Ionised concentrations in Ca^{2+} and Mg^{2+} buffers must be measured not calculated

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What is new?

1) The $[Ca^{2+}]/[Mg^{2+}]$ in buffers are usually calculated using one of seven programs. These all give different values, thus $[Ca^{2+}]/[Mg^{2+}]$ must be measured.
2) The Ligand Optimisation Method (LOM) is an accurate method to do this. The limitations of the method are described.
3) The LOM has been generalised to include calibration of fluorochromes and photoproteins and is the method to measure intracellular equilibrium constants.
4) If parameters based on calculated values in Ca^{2+}/Mg^{2+} buffers are used when modelling the intracellular regulation of Ca^{2+}/Mg^{2+}, conclusions reached from such studies are relative not absolute, and must now be re-examined.

Abstract

Modelling intracellular regulation of Ca^{2+}/Mg^{2+} is now an established part of physiology. However, conclusions drawn from such studies depend on accurate measurement of intracellular $[Ca^{2+}]$, $\Delta[Ca^{2+}]$ and the pK' values for the intracellular binding of Ca^{2+}/Mg^{2+}. Calculation of $[Ca^{2+}]/[Mg^{2+}]$ in buffers is normal. The seven freely available programs all give different values for the $[Ca^{2+}]/[Mg^{2+}]$ in the buffer solutions, varying by up to a factor of 4.3.

Because of this, concentrations must be measured. There are two methods to do this, both based on the Ligand Optimisation Method (LOM). Method 1) Calibration solutions from 0.5 mmol/l to 4 mmol/l and 2) Calibration solutions from 0.1 µmol/l to 2 mmol/l. Both methods can be used to calibrate Ca^{2+}/Mg^{2+}-electrodes. Only Method 2 can be directly used to calibrate fluorochromes and photoproteins. Software in the statistical program "R" to calculate the $[Ca^{2+}]/[Mg^{2+}]$ in buffers is provided for both methods. The LOM has now been generalised for use with electrodes, fluorochromes and photoproteins making it the ideal method to determine the pK' values for intracellular binding of Ca^{2+}/Mg^{2+}.

The $[Ca^{2+}]/[Mg^{2+}]$ in buffers must be routinely measurement; best done by calibrating electrodes with the LOM and software written in "R". If $[Ca^{2+}]/[Mg^{2+}]$ in buffers are calculated, the parameters used in modelling show the same degree of variability as the software programs. Uncritical acceptance of such parameters means that conclusions reached from such studies are relative not absolute, and must now be re-examined.

Keywords; Ca^{2+} and Mg^{2+} buffers; measurement of $[Ca^{2+}]$ and $[Mg^{2+}]$ in buffers; fluorochromes; photoproteins; Ligand Optimisation Method; Ca^{2+}/Mg^{2+} electrodes

Units and Definitions

Solutions	
$[X^{n+}]$, $[X]_T$	Ionised and total concentrations of cations, mmol/l
$\Delta[X^{2+}]$	Changes in the ionised calcium and magnesium concentrations, mmol/l
$[X^{n-}]$, $[X]_T$	Ionised and total concentration of anions, mmol/l
Calibration and buffer solutions	
C1 to Cn	Calibration solutions C1 to Cn
B1 to B10	Buffer solutions B1 to B10
$[Ligand]_N$, $[Ligand]_T$	Nominal and total Ligand concentration, mmol/l
[X-Bound]	$[X^{2+}]$ bound to ligand, $([X]_T - [X^{2+}])$, mmol/l
Dissociation Constants (non-standard terminology, McGuigan et al., 2017)	
K'	Apparent equilibrium constant at the pH_a, temperature and ionic strength of the solution, mol/l
pK'	$-\log(K')$
pH	
pH_a	Measured pH in terms of activity, $pH_a = -\log([H^+]\gamma_{H^*})$
pH_c	pH in terms of concentration, $pH_c = -\log([H^+])$
γ_{H^+} γ_{Cl^-}	Single ion activity coefficients for H^+ and Cl^-
Electrodes	
E^0	Constant of the recording system, mV
E	Measured potential, mV
Σ	Lumped interference, mol/l, a constant
s	Slope of the electrode, mV/decade
Equations	
Nernst	$E = E^0 + s\log(10^{-pX})$
Nicolsky-Eisenman	$E = E^0 + s\log(10^{-pX} + \Sigma)$

Fluorochromes	
F	Measured fluorescence
F_{Min}, F_{Max}	Maximum and minimum fluorescence
F_N	Fluorescence normalised to lie between 0 and 1.
Aequorin	
L/L_{Max}	Fractional luminescence
K_1	Binding constant for Ca^{2+} on Aequorin, mol^{-1}
K_2	Binding constant on Aequorin that does not bind Ca^{2+}, dimensionless

1 Introduction

Modelling the intracellular behaviour of Ca^{2+} and Mg^{2+} is important in physiology, especially in cell signalling. The validity of conclusions drawn from such studies depends on the accuracy of the measured values for resting $[Ca^{2+}]/[Mg^{2+}]$, their changes and the pK' values for the intracellular binding of Ca^{2+}/Mg^{2+}. However, the resting values, their changes and the pK' values depend on the accuracy of the calibration buffers for Ca^{2+}/Mg^{2+}. Regarding such buffers, it is often ignored that it was only in 1980 that Tsien & Rink pointed out that measured pH was in terms of activity, not concentration. Four years later in 1984 Miller & Smith showed that EGTA was less than 100% pure. This means measurements of $[Ca^{2+}]$, $\Delta[Ca^{2+}]$ and pK' values for intracellular binding before 1984 are inaccurate.

In this paper we first discuss the origin of the word “buffer” and describe how buffer solutions are manufactured to calibrate Ca^{2+}/Mg^{2+}-electrodes. We explain why the seven software programs commonly used to calculate the $[X^{2+}]$ always give different values for $[X^{2+}]$ in the 10 buffers. We illustrate the problems this causes when 1) measuring intracellular $[Ca^{2+}]$ and $\Delta[Ca^{2+}]$ and 2) measuring pK' values for intracellular Ca^{2+} binding. Since calculations give variable results, the $[X^{2+}]$ in buffers must be measured. We consider the two methods, both based on the Ligand Optimisation Method (LOM) to do this. The LOM was originally developed for electrodes but has now been generalised for use with fluorescent probes and photoproteins. We show that the parameters used in modelling show the same degree of variability as that of these software programs. This has consequences for the conclusions reached when modelling the intracellular regulation of Ca^{2+}/Mg^{2+} in health and disease.

The Supporting Information to this paper provides a “do it yourself” guide to the measurements of $[X^{2+}]$ in Ca^{2+}/Mg^{2+} buffers using electrodes.

2 Calcium and Magnesium buffer solutions

2.1 Why the word “buffer”?

The first reported action of a buffer was by Fernbach & Hubert (1900) for phosphate mixtures. The article was in French and they used the word “tampon”, meaning that it “dampened” changes in the [H^+] caused by the addition of H^+ or OH^- ions to the solution. This was translated into German by Sörensen (1909, page 187) as “Puffer”, meaning damping device, station or carriage buffer. A literature search suggested “Puffer Lösung” was first translated as “buffer solution” by Dale (1913) and Clark (1913). Koppel & Spiro (1914) in their paper on the buffer capacity used the word “Moderatoren”, “moderators” instead of “Puffer”, because it moderates or dampens the changes for both the acidic and basic alterations. However, as pointed out by Ross & Boron (1980) in their English translation, both the paper and the word “moderator” were ignored. It was published in German just before the outbreak of the First World War; Max Koppel was killed in 1916 at Verdun and Karl Spiro moved to other fields. Their paper was simply forgotten.

Bayliss, in the first edition of his text-book “Principles of General Physiology” (1915, p203) pointed out a railway buffer is not a good way to describe the word tampon, as a railway buffer does not absorb the engine. He suggested “sponge” would be more appropriate, but made no concrete proposals. While comparing “buffer solution” with railway buffers is a misnomer, the word “buffer” meaning “damping” is now standard terminology. Buffering action should be thought of as the action of a “Ligand-ion-complex” as illustrated in Figure 1. It seems a pity that the suggestions of Koppel & Spiro (1914) of “moderators” did not gain acceptance, because it unambiguously describes the moderating action on the changes in the ionic concentration.

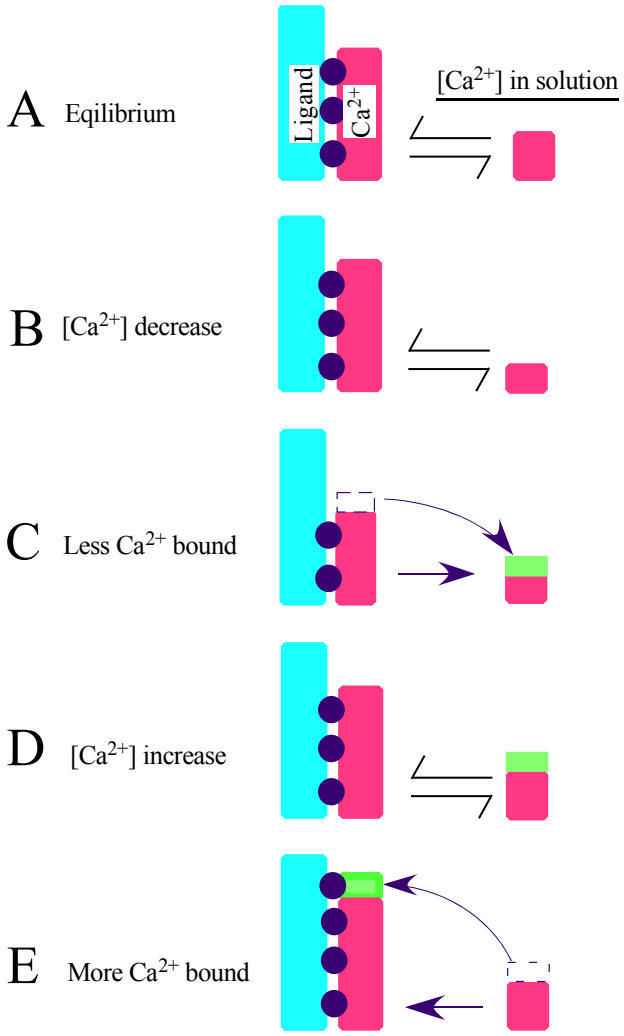


Figure 1. Function of the Ligand-calcium-complex as a buffer. A. Normal equilibrium state. B. A decrease in the $[Ca^{2+}]$ is compensated as shown in C, by less Ca^{2+} being bound. D. An increase in the $[Ca^{2+}]$ causes more to be bound as is illustrated in E.

3 Buffer solutions and the software programs for calculating the $[Ca^{2+}]$

3.1 Ca^{2+}/Mg^{2+} buffer solutions

$[X^{2+}]$ can be routinely set by dilution down to 0.5 mmol/l (pX, 3.301) but to fully calibrate Ca^{2+}/Mg^{2+}-electrodes over the required range, buffer solutions are necessary. The simplest method to manufacture such buffers is the ratio method in which two solutions are manufactured in the background solution at the same pH_a namely:

1) The ligand plus a $[X]_T$ equimolar to the $[Ligand]_N$.
2) The ligand alone.

These two solutions are then mixed in appropriate ratio to give the 10 buffer solutions. The ratios range from 7:1 to 1:9 so that the pX values of the 10 buffer buffers lie within pK' ± 1 for the ligand (McGuigan et al., 2006, Table 5; Table 1, page 15). The background solution for the 10 buffer solutions was in mmol/l, NaCl, 15, KCl 142.5, Hepes 5 and pH_a 7.2. The $[Na^+]$ and $[K^+]$ are based on measurements in ferret ventricular muscle (Reverdin et al., 1986). The pH_a of 7.2 was initially from Ellis & Thomas (1976); later measurements (Blatter & McGuigan, 1990) gave a value of 7.215. The ratio method has the advantage that the $[Na^+]_T$, $[K^+]_T$, $[Ligand]_T$ and pH_a in all 10 buffer solutions are identical, as is the pK' value.

3.2 Calculation of $[Ca^{2+}]$ in the 10 buffer solutions

To fully calibrate Ca^{2+}/Mg^{2+}-electrodes, calibration and buffer solutions are both required. In the original method the calibrating solutions, C1 to C7 ranged from 0.5 to 10 mmol/l. In these solutions, $[Ca^{2+}]$ equals $[Ca]_T$. In the buffers the $[X^{2+}]$ are either measured or calculated; calculation is usual. We have considered seven commonly used programs, shown in Figure 2 and the calculated $[Ca^{2+}]$ in the 10 buffer solutions for the seven programs all differ. The calculations are for EGTA Ca^{2+} buffers at a pH_a of 7.2 and the background solution. EGTA purity can vary from 92.8% (Tran et al., 2018) to 98.7% (Miller & Smith) and depends on supplier, batch and batch age. For the calculations a purity of 95% was assumed (3.8 mmol/l) To illustrate the importance of ligand purity, Visual Minteq was also used to calculate $[Ca^{2+}]$ with a $[EGTA]_N$ of 4 mmol/l (100% purity). Figure 2, updated from McGuigan et al., (Figure 6B, 2017) includes Visual Minteq because of its use by Neumaier et al., (2018).

The calculated $[Ca^{2+}]$ fall roughly into three groups. In buffer B1, Visual Minteq gives a value of 1.3 µmol/l ($[EGTA]_T$, 3.8 mmol/l) and 0.8 µmol/l ($[EGTA]_T$, 4.0 mmol/l). Chelator gives a value of 3.5 µmol/l, and a group of 4 programs give values of around 1.8 µmol/l. The $[Ca^{2+}]$ was also measured in these experiments using the Ligand Optimisation Method (McGuigan et al., 2017); shown as the red crossed squares and the red line in Figure 2. The measured values overlap the values calculated using the program of Fabiato & Fabiato (1979); fortuitous as in other calculations this was not the case (McGuigan, unpublished).

It may seem surprising that these programs all give different calculated $[Ca^{2+}]$, but this is due to; 1) Calculations for changes in ionic strength are based on single ion activity coefficients. These cannot be experimentally determined and assumptions have to made to calculate them; 2) pH_a (activity) has to be converted to pH_c (concentration). This is not possible, because of potential

changes at the reference electrode during measurement. In addition, assumptions have to be made to calculate the single ion activity coefficient for H^+-ions (see McGuigan et al., 2017); and 3) the tabulated dissociation constants for Ca^{2+} and H^+ binding to EGTA vary, and the calculated $[Ca^{2+}]$ in the buffers depends on which constants are used (Table 2, McGuigan et al., 2017).

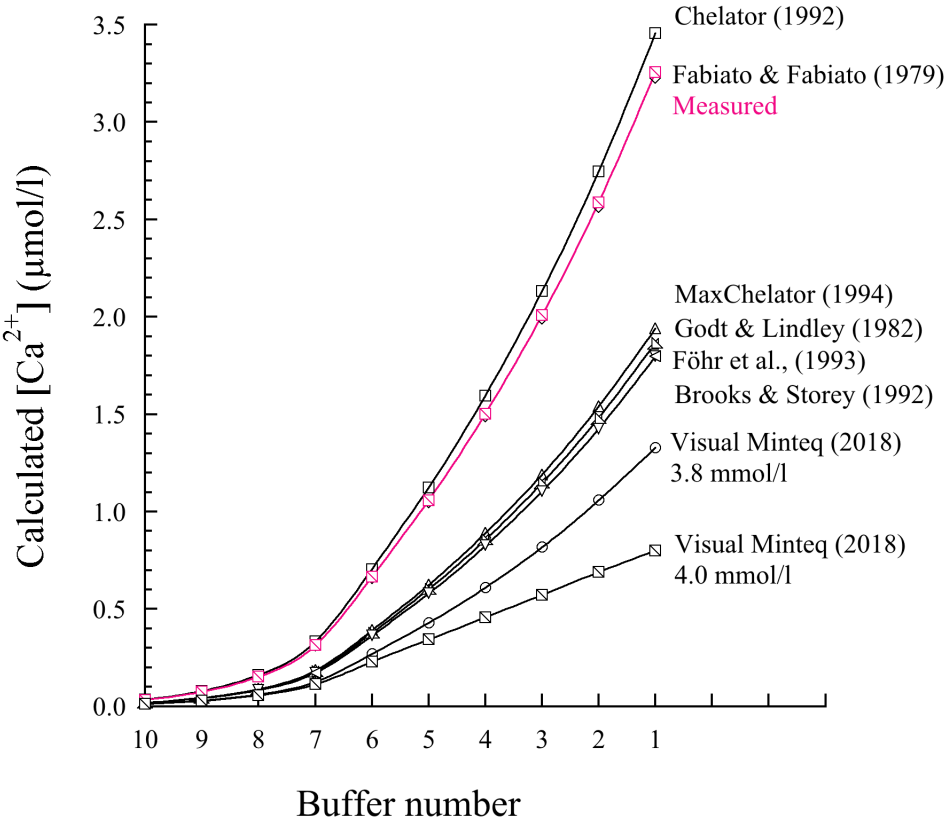


Figure 2. Comparison between calculated and measured $[Ca^{2+}]$ in the 10 buffer solutions; intracellular background solution and a pH_a of 7.2. The programs used were: Chelator (Schoenmakers et al., 1992), Fabiato & Fabiato (1979), MaxChelator (Bers et al., 1994), Godt & Lindley (1982), Föhr et al., (1993), Brookes & Storey (1992) and Visual Minteq (2018, version 3.1) for both 3.8 mmol/l and 4.0 mmol/l $[EGTA]_T$. The measured $[Ca^{2+}]$ are shown in red.

4 Consequences of calculating the $[Ca^{2+}]$ in the buffer solutions

4.1 Resting $[Ca^{2+}]$ and its physiological changes

As shown by Woods et al., (1986) using Aequorin, the hormone vasopressin causes the $[Ca^{2+}]$ in liver cells to spike and the frequency of the spikes is concentration dependent. To calibrate Aequorin either the measured values or the calculated values in the buffers could be used. Using measured buffer values, the assumption was made that the resting intracellular $[Ca^{2+}]$ was 250 nmol/l, and due to the action of vasopressin it spiked to an average value of 800 nmol/l. These results, together with the programs MaxChelator and Visual Minteq are illustrated in Figure 3A.

With MaxChelator, the resting [Ca^{2+}] was 147 nmol/l and the spike, 473 nmol/l; the corresponding values with Visual Minteq (4 mmol/l $[EGTA]_T$) were 94 nmol/l and 325 nmol/l. If EGTA purity is considered, the resting [Ca^{2+}] and spike with Visual Minteq were 101 nmol/l and 325 nmol/l; for clarity, not illustrated. These differences emphasise the problems associated with calculating the [X^{2+}] in buffer solutions.

4.2 K' values for Ca^{2+} /Mg^{2+} intracellular binding

A straightforward way to measure the dissociation constants, *e.g.* of X^{2+}, is to measure the [X^{2+}] in an appropriate background solution containing the ligand and various concentrations of $[X]_T$ (*e.g.* Zhang et al., 1997 for Mg^{2+}ATP). From such measurements, the K' value can be calculated from a plot of Bound/Free *e.g.* with Ca^{2+}:

$$[\text{Ca}-\text{Bound}] = \frac{[\text{Ca}^{2+}][\text{Ligand}]_T}{\left([\text{Ca}^{2+}] + K'\right)} \qquad [1]$$

However, the problem with such a method is to accurately measure the [Ca^{2+}]. To demonstrate the problem the K' value for the Ca^{2+} equilibrium constant for association with a ligand was assumed to be 500 nmol/l. In this ligand solution, the $[Ca]_T$ necessary to match the [Ca^{2+}] in the 10 measured [Ca^{2+}] buffers was then calculated from the following equation:

$$[\text{Ca}]_T = \frac{[\text{Ca}^{2+}]([\text{Ligand}]_T + K' + [\text{Ca}^{2+}])}{(K' + [\text{Ca}^{2+}])} \qquad [2]$$

(The derivation of equations [1] and [2] are given in Appendix to McGuigan *et al*., 2006, Section-1, pages 22 and 23.)

The calculation of the $[Ca]_T$ allowed a plot of Bound/Free for the K' value of 500 nmol/l, illustrated as the filled circles in Figure 3B. Plots are also shown using MaxChelator and Visual Minteq for $[EGTA]_T$ of 3.8 mmol/l and 4.0 mmol/l. All have been fitted with equation [1]. Experimentally, the regression coefficient has long been used to judge the goodness of fit for Equation [1] (Figure 1, Zhang et al., 1997, r = 0.9995). With the simulated data "r" was in all four cases 1.000000 not just for the assumed value of K' of 500 nmol/l, for which $[Ca]_T$ had been calculated. The reason for this, is not that this is a simulated experiment, but is a consequence of the form of equation [1]. In the equation the [Ca-Bound] is the difference between ($[Ca]_T$ – [Ca^{2+}]) and the [Ca-Bound] is then plotted against the [Ca^{2+}] that was used to calculate it. It is equivalent to a horizontal shift of Bound/Free plot along the [Ca^{2+}] axis. Such plots give the impression of being accurate, even when the calculated [Ca^{2+}] in the buffers are wrong. However, as Zhang et al.,

(1997) pointed out, ATP is a Mg^{2+} buffer and both purity and K' can be accurately measured using the LOM. Extensive measurements of K' values for Mg^{2+}-ATP with LOM were carried out by McGuigan et al., (2007). Methods involving equation [1] should no longer be used to estimate K' values. This has been superseded by the LOM.

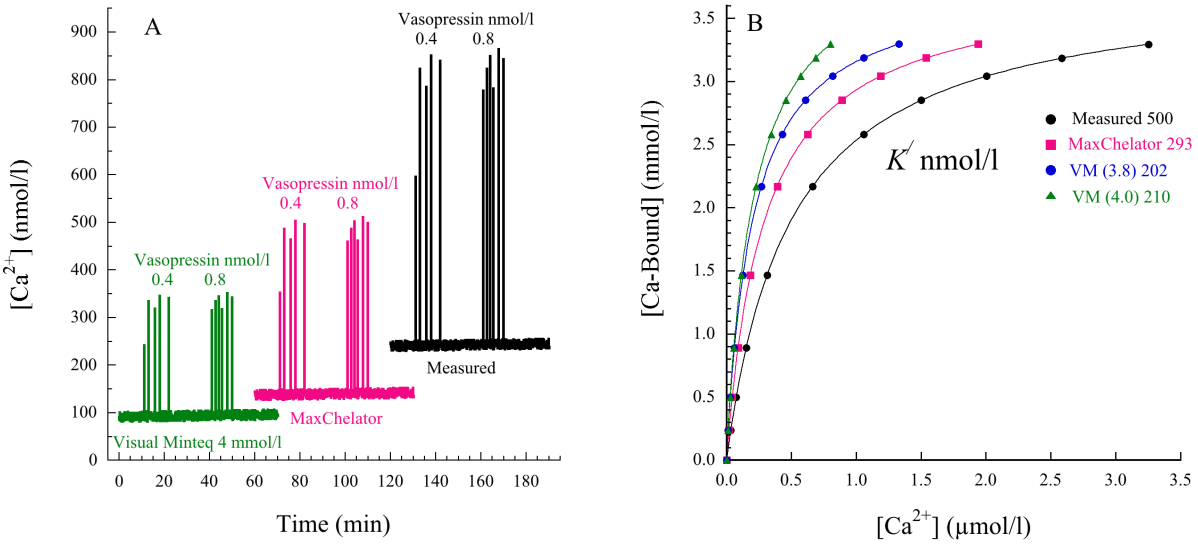


Figure 3 A. Computer generated action of vasopressin on liver cells, comparing measured $[Ca^{2+}]$ with the programs MaxChelator and Visual Minteq calculated with an $[EGTA]_T$ of 4.0 mmol/l. B. Estimated K' values for intracellular Ca^{2+} binding. Black circles, measured $[Ca^{2+}]$ in the buffers and K' had a value of 500 nmol/l. The calculated $[Ca^{2+}]$ were red squares, MaxChelator, blue circles and green triangles calculated with Visual Minteq with $[EGTA]_T$ of 3.8 mmol/l and 4.0 mmol/l respectively.

5 Measurement of $[Ca^{2+}]/[Mg^{2+}]$ in buffer solutions

5.1 Introduction

McGuigan et al., (2006) described 6 criteria for an ideal method to measure $[X^{2+}]$ in buffer solutions. From the seven methods investigated only two met all six criteria namely, the LOM (Lüthi et al., 1997) and setting concentrations by dilution from 2 mmol/l to 10^{-7} mol/l (Allen et al., 1977). While the LOM is a routine method, setting of concentrations by dilution requires special laboratory facilities (Lüthi et al., 1997).

5.2 Ligand Optimisation Method

5.2.1 Basis of the method

The method has been described in detail in Lüthi et al., (1997) and McGuigan et al., (2006, 2014, 2017) and only the principles of the method are described here. The LOM was developed to be used with Ca^{2+}/Mg^{2+}-electrodes in conjunction with calibration and buffer solutions in the appropriate range. The basis of LOM is twofold. 1) The electrode slope, *s* and the constant of the recording system E^0 are first determined in the 7 Calibrating solutions in which $[Ca^{2+}]$ equals $[Ca]_T$ (Table 5, McGuigan et al., 2006). 2) The electrode potentials in the 10 buffer solutions are measured. In the buffers the $[X]_T$ is known, but both pK' and $[Ligand]_T$ have to be estimated.

5.2.2 Ca^{2+}/Mg^{2+} Electrodes

At $[X^{2+}]$ greater than 0.5 mmol/l (pX 3.301), the response of the electrodes can be described to a first approximation, by the Nernst equation. In the buffer range the electrode deviates from a Nernst response (Figure 4C) and this response can be described by the Nicolsky-Eisenman equation. The value of Σ in the LOM is a constant, because the concentrations of the interfering ions in both calibrating and buffer solutions are identical. However, E^0 varies from experiment to experiment. In order to standardise the calibration curves, the potential in calibration solution C1, is defined as zero potential. From this definition, the Nernst and Nicolsky-Eisenman equations become:

$$E = -s\log(\frac{C1}{1000}) + s\log(10^{-pX}) \qquad [3]$$

$$E = -s\log(\frac{C1}{1000} + \Sigma) + s\log(10^{-pX} + \Sigma) \qquad [4]$$

5.2.3 Steps in the LOM

There are 4 steps in the method; the first three are illustrated in Figure 4A.

Steps

1) The slope *s* and the constant of the recording system E^0 are determined by fitting equation [3] to the measured potentials in the calibration solutions. This gave a value for *s*, of 28.532195 mV/decade. The K' value estimated by assuming a Nernstian response down to buffer B1 was pCa 6.2902 or 512.66 nmol/l.

2) The pCa/potential response of an electrode in the buffer range can be fitted with the Nicolsky-Eisenman equation [4]. This equation can be fitted to the buffer pCa/potential curve as the $[EGTA]_T$ is systematically reduced from the nominal value of 4 mmol/l to 3.65 mmol/l. At each concentration the regression coefficient is taken as a measure of the curve

fit. The regression coefficient passes through a maximum value, which was at an $[EGTA]_T$ of 3.7699 mmol/l (Figure 4B).

3) Both curves are then mathematically superimposed. The pK_l' and K_l' values are recalculated. Steps 2) and 3) are then repeated until two successive values of pK_l' differ by less than

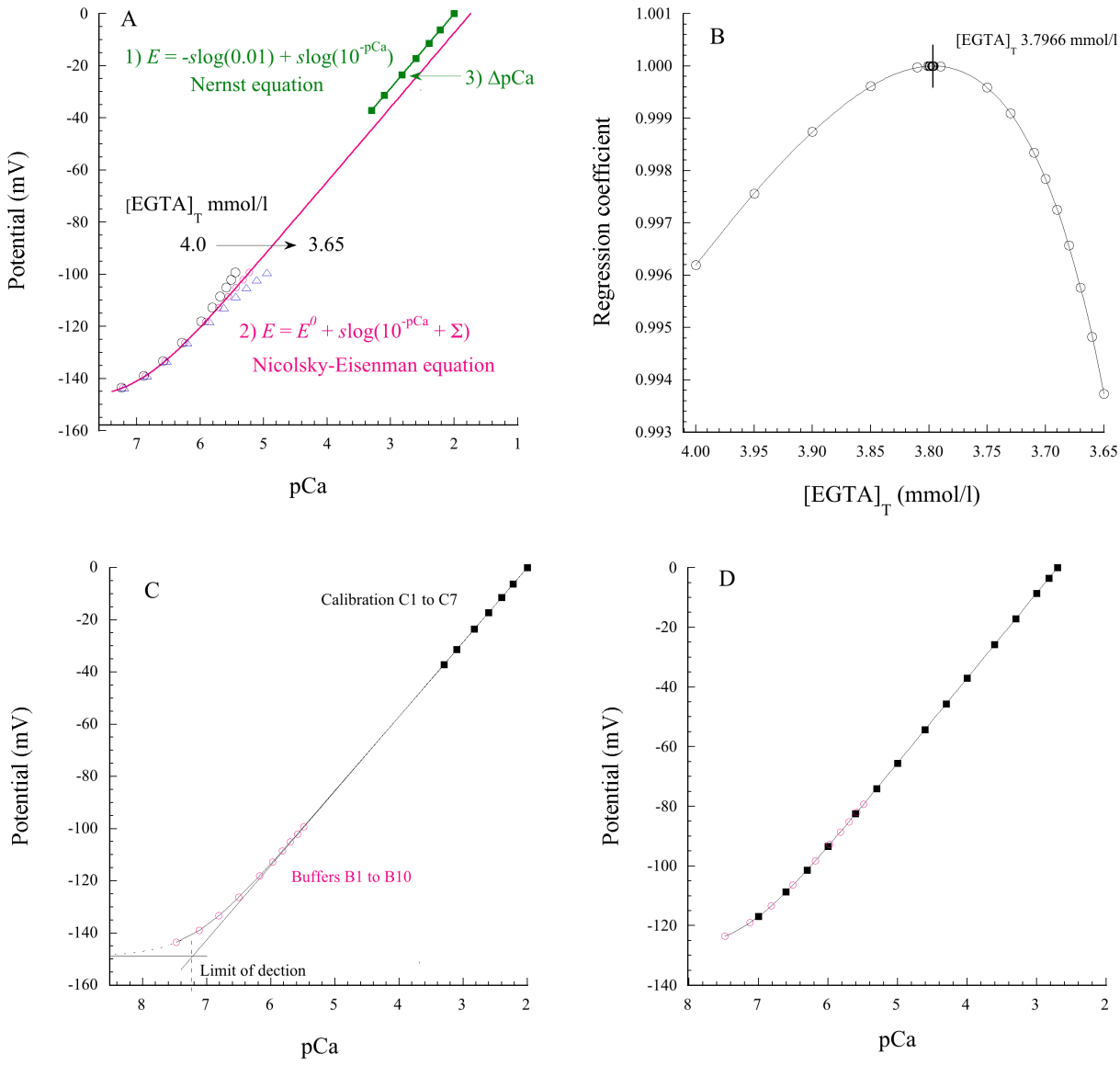


Figure 4 The plot is for a Ca^{2+}-electrode from McGuigan & Stumpff (2013). The slope *s* was 28.533 mV/decade and Σ 6.045*10^{-8} mol/l. The K_l' value was the measured value of 282.46 nmol/l (Figure 2). To standardise measurements, the potential in C1 (pCa, 2.0) is defined as zero potential. A. First 3 steps of the LOM. For details see text. B. Regression coefficient as the $[EGTA]_T$ was reduced from 4.0 mmol/l to 3.65 mmol/l. C. Final plot of the electrode, showing limit of detection. D. Calibration of the electrode with dilution from 2 mmol/l (pCa 2.699) to 0.1 µmol/l (pCa 7.000). Also shown are the 10 Ca^{2+}-EGTA buffers.

0.00001 pK' units. In this example it took 3 further iterations. The final values were, slope s, 28.532195 mV/decade, Σ, 6.042*10^{-8} mol/l and $[EGTA]_T$, 3.8000 mmol/l.

4) The original LOM was based on measuring s and E^0 using the Nernst equation. However, s is not independent of the value of Σ (McGuigan et al., 2014). This has now been incorporated into the calculations, by fitting the Nicolsky-Eisenman equation in Step 1 instead of the Nernst equation. Three more iterations gave the simulated initial parameters, namely, $[EGTA]_T$, 3.8000 mmol/l; slope s, 28.533 mV/decade and Σ 6.044*10^{-8} mol/l.

5.3 **Limitations of the LOM**

When LOM was originally developed (Lüthi et al., 1997) there were two initial limitations to the method:

1) The pK' values for Ca^{2+}/Mg^{2+} had to be the same in all the buffer solutions.
2) The pK' must be greater than 4. If pK' is less than 4, the ligand purity has to be independently measured. In such buffer ranges the ligand concentration can normally be determined by measuring the cation concentration (Figures 5C and D, McGuigan et al., 2006). Only the K' value has to be determined and this can be done by adjusting the value of K' until calibration and buffer curves overlap (cf. step 3 in Figure 4A)..

Since the introduction of LOM in 1997 it has become clear that there are two other limitations to the method, and these are set out below.

3) The binding of only one ion to a ligand can be studied. If there is simultaneous binding of two ions to the ligand *e.g.* Ca^{2+} and Mg^{2+} to HEDTA, the LOM cannot be used to measure the K' and the $[Ligand]_T$ (Tran et al., 2018).
4) The limit of detection of an electrode is defined as the pX value where the asymptote of the Nicolsky-Eisenman equation meets the Nernst equation, shown in Figure 4C (see also Lüthi et al.,1997, Figure 2A). For Ca^{2+}/Mg^{2+}-electrodes this value approximates to the lumped interference Σ (Lüthi et al., 1997) and in this case is equal to a pCa of 7.21, As illustrated in Figure 4C electrodes can measure down to (Σ +1), in this case to around pCa 8.0. The 10 buffers cover the range, pK' ± 1; thus, a ligand with a pK' value equal to the lumped interference Σ could be measured with the LOM. This would be the lower limit of measurement.

5.4 Three additional problems with the use LOM

5.4.1 Contamination

In calibration solutions contamination can be around 20 µmol/l due to using double distilled water in the manufacture of the solutions (Lüthi et al.,1997, Figure 3A). To reduce contamination to an absolute minimum, high resistivity (18.2 MΩ.cm) distilled water must be used and all solutions passed through a Chelex 100 resin column (Blinks et al., 1978; Lüthi et al., 1997). Blinks (1989, p 192) wrote: "With the use of Chelex-treated solutions, it is fairly easy to control Ca^{2+} concentrations down to about 10^{-6} M and possibly as low as 10^{-7} M without the use of Ca^{2+} buffers". However, this means using plastic labware and acid washing (see Table 4, Neumaier et al., 2018).

Professor John Blinks and colleagues at the Mayo Clinic (Allen et al., 1977) used calibration solutions ranging from pCa 7.5 (31.6 nmol/l) to pCa 2 (10 mmol/l) to calibrate Aequorin. They measured the pK' of Ca^{2+} binding to EGTA, EDTA and CDTA individually, by superimposing the buffer curve on the calibrated Aequorin curve. The problems of Ligand purity were unknown in 1977 but despite this, the experiments were ground-breaking. The idea of superimposing the calibration curve and the buffer curve is the basis for the LOM (Step 3, Figure 4A). It also means the use of LOM can be expanded to include not only calibrations in the mmolar range 4.0 mmol/l to 0.5 mmol/l, but also to include calibration solutions from 0.1 µmol/l to 2 mmol/l.

5.4.2 Accuracy of the buffer solutions

The manufacture of buffers by the ratio method although straightforward, requires care and technical skill. If a series of buffers is manufactured and the $[Ca^{2+}]/[Mg^{2+}]$ in each of the 10 buffers is measured, then the coefficient of variation (CV, $\frac{SD}{mean} * 100$) can be taken as a measure of accuracy of manufacture (Table 2, McGuigan & Stumpff, 2013). During the development of the LOM, the CV for EGTA buffers was 27.4% (McGuigan et al., 2007), later reduced to 7% or less for EGTA and BAPTA buffers due to an increase in the accuracy of weighing, pipetting, pH_a control and in the manufacture of the solutions (McGuigan & Stumpff, 2013). Such CV are possible and should be aimed for.

If the $[X^{2+}]$ in the buffer solutions are calculated, it is impossible to estimate the CV of the buffer solutions and there is no way to confirm the accuracy of such calculations.

5.4.3 Changes in the potential at the reference electrode.

It is routine to use a 3 mol/l KCl as a reference electrode. The potential of this electrode will only remain constant if the ionic strength and ionic concentrations are identical in all solutions. In Lüthi et al., (1997), the $CaCl_2$ in calibration solutions was varied from 10 mmol/l to 0.5 mmol/l. While calibration and buffer solutions have the same pH_a, $[K]_T$, $[Na]_T$ and $[HEPES]_T$ the $CaCl_2$ in all solutions varied. Because of these differences in the $[Ca]_T$ and $[Cl]_T$ in the solutions, the ionic strength varied from 188.7 mmol/l in calibration solution C1 to 156.2 mmol/l in buffer solution B10, a difference of 32.5 mmol/l.

These differences caused minor changes in potential at the reference electrode (Stumpff & McGuigan, 2014). In Figure 4A each phase of the calculation was carried out separately, which was why calibration solutions C1 to C7 were from 0.5 mmol/l to 10 mmol/l. In the most recent program (see page 18) the Nicolsky-Eisenman equation is optimised to fit both the calibration and buffer curves simultaneously. Because of this, the $[Ca]_T$ in calibration solution C1 has now been reduced from 10 mmol/l to 4 mmol/l and the $[Ca]_T$ in calibration solutions (C1 to C6) are 4.0, 2.5, 1.5, 1.0, 0.75 and 0.5 mmol/l respectively. The differences in $[Cl]_T$ and ionic strength are reduced to 28.2 mmol/l and 32.5 mmol/l respectively. The calculated changes in potential at the reference electrode are now less than 0.4 mV making it possible to routinely use a 3 mol/l KCl electrode as a reference electrode.

6 Calibration of Ca^{2+}/Mg^{2+}-electrodes, fluorochromes and photoproteins

6.1 Generalisation of the LOM

The two methods for measuring the pK' value and the $[Ligand]_T$ in X^{2+} buffer solutions, both in conjunction with the LOM are:

1) Method 1: Calibration in unbuffered solutions in the range from 0.5 mmol/l to 4 mmol/l.
2) Method 2: Calibration in unbuffered solutions from 0.1 µmol/l to 2 mmol/l.

Method 1 is the original method used to calibrate electrodes (Figure 4). With Method 2, using unbuffered calibrating solutions the LOM can be generalised to calibrate not only electrodes but also fluorochromes and photoproteins. Table 1 (an update of Table 5 in McGuigan et al., 2006) defines the calibration solutions for both methods. It also shows the buffer ratios and $[X]_T$ for both methods. Method 1 can be used in any laboratory. Method 2 requires special laboratory facilities, as described in detail in Blinks (1989) and in Lüthi et al., (1997).

6.2 Calibration of electrodes

Both Method 1 and Method 2 can be used. Figure 4C illustrates calibration with Method 1. Figure 4D illustrated calibration with dilution from 2 mmol/l down to 0.1 µmol/l as well as the buffer solutions. Calibration and buffer solutions overlap from pCa 5.5 down to pCa 7. This means that when using calibration solutions set by dilution alone, it would only be necessary to calibrate down to pCa 6.

Calculations for Mg^{2+} electrodes (*s,* 26.206 mV/decade and Σ, $1*10^{-5}$ mol/l, McGuigan et al., 2006) with the ligand EDTA (K' 3.8 µmol/l, McGuigan et al., 2007) gave similar results as that for the Ca^{2+}-electrode. Similar to the Ca^{2+}-electrodes with Method 1, calibration and buffers solutions did not overlap. With Method 2 the calibration curve and the buffer curves did overlap; indeed, the Mg^{2+}-electrode could be completely calibrated without the use of buffers (Lüthi et al., 1997).

Unlike Fluorochromes and Aequorin which require complicated and expensive equipment, electrodes require only a Ca^{2+}/Mg^{2+}-electrode, a 3 mol/l KCl reference electrode and a pH meter in mV mode. Laboratory glassware can be used if washed three times in high-resistivity distilled water (Lüthi et al., 1997; Tran et al., 2018).

Table 1: Calibrating and buffer solutions for Methods 1 and 2

Method 1			Method 2		
Calibration Number	$[X]_T$ (mmol/l)	pX	Calibration Number	$[X]_T$ (mmol/l)	pX
Calibration solutions					
C1	4.00	2.3979	C1	2.000	2.6990
C2	2.50	2.6021	C2	1.500	2.8239
C3	1.50	2.8239	C3	1.000	3.0000
C4	1.00	3.0000	C4	0.500	3.3010
C5	0.75	3.1249	C5	0.250	3.6021
C6	0.50	3.3010	C6	0.100	4.0000
			C7	0.050	4.3010
Buffer Solutions	Ratio	$[Ca]_T$	C8	0.025	4.6021
		(mmol/l)	C9	0.010	5.0000
1	7:1	3.5000	C10	0.005	5.3010
2	6:1	3.4286	C11	0.0025	5.6021
3	5:1	3.3333	C12	0.0010	6.0000
4	4:1	3.2000	C13	0.0005	6.3010

5	3:1	3.0000	C14	0.00025	6.6021
6	2:1	2.6666	C15	0.00010	7.0000
7	1:1	2.0000			
8	1:2	1.3333			
9	1:4	0.8000			
10	1:9	0.4000			

6.3 Calibration of Fluorochromes

With Ca^{2+}fluorochromes, Method 1 is not applicable because calibration solutions C1 (4.0 mmol/l, pCa 2.398) to C6 (0.5 mmol/l, pCa 3.301) lie on the flat part of the calibration curve (Figure 5A) and only Method 2 can be used. This is illustrated in Figure 5A for the fluorochrome OGB-1 using the values from Tran et al., (2018). Their calibration curve was fitted by the following equation:

$$F = F_{Min} + (F_{Max} - F_{Min})\frac{10^{-\mathrm{pCa}}}{(10^{-\mathrm{p}K'} + 10^{-\mathrm{pCa}})}$$

[5]

This equation can be normalised for the fluorescence to lie between 0 and 1 as follows:

$$F_N = \frac{10^{-\mathrm{pCa}}}{(10^{-\mathrm{pK}'} + 10^{-\mathrm{pCa}})} \text{ where } F_N = \frac{(F - F_{Min})}{(F_{Max} - F_{Min})} \qquad [6][7]$$

Figure 5A shows a calibration curve for OGB-1, this time down to a pCa of 7 because it is the most sensitive of the probes investigated by Tran et al., (2018, their Table 5, K' 0.26 µmol/l). To illustrate the use of LOM to determine ligand purity, a purity of 95% was used for the EGTA buffer solutions, and an assumed pK' of 6.0, instead of the measured pK' value of 6.824 (Tran et al., 2018), was used in the calculations. The calculated $[Ca^{2+}]$ is shown in the 10 buffer solutions for $[EGTA]_T$ of 4 mmol/l (open triangles) and 3.4 mmol/l (open circles). The best fit to the normalised equation [4] was with an $[EGTA]_T$ of 3.7803 mmol/l and this is shown as the red open squares. At a normalised fluorescence value of 0.5 the $[Ca^{2+}]$ is equal to the K' and to superimpose both buffer and calibration curves the shift along the pCa axis was calculated as the log difference, $\log(\frac{K' \text{ buffer}}{K' \text{ calibration}})$. In the example shown in Figure 5A this was 0.8329 pCa units. Two more iterations gave the correct values for both pK' of 6.824 and $[EGTA]_T$ of 3.8 mmol/l (Figure 5B).

Mg^{2+} fluorochromes have K' values in the mmolar range *e.g.* Magnesium Green has a K' value of 1 mmol/l (manufacturer). Calibration of this most sensitive Mg^{2+} fluorochrome does not

require Mg^{2+} buffers as it can be carried out by using Mg^{2+} calibration solutions between 5 mmol/l (pMg 2.301) and 1 µmol/l (pMg 6).

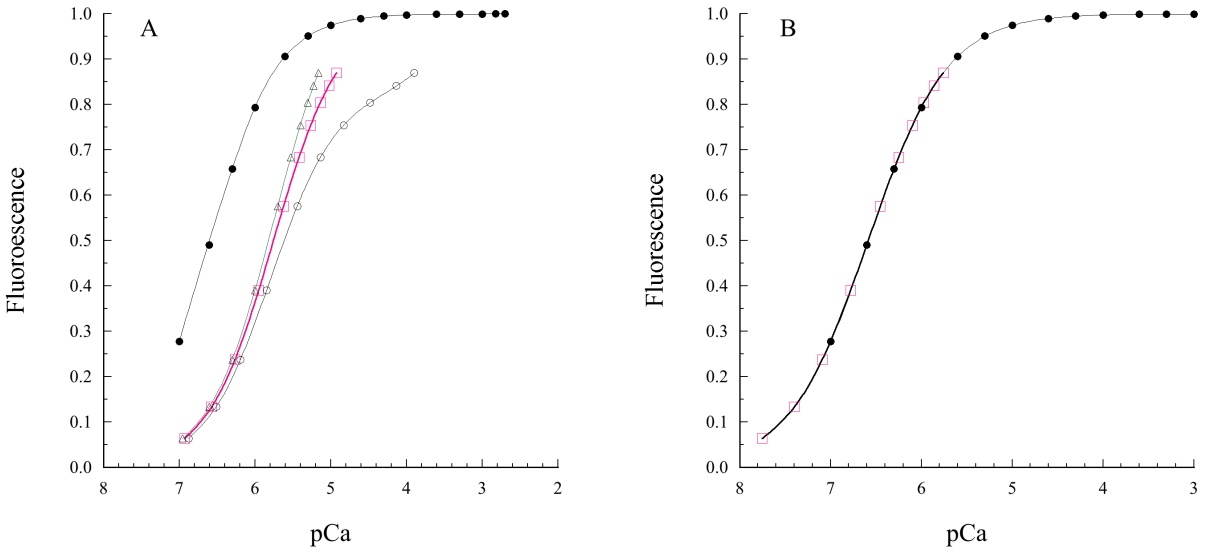
6.4 Calibration of photoproteins

The photoprotein most frequently used is Aequorin (Allen et al., 1977). Only Method 2 can be used in calibration and this is illustrated in Figure 5C, using the parameters from Allen et al., (1977). The curve was fitted by the equation for their model B namely:

$$\log\left(\frac{L}{L_{Max}}\right) = 3*1\log\left(\frac{1+K_R[Ca^{2+}]}{1+K_{TR}+K_R[Ca^{2+}]}\right) \qquad [8]$$

Where, $K_R = 7 *10^6$ mol^{-1} and $K_{TR} = 118$.

Allen et al., (1977) used the ratio method to manufacture the buffer solutions. To mimic their experiments, we have used the 10 EGTA buffer ratios from Table 1, but for 1 mmol/l EGTA. The importance of purity was tested by assuming a purity of 90% (Tran et al, 2018). The optimisation of the $[EGTA]_T$ is illustrated in Figure 5C for three values of $[EGTA]_T$, namely 1.0 mmol/l, 0.8829 mmol/l (best fit to equation [8]) and 0.85 mmol/l. The mid-point of the Aequorin calibration curve was at a L/L_{Max} of -3.113 and the pCa at this value was calculated for both the calibration curve and for a pK' of 6. The difference between the two pCa values allowed the buffer curve to be moved along the x-axis to superimpose both curves. In all, four iterations gave the correct values for pK' and $[EGTA]_T$ of 6.45 and 0.9 mmol/l respectively. The calibration curve and the superimpose buffer curve are illustrated in Figure 5D.



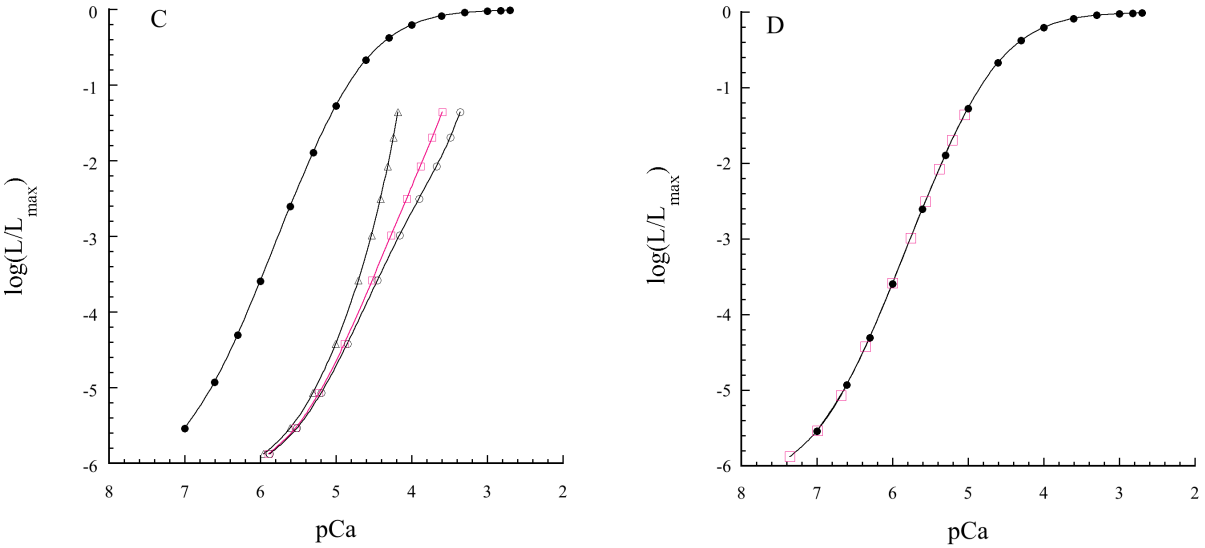


Figure 5. Filled black circles calibration solutions, open red squares EGTA buffers. A. Calibration curve for the Ca^{2+} fluorochrome OGB-1 using Method 2. The use of LOM to determine Ligand purity is also shown in the Figure. The $[EGTA]_T$ were 4 mmol/l (open triangles), 3.7803 mmol/l, the best fit to equation [6] (open red squares) and 3.4 mmol/l (open circles). B. Superimposition of the calibration and buffer curves for OGB-1. In both 5A and B the curves have been fitted with equation [6]. C. Calibration of Aequorin from pCa 7 to pCa 2 The points have been fitted by equation [8]. Also shown are the EGTA buffer values calculated for $[EGTA]_T$ of 1.0 mmol/l (open triangles), 0.8829 mmol/l (open red squares), best fit to equation [8] and 0.85 mmol/l (open circles). For clarity, the three EGTA buffer curves have been moved along the pCa by -1 pCa unit. D. Superimposed calibration curve and EGTA buffer curve. In both 5C and D the curves have been fitted with equation [8].

6.5 Automatic determination of pK', $[Ligand]_T$ and the $[X^{2+}]$ in the buffer solutions

With electrode data, computation in the original LOM was automated by defining an objective function and then minimizing it subject to constraints, as described in Kay et al., (2008). The computer program described there is now obsolete. Since then, the LOM has been improved (McGuigan at al., 2014) and computer programs, written in "R" (R Core Team, 2018), have been available. They were used successfully by Tran et al., (2018) and are available from the data repository: https://github.com/JWKay/LigOpt

For data obtained using fluorochromes or photoproteins, the new computer program LOME (also available in the data repository) has been written in "R" in order to automate the extended LOM computation that is illustrated in Subsections 6.3 and 6.4. Measurements of $[X^{2+}]$ in the

buffer solutions are given. The $[X^{2+}]$ in the buffers can be estimated as a function of pK' and $[Ligand]_T$ by using the following equation (McGuigan et al., 2006):

$$[Ca^{2+}] = \frac{-([Ligand]_T + K' - [Ca]_T) + \sqrt{([Ligand]_T + K' - [Ca]_T)^2 + 4K'[Ca]_T}}{2} \quad [9]$$

The unknown parameters pK' and $[Ligand]_T$ are then determined by minimizing the sum of the absolute values of the respective differences between the measured and the estimated $[X^{2+}]$ in the buffer solutions, subject to the constraints that pK' > 0 and also that $[Ligand]_T \leq [Ligand]_N$. To initialise the optimisation, an initial value for pK' is obtained from equation [10] and $[Ligand]_T$ is set to equal $[Ligand]_N$ (Appendix to McGuigan et al., 2006, Section-1 page 22):

$$K' = \frac{[Ca^{2+}]\{[Ligand]_T - ([Ca]_T - [Ca^{2+}])\}}{([Ca]_T - [Ca^{2+}])} \quad [10]$$

7 Discussion

7.1 Calculation and measurement of $[Ca^{2+}]/[Mg^{2+}]$ in buffer solutions

As shown in Figure 2 calculation gives answers varying from 0.8 µmol/l to 3.45 µmol/l in buffer solutions B1, or a difference of 4.3 times; not surprising as 1) the correction for ionic strength involve single ion activity coefficients which cannot be measured and 2) it is not possible to accurately convert measured pH_a (activity) to pH_c (concentration).

There are two Methods to measure the $[Ca^{2+}]/[Mg^{2+}]$ in buffer solutions both based on the LOM. In Method 1, the calibration solutions are from 4 mmol/l to 0.5 mmol/l and it can be used in any laboratory (Tran et al., 2018). Once the electrode is calibrated it can be used to calibrate fluorochromes and photoproteins. Method 2, in which calibration solutions range from 0.1 µmol/l to 2 mmol/l, requires special laboratory facilities but it is the "Gold Standard" for calibration.

7.2 Accuracy of $[Ca^{2+}]/[Mg^{2+}]$ buffer solutions and relation to IS units

Measurement of the $[X^{2+}]$ in the buffers means there is a measure of the precision, and a value of a CV of 7% or less should be aimed for (McGuigan, et al., 2007). Since calibration of Ca^{2+}/ Mg^{2+}-electrodes, fluorochromes and photoproteins depends on accurate buffers, it is crucial that the accuracy of these buffers is known. These concentrations can be related to SI units. If the $[Ca^{2+}]$/ $[Mg^{2+}]$ in buffers are calculated there is no way to verify the accuracy of the buffers. Moreover, the

calculated concentrations in the buffer solutions cannot be related to SI units, because of the assumptions involved in the calculations.

7.3 Consequences of inaccurate buffer solutions

The values for resting $[Ca^{2+}]$, $\Delta[Ca^{2+}]$ as well as the pK' values for binding of Ca^{2+} and Mg^{2+} to intracellular sites, estimated using calculated concentrations in buffers, are not accurate. This has consequences for both the measurement of the pK' values for fluorescent indicators and for modelling as was shown by Tran et al., (2018). These authors used Method 1 to measure the $[Ca^{2+}]$ in their buffer solutions and to measure the pK' values for 5 different Ca^{2+} indicators. The buffer solutions had similar composition, ionic strength, temperature and pH_a as in their experiments. They found there was no correlation between the measured values for the pK' and the values from the manufacturers or the literature (Tran et al., 2018, Table 5). The batch to batch variation of the values of the indicators was not due to impurities, but due to calculating the $[Ca^{2+}]$ in the buffers and ignoring the purity of EGTA. This has ramifications in modelling. When using the measured pK' values for the indicators they showed that the rise in $[Ca^{2+}]$ at the nerve endings was greater than previously calculated, and the extent of buffering less (their Figure 4).

8 Conclusions

We conclude that not much has changed since Weber & Murray in 1973, in their article in Physiological Reviews (footnote page 647), pointed out that the use of diverse constants by different investigators means different absolute values for resting $[Ca^{2+}]$ and $\Delta[Ca^{2+}]$. After 46 years the time has come to rectify this situation by routinely measuring $[Ca^{2+}]/[Mg^{2+}]$ in buffer solutions. The way to do this, is with Ca^{2+}/Mg^{2+}-electrodes combined with the software "R" as explained in detail in the Supporting Information.

Author contributions:

JMcG conceived and designed the work. All the authors contributed (i) to the acquisition, analysis and interpretation of data and (ii) to the writing of the paper. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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