ARSENAZO III FORMS 2:1 COMPLEXES WITH Ca AND 1:1 COMPLEXES WITH Mg UNDER PHYSIOLOGICAL CONDITIONS

ESTIMATES OF THE APPARENT DISSOCIATION CONSTANTS

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ABSTRACT Experiments to determine the apparent dissociation constants of the Ca and Mg complexes of arsenazo III clearly indicated that the predominant Ca complex contains one Ca ion and two dye molecules, although previous reports have either claimed or assumed 1:1 complexing. The evidence is based on the effects of varying [dye] as well as [Ca] and [Mg], and clear evidence for the formation of 1:1 complexes with Ca was obtained only at submicromolar [dye], whereas Mg formed 1:1 complexes exclusively. The implications of these findings with regard to the use of arsenazo III as an indicator of intracellular free [Ca] are discussed, with particular reference to its selectivity for Ca and the interference effects of other ions.

The metallochromic indicator dye arsenazo III, which undergoes an absorbance change on forming a complex with Ca and many other cations, has recently been used to follow changes in internal [Ca] in a variety of physiological preparations (1–6). In many applications it is expected to be superior to aequorin, particularly because its absorbance change is linear with changes in free [Ca] in the physiological range (2, 4), whereas there is still no general agreement on the relation between aequorin glow and free [Ca], although a square-law relation is often assumed.

The exact composition of the Ca-dye complex has not been thoroughly investigated, however, although the linearity with [Ca] shows that it contains only one Ca ion. The commonly quoted report that only 1:1 complexes are formed with Ca (7) does not have a strong theoretical foundation, and was primarily based on the observation that the absorbance spectrum of the Ca-dye complex contains "the same two peaks" as [Ca] and dye, ([L]), are varied over a wide range. Our initial calibration and in vivo experiments (4) suggested, however, that the composition of the complex was CaL_2, but these were done with impure dye (8) and so could not be conclusive. Results with pure arsenazo III presented here confirm the CaL_2 composition, whereas the dye-Mg complex is shown to be 1:1, i.e., MgL, and estimates

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of the apparent dissociation constants are also given. The methods used in this investigation are somewhat less straightforward than some more commonly used ones such as Job plots, but they provide a much greater insight into the behavior of arsenazo III as an indicator of relatively low free Ca levels, and they also show much more clearly how the existence of a CaL_2 complex has previously escaped detection.

Fig. 1 shows the results of calibration experiments on 98% pure arsenazo III (Sigma Chemical Co., St. Louis, Mo.), in the presence of 400 mM KCl and 100 mM morpholinopropane sulfonic acid (MOPS)[1] at pH 7.3 and 16°C, obtained with the pulsed-wavelength differential spectrophotometer described previously (4); these conditions were kept constant for all subsequent calibration experiments. Results are shown for 200 μM dye in a 0.5 cm path length and 100 μm dye in a 1 cm path length, so the number of dye molecules in the light path (and hence the absorbance in the absence of Ca) is the same. The free [Ca] was defined by ethylene glycoltetraacetic acid (EGTA) buffering (the total [EGTA] was equal to the total [L]), being proportional to the ratio of [CaEGTA] to free [EGTA], and it can be seen that the dye absorbance change is linearly related to this ratio, and hence to free [Ca], at both dye concentrations. The linearity is as expected for the formation of a complex containing only one Ca ion when only a small proportion of the dye is in the complexed form, but gives no information on the number of dye molecules in the complex. This information is given by the effect of changing the dye concentration on the slope of the response to Ca, as the following equations will make clear. For 1:1 complexing,

[1]A high buffer concentration was used because Ca binding by the dye is expected to displace protons (as with EGTA), and the dye absorbance is quite pH-sensitive. Results with a Ca electrode showed that MOPS had no detectable Ca-buffering effect.

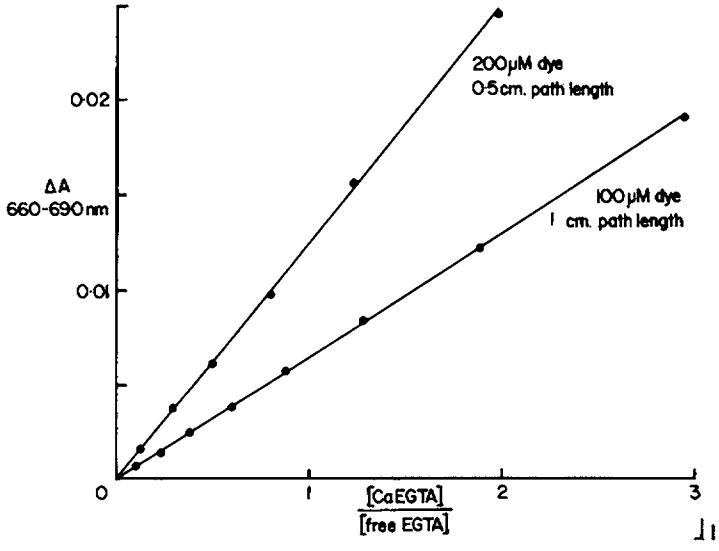

FIGURE 1 Differential absorbance change (660–690 nm) in response to changes in free [Ca] at dye concentrations of 100 and 200 μm. The optical path length at the higher concentration was halved to maintain a constant absorbance in the absence of Ca, but the slope of the absorbance change has almost doubled (actual ratio = 1.94). The result suggests that the complex formed contains two dye molecules (see text).

$$Ca + L = CaL, \text{ and } K_1 = [Ca][L]/[CaL]. \quad (1)$$

It is reasonable to suppose that the formation of a CaL_2 complex would proceed through a CaL intermediate, even if the steady-state concentration of CaL were very small compared to that of CaL_2, i.e.,

$$CaL + L = CaL_2, \text{ and } K_2 = [CaL][L]/[CaL_2]. \quad (2)$$

When considering the limiting case in which $[CaL] \ll [CaL_2]$, it is more useful to combine Eqs. 1 and 2, to give

$$Ca + 2L = CaL_2, \text{ and } K_1K_2 = [Ca][L]^2/[CaL_2]. \quad (3)$$

In the limiting condition where virtually all the dye is in the uncomplexed form (i.e., low [Ca]), the relative change in absorbance, $\Delta A/A$, which results from a change in (Ca) is, from Eq. 1

$$\Delta A/A = \Delta[CaL]/[L] = \Delta[Ca]/K_1, \quad (4)$$

and from Eq. 3

$$\Delta A/A = \Delta[CaL_2]/[L] = \Delta[Ca][L]/K_1K_2. \quad (5)$$

Eqs. 4 and 5 show that when the absorbance A is constant, the slope of the response to Ca is independent of [L] for 1:1 complexing, but increases with [L] when the complexing is 2:1. Thus Fig. 1 clearly shows that the predominant complex is CaL_2.

If the path length remains constant as [L] is increased, the dye absorbance A will also increase, so under these conditions Eq. 5 shows that the absorbance change will increase with the square of the dye concentration. This behavior has been observed in experiments on the *Aplysia* R15 bursting pacemaker neurons on both impure dye (4) and pure dye (unpublished experiments), demonstrating that 2:1 complexing also occurs in vivo.

The dissociation constants of 1:1 complexes are normally determined by use of the Lineweaver-Burk double reciprocal relation. If the complex is CaL, expressing [L] and [CaL] as fractions of the total dye concentration, we can substitute in Eq. 1, $[L] = 1 - [CaL]$, and hence derive

$$1/[CaL] = 1 + (K_1/[Ca]). \quad (6)$$

Thus when $1/\Delta A$ is plotted against $1/[Ca]$, a linear relation is obtained, intercepting the x-axis at $-K_1/[Ca]$. For a CaL_2 complex, a linear relation is not obtained, but it will be shown below that the deviation from linearity is relatively small when [Ca] is varied over a limited range, and it could easily pass undetected. Apparent linearity on Lineweaver-Burk coordinates is thus not necessarily sufficient evidence for 1:1 complexing.

Fig. 2 *a* shows the results of a Ca calibration experiment with 200 μM dye. A problem with determining [Ca] under these conditions is that most of the total Ca added may be complexed by the dye, and estimates of free [Ca] based on subtraction of the calculated amount complexed will be prone to error (and will also vary according to whether 1:1 or 2:1 complexing is assumed). The problem was avoided by determining free [Ca] directly with a Ca electrode (9), which gave a Nernstian response to [Ca] down to below 10 μM.

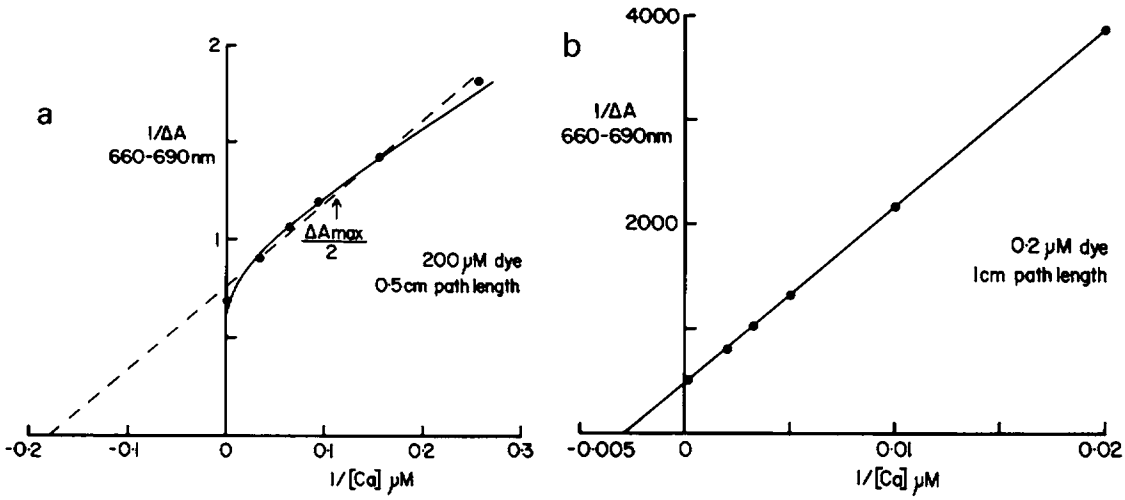

FIGURE 2 Double reciprocal plots of absorbance chance against free [Ca] at dye concentrations of 200 μM (*a*) and 0.2 μM (*b*). For a CaL_2 complex the relation is expected to be curved [solid line in (*a*)], but in practice the deviation from linearity (dotted) could pass undetected. At very low dye concentrations (*b*), however, the results become consistent with 1:1 complexing (see text).

The result in Fig. 2 *a* has been plotted on Lineweaver-Burk coordinates to illustrate that a reasonable fit to a linear relation can indeed be obtained (dotted line), although in this case the intercept will have no physical meaning. The relation that is expected for 2:1 complexing is given by the solid curve. It is obtained by expressing [L] and $[CaL_2]$ as fractions of the total dye concentration, and substituting in Eq. 3, $[L] = 1 - 2[CaL_2]$, which gives

$$4[CaL_2]^2 - (4 + K_1K_2/[Ca])[CaL_2] + 1 = 0. \tag{7}$$

Taking the appropriate root of this equation yields

$$\frac{1}{[CaL_2]} = \frac{8}{4 + K_1K_2/[Ca] - \sqrt{\{(4 + K_1K_2/[Ca])^2 - 16\}}}. \tag{8}$$

The K_1K_2 and the absorbance of the CaL_2 complex were selected to give the closest fit to the data points in the region of half-maximum absorbance, although the relation fits all the data points quite well.[2]

K_1K_2 clearly has the units of concentration squared, and it can be evaluated from Eq. 3 at the [Ca] for which $\Delta A = \Delta A_{max}/2$, when $[L] = 2[CaL_2]$. From Eq. 3 we now obtain

$$K_1K_2 = 2[Ca][L] = [Ca][\text{total L}]\ (\Delta A = \Delta A_{max}/2). \tag{9}$$

From the result in Figure 2 *a*, this gives $K_1K_2 = 8.9\,\mu M \times 200\,\mu M = 1.8 \times 10^{-9} M^2$. Similar values were obtained in two further experiments.

[2]At [Ca] below about 5 μM, the response time of the electrode became very slow, making accurate determination difficult, whereas at very high [Ca] the complexing may change to 1:1 (as discussed later), so at both extremes the data points may be expected to deviate from the theoretical curve. There was no evidence to suggest that the dye or its complex interfered with the electrode.

Although the best-fit straight line in Fig. 2*a* has no physical significance, the "K_1" it yields (5.6 μM) is fairly close to the [Ca] for $\Delta A = \Delta A_{max}/2$, which has the fortunate result that the calibration of dye absorbance changes based on the assumption of 1:1 complexing may not be seriously in error if the same dye concentration was always used.

Eq. 9 shows that the [Ca] for $\Delta A = \Delta A_{max}/2$ decreases with increasing [dye], so the K_1 determined when using Lineweaver-Burk coordinates is expected to vary in a similar manner, and on these coordinates this is a far better test for 1:1 or 2:1 complexing than is the apparent linearity of the relation. Such variation was indeed observed in the present experiments. The K_1 approximately doubled when the dye concentration was reduced to 100 μM, and increased to 140 μM at 2.5 μM dye. At lower concentrations, however, there was little further shift in the *x*-axis intercept, and values of 270 μM at 0.5 μM dye and 330 μM at 0.2 μm dye (see Fig. 2*b*) were obtained. This finding suggests that a 1:1 complex, CaL, is now predominating, but that K_1 is at least 3×10^{-4} M. The values of K_1 and K_1K_2 given here imply that K_2 is approximately 6×10^{-6} M, which is nearly two orders of magnitude lower than K_1. This difference suggests that a Ca ion binds the second arsenazo molecule much more readily than the first, which greatly favors formation of CaL_2 over CaL, as observed in Fig. 1. The difference is also great enough for the determination of K_1K_2 in Fig. 2*a* to have been scarcely affected by possible errors due to formation of the CaL complex, although formation of CaL is expected to be more significant at very high [Ca], as is apparent from Eqs. 1 and 2.[3]

The results from the Mg calibration experiments are very straightforward, and are shown in Fig. 3. Excellent linearity and identical *x*-axis intercepts were observed on Lineweaver-Burk coordinates for dye concentrations of 5μM and 100 μM, which clearly demonstrates that only a 1:1 Mg-dye complex, MgL, is formed under these conditions. The K_1 is 7.0 mM.[4]

Since arsenazo III thus forms a 2:1 complex with Ca and a 1:1 complex with Mg, the Ca:Mg selectivity (i.e., the absorbance change for a step change in free [Ca] relative to that for an equal change in free [Mg]) will increase with the dye concentration. For a constant background [Mg], however, its interference with the Ca sensitivity of the dye is expected to be independent of the dye concentration. Consideration of the three-way equilibrium between free L, MgL, and CaL_2 predicts that a constant background [Mg] will reduce the absorbance change for a step change in [Ca] for two reasons: first, formation of MgL will reduce the free [L]; and second, when free [L] is reduced by formation of CaL, there will be a corresponding reduction in [MgL]. This will give rise to a negative component in the over-

[3]Our spectrophotometer does not produce continuous spectra, but measurements at 690, 630, 600, 570, and 540 nm suggested that both the CaL_2 and the CaL complexes had absorbance maxima near 660 and 600 nm, the 660 nm peak being the larger in the CaL_2 complex and the 600 nm peak being the larger in the CaL complex. Attempts to determine the proportions of the two complexes from the relative absorbance at these two wavelengths, however, did not give meaningful results, and possibly a number of other factors (including, but not necessarily restricted to, changes in pH) may influence the relative amplitudes of the two absorbance peaks, but this point was not investigated further. At high [Ca], the molar absorbance was about 18,000 at 660 nm and 24,000 at 600 nm, but these wavelengths do not necessarily correspond to the peaks in the absorbance spectrum, which could be somewhat higher. Molar absorbance at the 570 nm isosbestic wavelength was 30,000.

[4]In a previous investigation (10), Ca-contamination problems were encountered with the Mg-containing stock solutions. The present experiments used $MgCl_2$ (from Fisher Scientific Co., Pittsburgh, Pa.), which was stated to contain 0.002% Ca, so a 10 mM solution would contain only 0.2 μM Ca. In any event, the results in Fig. 2 are inconsistent with any significant Ca contamination, which would cause a [L]-dependent shift in the *x*-axis intercept, among other effects.

all absorbance change, and its contribution will vary with the wavelength(s) used for measurement, since the absorbance spectra of the Mg and Ca complexes of the dye are different (2, 4, 5).

The relative reduction in sensitivity to Ca by both these effects will vary with [Mg], but at a constant [Mg] it is expected to be independent of the total dye concentration. With [Mg] in the range expected inside cells, the sensitivity reduction can be quite high, and for 2.5 mM [Mg], the reduction in the 660–690 nm differential absorbance change in response to a step change in free [Ca] was nearly threefold (based on the results with 100 μM dye). This value is about that expected from the dissociation constants and molar absorbances (at 660 and 690 nm) of the CaL_2 and MgL complexes, but paradoxically it is greater than would have been predicted on the basis of 1:1 complexing of Ca, on account of the square-law (rather than linear) relation between free [L] and the absorbance change for a change in free [Ca].

No data on the effects of pH will be presented here, but since the groups that comprise the Ca-binding site of the dye can be protonated, and this also causes an absorbance change (4, 5), the effects of pH are expected to be analogous to those of Mg, namely that the Ca:H selectivity of the dye would increase with [L], and that the relative sensitivity of the dye to Ca would change in an [L]-independent manner with pH.

These considerations suggest that while use of a fairly high dye concentration is desirable to achieve high Ca selectivity, the interference effects of other ions will remain serious, and must be taken into account if the dye absorbance changes are to be quantified in terms of changes in free [Ca]. The highest usable dye concentration in any system is limited by the point at which its Ca buffering capacity (which is related to $[CaL_2]$ and is thus expected to increase with $[L]^2$) becomes high enough to reduce the changes in free [Ca], and our experiments show that in *Aplysia* neurons this begins to become noticeable at dye concentrations above about 0.3 mM.

The apparent dissociation constants of the Ca and Mg complexes of the dye in 400 mM

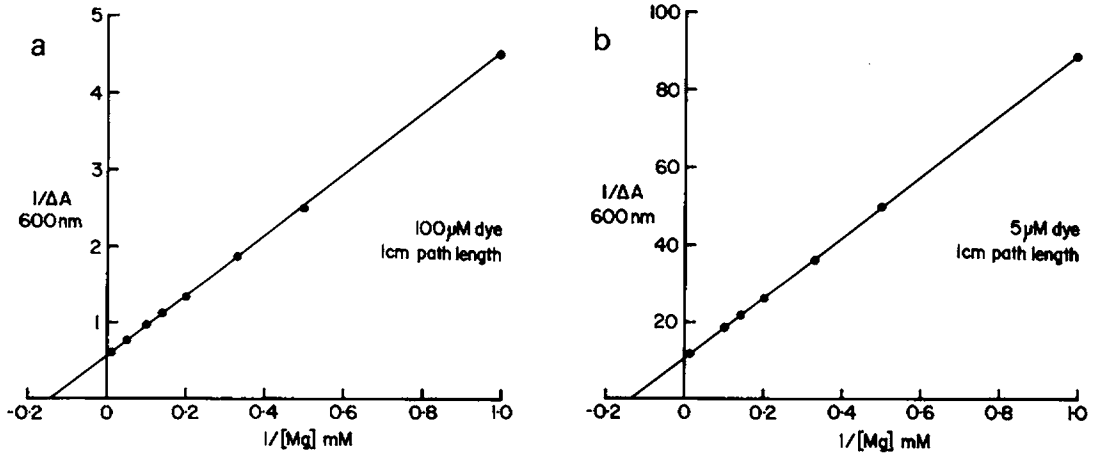

FIGURE 3 Double reciprocal plots of absorbance change against [Mg] at dye concentrations of 100 μM (*a*) and 5 μM (*b*). The identical *x*-axis intercepts suggest that the composition of the complex is MgL with an apparent K_1 of 7.0 mM.

TABLE I

APPARENT DISSOCIATION CONSTANTS OF ARSENAZO III AND EGTA IN 400 mM KCl AT pH 7.3 AND 16°C

Complex	Apparent dissociation constant
CaL	$>3.0 \times 10^{-4}$ M
CaL_2	1.8×10^{-9} M^2
MgL	7.0×10^{-3} M
Ca-EGTA*	3.7×10^{-8} M

*Indirect estimate from the dissociation constant of the CaL_2 complex.

KCl at pH 7.3 and 16°C are shown in Table I. A value for the apparent dissociation constant of the CaEGTA complex has also been given, and it has been calculated from the data in Fig. 1, by using the apparent dissociation constant of the CaL_2 complex. The value obtained (0.04 μM) is equivalent to a true CaEGTA stability constant (11, 12) of $10^{11.16}$, and it compares favorably with a recent direct determination with a Ca electrode (12), where a value of $10^{11.00}$ in 200 mM KCl was obtained. The agreement suggests that the apparent dissociation constant of the CaL_2 complex given here is in the correct range.

Construction of molecular models suggested that the CaL_2 complex has the same structure as that of the general L_2 form proposed by Budesinsky (13), in which the dye molecules are at right angles, giving a cubic complex with a coordination number of eight. Many related dyes may be able to form such a complex, and it has been reported that chlorphosphonazo III forms 2:1 complexes with Ca (14), so for this entire group of molecules, 2:1 complexing of Ca could perhaps be the rule rather than the exception, and this point is certainly worthy of further investigation.

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