THE IONIZATION OF CALCIUM, MAGNESIUM, AND STRONTIUM CITRATES*

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Since the postulation by Greenwald (1) of a citrate-like substance in blood, capable of holding calcium in a non-ionized form, the behavior of calcium in the presence of citrate has received considerable attention from investigators primarily interested in the state of calcium in the living organism. The subject has also assumed considerable importance in the field of the chemistry of milk.

The hypothesis that citrate forms a complex ion with calcium, invoked by Sabbatani (2) in 1901 to explain the effect of citrate in preventing the coagulation of blood, has received support from the work of Shear and Kramer (3), Shear, Kramer, and Resnikoff (4), Greenberg and Greenberg (5), Hastings, Murray, and Sendroy (6), Clark, Percival, and Stewart (7), Shelling and Maslow (8), Peretti (9), Minot, Dodd, and Bryan (10), Mulli and Standenath (11), and others. On the other hand, Salant and Hecht (12) and Salant and Swanson (13) failed to find support for this hypothesis in certain biological experiments.

Previous investigations have failed to lead to definite conclusions as to the nature of the reaction occurring between calcium and citrate in solution, and have furnished insufficient information for a quantitative analysis of this reaction, owing chiefly to the fact that no satisfactory method for the direct observation of calcium ion concentrations has been available. A new approach


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to the problem was made possible by the introduction of such a method by two of the authors (14) and the present paper includes the results obtained by this method, coupled with corroborative evidence secured by indirect methods, both in the hands of the authors and of others.

Symbols

In addition to the usual chemical symbols, the following are employed in this paper.

\[ [ ] = \text{concentration, in moles per kilo of H}_2\text{O} \]
\[ \mu = \text{ionic strength} \]
\[ p = \text{negative logarithm} \]
\[ \text{Cit}^3^- = \text{trivalent citrate ion, C}_6\text{H}_5\text{O}_7^- \]
\[ \text{CaCit}^- = \text{negatively charged calcium citrate complex ion, CaC}_6\text{H}_5\text{O}_7^- \]

Theoretical

The hypothesis which this paper seeks to substantiate may be stated as follows:

Calcium citrate, \( \text{Ca}_2\text{Cit}_2 \), ionizes in two stages. The primary dissociation may be represented by the following equation:

\[ \text{Ca}_2\text{Cit}_2 = \text{Ca(CaCit)}_2 \rightleftharpoons \text{Ca}^{++} + 2\text{CaCit}^- \] (1)

This dissociation appears to be complete, or very nearly complete, in dilute solutions, in accordance with the theory of strong electrolytes. For this to be true the concentration of \( \text{Ca}^{++} \) in any dilute solution of \( \text{Ca}_2\text{Cit}_2 \) must be equal to, or greater than, one-third of the total calcium present; or, to state the more general case for varying mixtures of calcium and citrate,

\[ \text{Total Ca} - \text{Ca}^{++} \cong \text{total citrate} \] (2)

These conditions have been found to be fulfilled.

The secondary dissociation may be represented by the equation

\[ \text{CaCit}^- \rightleftharpoons \text{Ca}^{++} + \text{Cit}^3^- \] (3)

This dissociation is incomplete, and is that of a weak electrolyte. The mass law equation for this dissociation may be written

\[ \frac{[\text{Ca}^{++}] \times [\text{Cit}^3^-]}{[\text{CaCit}^-]} = K \] (4)\(^1\)

\(^1\) It will be noted that \( [\text{Ca}^{++}] \) includes \( \text{Ca}^{++} \) derived not only from the secondary dissociation of \( \text{CaCit}^- \), but also from the primary dissociation (Equation 1) and from any other calcium salt in the solution.
or in logarithmic form

\[(5) \quad p\text{Ca}^{++} + p\text{Cit}^- - p\text{CaCit}^- = p\text{K}_{\text{CaCit}^-}\]

Proof of the correctness of this hypothesis rests upon the finding of constant values for \(p\text{K}_{\text{CaCit}^-}\). Other hypotheses as to the nature of the combination of calcium with citrate, and of its ionization, which we have examined, were inconsistent with this finding.

Determination of \(p\text{K}_{\text{CaCit}^-}\) depends upon the possibility of securing data concerning the concentration of \(\text{Ca}^{++}\), \(\text{Cit}^-\), and \(\text{CaCit}^-\) in varying mixtures of calcium and of citrate. With total calcium and \(\text{Ca}^{++}\) concentrations known, \(\text{CaCit}^-\) may be calculated by difference. At reactions above pH 7.0, the citrate in solution may be regarded as being entirely in the form of \(\text{Cit}^-\) or its salts (15). With total citrate and \(\text{CaCit}^-\) known, \(\text{Cit}^-\) concentrations may be obtained by difference. The determination of \(p\text{K}_{\text{CaCit}^-}\), therefore, can be made to depend upon determination of the concentrations of total calcium, total citrate, and \(\text{Ca}^{++}\).

By substituting \((\text{total Ca} - [\text{Ca}^{++}])\) for \([\text{CaCit}^-]\) and \((\text{total Cit} - \text{total Ca} + [\text{Ca}^{++}])\) for \([\text{Cit}^-]\) Equation 4 takes the form

\[(6) \quad \frac{[\text{Ca}^{++}] \times ((\text{total Cit}) - ([\text{total Ca}] + [\text{Ca}^{++}]))}{([\text{total Ca}] - [\text{Ca}^{++}])} = K\]

If we solve for \([\text{Ca}^{++}]\),

\[(7) \quad [\text{Ca}^{++}] = \frac{\sqrt{4K(\text{total Ca}) + ([\text{total Cit}] - ([\text{total Ca}] + K)^2 - ([\text{total Cit}] + ([\text{total Ca}] - K)^2)}}{2}\]

It will be noted that in the above equations stoichiometric designations have been used throughout. It appears probable that \(\text{Ca}^{++}\) values obtained by the methods used are stoichiometric, and this assumption is followed when values of \(\text{CaCit}^-\) and \(\text{Cit}^-\) are determined by difference, the values for total calcium and total citrate being stoichiometric. The methods used for estimation of \(\text{Ca}^{++}\) concentrations also imply that the salts \(\text{CaCl}_2\) and \(\text{Ca(HCO}_3\text{)}_2\) may be regarded as completely dissociated. Proof to the contrary would require correction of the values for \(\text{Ca}^{++}\) in the presence of citrate, and a corresponding change in the values for \(p\text{K}_{\text{CaCit}^-}\).
Determinations of the constant $pK_{CaCit^-}$ have been carried out in solutions of approximately constant ionic strength and varying calcium and citrate concentrations. The constant has also been determined, with a lesser degree of accuracy, for the similar combinations of magnesium and strontium with citrate.

**EXPERIMENTAL**

**Methods**

**Total Calcium**—Total calcium has been determined by the method of Kramer and Tisdall (16) as modified by Clark and Collip (17). In the case of solutions used in the direct method for Ca$^{++}$ estimations, reliance has been put on stock calcium solutions accurately standardized by gravimetric analysis and accurately diluted, the Kramer and Tisdall method being used as a check against contamination with calcium from other sources or loss of calcium by precipitation.

**Total Citrate**—The citrate content of the solutions was determined by a gasometric method with the Van Slyke and Neill (18) manometric apparatus. It was found that oxidation in the cold with $0.1\text{ N} \cdot \text{KMnO}_4$ in strongly acid solution gave a constant amount of CO$_2$ per mole of citrate. For the conditions of our analyses, 4.62 moles of CO$_2$ per mole of citrate were obtained. This is in substantial agreement with the results obtained by Kuyper (19) using the same method. Gasometric determinations on sodium citrate solutions were checked from time to time by gravimetric sodium determinations. For the solutions used for direct estimation of Ca$^{++}$ reliance was put on accurately standardized stock solutions, accurately diluted.

**Carbon Dioxide**—CO$_2$ analyses were made with the Van Slyke and Neill manometric gas apparatus.

**Hydrogen Ion Concentration**—The pH of solutions was determined colorimetrically, with phenol red as indicator and bicolor standards. These were checked against phosphate buffers which had been standardized electrometrically.

**Sodium**—Sodium was determined gravimetrically by the Butler and Tuthill method (20).

**Chlorides**—Chlorides were determined gravimetrically, by precipitation as AgCl.
Calcium Ions. Direct Method—The method is that described by McLean and Hastings (14) and consists essentially in the direct comparison and matching of known solutions with solutions of similar composition, but with unknown Ca\(^{++}\) concentrations, the criterion for equal Ca\(^{++}\) concentration being an identical response of the amplitude of contraction of the ventricle of the isolated heart of the frog, as recorded on a drum.

Equilibration with Solid CaCO\(_3\)—The solutions used in this series were isotonic with mammalian plasma and had an ionic strength of approximately \(\mu 0.160\), NaCl being replaced in the solutions by varying amounts of isotonic sodium citrate solutions.

The solutions were brought into equilibrium with gas composed of 5.5 per cent CO\(_2\) and 94.5 per cent O\(_2\), and solid CaCO\(_3\) was added (approximately 1 gm. per 100 cc.). Equilibration in closed vessels was then carried out in a water bath at 38\(^\circ\), after which the solid phase was removed by centrifugation and the supernatant liquid analyzed for total citrate, total calcium, CO\(_2\), and pH.

By bringing solutions containing varying amounts of citrate into equilibrium with solid CaCO\(_3\) at a fixed CO\(_2\) tension the Ca\(^{++}\) concentration could be calculated from the relation

\[
[\text{Ca}^{++}] \times [\text{CO}_3^{--}] = K_{s.p,\text{CaCO}_3} = 4.0 \times 10^{-8} \text{ at } \mu 0.160
\]

the value for the constant being that found by Hastings, Murray, and Sendroy (6).

To determine the Ca\(^{++}\) concentration by this method two criteria must be satisfied: (1) equilibrium between the liquid and solid phases must be established; (2) the concentration of CO\(_3^{--}\) must be known.

1. Starting with no calcium in solution, we found that equilibrium was reached within 1 hour. Longer equilibration up to 31 hours did not alter the amount of CaCO\(_3\) dissolved. This equilibrium was not altered by changing the solid phase during the equilibration. Much time and effort were spent trying to reach equilibrium in citrate-containing solutions which were originally supersaturated with CaCO\(_3\), before it was found that this is impossible within a reasonable length of time. Solutions equilibrated for a month remained definitely supersaturated. Experiments on the solubility of CaCO\(_3\) in citrate-containing...
solutions are therefore definitely limited to solutions initially undersaturated with respect to CaCO₃.

It may be noted in passing that it is well known that the presence of small amounts of certain chemical substances in solution results in delaying equilibrium between supersaturated solutions of salts and their corresponding solid phases. A systematic study of this phenomenon, which is obviously of biological importance, is now being carried out, between calcium salts and solutions containing substances which are present in biological fluids and which delay equilibrium.

2. The CO₃⁻ concentration was calculated from the pH and CO₂ content, by the constants and tables of Hastings, Murray, and Sendroy (6).

Numerical Value of pK_{CaCit⁻} by the Frog Heart Method

From the earliest experiments it was found possible, with given standard solutions of CaCl₂ and Na₂Cit, to determine values for pK_{CaCit⁻}, by use of the frog heart method, within a limit of error of approximately ±0.02, which indicates a high degree of accuracy for the frog heart method under the conditions of these experiments. In the accompanying paper (14) it was pointed out that the method is most accurate with solutions of which the composition can be accurately controlled, and in a series of six solutions with the calcium concentrations unknown to the observer the maximum error was 0.025 mM per liter and the average deviation ±0.019 mM per liter.

Solutions containing citrate provide equally favorable conditions for observations by this method. There are no interfering pressor effects, such as are seen in the use of biological fluids. A favorable range can be chosen, and with a particularly sensitive preparation Ca++ concentrations can be estimated to within 0.02 to 0.03 mM per liter. In the series of thirty-eight observations used in arriving at the numerical value of pK_{CaCit⁻} (Fig. 1) the maximum deviation of observed from calculated Ca++ concentration was 0.07 mM per liter and the average deviation ±0.014 mM per liter. In a series of 87 observations a deviation as large as 0.10 mM per liter has been encountered only once.

All determinations of pK_{CaCit⁻} by the frog heart method were carried out under the following conditions.
Temperature—This was room temperature, usually 22–23°C. No change in values for pH\textsubscript{CaCl} could be demonstrated by varying the temperature from 15–25°C, and solutions of equal physiological activity with respect to the frog's heart at 25°C were also of equal activity with respect to the rabbit's heart at 38°C, indicating that between 15–38°C the influence of temperature upon the ionization of calcium citrate is small.

The hydrogen ion concentration was approximately 7.4.

Ionic Strength, Osmotic Pressure, and Sodium Content of Solutions—All solutions used were isotonic with mammalian plasma. The standard solutions of reference had an ionic strength of \(\mu\) 0.154 to \(\mu\) 0.155, and a sodium content of approximately 0.146 mole per liter. With the replacement of sodium chloride by isotonic citrate and calcium solutions, the ionic strength rises, owing to the effect of the divalent and trivalent ions. For this reason, determination of the constant pH\textsubscript{CaCl} was carried out at calcium concentrations not to exceed 4.0 mM per liter. Under these conditions the sodium content of the unknown solutions varied from 0.146 to 0.148 mole per liter, and the ionic strength from \(\mu\) 0.155 to \(\mu\) 0.163.

Standard Solutions

An error of 1 per cent in the concentration of citrate in the solutions leads to an error of approximately 0.01 in the value of pH\textsubscript{CaCl}. The error in the constant introduced by an error in the calcium concentration is slightly less, owing to the fact that the same stock solutions are used in the known and unknown solutions. Solutions of sodium citrate may gradually change slightly with respect to their capacity to bind calcium, without change in the yield of CO\textsubscript{2} on gasometric analysis. For example, stock solutions of sodium citrate and calcium chloride which gave a value of 3.22 for pH\textsubscript{CaCl} on September 20, 1933, gave a value of 3.15 on December 18, 1933, after standing at room temperature during the intervening time, indicating a loss of about 7 per cent in calcium-binding power. Gasometric analysis showed no change in yield of CO\textsubscript{2}. The same solution of calcium chloride, with a fresh solution of citrate, again gave a value of 3.22 for pH\textsubscript{CaCl}. This change has not been further investigated.

It was regarded as desirable to determine the value for pH\textsubscript{CaCl}
as accurately as possible by the frog heart method, by careful control of the standard solutions. To this end the following standard solutions were prepared, and used while fresh, and only the results so obtained have been taken into account in arriving at the value of the constant.

Calcium Chloride. Solution A—A solution of CaCl₂, c.p., was made up to approximately 110 mM per liter. On analysis 114.4 mM per liter (macrogravimetric) of Ca and 226.2 mM per liter (macrogravimetric) of Cl were found. A probable value of 114.4 mM per liter for Ca was adopted.

Solution B—Pure calcium carbonate (calcite) was dried to constant weight. 2.0014 gm. of this substance (20.0 mM) were dissolved in a slight excess of HCl, the excess of HCl was neutralized with NaOH, and the whole diluted to 200 cc. with distilled water. On analysis (macrogravimetric), 99.85 mM per liter of Ca were found. A probable value of 100.0 mM per liter for Ca was adopted.

Sodium Citrate. Solution X—A solution of trisodium citrate was made up to approximately 77 mM per liter. On analysis 75.0 mM per liter (gasometric) of citrate and 219.0 mM per liter (macro-
gravimetric) of Na were found. A probable value of 73.0 mM per liter for citrate was adopted.

**Solution Y**—A solution of citric acid (c.P.) was titrated with 0.5 N NaOH until the end-point with phenol red showed complete neutralization of the citric acid (pH 7.0). Sufficient water was then added to dilute the Na concentration to 231 mM per liter. On analysis 75.7 mM per liter (gasometric) of citrate and 229.0 mM per liter (macrogravimetric) of Na were found. A probable value of 76.0 mM per liter for citrate was adopted.

![Fig. 1. Distribution of values found for pKCaCit- by the frog heart method.](http://www.jbc.org/)

The standard calcium and citrate solutions were then paired and pKCaCit- was determined by the frog heart method. A typical protocol is shown in Table I.

The distribution of values found for pKCaCit- by the frog heart method in the series of four experiments with the above standard solutions is shown in Fig. 1. Calculation from these values, by averaging the values for $K$, gives a mean value of $3.215 \pm 0.018$ for pKCaCit-. In order to avoid introducing an insignificant figure into the constant, this has been rounded off, and a value of $3.22 \pm 0.025$ adopted for pKCaCit- under the conditions of temperature, pH, and ionic strength as described.
The mean values for $pK_{\text{CaCit}^-}$ obtained from different experiments show a slight variation. For example, the mean value for $pK_{\text{CaCit}^-}$ from $\text{CaCl}_2$ (A) and $\text{Na}_2\text{Cit}$ (X), as shown in Fig. 1, is 3.22, while the corresponding value from $\text{CaCl}_2$ (B) and $\text{Na}_2\text{Cit}$ (X) is 3.20. The difference corresponds, under the conditions of these experiments, to an average deviation of 0.02 mM per liter in observed Ca$^{++}$ concentrations, is within the limit of accuracy as arrived at for the constant, and is attributed to experimental error.

With $pK_{\text{CaCit}^-}$ known, Ca$^{++}$ may be calculated from total Ca and total citrate (Equation 7). Fig. 2 is a Cartesian nomogram prepared to facilitate this calculation, the value of 3.22 being taken for $pK_{\text{CaCit}^-}$.

The nomogram is most easily constructed with the aid of its general regression equation

\[ [\text{Total Ca}] = \frac{[\text{Ca}^{++}] \times [\text{total Cit}]}{[\text{Ca}^{++}] + K} + [\text{Ca}^{++}] \]
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which is obtained by solving Equation 6 for [total Ca], and from which the slope of iso-Ca++ lines is readily calculated.

As noted above, values for Ca++ concentrations observed by the frog heart method agree very closely (average deviation ±0.014 mM per liter) with those calculated by use of the nomogram, provided that the calcium and citrate concentrations are accurately known (±0.5 per cent).

Table II

Equilibration of Solutions Containing Citrate with CaCO₃

Temperature, 38°; time, 30 hours.

<table>
<thead>
<tr>
<th>Solution No.</th>
<th>Determined values</th>
<th>Ca++</th>
<th>Calculated values*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>mM per l.</td>
<td>mM per l.</td>
</tr>
<tr>
<td>1</td>
<td>7.37</td>
<td>24.03</td>
<td>1.34</td>
</tr>
<tr>
<td>2</td>
<td>7.48</td>
<td>23.50</td>
<td>1.04</td>
</tr>
<tr>
<td>3</td>
<td>7.34</td>
<td>26.01</td>
<td>2.17</td>
</tr>
<tr>
<td>4</td>
<td>7.27</td>
<td>25.10</td>
<td>2.46</td>
</tr>
<tr>
<td>5</td>
<td>7.42</td>
<td>26.76</td>
<td>2.85</td>
</tr>
<tr>
<td>6</td>
<td>7.52</td>
<td>28.02</td>
<td>3.23</td>
</tr>
<tr>
<td>7</td>
<td>7.48</td>
<td>27.81</td>
<td>3.15</td>
</tr>
</tbody>
</table>

Means: 0.06

* Assuming Ca++ as calculated from Equation 8.

pK_{CaCit} As Determined by Equilibration Experiments

The values for pK_{CaCit} as determined by equilibration experiments with CaCO₃ as the solid phase are shown in Table II.

It will be noted from Table II that the concentrations of Ca++ as calculated from the solubility product of CaCO₃ (Equation 8) are slightly lower than those calculated from the mass law relationship between calcium and citrate (Equation 7).

This results in the value for pK_{CaCit} being slightly higher, as determined by the equilibration method, than that determined...
by the frog heart method (3.31 as compared with 3.22). Considering the fact that the two values were obtained by totally different methods, and at different temperatures, and that the difference depends upon very small differences in Ca++ concentrations, as arrived at by different methods of calculation, the authors are not inclined to attach significance to the difference between the two values.

**Specificity of the Frog Heart Method**

Agreement of the values for $pK_{Ca^{2+}}$ found by the frog heart method with those found by equilibration experiments indicates also that the frog heart is unaffected by CaCit$^-$ or by Cit$^-$ in the concentrations found in our solutions, since if it were sensitive to these constituents the results by the two methods could not be in agreement.

Additional evidence to the same effect is found in the observation that solutions containing total calcium concentrations up to 30.0 mM per liter and correspondingly high citrate concentrations ($\pm 40.0$ mM per liter) are found to be physiologically isoactive with solutions containing, for example, 1.0 mM per liter of calcium and without citrate. Such a solution gives a value for $pK_{Ca^{2+}}$ of about 3.32, on the assumption that its Ca$^{2+}$ concentration is the same as that of the solution without citrate with which it is isoactive, and it is assumed that this slight change in $pK_{Ca^{2+}}$ is due to the known effect of the increased ionic strength ($\mu \pm 0.200$) upon the physiological activity of Ca$^{2+}$. A solution containing $30$ mM per liter of total calcium, isoactive with $1.0$ mM per liter of Ca$^{2+}$, must contain not less than $29.0$ mM per liter of calcium in combination with citrate (CaCit$^-$), and a high concentration ($11.0$ mM) of Cit$^-$.

It seems apparent that these ions, even in these high concentrations, are exerting no appreciable effect upon the heart, since any assumption to the contrary cannot be reconciled with approximately constant values for $pK_{Ca^{2+}}$ or with physiological isoactivity with solutions of low Ca$^{2+}$ concentration. It is therefore concluded that in these experiments the frog heart method is specific for Ca$^{2+}$, and that CaCit$^-$ and Cit$^-$ are inert with respect to the preparation.
That magnesium forms with citrate a compound resembling that of calcium with citrate, and with a degree of dissociation of the same order of magnitude, is apparent from the conductivity measurements of Mulli and Standenath (11).

It was found possible to utilize the frog heart method for indirect determination of $pK_{\text{MgCit-}}$. Mg$^{++}$ up to concentrations of 5.0 mM per liter has a very slight effect upon the amplitude of contraction of the frog's heart. When, however, magnesium was added to solutions containing both calcium and citrate, a marked increase in amplitude of contraction occurred. This effect was attributed to the combination of magnesium with citrate, thereby liberating Ca$^{++}$.

On the basis of this hypothesis the calculation of $pK_{\text{MgCit-}}$ was carried out. Solutions of known total magnesium, total calcium, and total citrate were prepared and their Ca$^{++}$ concentrations were estimated by the frog heart method. The following equations were used.

\begin{align*}
(10) \quad \text{CaCit}^- &= \text{total Ca} - \text{Ca}^{++} \\
(11) \quad p\text{Cit}^- &= pK_{\text{CaCit-}} - p\text{Ca}^{++} + p\text{CaCit}^- \\
(12) \quad \text{Cit}^- &= - \text{antilog } p\text{Cit}^- \\
(13) \quad \text{MgCit}^- &= \text{total Cit} - \text{CaCit}^- - \text{Cit}^- \\
(14) \quad \text{Mg}^{++} &= \text{Mg} - \text{MgCit}^- \\
(15) \quad pK_{\text{MgCit-}} &= p\text{Mg}^{++} + p\text{Cit}^- - p\text{MgCit}^- 
\end{align*}

After these calculations were found to give approximately constant values for $pK_{\text{MgCit-}}$, each experiment was planned to give approximately equal values for Mg$^{++}$ in the unknown solutions, and a concentration of MgCl$_2$ equivalent to the calculated Mg$^{++}$ concentration in the unknown solutions was added to the standard known solutions. The protocol of a typical experiment is given in Table III.

It is not to be expected that the method will yield values for $pK_{\text{MgCit-}}$ of such a high degree of constancy as those for $pK_{\text{CaCit-}}$, owing to the fact that a small error in the observed Ca$^{++}$ concentrations introduces a large error in the value for $pK_{\text{MgCit-}}$. Fig. 3 shows the results of all observations made with Mg$^{++}$ concentrations compensated for in the standard solutions. Averaging the values for $K$ gives a mean value of 3.25 ± standard deviation 0.19.
for $pK_{Mg}Cit^-$. It appears that this value is indistinguishable from that of $pK_{Ca}Cit^-$, on the basis of these experiments, and we have consequently adopted 3.22 as the value for both constants.

**TABLE III**

_Determination of $pK_{Mg}Cit^-$ by the Frog Heart Method_

The standard solutions of reference contained 2.5 mM per liter of MgCl₂. $pK_{Ca}Cit^-$ for solutions used = 3.23.

<table>
<thead>
<tr>
<th>Solution No.</th>
<th>Total Ca mM per l.</th>
<th>Total Mg mM per l.</th>
<th>Total citrate mM per l.</th>
<th>Observed $Ca^{++}$ mM per l.</th>
<th>Calculated $Ca^{++}$ mM per l.</th>
<th>Calculated $Mg^{++}$ mM per l.</th>
<th>$pK_{Mg}Cit^-$</th>
<th>Calculated pKm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.94</td>
<td>3.00</td>
<td>0.90</td>
<td>0.14</td>
<td>0.09</td>
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<td>0.85</td>
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<td>3.75</td>
<td>2.04</td>
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<td>3.25</td>
<td>0.85</td>
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<td>3.20</td>
<td>0.71</td>
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<td>0.85</td>
</tr>
<tr>
<td>4</td>
<td>2.09</td>
<td>6.00</td>
<td>5.50</td>
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<td>1.48</td>
<td>6.38</td>
<td>3.22</td>
<td>0.90</td>
</tr>
</tbody>
</table>

**Fig. 3.** Distribution of values found for $pK_{Mg}Cit^-$ by the frog heart method.

_Calculation of $Ca^{++}$ Concentrations in Mixtures of Calcium, Magnesium, and Citrate_

With identical values for $pK_{Ca}Cit^-$ and $pK_{Mg}Cit^-$, and with both calcium and magnesium in equilibrium with Cit⁻, a mass law equation may be written as follows:

\[
\frac{([Ca^{++}] + [Mg^{++}]) \times [Cit^-]}{([CaCit^-] + [MgCit^-])} = 10^{-3.22}
\]
(\([\text{Ca}^{++}] + [\text{Mg}^{++}]\)) may be calculated from Equation 7, or from the nomogram (Fig. 2), by substituting \((\text{[Ca}^{++}] + [\text{Mg}^{++}]\)) for \([\text{Ca}^{++}]\) and \(((\text{total Ca}) + (\text{total Mg}))\) for \([\text{total Ca}]\). Since in any mixture of calcium, magnesium, and citrate, \(\text{Ca}^{++}\) and \(\text{Mg}^{++}\) will be present in the same proportions as total calcium and total magnesium,

\[
[\text{Ca}^{++}] = \frac{(\text{total Ca})}{(\text{total Ca} + \text{total Mg})} \times ([\text{Ca}^{+}] + [\text{Mg}^{+}])
\]

The final column of Table III includes \(\text{Ca}^{++}\) concentrations calculated by this method, for comparison with observed values. \(\text{Mg}^{++}\) concentrations may, of course, be similarly calculated.

**Strontium Experiments**

As has been shown by Ringer (21) and others, strontium is capable of replacing calcium in solutions used for maintaining the contractions of the isolated heart of the frog. It was therefore a simple matter, first, to determine that strontium is bound by citrate in a manner similar to that of the binding of calcium, and second, to determine \(\text{pK}_{\text{SrCl}^{-}}\), the determinations of \(\text{Sr}^{++}\) concentration being made by the same method as that described for calcium, but with strontium substituted for calcium in the solutions. One precaution, however, had to be observed. \(\text{SrCO}_3\), being less soluble than \(\text{CaCO}_3\), precipitates rapidly from the van Dyke-Hastings solution. The final observations on strontium were therefore made on solutions (known and unknown) containing no \(\text{HCO}_3^{-}\)ions. A value of 2.70 ± standard deviation 0.06 was obtained for \(\text{pK}_{\text{SrCl}^{-}}\), but this must be regarded as a first approximation only.

**Corroborative Evidence**

**Biological Experiments**—Clark, Percival, and Stewart (7) have made quantitative determinations of the effect of varying concentrations of calcium and of citrate upon the frog's heart, using another method of recording physiological activity. Their preparations, once standardized, were assumed, under the conditions of their experiments, to remain constant in reactivity throughout a series of observations, an assumption which we have found to hold only approximately true. From their data they have estimated the
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composition of various mixtures of calcium and citrate which should be physiologically isoactive, but appear to have made no direct comparisons as a test of their conclusions.

We have tested solutions made up on the basis of their calculations, and have found them to be only approximately isoactive, the deviation in each case being as predicted from our mass law equation. We have also calculated pK_{CaCit} from their data, and find, after exclusion of three calculated isoactive solutions, values from 3.23 to 3.82, with a mean of 3.51 ± standard deviation 0.23.

Salant and Hecht (12) studied the influence of citrate on isolated mammalian and frog hearts. They demonstrated a depressing effect upon the amplitude of contraction of the heart, but came to the conclusion that "the theory that the action is due to the formation of non-ionized calcium salts is . . . untenable for calcium . . . citrate can be utilized by the heart."

This conclusion was apparently based upon experiments in which the calcium of Locke's solution, used for perfusion of the isolated heart of the cat, was replaced by an equal concentration of calcium in the form of Ca_3Ci_2, and in which a relatively slight effect upon the amplitude of contraction was observed. The authors do not state the concentration of calcium used in their Locke's solution, but if we assume that they used 0.024 per cent CaCl_2, as used by Locke, this would correspond to 2.16 mM of calcium per liter. An equal concentration of calcium in the form of Ca_3Ci_2 would give a concentration of 1.44 mM per liter of citrate. From the nomographic chart (Fig. 2) it will be seen that this combination of calcium and citrate concentrations will result in a Ca^{++} concentration of 1.15 mM per liter. This concentration of Ca^{++} is near the upper limit of the range of sensitivity of the mammalian heart to changes in Ca^{++} concentration, and the failure of the heart to respond more strikingly to a change in concentration of Ca^{++} from 2.16 to 1.15 mM per liter was apparently due to the fact that the heart was relatively insensitive in this range.

Electrodialysis—Greenberg and Greenberg (5) have carried out electrodialysis experiments on serum and on ultrafiltrates from serum, and have found that when relatively large concentrations of sodium citrate are added part of the calcium migrates to the

---

2 Two of these gave values that could not be used in our equations. The third (4.24) was excluded as outside the range of probability.
<table>
<thead>
<tr>
<th>Solution No.</th>
<th>Ca\textsubscript{2}Cit\textsubscript{2}</th>
<th>Total Ca</th>
<th>Total Cit</th>
<th>Specific conductivities</th>
<th>Equivalent conductivities</th>
<th>Ca\textsuperscript{++}</th>
<th>CaCit\textsuperscript{−}</th>
<th>Cit\textsuperscript{−}</th>
<th>Ca\textsuperscript{++}</th>
<th>Cit\textsuperscript{−}</th>
<th>CaCit\textsuperscript{−} + Cit\textsuperscript{−}</th>
<th>Equivalent conductivities</th>
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<td></td>
<td>m.-eq. per l.</td>
<td>m-\textsubscript{M} per l.</td>
<td>m-\textsubscript{M} per l.</td>
<td>$m\text{hos} \times 10^3$</td>
<td>$\times 10^3$</td>
<td>m-\textsubscript{M} per l.</td>
<td>m.-eq. per l.</td>
<td>m-\textsubscript{M} per l.</td>
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<td>2.180</td>
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</table>
Ionization of Ca, Mg, and Sr Citrates

anode compartment, indicating that part of the calcium is present in the form of a complex, negatively charged ion.

Conductivity Measurements—Conductivity measurements on solutions containing varying proportions of calcium and citrate, together with other ions, have been made by Shear and Kramer (3), Shear, Kramer, and Resnikoff (4), and Mulli and Standenath (11). The results show a marked deviation from the conductivity predicted on the assumption of simple mixtures of these substances, and lead to the conclusion that complex calcium citrate ions or molecules are formed in the solutions.

Wiley (22) has made conductivity measurements upon solutions containing only Ca₃Cit₂. Table IV includes the results of his observations, to which have been added our calculations of equivalent conductivities on the basis of the ions calculated to be present in the solutions, with a value of 3.22 for pK_{CaCit⁻}. The approximate constancy of these equivalent conductivities supports our hypothesis as to the ionization of Ca₃Cit₂.

SUMMARY

1. When calcium and citrate are present in solution, a part of the calcium present is bound in a complex, negatively charged ion.

2. The conditions found in such solutions may be described by the mass law equation (\([Ca^{++}] \times [Cit^=]/[CaCit^-] = K\). Or, in logarithmic form, pCa^{++} + pCit = pCaCit^- = pK_{CaCit⁻}.

3. At 22°, pH 7.4, and total ionic strength of \(\mu\) 0.155 to \(\mu\) 0.163, a value of 3.22 ± 0.025 for pK_{CaCit⁻} has been found.

4. The primary dissociation of calcium citrate may be expressed as Ca(CaCit)₂ \(\rightleftharpoons\) Ca^{++} + 2CaCit⁻. Under the theory of strong electrolytes, this dissociation may be considered to be complete.

5. The secondary dissociation of calcium citrate may be written as CaCit⁻ \(\rightleftharpoons\) Ca^{++} + Cit^=.

6. A probable structural formula for Ca₃Cit₂ is suggested.

7. The dissociation of magnesium citrate resembles that of calcium citrate. By an indirect method a value of approximately 3.22 for pK_{MgCit⁻} has been derived.

8. The dissociation of strontium citrate resembles that of calcium and magnesium citrates. A value of 2.70 ± 0.06 for pK_{SrCit⁻} has been found as a first approximation.

9. The frog’s heart is insensitive to CaCit⁻ and Cit^=.
CONCLUSIONS

A value of $3.22 \pm 0.025$ has been derived for the constant $pK_{\text{CaCit}^-} = p\text{Ca}^{++} + p\text{Cit}^\equiv - p\text{CaCit}^-$. The use of this constant as a means of predicting observed $\text{Ca}^{++}$ concentrations in varying mixtures of calcium and of citrate, and the failure of any other hypothesis to fit the observed facts are regarded as sufficient proof of the correctness of the hypothesis advanced at the beginning of this paper.

The evidence presented throws some light upon the probable structural formula of calcium citrate. Since the evidence is in favor of dissociation in two steps only, the first step being the complete dissociation of a strong electrolyte, and the second step the partial dissociation of a weak electrolyte, the following is offered as the most probable structural formula.

```
H_2C-C-O-Ca-O-C-CH_2

HO-C-C  O  O  C-C-OH

Ca  Ca

H_2C-C  O  O  C-CH_2
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This conception of the structure of calcium citrate may have some bearing on the nature of the combination of calcium with other substances having spatially neighboring pairs of carboxyl groups.
BIBLIOGRAPHY

THE IONIZATION OF CALCIUM, MAGNESIUM, AND STRONTIUM CITRATES
A. Baird Hastings, Franklin C. McLean, Lillian Eichelberger, James Lowell Hall and Esther Da Costa


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