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Membrane Fusion Techniques

Part B

EDITED BY

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[12] Calculation and Control of Free Divalent Cations in Solutions Used for Membrane Fusion Studies

By KARL J. FÖHR, WOJCIECH WARCHOL, and MANFRED GRATZL

Introduction

The investigation of intracellular processes requires aqueous media that mimic the intracellular fluid. The adjustment of the free Ca^{2+} concentration in these media is of critical importance because of the essential role of Ca^{2+} in the control of exocytotic membrane fusion (see, e.g., [11] in this volume). It is evident that precisely defined free Ca^{2+} concentrations in the submicromolar range cannot be easily obtained by adding the salts to a solution, because laboratory equipment, distilled water, and chemicals are contaminated with Ca^{2+}. In addition, Ca^{2+} binding to cellular constituents and membranes as well as active sequestration by cellular organelles must be taken into account. Therefore divalent cations must be buffered, as is routinely done for protons. One common problem is the choice of appropriate ligands to buffer free Ca^{2+} at a given value, and the calculation of complex media that contain more than one ligand and more than one metal ion. In this chapter a computer program is described that allows the calculation of multiple equilibria between different ligands and metal ions. Although the media prepared according to the calculation generally give concentrations in good agreement with measured values, the calculation should always be controlled. For this purpose, an easy and inexpensive procedure for the preparation of ion-selective electrodes is then described.

Calculation of Ligand–Metal Equilibria

Special ligands have been developed for buffering metal ions.[1] The cation-binding sites of these ligands also bind protons. Thus the addition of acid to aqueous solutions of metal–ligand complexes leads to an increase of free metal ions. Conversely, alkalinization results in stronger binding

[1] G. Schwarzenbach, H. Senn, and G. Anderegg, *Helv. Chim. Acta* **40**, 1886 (1957).

between ligands and metal ions and, consequently, in a decrease in the free metal ion concentration. This competition between protons and metal ions is not a serious problem because the pH is generally buffered to a fixed value that can be used for the purposes of calculation as a constant. To simplify the mathematical calculation, so-called "apparent association constants" with different definitions have been introduced.[2-5] The term *apparent association constants* is used in this chapter for recalculation of absolute metal–ligand association constants for a fixed pH value.[2]

The absolute association constants required for the calculation of metal buffers were originally determined at an ionic strength I of 0.1 and a temperature T of 20°.[1,3,6] Biological experiments are often carried out at different temperatures and ionic strengths. Mathematical procedures have been proposed to adjust the absolute association constants for the desired conditions.[7,8] It should be remembered that absolute association constants are listed in terms of concentrations, whereas pH measured with a glass electrode is determined in terms of activity.[6,9] To obtain the same units, either the proton activity can be converted to concentration[10] or metal association constants can be expressed in terms of activities.[11] Alternatively, mixed binding constants may be used.[4,6,9]

It is often necessary to buffer Ca^{2+} in the submicromolar range and Mg^{2+} in the millimolar range, that is, at concentrations occurring in the cytosol of living cells. In Fig. 1 a computer program is described to calculate such a metal buffer with ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) as a ligand. Briefly, in Part I of Fig. 1 the absolute association constants (for $I = 0.1$ and $T = 20°$)[6] and the considered equilibria are listed. Part II (Fig. 1) contains the input of the final parameters (free divalent metal ions and total amount of ligands), including conversion of proton activity to proton concentration.[10,11] Part III (Fig. 1) calculates the apparent association constants (according to Ref. 2) followed by that of the free ligand concentration. From the free ligand

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[3] A. Fabiato and F. Fabiato, *J. Physiol. (London)* **75,** 463 (1979).
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[5] N. Stockbridge, *Comput. Biol. Med.* **17,** 299 (1987).
[6] A. E. Martell and R. M. Smith, "Critical Stability Constants," Vol. 1, Plenum, New York, 1974.
[7] O. Scharf, *Anal. Chim. Acta* **109,** 291 (1979).
[8] S. M. Harrison and D. M. Bers, *J. Am. Physiol.* **256,** C1250 (1989).
[9] R. Y. Tsien and T. J. Rink, *Biochim. Biophys. Acta* **599,** 623 (1980).
[10] D. Ammann, T. Bührer, U. Schefer, M. Müller, and W. Simon, *Pflügers Arch.* **409,** 223 (1987).
[11] A. C. H. Durham, *Cell Calcium* **4,** 33 (1983).

concentration and the apparent association constants the concentration of each metal–ligand complex is calculated. Finally, the sum of each metal species (free and complexed forms) is calculated to give the total amount of required metals to prepare the medium of interest. This procedure forms the basis for calculating complex media and has the advantage over other published programs[3,5,13] that no iterative calculation is required.

The example in Fig. 1 shows the computation to buffer free Ca^{2+} in the submicromolar range. To investigate the Ca^{2+} dependency of biological processes appropriate buffers in the range between 0.1 and 100 μM are required. Calcium ion buffering in the higher micromolar range can be achieved by lowering the pH (Fig. 2a). Alternatively, if the pH must be kept constant, other ligands must be selected. For this purpose, ligands like HEDTA or nitrilotriacetic acid (NTA) are applicable[9] although these ligands, like ethylenediaminetetraacetic acid (EDTA), do not discriminate as well as EGTA between Ca^{2+} and Mg^{2+} (Fig. 2b). Furthermore, in some experiments, naturally occurring ligands such as ATP or GTP must also be taken into account. To follow these requirements the program must be enlarged. This can be done by a simple routine that calculates the additional apparent association constants of the new ligand metal complexes. Thereafter, the concentration of the new ligand–metal complexes can be calculated and summarized as described above.

The computer program developed by the authors considers nine different ligands (EDTA, EGTA, HEDTA, NTA, ATP, ADP, GTP, phosphate, and creatine phosphate), and corrections for temperature and ionic strength. The program calculates either the total amount of metals to give the desired free metal concentrations (Ca^{2+}, Mg^{2+}) or, in the reversed mode, it calculates the free metal concentration for a given total amount of metals and the selected mixture of ligands. Furthermore, an option exists for calculating the apparent association constants under different conditions (pH, T, I), in order to choose the appropriate ligands for the experimental purposes. A further option illustrates the complex situation by drawing buffer curves (see Fig. 2b). In addition, absolute association constants, enthalpy values for temperature correction, and Debye–Hückel parameters for correction of ionic strength can be changed and saved as a separate file. (The program may be obtained from the authors on request.)

Despite the sophisticated calculation of metal buffers as described above, the media prepared do not necessarily have the desired free divalent metal concentrations. Apart from uncertainties in the absolute association

[12] P. C. Meier, D. Ammann, W. E. Morf, and W. Simon, *in* "Medical and Biological Applications of Electrochemical Devices" (J. Koryta, ed.), p. 13. 1980.

[13] A. Fabiato, this series, Vol. 157, p. 378.

rem Part I
rem Main forms present in a solution containing:
rem the Ligand EGTA (L), Ca and Mg: L_{free} + Ligand-Metal-Complexes + $Metal_{free}$
rem $Ligand_{free}$: L, HL, H2L, H3L, H4L (L = $EGTA^{4-}$)
rem Ligand-Metal-Complexes : CaL, CaHL, MgL, MgHL
rem $Metall_{free}$: Ca_{free}, Mg_{free}

rem absolute association constants (log K values for
rem T = 20°C, I = 0.1); for other conditions the
rem absolute association constants must be recalculated

equilibria

$K_{H1} = 10^{9.47}$	rem: H + L -- HL
$K_{H2} = 10^{8.85}$	rem: H + HL -- H2L
$K_{H3} = 10^{2.66}$	rem: H + H2L -- H3L
$K_{H4} = 10^{2.0}$	rem: H + H3L -- H4L
$K_{Ca1} = 10^{10.97}$	rem: Ca + L -- CaL
$K_{Ca2} = 10^{3.79}$	rem: H + CaL -- CaHL
$K_{Mg1} = 10^{5.21}$	rem: Mg + L -- MgL
$K_{Mg2} = 10^{7.62}$	rem: H + MgL -- MgHL

rem Part II
rem Input: desired conditions: $Metal_{free}$, $Ligand_{total}$, pH

$Ca_{free} = 10^{-7}$	rem: Ca = Ca^{2+}
$Mg_{free} = 10^{-3}$	rem: Mg = Mg^{2+}
$L_{total} = 10^{-2}$	
pH = 7.2	rem: (activity)
$H = 10^{-7.089}$	rem: (concentration)

rem Part III
rem Calculation of apparent association constants (depending on H)

$Sum = 1 + K_{H1}*H + K_{H1}*K_{H2}*H^2 + K_{H1}*K_{H2}*K_{H3}*H^3 + K_{H1}*K_{H2}*K_{H3}*K_{H4}*H^4$ rem: Calculated values
rem: 14108

			log K_{app}
$K_{appCa1} = K_{Ca1} / Sum$	rem:	6614605.5	6.82
$K_{appCa2} = K_{Ca1} * K_{Ca2} * H / Sum$	rem:	3322.8	3.52
$K_{appMg1} = K_{Mg1} / Sum$	rem:	11.5	1.06
$K_{appMg2} = K_{Mg1} * K_{Mg2} * H / Sum$	rem:	39	1.59

rem Calculation of free Ligand concentration (L_{free})

$L_{free} = L_{total} / (1 + K_{appCa1} * Ca_{free} + K_{appCa2} * Ca_{free} + K_{appMg1} * Mg_{free} + K_{appMg2} * Mg_{free})$ rem: 5.84^{-3} (Mol/l)

rem Calculation of Ligand-Metal-Complexes (CaL, CaHL, MgL, MgHL)

$CaL = L_{free} * K_{appCa1} * Ca_{free}$	rem:	3.863^{-3}	(Mol/l)
$CaHL = L_{free} * K_{appCa2} * Ca_{free}$	rem:	1.941^{-6}	(Mol/l)
$MgL = L_{free} * K_{appMg1} * Mg_{free}$	rem:	6.713^{-5}	(Mol/l)
$MgHL = L_{free} * K_{appMg2} * Mg_{free}$	rem:	2.280^{-4}	(Mol/l)

rem Output: required total metal concentrations to achieve the desired free metal concentrations

$Ca_{total} = Ca_{free} + CaL + CaHL$	rem:	3.864^{-3}	(Mol/l)
$Mg_{total} = Mg_{free} + MgL + MgHL$	rem:	1.295^{-3}	(Mol/l)

FIG. 1. Program to calculate metal buffers.

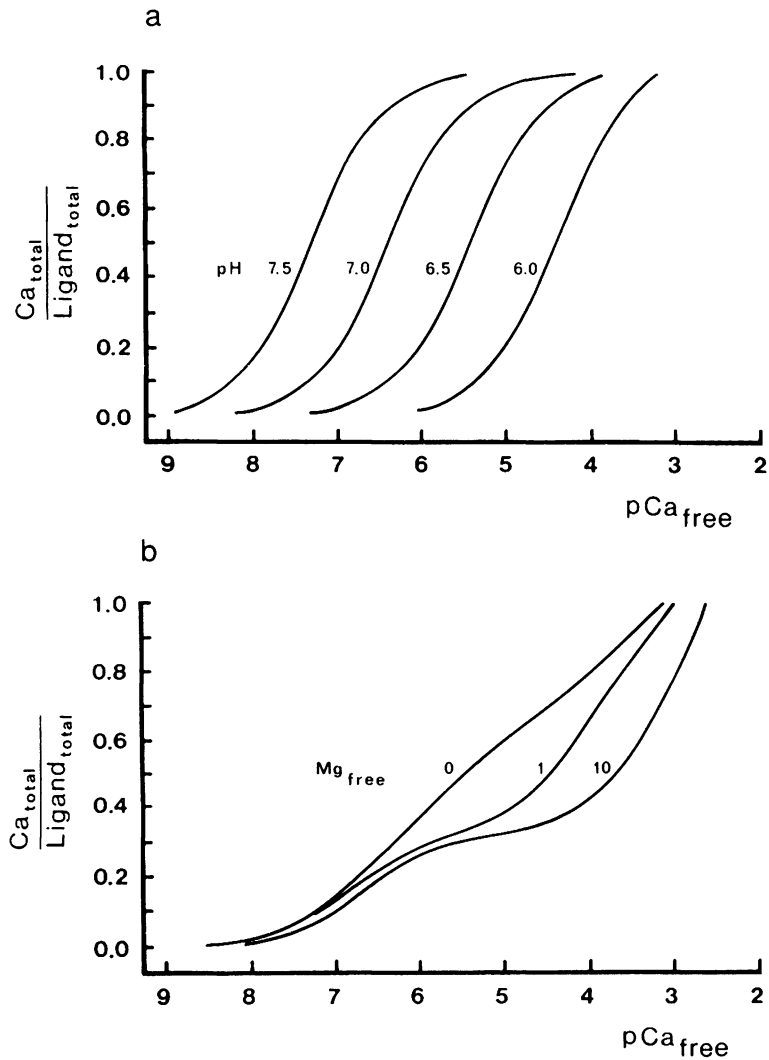

FIG. 2. Calcium ion buffering with selected media. (a) Calcium ion binding to ligands such as EGTA strongly depends on pH. The optimal buffer capacity for a given pH value occurs at a ratio (Ca_{total} to $Ligand_{total}$) of 0.5, which corresponds to the apparent association constant. From these curves it can be deduced that Ca^{2+} buffering is assured at ratios between 0.2 and 0.8. (b) Calcium ion buffer curve for more complex media (5 m*M* EGTA, 5 m*M* HEDTA, 5 m*M* NTA; pH 7.2) to cover a wide range of free Ca^{2+} concentrations at different Mg^{2+} concentrations (0, 1, or 10 m*M*). In the absence of Mg^{2+} almost constant Ca^{2+} buffering capacity can be obtained between pCa 6.8 and pCa 4. In the presence of increasing amounts of free Mg^{2+} progressively reduced Ca^{2+} buffering exists, as indicated by the shoulder around 10 μM free Ca^{2+}.

constants (as documented by differences in the published constants), the purity of the ligands[14] and errors in pH measurements[15] may contribute to a difference between calculated and actual free metal ion concentrations. Therefore the prepared media should be checked by ion-specific electrodes.

Estimation of Free Metals with Ion-Selective Electrodes

Ion-selective electrodes are valuable tools to measure the activity of ions. To become familiar with the chemical, physical, and mathematical background of ion-selective electrodes the reader should consult previous publications.[16-18]

Ion-selective electrodes may be obtained from different suppliers or can be made in a laboratory workshop. The equipment for ion-selective electrodes is analogous to that of a pH electrode: it consists of ion-selective and reference half-cells connected to a recording system (conventional pH meter).

The most important part of the ion-selective half-cell is the ion-selective membrane. The Ca^{2+}-selective polyvinyl chloride (PVC) membranes are made according to Schefer *et al.*[19] and Mg^{2+}-selective membranes are made according to Hu *et al.*[20] For the preparation of Ca^{2+}-selective membranes the neutral ligand ETH129 was chosen because of its low detection limit and high selectivity over other ions.[10,19] All chemicals necessary for the preparation of ion-selective membranes are commercially available from Fluka (Buchs, Switzerland). The Ca^{2+}-selective (Mg^{2+}-selective: values in parentheses) membranes are made by dissolving 102.1 mg (120.1 mg) polyvinyl chloride, 204.1 mg (238 mg) *o*-nitrophenyloctyl ether, 1.75 mg (2.25 mg) potassium tetrakis(4-chlorophenyl)borate, and 3.1 mg ETH129 (3.64 mg ETH5124) in 5 ml tetrahydrofuran. When fully dissolved the fluid is poured into an appropriate glass petri dish, 3 cm in diameter, which should be partly covered to assure slow evaporation of the solvent overnight. The remaining PVC membrane can be stored in the

[14] D. J. Miller and G. L. Smith, *J. Am. Physiol.* **246,** C160 (1984).
[15] J. A. Illingworth, *Biochem. J.* **195,** 259 (1981).
[16] W. E. Morf and W. Simon, *in* "Ion-selective Electrodes in Analytical Chemistry" (H. Freiser, ed.), Vol. 1, p. 211. Plenum, New York, 1978.
[17] A. K. Covington, "Ion-Selective Electrode Methodology," Vols. 1,2. CRC Press, Boca Raton, FL, 1979.
[18] K. Cammann, "Working with Ion-Selective Electrodes." Springer-Verlag, Berlin and New York, 1979.
[19] U. Schefer, D. Ammann, E. Pretsch, U. Oesch, and W. Simon, *Anal. Chem.* **58,** 2282 (1986).
[20] Z. Hu, T. Bührer, M. Müller, B. Rusterholz, M. Rouilly, and W. Simon, *Anal. Chem.* **61,** 574 (1989).

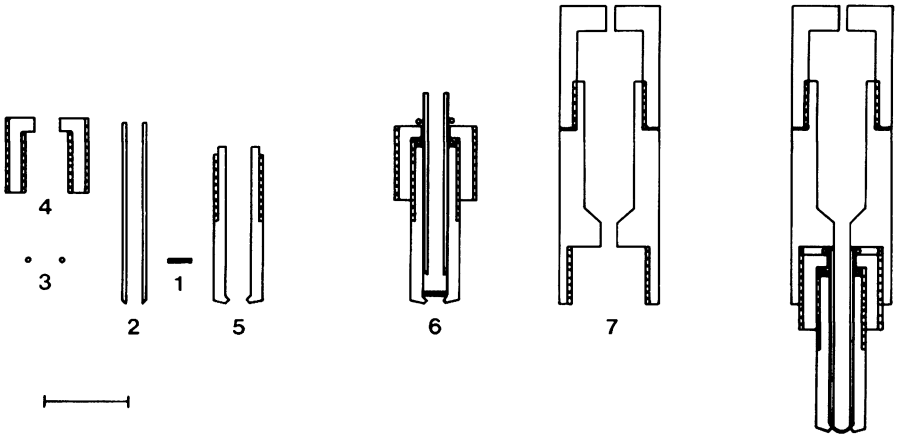

FIG. 3. Design of the ion-selective half-cell. The ion-selective membrane (1) is inserted into the electrode body as described in text. (3) indicates sections through O rings used for sealing. Hatched areas are threads. Bar: 1 cm.

refrigerator at 4° for about 1 year. The major problem in the construction of the electrode body is to separate the sample solution completely from the internal filling solution by the ion-selective membrane. This can be done by using an O ring,[17] by using adhesives,[21] or by mechanical clamping.[16] The electrode body developed and used in the authors' laboratory is shown in Fig. 3. The ion-selective membrane [(1), Fig. 3] is inserted in the tip of the electrode body [(5), Fig. 3] and gently squeezed with piece (2) by screwing piece (6) into piece (7). Afterward the ion-selective half-cell is filled with a syringe with either 10 m*M* $CaCl_2$ or 10 m*M* $MgCl_2$.

Tubing or a glass capillary with a salt bridge [1% (w/v) agar in 3 *M* KCl] at the tip may be used as a reference electrode. The reference electrode is filled with 3 *M* KCl (saturated with AgCl). Commercially available silver wires chlorinated electrically (1.5 V for 30 min as an anode in a solution containing about 100 m*M* Cl^-) are placed in the electrode filling solutions and connected with the pH (voltage) meter.

Half-cells prepared in this way are stored in the filling solution overnight. Prior to use, the electrodes should be equilibrated for about 2 hr in the experimental medium. The electrode may be checked rapidly by several changes of the experimental solutions containing 0 Ca^{2+} (medium containing 1 m*M* EGTA) and 1 m*M* Ca^{2+}. Calibration of the electrodes between pCa 2 and pCa 5 dilutions of neutral $CaCl_2$ (e.g., Orion, Lorch,

[21] H. Affolter and E. Sigel, *Anal. Biochem.* **97**, 315 (1979).

Germany) or $MgCl_2$ in experimental solution without any ligands is suitable. For lower metal concentrations the calibration curve (plotted in a semilogarithmic way) may be extended by extrapolation. Alternatively, Ca^{2+} buffers in experimental media with EGTA as the only ligand are suitable.[9,10,22] Then the electrodes can be used to control complex media as calculated at the beginning of this chapter. The electrodes can also be applied for the analysis of intracellular Ca^{2+} uptake and Ca^{2+} release from permeabilized cells.[23-26]

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[25] R. Engling, K. J. Föhr, T. P. Kemmer, and M. Gratzl, *Cell Calcium* **12,** 1 (1991).
[26] K. J. Föhr, Y. Wahl, R. Engling, T. P. Kemmer, and M. Gratzl, *Cell Calcium* **12,** 735 (1991).