STOICHIOMETRY OF THE REACTIONS OF CALCIUM WITH THE METALLOCHROMIC INDICATOR DYES ANTIPYRYLAZO III AND ARSENAZO III

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ABSTRACT A method for determining the stoichiometry of one-product reactions involving a metal ion and an organic ligand is presented and applied to the reactions of calcium and magnesium with the metallochromic dyes Antipyrylazo III and Arsenazo III. The method consists of fitting titration data, obtained in solutions buffered for the metal, with theoretical functions that include: (*a*) the dependence of product concentration on the concentration of both reactants, (*b*) the relationship between metal ion concentration and total amount added in the presence of the buffer, and (*c*) a correction for the amount of metal ion that binds to the organic ligand. It is shown that the products of the reactions of Antipyrylazo III with calcium and magnesium are CaD_2 and MgD, respectively. The product formed between calcium and Arsenazo III at $[Ca^{2+}]$ over 20 μM is Ca_2D_2, other products accumulating at lower $[Ca^{2+}]$. The kinetics of the Antipyrylazo III:Ca reaction are rapid under conditions in which this dye has been applied to measure calcium transients in skeletal muscle fibers. The present results provide a calibration for previous studies with Antipyrylazo III in muscle fibers.

INTRODUCTION

Absorbance changes in muscle fibers loaded with the metallochromic indicator dye Antipyrylazo III (Ap III) (1) have been interpreted as measuring changes in myoplasmic free calcium $[Ca^{2+}]$ (2–4). Kovács et al. (2) ascribed all the kinetic features of these signals to the time dependence of $[Ca^{2+}]$ under the explicit assumptions that the measurement provided by Ap III is both a linear and time-independent function of $[Ca^{2+}]$. Except for the indirect argument that the kinetics of the myoplasmic Ap III signal are relatively simple (2), these assumptions were not further tested. Recent reports by Palade and Vergara (4 and personal communications) have, however, demonstrated complexities in the Ca:Ap III reaction: the equilibrium titration could only be explained by assuming the existence of several Ca:dye complexes, and the reaction exhibited multiphasic kinetics at high calcium concentrations.

The studies reported here were initially conducted with the purpose of verifying the assumptions in reference 2 regarding the linearity with $[Ca^{2+}]$ and time independence of the Ca:Ap III reaction. In trying to characterize the absorbance change as a function of $[Ca^{2+}]$ in calibrating solutions we realized that, owing to the calcium-dye reaction, the $[Ca^{2+}]$ cannot be known exactly unless the stoichiometry of the reaction is known. A method for studying the stoichiometry of these reactions was thus devised with which we could show clearly that the stoichiometry of the Ca:Ap III reaction is 1:2 over a broad range of $[Ca^{2+}]$ and dye concentrations [D], and confirm the hypothesis of linearity in reference 2. The same method showed that the Mg:Ap III reaction is 1:1.

The straightforward results obtained with Ap III and the recent conflicting reports (4–8) about the stoichiometry of the widely used dye Arsenazo III (Ar III) (9) prompted us to apply our methods to this dye. In agreement with previous reports by Palade and Vergara (4), our results show that there is a clear predominance of a Ca_2Ar_2 complex, other complexes accumulating at low (micromolar) $[Ca^{2+}]$.

MATERIALS AND METHODS

AP III was obtained from ICN K&K Laboratories Inc., Plainview, N.Y. According to Scarpa et al. (1), it has a major impurity, ~15% in weight fraction, which does not react with calcium to give a detectable colored product. Our present results likewise show that the commercially available AP III reacts as a single metallochromic species. Ar III was obtained from Sigma Chemical Co., St. Louis, Mo., as a sodium salt, 98% pure.

To obtain reproducible results our reaction media were heavily buffered for calcium. We used HEDTA[1] (98%, Sigma Chemical Co.) with a dissociation constant (K_L) of 6.46×10^{-6} M at 0.1 M ionic strength and pH = 6.9[2] or NTA (99.5%, Sigma Chemical Co.; $K_L = 324 \times 10^{-6}$ M), depending on the $[Ca^{2+}]$ desired. EGTA ($K_L = 0.776 \times 10^{-7}$ M) (11) was not used as it has minimal buffer capacity in most of the titration range of these dyes.

In addition to 15 mM of either HEDTA or NTA the reaction medium contained 70 mM KCl, 20 mM Tris maleate buffer, and various concentrations of dye. For the Ar III calibrations at 0.5 M ionic strength, 430 mM KCl and 50 mM Tris maleate buffer were used. Calcium was added as a small aliquot of 1 M $CaCl_2$, the pH was then reset to 6.900 ± 0.005 (read in an Orion Research Inc., Cambridge, Mass., model 801 digital pH meter), the volume was set to a fixed final value by adding water, and the absorbance was read in a Bausch & Lomb Spectronic 100 spectrophotometer (Bausch & Lomb Inc., Scientific Optical Products Div., Rochester, N.Y.), in 0.1-, 0.2-, or 1-cm cuvettes, depending on [D]. The measurements were carried out at room temperature, 20–25°C.

The absorbance (A_λ) of each solution was determined at two or three wavelengths (λ). Absorbance changes (ΔA_λ) due to calcium addition were calculated as differences between A_λ values for calcium-containing and calcium-free solutions. Each ΔA_λ value was normalized to the absorbance (A_{ref}) of the calcium-free solution at a reference wavelength, so that all absorbances given here correspond to the same total number of dye molecules.

Analysis of Data

In order that a set of A_λ-vs.-$[Ca^{2+}]$ data correspond to a single product reaction, two criteria must be satisfied: (*a*) the ratios $\Delta A_{\lambda_1}/\Delta A_{\lambda_2}$ of absorbance changes at any two different wavelengths λ_1 and λ_2 must be independent of $[Ca^{2+}]$ (failure to satisfy this criterion demonstrates formation of more than one product); and (*b*) the experimental relationship ΔA_λ vs. $[Ca^{2+}]$ determined at any wavelength must be proportional to the theoretical relationship [product] vs. $[Ca^{2+}]$ for some particular calcium:dye stoichiometry. The second criterion thus encompasses the first.

The first of these criteria was seen to hold perfectly for the Mg:Ap III reaction at all concentrations tested. It held very well for Ca:Ap III except at $[Ca^{2+}] \geq 1$ mM. Small deviations were also detected at low dye and very low $[Ca^{2+}]$. For the Ca:Ar III reaction it only held at $[Ca^{2+}] > 5\ \mu M$ or $[Ca^{2+}] > 20\ \mu M$, depending on the ionic strength. Accordingly, an excellent fit of an equation corresponding to a

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[1] *Abbreviations used:* HEDTA, *N*-2-hydroxyethyl ethylenedinitrilo-*N*, *N'*, *N'*-triacetic acid; NTA, nitrilotriacetic acid.

[2] These K_L values were calculated from the values of affinity constants for Ca:L and the various protonation reactions given by Martell and Smith (10). In the case of NTA, which also gives a $Ca{:}L_2$ complex, it was found that the amount of this complex was negligible at the concentrations used here. Measurements duplicated with the two buffers showed a small systematic difference, the ΔA being smaller with NTA as shown in Fig. 1. This is evidence that one or both sets of affinity constants may be in error by a small amount.

simple stoichiometry was obtained for Mg:Ap III at all concentrations; a very good fit was obtained for the Ca:Ap III except at $[Ca^{2+}] \geq 1$ mM and a good fit was obtained also for the Ca:Ar III reaction for calcium concentrations greater than either 5 or 20 μM, depending on ionic strength. In the ranges of concentrations where criterion (*a*) did not hold, the fits were not good.

Four possible stoichiometries (Ca:D) for the Ap III and Ar III reactions were considered: 1:1, 1:2, 2:1, and 2:2. Each of these provides a different theoretical relationship of [product] vs. $[Ca^{2+}]$ that was fit to the experimental data. The procedure will be exemplified with equations applicable to the 1:2 stoichiometry. Concentration brackets and valences have been omitted (e.g., $Ca \equiv [Ca^{2+}]$).

At equilibrium,

$$CaD_2 = \frac{D_T}{2} + \frac{K}{8Ca} - \left[\left(\frac{K}{8Ca}\right)^2 + \frac{D_T K}{8Ca}\right]^{1/2}, \tag{1}$$

where D_T represents total dye and K the second-order dissociation constant.

The absorbance change ΔA_λ due to this reaction would be proportional to the concentration of CaD_2, with the maximum change $\Delta A_\lambda\text{max}$ for full dye reaction with calcium being proportional to half the total dye concentration. With the aid of Eq. 1, the normalized absorbance change $\Delta A_\lambda/A_{ref}$ is thus given by

$$\frac{\Delta A_\lambda}{A_{ref}} = \frac{\Delta A_\lambda\text{max}}{A_{ref}}\left[1 + \frac{K}{4D_T \cdot Ca} - \left(\frac{K^2}{16Ca^2 \cdot D_T^2} + \frac{K}{2Ca \cdot D_T}\right)^{1/2}\right] = f(Ca, D_T). \tag{2}$$

This theoretical relationship contains only two parameters, a maximum ordinate $\Delta A_\lambda\text{max}/A_{ref}$ and a dissociation constant K. Note that for the 1:2 stoichiometry $\Delta A_\lambda\text{max}/A_{ref}$ is equal to $(\epsilon_{CaD_2} - 2\epsilon_D)/\epsilon_{ref}$, where ϵ_{CaD_2} and ϵ_D are the molar extinction coefficients for CaD_2 and D at wavelength λ, and ϵ_{ref} is the molar extinction coefficient for D at the reference wavelength.

To fit an equation such as Eq. 2 to our data, the free calcium concentration corresponding to each total calcium concentration C_T must be determined. This involves correcting C_T for dye-bound calcium and using the resulting value to calculate the free calcium. If C'_T is the total calcium not bound to dye, which includes both free calcium and calcium bound to the buffer, the free calcium will be given by

$$Ca = \tfrac{1}{2}\{C'_T - K_L - L_T + [(C'_T - K_L - L_T)^2 + 4K_L C'_T]^{1/2}\} \equiv g(C'_T), \tag{3}$$

where L_T is the total concentration of calcium buffer. C'_T can be calculated from the total calcium, C_T, by subtracting the calcium bound to the dye. The evaluation of the dye-bound calcium requires knowledge of the stoichiometry of the reaction and the maximum value of the absorbance change, which is one of the two theoretical parameters in Eq. 2. For the 1:2 stoichiometry,

$$C'_T = C_T - CaD_2 = C_T - \frac{D_T}{2} \cdot \frac{\Delta A_\lambda}{\Delta A_\lambda\text{max}} \equiv h(C_T, D_T). \tag{4}$$

Sequential substitutions of Eq. 4 in 3 and Eq. 3 in 2 provide a final theoretical expression for ΔA_λ as a function of total calcium[3]

$$\frac{\Delta A_\lambda}{A_{ref}} = f\{g[h(C_T, D_T)], D_T\} \equiv F(C_T, D_T). \tag{5}$$

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[3] A novel feature of the theoretical function for fit is that the definition provided by Eq. 5 is implicit at ΔA_λ appears also on the second member through Eq. 4. This complicates the actual calculation of the thoretical function and the computation of the partial derivatives involved in the fitting procedure. Considerable simplification was obtained by considering that the correction for dye-bound calcium, Eq. 4, depends on the measured ΔA_λ values, not the theoretical ones. Accordingly, the definition (Eq. 5) was no longer implicit and the partial derivatives could be calculated as for an explicit function. Although this simplification merits a more careful theoretical examination, its use is justified as the programs thus written converted rapidly to correct fits when tested on simulated data.

Analogous functions F may be constructed for each stoichiometry. In all cases the function g, which does not depend on stoichiometry, would be given by Eq. 3.

For 1:1 stoichiometry, the function f would be

$$\frac{\Delta A_\lambda}{A_{\text{ref}}} = \frac{\Delta A_\lambda \text{max}}{A_{\text{ref}}} \cdot \frac{Ca}{Ca + K} \equiv f(Ca), \tag{6}$$

and the function h would be

$$C'_T = C_T - CaD = C_T - D_T \frac{\Delta A_\lambda}{\Delta A_\lambda \text{max}} \equiv h(C_T, D_T). \tag{7}$$

For 2:2 stoichiometry, the function f would be

$$\frac{\Delta A_\lambda}{A_{\text{ref}}} = \frac{\Delta A_\lambda \text{max}}{A_{\text{ref}}} \left[1 + \frac{K}{4D_T \cdot Ca^2} - \left(\frac{K^2}{16D_T^2 \cdot Ca^4} + \frac{K}{2D_T \cdot Ca^2}\right)^{1/2}\right]. \tag{8}$$

In this case h would be given by Eq. 7, as the correction for dye-bound Ca is the same for 1:1 and 2:2 stoichiometries.

The functions F for each of the four simpler stoichiometries were fit to the data with iterative nonlinear least-squares fit programs written according to reference 12, and run in a PDP8/E computer (Digital Equipment Corp., Marlboro, Mass.). The input data were all the values $\Delta A_\lambda/A_{\text{ref}}$ measured at a given D_T, the corresponding C_T values, and the three parameters D_T, L_T, and K_L. The actual values of $[Ca^{2+}]$ were calculated by Eq. 3 in each iteration. When the convergence criterion was satisfied, the program printed out the experimental data $\Delta A_\lambda/A_{\text{ref}}$ as a function of the calculated $[Ca^{2+}]$ in addition to the best-fit parameters. The $\Delta A_\lambda/A_{\text{ref}}$ data were subsequently divided by the value of $\Delta A_\lambda\text{max}/A_{\text{ref}}$ determined from the fit and thus converted to $\Delta A_\lambda/\Delta A_\lambda\text{max}$, i.e., saturation fraction. These values and the best fit theoretical curves were plotted as a function of $[Ca^{2+}]$, either on linear or semilogarithmic scales, thus originating all figures presented here.

The statistical evaluation of the fit included calculation of the covariance matrix and standard deviations (σ) for each of the best fit parameters (13). A 95% confidence interval for these includes $\pm 3\,\sigma$ instead of ± 2, owing to the nonlinear nature of the problem (14).

For a stoichiometry to be considered correct, the fits obtained at different dye concentrations should be internally consistent; that is, the best fit parameters should be the same at all dye concentrations. As an equivalent criterion, the sets of data obtained at different D_T values were jointly fit by functions F. This was done by a straightforward modification of the numerical method of nonlinear least-squares fit by considering F as a surface, a function of the two variables D_T and C_T. The solid lines in the figures are the sections of the best-fit surfaces by the constant D_T planes at the values of D_T used.

RESULTS

Ca:Ap III

Figs. 1 and 2 summarize our results concerning the Ca:Ap III reaction. Fig. 1 shows absorbance changes at 720 and 520 nm for a range of $[Ca^{2+}]$ from 1×10^{-6} M to 0.8×10^{-3} M and for three dye concentrations, 50, 187.5, and 750 μM. ΔA values determined at $[Ca^{2+}]$ levels above this range did not fulfill criterion (*a*), showing an actual decrease of ΔA_{720} when ΔA_{550} was still increasing with $[Ca^{2+}]$ and were not used. Absorbance changes at a given calculated $[Ca^{2+}]$ tended to be systematically larger with HEDTA as calcium buffer (circles in Fig. 1) than with NTA (squares). This indicates a slight inconsistency in the affinity constants used for the two calcium buffers.

These data were fit with functions F corresponding to the four simplest stoichiometries.

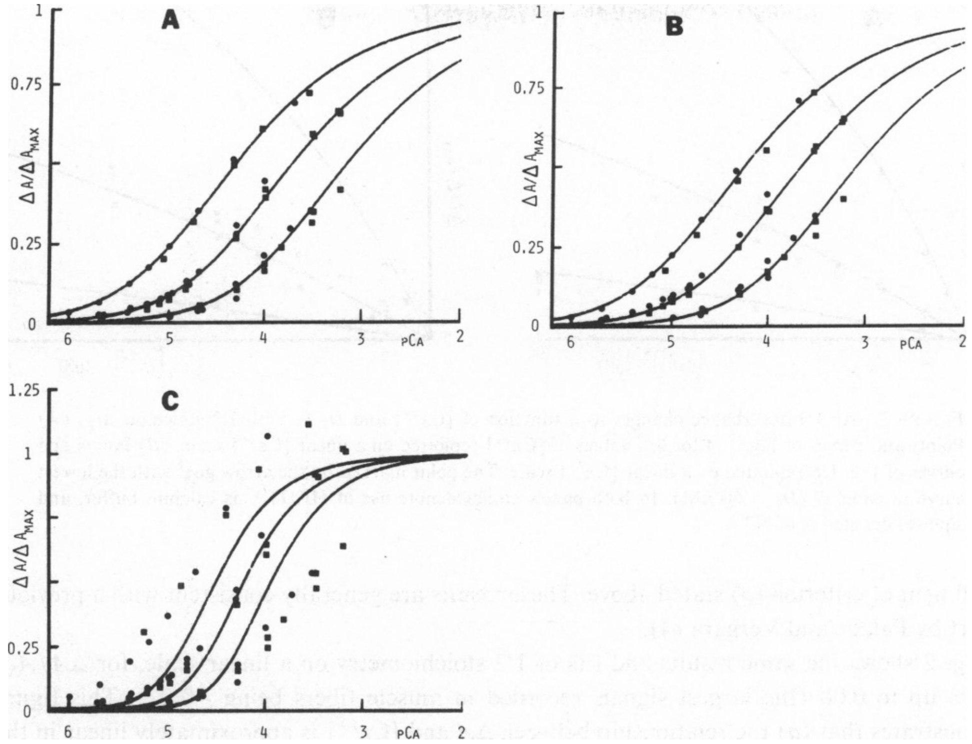

FIGURE 1 AP III absorbance changes as a function of [Ca^{2+}] and D_T fit with 1:2 and 2:2 stoichiometries. (*A*) Results at 720 nm and fit with 1:2 stoichiometry. The [Ca^{2+}] values, represented logarithmically on the abscissa, were calculated as explained in the text. Each absorbance change was normalized to the maximum change by dividing the measured values $\Delta A_{720}/A_{550}$ by the value of $\Delta A_{720}\mathrm{max}/A_{550}$ obtained from the best fit of the function $F(C_T, D_T)$ for 1:2 stoichiometry (see text) to all data. The curves, from left to right, are sections of the best fit F by the three planes $D_T = 750$, 187.5, and 50 μM, which were the dye concentrations used. At [Ca^{2+}] = 0, $\epsilon_{550} = 2.55 \times 10^4$ M^{-1} cm^{-1}. Best-fit parameters: $\Delta A_{720}\mathrm{max}/A_{550} = 0.322 \pm 0.005$ and $K = 3.69 \pm 0.18 \times 10^{-8}$ M^2. Standard error of fit = 0.018. $\Delta\epsilon_{720} = 2 \times \epsilon_{550} \times \Delta A_{720}\mathrm{max}/A_{550} = 1.64 \times 10^4$ M^{-1} cm^{-1}. (*B*) Same as (*A*), but for absorbance changes recorded at 550 nm. Best-fit parameters: $\Delta A_{550}\mathrm{max}/A_{550} = 0.358 \pm 0.007$. $K = 4.13 \pm 0.22 \times 10^{-8}$ M. Standard error of fit = 0.025. $\Delta\epsilon_{550} = 1.82 \times 10^3$ M^{-1} cm^{-1}. (*C*) Results at 720 nm fit with 2:2 stoichiometry. The raw data are the same as in (*A*) although their representation on the abscissa is slightly different because of the different correction for dye-bound calcium involved in fitting a different stoichiometry. The ordinate was normalized to the best-fit parameter $\Delta A_{720}\mathrm{max}/A_{550} = 0.209$. Standard error of the fit = 0.150. In all panels circles denote use of HEDTA as calcium buffer and squares denote use of NTA. Path lengths: 1 cm for measurements in 50 μM D_T, 0.2 cm for 187.5 μM D_T, 0.1 cm for 750 μM D_T. Reaction medium given in the text.

Fig. 1 presents examples of best fits for 1:2 Ca-dye stoichiometry at 720 and 550 nm (*A* and *B*) and 2:2 stoichiometry at 720 nm (*C*). The curves in Fig. 1 are sections of the best-fit surfaces at the three values of D_T used. The 1:2 stoichiometry gives a very good fit, whereas the 2:2 stoichiometry obviously cannot fit the data (*C*). The 1:1 and 2:1 stoichiometries gave even worse fits, which are not shown. The best-fit parameters for the 1:2 model are given in the figure legend. The estimates of the dissociation constant, 3.69×10^{-8} M from the data at 720 nm and 4.13×10^{-8} M at 550 nm are not significantly different, which is equivalent to

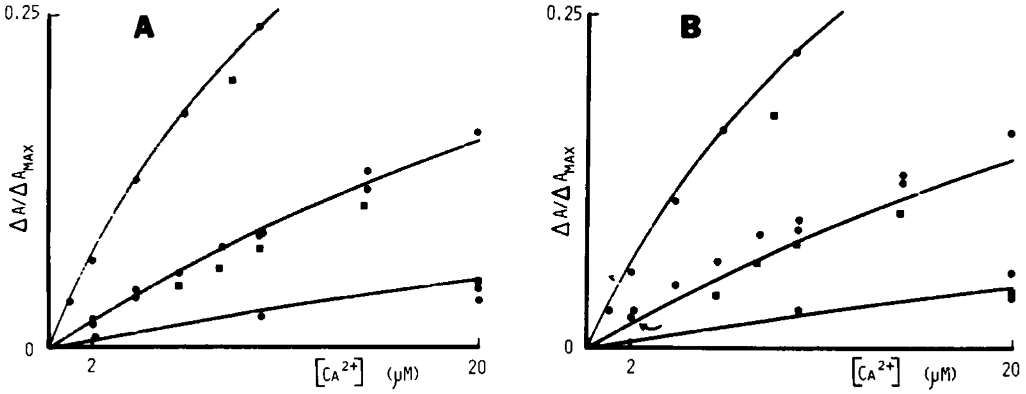

FIGURE 2 Ap III absorbance changes as a function of $[Ca^{2+}]$ and D_T fit with 1:2 stoichiometry. (*A*) Points and curves of Fig. 1 *A* for low values of $[Ca^{2+}]$ replotted on a linear $[Ca^{2+}]$ scale. (*B*) Points and curves of Fig. 1 *B* replotted on a linear $[Ca^{2+}]$ scale. The point marked by the arrow goes with the lowest curve in panel *B* (D_T = 50 μM). In both panels circles denote use of HEDTA as calcium buffer, and squares denote use of NTA.

fulfillment of criterion (*a*) stated above. These results are generally consistent with a previous report by Palade and Vergara (4).

Fig. 2 shows the same results and fits of 1:2 stoichiometry on a linear scale, for $\Delta A/A_{550}$ values up to 0.08 (the largest signals recorded in muscle fibers being ~0.07). This figure demonstrates that (*a*) the relationship between ΔA and $[Ca^{2+}]$ is approximately linear in the range of absorbance changes found in muscle fiber experiments, and (*b*) the slope is proportional to the dye concentration, a general property of the 1:2 stoichiometry that can be derived from Eq. (1).

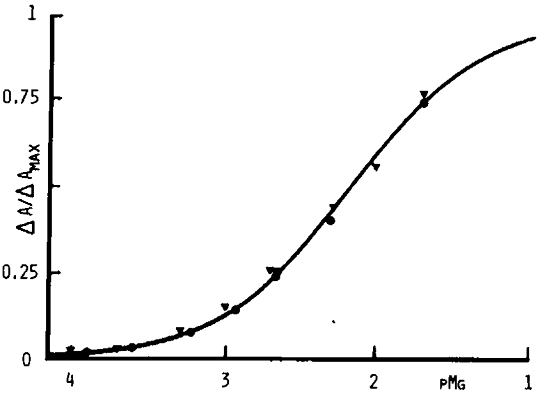

FIGURE 3 Ap III absorbance changes at 600 nm as a function of $[Mg^{2+}]$ and D_T fit with 1:1 stoichiometry. $[Mg^{2+}]$ values were calculated as explained in the text and are represented logarithmically on the abscissa. Circles, measurements at D_T = 750 μM; squares, at 75 μM. The function $F(Mg_T, D_T)$ for 1:1 stoichiometry was fit to all the points. The curve, the intersection of the best-fit surface *F* with the planes D_T = constant, is independent of D_T. Best-fit parameters: $\Delta A_{600}\text{max}/A_{600}$ = 0.831 ± 0.027. K_D = 6.702 ± 0.509 × 10^{-3} M. Standard error of fit = 0.019. Path lengths: 1 cm for 75 μM D_T and 0.1 cm for 750 μM D_T. ϵ_{600} = 2.03 × 10^4 M^{-1} cm^{-1}. $\Delta\epsilon_{600}$ = 1.69 × 10^4 M^{-1} cm^{-1}. Reaction medium given in the text.

The straightforward result obtained for the equilibrium properties of the Ca:Ap III reaction suggested that the kinetics of the reaction should also be simple, and that the results of Palade and Vergara (4) showing multiple phases might be attributed to the fact that they used very high [Ca^{2+}], with appreciable advancement of the reaction to further steps. To test this interpretation, absorbance records were obtained by Dr. John W. Stewart in a stopped-flow instrument (Aminco DW'2a spectrophotometer with Aminco-Morrow stopped-flow apparatus; American Instrument Co., Silver Springs, Md.). Upon mixing dye and calcium to a final D_T of 0.75 mM and a final [Ca^{2+}] that produced a $\Delta A_{720}/A_{550}$ of 0.04, a typical value for muscle fiber experiments, the entire absorbance change was completed during the mixing time of the instrument (3–6 ms) and thereafter remained constant for 75 s, the longest recording interval tested. The assumptions about linearity and time independence of the Ca:Ap III reaction (2) are thus seen to hold. The reaction takes <3–6 ms to be completed and

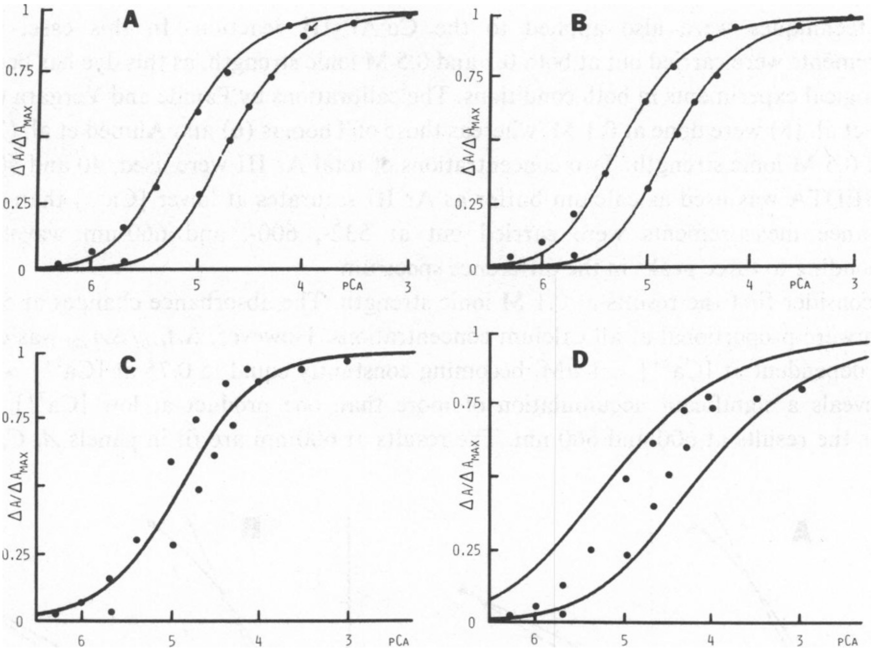

FIGURE 4 AR III absorbance changes as a function of [Ca^{2+}] and D_T fit with various stoichiometries. Ionic strength = 0.1 M. (*A*) Results at 600 nm, fit with 2:2 stoichiometry. The ordinate was normalized to the maximum change by dividing the measured values $\Delta A_{600}/A_{552}$ by the factor $\Delta A_{600}\mathrm{max}/A_{532}$ obtained from the best fit. Measurements at D_T = 40 μM and 400 μM. The curves are sections of the best fit of the function $F(C_T, D_T)$ for 2:2 stoichiometry by the planes at D_T = 400 μM (left) and 40 μM (right). The point marked by an arrow corresponds to 400 μM D_T. $\epsilon_{532} = 2.57 \times 10^4\ M^{-1}\ cm^{-1}$. Best fit parameters: $\Delta A_{600}\mathrm{max}/A_{532} = 0.890 \pm 0.008$; $K = 1.74 \times 10^{-14} \pm 0.11\ M^3$. Standard error of fit = 0.018. $\Delta\epsilon_{600} = 2 \times \epsilon_{532} \times \Delta A_{600}\mathrm{max}/A_{532} = 4.58 \times 10^4\ M^{-1}cm^{-1}$. (*B*) Same as (*A*) but at 600 nm. Best fit parameters: $\Delta A_{660}\mathrm{max}/A_{532} = 0.641 \pm 0.007$; $K = 1.29 \pm 0.09 \times 10^{-14}\ M^3$. Standard error of fit = 0.022. $\Delta\epsilon_{660} = 3.30 \times 10^4\ M^{-1}\ cm^{-1}$. Best-fit parameters at 532 nm (results not shown): $\Delta A_{532}\mathrm{max}/A_{532} = 0.438 \pm 0.004$; $K = 1.57 \times 10^{-14} \pm 0.09\ M^3$. Standard error of fit 0.018 $\Delta\epsilon_{532} = 2.25 \times 10^4\ M^{-1}\ cm^{-1}$. (*C*) Data of (*A*) fit with 1:1 stoichiometry. The curve, intersection of the best-fit surface *F* with the planes D_T = constant, is independent of D_T. Ordinate normalized to the best-fit parameter $\Delta A_{600}\mathrm{max}/A_{532} = 0.903$. Standard error of fit = 0.101. (*D*) Data of (*A*) fit with 1:2 stoichiometry. Ordinate normalized to best-fit parameter $\Delta A_{600}\mathrm{max}/A_{532} = 1.085$. Standard error of fit = 0.120.

should not contribute any spurious slow phase during the 50–500-ms time span of the muscle records.

Mg:Ap III

The equilibrium properties of the Mg:Ap III reaction were studied with the same techniques, in a medium containing 70 mM KCl, 20 mM Tris maleate, 0.1 mM EGTA to eliminate the contaminant Ca^{2+} and either 75 or 750 μM Ap III. The normalized absorbance changes at 600 nm were perfectly fit with the function F for 1:1 stoichiometry, as shown in Fig. 3. This figure includes two sections of the theoretical surface F by the planes $D_T = 75\ \mu M$ and $D_T = 750\ \mu M$, which in the 1:1 case are coincident. The dissociation constant obtained for the Mg:Ap III reaction was 6.7 mM. ΔA was also monitored at 510 nm, for $D_T = 75\ \mu M$, and criterion (*a*) was found to hold.

Ca:Ar III

These techniques were also applied to the Ca:Ar III reaction. In this case, sets of measurements were carried out at both 0.1 and 0.5 M ionic strength, as this dye has been used for biological experiments in both conditions. The calibrations by Palade and Vergara (4) and Ogawa et al. (8) were done at 0.1 M, whereas those of Thomas (6) and Ahmed et al. (7) were done at 0.5 M ionic strength. Two concentrations of total Ar III were used, 40 and 400 μM. Only HEDTA was used as calcium buffer as Ar III saturates at lower [Ca^{2+}] than Ap III. Absorbance measurements were carried out at 532-, 600-, and 660-nm wavelengths, corresponding to three peaks in the difference spectrum.

We consider first the results at 0.1 M ionic strength. The absorbance changes at 532 and 600 nm were proportional at all calcium concentrations. However, $\Delta A_{660}/\Delta A_{600}$ was concentration dependent at [Ca^{2+}] $< 1\ \mu M$, becoming constantly equal to 0.75 at [Ca^{2+}] $> 5\ \mu M$. This reveals a significant accumulation of more than one product at low [Ca^{2+}]. Fig. 4 presents the results at 600 and 660 nm. The results at 600 nm are fit in panels *A*, *C*, and *D*

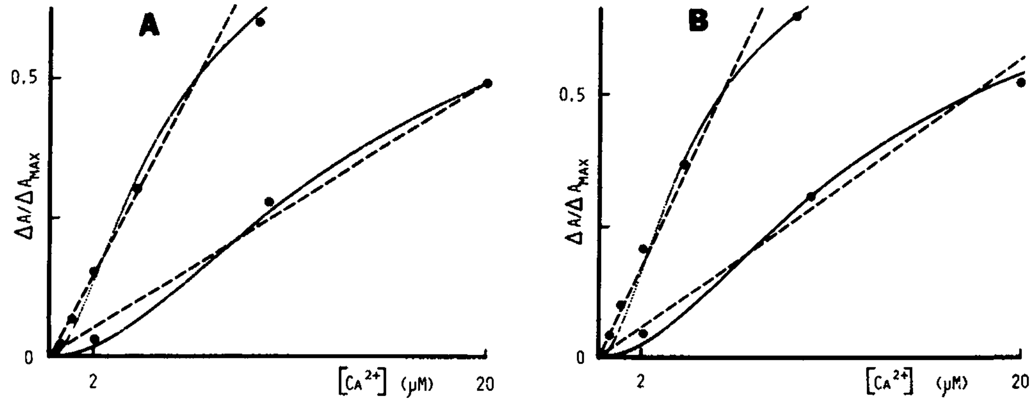

FIGURE 5 Ar III absorbance changes as a function of [Ca^{2+}] and D_T fit with 2:2 stoichiometry. Ionic strength 0.1 M. (*A*) Points and curves of Fig. 4 *A* for low values of [Ca^{2+}] replotted on a linear [Ca^{2+}] scale; 0.5 in the ordinate scale corresponds to half saturation, or a $\Delta A_{600}/A_{532} = 0.445$; the dashed lines are determined by the origin and the half-saturation point on the theoretical curves. The ratio of the slopes of the two dashed lines is $\sqrt{10}$. (*B*) Corresponding data and curves at 660 nm.

with the function F corresponding to 2:2, 1:1, and 1:2 stoichiometries, respectively. The curves are sections of the best-fit surfaces F by planes at $D_T = 400$ and 40 μM. The 2:2 stoichiometry provides a good description, whereas the other stoichiometries are totally inadequate, as was the 2:1 stoichiometry, which is not shown. Comparison of the three fits (*A*, *C*, and *D*) clearly demonstrates that consideration of the dependence of the reaction on both the concentration of calcium and dye is crucial in the evaluation of stoichiometry models. For each D_T considered separately the [Ca] dependence was not too far from a 1:1 or a 1:2 curve. The 1:1 stoichiometry, however, fails to account for the dye-concentration dependence of the affinity, and the 1:2 model exaggerates this dependence. Any of these models might have been approved by a separate fitting of the data at different D_T values.

Fig. 4*B* shows the 2:2 fit at 660 nm. The best-fit third order dissociation constant was consistently estimated to be $\sim 1.5 \times 10^{-14}$ M^3 at the three wavelengths explored. Individual values and error estimates are given in the figure legend.

As expected from the absorbance ratios, the fit is not very good at $[Ca^{2+}] < 5$ μM,

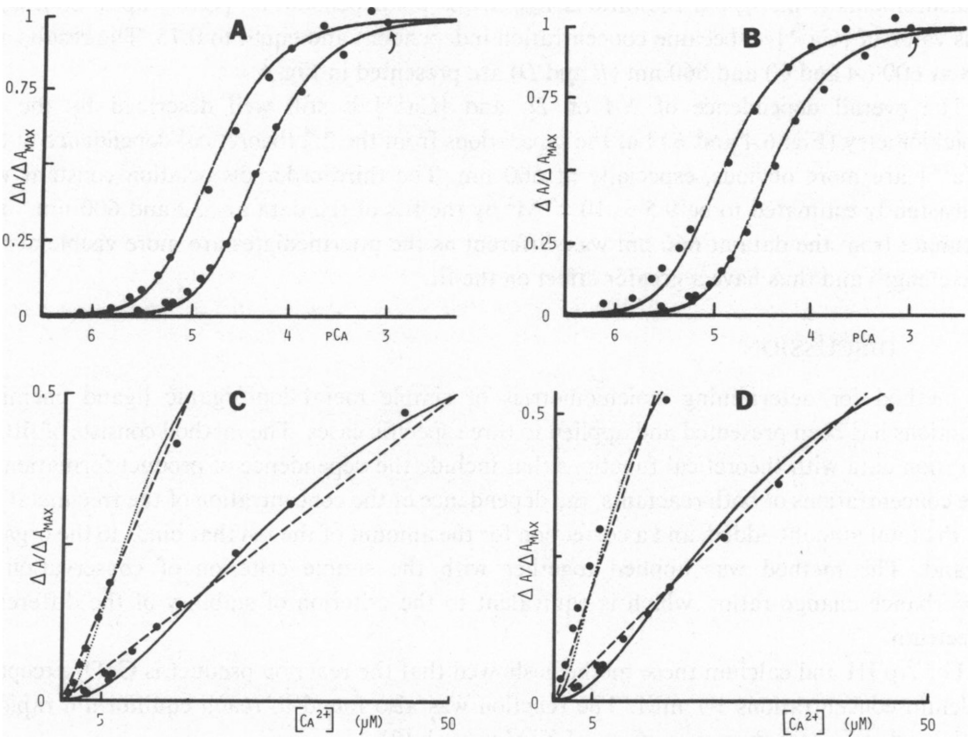

FIGURE 6 Ar III absorbance changes as a function of $[Ca^{2+}]$ and D_T fit with 2:2 stoichiometry. Ionic strength 0.5 M. (*A*) Results at 600 nm. The ordinate was normalized as in previous figures. Measurements at $D_T = 40$ μM and $D_T = 400$ μM. The curves are as in Fig. 4 *A* and B. The point marked by an arrow corresponds to the lower curve. Reference wavelength: 532 nm. $\epsilon_{532} = 2.36 \times 10^4$ M^{-1} cm^{-1}. Best-fit parameters: $A_{600}\max/A_{532} = 0.855 \pm 0.010$. $K = 1.03 \pm 0.006 \times 10^{-13}$ M^3. Standard error of fit = 0.033. $\Delta\epsilon_{660} = 2.97 \times 10^4$ M^{-1} cm^{-1}. (*C*) Points and curves of (*A*) for low $[Ca^{2+}]$ replotted on a linear scale. Dashed lines as in Fig. 5. (*D*) Same as (*C*) at 660 nm. Best-fit parameters at 532 nm (results not shown): $\Delta A_{532}\max/A_{532} = 0.416 \pm 0.005$. $K = 0.98 \pm 0.07 \times 10^{-13}$ M^3. Standard error of fit = 0.026. $\Delta\epsilon_{532} = 1.96 \times 10^4$ M^{-1} cm^{-1}. Path lengths as in Fig. 3.

particularly at 660 nm. The fact that this deviation occurs at low [Ca^{2+}] and is more noticeable at $D_T = 400$ μM suggests that another significant product in this range is CaD_2, as proposed by Palade and Vergara (4). The separation from the 2:2 stoichiometry that is observed at low [Ca^{2+}] tends to linearize the ΔA-vs.-[Ca^{2+}] relationship. It can be seen in the linear scale plots (Fig. 5) that the measured relationship has some upward concavity, as previously shown by Ogawa et al. (8), although less than predicted by the 2:2 theory (continuous curves). For [Ca^{2+}] up to the half-saturating value the equilibrium $\Delta A_\lambda/A_{ref}$-vs.-[Ca^{2+}] relationship for this dye could be rather closely approximated by straight lines passing through the origin and the half-saturation point of the curve. The 2:2 stoichiometry theory requires specifically that these straight lines have slopes proportional to the square root of D_T (see Appendix). This is at variance with 1:2 stoichiometry (slopes proportional to D_T, Fig. 2) and 1:1 stoichiometry (slopes independent of D_T), and is well followed by the experimental results.

At 0.5 M ionic strength the dye had less affinity for calcium than at 0.1 M ionic strength (9). The absorbance change ratio $\Delta A_{532}/\Delta A_{600}$ kept the same value as in the 0.1 M measurements (~0.50), but the ratio $\Delta A_{600}/\Delta A_{600}$ was dependent on [Ca^{2+}] up to 20 μM; at this value of [Ca^{2+}], it became concentration independent and equal to 0.75. The results and fits at 600 (*A* and *C*) and 660 nm (*B* and *D*) are presented in Fig. 6.

The overall dependence of ΔA on D_T and [Ca^{2+}] is still well described by the 2:2 stoichiometry (Fig. 6*A* and *B*) but the separations from the 2:2 theoretical dependence at low [Ca^{2+}] are more obvious, especially at 660 nm. The third-order dissociation constant was consistently estimated to be 9.5×10^{-14} M^3 by the fits of the data at 532 and 600 nm.[4] The estimate from the data at 660 nm was different as the intermediates are more visible at this wavelength and thus have a greater effect on the fit.

DISCUSSION

A method for determining stoichiometries of simple metal ion-organic ligand chemical reactions has been presented and applied to three specific cases. The method consists of fitting titration data with theoretical functions that include the dependence of product formation on the concentrations of both reactants, the dependence of the concentration of the free metal ion on the total amount added, and a correction for the amount of the ion that binds to the organic ligand. The method was applied together with the simple criterion of conservation of absorbance change ratios, which is equivalent to the criterion of stability of the difference spectrum.

For Ap III and calcium these methods showed that the reaction product is CaD_2 except at calcium concentrations >1 mM. The reaction was also found to reach equilibrium rapidly, which substantiates the assumptions of Kovács et al. (2).

The Mg:Ap III reaction, which was found to be 1:1, provided a test of our methods in another simple situation. It also illustrated the similarity of Ap III and Ar III (6) in terms of their reactivity with magnesium.

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[4] These estimate rely on a value of 9.46×10^{-6} M for K_{HEDTA} at 0.5 M ionic strength. This value was calculated by correcting the value at 0.1 M for the change in ionic strength with a mean activity coefficient at 0.5 M ionic strength calculated according to the Debye-Hückel theory (11).

The Ca:Ap III and Mg:Ap III reactions having been characterized, it is possible to derive equations for the equilibrium of the dye in the presence of both Ca^{2+} and Mg^{2+}, and to use these results as a calibration for measurements in muscle.

In the presence of calcium only, the equilibrium condition is

$$K = \frac{Ca(D_T - 2CaD_2)^2}{CaD_2}, \tag{9}$$

from which Eq. 1 can be derived by solving for CaD_2. When both Ca and Mg are present, the equations are

$$K = \frac{Ca \cdot D^2}{CaD_2}, \tag{10}$$

$$K_{Mg} = \frac{Mg \cdot D}{MgD}, \tag{11}$$

$$D_T = D + 2CaD_2 + MgD. \tag{12}$$

It is convenient to introduce

$$\alpha \equiv \frac{K_{Mg}}{Mg + K_{Mg}},$$

which is the magnesium-free fraction of the calcium-free dye. Then, from Eqs. 10, 11, and 12, we obtain

$$\frac{K}{\alpha^2} = \frac{Ca(D_T - 2CaD_2)^2}{CaD_2}. \tag{13}$$

Eq. 13 is analogous to Eq. 9, which demonstrates that the same equilibrium equations of 1:2 stoichiometry, Eqs. 9 and 1, hold when magnesium is present, with an apparent second-order dissociation constant K', which is related to the constant K for Mg-free solution by

$$K' = \frac{K}{\alpha^2} = K\left(\frac{Mg + K_{Mg}}{K_{Mg}}\right)^2. \tag{14}$$

With these results an appropriate calibration for the muscle fiber experiments may be constructed. Eq. 2 would be the calibration function, relating normalized absorbance changes to free-calcium concentration, but the apparent dissociation constant, $K' = 1.33 \times K$, should be used instead of K in the presence of 1 mM $[Mg^{2+}]$. This calibration function is a function of both $[Ca^{2+}]$ and D_T. For a $D_T = 0.750$ mM, which is a reasonable estimate of the value attained in cut muscle fibers after 2–3 h of exposure to a 1-mM Ap III-containing solution in the end compartments (2), the upper curve of Fig. 2 *A* would be the calibration curve. The change in apparent dissociation constant due to the free Mg present in the fibre (~1 mM) may be incorporated by multiplying the abscissa values of Fig. 1 *C* by 1.33. The ordinate units of saturation fraction in Fig. 7 *C* can be converted to units of $\Delta A_{720}/A_{550}$ with the normalization factor $\Delta A_{720}\text{max}/A_{550} = 0.322$, which corresponds to 100% saturation. After these considerations, the maximum values of $\Delta A_{720}/A_{550}$ reported for skeletal muscle fibers under voltage clamp (~0.07) (2) correspond to a $[Ca^{2+}]$ of ~10 μM and a dye-bound Ca of ~80 μM.

Scarpa et al. (1) give estimates of first order Ca:Ap III dissociation constants in the presence of various $[Mg^{2+}]$. In order to compare these with our results, the first-order approximation to 1:2 stoichiometry developed in the Appendix is used. Eq. A3 relates an apparent first order dissociation constant $K^{(1)}$ to the second order dissociation constant K of the reaction. It is possible to calculate an apparent first order dissociation constant in the presence of magnesium, $K^{(1)\prime}$, by using K' from Eq. 14 instead of K in Eq. A3:

$$K^{(1)\prime} = \frac{K}{2D_T}\left(\frac{Mg + K_{Mg}}{K_{Mg}}\right)^2 = K^{(1)}\left(\frac{Mg + K_{Mg}}{K_{Mg}}\right)^2. \tag{15}$$

Scarpa et al. (1) found ratios of 1.3, 1.6, and 2.6 for $K^{(1)\prime}/K^{(1)}$ in the presence of 1, 2.5, and 5 mM Mg respectively. Our K_{Mg} value together with Eq. 15 predicts values of 1.33, 1.92, and 3.13. Considering that the actual stoichiometry of the Ca:Ap III reaction is not 1:1, the agreement seems reasonable.

Our results with Ar III (40 or 400 μM) and calcium demonstrate a predominant 2:2 stoichiometry for $[Ca^{2+}]$ above 5 or 20 μM at 0.1 or 0.5 M ionic strength, respectively. At relatively lower levels of calcium, such as those encountered in biological measurements, we found evidence for accumulation of other products in addition to Ca_2D_2. At these low levels of calcium our measurements revealed an equilibrium $\Delta A/A_{ref}$-vs.-$[Ca^{2+}]$ relationship that had a small upward concavity but which still could be described approximately by a straight line. In both 0.1 m and 0.5 M ionic strength the slope of this straight line was proportional to the square root of D_T, a result which is close to the relationship predicted for 2:2 stoichiometry (see Appendix).

Our results must be compared with various recent studies of Ar III in calibration conditions. Our results with 0.1 M ionic strength are consistent with those of Vergara and Palade (4). These authors carried out a more complete analysis, interpreting their measured absorbance changes as being due to sequential formation of CaD_2 and Ca_2D_2. A third-order dissociation constant of 0.8×10^{-14} M^3 can be obtained from their best-fit affinity constants, in rough agreement with our estimate of 1.5×10^{-14} M^3 at 0.1 M ionic strength. Our results at 0.1 M ionic strength are also consistent with a report of Ogawa et al. (8), which shows upward concavities in ΔA-vs.-Ca curves. Ogawa et al. also found first order binding constants at various D_T that were proportional to $D_T^{1/2}$, as predicted by our first-order approach to 2:2 stoichiometry (Appendix). The observation that led these workers to assume a 1:1 stoichiometry is that ΔA in the presence of 1 mM Ca and various D_T is proportional to D_T. This would actually be true for any stoichiometry at micromolar D_T as all the dye would be bound to calcium.

Our results at 0.5 M ionic strength are not in conflict with those of Thomas (5, 6), who used 100 or 200 μM Ar III and submicromolar $[Ca^{2+}]$, and obtained a relationship between normalized ΔA_{660} and $[Ca^{2+}]$ that is clearly consistent with CaD_2 formation and inconsistent with Ca_2D_2 (Fig. 1 of reference 6). Although we have not explored such low $[Ca^{2+}]$ levels, we did observe obvious deviations from 2:2 stoichiometry at the lowest calcium concentrations we used. Simulations with a sequential reaction scheme of the type proposed by Palade and Vergara (4) in which CaD_2 is formed as a precursor of Ca_2D_2 indicate that the conditions of

Thomas's Fig. 1 (6) would result in almost exclusive formation of CaD_2, in agreement with his interpretation. However, at the higher $[Ca^{2+}]$ levels used in his Fig. 2 *a* (6), our results and analysis do not support the 1:2 stoichiometry but indicate a clear predominance of Ca_2D_2. In fact, the data for 200 μM dye and $[Ca^{2+}] > 3$ μM that Thomas presented in Fig. 2 *a* (6) provide little distinction between CaD_2 and Ca_2D_2 as predominant product. Finally, Thomas's double reciprocal plot in 0.2-μM dye (Fig. 2 *b* of reference 6) does provide clear evidence for a predominance of CaD at submicromolar dye. Since the lowest dye level we investigated was 40 μM, CaD was presumably negligible under our conditions. The overall conclusion from our results and those of Thomas is that either CaD_2 or Ca_2D_2 will predominate at Ar III levels above a few micromolar and that CaD is seen only at very low dye. Palade and Vergara (4) reached a similar conclusion regarding calcium and Ar III at 0.1 M ionic strength.

The preceding is in direct conflict with the conclusion of Ahmed et al. (7) that the Ca:Ar III stoichiometry is 1:1 for 100–1,000 μM D_T at 0.5 M ionic strength. Their observation that plots of normalized ΔA vs. $[Ca^{2+}]$ at 100, 200, and 1,000 μM total dye approximately coincided (Fig. 3 in Ref. 7) would be clearly inconsistent with a reaction higher than first order in dye. This observation, however, is in direct conflict with the results that Thomas presented in an identical format in his Fig. 1 (6), and also in conflict with our results. At the lowest calcium levels we investigated, which correspond to the upper part of the range of $[Ca^{2+}]$ in Fig. 3 of Ahmed et al. (7), we observed a definite dependence of normalized absorbance on dye concentration. Both random measurement errors and a systematic tendency to overestimate the higher values of $[Ca^{2+}]$ owing to lack of correction for calcium removal by binding to dye, which would be most important at high dye, may have obscured part of the dye dependence of normalized absorbance in the results of Ahmed et al. (7).

A pertinent final conclusion from this survey of published reports is that the analyses of stoichiometry based on measurements over restricted ranges of concentrations of both reactants may lead to oversimplification and yield contradictory results. Our present method does not have this drawback but is still an oversimplification, as it assumes a single product and does not take intermediate compounds into account.

APPENDIX

First-order Approximations to 1:2 and 2:2 stoichiometry

Let Y represent the saturation fraction of the dye, i.e., CaD/D_T for 1:1 stoichiometry, $2CaD_2/D_T$ for 1:2 or $2Ca_2D_2/D_T$ for 2:2. Then the dissociation constant for 1:1 stoichiometry satisfies

$$K = \lim_{Ca \to 0} \left(\frac{dY}{dCa}\right)^{-1}. \tag{A1}$$

Eq. A1 provides the starting point for a first-order aproximation to 1:2 stoichiometry. An apparent first-order dissociation constant for a 1:2 reaction may be defined as

$$K^{(1)} \equiv \lim_{Ca \to 0} \left(\frac{dY}{dCa}\right)^{-1}. \tag{A2}$$

Text Eq. 1 divided by D_T is the relationship between Y and Ca from which the limit in Eq. A2 can be calculated, giving

$$K^{(1)} = K/2D_T. \tag{A3}$$

At low Ca the 1:2 stoichiometry is thus equivalent to 1:1 binding with an affinity constant proportional to the concentration of dye.

A similar approach is not applicable in principle to the 2:2 stoichiometry, as the initial slope $\mathrm{d}Y/\mathrm{d}Ca$ is zero in this case. The theoretical curve at concentrations below half saturation, however, is very close to a straight line through the origin and the half-saturation point on the curve. For 2:2 stoichiometry

$$K = \frac{Ca^2 \cdot D^2}{Ca_2D_2}. \tag{A4}$$

At half saturation $D = 2Ca_2D_2 = D_T/2$ and $Ca = (K/D_T)$.$^{1/2}$ A straight line through the origin and the point $Ca = (K/D_T)$,$^{1/2}$ $Y = 1/2$, has a slope

$$\frac{\Delta Y}{\Delta Ca} = \left(\frac{D_T}{4K}\right)^{1/2}. \tag{A5}$$

The data points for Arsenazo, Figs. 5 and 6 *C* and *D* fall above the theoretical curve for 2:2 stoichiometry and closer to such a straight line, which thus constitutes a good semiempirical description of the results:

$$\frac{\mathrm{d}A}{\mathrm{d}Ca} = \Delta A\mathrm{max}\left(\frac{D_T}{4K}\right)^{1/2} = \Delta\epsilon \cdot \frac{D_T{}^{3/2}}{4K^{1/2}}. \tag{A6}$$

The authors wish to express their gratitude to Dr. John W. Stewart, Department of Radiation Biology and Biophysics, University of Rochester, for performing the stopped-flow measurements and Drs. Philip Palade and Julio Vergara for providing a preprint of the paper cited as reference 4.

This work was supported by grants from the U.S. Public Health Service (NS 13842) and the Muscular Dystrophy Association.

Received for publication 1 April 1981 and in revised form 11 June 1981.

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