing: their results are in good agreement with those reported here.

The isotopic studies afford direct proof of the magnitude of the errors and justify the conclusion that the major part of the discrepancy between the flame-photometric and chemical results should be

attributed to error in the chemical method. The evidence further suggests that the normal values for serum calcium are some 3–4 % higher than the figures given by King (1951).

SUMMARY

1. A flame-spectrophotometric method of estimating calcium in biological fluids is described.

2. Spectral interference due to sodium is mostly eliminated by measuring the calcium emission at 423 mμ.

3. Phosphate interference is overcome by diluting the samples with excess of phosphate.

4. Studies with radioactive calcium (^{45}Ca) show that about 7 % of calcium in serum is lost during washing and precipitation in the procedure of Kramer & Tisdall (Tisdall, 1923).

5. Magnesium is coprecipitated in this procedure in a variable amount (range 0–11 %), averaging 3 %.

6. It is concluded that the accepted normal serum-calcium figures are approximately 4 % too low.

The author wishes to express his thanks to Professor King for suggesting this investigation, and for subsequent advice and criticism.

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Acidic Peptides of the Lens

2. THE USE OF ION-EXCHANGE RESINS AS MOLECULAR SIEVES*

BY S. G. WALEY
Nuffield Laboratory of Ophthalmology, University of Oxford

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A tripeptide, named ophthalmic acid, has recently been isolated from calf lens (Waley, 1956). Further work on the structure of ophthalmic acid needed more material than was available from the isolation already carried out. The original method used dialysis, followed by zone electrophoresis, to obtain acidic compounds of low molecular weight; large volumes of solution have to be concentrated to isolate the diffusible compounds, and zone electrophoresis is limited to handling fairly small amounts of material. The procedure described below avoids these drawbacks, has permitted the isolation of ophthalmic acid in the crystalline state, and has allowed the identification of another peptide, norophthalmic acid. This procedure utilizes ion-exchange resins to effect separations based on molecular size as well as ionic charge.

* The paper by Waley (1956) is to be regarded as Part 1.