DETERMINATION OF SERUM CALCIUM BY PRECIPITATION WITH OXALATE

A COMPARATIVE STUDY OF FACTORS AFFECTING THE RESULTS OF SEVERAL PROCEDURES

By JULIUS SENDROY, JR.

(From the Department of Experimental Medicine, Loyola University School of Medicine, and Mercy Hospital, Chicago)

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Most of the methods for analysis of serum calcium have been based on the principle of precipitation and isolation of calcium as the insoluble oxalate. In the successive analytical steps involved, (a) the preparation of the serum sample for (b) its precipitation as calcium oxalate, (c) the isolation of the pure precipitate, and (d) its quantitative measurement, many variable factors may affect the results and must be empirically controlled.

The final step (d), the measurement of the calcium, usually and most conveniently as oxalate, can be made the least variable and most accurate part of the analysis. The dissolved oxalate can be estimated by titration with potassium permanganate or ceric sulfate, or by the gasometric method of Van Slyke and Sendroy (43, 45, 36), with an error of 0.5 to 1 per cent. The washing of the calcium oxalate precipitate in the course of its isolation (c), usually by centrifugation, is a strictly empirical and arbitrary procedure (6, 4, 45, 49, 35), which can nevertheless be carried out to yield constant, reproducible results. Error in this step alone can be limited to a maximum of 1 per cent.

Concerning the initial steps (a) and (b) there is much less certainty. The serum calcium may be precipitated by the addition of oxalate (Procedure 1) to serum directly (without further treatment other than dilution with water), as proposed by Príbram (32) in 1871, and others (47, 5, 6, 17); (Procedure 2) to the dissolved ash of serum, as applied to microanalysis by de Waard (46) and Kramer and Howland (16); or (Procedure 3) to a protein-free filtrate of serum, as was first done by Lyman (22) and Halveson and Bergeim (9).

Were the results obtained under such widely different conditions of precipitation equal in accuracy, the method of choice would be determined largely by convenience or the amount of sample available. However, when comparative analyses of the same serum samples are carried out in the three ways mentioned, the method of washing and measuring the oxalate being the same throughout, large differences in results may be found, in the same laboratory and by the same analyst. Despite many studies
devoted to a consideration of such discrepancies, there is no agreement as to the optimum conditions for the quantitative precipitation of serum calcium as oxalate, nor has a satisfactory explanation been found to reconcile the differences in results obtained by various methods, and by different investigators.

Indicative of the situation are the following references to comparative investigations in which the preparation of the sample and the conditions affecting the precipitation of serum calcium were studied as variable factors. Results by ashing and by direct precipitation have been found to be the same (32, 46, 47, 6, 10, 2, 29, 39), higher for direct precipitation (3, 50, 48), and also lower (30, 11, 41, 2). In comparison with results obtained by ashing, the deproteinization methods have been reported to give values the same (18, 26), higher (9, 6, 38, 20, 10, 40, 29), and also lower (6, 19, 2). Higher values for calcium of trichloroacetic acid filtrate compared with directly precipitated serum calcium have been found (45, 40, 29, 12) and likewise, lower ones (6, 2). Příbram (32) and Blühdorn and Genek (1) could find no calcium in the ash of serum from which calcium had been directly precipitated, nor could de Waard (47) find any in the filtered serum after direct precipitation. Van Slyke and Sendroy (45), however, found calcium in the trichloroacetic acid filtrate of decalcified serum.

Since the papers cited embrace a wide variety of techniques for each of the four steps in analysis mentioned above, no inferences even as to the general trend of the values for any group of similar procedures may be drawn. Obviously, conclusive evidence concerning the validity of these conflicting groups of results, and an explanation of the origin of the discrepancies involved, are desirable (25).

The accuracy and precision of the gasometric method for calcium (43, 45) were well suited for the purposes of the present investigation. The maintenance of a uniformity of sample size, of washing technique, and of calcium oxalate measurement eliminated these factors as variables in a study concerned mainly with the factors governing the precipitation of serum calcium in different media and under various conditions. Control analyses of known salt solutions, paralleling those of serum at every step, and for each of the factors studied, served as standards of absolute accuracy. The results indicate that it is possible to obtain complete agreement in analyses of the same serum sample, whether calcium is precipitated directly from the serum, from the ash, or from the deproteinized filtrate, provided that appropriate corrections are made in each case. Many of the discrepancies reported in the literature are merely artifacts, arising from an accumulation of errors, no one of which is ordinarily detectable in blank analyses by the usual procedures heretofore followed.
Procedure

The techniques used, with some modifications of our own, were similar to ones described in the literature. For the sake of clarity, and because some of the discrepancies cited may owe their origin to certain details of procedure, an exact description of the analytical methods employed is given in the following.

(a) Preparation of Serum Sample and (b) Precipitation of Calcium

With few exceptions, all analyses were performed in triplicate, the samples used being 1 cc. of serum (or its equivalent). The sample, in a conical bottom 12 cc. centrifuge tube, was distributed in a fluid volume of 6 cc. in every analysis. The calcium was precipitated, at room temperature, by the addition of 1 cc. of saturated ammonium oxalate. Precipitation ("digestion") took place overnight, or for a period of about 16 hours.

The blanks for all analyses reported in this paper were uniformly prepared by the addition of 1 cc. of saturated ammonium oxalate to 6 cc. of distilled water.\(^1\)

Procedure 1. Calcium Precipitation Directly in Diluted Serum—To 1 cc. of serum, there were added 5 cc. of distilled water, followed, after mixing, by 1 cc. of saturated ammonium oxalate. The contents of the centrifuge tube were thoroughly mixed.

Procedure 2. Calcium Precipitation in Dissolved Ash of Serum—To 1 cc. of serum in a 15 cc. platinum dish, 2 cc. of 1 N HCl were added; the mixture was evaporated to dryness on a steam bath, then placed in an oven at 110° for 15 to 20 minutes. The dish was transferred to a muffle furnace initially at the same temperature. The furnace was gradually heated to 500°, at which temperature the sample was then ashed for 2 hours.\(^2\) After cooling, the ash was dissolved in 2 cc. of 1 N HCl and the solution evaporated on a steam bath.\(^3\) The dish was again cooled and the ash dissolved in 2 cc. of 1 N HCl. The solution was quantitatively transferred to a centri-

\(^1\) These "water" blanks served (a) to correct for possible failure to remove ammonium oxalate completely in washing, and (b) to provide necessary corrections for the gasometric measurement (44, 36).

\(^2\) If the furnace is kept at 500° for the duration of the day's work, the initial heating of the dried serum must be done with especial care to prevent mechanical spattering of the ash as water of combustion is evolved. With the furnace door open, the dish is held by tongs and alternately moved in and out of the furnace until the fumes, which should be evolved from the material gently, are no longer visible. The dish is then heated at 500°.

\(^3\) A white ash is usually obtained at this point. On the rare occasions when visible particles of carbon remain, the ashing at 500° should be repeated for \(\frac{1}{2}\) to 1 hour.
DETERMINATION OF SERUM CALCIUM

fuge tube, followed by three portions of 1 cc. of water used to wash the walls of the platinum dish.

To the 5 cc. of solution in the centrifuge tube, 1 cc. of freshly prepared 20 per cent sodium acetate, 2 drops of 0.1 per cent brom-cresol green indicator, and 1 cc. of saturated ammonium oxalate were added (45). After the mixture was stirred with a thin, footed glass rod, a few drops (usually 6) of 1:1 ammonia were added to obtain a color matching that of a similar volume of 0.067 M KH₂PO₄ solution, of pH about 4.5, containing the same amount of indicator. The stirring rod was washed off into the tube with a few drops of water.

Procedure 3. Calcium Precipitation in Protein-Free Filtrate of Serum—

To 1 measured volume of serum in a volumetric flask of 5-fold volume, were added 3 volumes of water, then, drop by drop, with gentle whirling, 1 volume of freshly prepared 20 per cent trichloroacetic acid solution (45). After the addition of a drop of caprylic alcohol, and water to the mark, the material was mixed and allowed to stand 1/2 hour. The mixture was then centrifuged for 10 minutes at 2200 R.P.M. The supernatant fluid was filtered through a sintered glass filter funnel, Jena No. 3G4, No. 3G3, or Pyrex No. 30F. Samples of 5 cc. of filtrate were transferred to centrifuge tubes and the calcium precipitated exactly as described above for 5 cc. volumes of dissolved ash. Usually, less (about 2 drops) 1:1 ammonia was needed to obtain a final pH of 4.5.

(c) Purification and Isolation of Precipitated Calcium Oxalate

The procedure followed was a modification of that previously described ((45) p. 221). After standing for at least 16 hours, samples and blanks alike were centrifuged for 5 minutes at 2600 R.P.M. By means of an upturned capillary, the tip of which was held below the surface of the liquid, all but 0.2 to 0.3 cc. of the supernatant fluid was slowly siphoned off. The precipitate was washed with 2 per cent ammonia water as follows: 1 cc. was slowly admitted from a pipette to wash the entire inner surface of the tube, which was then slanted while an additional 2 cc. were allowed to flow in quickly. The supernatant wash fluid was then carefully stirred with a thin, footed glass rod, the end of which was permitted to come no nearer than within 0.5 cc. of the bottom of the tube. Prior to withdrawal from

4 Büll's (3) results probably explain the variation in the time required for direct precipitation by different workers. He found that while calcium could be completely precipitated in some sera in a half hour others required at least 16 hours, depending on the viscosity and protein content of the sample.

5 The ammonia wash water should be freshly prepared for each day's use, from the concentrated material (21).

6 This washing procedure avoids disturbance of the precipitated calcium oxalate by a violent inrush of wash water, which nevertheless is well mixed with the 0.2 to 0.3
the tube, the rod was washed off with a few drops of ammonia water. After 5 minutes centrifugation at 2600 R.P.M., the washing, centrifugation, and withdrawal of fluid were repeated twice as above, except that the supernatant was not again stirred with the glass rod.

(d) Quantitative Measurement of Precipitated Calcium Oxalate

The gasometric procedure of Van Slyke and Sendroy (45 pp. 221–224) was followed, modified as described elsewhere (36). The c correction was obtained from the analysis of the blank, prepared, washed, and analyzed as above.

EXPERIMENTAL

Analyses of Salt Solutions of Known Calcium Content

Salt solutions of known calcium content were analyzed under conditions of controlled accuracy, by the same techniques as are outlined above for serum samples. The dilute solutions used, approximating the inorganic pattern of serum, were prepared from stock solutions at frequent intervals, exactly as previously described (35 p. 253), and contained, in milliequivalents per liter, CaCl₂ 5.0, MgCl₂ 3.0, NaCl 154, KH₂PO₄ 1.2. Occasionally magnesium or phosphate, or both, were omitted. In this work, sixteen stock salt solutions were used over a period of 8 months.

The results are summarized in Table I. All values obtained are included in the calculations. The statistical treatment follows methods described by Mainland (24); the t and x tables of Fisher (8) are used as criteria of significant differences.

Series 1. Analyses by Direct Precipitation—The mean of 5.01 milliequivalents of calcium per liter indicates the accuracy and reliability of this technique. The difference of the mean by +0.01 milliequivalent from the known value is not statistically significant.

Series 2. Analyses by Precipitation from Dissolved Ash of Sample—

(a) These were carried out as described for serum. The difference of the mean by +0.11 milliequivalent from the known value is highly significant (Table I).

(b) In another group of analyses, actual ashing was not performed, but cc. of supernatant fluid left above the crystals. Mixing tests with colored solutions and periodic analyses of known calcium solutions (Table I) have established the correctness of this empirical procedure.

As in all analyses of salt solutions reported in Table I, the calcium in these samples, also, was precipitated at pH 4.5. This adjustment, not made when serum calcium was directly precipitated, followed the addition of oxalate, and usually required the addition of 1 drop of 1 N HCl.
the calcium in 1 cc. samples was precipitated in the presence of all of the reagents used in the ashing procedure. A mean of 5.13 milliequivalents of calcium per liter was obtained.

The difference of 0.02 milliequivalent between the means of groups (a) and (b) might be attributed to the elimination in the ashing group (a) of some organic contaminant reacting with Ce(SO₄)₂ to produce CO₂. However, the difference here is statistically not significant. It is clear that

<table>
<thead>
<tr>
<th>No. of analyses</th>
<th>Series 1, direct pptn.</th>
<th>Series 2a, ashing</th>
<th>Series 2b, reagents for ashing</th>
<th>Series 3a, CeCl₃COOH &quot;filtrate&quot;</th>
<th>Series 3b, CeCl₃COOH &quot;filtrate&quot; ashed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (= M)</td>
<td>5.01</td>
<td>5.11</td>
<td>5.13</td>
<td>5.18</td>
<td>5.16</td>
</tr>
<tr>
<td>&quot; error (= ΔM)</td>
<td>±0.01</td>
<td>±0.11</td>
<td>±0.13</td>
<td>±0.18</td>
<td>±0.16</td>
</tr>
<tr>
<td>&quot; deviation</td>
<td>±0.040</td>
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<td>±0.046</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>±0.047</td>
<td>±0.042</td>
<td>±0.059</td>
<td>±0.069</td>
<td>±0.065</td>
</tr>
<tr>
<td>&quot; error of mean (= εM)</td>
<td>±0.0069</td>
<td>±0.0140</td>
<td>±0.0132</td>
<td>±0.0120</td>
<td>±0.0196</td>
</tr>
<tr>
<td>t = ΔM/εM, referred to known</td>
<td>1.45</td>
<td>7.9</td>
<td>9.9</td>
<td>15.0</td>
<td>8.2</td>
</tr>
</tbody>
</table>

| Standard error of difference (= εD) between means, referred to Series 1 | ±0.0154 | ±0.0144 | ±0.0138 | ±0.0208 |
| x = ΔM/εD, referred to Series 1 | 6.5 | 8.3 | 12.3 | 7.2 |

<table>
<thead>
<tr>
<th>Standard error of difference (= εD) between pairs of means</th>
<th>Significance ratio of t or x</th>
<th>Pairs of means compared</th>
</tr>
</thead>
<tbody>
<tr>
<td>±0.0219</td>
<td>t = 0.91</td>
<td>Series 2a and 2b</td>
</tr>
<tr>
<td>±0.0230</td>
<td>x = 0.87</td>
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</tr>
<tr>
<td>±0.0178</td>
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</tr>
<tr>
<td>±0.0249</td>
<td>t = 2.01</td>
<td>&quot; 2a &quot; &quot; 3b</td>
</tr>
<tr>
<td>±0.0219</td>
<td>x = 2.28</td>
<td>&quot; 2a + 2b and 3a + 3b</td>
</tr>
</tbody>
</table>

The results of Analyses of (Serum-Salt) Solutions Containing 5.00 Milliequivalents of Calcium per Liter

The results are given in milliequivalents per liter.

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<tr>
<th>No. of analyses</th>
<th>Series 1, direct pptn.</th>
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| Standard error of difference (= εD) between means, referred to Series 1 | ±0.0154 | ±0.0144 | ±0.0138 | ±0.0208 |
| x = ΔM/εD, referred to Series 1 | 6.5 | 8.3 | 12.3 | 7.2 |

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the average error of +0.12 milliequivalent (for (a) and (b)) obtained by the ashing technique is caused by the use of those additional reagents not required in the direct precipitation method.

Series 3. Analyses by Precipitation in Presence of Reagents Used for Protein-Free Filtrates—(a) These were carried out as described for trichloroacetic acid filtrates of serum. Since no protein was present in these samples, the centrifugation and filtration through sintered glass were
omitted after it had been shown that these steps per se had no effect on the results. The difference of +0.18 milliequivalent from the known value is highly significant (Table I).

(b) In another group, 5 cc. samples of trichloroacetic acid “filtrate” were first dried with 2 cc. of 1 N HCl and ashed exactly as described for serum and Series 2a of salt solutions above. A mean of 5.16 milliequivalents per liter was obtained. Statistically, as for Series 2a and 2b, the difference of 0.02 milliequivalent between the means for Series 3a and 3b is not significant. However, both means (5.18 and 5.16 milliequivalents), obtained by the “deproteinization” technique, are decidedly significant in difference from the known value (5.00), or the mean for Series 1. Furthermore, comparison of the means of appropriate unashed (Series 2b and 3a) or ashed (Series 2a and 3b) pairs, and of the combined groups (Series 2a + 2b) and (Series 3a + 3b), indicates that the increase in average error of 0.05 milliequivalent per liter, of Series 3 with trichloroacetic acid, over that of Series 2 without it, is fairly significant. Since the reagents used in both series were otherwise the same, this increase is attributable to the effect of trichloroacetic acid.

Individual Factors Responsible for Errors in Calcium Analyses of Salt Solutions and Serum

The foregoing results have been quantitatively confirmed, by direct, independent experiments designed to determine the various individual factors involved in serum calcium analyses as they have been carried out in the past by ourselves and others.

Effect of Low Calcium Concentration in Sample Analyzed—From the results with serum-salt solutions (Table I, Series 2 and 3), it might reasonably be expected that the calcium-raising effect of the reagents could be eliminated by the use either of reagents previously tested as calcium-free, or of suitable “reagent” blanks instead of the “water” blanks used in these analyses. However, no calcium has ever been detected in our individual reagents by qualitative tests, either in this or in previous work (45), nor have “reagent” blanks ever been found quantitatively different from “water” blanks.

An explanation for the failure to detect small amounts of calcium, as an impurity or otherwise, is afforded by a series of experiments, comprising 1 cc. analyses of twenty serum-salt solutions diluted to contain calcium from 0.1 to 2.5 milliequivalents per liter (2 to 50 per cent of normal serum concentration), performed under the conditions outlined above for direct precipitation (Series 1). Measurements of calcium were made gasometrically (45) and photoelectrically (35). The results deviated from stoichio-
metrical yields for samples containing less than 1.80 milliequivalents per liter, below which concentration they were accurately expressed by the straight line equation,

\[ \text{m.eq. Ca per liter sample present} = 0.86x + 0.25 \]

where \( x \) = milliequivalents of Ca per liter of sample found by analysis. Thus, under the conditions of analysis, up to amounts corresponding to a concentration of 0.25 milliequivalent per liter of the original sample, no calcium at all is detectable, and therefore none would be found in analyses of "reagent" blanks.\(^8\) Calcium as an impurity in the reagents to the extent of 10 per cent of normal serum calcium (0.50 milliequivalent per liter) would appear as an error of only 5.8 per cent in the "reagent" blank. Obviously, to measure the error from reagents, analyses should be made of samples at a concentration above 1.80 milliequivalents per liter, by the addition of known amounts of calcium, with "water" blanks for control.\(^9\)

Effect of Filtration through Filter Paper—Early in this work, in comparative analyses of serum, values for trichloroacetic acid filtrates passed through "ashless" filter papers were always higher (from 1 to 24 per cent) than the results by ashing or direct precipitation (Table II, Samples 1 to 20). These observations, which confirmed previous work (45), led to an extensive inquiry into the effect of filtration through filter papers, and the elimination of their use in microanalysis of calcium.

50 cc. volumes of mixtures of known serum-salt solutions and trichloroacetic acid (as in Series 3, Table I) were passed once through a 9 cm. filter paper, then analyzed directly as "filtrate" (a). The filter papers tested were Munktell's No. 00 "ashless," and Whatman's No. 2 and No. 5. Several of the "filtrates" were also analyzed after ashing (b). In such cases the results of groups (a) and (b) were in agreement, indicating the absence of any extraneous organic reduction of Ce(SO\(_4\))\(_2\) in passage through the filter.

Analyses of such "filtrates," in a test of twenty-four individual No. 00

\(^8\) Under the conditions of analysis, the actual concentrations in the diluted solutions from which calcium is precipitated will, of course, be one-seventh that of the original sample.

\(^9\) The above empirical results are quantitatively not strictly in accord with theoretical considerations based on the assumption of CaC\(_2\)O\(_4\) equilibrium between solid and liquid phases. However, the deviations are in no way related to inadequacies of measurement. Although the calcium in 1 cc. of 0.1 milliequivalent of solution would yield only 3 mm. in the gasometric measurement (45), the photoelectric method (35) is wholly adequate for the determination of corresponding amounts, i.e. 2 \( \gamma \), of calcium precipitated as oxalate from smaller volumes of more concentrated solutions. It is highly probable, since no solid CaC\(_2\)O\(_4\) was initially present, that supersaturation, with complete or partial failure to precipitate the calcium present, was responsible for the progressively greater deficiency in stoichiometrical yields from the more dilute samples.
papers selected at random, after correction for the reagent error, gave an average of 0.0016 (±0.0010) milliequivalent of "extra calcium" extracted per paper. This corresponds to an error of +3.2 per cent in the filtrate analysis of the equivalent of 1 cc. of normal serum. However, two of the papers gave errors of as much as 17 per cent. For two No. 2 papers and four No. 5 papers, the average extractions were 0.0105 and 0.0228 milliequivalent of calcium per paper, respectively.

In other experiments, the filter papers themselves were ashed and analyzed. The yields were 0.0012 (±0.00025), 0.0112, and 0.0256 milliequivalent of calcium per paper for eight No. 00, two No. 2, and two No. 5 papers, respectively. The ash weight given by the manufacturers, if calculated wholly as CaO, would correspond to +0.0011, +0.0419, and +0.0479 milliequivalent of calcium per paper, respectively.

Thus, there can be no doubt that varying, significant amounts of calcium may be extracted from filter paper by acidified serum filtrates. The failure of previous workers to notice this error is understandable. A priori, it would not seem necessary to pass a protein-free "blank" solution through an "ashless" filter paper, especially if the reagents had been tested "calcium-free" in the usual qualitative manner. Moreover, results of this and a preceding section show that even if such control were attempted the error (averaging +3.2 per cent) from most "ashless" papers would be undetectable or inaccurately determined in the usual "blank" analysis, in which errors up to 5 per cent would be completely masked. Finally, since the variability of the individual paper correction, however accurately determined, makes its application impossible, it is clear that filter paper should not be used in micro calcium analysis.

Effects of Individual Reagents—To verify the reagent errors indicated by the foregoing results on ashing (Series 2) and protein-free filtrate (Series 3), direct analyses were performed of each of the reagents used, with samples large enough to afford reliable values for calcium.

Samples of 2 cc. of the 1:1 ammonia solution used (6 and 2 drops in Procedures 2 and 3, respectively) were concentrated and dried on a steam bath, in platinum dishes. Analyses of the washings, with known calcium added as for Series 1 in Table I, showed no extra calcium. Samples of 2 cc. of the concentrated HCl from which was made the 1 N HCl used (6 cc. in Procedure 2), were likewise analyzed and showed a small calcium content, of doubtful significance, equivalent to +0.01 milliequivalent per liter for 6 cc. of 1 N HCl. Tests have shown that neither brom-cresol green nor caprylic alcohol affects the results.

Since a maximum error of only +0.01 milliequivalent per liter could be attributed to the other reagents of Series 2, it would seem, from Table I, that an error of +0.11 milliequivalent per liter in calcium values would arise from the use of 1 cc. of 20 per cent sodium acetate. To account more
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directly for error from this source alone, 2 gm. portions of the fused crystals (NaC2H3O2·3H2O) were dissolved in 2 cc. of concentrated HCl and water, and the solution evaporated to dryness. Analyses were carried out with known calcium added, as for Series 1 in Table I. Immediately upon the addition of oxalate, a copious white precipitate was obtained. Reacting with Ce(SO4)2, this gave varying yields of CO2, corresponding to as much as 100 per cent more oxalate than that expected from the calcium added.

Obviously, this could not be all calcium oxalate. That it represented a coprecipitation effect with a reduction in solubility of ammonium or sodium oxalate was indicated when equivalent amounts of NaCl (0.85 gm.) were similarly treated, no calcium being added. 48 hours after the addition of oxalate, a heavy white precipitate was formed, which was then washed several times with absolute alcohol. The material was soluble in water and gave an immediate white precipitate upon the addition of CaCl2. This precipitate was insoluble in water, easily soluble in 1 N H2SO4, and was identified microscopically as calcium oxalate.

Thus, at high concentrations of a sodium salt, not necessarily acetate, and in the absence of calcium, large amounts of non-calcium oxalate may be precipitated. At lower concentrations, under the conditions prescribed for stabilizing pH in analyses of ash and of protein-free filtrates, a similar phenomenon may possibly occur, the precipitated sodium or ammonium oxalate escaping solution in the subsequent washing procedure. This would be especially so were the contaminating precipitates occluded to the precipitated CaC2O4. In "reagent" blanks, in the absence of calcium, no trace of extra oxalate has been found. Similar types of contamination by the coprecipitation of sodium oxalate with calcium have been noted (7, 31, 33). Excess cations and acetate have also been cited as complicating factors in calcium analysis (2, 15, 14).

Although the extra oxalate was found only when calcium was precipitated, little calcium could be found in the sodium acetate itself. In a repetition of the above experiments with 2 gm. samples of sodium acetate, with added calcium, the supernatant fluid above the heavy precipitate was withdrawn as usual, and the precipitate dissolved in 1 cc. of 1 N HCl, then reprecipitated overnight. Following this, withdrawal of supernatant fluid, solution, and reprecipitation were again repeated, and the diminished bulk of precipitate washed and analyzed as usual. After correction for HCl and reprecipitation,10 an increase of +0.23 milliequiva-

10 In an independent series of analyses, when directly precipitated, unwashed calcium from known salt solutions was dissolved after withdrawal of the supernatant mother liquor, and then reprecipitated, the results were low by 0.10 milliequivalent per liter.
lent of calcium per liter was found, corresponding to a content of 0.02 milliequivalent of calcium per liter as an impurity in the 0.2 gm. of acetate used in procedures of Series 2 and 3. Apparently, in these analyses, an error of +0.09 milliequivalent per liter must be ascribed to the coprecipitation of non-calcium oxalate.

From Table I (Series 3a and 3b), an average error of +0.05 milliequivalent per liter in calcium values would arise from the use of 20 per cent trichloroacetic acid (1 cc. per 5 cc. of protein-free filtrate sample). A direct study of this point was made in analyses of the two lots of trichloroacetic acid used throughout this work. Samples of 50 cc. of 20 per cent solutions were boiled down to 5 to 10 cc., dried and heated on steam and sand baths, and finally ashed in an oven at 500°. The analyses were finished as in Series 2a with known calcium added in some of the determinations. In agreement with results in Table I, the excess calcium found corresponded to a content of 0.04 to 0.05 milliequivalent of Ca per liter, as an impurity in the 0.2 gm. of trichloroacetic acid used in Series 3.

Thus, for our ashing technique, a total correction of −0.12 milliequivalent per liter, or 2.4 per cent of normal serum values, is applicable to all results. The correction covers errors arising from calcium in 1 N HCl (+0.01 milliequivalent), calcium in sodium acetate (+0.02 milliequivalent), and the coprecipitation effect of sodium acetate (+0.09 milliequivalent). For analyses by deproteinization by trichloroacetic acid, the error of +0.05 milliequivalent for calcium present in the protein precipitant, added to the error from sodium acetate, makes the total correction −0.16 milliequivalent per liter, or 3.2 per cent of normal serum values. The individual corrections account for, and their sum total is in agreement with, the results recorded in Table I.

Effect of pH at Which Calcium Is Precipitated As Oxalate—The necessity of adjusting solutions to a pH optimum for CaC₂O₄ precipitation in the presence of other possibly interfering electrolytes (27, 28, 37) has often been emphasized. Thus, it has generally been the practice, in ashing and deproteinization procedures, to precipitate calcium oxalate at approximately pH 5.0. However, the factor of pH in the direct precipitation of calcium from serum has been neglected. One would expect such samples to be somewhat more alkaline than the upper limit of pH 5.6 (37). In fact, by glass electrode measurements of diluted sera (fresh and stale) after addition of oxalate, we have found pH values varying between 7.1 and 7.5. In view of this, and because McCrudden (28) and Shohl (37) carried out their studies under analytical conditions quite different from ours, the effect of pH in our analyses was studied.

In a series of analyses of known serum-salt solutions and sera, calcium
DETERMINATION OF SERUM CALCIUM

was precipitated directly, at various pH levels between 4.2 and 7.5. The pH values were set by adjustment with dilute HCl or ammonia water, and measured with the glass electrode (23). The calcium values varied only within the limits of analytical error, without relationship to pH. Thus, under the conditions of analysis described, calcium may be quantitatively precipitated from serum and from fluids of approximately the same electrolyte pattern as serum, without further adjustment, at pH within the limits of 4.0 to 7.5.

Analyses of Serum Samples

Human serum was obtained from clotted or defibrinated blood freshly drawn from ambulant dispensary patients. Samples of 1 cc. or its equivalent were analyzed according to the techniques described ("Procedure"). The corrections found (mean errors of Table I) for the analyses of known salt solutions by Procedures 1, 2, and 3, namely -0.01, -0.12, and -0.17 milliequivalent per liter, respectively, were applied to these results for serum. If the individual factors responsible for errors in serum analyses were the same as in salt solutions, corrected results for serum would show complete agreement among the three methods. If otherwise, discrepancies between such corrected results and the true values could then be ascribed to the effect of serum constituents other than the inorganic salts. Since organic constituents are eliminated in the ashing technique, the results (corrected) by that method were accepted as the standard, true values for calcium in serum.

Analyses by Direct Precipitation and by Ashing—For serum, analyses by direct precipitation were higher than by ashing (Table II, Series 1 and 2a), indicative not of an increased precipitation of calcium in the former method, but rather of a further reduction of the Ce(SO₄)₂ oxidant. The average discrepancy of +0.04 milliequivalent per liter (+0.8 per cent of normal) for twenty-eight analyses is just barely significant, and is attributable to the precipitation of calcium in the presence of reducing substances present in serum.

That the contaminating effect is not simply the result of an oxidation by Ce(SO₄)₂ of organic matter of serum retained after washing is indicated by the following. Serum blanks, consisting of 1 cc. of serum + 5 cc. of H₂O + 1 cc. of 2 per cent NaCl were compared with blanks of 6 cc. of H₂O + 1 cc. of 2 per cent NaCl. Contrary to previous results with two washings of serum mixtures twice as concentrated ((45) pp. 227–228), under our conditions of washing no serum effect was found. The reducing effect of serum occurs, therefore, only when the CaC₂O₄ is precipitated in the pres-

11 We are indebted to Miss Hester E. Reynolds and physicians of the Mercy Hospital–Loyola University Clinics for this material.
Table II
Results of Analyses of Calcium in Serum; Values Corrected for Mean Errors Found for Salt Solutions (Table I)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Analysis by ashing, Series 2a</th>
<th>Deviation from ashing (Series 2a) of analysis of Serum by direct pptn., Series 1</th>
<th>CCl₃COOH filtrate,* Series 3a</th>
<th>Ash of CCl₃COOH filtrate,* Series 3b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m.eq. per l.</td>
<td>m.eq. per l.</td>
<td>m.eq. per l.</td>
<td>m.eq. per l.</td>
</tr>
<tr>
<td>1</td>
<td>4.94</td>
<td>+0.01</td>
<td>+0.49</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.05</td>
<td>-0.07</td>
<td>+0.13</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.14</td>
<td>-0.02</td>
<td>+0.28</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.96</td>
<td>+0.10</td>
<td>+0.19</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.88</td>
<td>+0.09</td>
<td>+0.19</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.92</td>
<td>-0.03</td>
<td>+0.55</td>
<td>-0.70</td>
</tr>
<tr>
<td>7</td>
<td>4.69</td>
<td>+0.08</td>
<td>+0.55</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.73</td>
<td>+0.11</td>
<td>+0.41</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4.73</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>5.00</td>
<td>-0.01</td>
<td>+0.86</td>
<td>+0.72</td>
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<td>11</td>
<td>4.85</td>
<td>+0.13</td>
<td>+0.14</td>
<td>+0.22</td>
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<tr>
<td>12†</td>
<td>4.05</td>
<td>+0.08</td>
<td>+0.26</td>
<td>+0.25</td>
</tr>
<tr>
<td>13</td>
<td>5.04</td>
<td>+0.07</td>
<td>+0.16</td>
<td>+0.11</td>
</tr>
<tr>
<td>14†</td>
<td>5.04</td>
<td>0.00</td>
<td>+0.18</td>
<td>+0.21</td>
</tr>
<tr>
<td>15†</td>
<td>4.88</td>
<td>+0.05</td>
<td>+1.12</td>
<td>+1.24</td>
</tr>
<tr>
<td>16</td>
<td>4.95</td>
<td>+0.01</td>
<td>+0.14</td>
<td>+0.15</td>
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<tr>
<td>17†</td>
<td>4.88</td>
<td>+0.06</td>
<td>+0.29</td>
<td>+0.25</td>
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<tr>
<td>18†</td>
<td>4.85</td>
<td></td>
<td>+0.61</td>
<td></td>
</tr>
<tr>
<td>19†</td>
<td>4.85</td>
<td></td>
<td>+0.54</td>
<td>+0.50</td>
</tr>
<tr>
<td>20</td>
<td>5.00</td>
<td>+0.07</td>
<td>+0.54</td>
<td>+0.50</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>+0.043</td>
<td>+0.36</td>
<td>+0.41</td>
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<tr>
<td>21</td>
<td>5.04</td>
<td>-0.01</td>
<td>+0.12</td>
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<td>22†</td>
<td>4.84</td>
<td>+0.19</td>
<td>+0.14</td>
<td></td>
</tr>
<tr>
<td>23†</td>
<td>4.93</td>
<td>+0.16</td>
<td>+0.12</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>5.16</td>
<td>+0.07</td>
<td>+0.01</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>4.89</td>
<td>+0.07</td>
<td>+0.17</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>4.99</td>
<td>+0.08</td>
<td>+0.14</td>
<td></td>
</tr>
<tr>
<td>27†</td>
<td>4.79</td>
<td>-0.02</td>
<td>+0.07</td>
<td>+0.13</td>
</tr>
<tr>
<td>28†</td>
<td>4.95</td>
<td>-0.04</td>
<td>+0.09</td>
<td>+0.08</td>
</tr>
<tr>
<td>29†</td>
<td>4.96</td>
<td>-0.07</td>
<td>+0.19</td>
<td>+0.15</td>
</tr>
<tr>
<td>30‡</td>
<td>4.85</td>
<td>-0.02</td>
<td>+0.15</td>
<td>+0.16</td>
</tr>
<tr>
<td>31‡</td>
<td>4.91</td>
<td>+0.04</td>
<td>+0.04</td>
<td>+0.08</td>
</tr>
<tr>
<td>32</td>
<td>4.69</td>
<td>+0.02</td>
<td>+0.15</td>
<td>+0.12</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>+0.026</td>
<td>+0.12</td>
<td>+0.12</td>
</tr>
</tbody>
</table>

* The filtrates for the first group (Samples 1 to 20) were obtained by filtration through "ashless" filter paper; for the second group (Samples 21 to 32) by filtration of the centrifuged supernatant through a sintered glass filter.
† Fatty, more or less opaque serum.
‡ Contained 1 per cent by volume of hemolyzed erythrocytes.
Determination of Serum Calcium

ence of serum and is probably caused by occlusion or adsorption of protein on the precipitated crystals.

Analyses by Deproteinization and by Ashing—In the first group (Table II, Series 2a and 3a, Samples 1 to 20), filtrates were obtained by separation from the coagulated protein through Munktell's (9 cm.) No. 00 "ashless" filter paper. In the second group (Samples 21 to 32), filtrates were obtained by centrifugation, and filtration through sintered glass, as described under Procedure 3. In a test of the possibility of error in the precipitation of calcium oxalate in the presence of organic substances derived from serum and present in the trichloroacetic acid filtrate of serum (20, 29), the results for both groups were checked by analyses (Table II, Series 3b) of ashed portions of the same protein-free filtrates.

The corrected results show that filtrates through paper (first group) gave widely varying results, all higher (by 0.03 to 1.12 milliequivalents per liter) than the true values found by direct ashing of the serum. The increment (from 1 to 24 per cent) was not only widely different among all the samples, but large variations were sometimes found among portions of the same filtrate poured through different papers. Furthermore, the average increment of +0.36 milliequivalent per liter was not significantly different from that of +0.41 found by ashing the same filtrates. These positive errors, therefore, occur during the steps of deproteinization and filtration, and are not of organic origin, but definitely represent calcium.

In the second group of Table II, the error of the filtrate analyses was reduced, both in range of variation, and in average, to +0.12 milliequivalent per liter, statistically significant in difference from the true, directly ashed values. Ashing of the same filtrates, not poured through filter paper, gave values in complete agreement, also indicating an average error of about 2.5 per cent incurred in deproteinization (and filtration). Again, this increment actually represents calcium, and not an organic reduction of Ce(SO₄)₂ in measurement.

The difference in results for filtrate (Series 3a and 3b) for the two groups of Table II represents calcium acquired as an impurity in filtration through filter paper. The error of +0.12 milliequivalent per liter found when filter paper was not used must be regarded as common to both groups of measurements, and represents an increase in concentration of filtrate calcium incurred in the deproteinization process itself. In direction and in magnitude, this error corresponds to the effect of volume displacement of fluid by protein precipitate, as found in other methods in which aliquot portions of protein-free filtrates are used for analysis (42, 34, 13).

Analyses of Previously Decalcified Serum with Known Calcium Added—These experiments served (1) to check the reagent errors for serum due to ashing and deproteinization, with elimination of the complicating factor.
of *volume displacement* from the latter, and (2) to prove conclusively that calcium can be completely removed from serum by direct precipitation.

In proportions previously used ((15) p. 228) 2 volumes of serum, 3 of water, and 1 of ammonium oxalate were mixed, and the calcium precipitated overnight in two tubes. The dilute sera were then centrifuged and the supernatant fluids poured through sintered glass filters. To the ash and to the trichloroacetic acid filtrate of portions of this decalcified material equivalent to 1 cc. of serum, were added known amounts of calcium. Analyses were performed according to Procedures 1 and 3. As an additional control, errors due to the deproteinization reagents were also determined. The known salt solutions were analyzed in each case, in the presence of the same reagents used in the analysis of the decalcified serum sample filtrate, exactly as described for Procedure 3a.

### Table III

Analyses of Ash and Trichloroacetic Acid Filtrates of Previously Decalcified Serum with Known Calcium Subsequently Added (5.00 Milliequivalents per Liter)

The results are expressed in milliequivalents per liter.

<table>
<thead>
<tr>
<th>Calcium added to</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
<th>Sample 6</th>
<th>Sample 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Ash of decalcified serum.</td>
<td>5.14</td>
<td>5.10</td>
<td>5.12</td>
<td>5.13</td>
<td>5.13</td>
<td>5.28</td>
<td>5.16</td>
</tr>
<tr>
<td>II. Trichloroacetic acid filtrate of decalcified serum</td>
<td>5.16</td>
<td>5.11</td>
<td>5.15</td>
<td>5.08</td>
<td>5.17</td>
<td>5.28</td>
<td>5.19</td>
</tr>
<tr>
<td>III. Deproteinization reagent blank</td>
<td>5.19</td>
<td>5.13</td>
<td>5.08</td>
<td>5.13</td>
<td>5.18</td>
<td>5.32*</td>
<td>5.15*</td>
</tr>
</tbody>
</table>

* Ash of deproteinization reagent blank, + Ca = 5.32 for Sample 6; 5.15 for Sample 7.

Table III constitutes a complete verification of the results presented in previous sections. In Line III, with the exception of Sample 6, the error for deproteinization reagents is in statistical agreement with the average error of +0.16 milliequivalent per liter previously found (Table II). The agreement of results in Line II with those in Line III indicates that the error for deproteinization reagents remains the same, even in the presence of serum protein-free filtrate. Likewise, the results of the ashed serum, Line I, are in statistical agreement with the error for the ashing reagents of +0.12 milliequivalents per liter.

We have no explanation for these results, which indicate a consistently high error for the three different types of analyses of Sample 6 carried out the same day. Failure of complete preliminary decalcification of the serum is not a factor, since the same error was found for deproteinization reagents used with salt solution alone (Line III). Use of the same reagents for simultaneous serum analyses (Table II, Sample 30) resulted in little more than the expected average error.
equivalent found for salt solutions (Table I, Series 2a). The results of Table III show clearly that the native calcium in the original whole serum was completely precipitated in the preliminary, direct decalcification.

Procedure 3 for trichloroacetic acid filtrates was also carried out on decalcified serum to which no calcium was subsequently added, with filtration through Munktell's No. 00 filter paper instead of sintered glass. The "extra calcium recovered" from these filtrates (from 0.22 to 0.80 milliequivalent per liter) was of the order of magnitude previously found (45), and now proved to represent a summation of filter paper and reagent errors, and not residual calcium unprecipitated from serum by the direct (decalcification) procedure.

**DISCUSSION**

The foregoing results are indicative of the origin of most of such errors in analyses of serum calcium as occur in the precipitation of calcium oxalate under different conditions. Failure of previous workers to reconcile results by such different methods may be explained in most cases by the fact that some of these errors have hitherto been unrecognized, while others, although anticipated, have escaped detection or adequate control by the usual tests or blank analyses for contaminants. Parallel and comparative analyses of known calcium solutions, however, furnish corrections suitable for each type of analysis and lead to complete agreement in results, whether the serum calcium is precipitated directly, from the ash, or from the deproteinized sample.

Some of these errors are common to all analyses of the same type, regardless of the sample used. Filter papers have been found to contain calcium. Consequently, their use leads to contamination of the sample. Because the error varies from one paper to another, filtrates or supernatant fluids in micro calcium analysis should be centrifuged, or passed only through sintered glass. In our experiments, the conditions of which correspond to those of prevailing techniques, calcium has not been quantitatively precipitated, if at all, when present in concentrations less than 0.26 milliequivalent per liter (1.80 milliequivalents per liter of original sample). This observation, therefore, not only accounts for the failure to detect in blank analyses calcium present in small amounts as an impurity in reagents, but also casts doubt on the validity of most serum calcium values in the literature lower than 3 mg. per 100 cc. of original sample. It is clear that errors of contamination from reagents, whether from calcium as an impurity or oxalate as a coprecipitate, must be evaluated under actual analytical conditions in the presence of calcium in known, normal serum concentration.

Other errors occur only in serum analyses. Proteins in serum give rise to additional errors not reproducible in, or controllable by the analysis of
known salt solutions. The reduction of the oxidant by protein in the
direct precipitation method and the effect of volume displacement in the
deproteinization procedure cause positive errors of the order of magnitude
of 0.8 and 2.4 per cent, respectively. Correction for these errors may be
applied only on the basis of many comparisons of results (all corrected for
reagents) with those obtained by ashing. For analysis of serum calcium,
although the ashing procedure is theoretically the best for standardization
purposes, the method of choice involving the minimum of technical detail
and correction for error appears to be the direct precipitation procedure.
Analyses of deproteinized serum are the least satisfactory in that a greater
number of errors may occur.

That a variation in the foregoing errors, or the occurrence of others not
found or considered in this work, may arise from differences in the chemical
composition of pathological sera remains a possibility, despite the fact
that within the range of pathology presented by clinical material from
dispensary patients no such factor of error in any of the methods used has
been observed in our experiments.13

SUMMARY

A comparative study has been made of the factors of analytical error
involved in the precipitation of serum calcium as oxalate, under various
conditions. Analyses of known salt solutions and of serum, by direct
precipitation, ashing, and deproteinization techniques, indicate the fol-
lowing as sources of error: contamination by calcium from filter paper and
reagents and by non-calcium oxalate in coprecipitation, the reducing ac-
tion of serum in direct precipitation, and the fluid volume displacement of
proteins in deproteinization. Together with an explanation of their origin,
methods of eliminating these errors or of evaluating them accurately have
been presented. The application of such corrections quantitatively recon-
ciles the apparent discrepancies among the three methods. In these
analyses, calcium oxalate may be precipitated at pH from 4.0 to 7.5.
For both accuracy and simplicity, the technique of direct precipitation is,
in most cases, the method of choice for the determination of serum cal-
cium as oxalate.

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13 It has been reported that fatty or opaque sera may not be satisfactorily analyzed
by the direct precipitation method (Tingey (40)). Our data (Table II), although
they reveal no such trend, are too few in this respect for any conclusion. The same
may be said of the effect of hemolysis (Table II), to which Muller (29) has ascribed
large errors in the analysis of trichloroacetic acid filtrates.
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DETERMINATION OF SERUM CALCIUM BY PRECIPITATION WITH OXALATE: A COMPARATIVE STUDY OF FACTORS AFFECTING THE RESULTS OF SEVERAL PROCEDURES

Julius Sendroy, Jr.

*J. Biol. Chem.* 1944, 152:539-556.

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