CHELATOR: An Improved Method for Computing Metal Ion Concentrations in Physiological Solutions

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ABSTRACT

An algorithm is presented for the calculation of metal ion concentrations from given total metal concentrations (and vice versa) in physiological media containing metal-chelating compounds. In such media, conditions differ from those used for stability constant determination of metal-chelator equilibria; therefore calculated metal ion concentrations are incorrect. We recompute stability constants to reflect the effects of ionic strength and temperature of physiological solutions. Twelve different equilibria can be considered per metal-chelator pair. The computer program also calculates the contribution of ionized species of metals, chelator, complexes and pH buffers to ionic strength. Measurements with a Ca-selective electrode and with fura-2 show that calculated ionic Ca\(^{2+}\) concentrations are correct from 10 nM up to the millimolar range. The importance of the correct calculation of metal ion concentrations in physiological experiments is demonstrated by data, and derived kinetic parameters, on Na\(^+\)/Ca\(^{2+}\) exchange and the ATP-dependent Ca\(^{2+}\) pump of enterocyte plasma membrane vesicles. The program is written in Turbo Pascal\textsuperscript{®} and will run on IBM-compatible computers. It is menu-driven and supports the use of a Microsoft\textsuperscript{®} mouse.

INTRODUCTION

The study of many biochemical and physiological processes requires low and accurately controlled metal ion concentrations. Because intracellular metal ions are, for instance, involved in the regulation of enzyme activities (7,21) or function as a second messenger to hormonal stimulation (2), an accurate control of their concentration is essential in studies on such mechanisms. This also holds true for toxicological studies on the effects of low intracellular concentrations of heavy metal cations (19,25,26). Although the computer program we present can be used for the calculation of ionic concentrations in many species, we focus here on the calculation of Ca\(^{2+}\) ion concentrations, since the intracellular role and the in vitro control of ionic Ca\(^{2+}\) levels have been widely studied (e.g., References 2,5).

Submicromolar Ca\(^{2+}\) ion concentrations, which occur in the cytosol of all cells studied so far, may be produced through the use of chelating substances (chelators). A more constant buffering capacity over a range of Ca\(^{2+}\) concentrations may be obtained by mixing chelators (15). Thus, use of a combination of chelators with distinct calcium affinities, e.g., EGTAT plus HEDTA [N-(2-hydroxyethyl)-ethylendiamine-N\(^\prime\),N\(^\prime\),N\(^\prime\)-triacetic acid] plus NTA [nitrilotriacetic acid] (8,9,24–26), allows accurate control of Ca\(^{2+}\) concentration over a wide range. However, with increasing numbers of chelators, equilibrium calculations become increasingly complex.

During the last decade, several methods were published to perform such calculations (e.g., References 3, 5, 6, 12, 23, 24). The composition of the solutions under study ranged from simple (12) to complex (3,24). Pivotal for all calculations is the correct choice for the stability constants of the equilibria under consideration. The stability constants have to account for experimental conditions, i.e., ionic strength and temperature, which influence metal-chelator equilibria.

Physiologically relevant conditions (e.g., an iso-osmotic NaCl-based solution at 37°C with a pH of 7.4) differ from those commonly used to determine stability constants: a 0.1 M KNO\(_3\) solution at 20°C with a pH of 7.0 (14,20). The biologist then has the choice to a) determine the stability constant himself for his particular experimental conditions, b) use a published stability constant measured at his experimental conditions or c) correct the published stability constant (measured at different conditions) to reflect the change in experimental conditions. In a simple one metal-one chelator
system (e.g., Ca\(^{2+}\) and EGTA), changes in apparent Ca-affinity of the chelator, as a result of the particular experimental conditions, can be measured and used for further calculations (12). However, to determine the change in a single stability constant in a complex solution may be very complicated or impossible. Compilations of stability constants do not list many constants measured at biologically relevant conditions, since few such determinations have been performed or described adequately (14). Thus, correcting the “standard” stability constant to reflect changes in ionic strength and temperature is the most feasible option when equilibration in multi-metal, multi-chelator solutions have to be computed. Calculations using stability constants properly corrected for effects of ionic strength and temperature have been shown to accurately predict experimental results when applied to a solution containing calcium and EGTA (13). We here extend this model to include additional reactions, which can become important when other metals or chelators are to be considered.

At nonzero ionic strength, the chemical activity of all ions, including protons, is related to their concentration through an activity coefficient \( \gamma (a = c \cdot \gamma) \). The pH then is the negative logarithm of the \( H^+ \) activity, not of the \( H^+ \) concentration. Stability constants for complex formation are expressed in terms of \( H^+ \) concentration (14,23). Accordingly, \( H^+ \) activity (derived from the pH) must be converted to \( H^+ \) concentration before performing equilibrium calculations, or mixed constants should be used. Though some were familiar with these limitations (18), others appear to have overlooked this point (3,24). The much-used algorithm of Fabiato and Fabiato (6), for instance, is incomplete without the correction published by Fabiato (4).

Most methods published so far calculated the equilibria of the complex formation of either protons or metal ions with chelator molecules without considering other possible reactions (5,23,24). Binding of a second metal ion to a metal-chelator complex or deprotonation of an \( H_2O \) molecule of the water shell of the complex are examples of reactions known to occur, particularly when multivalent metal ions are concerned (14). To the best of our knowledge, only Chang et al. (3) incorporated numerous side reactions in their calculations. Unfortunately, their program does not correct stability constants for “non-standard” conditions, and it is limited to solutions containing at most three chelators. Here we describe a computer program that supports up to 15 different metals and chelators, corrects stability constants for effects of ionic strength and temperature and can consider 12 different reactions per particular metal–chelator pair. We found extremely good agreement between calculated and measured Ca\(^{2+}\) concentrations ranging from 10 nM up to 0.1 mM. Also, apparent kinetic anomalies of Ca\(^{2+}\) transport mechanisms can now be explained.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals used were of analytical grade. Fura-2 was obtained from Molecular Probes (Eugene, OR). EGTA, HEDTA, NTA, ATP (Tris salt), HEPES and Tris were obtained from Sigma Chemical, St. Louis, MO. All other chemicals were from E. Merck, Darmstadt, FRG.

**Basic Calculations**

In a solution containing both chelators and metals, the total amount of chelator molecules equals the sum of all chelator molecules complexed to the various metals and protons and of the unbound molecules. A similar relationship holds true for all metal species: the total concentration of a metal species equals the sum of all concentrations of complexed forms, plus the concentration of the ionic form. These relationships may be represented by the following equations:

\[
T_{Ci} = F_{Ci} + B_{Ci}
\]

(eq. 1)

where \( T_{Ci} \) stands for the total concentration of the \( i \)th chelator in the system, \( F_{Ci} \) for the concentration of unbound chelator \( i \) and \( B_{Ci} \) for the concentration of bound chelator \( i \). In the same way,

\[
T_{Mi} = F_{Mi} + B_{Mi}
\]

(eq. 2)

where \( M \) stands for the \( J \)th metal in the system.

The equilibrium reaction describing the formation of the complex of one chelator and one metal species is characterized by its stability constant, which is measured at a specific ionic strength and temperature. We have taken most of the stability constants involved in our calculations from the series compiled by Martell and Smith (14), which presents a concise set of unequivocal data. Where this series was incomplete, we consulted the compilation by Sillén and Martell (20).

The computer program described here can include the following reactions in its calculations:

**Equilibrium Stability Constant**

- \( H + C \Leftrightarrow HC \) \( K_{A1} \)
- \( H + HC \Leftrightarrow H_2C \) \( K_{A2} \)
- \( M + C \Leftrightarrow MC \) \( K_B \)
- \( M + HC \Leftrightarrow MHC \) \( K_C \)
- \( M + MC \Leftrightarrow M_{2}C \) \( K_D \)
- \( M + MHC \Leftrightarrow M_{2}HC \) \( K_E \)
- \( MC \cdot H_2O \Leftrightarrow MC \cdot OH + H \) \( K_F \)
- \( MC(OH) \cdot H_2O \Leftrightarrow MC(OH)_2 \cdot H \) \( K_G \)
- \( M_{2}C + OH \Leftrightarrow M_{2}C(OH) \) \( K_H \)
- \( M_{2}C(OH) + OH \Leftrightarrow M_{2}C(OH)_2 \) \( K_I \)
- \( MHC \cdot H_2O \Leftrightarrow MHC(OH) + H \) \( K_J \)
- \( MHC(OH) \cdot H_2O \Leftrightarrow MHC(OH)_2 \cdot H \) \( K_K \)
- \( 2MC(OH) \Leftrightarrow \{MC(OH))_2 \) \( K_L \)
- \( 2C \Leftrightarrow C_2 \) \( K_M \)
- \( M + C_2 \Leftrightarrow MC_2 \) \( K_N \)

where \( H_2O \) represents water molecules in the water shell of the complexes and \( (OH) \) represents shell hydroxide ions. Since various metals and chelators in the system may have different electrical charges, they were omitted for clarity.

The equilibrium concentration of a chelator–metal complex is given by the free concentrations of chelator and metal,
and their stability constant. Thus, we may express $B_{C2}$ as a function of $F_{C2}$. Then, $T_{C2}$ becomes solely a function of $F_{C2}$. Using coefficient matrices to gather the chelator-metal interactions into two groups, we may rewrite equation 1 as

$$T_{C2} = F_{C2} \cdot M_{1} + F_{C2}^2 \cdot M_{2},$$

(eq. 3)

where $M_{1}$ and $M_{2}$ are coefficient matrices, which are defined as follows:

$$M_{1} = \frac{1}{2} \sum_{i=1}^{x} \sum_{j=1}^{y} \left[ K_{A_{i} F} \cdot \left( K_{C_{j} F} - K_{C_{j} F}^{-1} \right) \right] + \sum_{i=1}^{x} \sum_{j=1}^{y} \left[ K_{A_{i} C_{j}} \cdot \left( K_{C_{j} F}^{-1} - K_{C_{j} F} \right) \right],$$

and

$$M_{2} = \sum_{i=1}^{x} \sum_{j=1}^{y} \left[ K_{A_{i} F} \cdot K_{C_{j} F}^{-1} \right] + \sum_{i=1}^{x} \sum_{j=1}^{y} \left[ K_{A_{i} C_{j}} \cdot K_{C_{j} F} \right].$$

The second part of the calculation is concerned with calculating total metal concentrations. To this end parts of the matrices $M_{1}$ and $M_{2}$ can be rewritten as separate matrices $A_{1}$ to $A_{4}$, which are used in the following calculation:

$$A_{1} = \left[ K_{C_{j} F}^{-1} \right],$$

$$A_{2} = \left[ K_{C_{j} F} \right],$$

$$A_{3} = \left[ K_{C_{j} F}^{-1} \right],$$

$$A_{4} = \left[ K_{C_{j} F}^{-1} \right].$$

The relationship between $T_{M}$ and $F_{M}$ is similar to that of $T_{C}$ and $F_{C}$:

$$T_{M} = F_{M} \cdot M_{3} + F_{M}^2 \cdot M_{4},$$

(eq. 5)

where

$$M_{3} = \sum_{i=1}^{c} F_{C_{i}} \cdot A_{1_{i}} + \sum_{i=1}^{c} F_{C_{i}}^2 \cdot A_{4_{i}}.$$

The free chelator concentrations calculated from equation 4 can now be inserted in these formulas. Equation 5 will then yield the total metal concentrations required to obtain the desired metal ion concentrations.

Also, a metal ion concentration may be calculated from a given total metal concentration by performing the above calculations in an iterative procedure. The total metal concentrations are used as a first estimation for the metal ion concentrations. The program then compares the outcome of the calculations with the known total metal concentration and, if the approximation of the respective metal ion concentration by the square root of the ratio, total metal concentration vs. metal ion concentration.

### Correction for Ionic Strength Effects

Most tabulated stability constants have been determined at the standard ionic strength of 0.1 M KNO₃. At this ionic strength, the activity of ions is smaller than their concentration. This can be expressed as $a = c^*$, where $c$ is the activity coefficient (here $0 < c^* < 1$). This also holds true for $H^+$ ions: the $pH$ is no longer the negative logarithm of the $H^+$ concentration, but of the $H^+$ activity. The activity coefficient $c^*$ at 0.1 M ionic strength (and 20°C) is 0.77, so the pH of a solution containing $10^{-2}$ M H⁺ ions would not be 7.0, but 7.11.
Table 2. Apparent Stability Constants of Combinations of Metals and Chelators Corrected for Use at 37°C and 165 mM Total Ionic Equivalents

<table>
<thead>
<tr>
<th>Metals</th>
<th>Chelators</th>
<th>ATP</th>
<th>EGTA</th>
<th>HEDTA</th>
<th>NTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K2: 3.80</td>
<td>K2: 8.64</td>
<td>K2: 5.15</td>
<td>K2: 2.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K3: 2.58</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>K1: 3.69</td>
<td>K1: 10.32</td>
<td>K1: 8.08</td>
<td>K1: 6.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K2: 1.94</td>
<td>K2: 5.09</td>
<td>K2: 1.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>K1: 4.00</td>
<td>K1: 5.09</td>
<td>K1: 5.67</td>
<td>K1: 5.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K2: 2.07</td>
<td>K2: 3.13</td>
<td>K2: 1.27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
|        | K1, K2 and K3 represent stability constants for the binding of the metal to the unprotonated, mono- and diprotonated chelator, respectively. Electrical charges were omitted for clarity.

Conversely, a solution with a pH of 7.0 contains 1.3 × 10⁻⁷ M H⁺ ions. This effect becomes increasingly important at higher ionic strengths (as γ decreases more) and even more so for chelators that bind more than one proton, such as EGTA.

The activity of all ions not only decreases with increasing ionic strength (note: at high unphysiological ionic strength, γ will increase again and can even exceed the value of 1) but also with increasing electrical charge. For divalent cations and anions, the activity equals the concentration multiplied by γ², for trivalent ions a = c × γ³, etc. Since most of the metals and chelators involved in the calculations presented here have multiple electrical charges, correcting for effects of ionic strength is vital for a correct calculation of metal ion concentrations.

A semi-empirical form of the Guggenheim-Davies extension of the Debye-Hückel limiting law was used to correct the stability constants K for the effects of ionic strength (1,12):

\[ \log K' = \log K + F \cdot \log f_i - \log f'_i \]

where K' is the constant after and K the constant before correction, f_i the activity coefficient of ion i at the tabulated ionic strength and f'_i that for the desired conditions. Harrison and Bers have described the factor F as 2xy, where x is the valence of the metal cation and y is the valence of the chelator anion (12,13). This formula, however, may only be used when referring to reaction B (M + C → MC; see above). We calculate F by subtracting the sum of the squares of the electrical charges of the species on the left-hand side of the equilibrium sign from the squares of the electrical charges of the species on the right-hand side (1): e.g., for reaction C, \[ F = (M + C +1)^2 - M^2 - (C + 1)^2 = 2MC + 2M \]. So, F is calculated according to different formulas, depending on which stability constant is being corrected, for effects of ionic strength (Table 1). The activity coefficient f_i is calculated by:

\[ \log f_i = (1.8246 \times 10^6 \times 10^6)^{1/3} \times \left( \frac{T}{1 + \frac{1}{T}} \right) - 0.25 \times 1, \]

where T is the relative dielectric constant of water (we correct this constant for the temperature used), T the absolute temperature and 1 the ionic strength in ionic equivalents (12).

The program calculates the various degrees in which ionized species of metals, chelators, metal-chelator complexes and pH buffers contribute to the ionic strength of the solutions. To this end the pKa of the pH buffers is also corrected for effects of ionic strength.

**Correction for Temperature Effects**

Metal-chelator equilibria are influenced by temperature. Forward and backward reactions of the equilibrium are accelerated at different rates with higher temperatures. Stability constants of metal-chelator equilibria are usually determined at 20°C, which means that they have to be corrected before they can be used for calculations involving other temperatures. The shift in the overall stability constant as a response to temperature changes can be expressed by a ΔH-value, reflecting the change in enthalpy of the reaction.

The effect of temperature is calculated by using Van't Hoff's Isochor (12):

\[ \log K' = \log K + \frac{\Delta H}{R} \left( \frac{1}{T_2} - \frac{1}{T_1} \right), \]

where R is the universal gas constant (8.314 × 10⁻³ J·mol⁻¹·K⁻¹) and ΔH is expressed in J·mol⁻¹. Van't Hoff's Isochor was also used to correct K_w (the ion product of water) for temperature effects. This correction implicitly assumes that ΔH remains unchanged for T and T'. Table 2 shows stability constants corrected for ionic strength and temperature that were used in the calculations presented below.

**Features of the Computer Program**

Figure 1 shows an example of the program-user interface. We have subdivided the user input into logical steps through the use of pulldown menus, which may be operated through the keyboard or by a Microsoft®-compatible mouse. By using such a program structure, the user does not have to be familiar with the actual calculations or worry about possible syntax errors in input files.

The program makes use of a datafile to store information about all compounds and media composition. Every single user may make personal datafiles. All stability constant data may be edited, modified and stored. Also, the specified media composition, ionic strength, temperature and pH are stored in the datafile. Thus, routine calculations can be performed quickly by retrieving the specific datafile and proceeding straight to the calculations. The program produces a printout.

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Figure 1. Use of Chelator to calculate metal ion concentrations. This figure shows the pulldown menu in which a combination of chelators is selected. It demonstrates the way in which the entire program is subdivided into pulldown menus. The user may manipulate the menus using either keyboard arrow keys or a Microsoft®-compatible mouse.
of the calculation results, including all stability constant and media composition data, upon demand.

Measurement of Ionic [Ca\(^{2+}\)]

Ionic Ca\(^{2+}\) concentrations above 0.5 \(\mu\)M were measured with a Ca\(^{2+}\) selective electrode (IS 561-Ca; Philips, Eindhoven, The Netherlands) in combination with a double junction reference electrode (RH 44/2-SD/1; Philips). The inner compartment of the reference electrode contained saturated KCl, the outer compartment 100 mM KCl. The Ca-electrode was calibrated with chelating compound-free solutions with Ca\(^{2+}\) concentrations down to 1 \(\mu\)M, which contained 150 mM KCl, 1.5 mM MgCl\(_2\) and 20 mM HEPES, adjusted to pH 7.4 at 37°C with Tris. The water used in the preparation of the solutions was purified by reverse osmosis followed by distillation and subsequent treatment by the Milli-Q\(^\circ\) water purification system (Millipore, Bedford, MA). Calcium contamination of the HEPES/Tris-buffered solution was measured by Coupled Plasma Atomic Emission spectrometry ( Plasma 200; Instrumentation Laboratory, Andover, MA) and amounted 1.2 \pm 0.2 \(\mu\)M.

Solutions of a desired Ca\(^{2+}\) concentration (0.5–10 \(\mu\)M) were prepared in 150 mM KCl, 1.5 mM ionic Mg\(^{2+}\) (total Mg ranged from 4.971 to 4.717 mM), 3 mM ATP, 0.5 mM EGTA, 0.5 mM HEDTA, 0.5 mM NTA and 20 mM HEPES, adjusted to pH 7.4 at 37°C with Tris. Concentrations of total Ca and total Mg required to obtain desired Ca\(^{2+}\) ion concentrations in the above metal-buffering solution were calculated with the computer program presented here. The Ca\(^{2+}\) ion concentrations of these metal-buffered solutions were measured with the Ca\(^{2+}\) selective electrode, as described above.

Solutions containing Ca\(^{2+}\) concentrations in a 10–800 nM range were prepared in an analogous way in the Ca\(^{2+}\) buffering solution described above, to which the fluorescent Ca\(^{2+}\)-indicator fura-2 was added to a final concentration of 0.25 \(\mu\)M. The fluorescence of these solutions was measured at 510 nm (slit 10 nm) at excitation wavelengths 340 and 380 nm (slit 5 nm) with an Aminco SPF 500 spectrophotometer, operating in the ratio mode. The calibration of the fluorescence signal in terms of the Ca\(^{2+}\) ion concentration was done according to Grynkiewicz et al. (11), assuming that fura-2 binds Ca\(^{2+}\) with a dissociation constant of 224 nM. All measurements of Ca\(^{2+}\) were performed at 37°C.

RESULTS AND DISCUSSION

The calibration curve of the Ca\(^{2+}\) selective electrode used in this study is shown in Figure 2. The Ca-electrode showed Nernstian behavior over the entire range of Ca\(^{2+}\) concentrations applied. The slope of the electrode response was 30.13 mV/decade change in Ca\(^{2+}\) ion concentration, which almost equals the value of 30.08 mV/decade predicted by the Nernst equation. Furthermore, the figure shows that the data points referring to Ca\(^{2+}\) concentrations of the chelating agents containing solutions, which were calculated with our computer program, do not deviate significantly from the calibration curve down to 0.5 \(\mu\)M ionic Ca\(^{2+}\). The electrode began to deviate from Nernstian behavior below 0.5 \(\mu\)M (data not shown).

Ionic Ca\(^{2+}\) concentrations from 0.83 \(\mu\)M down to 10 nM, prepared in the solution containing EGTA, HEDTA, NTA and ATP, were measured fluorimetrically with fura-2. Figure 3 shows that over a range of Ca\(^{2+}\) concentrations from

![Figure 2. Calibration curve of the Ca-electrode used for measurement of Ca\(^{2+}\) ion concentrations prepared in a buffer containing Ca\(^{2+}\)-chelators. The electrode potential V\(_{Ca}\) of chelator-free (filled circles) and Ca\(^{2+}\)-chelator-containing (open squares) solutions is plotted against pCa (pCa = -log ([Ca\(^{2+}\)])) The Ca\(^{2+}\) ion concentration of the buffer containing the Ca\(^{2+}\)-chelator was calculated with Chelator. The solid line represents the result of linear regression of these data. The bars represent the SEM of triplicates. The slope of the regression line is 30.13 mV/decade.](image)

![Figure 3. Verification of Ca\(^{2+}\) ion concentrations calculated with Chelator. Ca\(^{2+}\) ion concentrations measured with the Ca-electrode (open squares) or fluorimetrically with fura-2 (open triangles) are plotted against the Ca\(^{2+}\) ion concentration calculated with Chelator. The solid line represents the result of linear regression of these data; the bars represent the SEM of triplicates. The slope of the regression line is 1.0.](image)
10 nM to 10 μM, the measured Ca²⁺ ion concentration was (within experimental error) equal to that calculated by Chelator.

Figure 4 shows that ignoring several criteria mentioned above can lead to substantial errors in calculating metal ion concentrations. Not interpreting pH as the logarithm of the H⁺ activity, but of the H⁺ concentration, underestimates the binding of H⁺ ions to EGTA. As a result of this, a calculation routine [e.g., the method described by van Heeswijk et al. (24)] that incorrectly computes H⁺ concentration, calculates that more EGTA, which is responsible for most of the Ca²⁺ chelating action in the nanomolar concentration range, is available to bind Ca²⁺. Our calculations and the Ca²⁺ measurements described above clearly show that, when such a method is used, ionic Ca²⁺ is underestimated in this concentration range.

What effect can incorrect calculations of metal ion concentrations have on the interpretation of kinetic studies on Ca²⁺ affinities of Ca²⁺-transporting membrane proteins? Our experimental work involves the characterization of Ca²⁺ transport mechanisms in the intestine of the freshwater fish Oreochromis mossambicus. To this end we isolate membrane vesicles from the basolateral side of the epithelial cells and perform ⁴⁵Ca uptake studies in Ca²⁺-buffered solutions. When we used the calculation routine published by van Heeswijk et al. (24), we found that the ATP-dependent Ca²⁺ pump in these membranes displayed a Vₘₐₓ of 0.63 ± 0.04 nmol · min⁻¹ · mg⁻¹ and a Kₘ of 27 ± 4 nM (8). At intracellular Ca²⁺ concentrations around 85 nM (19), the Ca²⁺ pump would therefore be operating near its maximum velocity. The pump would respond rather slowly to changes in intracellular Ca²⁺ levels and would not be functional in regulating intracellular Ca²⁺ concentration. When we performed the experiments again, using Ca²⁺-buffered solutions calculated with Chelator, we found a similar Vₘₐₓ (0.81 ± 0.05 nmol · min⁻¹ · mg⁻¹) but a quite different Kₘ, i.e., 88 ± 17 nM. This value is much more plausible for a mechanism thought to be involved in intracellular Ca²⁺ regulation, since intracellular substrate concentrations are often found to approximate to the Kₘ of the enzyme involved in its regulation (16).

Figure 5 shows the effects of an incorrect calculation of Ca²⁺ ion concentrations on the kinetic analysis of the Ca²⁺ dependence of Na⁺/Ca²⁺ exchange activity in these membrane vesicles. We previously found a complex kinetic behavior that predicted more than one Ca²⁺ site (8). We described this kinetic behavior using a double Michaelis-Menten relationship, where the two Kₘ values were 181 nM and 3.3 μM. The correct calculation of Ca²⁺ concentrations with Chelator has a drastic effect on this behavior. Na⁺/Ca²⁺ exchange activity now obeys a single Michaelis-Menten relationship, displaying a Kₘ of 1.21 ± 0.06 μM (19). This value, and the kinetic behavior, are comparable to data found in the literature (e.g., Reference 17). However, in most of the latter studies on Na⁺/Ca²⁺ exchange, no Ca²⁺ chelators were used to control ionic Ca²⁺ levels. The apparent fall in Ca²⁺ affinity does not mean that Na⁺/Ca²⁺ exchange is not involved in Ca²⁺ homeostasis in these cells: the high Vₘₐₓ together with the stimulatory effect of an inside-negative cell membrane potential on the electrogenic exchanger, more than compensates for the relatively high Kₘ. This still designates Na⁺/Ca²⁺ exchange as the primary candidate for the regulation of intracellular Ca²⁺ levels and as the transport protein chiefly responsible for transcellular Ca²⁺ uptake in this particular epithelium.

Figure 4. Errors in calculating metal ion concentrations. Calcium ion concentrations ([Ca²⁺]₀) were calculated with Chelator for a solution of 0.165 M total ionic strength (in ionic equivalents) at 37°C containing as metal chelators 3 mM ATP, 0.5 mM EGTA, 0.5 mM EDTA and 0.5 mM NTA. Then, the total metal concentrations served to calculate metal ion concentrations ([Ca²⁺]₀) according to a method that does not implement several corrections (24). The ratio of [Ca²⁺]₀/[Ca²⁺]₀ calculated is plotted as a function of [Ca²⁺]₀ calculated. The largest and most serious differences are observed in the nanomolar range of metal ion concentrations, which is critical for the exact determination of the Ca²⁺ affinities of intracellular Ca²⁺ acceptors. The errors in the older method result mainly from an incorrect interpretation of pH values, but also from the use of stability constants that were not all corrected for the experimental ionic strength and temperature.

Figure 5. Correct calculation of Ca²⁺ ion concentrations results in an apparent change in kinetic behavior of Na⁺/Ca²⁺ exchange. This is a Lineweaver-Burk plot of the Ca²⁺ dependence of Na⁺/Ca²⁺ exchange activity in isolated basolateral plasma membrane vesicles from intestinal cells of Oreochromis mossambicus. Filled squares and dashed line denote the Na⁺/Ca²⁺ exchange activity (n = 15) as published by Flik et al. (8) that was determined using Ca²⁺ ion concentrations calculated by the method of van Heeswijk et al. (24). The kinetic behavior could only be described adequately by a double Michaelis-Menten relationship with Kₘ's of 181 nM and 3.3 μM. When the experiment was performed using Ca²⁺ concentrations calculated with Chelator (19), the data (filled circles and solid line) obeyed a single Michaelis-Menten relationship with a Kₘ of 1.21 ± 0.06 μM (n = 5).
CONCLUSIONS

We must stress that, although in this paper we focus on the control of Ca\(^{2+}\) ion concentrations, the program is equally well suited to calculate ionic concentrations of any other metal species. We have used it successfully in in vitro studies on the effects of Cd\(^{2+}\) on Ca\(^{2+}\) transporting membrane proteins (19), demonstrating the applicability of the computer program in toxicological studies. Since changes in binding equilibria induced by differences in ionic strength become progressively important with increasing valencies of the metal ions involved, studies on the effects of such metals may benefit especially from the use of Chelator.

The measurements using turn-2 fluorescence and the selective Ca\(^{2+}\) electrode show that this metal ion buffering system behaves according to our calculations from 10 nM Ca\(^{2+}\) up to the micromolar range. The mixture of BGTa, HEDTA and NTA indeed appears to be well suited to accurately control Ca\(^{2+}\) concentrations over a wide range. The correlation between calculated and measured Ca\(^{2+}\) concentrations is excellent. It appears that, provided that all equilibria involved in metal-chelator binding are carefully computed, metal ion concentrations can be correctly calculated by a computer program, such as Chelator. This, of course, is a prerequisite for performing and analyzing experiments involving the effects of low metal ion concentrations on physiological processes.

CHELATOR is available free on a 3 1/2" diskette (720 kB), together with printed documentation concerning its use, from Theo J.M. Schoemakers. An update incorporating frequent suggestions of registered users will be made available in due course (ca. one year). This program is also available from the BioTechNet software library under the filename PACKCHEL.EXE. For downloading instructions, see BioTechNet Network News in this issue.

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