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# Calibration Methods and Avoidance of Errors in Measurement of Intracellular pH ($pH_{cyt}$) Using the Indicator Bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) in Human Platelets

**Peter A. Valant[1,2] and Duncan H. Haynes[1]**

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Determinations of $pH_{cyt}$ in suspensions of human platelets using BCECF [bis(carboxyethyl)-5(6)-carboxyfluorescein] can be seriously biased by leakage of the fluorescent indicator. Two methods ("pH jump" and "$Mn^{2+}$") are presented for determining the fraction of external indicator ($B_{ext}$) and eliminating this error. Both methods rely on rapid perturbations (pH jump or $Mn^{2+}$ addition), which affect the fluorescence of the external dye immediately and the intracellular dye more slowly. Identical values of $B_{ext}$ are reported. Failure to correct for dye leakage can result in overestimation of $pH_{cyt}$ by as much as 0.4 unit at physiological external pH ($pH_{ext}$). Two methods of calibration of the cytoplasmic signal were compared after correcting for $B_{ext}$: the "digitonin lysis" method and the "nigericin calibration" method. In the digitonin method the dye is released at the end of the experiment and the dependence of its fluorescence is determined as a function of pH. The method assumes that the fluorescence and titration characteristics of the dye in the cytoplasm are not different from those in solution. It gives $pH_{cyt} = 6.75 \pm 0.07$ for $pH_{ext} = 7.3$. In the nigericin method, 150 m*M* external $K^+$ and 10 $\mu M$ nigericin are used for the purpose of setting $pH_{cyt} = pH_{ext}$ to accomplish an *in situ* calibration. The method was complicated by extra leakage induced by nigericin. Assuming that the ionophore could equilibrate pH in the alkaline range, the fluorescence of the anionic form of BCECF in the cytoplasm would be 15% lower than in solution and $pH_{cyt}$ would be 0.3 unit higher than presented above. A number of observations favor the digitonin lysis method of calibration. The fluorescence polarization of BCECF in platelets is small and indistinguishable from that in solution ($0.000 \pm 0.022$). The spectrofluorimetric characteristics of the intracellular dye are identical to those in solution (150 m*M* NaCl or KCl). There was no evidence for self-quenching or binding to cellular elements for cytoplasmic BCECF concentrations up to 1.8 m*M*. The following agents are capable of introducing error: (1) the $Na^+$ substitute *N*-methyl-D-glucamine doubles the $K_d$ and decreases by 13% the $\Delta F_{max}$ of BCECF; (2) the $Na^+/H^+$ exchange inhibitor amiloride quenches BCECF fluorescence and is intrinsically fluorescent; and (3) bovine serum albumin (used to remove nigericin) quenches external BCECF with kinetics mimicking acidification of the cytoplasm.

**KEY WORDS:** Extracytoplasmic bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence; human platelet suspensions; cytoplasmic pH calibration; fluorescence polarization; fluorescence quenching.

[1] Department of Molecular and Cellular Pharmacology (R-189), University of Miami School of Medicine, P.O. Box 016189, Miami, Florida 33101.

[2] To whom correspondence should be addressed.



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## INTRODUCTION

The pH-sensitive fluorescent indicator bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)[3] [1] has been used extensively to measure cytoplasmic pH ($pH_{cyt}$) of many cell types including platelets [2]. Accurate determination of $pH_{cyt}$ is important since membrane-bound pumps and channels and many intracellular regulatory processes are sensitive to changes in $pH_{cyt}$.

Several studies [2–4], including the present one, have shown that BCECF leaks considerably into the extracellular medium of platelet suspensions. Accurate calibration of the cytoplasmic fluorescence against known values of pH is also problematic. Although qualitative information can be obtained without taking the aforementioned dye leakage into account, calculated $pH_{cyt}$ values will be biased in the direction of the external pH ($pH_{ext}$). Indicators suitable for dual-wavelength (ratiometric) measurement are equally susceptible to this bias when cells are studied in suspension but less so when studied by single-cell fluorescence methods. Additions of drugs or agents capable of interacting with indicator can produce artifacts which are both qualitatively and quantitatively misleading for all modes of experimentation.

The literature on the use of BCECF in cell suspensions has numerous examples in which the problem of dye leakage is ignored, many examples in which it is addressed by attempts to remove external dye, and some examples in which it is addressed by making corrections for dye leakage. Davies *et al.* [3] found that 35.5 ± 9.3% of the loaded BCECF leaked from their platelets. Their method of addressing the problem involved centrifugation of samples of platelet suspensions and determination of BCECF fluorescence in the supernatant. Livne *et al.* [2] have employed gel filtration of platelet suspensions as an alternative method of separating platelets from their external medium. However, since the process of dye leakage is continuous, more dye will be released into the medium immediately after the platelets have been separated. Thus, these separation techniques do not allow quantitation of the amount of external dye at the instant at which $pH_{cyt}$ is to be determined. Furthermore, it is not always feasible or desirable to use these separation techniques, since they might increase the fragility of the cells and thus generate further problems in interpretation of results.

The present communication presents in detail two methods to separate the fluorescence of extracellular BCECF ($F_{ext}$) from the fluorescence of cytoplasmic BCECF ($F_{cyt}$) in platelet suspensions. Both methods rely on rapid perturbations of the external medium that appear as instantaneous or vertical shifts in the fluorescence trace. The first method is termed "pH jump." Simpson and Rink [4] have reported the use of an "immediate shift" in BCECF signals seen upon jumping the pH of platelet suspensions by approximately 0.5 pH unit but did not give further detail about how and when their method was applied. The second method in the present study is based on the addition of $Mn^{2+}$, which causes instantaneous quenching of external dye. Both methods are described further under Methods. These procedures allow determination of the fraction of external dye ($B_{ext}$) at any given instant in a platelet suspension and thus permit elimination of its contribution to the measured fluorescence ($F$).

Once the signal from cell-associated BCECF is isolated, the problem becomes calibration. Two methods of calibration of BCECF have been reported in the literature. (A) One procedure is based on the premise that the cytoplasm does not alter the behavior of the dye observed *in vitro*. This procedure is termed the "digitonin-lysis" method, since it uses the fluorescence signal obtained following cell lysis with detergents (i.e., digitonin or Triton X [1,4]). (B) The second method, termed "nigericin calibration," assumes that there may be differences in the reporting behavior of the dye due to effects of the cytoplasmic environment [5]. High

[3] Abbreviations used: $pH_{cyt}$, cytoplasmic pH; BCECF, 2′, 7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; BCECF/AM, pentaacetoxymethyl ester; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES 2-[*N*-morpholino]ethanesulfonic acid; NMDG, *N*-methyl-D-glucamine; [BCECF], BCECF concentration, $[BCECF]_{cyt}$, intracellular BCECF concentration; $F_{min}$, extracellularly measured minimal fluorescence of fully protonated dye; $F_{max}$, extracellularly measured maximal fluorescence of fully deprotonated dye; $F_{ext}$, extracellular fluorescence; $F_{cyt}$, cytoplasmic fluorescence; $F$, observed fluorescence; $F_{\parallel}$, $F_{\perp}$, $I_{\parallel}$, $I_{\perp}$, $T$, and $P$, see Eq. (1) and Methods; $\alpha$, degree of advancement of BCECF deprotonation; $K_d$, dissociation constant of the dye for $H^+$; $\Delta F_{max} = F_{max} - F_{min}$; $B_{ext}$, fractions of extracellular dye; $\alpha_{cyt}$, fraction of deprotonated dye in the cytoplasm; $\alpha_{ext}$, fraction of deprotonated dye in the external medium; $F_{dig}$, fluorescence after cell lysis by digitonin; $\Delta F_{max,\,cyt}$, $\Delta F_{max}$ for the cytoplasmic compartment; $[K^+]_{cyt}$, cytoplasmic $K^+$ activity; $[H^+]_{cyt}$, cytoplasmic hydrogen ion activity; $[H^+]_{ext}$, extracellular hydrogen ion activity; $pH_{ext}$, extracellular pH; $\Delta\alpha_{ext}$, difference between two values of $\alpha_{ext}$; $B_{cyt}$, fraction of dye in the cytoplasmic compartment; $F_{dig,\,pH=11}$, fluorescence at pH 11 after cell lysis by digitonin; $RF$, residual fluorescence after quenching by $Mn^{2+}$; $RF_{max}$, maximal residual fluorescence after $Mn^{2+}$ addition; $RF_{min}$, minimal residual fluorescence after $Mn^{2+}$ addition; $\Delta F_{Mn}$, fluorescence drop on addition of 62.5 m*M* $MnCl_2$ to intact platelets.

concentrations (ca. 10 μ*M*) of nigericin, which exchanges $K^+$ for $H^+$ [7], are used in a high-$K^+$ medium [5] in order to set $pH_{cyt} = pH_{ext}$ over a wide range of values of the latter. When this condition is fulfilled, it constitutes an *in situ* calibration of the indicator. The literature also describes a method which uses monensin, which catalyzes $Na^+$ for $H^+$ exchange [7], the presence of external $Na^+$ and ouabain, an inhibitor of the $Na^+/K^+$ ATPase [6]. To the best of our knowledge, ours is the first study which compares values of $pH_{cyt}$ obtained by the digitonin and nigericin methods after taking into account the effects of dye leakage. The digitonin lysis calibration is shown to be more reliable for pH studies on platelet suspensions.

The question whether the cytoplasmic environment alters BCECF behavior is central to determining the reliability of the digitonin vs nigericin calibration procedures. Therefore, the present communication examines whether the pH reporting behavior of BCECF in solution is altered by the intracellular environment. Such alterations might be possible as a result of self-interactions of BCECF (e.g., self-quenching) or interactions with cytoplasmic elements. A variety of techniques (e.g., spectrophotometric tests for self-quenching of fluorescence and fluorescence polarization) is available to address this type of question. One might expect that self-interactions of BCECF causing self-quenching of fluorescence would be increasingly probable as cytoplasmic concentrations of the dye ($[BCECF]_{cyt}$) are raised. The existing papers on BCECF in platelets and other cells do not report the values of $[BCECF]_{cyt}$. The present study shows that cytoplasmic concentrations of ca. 1 m*M* are obtained by incubating with 8.4 μ*M* BCECF/AM for 45 min, a concentration and time within the usually reported range. A relevant question is whether self-quenching of fluorescence could result from either resonance energy transfer or self-complexation among indicator molecules at this concentration. Also, resonance energy transfer can occur with many fluorophores, when they are dissolved in the millimolar concentration range, when they are at sufficient proximity (≤100Å) to interact in the excited state, and when there is a high degree of overlap in the excitation and emission spectra [8]. Self-complexation in the ground state to give multimeric stacked aggregates is well-known for aromatic dye molecules. In either case, the presence of a few quenched molecules would provide a "sink" for the excited state energy in the communicating molecules and result in the loss of fluorescence [9]. The present study applies spectrophotometric methods to assess whether self-quenching of BCECF fluorescence occurs both *in vitro* and *in situ*. In addition, fluorescence polarization experiments are done to address the question of possible binding of the dye to cytoplasmic elements. Finally, we report that certain agents used in studies of pH regulation are capable of substantial interaction with BCECF.

## METHODS

### Materials, Instrumentation, and Platelet Preparation

#### *Chemicals*

Dimethyl sulfoxide (DMSO) was from Aldrich Chemical Co., Milwaukee, WI. 2′, 7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) pentaacetoxymethyl ester (/AM), BCECF (free acid), bovine serum albumin (BSA; fraction V; fatty acid-, nuclease-, and protease-free) and nigericin were obtained from Calbiochem, La Jolla, CA. Amiloride hydrochloride, $NH_4Cl$, digitonin, glucose, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 2-[*N*-morpholino]ethanesulfonic acid (MES), *N*-methyl-D-glucamine (NMDG), and KOH were supplied by Sigma Chemical Co., St. Louis, MO. $CaCl_2$, KCl, $MnCl_2$, NaCl, and $NaHCO_3$ were purchased from Mallinckrodt Inc., Paris, KY.

#### *Solutions*

The composition of the Tyrode solution used for platelet isolation, loading, and short term storage was as follows: 135 m*M* NaCl, 2.7 m*M* KCl, 0.36 m*M* $NaH_2PO_4$, 11.9 m*M* $NaHCO_3$. In experiments measuring BCECF fluorescence in platelets, the composition of the Tyrode was modified by the omission of $HCO_3^-$ or by isosmolar substitution of $Na^+$ by $NMDG^+$ or $K^+$. In all cases the Tyrode was buffered with 25 m*M* HEPES or MES (for values of $pH_{ext}$ <6.5). Heavy buffering was necessary to avoid alkalinization resulting from loss of $CO_2$ from the external medium containing $HCO_3^-$. The process is accelerated by continuous stirring of the platelet suspension during the fluorescence experiments. Unless otherwise specified, all Tyrode solutions contained 10 m*M* glucose. All solutions were titrated to the desired pH by the addition of HCl, NaOH, KOH, or NMDG.

Stock solutions of BCECF/AM and of amiloride-HCl were prepared in DMSO. Stock solutions of monensin and nigericin were prepared in ethanol. The final DMSO and ethanol concentrations were always ≤1% or ≤0.3%, respectively, and were found not to have nonspecific effects on the intact platelet suspensions.

*Fluorometry*

Fluorescence was measured with a Perkin Elmer (Model MPF3-L) fluorescence spectrophotometer. The excitation wavelength was set at 495 nm and the emission wavelength at 530 nm, with excitation and emission slits 8 nm wide. A horizontally oriented polarization filter was present on the excitation pathway to reduce light-scattering artifacts of the platelet suspension [10].

Fluorescence polarization ($P$) of free and platelet-associated BCECF was also measured. Simple formulae [9,17] have been given for the calculation of this parameter from fluorescence intensity measured with crossed vs parallel polarizing filters with instruments with isotropic light sources [9]. These formulae require some correction for use in grating fluorometers due to polarization dependence of transmission of light through their optics and monochromators. Our analysis of signal intensity ($I$) of aqueous BCECF for parallel orientation showed that when both polarizers are oriented horizontally to the plane of the instrument, the measured fluorescent intensity is 0.573 as large as when both polarizers are oriented vertically. Thus, the optical systems for excitation and emission each discriminate against horizontally polarized light, with a transmission factor ($T$) of 0.757 (no discrimination = 1.0). Comparison of the two combinations of crossed polaroids showed that the discrimination factor of both monochromators was equal for their respective BCECF wavelengths. Thus fluorescence polarization ($P$) was measured as

$$P = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}} = \frac{I_{\parallel} - (I_{\perp}/T)}{I_{\parallel} + (I_{\perp}/T)} \quad (1)$$

where $F$ is the conventionally defined unbiased fluorescence, $I$ is the fluorescence intensity actually measured, and $\parallel$ and $\perp$ have their usual meaning.

*BCECF Loading and Platelet Preparation*

Platelets were isolated as described previously [11]. Unless stated otherwise, washed suspensions of $2 * 10^8$ platelets/ml were incubated with 8.4 $\mu M$ BCECF/AM for 45 min at room temperature. The platelet suspensions were then centrifuged at 400$g$ and the pellets were resuspended in a small volume of $Na^+$ Tyrode. The suspension was stored in the dark at room temperature and its platelet concentration was determined turbidimetrically. Periodically, turbidimetric determinations of platelet concentration were calibrated against a Coulter counter [11]. Small aliquots (40–110 $\mu$l) of the suspension were then introduced into various media at room temperature or after preequilibration of the media to 37°C. The final volume was 2.0 ml, yielding a final concentration of $1.6 * 10^7$ platelets/ml. Fluorescence was monitored at 37°C within a minute of the final platelet dilution.

The intracellular concentration of BCECF ($[BCECF]_{cyt}$) was calculated by comparing the fluorescence measured after digitonin lysis with that of an alkaline standard and by using a value of 10 fl for platelet volume, determined by Coulter counting. Preincubation of platelets with 8.4 $\mu M$ BCECF/AM yielded values of $[BCECF]_{cyt}$ ranging between 0.3 and 1.0 mmol/liter cell volume.

## Methods of Determination of $pH_{cyt}$

*Equations Used in Calibration of the Cytoplasmic BCECF Fluorescence Signal and in Elimination of the Extracellular Component*

According to the generally used method described by Rink *et al.* [1], the dye is assumed to reside in a single compartment (the cytoplasm). In such a case, the observed fluorescence signal ($F$) would be directly translated into $pH_{cyt}$ using Eq. (2):

$$F = F_{min} + ((F_{max} - F_{min}) * (K_d/(K_d + [H^+]))) \quad (2)$$

where $F_{min}$ is the extracellularly measured minimal fluorescence given by the fully protonated dye, $F_{max}$ is the extracellularly measured maximal fluorescence given by the fully deprotonated dye, and $K_d$ is the dissociation constant of BCECF for $H^+$ ($=1.07 * 10^{-7} M$ [1]). The values of $F_{max}$ and $F_{min}$ can be determined by either the "digitonin lysis" method [1] or the "nigericin calibration method" [5], which are described in the next section. One can define

$$\Delta F_{max} = F_{max} - F_{min} \quad (3)$$

The last right-hand term in Eq. (2) represents the degree of advancement ($\alpha$) of the deprotonation reaction at a given pH, such that

$$\alpha = K_d/(K_d + H^+) \quad (4)$$

Leakage of dye from platelets in suspension will corrupt measurements of $pH_{cyt}$ if only the above equations are used. The extracellular dye is "counted" as intracellular dye and the calculated cytoplasmic pH is biased in the direction of the external pH. Proper calculation of $pH_{cyt}$ requires elimination of the extracellular fluorescence contribution. This can be done using the following equations, which describe the measured fluorescence in terms of the contributions from the external and cytoplasmic compartments. The fraction of total dye

which is external ($B_{ext}$) is determined and used to subtract the contribution from extracellular fluorescence ($F_{ext}$) from the measured fluorescence ($F$) to arrive at the fluorescence from the BCECF in the cytoplasm ($F_{cyt}$). Methods for determining $B_{ext}$ are described in a later subsection. Thus

$$F = F_{cyt} + F_{ext} \quad (5)$$

The two right-hand terms in Eq. (4) can be expressed in terms of the fraction of external dye ($B_{ext}$) as shown below:

$$F_{cyt} = (1 - B_{ext}) * [\Delta F_{max} * \alpha_{cyt}) + F_{min}] \quad (6)$$

$$F_{ext} = B_{ext} * [(\Delta F_{max} * \alpha_{ext}) + F_{min}] \quad (7)$$

Substitution of the left-hand terms in Eq. (5) for those in Eqs. (6) and (7) then yields

$$F = \Delta F_{max} * [(1 - B_{ext}) * \alpha_{cyt} + (B_{ext} * \alpha_{ext})] + F_{min} \quad (8)$$

Determination of $pH_{cyt}$ requires a knowledge of $\Delta F_{max}$, $F_{min}$, $B_{ext}$, and $\alpha_{cyt}$. The value of $\alpha_{ext}$ is determined from the known $pH_{ext}$. The values of $\Delta F_{max}$ and $F_{min}$ can be determined from manipulations which put all of the dye into the same state at high and low pH, respectively. In the digitonin lysis method this is accomplished by releasing the dye from the cells into a medium of high or low pH. This method contains the implicit assumption that the fluorescence characteristics of the dye in the cytoplasm are identical to those in the external medium. In the nigericin calibration method it is assumed that the ionophore in the presence of a high external $K^+$ concentration will make $pH_{cyt}$ equal to $pH_{ext}$ allowing for determination of $\Delta F_{max}$ and $F_{min}$ values which may be unique to the cytoplasm. In the following steps we derive equations useful for the digitonin lysis method. The same formalism can be used for the nigericin method (using the term $F_{nigericin}$), with slight modification regarding $\Delta F_{max}$ and $F_{min}$.

Let $F_{dig}$ represent the fluorescence obtained after digitonin lysis of BCECF-laden platelets. When the dye is released into the external medium, its degree of deprotonation will be $\alpha_{ext}$, determined by the external pH. Thus,

$$F_{dig} = (\Delta F_{max} * \alpha_{ext}) + F_{min} \quad (9)$$

Comparison of Eqs. (7) and (9) shows that

$$F_{ext} = B_{ext} * F_{dig} \quad (10)$$

where the $B_{ext}$ value pertains to conditions before lysis and remains to be determined. Substituting Eqs. (6) and (10) into Eq. (5),

$$F = (1 - B_{ext}) * [(\Delta F_{max} * \alpha_{cyt}) + F_{min}] + (B_{ext} * F_{dig}) \quad (11)$$

and solving for $\alpha_{cyt}$, one obtains

$$\alpha_{cyt} = \frac{F - [(1 - B_{ext}) * F_{min} + (B_{ext} * F_{dig})]}{(1 - B_{ext}) * \Delta F_{max}} \quad (12)$$

Equation (12), used in conjunction with Eq. (13) (given below) represents the key equation used in the determination of $pH_{cyt}$. One the right-hand side of Eq. (12) the intracellular fluorescence due to fully protonated BCECF is given by the first product inside the square brackets. The extracellular fluorescence is given by the second product. In Eq. (12) the sum of these is subtracted from the observed fluorescence ($F$) to yield pH-sensitive intracellular fluorescence. The denominator gives the intracellular $\Delta F_{max}$ ($\Delta F_{max, cyt}$). To obtain the internal pH, the value of $\alpha_{cyt}$ obtained in Eq. (12) is substituted into the equation below:

$$pH_{cyt} = -\log[((1 - \alpha_{cyt})/\alpha_{cyt}) * K_d] \quad (13)$$

Thus one can calculate $pH_{cyt}$ from the above-described and above-measured parameters, once $B_{ext}$ is determined. This can be accomplished using either the "pH jump" method or the "$Mn^{2+}$ quench" methods described below.

*The pH Jump Method of Determination of $B_{ext}$*

This method is illustrated in Fig. 1. The method is based on the fact that the extracellular dye responds instantaneously to changes in extracellular pH ($pH_{ext}$) and that the intracellular dye does not. Figure 1A presents, on the same time axis, two experiments in which the pH was jumped to 9.0 or 5.0, respectively, by rapidly adding base or acid to the platelet suspension. The $pH_{ext}$ is measured with a pH electrode before and after the pH jump. At these extremes 99 and 99% of the extracellular BCECF is deprotonated and protonated, respectively. Upon digitonin lysis, further changes in fluorescence occur. As indicated in the right-hand portion of Fig. 1A, the value of $\Delta F_{max}$ is given by the difference in amplitudes of the postdigitonin values, with a small correction (involving $\alpha_{ext}$ and $\Delta\alpha_{ext}$) for incomplete deprotonation and protonation at the two extremes of pH. Similarly, the value of $\Delta F_{max} * B_{ext}$ is determined from the instantaneous amplitude observed upon pH jump in the left-hand portion of Fig. 1A. The value of $B_{ext}$ is calculated simply as the ratio of these two amplitudes $(\Delta F_{max} * B_{ext})/\Delta F_{max})$. This completes the information necessary to calculate $pH_{cyt}$ using Eq. (12) and (13).

Figure 1B presents the same traces with the instan-

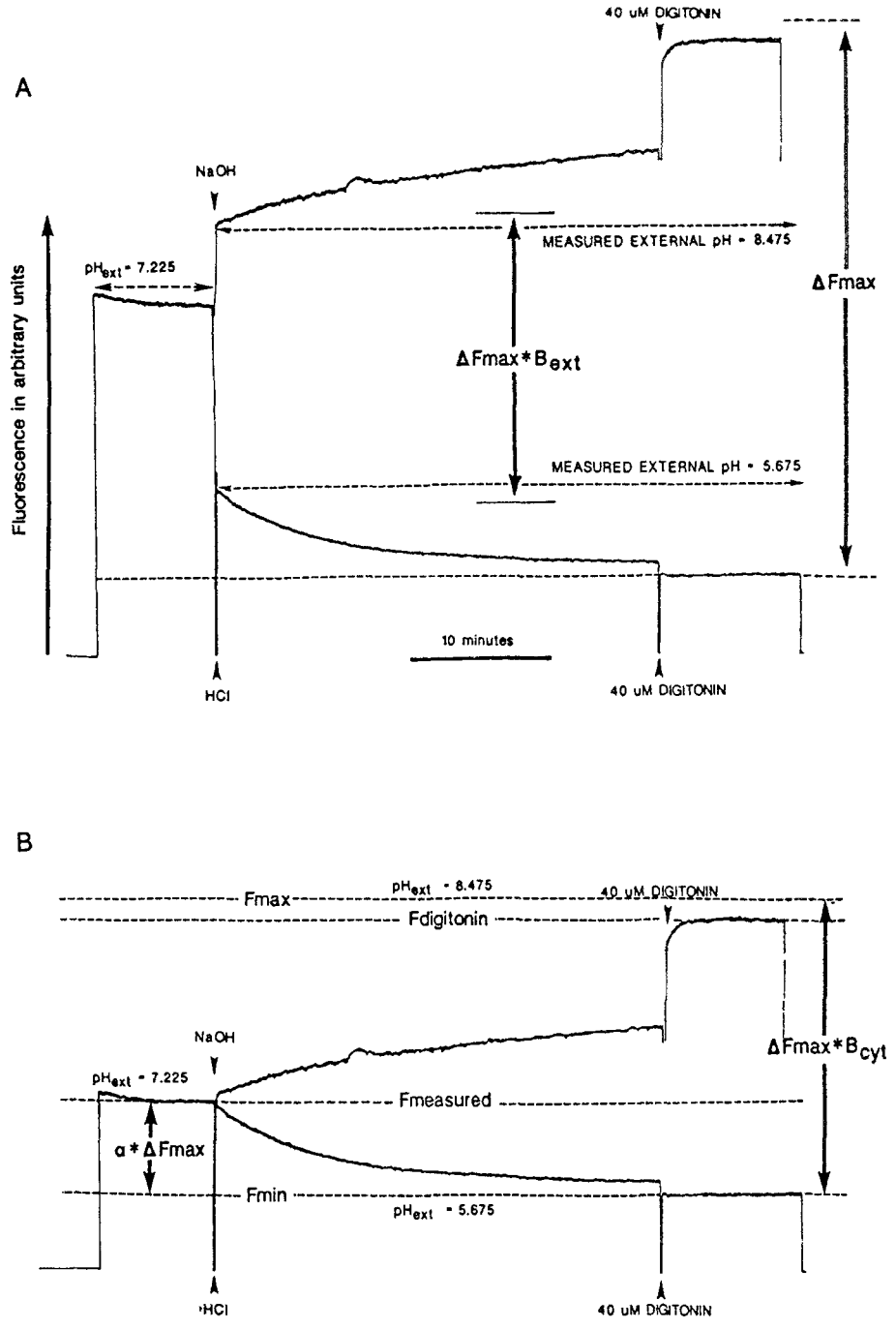


**Fig. 1.** The pH jump method of correction for fluorescence contributed by extracellular BCECF. (A) The fluorescence traces from two experiments on the same time axis. In each experiment platelets were introduced into $Na^+$ Tyrode at pH 7.23 and the fluorescence was allowed to reach a steady value. In one experiment, the external pH was rapidly jumped to pH 8.47 by the addition of NaOH. At this pH, 97.0% of the dye is deprotonated. In the parallel experiment, the external pH was rapidly jumped to 5.67 by the addition of HCl. At this pH, 95.2% of the dye is protonated. In both cases, this resulted in both an instantaneous and time-resolved changes in fluorescence. Subsequent cell lysis with digitonin (40 $\mu M$) caused further changes in fluorescence. The sum of instantaneous amplitudes represents 92.2% of the difference between $F_{max}$ and $F_{min}$ for extracellular BCECF. The arrow shows the extent of $\Delta F_{max} * B_{ext}$ after a small correction. The time-resolved changes in fluorescence after a pH jump and digitonin addition were considered to be of cytoplasmic origin. (B) Trace from A with instantaneous changes graphically subtracted. The span of amplitude after the digitonin additions indicated by the arrows shows $\Delta F_{max} * B_{cyt}$ after a small correction. In this case, $B_{ext} = 53\%$.

taneous extracellular changes in fluorescence (due to extracellular BCECF) subtracted. Figure 1 shows that $pH_{cyt}$ decreases slowly after an acid jump and increases slowly after an alkaline jump. The $pH_{cyt}$ values never reach the extremes of the $pH_{ext}$ values, as seen by the further effect of digitonin lysis. It is noted that the pH jump method cannot be used in the presence of nigericin or monensin since these give rapid movement of $H^+$ rendering changes in protonation of internal BCECF indistinguishable from changes in protonation of external BCECF.

*$Mn^{2+}$ Quenching Method for Determination of $B_{ext}$*

Like the pH jump method, the $Mn^{2+}$ quench method is based on the fact that the instantaneous change in fluorescence caused by the addition of $Mn^{2+}$ reflects instantaneous reactions with extracellular BCECF. In contrast, reactions involving cytoplasmic BCECF require more time due to the delay caused by slow permeation of $Mn^{2+}$ across the cell membrane.

The paramagnetic ion $Mn^{2+}$, when in proximity to a fluorophore such as BCECF, increases the rate of radiationless transitions from the excited state, such that no fluorescence or diminished fluorescence is seen [12–14]. $Mn^{2+}$ has been used by McDonough and Button [15] to quench extracellular fura2 fluorescence. BCECF quenching occurs in the $[Mn^{2+}]$ range of 20 to 100 m*M*. This is consistent with a Stern–Volmer collisional quenching mechanism [16]. The concentration of 62.5 m*M* chosen because it was close to that which gave half-maximal quenching in the neutral pH range. Since in most instances BCECF will be present in both charged (deprotonated) and uncharged (protonated), the extent of $Mn^{2+}$ quenching is pH dependent.

Figure 2A shows that the addition of 62.5 m*M* $MnCl_2$ to a suspension of intact platelets results in an instantaneous decrease in fluorescence ($\Delta F_{Mn}$), reflecting the rapid quenching of extracellular BCECF. A separate digitonin lysis and calibration experiment (Fig. 2B) establishes $F_{min}$, $F_{max}$ and $\Delta F_{max}$ values. The fraction of BCECF external can be determined in separate *in vitro* experiments.

It is useful to be able to apply the $Mn^{2+}$ method over a range of pH values. Figure 3 presents *in vitro* experiments showing the pH dependence of RF (residual fluorescence), the fraction of fluorescence remaining immediately after the addition of 62.5 m*M* $Mn^{2+}$. The points in the curve were best fit by the following semiempirical equation:

$$RF = \frac{RF_{max} * [H^+]}{K_d + [H^+]} + RF_{min} \qquad (14)$$

where $RF_{max}$ and $RF_{min}$ represent the maximal and minimal values of residual fluorescence, respectively, measured after the addition of 62.5 m*M* $Mn^{2+}$. The fraction of fluorescence quenched by $Mn^{2+}$ is given by $1-RF$ and is half-maximal when $RF = 0.5$. Thus, the $K_d$ ($= 0.45 * 10^{-7}$) in Eq. (14) gives the apparent $[H^+]$ at which $Mn^{2+}$ quenching is half-maximal. As might be expected, this value is close to the apparent $K_d$ of BCECF for $H^+$ ($1.07 * 10^{-7}$).

When platelets are lysed at a given external pH, all the cytoplasmic dye is released into the external medium and the corresponding fluorescence, $F_{dig}$, is obtained. Because all of the cytoplasmic dye has become external, the addition of 62.5 m*M* $Mn^{2+}$ after cell lysis would produce the maximal quenching achievable for a given platelet suspension at a given $pH_{ext}$. The $pH_{ext}$ will determine what fraction ($1-RF$) of the external dye is quenchable. Thus, the maximal decrease in fluorescence achievable by $Mn^{2+}$ quenching is given by the product of the following two terms: (1-*RF*), the fraction quenched at that pH, and $F_{dig}$, the value of fluorescence given by the platelet lysate at the same pH (i.e., when all of the dye is extracellular). It follows that the fraction of external dye when the platelets are intact can be calculated by

$$B_{ext} = \frac{\Delta F_{Mn}}{(F_{dig}) * (1 - RF)} \qquad (15)$$

where $\Delta F_{Mn}$ represents the fluorescence decrease resulting from quenching of external dye when the platelets are intact and the denominator represents the corresponding decrease in fluorescence when the platelets have been lysed with digitonin at the particular external pH. The value of $B_{ext}$ can then be entered into Eq. (12) for the determination of cytoplasmic pH.

Readers wishing to apply the $Mn^{2+}$ quenching method are advised that at levels of about 60 m*M*, the cation precipitates slowly as a hydroxide complex in the alkaline range, giving rise to a light-scattering artifact. Also, we do not advise further experimentation after $Mn^{2+}$ addition; the cation may affect the behavior of pumps and channels located in the plasma membrane. Of the two methods, the pH jump method is the most convenient. It is shown under Results Section that the two methods give identical estimates of $B_{ext}$. The $Mn^{2+}$ quench method becomes the method of choice in the presence of $H^+$ translocating ionophores, such as nigericin and monensin. As noted earlier, these ionophores speed up the $H^+$ equilibration across the plasma membrane to a

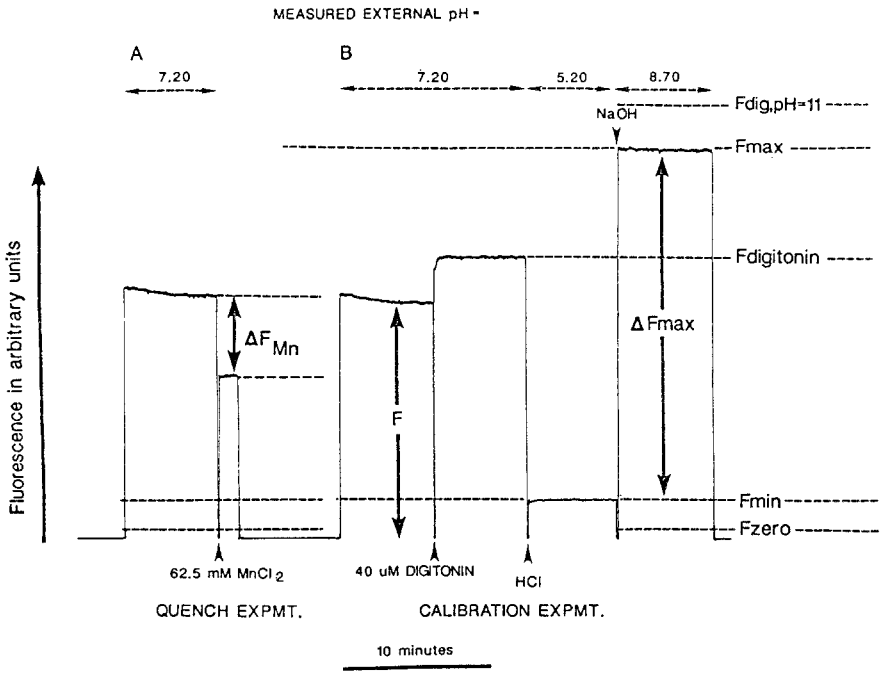


**Fig. 2.** The $Mn^{2+}$ quench method for determination of fluorescence contributed by extracellular BCECF. The experiments shown in both A and B are done in parallel to determine $B_{ext}$. (A) An experiment performed to determine the instantaneous drop in fluorescence ($\Delta F_{Mn}$) resulting from the addition of 62.5 m*M* $MnCl_2$. (B) The digitonin-lysis calibration experiment done to determine $F_{dig}$ and $\Delta F_{max}$. The values of $F_{dig}$, $\Delta F_{Mn}$, and *RF* (obtained as shown in Fig. 3) are entered into Eq. (15) to determine $B_{ext}$. In this case, $B_{ext}$ = 68%.

sufficient extent to render intracellular dye indistinguishable from extracellular dye.

### *Tests for Self-Quenching of BCECF Fluorescence* in Vitro *and* in Situ

In this study we define self-quenching as a dye concentration-dependent loss of fluorescence due to self-interaction (ground-state complexation, excited-state interaction, collisional quenching). We do not include under this term trivial quenching ("inner filter effect" [9]), which can be observed when the optical density at the excitation or emission wavelength is greater than 0.1. Self-quenching of BCECF was tested *in vitro* by determining whether fluorescence was proportional to BCECF concentration over a large range. BCECF concentration was varied by performing serial 1:3 dilutions of a concentrated (13.3 m*M*) aqueous stock solution of BCECF and by monitoring excitation spectra (at 530 nm) and emission spectra (at 470 nm) in 1- to 5-μl vertically oriented glass capillary tubes having an internal diameter of 148 μm (Clay Adams No. 4614). Using an experimentally determined extinction coefficient ($4.4 * 10^5\ M^{-1}\ cm^{-1}$), the optical density at each concentration was calculated from Beer's law. To correct for the inner filter effect [9] at each concentration, an average transmission factor was calculated graphically and applied to the measured fluorescence.

Self-quenching was tested *in situ* using similar principles. Platelets were loaded with variable concentrations of BCECF (0.02 to 2.4 mmol/liter cell volume) and were tested for proportionality between BCECF fluorescence and intracellular concentration.

### *Statistics*

Curve fitting was performed with the computer program ASYSTANT (Macmillan Software Company, 1986).

## RESULTS

### Corrections for Dye Leakage

Leakage of BCECF from platelet suspensions was a continuous but variable process. The amount of exter-

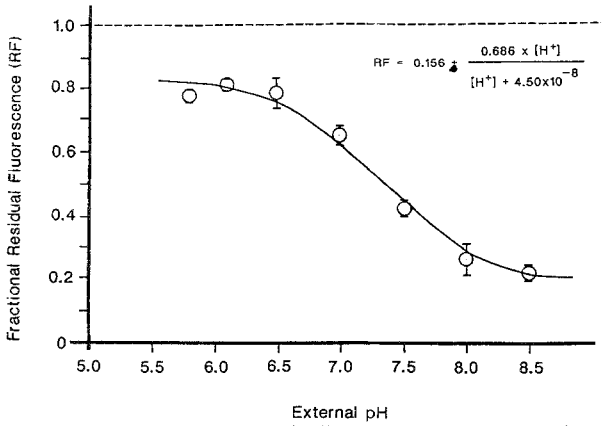


**Fig. 3.** Effect of $MnCl_2$ on BCECF fluorescence as a function of pH. The calibration curve shown was obtained by adding 62.5 m*M* $MnCl_2$ to solutions of 0.1 μ*M* BCECF in $Na^+$ Tyrode. The pH of the latter had been adjusted with NaOH to pH values ranging between 5.0 and 8.5. The fluorescence (*RF*, or residual fluorescence) was measured immediately after mixing. Prompt measurement was necessary since slower changes were observed in the signal resulting from the precipitation of manganese hydroxide which occur at alkaline pH. The experiment was repeated for each pH value. The dashed line represents the level of unquenched fluorescence.

nal dye released from platelet suspensions progressively increased during the course of experimentation. The initial amount of dye released into the medium varied among platelet preparations from different donors. Also, some of the manipulations and interactions during a single experiment cause increased leakage. In approximately 200 experiments with approximately 40 preparations, the fraction of total dye leaked into the external medium ($B_{ext}$) ranged from 13% at 30 min postloading to 70% at 6 h postloading. Table I presents calculations of $pH_{cyt}$ for platelet samples with leakage within this range and shows that $pH_{cyt}$ can be considerably misestimated when leakage is not taken into account. The third column shows average $pH_{cyt}$ values of 7.07 ± 0.04 for average $pH_{ext}$ values of 7.28. The fourth and fifth columns show that when the "pH jump" and "$Mn^{2+}$ quench" methods are used to correct for leakage, values of 6.72 ± 0.09 and 6.78 ± 0.07 (respectively) are obtained. Not only are the two corrected values in excellent agreement, but also they are 0.4 unit lower than the uncorrected values. We believe that this observation may be significant to a number of studies in which the problem of leakage has not been addressed.

## Comparison of Results by "Nigericin pH Clamp" and "Digitonin-Lysis" Methods

Table II compares the nigericin calibration and digitonin lysis methods at a high external $K^+$ concentration, a condition appropriate to the former method. The second and third columns show that the nigericin addition integral to the former method causes a substantial increase in external BCECF. This was a consistent finding. It represents a serious drawback to this method as it is generally used (i.e., constructing fluorescence vs pH graphs for the system as a whole and then reading off $pH_{cyt}$ from fluorescence measured in the experiment). The fourth column shows the $pH_{cyt}$ values calculated using the nigericin method without correction for leakage. The fifth column shows $pH_{cyt}$ values calculated by the nigericin method corrected for leakage (cf. Methods of Determination of $pH_{cyt}$). The correction results in lower values, particularly at high external pH. The final column presents $pH_{cyt}$ values calculated using the digitonin lysis method. The values calculated by the corrected nigericin method are 0.2–0.3 unit higher than those calculated by the digitonin lysis method. An important contribution to this difference is that the $F_{max}$ values determined by the nigericin method are 15% smaller than the corresponding values obtained by the digitonin method.

**Table I.** Comparison of Corrected and Uncorrected Values of $pH_{cyt}$ Obtained by the "Digitonin Lysis" Method[a]

| Expt. No. | $pH_{ext}$ | Calculated $pH_{cyt}$ | | |
|---|---|---|---|---|
| | | Uncorrected | Corrected by pH jump | Corrected by $Mn^{2+}$ quench |
| 1 | 7.30 | 7.14 | 6.80 | 6.83 |
| 2 | 7.30 | 7.10 | 6.73 | 6.81 |
| 3 | 7.23 | 6.96 | 6.63 | 6.70 |
| Mean ± SD | 7.28 ± 0.04 | 7.07 ± 0.03 | 6.72 ± 0.09 | 6.78 ± 0.07 |

[a] $B_{ext}$, the fraction of BCECF in the external medium, ranged between 0.27 and 0.65. It was determined by the pH jump and the $Mn^{2+}$ quench methods, as described in the text.

**Table II.** Comparison of $pH_{cyt}$ Values Calculated Using "Digitonin Lysis" vs "Nigericin Calibration" Methods in $K^+$ Medium[a]

| $pH_{ext}$ | $B_{ext}$ Before nigericin | $B_{ext}$ After nigericin | $pH_{cyt}$ based on $F_{nigericin}$ Uncorrected for $B_{ext}$ | $pH_{cyt}$ based on $F_{nigericin}$ Corrected for $B_{ext}$ | $pH_{cyt}$ based on $F_{dig}$ corrected for $B_{ext}$ |
|---|---|---|---|---|---|
| 6.75 | 0.33 | 0.47 | 6.71 ± 0.04 | 6.67 ± 0.07 | 6.40 ± 0.13 |
| 7.05 | 0.46 | 0.72 | 6.98 ± 0.01 | 6.93 ± 0.02 | 6.61 ± 0.03 |
| 7.98 | 0.35 | 0.42 | 7.35 ± 0.09 | 7.10 ± 0.01 | 6.90 ± 0.15 |

[a] Platelets were introduced into a $HCO_3^-$-free $K^+$ medium (149.6 m*M* $K^+$ and 0.4 m*M* $Na^+$) and allowed to reach a steady fluorescence. Then 10 μ*M* nigericin was added, and the resulting fluorescence change allowed to stabilize. Values of $pH_{cyt}$ were corrected for the fraction of external BCECF ($B_{ext}$) by the $Mn^{2+}$ quench method and are expressed as the mean ± SD. Values of $\Delta F_{max}$ determined by the "nigericin clamp" procedure were 0.85 ± 0.04-fold lower than those determined by the digitonin lysis method, as a result of a decrease in values of $F_{max}$. The presented values of $B_{ext}$ are from one typical experiment.

## Self-Quenching of BCECF in Solution

Table III summarizes the results of an experiment designed to test for self-quenching of BCECF *in vitro*. If self-quenching is absent, fluorescence would be expected to be strictly proportional to BCECF concentration ([BCECF]). If self-quenching were present, fluorescence should be less than proportional to [BCECF] and might actually decrease when [BCECF] is raised above a certain critical value. Table III shows that rough proportionality is observed in the BCECF concentration range of 0.18 to 0.49 m*M*. In the BCECF concentration range of 0.49 to 4.4 m*M*, proportionality breaks down, and between 4.4 and 13.3 m*M* a decrease is actually observed. Thus, self-quenching of BCECF *in vitro* begins at 0.5 m*M* and becomes very serious at concentrations above 1.5 m*M*.

**Table III.** Self-Quenching of BCECF Fluorescence *in Vitro*[a]

| [BCECF], m*M* | OD | Average transmission correction factor | Fluorescence Uncorrected | Fluorescence Corrected |
|---|---|---|---|---|
| 0.0182 | 0.012 | 1.0 | 17.2 | 17.2 |
| 0.0549 | 0.036 | 1.0 | 28.9 | 28.9 |
| 0.165 | 0.109 | 1.0 | 66.2 | 66.2 |
| 0.494 | 0.326 | 0.736 | 261 | 355 |
| 1.48 | 0.978 | 0.457 | 195 | 425 |
| 4.44 | 2.93 | 0.202 | 107 | 532 |
| 13.3 | 8.80 | 0.145 | 7.80 | 53.9 |

[a] Experiments were done with glass capillary tubes with an internal diameter of 148 μm. Correction of fluorescence is based on the average transmission factor, which was determined from optical density (OD) as described under Methods of Determination of $pH_{cyt}$.

## Lack of Self-Quenching of BCECF in Platelets

We applied the same tests for self-quenching in platelets, testing whether proportionality is maintained between BCECF fluorescence and BCECF concentration in the cytoplasm as the latter is increased. Figure 4 shows the protocol by which the fluorescence from the cytoplasm is measured. BCECF-loaded platelets are added to the medium at $pH_{ext}$ = 7.3, then allowed to come into steady state, and the fluorescence arising from the cytoplasmic dye ($F_{cyt,\ resting}$) is measured. Next the pH of the external medium is jumped to 8.25, the platelets are allowed to establish a new steady-state $pH_{cyt}$, and then 20 μ*M* monensin is added to alkalinize the cytoplasm further. The $pH_{cyt}$ values were calculated using the digitonin lysis method of correcting for leakage. The experiment in Fig. 4 was repeated with platelet samples loaded with variable amounts of BCECF. Table IV shows the dependence of $F_{cyt,resting}$ on the cytoplasmic BCECF concentration ($[BCECF]_{cyt}$). The ratio $F_{cyt,resting}/[BCECF]_{cyt}$ remains constant when $[BCECF]_{cyt}$ is varied from 18 μ*M* to 1.8 m*M*. This demonstrates that self-quenching of BCECF does not occur for the tested concentrations of 1.8 m*M* or less. As expected, the calculated $pH_{cyt}$ values remain constant (final column, Table IV). Table V presents a similar test for the alkalinized condition (monensin plus high external pH in Fig. 4). This condition maximizes the anionic form, which produced the measured signal from which $pH_{cyt}$ is calculated. Again, cytoplasmic fluorescence remains proportional to cytoplasmic BCECF concentration through the entire cytoplasmic BCECF concentration range (18 μ*M* to 2.4 m*M*), giving no evidence for self-quenching.

It is of interest to compare the *in vitro* and *in situ* results. The previous subsection showed that BCECF is

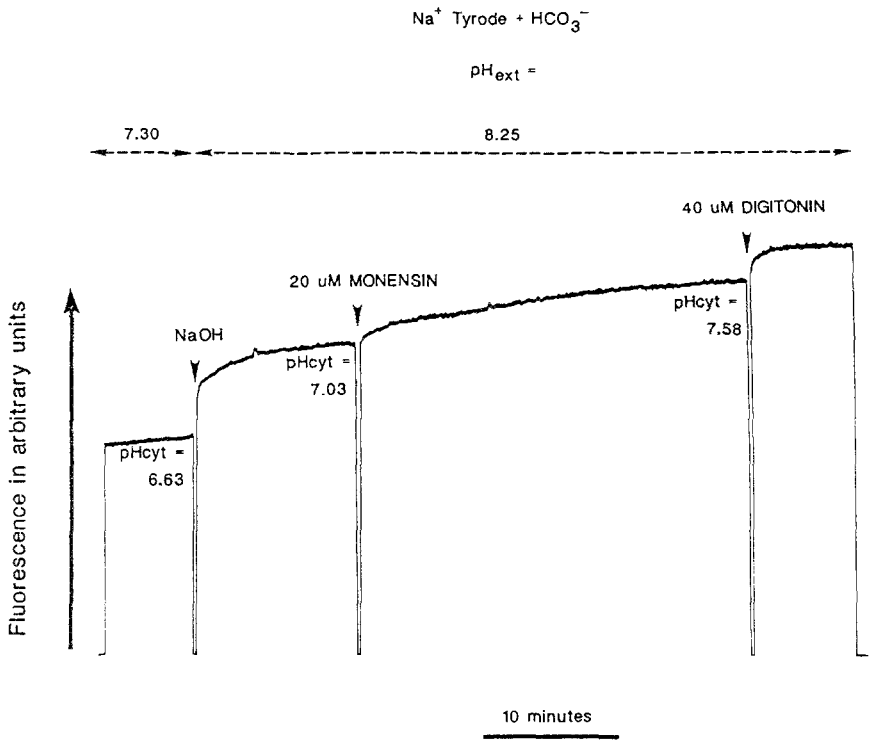


**Fig. 4.** Alkalinization of the cytoplasm by monensin after an alkaline pH jump.

**Table IV.** Lack of Intracellular Self-Quenching Shown by Analysis of the Ratio $F_{cyt,resting}/[BCEF]_{cyt}$ at Various Cellular BCECF Concentrations[a]

| [BCECF/AM], $\mu M$ | $[BCECF]_{cyt}$, $\mu$mol/liter cell volume | $F_{cyt,resting}$ | $F_{cyt,resting}/[BCECF]$ | $pH_{cyt}$ |
|---|---|---|---|---|
| 0.10 | 18.1 | 4.8 | 0.221 | 6.75 |
| 1.0 | 103 | 23.8 | 0.231 | 6.80 |
| 1.0 | 202 | 52.4 | 0.259 | 6.90 |
| 8.4 | 383 | 97.5 | 0.255 | 6.89 |
| 8.4 | 950 | 208 | 0.219 | 6.75 |
| 168 (75 min) | 1441 | 294 | 0.204 | 6.69 |
| 168 (120 min) | 1803 | 381 | 0.211 | 6.72 |

[a] Platelets which had been preincubated with varying concentrations of BCECF/AM were subjected to the protocol shown in Fig. 4. The mean values of $pH_{ext}$ prior to and after the alkaline pH jump were 7.28 ± 0.02 and 8.03 ± 0.13, respectively. The data shown are a composite of results obtained from two different platelet preparations. The value of $F_{cyt,resting}$, the cytoplasmic fluorescence at $pH_{ext}$ = 7.28, was calculated from knowledge of what $F_{dig}$ would have been at $pH_{ext}$ = 7.28. The latter value of $F_{dig}$ was calculated from Eq. (9) after $\Delta F_{max}$ and $F_{min}$ had been determined. Values of $F_{cyt,resting}$ were corrected for dye leakage. The average intracellular concentration of dye ($[BCECF]_{cyt}$) was determined from $F_{dig}$ (after correction for dye leakage) and the same BCECF standard. Calculated values of $F_{cyt,monensin}/[BCEF]_{cyt}$ are presented above as a function of $[BCECF]_{cyt}$.

self-quenched in macroscopic solution at concentrations of 0.5 m*M* and higher. The *in situ* results show that BCECF is not self-quenched in the microscopic environment of the cytoplasm even at concentrations as high as 2.4 m*M*. It is possible that the microscopic environment of the cytoplasm is not conducive to stacking or

**Table V.** Lack of Intracellular Self-Quenching by Analysis of the Ratio $F_{cyt,monensin}/[BCEF]_{cyt}$ at Various Cellular BCECF Concentrations[a]

| [BCECF/AM], μ*M* | $[BCECF]_{cyt}$, μmol/liter cell volume | $F_{cyt}$ after monensin | $F_{cyt,monensin}/[BCECF]_{cyt}$ |
|---|---|---|---|
| 0.10 | 18.1 | 6.1 | 0.337 |
| 1.0 | 103 | 35.6 | 0.346 |
| 1.0 | 176 | 57.2 | 0.325 |
| 8.4 | 383 | 132 | 0.345 |
| 8.4 | 963 | 332 | 0.348 |
| 168 (75 min) | 1980 | 682 | 0.344 |
| 168 (120 min) | 2380 | 732 | 0.308 |

[a] The results shown represent the same set of data obtained by the protocol in Fig. 4 and shown in Table IV except for the monensin plus high-pH alkalinized condition, which gave values of $F_{cyt,monensin}$. The steady values of $F_{cyt,monensin}$ were determined after correction for dye leakage. Calculated values of $F_{cyt,monensin}/[BCEF]_{cyt}$ are presented as a function of $[BCECF]_{cyt}$.

self-associating processes which may be responsible for self-quenching in macroscopic solution.

## Fluorescence Polarizations of BCECF in the Cytoplasm and Solution Are Identical

The question whether BCECF binds to cytoplasmic components was addressed by the classical method of fluorescence polarization. Binding of small fluorophores such as BCECF to macromolecules or other slowly diffusing components would increase the fluorescence polarization [*P*; cf. Eq. (1)]. Table VI presents *P* values for a suspension of BCECF-laden platelets which were perturbed in sequential fashion. The second column in the table gives the origin of the signals (cytoplasmic vs external). The first row gives values for resting platelets with 35–44% of the fluorescence signal arising from extracellular dye. The *P* value of $0.000 \pm 0.020$ was calculated. This is not different from that for BCECF in aqueous solution (last row). There was also no difference when *P* of the cytoplasmic BCECF was assessed by the change in fluorescence observed by acidifying the cytoplasm using nigericin (second row), which transports $H^+$ inward in exchange for outward $K^+$ movement.

Table VI (third row) also shows that BCECF in the external medium has a low *P* value, as expected. This was assayed by the addition of BSA to the platelet suspension which binds and quenches the external dye.

## Spectral Characteristics of Cytoplasmic and Extracellular BCECF Are Identical

Rink *et al.* [1] reported a 5-nm "red shift" in the excitation spectrum of BCECF when the dye is released from porcine lymphocytes by detergent lysis. They commented that this would result in underestimation of $pH_{cyt}$ by 0.1–0.15 pH unit if not taken into account. We have taken excitation spectra (at 530-nm emission) before and after cell lysis with 40 μ*M* digitonin and have failed to see any change in excitation maximum of the cytoplasmic dye with nigericin, monensin, or pH perturbation.

**Table VI.** A Fluorescence Polarization Study Based on Sequential Perturbation of Cytoplasmic and External BCECF Fluorescence and Standardization Against BCECF Fluorescence in Solution[a]

| Condition addition | Fluorescence (*F*) or (±)ΔFluorescence: Origin of measured *F* | $F_{\parallel}$ | $F_{\perp}$ | *P* |
|---|---|---|---|---|
| Resting platelets | Cytoplasm & external | 127 | 127 | $0.000 \pm 0.020$ |
| +0.5 μ*M* nigericin | Cytoplasm | −19.3 (Δ) | −18.1 (Δ) | $0.032 \pm 0.025$ |
| +BSA (7.4 mg/ml) | External | −39.0 (Δ) | −37.3 (Δ) | $0.022 \pm 0.020$ |
| Fluorescence of aqueous BCECF *in vitro* | Solution (control standard) | 52.5 | 50.3 | $0.021 \pm 0.015$ |

[a] Platelets (containing 0.87 mmol BCECF/liter cell volume) were introduced into $HCO_3^-$-free $Na^+$ medium at pH 7.20 and subjected to the above conditions or additions in a single experimental run. The $F_{\perp}$ values are corrected for monochromator bias according to Eq. (1). Parallel and perpendicular measurements were made after steady state had been achieved after each sequential perturbation (first three rows). The ± values indicate the uncertainty of *P* values, based on the uncertainty of *F* values. Quenching of BCECF fluorescence by BSA was verified in solution. At the time of the experiment 35–43% of the dye was extracellular, as determined by the $Mn^{2+}$ quench method.

### *N*-Methyl-D-Glucamine Affects BCECF Behavior; $Na^+$, $K^+$, $Mg^{2+}$, and Digitonin Do Not

*N*-Methyl-D-glucamine ($NMDG^+$) has been widely used as one of the substitutes for external $Na^+$. In the course of a $NMDG^+$-for-$Na^+$ substitution experiment, we made the unexpected finding that digitonin lysis caused an *increase* in fluorescence at acidic $pH_{ext}$ values (i.e., pH 5.5 to 6.5). This was in contrast to the decrease in fluorescence always observed in the presence of $Na^+$ or $K^+$ under similar conditions. This prompted a systematic examination of the titration behavior of BCECF in the presence of $NMDG^+$, $Na^+$, and $K^+$ in solution.

Figure 5 shows the titration curve obtained when the fluorescence of the dye in the digitonin lysate is plotted against the pH of the medium. The data show that in both $Na^+$ and $K^+$ solutions the best fit conforms to Eq. (2), with $K_d = 1.07 * 10^{-7}$ and with the $F_{min}$ and $F_{max}$ values given for the figure. The $K_d$ value is in agreement with those of Rink *et al.* [1]. However, the titration curve is shifted to be left in the presence of $NMDG^+$ and the maximum value of fluorescence is somewhat lower. Inspection of Fig. 5 shows that this is important since $pH_{cyt}$ might otherwise be underestimated by 0.3 unit. Repetition of the above-described titrations with BCECF free acid (not shown) gave essentially identical results. This indicates that digitonin does not affect BCECF behavior.

Figure 5 shows that $NMDG^+$-for-$Na^+$ substitution shifts the $K_d$ to $2.00 * 10^{-7}$ and that $F_{max}$ is 13% lower than the corresponding value for $Na^+$ and $K^+$. This suggests that $NMDG^+$ may complex BCECF with a low affinity. To test this explanation, the $NMDG^+$ concentration dependence of its effects on BCECF fluorescence was studied in the presence of physiological NaCl at three pH values. Double-reciprocal plots (not shown) of the perturbation vs $[NMDG^+]$ gave straight lines. Table VII presents the $F$ values for the fully titrated states and

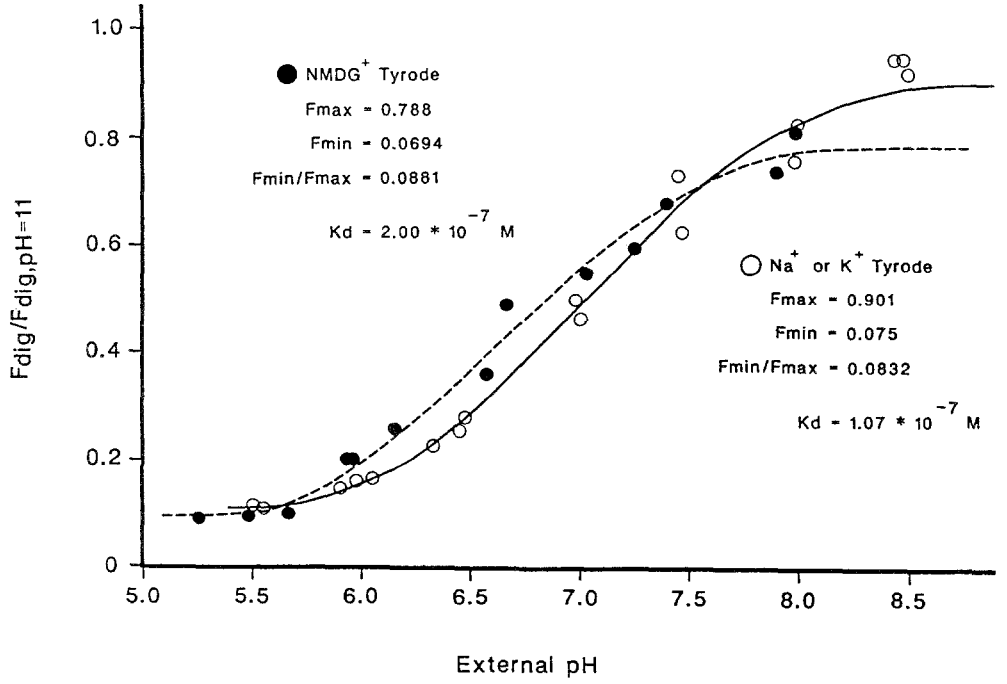


**Fig. 5.** Calibration of BCECF fluorescence against pH. The presented date were obtained with digitonin lysate at the end of platelet experiments. The fluorescence was measured after digitonin lysis ($F_{dig}$), the pH increased to 11, and the fluorescence again measured ($F_{dig,pH=11}$). Each measurement was self-standardized by taking the ratio of $F_{dig}/F_{dig,pH=11}$, in which the value of $F_{dig}$ was determined in $Na^+$ or $K^+$ media. Plots of the ratio of $F_{dig}/F_{dig,pH=11}$ vs external pH after lysis were fitted to the equation $F_{dig}/F_{dig,pH=11} = \alpha \times (F_{max} - F_{min}) + F_{min}$ to obtain the best values for $K_d$ and for the ratio $F_{min}/F_{max}$. The solid line fitted to the open circles: $Na^+$ – or $K^+$-substituted Tyrode. The dashed line fitted to the filled circles: $NMDG^+$-substituted Tyrode. Identical results were obtained for BCECF solution (in the absence of both digitonin and lysed platelets). Analysis of BCECF behavior (not shown) based on a larger number of samples showed that $NMDG^+$ also decreases values of $\Delta F_{max}$ by 13% relative to those obtained in $Na^+$ and $K^+$.

**Table VII.** $NMDG^+$ Concentration Dependence of BCECF Fluorescence in Physiological NaCl Solution[a]

| pH | $F_0$ | $\Delta F_{max}$ | $F_{end}$ | $EC_{50}$ (m*M*) |
|---|---|---|---|---|
| 6.7 | 26.0 | +25.0 | 51.0 | 420 |
| 7.2 | 42.0 | +12.5 | 54.5 | 227 |
| 8.0 | 58.0 | −2.5 | 55.5 | 91 |

[a] Titrations with $NMDG^+$ were performed as described in the text. $F_0$ is the value before titration. $\Delta F_{max}$ is the maximal change determined from extrapolation of the double-reciprocal plots. $F_{end}$ is the sum of $F_0$ and $\Delta F_{max}$. The experimental uncertainty of individual $F$ measurements is ca. 1 unit. $EC_{50}$ is the $NMDG^+$ concentration giving half-maximal change.

$EC_{50}$ values for the perturbation. The $F_0$ values give the pH-dependent fluorescence before titration with $NMDG^+$ and correspond to the $Na^+$ values in Fig. 5. The $F_{end}$ values obtained with complete titration with $NMDG^+$ are essentially invariant with pH. This is in agreement with complexation of the deprotronated form of BCECF by $NMDG^+$ to give a complex with pH-independent fluorescence which is about 4% less than that of the free anionic form of the indicator. The $EC_{50}$ data, giving the $NMDG^+$ concentration for half-maximal change, are in agreement with this interpretation. At pH 8.0 where BCECF is 91% deprotonated, the $EC_{50}$ is 91 m*M*. At pH 7.2 and 6.7 where BCECF is 67 and 33% deprotonated, the $EC_{50}$ values are correspondingly higher. Finally, the twofold increase in apparent dissociation constant of $H^+$ from BCECF in 150 m*M* $NMDG^+$ can also be explained by complexation by the cation, with a $K_d$ of ca. 91 m*M*.

Since cytoplasmic levels of free $Mg^{2+}$ may range between 0.1 and 1.2 m*M* [1, 11, 18], the effect of adding 1.0 m*M* $Mg^{2+}$ to 0.1 μ*M* BCECF *in vitro* was tested at various values of external pH (experiments not shown). At pH 8.5 only a 1–2% decrease in fluorescence was observed. At lower pH values, e.g., 7.0 or 5.5, the addition of 1.0 mM $MgCl_2$ had no effect.

### Quenching of BCECF Fluorescence by Amiloride and Bovine Serum Albumin (BSA)

Amiloride and its more potent derivatives have been used extensively as inhibitors of the plasmalemmal $Na^+/H^+$ exchanger [19]. In our use of amiloride as an inhibitor of this activity in platelets, we have observed that is quenches BCECF fluorescence and can cause serious misestimation of intracellular pH if this is not taken into account. When 1 m*M* amiloride is added to BCECF in solution (i.e., $K^+$ or $Na^+$ medium), it causes an instantaneous decrease in BCECF fluorescence by 45–50% (not shown). Measurements of optical density of the 1 m*M* amiloride solution showed that the absorbance at the BCECF excitation and emission wavelengths is lower than 0.05. Thus the quenching of BCECF fluorescence is not due to an inner filter effect [9] or radiationless energy transfer. The quenching phenomenon was studied systematically in physiological saline solution at [BCECF] = 3.8 μ*M*. A double-reciprocal plot of percentage quenching and amiloride concentration was linear for amiloride concentrations between 100 μ*M* and its solubility limit of 1.8 m*M*, extrapolating to 100% quenching. A $K_Q$ ($EC_{50}$) value of 0.7 m*M* was calcualted. This result could be consistent with Stern–Volmer quenching or ground-state complexation.

We also report that amiloride is itself fluorescent but at different wavelengths than BCECF. It has absorption peaks at 290 and 360 nm, which correspond to uncorrected excitation peaks at 302, 342, and 385 nm on our instrument. The emission peak is at 415 nm. It will therefore add its fluorescence to that of probes having overlapping emission peaks, such as quin2.

We have also observed that the addition of bovine serum albumin (BSA), which has been used as a means of removing ionophores in studies of cytoplasmic pH regulation using BCECF [2, 20], can cause serious errors in interpretation. When a portion of the dye exists outside of the cell, BSA addition causes a decrease in fluorescence associated with binding and quenching of that fraction of the dye. The data in Table VI illustrate this point. Experiments in solution showed that 5 mg/ml BSA quenches the fluorescence of 0.1 μ*M* BCECF by 30–40%, with a halftime of 1 min (experiments not shown). An important observation is that this process is not instantaneous.

Since amiloride and BSA have been used together in studies of pH regulation in cell suspensions [2, 20], we performed tests to determine whether amiloride binds to BSA. Equilibrium dialysis experiments (not shown) showed that 5 mg/ml BSA was not able to bind a measurable fraction of 1.0 m*M* amiloride (amiloride was assayed fluorometrically).

### Lack of Effect of DMSO and Ethanol

Since DMSO and ethanol were used as vehicles for inhibitors or ionophores, it was important to examine their individual effects on BCECF fluorescence *in vitro*. The addition of 1% DMSO or 1% ethanol to 0.1 μ*M* BCECF at pH 7.3 caused a 2.7% decline in fluores-

cence. Similar effects were seen with intact platelet suspensions.

## DISCUSSION

### Correction for the BCECF Leakage Artifact

Leakage of cytosolic indicator dyes is a pervasive problem which varies in seriousness with indicator, cell type, and experimental conditions. In single-cell measurements with indicators which have spectral shifts (e.g., Fura-2), it is a less serious problem because the degree of complexation can be determined from the ratio of fluorescence using two wavelengths. In measurements of cell suspensions ratiometric methods do not eliminate the problem since external dye in the medium contaminates the signal. Measurements of internal pH in platelets using BCECF can be frustrated by progressive loss of dye from the cytoplasm and its accumulation in the external medium. If not taken into account, this leakage will cause misestimation of $pH_{cyt}$ by as much as 0.4 pH unit. In contrast to BCECF, the $Ca^{2+}$ indicator quin2 shows very little leakage [11, 21]. We conclude that leakage is a property of BCECF and platelet combination and that it must taken into account to obtain correct results. We suggest that all reports of cellular behavior using entrapped fluorimetric indicators must address the problem of leakage in such a way as to ensure the reader that the reported pH (or $Ca^{2+}$) changes are not due leakage or changes in leakage.

The present communication has described two methods which can be used to determine the concentration of BCECF outside the cells at any point during an experiment: the "pH jump" and the "$Mn^{2+}$ quench." Both report identical quantities of external dye, allowing the corresponding fluorescence signal to be subtracted and removed from the calculation of $pH_{cyt}$. The following cautions must be stated: The pH jump method is not possible if the cells are exposed to nigericin or monensin at concentrations $\geq 0.25$ $\mu M$ because $H^+$ equilibration across the membrane will become "instantaneous" through mediation of these ionophores. When the $Mn^{2+}$ quench method is used, experimentation should be halted after measuring the instanteous change. Subsequent behavior may reflect effects of the cation on the plasma membrane. Judicious use of these methods at selected points in repeated experiments will aid in arriving at correct interpretations of the fluorescence signals in terms of cellular processes.

### Comparison of "Nigericin Calibration" and "Digitonin Lysis" Methods

After subtraction of the extracellular component to yield the intracellular signal, the next step is proper calibration of the latter. As stated earlier, the literature described two methods of calibration: (i) the nigericin method and (ii) the digitonin lysis method. Our experimentation shows the digitonin to be more suitable. The nigericin method would seem attractive because it represents an *in situ* calibration of the dye. According to the method, the total fluoroscence of a cell suspension is determined in the presence of 150 m$M$ KCl and nigericin ($F_{nigericin}$) over a wide range of $pH_{ext}$. Is is assumed that nigericin sets $pH_{cyt} = pH_{ext}$. As the method has been generally practiced, $pH_{cyt}$ is read directly from plots of $F_{nigericin}$ vs $pH_{ext}$. The problem with this simple procedure is that it overlooks the problem of dye leakage, which we have seen, is exacerbated by nigericin itself. This renders the nigericin method, as generally practiced, a hybrid between *in situ* and extracellular calibration.

In our tests of the nigericin method we subtracted the extracellular component of the fluorescence. We then observed appreciable differences between $pH_{cyt}$ values calculated using the nigericin and the digitonin methods. The nigericin method consistently gave higher values of $pH_{cyt}$ (by 0.2–0.3 pH unit). Part of this difference can be explained by the observation that values of $F_{max}$ obtained by the nigericin method are 15% lower than those obtained by digitonin lysis. The pivotal question thus becomes whether the cytoplasm really does decrease the fluorescence of the deprotonated form of the dye or whether nigericin has failed to set $pH_{cyt} = pH_{ext}$ at very alkaline values. We consider the second alternative to be more likely. At a high pH nigericin would be working at less than one-tenth of its $K_m$ from protons. It is not clear that nigericin-mediated $H^+$ export can keep up with $H^+$ production via oxidative phosphorylation and glycolysis. Furthermore, our spectroscopic studies have given no evidence that the properties of BCECF in the cytoplasm are different from those in solution. The fluorescence polarization and excitation and emission spectra in the cytoplasm are identical to those in solution, thus giving no evidence for binding to cytoplasmic proteins or membranes. The fluorescence and titration behavior of BCECF in solution are independent of whether $K^+$ or $Na^+$ is used to supply physiological ionic strength. The presence or absence of a physiological concentration of $Mg^{2+}$ has no effect. As stated earlier, digitonin has no effect on the spectral characteristics of the dye. We thus consider the method of choice to be the digitonin

lysis method for calibration, in conjunction with the pH jump or $Mn^{2+}$ quenching methods for the subtraction of extracellular dye contributions during the experiment.

### Artifacts from Direct Interactions of Agents with BCECF

The present study also describes a number of possible artifacts which should be avoided. Failure to correct for the effect of $NMDG^+$ on BCECF fluorescence (150 m*M* $NMDG^+$ increased fluorescence at a fixed pH and decreased $\Delta F_{max}$) will underestimate $pH_{cyt}$ by about 0.3 pH unit via the digitonin lysis procedure. The finding that amiloride quenches BCECF fluorescence points to the need of using extreme caution in interpreting effects of amiloride on $pH_{cyt}$. Because amiloride is a weak base ($pK_a$ = 8.67 [22]) and also is cell membrane permeable [23], it can also quench the fluorescence of cytoplasmic BCECF. Such quenching would mimic cytoplasmic acidification. The same may apply to its more potent analogues. In addition, amiloride has its own fluorescence, which will add on to that of dyes with overlapping emission peaks. The effect of BSA (significant quenching of extracellular BCECF) can also give the appearance of a time-resolved acidification in cases where significant amounts of dye have leaked from cell suspensions. It is necessary to keep in mind that fluorescence is a complex process subject to many influences. When it is used as a tool for delineating cellular processes, the burden of proof is on the investigator.

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