*Journal of Fluorescence, Vol. 3, No. 3, 1993*

# A New Calibration Equation for Ratiometric Fluorescent Ion Indicators: Application to Fura-2

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*Received October 18, 1993*

The absolute values of intracellular ion concentrations as monitored by specific fluorescent indicators are determined by using calibration curves obtained under *in vitro* and *in vivo* conditions. In the derivation of the calibration curve by Grynkiewicz et al [(1985) *J. Biol. Chem* **260,** 3440] it is implicitly assumed that the observed fluorescence signal is directly related to the concentrations of the free dye and the dye-ion complex in the ground state. We modified the calibration equation so that ion binding and dissociation in the excited state are taken into account. The extended calibration equation assumes the knowledge of the rate constants in the excited state. Expressions for the calibration curve assuming the absence or presence of an excited-state reaction are compared for the $Ca^{2+}$ indicator Fura-2. The excited-state rate constants are determined by global compartmental analysis of time-resolved fluorescence decays of Fura-2 collected at various excitation and emission wavelengths using different $Ca^{2+}$ concentrations. It is found that for Fura-2 there is negligible interference of the excited-state reaction so that the original calibration can used.

**KEY WORDS:** Ratiometric ion indicators; Fura-2; calibration equations.

## INTRODUCTION

Several laboratories have developed fluorescent indicators undergoing changes in the excitation and/or the emission spectrum upon binding with specific ions. Using the ratio of the fluorescence intensities measured at two wavelengths, the ion concentration can be determined from a calibration equation [1]. This calibration equation is briefly reviewed and modified to take excited-state processes into account. The effect of the excited-state processes on the determination of $Ca^{2+}$ concentration using the indicator Fura-2 is discussed.

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## ORIGINAL CALIBRATION EQUATION

Consider a mixture of free and ion-bound indicators at ground-state concentrations $x_1$ and $x_2$, respectively. The steady-state fluorescence intensity due to excitation within the wavelength interval $\Delta\lambda^{ex}$ around $\lambda^{ex}$ and observed over the emission wavelength interval $\Delta\lambda^{em}$ around $\lambda^{em}$ will be denoted $F(\lambda^{ex}, \lambda^{em})$. Under the conditions that the free and ion-bound dyes do not interact in the excited state and that the absorbance is low, $F(\lambda^{ex}, \lambda^{em})$ is given by

$$F(\lambda^{ex},\lambda^{em}) = s_1(\lambda^{ex},\lambda^{em})x_1 + s_2(\lambda^{ex},\lambda^{em})x_2 \quad (1)$$

where the proportionality factors $s_i(\lambda^{ex}, \lambda^{em})$, $i = 1, 2$, are given by

$$s_i(\lambda^{ex},\lambda^{em}) = 2.3\, dq(\lambda^{ex},\lambda^{em})I_o(\lambda^{ex})\epsilon_i(\lambda^{ex})c_i(\lambda^{em})/k_{oi} \quad (2)$$

and $d$ is the optical path length (length of the excitation light path in the sample); $q(\lambda^{ex}, \lambda^{em})$, the instrumental



1053-0509/93/0900-0169$07.00/0 

factor; $I_O(\lambda^{ex})$, the excitation light flux within the considered excitation wavelength inverval; and $\epsilon_i(\lambda^{ex})$, the extinction coefficient for species $i$ for the excitation wavelength interval $\Delta\lambda^{ex}$ around $\lambda^{ex}$.

$$c_i(\lambda^{em}) = k_{F_i} \int_{\Delta\lambda^{em}} \rho_i(\lambda^{em}) d\lambda^{em} \tag{3}$$

where $k_{Fi}$ is the radiative deactivation rate constant of $i^*$, the excited form of species $i$; $\rho_i(\lambda^{em})$ is the spectral emission density, normalized to the total emission band; and $k_{oi}$ is the sum of the radiative and the nonradiative rate constants of $i^*$.

The proportionality factors $s_1$ and $s_2$ are determined from the fluorescence intensities of calibration solutions: $s_1$ is obtained at a low ion concentration ($x_2 \approx 0$), while $s_2$ is determined from a solution of ion-saturated dye ($x_1 \approx 0$).

Let $F_1$ and $F_2$ be the intensities obtained at the excitation (or emission) wavelengths $\lambda_1$ and $\lambda_2$,

$$F_1 = s_{11}x_1 + s_{21}x_2 \tag{4}$$

$$F_2 = s_{12}x_1 + s_{22}x_2 \tag{5}$$

where $s_{ij}$, $i,j = 1,2$, indicate the proportionality factors obtained for species $i$ at wavelength $\lambda_j$. The fluorescence ratio $R = F_1/F_2$ is related to the ion concentration [M] by

$$[M] = K_d \left(\frac{R-(s_{11}/s_{12})}{(s_{21}/s_{22}) - R}\right)\left(\frac{s_{12}}{s_{22}}\right) \tag{6}$$

where $K_d$ is the dissociation constant [1].

For known values of $K_d$ and $s_{11}$, $s_{12}$, $s_{21}$, and $s_{22}$, Eq. (6) can be used to calculate the ion concentration of the sample by measuring the ratio $R$.

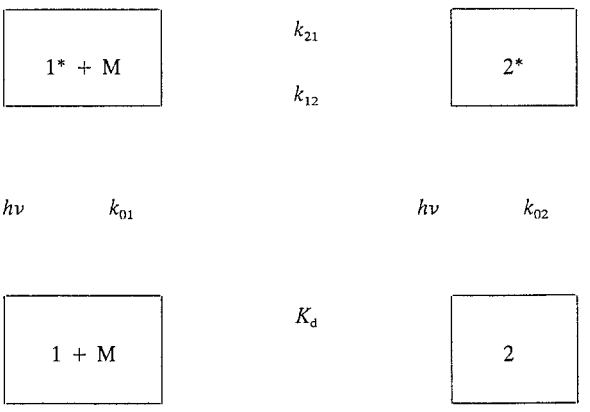
## NEW CALIBRATION EQUATION

A new calibration equation can be derived taking into account possible excited-state reactions (see Scheme I). The concentration of the free and ion-bound dyes in the excited state are denoted by $x_1^*$ and $x_2^*$, respectively. The rate equations for $x_1^*$ and $x_2^*$ are given by

$$\frac{dx_1^*}{dt} = -(k_{01} + k_{21}[M])x_1^* + k_{12}x_2^* + b_1 I_o \tag{7}$$

$$\frac{dx_2^*}{dt} = k_{21}[M]x_1^* - (k_{02} + k_{12})x_2^* + b_2 I_o \tag{8}$$

At low absorbance, $b_i$ can be approximated as




Scheme I

$$b_i \approx 2.3\, d\epsilon_i x_i \tag{9}$$

Setting the time derivatives in Eqs. (7) and (8) to zero yields the steady-state expressions for $x_1^*$ and $x_2^*$. The corresponding expression for the fluorescence intensity is denoted $F^*$ and is given by

$$F^* = p_1x_1 + p_2x_2 \tag{10}$$

where

$$p_1 = \frac{2.3dqI_o}{\Delta}\,\varepsilon_1\,[c_1(k_{02} + k_{12}) + c_2k_{21}[M]] \tag{11}$$

$$p_2 = \frac{2.3dqI_o}{\Delta}\,\varepsilon_2\,[c_1k_{12} + c_2(k_{01} + k_{21}[M])] \tag{12}$$

with

$$\Delta = k_{01}(k_{02} + k_{12}) + k_{02}k_{21}[M] \tag{13}$$

It must be emphasized that $p_1$ and $p_2$ depend on [M], which is in contrast to $s_1$ and $s_2$. This dependence on [M] remains when the ratio of the fluorescence intensities is taken. In general $p_1$, respectively $p_2$, will be different from $s_1$, respectively $s_2$. $p_1 = s_1$ at [M] = 0; $p_2 = s_2$ at very high values of [M]. Considering the fluorescence intensity $F^*$ at two excitation or emission wavelengths, $\lambda_1$ and $\lambda_2$, $F_1^*$ and $F_2^*$, allows one to calculate the ratio $R^* = F_1^*/F_2^*$. Just as for $R$, $R^*$ is independent of the indicator concentration. In general, only the extreme values of $R$ and $R^*$ coincide. The equivalent of Eq. (6) becomes

$$[M] = K_d \left(\frac{R^* - p_{11}/p_{12}}{p_{21}/p_{22} - R^*}\right)\left(\frac{p_{12}}{p_{22}}\right) \tag{14}$$

where $p_{ij}$, $i,j = 1,2$, is the factor corresponding with $x_i$

and $\lambda_j$. For the setup using two excitation wavelengths, $\lambda_1^{ex}$ and $\lambda_2^{ex}$, and one emission wavelength, Eq. (14) can be rewritten as

$$[M] = K_d \left( \frac{R^* - \dfrac{\varepsilon_1(\lambda_1^{ex})\, I_o(\lambda_1^{ex})}{\varepsilon_1(\lambda_2^{ex})\, I_o(\lambda_2^{ex})}}{\dfrac{\varepsilon_2(\lambda_1^{ex})\, I_o(\lambda_1^{ex})}{\varepsilon_2(\lambda_2^{ex})\, I_o(\lambda_2^{ex})} - R^*} \right) \frac{\varepsilon_1(\lambda_2^{ex})\,[c_1(\lambda^{em})\,(k_{02} + k_{12}) + c_2(\lambda^{em})\,k_{21}\,[M]]}{\varepsilon_2(\lambda_2^{ex})\,[c_1(\lambda^{em})\,k_{12} + c_2(\lambda^{em})\,(k_{01} + k_{21}\,[M])]} \tag{15}$$

When one excitation wavelength and two emission wavelengths, $\lambda_1^{em}$ and $\lambda_2^{em}$, are used, one obtains from Eq. (14)

$$[M] = K_d \left( \frac{R^* - \dfrac{q(\lambda_1^{em})\,[c_1\,(\lambda_1^{em})\,(k_{02} + k_{12}) + c_2\,(\lambda_1^{em})\,k_{21}\,[M]]}{q(\lambda_2^{em})\,[c_1\,(\lambda_2^{em})\,(k_{02} + k_{12}) + c_2\,(\lambda_2^{em})\,k_{21}\,[M]]}}{\dfrac{q(\lambda_1^{em})\,[c_1\,(\lambda_1^{em})\,k_{12} + c_2\,(\lambda_1^{em})\,(k_{01} + k_{21}\,[M])]}{q(\lambda_2^{em})\,[c_1\,(\lambda_2^{em})\,k_{12} + c_2\,(\lambda_2^{em})\,(k_{01} + k_{21}\,[M])]} - R^*} \right) U^*(\lambda^{ex},\lambda_2^{em}) \tag{16}$$

with

$$U^*(\lambda^{ex},\lambda_2^{em}) = \frac{\varepsilon_1(\lambda^{ex})\,[c_1(\lambda_2^{em})\,(k_{02} + k_{12}) + c_2(\lambda_2^{em})\,k_{21}\,[M]]}{\varepsilon_2(\lambda^{ex})\,[c_1(\lambda_2^{em})\,k_{12} + c_2\,(\lambda_2^{em})\,(k_{01} + k_{21}\,[M])]} \tag{17}$$

## APPLICATION TO FURA-2

The photophysical properties of the dual-excitation wavelength probe Fura-2 have been studied by Van den Bergh et al. (unpublished results). Fluorescence decay curves of Fura-2 in BAPTA at different concentrations of $Ca^{2+}$, ranging from 100 n$M$ to 0.1 $M$, were measured at two excitation wavelengths (350 and 370 nm) and two emission wavelengths (490 and 510 nm) using time-correlated single-photon timing. The resulting fluorescence decay surface was analyzed by global compartmental analysis [2]. The following values for the rate constants were obtained at room temperature: $k_{01} = (1.239 \pm 0.004) \times 10^9\ s^{-1}$, $k_{21} = (1.03 \pm 0.07) \times 10^{11}\ M^{-1}\ s^{-1}$, $k_{02} = (5.509 \pm 0.008) \times 10^8\ s^{-1}$, and $k_{12} = (2.2 \pm 0.3) \times 10^7\ s^{-1}$. The $pK_d$ values in the ground and excited state are 6.8 and 3.7, respectively. Also, the relative $c_i$ values were determined.

These values were used in the evaluation of Eqs. 6 and 15. For physiologically relevant concentration values of $Ca^{2+}$ the calculated ratio versus -log[$Ca^{2+}$] curves coincide. Therefore, it can be concluded that for Fura-2, the excited-state process has no effect in the determination of intracellular $Ca^{2+}$ levels.

## REFERENCES

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