STOICHIOMETRIES OF ARSENAZO III-Ca COMPLEXES

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ABSTRACT The equilibrium interactions of the metallochromic indicator arsenazo III with calcium at physiological ionic strength and pH were investigated spectrophotometrically and with the aid of a calcium electrode. Evidence suggests the formation of more than one dye-calcium complex. The analysis of data obtained over a 10,000-fold range of dye concentrations concludes that at the concentrations used for in vitro biochemical studies (10–100 μM), arsenazo III absorbance changes in response to calcium binding primarily involve the formation of a complex involving two dye molecules and two calcium ions. At millimolar dye concentrations, typical of physiological calcium transient determinations in situ, a second complex involving two arsenazo III molecules and one calcium ion is additionally formed. A third complex, involving one arsenazo III molecule and one calcium ion, is formed at very low dye concentrations. The results reported here suggest that equilibrium calibrations of the dye with calcium cannot be used directly to satisfactorily relate transient absorbance changes in physiological preparations to calcium concentration changes since several stoichiometrically distinct complexes with different absorbances could be formed at different rates. The results of this study do not permit the elucidation of a unique kinetic scheme of arsenazo III complexation with calcium; for this, in vitro kinetic analysis is required. Results of similar analysis of the dye interaction with magnesium are also reported, and these appear compatible with a much simpler model of complexation.

INTRODUCTION

Metallochromic indicators such as arsenazo III are well-known analytical tools for the quantitation of divalent metal cation concentrations in solution. While these compounds have been known to form several stoichiometrically distinct complexes with different divalent cations depending in part on pH (Budesinsky, 1967), such chemical anomalies can often be circumvented by the analytical chemist who can perform determinations under equilibrium conditions and alter the pH to suit the determination in question. Biochemists who wish to employ such indicators to detect relatively slow changes in calcium concentration (Scarpa, 1979) operate under a slightly greater handicap since the pH range over which the determinations must be performed is defined by the biological material being assayed. Provided that the changes in calcium concentration are slow enough to approach a quasi-equilibrium condition with respect to rapid dye-calcium interaction, even a dye that forms several different complexes with calcium may be useful if an adequate calibration curve is developed over the narrow range of dye and calcium (free or total) concentrations of interest. In such cases determination of dissociation constant(s) and stoichiometries need not necessarily be performed, but it is incumbent upon each investigator to carefully internally calibrate his own measurements.

However, a far more serious situation with dyes that form several different complexes with a metal ion arises when the time course of calcium concentration changes approaches that of the kinetics of dye-calcium interaction. Under such conditions the dye can no longer be assumed to be at equilibrium with calcium throughout the measuring interval. The operational limitations of certain probes include the fact that fast transient events simply may not be satisfactorily calibrated by equilibrium methods alone. This paper addresses the question of whether arsenazo III interacts with calcium forming several complexes of multiple stoichiometries at dye concentrations and pH approximating those encountered in physiological myoplasmic calcium transient determinations (Miledi et al., 1977*a, b, c,* 1979, 1980, 1981; Baylor, et al., 1979*a,b,* 1982; Palade, 1979; Palade and Vergara, 1981, 1982*a;* Rakowski and Best, 1982). In confirmation of some previous reports (Thomas, 1979; Ogawa et al., 1980; Palade and Vergara, 1981; Dorogi and Neumann, 1981*b*) our results provide clear indications of multiple complex formation. They conflict, however, with numerous reports suggesting that only a single complex is formed between arsenazo III and calcium (Ohnishi, 1979; Chiu and Haynes, 1980; Ahmed et al., 1980; Bauer, 1981; Brown and Rydqvist, 1981).

We analyze in this paper a number of specific chemical schemes involving stoichiometrically distinct complexes and provide best-fit parameters for each of them by fitting the experimental data using nonlinear least-square numerical methods. Formation of three well-identified complexes is required to account for the experimental data. A prelimi-

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nary report of these findings was presented (Palade and Vergara, 1982*b*).

MATERIALS AND METHODS

Spectrophotometric absorbance measurements were performed on a Hitachi (model 100-60; Hitachi Ltd., Tokyo, Japan) and a Hewlett-Packard (model 8450A; Hewlett-Packard Co., Palo Alto, CA) spectrophotometers. Difference spectra for the figures were generated by subtracting the absorbance spectra recorded from divalent ion-containing dye solutions from those of the same solutions in the absence of added divalent cations. Cuvettes of 10 cm, 4 cm, 1 cm, 1 mm and 0.1 mm were employed to examine a wide range of dye concentrations. Spectrophotometric determinations were performed by adding small aliquots of concentrated $CaCl_2$ (in some experiments $MgCl_2$) to a bulk dye-containing solution and transferring samples of this solution into cuvettes. At least 2 min were allowed to elapse between an addition and a subsequent absorbance measurement. All measurements were performed at room temperature (20–22°C).

The solutions were made with double deionized water exhibiting >15 MΩ resistance. The chemicals used were specially ordered to minimize contamination: Ultrex KCl (J. T. Baker Chemical Co., Phillipsburg, NI), ultrapure KOH (Alfa Products, Thiokol/Ventron Div., Danvers, MA), Ultrol MOPS (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA), ultrapure $CaCl_2$ (Alfa Products, Thiokol/Ventron Div.), and >98% arsenazo III (Sigma Chemical Co., St. Louis, MO), Na salt. $MgCl_2$ was obtained in concentrated solution from Sigma Chemical Co. Calcium chloride stock solution was calibrated by atomic absorption. Absorbance measurements were performed over a 10,000-fold range of dye concentrations (0.57 μM to 5.75 mM) titrated with calcium (or Mg) in the presence of 100 mM KCl and 20 mM KMOPS, adjusting the pH to 6.88. The pH was adjusted using concentrated KOH or HCl after each divalent ion addition and prior to spectrophotometric determinations. Absorbance values at 532, 600, and 660 nm (532, 620, and 660 nm for magnesium)[1] obtained for each of the divalent ion additions at every dye concentration (differing from each other by approximately a tenfold difference in dye concentration), were stored digitally in a data file for numerical analysis.

The spectrophotometric absorbance data were corrected for the ≤2% volume changes due to divalent ion additions and for calcium contamination of the stock salt solution (~1 μM) and the calcium content of the dye samples as reported by the manufacturer (Sigma Chemical Co.). Calcium electrode determinations were performed at certain dye concentrations with calcium electrode (model 932000; Orion Research Inc., Cambridge, MA) under identical conditions to those described for the absorbance measurements. At least 5 min were allowed to elapse after the calcium additions before reading the electrode potential with a digital electrometer (Select Ion 2000 ion analyser; Beckmann Instruments, Inc., Fullerton, CA). The electrode was calibrated with buffered calcium solutions using EGTA, EDTA, and HEDTA as calcium chelators (Bers, 1982). These calibrations demonstrated that the particular electrode used in these experiments responded in a fully Nerstian manner down to at least 0.3-μM free ionized calcium (D. M. Bers and J. Vergara, unpublished results). Scatchard plots of the calcium electrode titrations in the presence of dye as a calcium chelator were used to estimate the precise concentration of ligand in those solutions (see Fig. 2 and text below).

Analysis

Computer analysis involved simultaneous fittings of the spectral data, at three wavelengths and five dye concentrations, to mathematical models of the equilibrium reactions. An example of the detailed mathematical analysis of one of the models investigated is included here for clarification. This specific example involves the sequential formation of three complexes: AC, A_2C, and A_2C_2. Formation of less than three complexes can be modeled as a special case of this general scheme by fixing certain equilibrium constants to values that would yield negligible amounts of some of the complexes.

The general nomenclature for this scheme is as follows

$$A + C \underset{K_1}{\longleftrightarrow} AC \overset{A}{\underset{K_2}{\longleftrightarrow}} A_2C \overset{C}{\underset{K_3}{\longleftrightarrow}} A_2C_2,$$

in which A represents free dye; C, free calcium; AC, a 1:1 dye-calcium complex; A_2C, a 2:1 dye-calcium complex; A_2C_2, a 2:2 dye-calcium complex; K_1, K_2, and K_3, the equilibrium association constants for the partial reactions. The equilibrium equations derived for this scheme are $K_1 = [AC]/[A][C]$, $K_2 = [A_2C]/[AC][A]$, $K_3 = [A_2C_2]/[A_2C][C]$, and the conservation equations, $[A_T] = [A] + [AC] + 2[A_2C] + 2[A_2C_2]$ and $[C_T] = [C] + [AC] + [A_2C] + 2[A_2C_2]$, in which $[A_T]$ represents the total dye concentration and $[C_T]$ the total calcium concentration.

These general equations cannot be solved in an exact form for any of the complexes, but excellent approximated solutions can be obtained by numerical methods. We have used two independent numerical methods to solve them, generally employing the second to conserve computational time: (*a*) the system of five nonlinear equations with five unknowns (the concentrations of the five different species) was solved using Brown's algorithm (Brown, 1969) based on Gaussian elimination. (*b*) The equations were reduced to a fifth-order polynomial equation in one of the unknowns (e.g., A), yielding for the particular case of the sequential model

$$\begin{aligned} K_1^2K_2^2A^5 &+ [(2C_T - A_T)K_1^2K_2^2 + K_1^2K_2 - 2K_1K_2K_3]A^4 \\ &- [(A_T - C_T)K_1^2K_2 - 4(A_T - C_T)K_1K_2K_3]A^3 \\ &- [K_1 + 2K_1K_2K_3(A_T - C_T)^2 + 2C_TK_1K_2]A^2 \\ &+ [(A_T - C_T)K_1 - 1]A + A_T = 0. \end{aligned}$$

This equation can be used to generate the free dye concentration for a fixed total dye and total calcium concentration and any choice of association constants. The concentrations of the other species are then obtained by substitution of the determined free dye concentration into the appropriate equations above. This polynomial equation was solved numerically with an iterative Newton-Raphson algorithm (IBM/360 Scientific Subroutine Package, 360A-CM-03X; IBM Instruments, Inc., IBM Corp., Danbury, CT) for each particular set of parameters (association constants). To compare spectrophotometrical data with model predictions, nine additional variables were introduced: the extinction coefficients at each of three wavelengths for each of the three calcium-dye complexes. The free dye extinction coefficients at these wavelengths were experimentally determined. The absorbances, at every wavelength, were computed for each set of solutions of the previously described equations, multiplying the extinction coefficients by the concentrations of the respective species.

For the purposes described in this paper these equations and subroutines were incorporated into a fitting program that enabled the computer to vary parameters and compare predicted absorbances with experimental determinations. The values of the association constants and extinction coefficients were obtained from a nonlinear least-squares fit of the experimental spectrophotometric determinations. The fitting process was performed minimizing a residual equation in which the absorbances obtained experimentally were compared with those predicted theoretically by the model. The least-squares fit program used for that purpose was based on the Levenberg-Marquardt algorithm (Levenberg, 1944; Brown and Dennis, 1972). This program varied the three association constants of the model equations and the extinction coefficients included in the residual function until a convergence to a minimum was obtained. This process was simultaneously applied to the absorbance data obtained

[1]These wavelengths were chosen to correspond to those formerly employed in physiological experiments involving muscle fibers (Palade, 1979; Palade and Vergara, 1981, 1982*a*).

at the three wavelengths selected (e.g., 660 nm, 600 nm, and 532 nm) and the five dye concentrations used. The analysis was performed using a Nova 3/12 minicomputer (Data General Corp., Westboro, MA) with 64 K words of memory and a floating-point processor. The convergence to a set of fitted parameters typically required ~20 min in this system and was accompanied by statistical measures of dispersion, from which we have selected the sum of the square deviations (SSQ) and the percent misfit of the theoretical prediction to data (R-factor; see Hamilton, 1964, and Clausen et al., 1979).

Concentrations of individual complexes and/or absorbances, predicted by a given model at each total dye concentration, were calculated as a function of the total calcium concentration using the best-fit values obtained for the equilibrium association constants and extinction coefficients. The curves generated in this manner were plotted together with the experimental data points in some of the figures presented below.

RESULTS

Calcium-Arsenazo III Interaction

The difference spectrum exhibited by a dye that binds a divalent cation to form one unique dye-calcium complex should be evidenced by isosbestic points and a characteristic shape change that remain constant as the metal ion concentration is raised. Deviations from this behavior suggest the formation of multiple complexes with different spectral characteristics. A titration of a relatively low concentration (57 μM) of arsenazo III with increasing amounts of calcium is shown in Fig. 1 *A*. The isosbestic points remain sharp throughout the titration and the shape changes remain constant. At this dye concentration, typical of those used for in vitro biochemical studies, the difference spectrum shows no clear evidence of multiple complex formation. In contrast, Fig. 1 *B* shows a titration of an approximately 100-fold higher dye concentration (5.75 mM) with calcium. This concentration is only slightly higher than those used in some experiments involving injection of the dye into physiological preparations (Palade and Vergara, 1982*a*). Note that throughout part of the titration the absorbance increase in the 660-nm region is larger than that in the 600-nm region, although at higher calcium concentrations the absorbance difference at 600 nm becomes larger. Intermediate dye concentrations show this effect less clearly. This result suggests that more than one dye-calcium complex is formed at high arsenazo III concentrations.

Initial attempts to determine the stoichiometric composition of the predominant arsenazo III-calcium complex were made using the method of continuous variation (Job, 1928). This is a standard chemical procedure for determining the ratio between the number of ligands and the number of metal ions in a complex under study. The procedure depends on the principle that proportionally more of the complex will be formed at the correct stoichiometric ratio of ligand to metal than at any other ratio, provided that the sum of the total ligand and metal concentrations is held constant. In our case, with a constant sum of 0.8 mM total [arsenazo III] plus total [calcium], the difference spectra maxima were largest at a ratio of

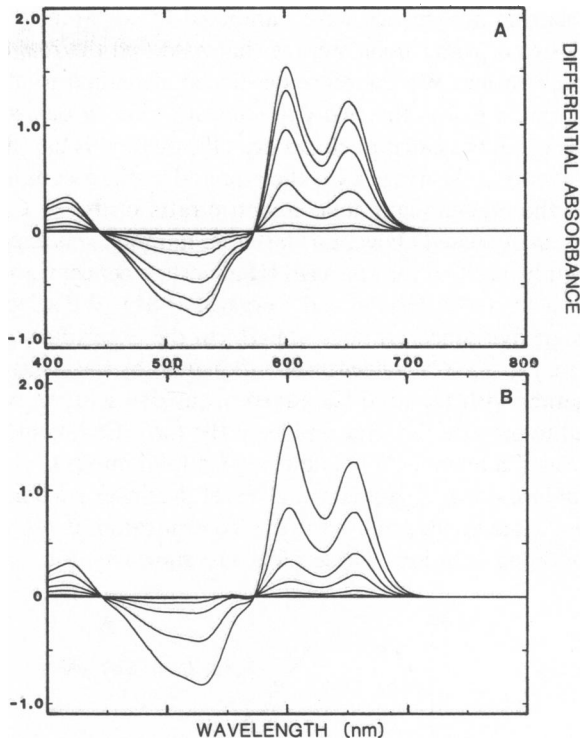


FIGURE 1 Spectrophotometric evidence suggestive of multiple complex formation between arsenazo III and calcium. (*A*) Titration of 57.4 μM arsenazo III in a 1-cm pathlength cuvette in 100 mM KCl, 20 mM KMOPS, pH 6.88 with the following values of added total calcium: 2.0, 10.0, 20.0, 50.0, 100.0, and 2,000 μM. Results are displayed as a difference spectrum, by subtracting the absorbance of the free dye from the spectra of those samples to which calcium had been added. (*B*) Titration of 5.76 mM arsenazo III in a 0.1-mm pathlength cuvette in 100 mM KCl, 20 mM KMOPS with the following values of added total calcium: 0.05, 0.2, 0.75, 1.5, 3.0, and 10.0 mM. Results are displayed as for Fig. 1 *A*.

total dye/total calcium = 1 (i.e., 400 μM of each). This result (not shown) confirms that recently reported by Ohnishi (1979). However, the method of continuous variation cannot discriminate simply between a complex involving one ligand and one metal ion and a complex involving two ligands and two metal ions. Furthermore, the use of the method of continuous variation can be misleading when more than one complex may be formed (Jones, 1964). Therefore claims that such results demonstrate the formation of a one dye-one calcium complex (Ahmed et al. 1980; Brown and Rydqvist, 1981) must be considered premature. More sophisticated data analysis using numerical methods was deemed necessary.

To minimize the number of parameters fitted by computer analysis, the total dye concentrations and the free dye extinction coefficients were fixed rather than varied. Unfortunately, the range of value for these extinction coefficients determined experimentally in the literature (Kendrick et al., 1977; Ohnishi, 1979; Ahmed et al., 1980) for arsenazo III is very wide, most performed in solutions distinctly different from those utilized here. Our own attempts to measure these values by weighing out dye and

measuring absorbance were hampered by apparent gains or losses of water upon storage that modified the real dye concentration. We therefore developed a method to more accurately assess the real dye concentration in our solutions using the calcium electrode. This method is based on the fact that the dye is a Ca chelator and on the assumption that the predominant stoichiometric ratio of dye to Ca is 1:1. As discussed above, this latter assumption was experimentally verified for arsenazo III at certain concentrations (Ohnishi, 1979; Palade and Vergara, 1981). We selected similar dye concentrations to perform the experiments. In these, the free Ca concentration measured by the electrode together with the total Ca added to the dye solution were used to prepare Scatchard plots of the form Ca bound/Ca free vs. Ca bound; Ca bound being Ca total minus Ca free. Standard linear-regression analysis of the linear portions of these plots yielded the total dye concentraton of the dye containing solution. These plots are shown in Fig. 2, in

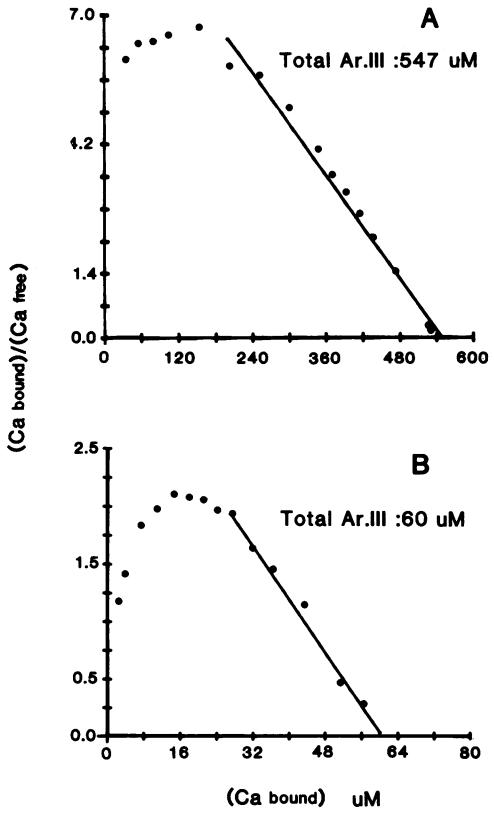


FIGURE 2 Determination of the concentration of arsenazo III in solution. (*A*) Scatchard plot analysis of a titration of a solution prepared to be ~400 μM dye with increasing calcium. Readings from a calcium electrode were used to determine free calcium concentrations. Bound calcium was calculated as the difference between calcium added and free calcium. (*B*) A similar titration with one-tenth this dye concentration. The discrepancy between the two determinations was deemed acceptable relative to the 50% discrepancy with respect to calculations based on weight. We assumed an average 575 μM value to be correct for the higher dye concentration and assigned the lower dye concentration a value of 57.4 μM. All determinations performed as in Materials and Methods section. The solid lines represent the least-square fits over the range of (Ca bound) shown. *Ar. III*, arsenazo III.

which real dye concentrations of 547 and 60 μM were calculated from dye solutions nominally thought to be 400 and 40 μM, respectively, by weight. Since the solution with lower dye concentration was a precise dilution of the more concentrated solution, we estimated the correct dye concentrations by averaging the two determinations. These corrected values (575 and 57.4 μM)[2] were used to determine extinction coefficients for the free dye at the wavelengths spectrophotometric data had been recorded in the absence of added divalent cations.

Modeling of Spectrophotometric Data

In the following sections we will consider the possible formation of the following complexes: a complex involving two dye molecules and two calcium ions (A_2C_2) (Palade and Vergara, 1981; Rios and Schneider, 1981); the simplest one-dye, one-calcium complex (AC); a complex involving two dye molecules and one calcium ion (A_2C) (Thomas, 1979; Ogawa et al., 1980); and finally an AC_2 complex (Dorogi and Neumann, 1981*b*) in which one dye molecule binds to two calcium ions. We will present the results of our analysis starting with the simplest combinations of these complexes and progressively introducing more elaborate schemes.

One-Complex Schemes

In the case of simple models involving formation of only one complex, the variables fitted included three extinction coefficients for the calcium-dye complex and one equilibrium constant. The results of these fittings are shown in Figs. 3 and 4. In reviewing these and similar subsequent figures, note that a perfect fit would have the dotted lines passing through the open circles, the dotted-and-dashed lines passing through the half-filled circles, dashed lines through filled circles, thin solid lines through small x's, and heavy solid lines through large X's. In all cases symbols represent data points and lines (curves) predictions of the model.

The fittings involving AC formation only (Fig. 3, upper row) fail most at the lowest dye concentrations (dotted lines, open circles, and dotted-and-dashed lines, half-filled circles) particularly because they cannot reproduce the shift to the left between the open and half-filled circles. Nor can this model accurately predict the steepness of the titration represented by the solid circles at an intermediate dye concentration (57.4-μM dye; dashed lines).

The fittings involving A_2C formation alone (Fig. 3, lower row) clearly fail to predict the experimental data in several ways. Note, however, that the shift to the left

[2]The corrected dye concentration is higher than the assumed one because we prepared solutions assuming the water content indicated by Sigma Chemical Co. Loss of water because of the Sigma determination would result in a higher dye concentration.

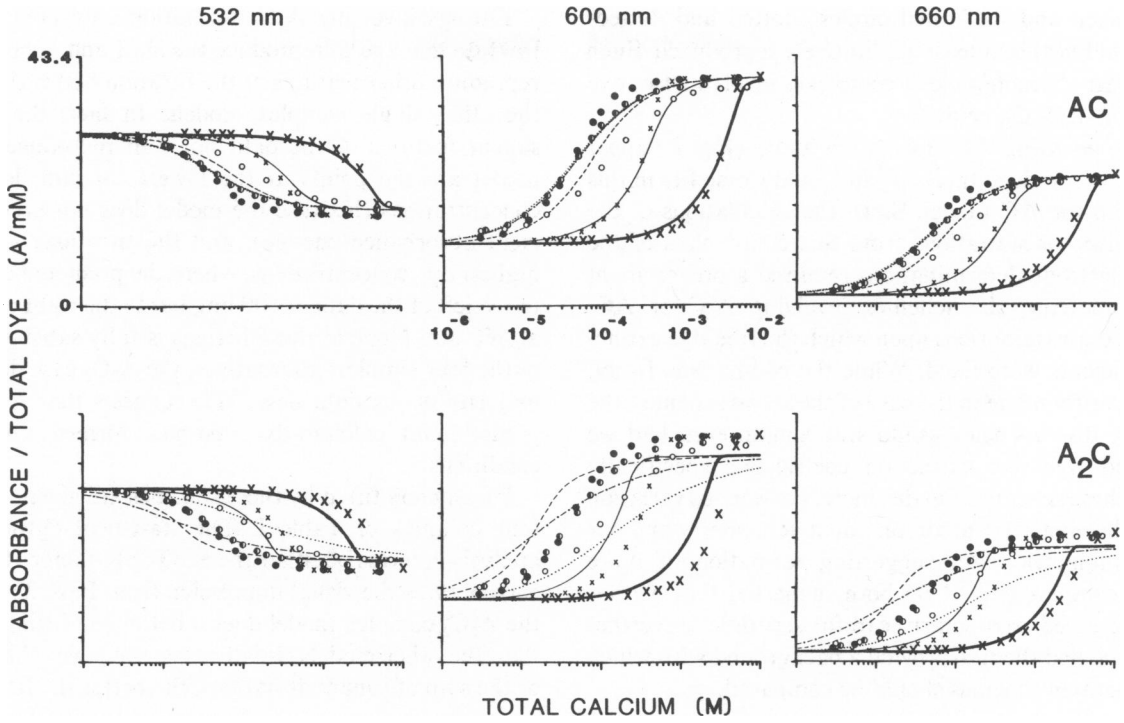


FIGURE 3 Comparison between experimental data and predicted absorbance changes accompanying arsenazo III-calcium complexation according to two simple one-complex models. The data points (O, x, X) and predicted curves (· · ·, -·-, ---, —, —)) shown are for determinations with 0.563 μM dye (O, · · ·), 5.76 μM dye (O, -·-), 57.4 μM dye (O, ---), 575 μm dye (x, —), and 5.76 mM dye (X, —). See Materials and Methods section for details. *Upper row:* Absorbance data at 532, 600, and 660 nm, respectively, and predictions based on a model involving formation of AC alone. *Lower row:* data and predictions based on a model involving formation of A_2C alone.

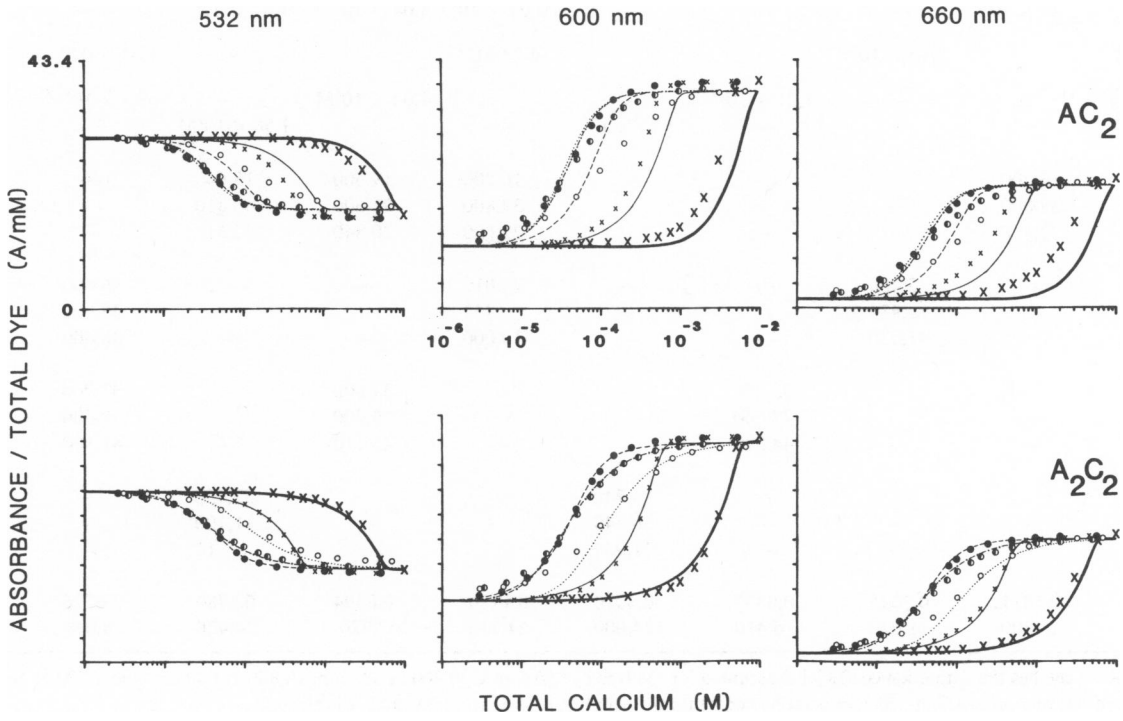


FIGURE 4 Comparison between experimental data and predicted absorbance changes accompanying arsenazo III-calcium complexation according to two other one-complex models. Experimental details and symbols as for Fig. 3. *Upper row:* data and predictions based on a model involving formation of AC_2 alone. *Lower row:* data and predictions based on a model involving formation of A_2C_2 alone.

between open and half-filled circles (dotted and dotted-and-dashed lines) is at least qualitatively reproduced. Such shifts appear characteristic of complexes involving two dye molecules (see A_2C_2, below).

Fittings involving AC_2 formation alone (Fig. 4, upper row) fail to reproduce this shift and yield worse fits in this regard than the AC model. Since the calculations of the effective dye concentrations from Scatchard plots of the calcium electrode determinations required a predominant 1:1 stoichiometry, the schemes involving A_2C or AC_2 invalidate our assumptions upon which the free-dye extinction coefficients were fixed. While the comparison is not, therefore, fairly made in the case of these two schemes, the qualitative discrepancies would still remain even had we determined free dye extinction coefficients specifically based on these schemes. Furthermore, continuous variation analysis showing a 1:1 predominant stoichiometry and our spectrophotometric data suggesting formation of more than one complex (Fig. 1 *B*) both suggested that neither scheme deserved particularly careful scrutiny. Nevertheless, we included them to provide a background with which more elaborated schemes should be compared.

Fittings involving A_2C_2 formation only (Fig. 4, lower row) do manage to reproduce the shift and appear able to reproduce other features of the titration better than any of the other single complex models. In fact, the only two salient features of the data not well reproduced by this model are the points at the lowest calcium, lowest dye concentrations (because the model does not predict sufficient absorbance change), and the titrations at the two highest dye concentrations, where the predicted curves fall to the left of the data at 600 nm, but to the right of the data at 660 nm. None of these fittings is fully satisfactory, but of the four simplest alternatives, the A_2C_2 case does fit the majority of the data best. This suggests that A_2C_2 is the predominant calcium-dye complex formed under most conditions.

Parameters fitted by these schemes are given in the first four columns of Table I. The statistical output of this analysis in terms of either the SSQ or R-factor (R) clearly corroborates the visual impression from Figs. 3 and 4 that the A_2C_2 complex model does a better job fitting the data than the other models. In all cases, the lower the R-factor or the sum of square deviations, the better the fit.

TABLE I
PARAMETERS DEDUCED FROM LEAST-SQUARES FITTINGS OF DATA OF ARSENAZO III COMPLEXATION OF CALCIUM WITH ONE- AND TWO-COMPLEX MODELS*

	Complexes considered								
	AC	A_2C	A_2C_2	AC_2	AC A_2C	AC A_2C_2	AC AC_2	A_2C A_2C_2	A_2C AC_2
$K_{AC}(M^{-1})$	2.24×10^4	—	—	—	2.27×10^4	8.04×10^3	2.02×10^4	—	—
$K_{A_2C}(M^{-2})$‡	—	9.95×10^9	—	—	$6.83\ M^{-1}$	—	—	1.41×10^9	3.27×10^9
$K_{A_2C_2}(M^{-3})$§‖	—	—	1.21×10^{14}	—	—	$1.02 \times 10^6 M^{-1}$	—	$9.1 \times 10^4 M^{-1}$	—
$K_{AC_2}(M^{-2})$¶	—	—	—	8.74×10^8	—	—	$1.56 \times 10^1 M^{-1}$	—	1.96×10^6
AC									
ϵ_{532}	16,200	—	—	—	16,200	19,400	15,640	—	—
ϵ_{600}	39,800	—	—	—	39,800	36,500	40,810	—	—
ϵ_{660}	22,840	—	—	—	22,600	20,840	23,610	—	—
A_2C									
ϵ_{532}	—	35,400	—	—	73,010	—	—	46,600	39,600
ϵ_{600}	—	72,930	—	—	952	—	—	47,900	62,800
ϵ_{660}	—	41,030	—	—	310,000	—	—	28,600	35,200
A_2C_2									
ϵ_{532}	—	—	33,200	—	—	31,600	—	32,900	—
ϵ_{600}	—	—	77,640	—	—	79,200	—	78,600	—
ϵ_{660}	—	—	44,230	—	—	45,110	—	41,100	—
AC_2									
ϵ_{532}	—	—	—	16,740	—	—	32,430	—	16,940
ϵ_{600}	—	—	—	38,740	—	—	11,100	—	38,600
ϵ_{660}	—	—	—	21,900	—	—	11	—	21,700
Statistics									
R	0.1811	0.3035	0.0837	0.4010	0.1776	0.0594	0.1780	0.0726	0.2022
SSQ	25,300	71,020	5,410	124,000	24,310	2,720	24,420	4,061	31,540

*With all these schemes the extinction coefficients assumed for the free dye (A) were 30,200 at 532 nm, 10,800 at 600 nm, and 1,520 at 660 nm.
‡In the case of AC and A_2C, a first-order association constant for formation of A_2C from AC and A is given.
§In the case of AC and A_2C_2, a first-order association constant is given for dimerization of AC to form A_2C_2.
‖ In the case of A_2C and A_2C_2, a first-order association constant is given for formation of A_2C_2 from A_2C and C.
¶In the case of AC and AC_2, a first-order association constant is given for formation of AC_2 from AC and C.

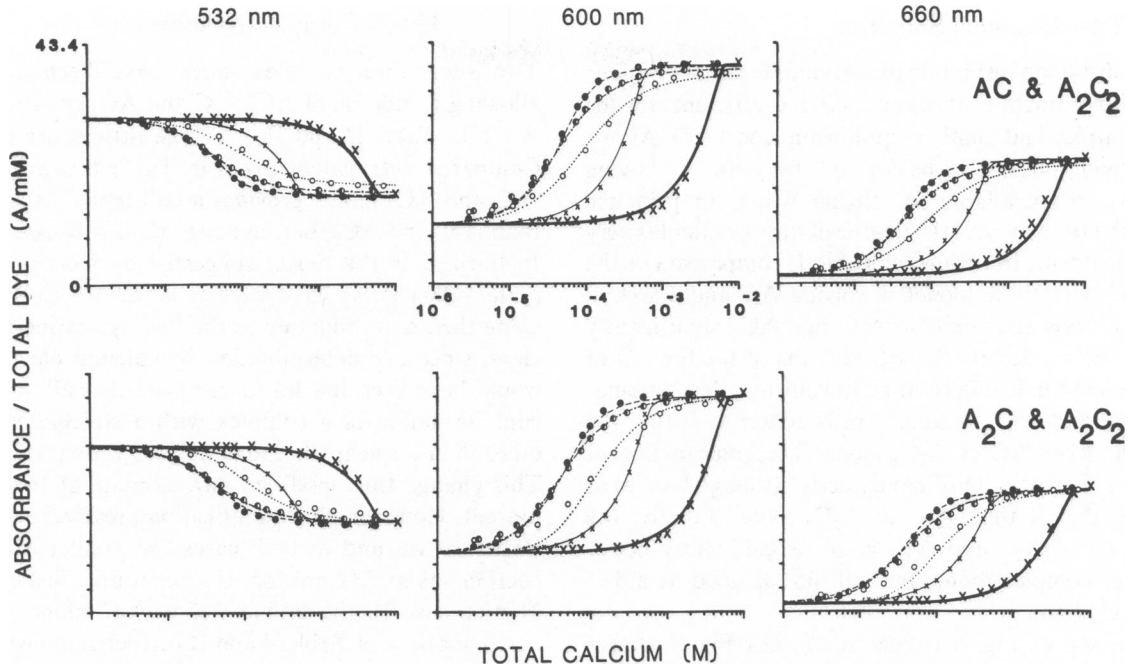


FIGURE 5 Comparison between experimental data and predicted absorbance changes accompanying arsenazo III-calcium complexation according to two different two-complex models. Experimental details and symbols as for Fig. 3. *Upper row:* data and predictions based on a model involving formation of AC and A_2C_2. *Lower row:* data and predictions based on a model involving formation of A_2C and A_2C_2.

TABLE II
PARAMETERS DEDUCED FROM LEAST-SQUARES FITTINGS OF DATA OF ARSENAZO III COMPLEXATION OF CALCIUM WITH THREE-COMPLEX MODELS*‡

	Complexes considered			
	AC, A_2, AC_2 (best fit)	AC, A_2C, AC_2 (Dorogi ϵ_A)	AC, A_2C, A_2C_2 (parallel)	AC, A_2C, A_2C_2 (sequential)
Parameters				
K_{AC}	$2.13 \times 10^4 M^{-1}$	$2.18 \times 10^4 M^{-1}$	$7.83 \times 10^3 M^{-1}$	$7.83 \times 10^3 M^{-1}$
K_{A_2C}	$2.30 \times 10^1 M^{-1}$	$2.87 \times 10^{-1} M^{-1}$	$1.99 \times 10^4 M^{-1}$	$1.97 \times 10^4 M^{-1}$
$K_{A_2C_2}$	—	—	$1.12 \times 10^6 M^{-1}$	$4.45 \times 10^5 M^{-1}$
K_{AC_2}	$7.61 \times 10^{-1} M^{-1}$	$4.91 \times 10^{-1} M^{-1}$	—	—
AC				
ϵ_{532}	15,820	14,010	19,600	19,600
ϵ_{600}	40,230	35,720	36,100	36,100
ϵ_{660}	22,900	20,200	21,200	21,200
A_2C				
ϵ_{532}	60,030	31,800	48,700	48,700
ϵ_{600}	23,800	70,600	43,440	43,440
ϵ_{660}	109,000	113,000	34,300	34,340
A_2C_2				
ϵ_{532}	—	—	32,320	31,320
ϵ_{600}	—	—	79,830	79,830
ϵ_{660}	—	—	44,400	44,400
AC_2				
ϵ_{532}	239,000	337,000	—	—
ϵ_{600}	26,920	4,093	—	—
ϵ_{660}	19,820	8,730	—	—
Statistics				
R	0.1774	0.1902	0.0474	0.0474
SSQ	24,300	27,900	1,730	1,730

*Extinction coefficients for the free dye as given in the first footnote of Table I, with the exception of those used in the second column, which were ϵ_{532} = 26,840, ϵ_{600} = 9,600, and ϵ_{660} = 1,350, as calculated from Dorogi and Neumann (1981*b*).
‡All association constants given are first order. Details of given models are discussed in the text.

Two-Complex Schemes

Schemes involving formation of two complexes involve four additional parameters (three extinction coefficients for the second complex and another equilibrium constant). Allowing a second complex to be formed, by virtue of having more variables available for fitting would in principle improve the fit; however, the degree of improvement is very model dependent. In reviewing Table I, comparison of the AC model with those models involving AC and a second complex reveals that neither A_2C nor AC_2 significantly improves the fit, despite the introduction of the four extra parameters in the fitting routine. In contrast, the combination of AC and A_2C_2 is significantly better at fitting the data than either AC or A_2C_2 alone. The combination of A_2C and A_2C_2 is also significantly better (though less so) at improving the fit than A_2C or A_2C_2 alone. Finally, the combination of A_2C and AC_2 is also significantly better than either complex alone, but still not as good as either AC or A_2C_2 alone.

Comparison of Fig. 5 (upper row) with Fig. 4 (lower row) reveals that the AC and A_2C_2 combination improves the fit at the lower dye concentrations compared with A_2C_2 alone. The inclusion of A_2C together with A_2C_2 (Fig. 5, lower row) instead helps the fit at the higher dye concentrations when compared with A_2C_2 alone. This provides a rationale for considering the possibility of forming three complexes.

Three-Complex Schemes

Two such three-complex cases have been considered, allowing formation of AC, A_2C and AC_2 or AC, A_2C and A_2C_2. In Table II and Fig. 6 these fittings are evaluated. Compared with values given in Table I clearly the AC, A_2C and AC_2 model provides a still better fit to the data than A_2C and AC_2, but no better than with AC and A_2C. In fairness to the model suggested by Dorogi and Neumann (1981*b*), we have refitted our data with their model using their determination of the free dye extinction coefficient, since our determination by calcium electrode data would have been invalid for a model that allowed significant formation of a complex with a stoichiometric ratio other than 1:1 near the end of a dye titration with calcium. This change thus modified our estimate of the total dye present. However, this modification provided a still worse statistical fit, and in both cases the predicted extinction coefficients at 532 nm for AC_2 seem unrealistically high. Neither case fits the data as well as A_2C_2 alone.

Comparison of Tables I and II further demonstrates that models involving formation of AC, A_2C and A_2C_2 yield significantly better fits than any combination of two of these complexes. The parallel model referred to in Table II allows AC dimerization to form A_2C_2, as well as addition of A to AC, resulting in A_2C. The sequential model referred to also allows A_2C formation from AC and A, but A_2C_2 is formed by the association of A_2C and C instead.

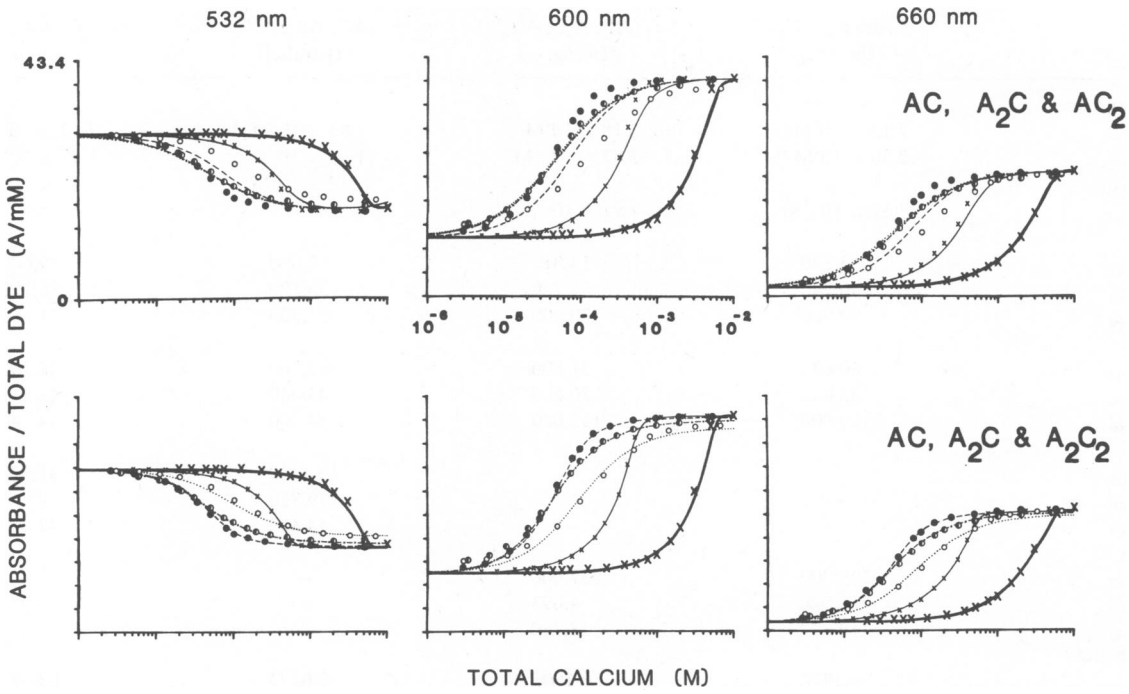


FIGURE 6 Comparison between experimental data and predicted absorbance changes accompanying arsenazo III-calcium complexation according to two three-complex models. Experimental details and symbols as for Fig. 3. *Upper row:* data and predictions based on a model involving formation of AC, A_2C and AC_2. See text for further details. *Lower row:* data and predictions based on a model involving formation of AC, A_2C and A_2C_2. See text for further details.

While AC, A_2C, and A_2C_2 could be formed by several different pathways, the best fits for the two pathways considered here appear to form equivalent amounts of each complex at each point, since they compute almost exactly the same extinction coefficients and fit with identical statistics. We would assume that a sequential model that formed A_2C_2 first and then lost a calcium to form A_2C should be capable of fitting the data as well.

Fig. 6 provides a visual comparison of the parallel Dorogi and Neumann (1981*b*) model of AC, A_2C and AC_2 (upper row) with that of the parallel scheme of AC, A_2C and A_2C_2 (lower row). Clearly the fit is far better for the scheme involving A_2C_2. While not shown, the sequential model for AC, A_2C and A_2C_2 provides exactly superimposable curves, as might be suspected from Table II. With the exception of a few points at the two lowest dye concentrations at 600 nm (open and half-filled circles), the AC, A_2C and A_2C_2 scheme provides a nearly perfect fit to all the data, certainly significantly better than all the previous models considered. A comparison with Fig. 5 shows that the addition of A_2C to the AC and A_2C_2 model has solved the problem of the predicted curves falling to the left of the data at 600 nm and to the right at 660 nm. Addition of AC to the A_2C and A_2C_2 scheme has aided in the fittings at low dye and low calcium concentrations. We therefore conclude that AC, A_2C and A_2C_2 are all required to account for the experimental data.

Reproduction of Ca^{++} Electrode Data by the Models Considered

In two of the spectrophotometric titrations, the free calcium concentration was monitored with a calcium electrode (those at 57.4 and 575 μM, Fig. 2). They provide the experimental basis for a final check on the accuracy of the proposed model for arsenazo III-calcium complexation. Using equilibrium association constants obtained from fittings of the spectrophotometric data, predicted values for the free calcium concentrations were calculated and compared with the experimental determinations. Since these determinations spanned several log units of concentration of calcium added, the data were plotted in terms of log (total calcium/free calcium) as shown in Figs. 7 and 8. Data points are shown as circles (open circles indicate 57.4 μM dye, filled circles, 575 μM dye) while model predictions are the continuous curves.

Fig. 7 demonstrates that no single complex scheme adequately fits the calcium electrode data. Qualitatively only the AC_2 and A_2C_2 schemes evince a declining shape in their curve at lower calcium concentrations, but only the A_2C_2 scheme comes close to quantitatively predicting the results. Figs. 7 (lower row) and 8 (upper row) demonstrate that the combinations of A_2C and AC_2, A_2C and A_2C_2, AC and A_2C, AC and AC_2, and even AC and A_2C_2 fail by a wide margin to accurately predict the calcium electrode

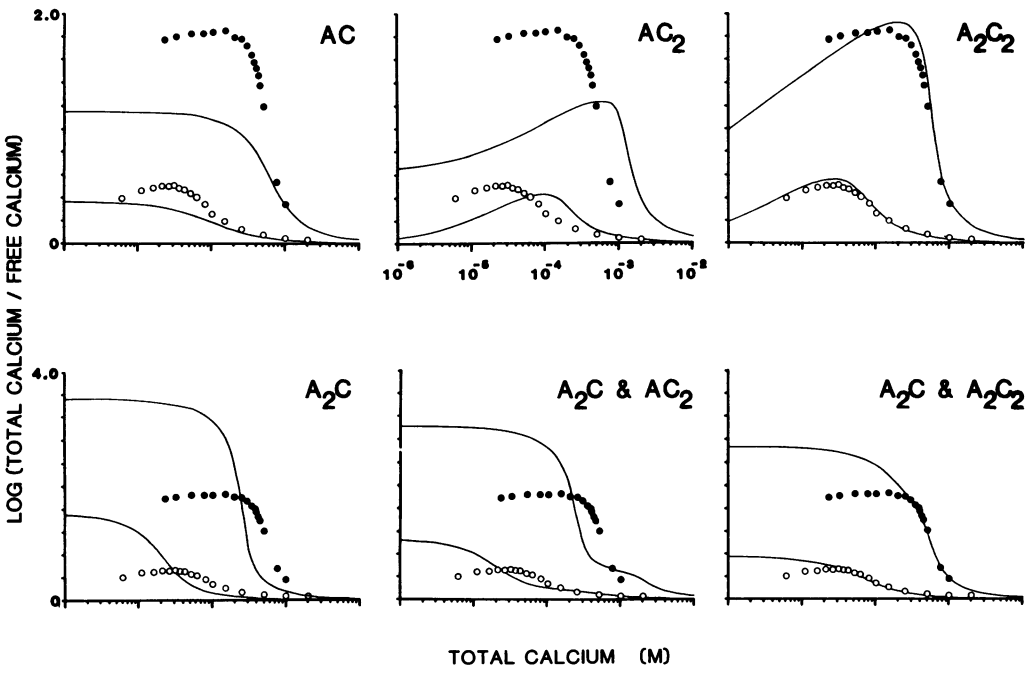


FIGURE 7 Comparison between experimental data and predicted free calcium concentration changes accompanying arsenazo III-calcium complexation according to several models. Experimental calcium electrode data and predictions of models generated from fittings of spectrophotometric data. Filled circles (●) represent data from a titration with 575 μM dye, open circles (○) data from a titrations with 57.4 μM dye. For details see Materials and Methods section. Free calcium levels for the models indicated were determined using the association constants given in Table I.

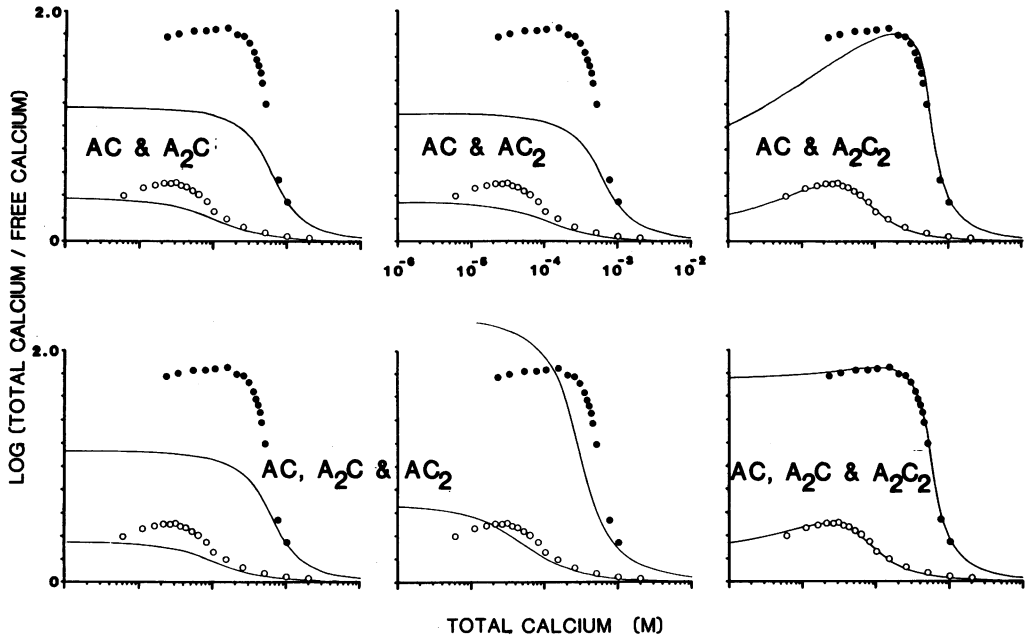


FIGURE 8 Comparison between experimental data and predicted free calcium concentration changes accompanying arsenazo III-calcium complexation according to several other models. Experimental calcium electrode data and predictions of models generated from fittings of spectrophotometric data using association constants given in Tables I and II. Other conditions as for Fig. 7. *Lower row:* the left most traces represent our best fitting using the association constants given in the first column of Table II, and the middle traces represent those using the constants given in the second column of Table II.

data. As shown in Fig. 8 (upper row) only AC and A_2C_2 comes close, but in the lower calcium ranges it fails to predict the electrode data from the high dye titration. Fig. 8 (lower row) also demonstrates that neither our best predictions of the Dorogi-Neumann scheme, nor the predictions obtained from using their determination of the extinction coefficient for the free dye agree with the data. Finally, the best fit of the spectrophotometric data, using AC, A_2C, and A_2C_2, is shown at the lower right of Fig. 8. The inclusion of A_2C relative to the AC and A_2C_2 scheme alone (Fig. 8, upper right) has corrected the discrepancy in the higher dye titration predictions without introducing significant distortions in the predictions of the low dye titration.

The plots shown in Figs. 7 and 8 were not fittings of the calcium electrode data, but merely predictions from parameters determined by the spectrophotometric data presented earlier. That the agreement is so good between the two sets of data and their predictions by the AC, A_2C and A_2C_2 model must certainly be considered strong evidence in favor of this model and against all the other models, which have failed to accurately predict either set of data. In Fig. 9 a demonstration is made of how the fractional dye distribution among the various complexes depends on the total dye concentration during titrations with calcium. AC, while the precursor for all more polymeric forms, is only a significant complex at 0.57 and 5.7-μM total dye; above these values its importance diminishes considerably. A_2C_2 is a significant complex at all dye

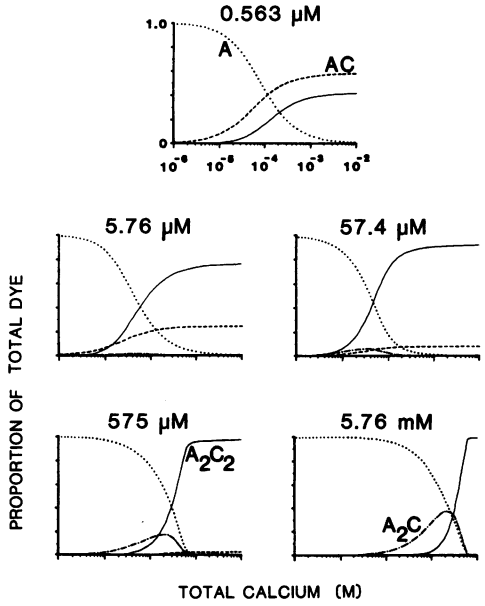


FIGURE 9 Predictions of the AC, A_2C and A_2C_2 model regarding the proportion of dye molecules in a given free or complex form simulating the experimental titration conditions. Predictions for five different total dye concentrations given above each plot are shown. Dotted curves (· · ·) represent free dye; dashed curves (---), AC; dotted-and-dashed curves (-·-), A_2C; solid curves (—), A_2C_2.

concentrations, while A_2C formation becomes increasingly significant from 57 μM to 5.75 mM. Dye concentrations around 50 μM thus seem to minimize contributions from complexes other than A_2C_2. This is a concentration range frequently used for much in vitro kinetic biochemical assay for Ca^{++}. We note that while we did not collect data with <2 μM added calcium, the free calcium ion concentration (at those added calcium levels, assuming 1 μM contaminating Ca^{++} in the medium and 0.004 μmol Ca^{++}/Mg arsenazo III) would have been 3.0 μM with 0.563 μM dye,

TABLE III
PARAMETERS DEDUCED FROM LEAST-SQUARES FITTINGS OF DATA OF ARSENAZO III COMPLEXATION OF MAGNESIUM WITH VARIOUS MODELS*

	Complexes considered				
	AM	A_2M ‡	A_2M_2 ‡§	AM_2 ‡‖	AM A_2M
Parameters					
K_{AM}	$3.79 \times 10^2 M^{-1}$	$1\ M^{-1}$	$10\ M^{-1}$	$10\ M^{-1}$	$3.70 \times 10^2 M^{-1}$
K_{A_2M}	—	$7.30 \times 10^8 M^{-1}$	—	—	$6.46 \times 10^1 M^{-1}$
$K_{A_2M_2}$	—	—	$2.67 \times 10^9 M^{-1}$	—	—
K_{AM_2}	—	—	—	$4.85 \times 10^4 M^{-1}$	—
AM					
ϵ_{522}	22,210	—	—	—	21,800
ϵ_{620}	20,900	—	—	—	21,100
ϵ_{660}	9,264	—	—	—	9,180
A_2M					
ϵ_{532}	—	52,400	—	—	68,200
ϵ_{620}	—	26,000	—	—	24,500
ϵ_{660}	—	10,600	—	—	17,800
A_2M_2					
ϵ_{532}	—	—	50,500	—	—
ϵ_{620}	—	—	29,430	—	—
ϵ_{660}	—	—	12,300	—	—
AM_2					
ϵ_{532}	—	—	—	23,800	—
ϵ_{620}	—	—	—	17,600	—
ϵ_{660}	—	—	—	7,610	—
Statistics					
R	0.0897	0.2758	0.2131	0.1300	0.0873
SSQ	2,431	23,000	13,720	5,110	2,304
AM § A_2M_2	AM AM_2	A_2M ‡§ A_2M_2	A_2M ‡ AM_2	AM ‖ A_2M A_2M_2	AM ‖ A_2M AM_2
$3.66 \times 10^2 M^{-1}$	$5.61 \times 10^2 M^{-1}$	$1 M^{-1}$	$1 M^{-1}$	$3.65 \times 10^2 M^{-1}$	$1.06 \times 10^3 M^{-1}$
—	—	$2.00 \times 10^9 M^{-1}$	$4.80 \times 10^3 M^{-1}$	$2.06 \times 10^1 M^{-1}$	$1.04\ M^{-1}$
$5.26 \times 10^1 M^{-1}$	—	$4.21 \times 10^{10} M^{-1}$	—	$4.56 \times 10^1 M^{-1}$	—
—	$1.12 \times 10^1 M^{-1}$	—	$4.42 \times 10^5 M^{-1}$	—	$5.69 \times 10^1 M^{-1}$
21,900	24,700	—	—	21,800	26,520
21,230	17,600	—	—	21,220	13,100
9,260	7,812	—	—	9,240	5,380
—	—	56,700	32,100	124,000	136,000
—	—	16,800	266,000	46,800	1,500,000
—	—	6,002	167,000	41,100	1,130,000
57,900	—	58	—	40,900	—
27,100	—	147,000	—	21,400	—
17,240	—	71,110	—	10,240	—
—	0	—	23,800	—	16,200
—	40,920	—	17,320	—	28,900
—	16,020	—	7,410	—	12,930
0.0887	0.0876	0.1443	0.1122	0.0867	0.0840
2,380	2,320	6,291	3,810	2,304	2,131

*Extinction coefficients for the free dye as given in the first footnote of Table I.
‡Modeled by allowing insignificant AM formation at equilibrium, with extinction coefficient for AM identical to those of A.
§Modeled assuming dimerization of AM to form A_2M_2.
‖Parallel scheme formulations: higher-order complexes formed directly from AM.

2.8 μM with 5.76 μM dye, 1.1 μM with 57.4 μM dye, 75 nM with 575 μM dye, and only 4.1 nM with 5.76 mM dye.

Magnesium-Arsenazo III Interaction

Titrations of arsenazo III with magnesium ion have also been explored spectrophotometrically over the same range of dye concentrations. Data were analyzed at 660, 620, and 532 nm in a manner previously described for the calcium titrations of the dye. Results are tabulated in Table III. Statistically it is clear that very little improvement is seen in the fittings relative to the most simple case, that of a simple 1:1 complexation. These small improvements in the statistics are also frequently accompanied by extremes of extinction coefficient determinations that render those models suspect. In any case, the degree of improvement seen is far less than in comparing some of the more complex models for calcium complexation with simpler models of complexation of that cation. This leads us to believe that the improvements in the case of the magnesium fittings may well reflect the fact that analysis will always be slightly more accurately represented by incorporation of additional variables. There appears to be no well-founded reason to consider arsenazo III-magnesium complexes other than of 1:1 stoichiometry under these experimental conditions of pH and ionic strength.

DISCUSSION

Arsenazo III has been shown to be a useful monitor of calcium ion concentration in a wide variety of systems, both in situ as well as in vitro. Much attention has been focused in the literature lately regarding the chemistry of the calcium-dye interaction. While most reports claim that the dye simply forms a single 1:1 complex with calcium at physiological pH and ionic strength (Kendrick et al., 1977; Scarpa, 1979; Ohnishi, 1979; Ahmed et al., 1980; Chiu and Haynes, 1980; Bauer, 1981; Brown and Rydqvist, 1981), a number of other reports suggest that arsenazo III is capable of forming more than one complex with calcium (Thomas, 1979; Ogawa et al., 1980; Palade and Vergara, 1981, 1982*b*; Dorogi and Neumann, 1981*b*; Rios and Schneider, 1981).

Formation of different absorbing species would occur at different rates and could seriously impair the deconvolution of transient absorbance changes recorded in situ into terms of calcium concentration changes. Our own earlier analysis (Palade and Vergara, 1981) of the interaction of the dye with calcium suggested that multiple complex formation was likely. Here we extend our analysis over a far broader range of dye concentrations to provide more incontestable evidence that this is the case. The difference spectrum of Fig. 1 *B* cannot be explained in any other way.

In addition to spectrophotometric determinations, Ca^{++} electrode determinations of free calcium concentrations were performed, avoiding the use of calcium buffering agents presumed to bind calcium more tightly than the dye. Even though data analysis may be simplified with the use of calcium buffers, we felt that assumptions regarding the relative lower affinity of the dye for calcium might not prove to be correct, especially at higher dye concentrations. We have endeavored to compensate for our avoidance of calcium buffers by employing the highest grade chemicals and water available to minimize contamination, and by carefully taking into account possible sources of contamination. We have considered more different schemes of stoichiometric relationships between the dye and calcium than previously addressed.

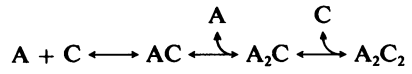
Our determinations of binding constants still must be considered applicable only under our fixed pH and ionic strength conditions in the absence of Mg^{++}. Others have shown that the calcium-dye interactions are affected by ionic strength and pH (Ogan and Simons, 1979; Chiu and Haynes, 1980; Brown and Rydqvist, 1981). Great care has been taken to monitor and adjust the pH during all titrations to ensure that changes in the difference spectra were not attributable to pH effects. No tendency was observed for the free-dye absorbance to change as a function of dye concentrations. Thus we have not considered possible dimerization of the free dye in any of our complexation schemes.

The numerical computational methods employed here make far fewer assumptions than those methods of analysis employed by other authors. While we assert that three complexes are required to explain our data, only certain models improve the fits significantly by allowing more variables to be fitted. This result, together with magnesium results demonstrating no significant improvement by incorporation of additional variables, suggest that our methods of analysis are extremely sensitive for the purposes employed and are not inherently biased in favor of providing better fits by allowing inclusion of extra complexes.

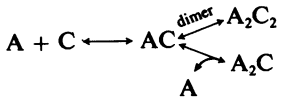
Two possible sources of error should be mentioned. First, as described in the legend of Fig. 2, Scatchard plot determinations at different dye concentrations do not yield entirely consistent results. While we chose to calibrate our dye concentrations using an averaged determination from the plots shown in Fig. 2, Fig. 9 clearly shows that the amount of A_2C present at some experimental points is not negligible. Nevertheless, we believe that the ~5% discrepancy between the assumed and either determined value is small relative to the discrepancy with respect to calculations based on weight (~50%), and should not pose a serious problem for the rest of the analysis presented here. Secondly, the commercial arsenazo III used in these experiments (the highest purity available) contained significant amounts of sodium, which would therefore have been present at higher concentrations in the titrations of higher dye concentrations. However, difference spectra such as shown in Fig. 1 are qualitatively unaltered by

addition of an equivalent amount of Na or by substitution of all the K by Na (not shown). Furthermore, multiple complex formation is evident in Fig. 1 *B* despite a constant Na concentration throughout that titration. We believe, therefore, that Na effects may only account for minor contributions to the absorbance changes discussed in this paper and would not affect our general conclusions. Our data suggest that the principal arsenazo III-calcium complex observed at intermediate dye concentrations in equilibrium studies is one involving two dye molecules and two calcium ions (Palade and Vergara, 1981; Rios and Schneider, 1981). Such a stoichiometry not only is consistent with results of the continuous variation method, but fits the data better than the suggestion of a 1:1 complex[3] with respect to the steepness of a given titration and shifts along the horizontal axis as a function of dye concentration. At very high (e.g., >1 mM) dye concentrations, a second absorbing species may be resolved when calcium levels are moderate. This form involves two dye molecules and is only readily formed at high dye to calcium ratios. Our results demonstrate that, in contrast to the 2:2 complex, the 2:1 complex exhibits a greater absorbance change (relative to the free dye) at 660 nm than at 600 nm. Since A_2C is formed from two free dye molecules, when it is formed exclusively, ΔA at any wavelength would be proportional to $\epsilon_{A_2C} - 2\epsilon_A$. Using the extinction coefficients determined from the fittings of our model, at 660 nm, $\epsilon_{A_2C} - 2\epsilon_A = 34{,}300 - 2(1{,}520) = 31{,}260$; while at 600 nm, $\epsilon_{A_2C} - 2\epsilon_A = 43{,}440 - 2(10{,}800) = 21{,}840$. We suggest (in agreement with Thomas, 1979) that this form contributes to transient absorbance changes recorded from physiological preparations injected with arsenazo III, especially in those cases where greater absorbance changes are recorded at 660 nm (or 650 nm) than at 600 nm (e.g., Baylor et al., 1979*a*, 1982; Palade and Vergara, 1981, 1982*a*). Thus our previous conclusions regarding formation of A_2C and A_2C_2 (Palade and Vergara, 1981) remain qualitatively[4] unaltered. The identification of these two complexes implies the existence of an intermediate form involving one dye molecule and one calcium ion. The data at submicromolar concentrations of dye, in fact, are better fit by allowing for the formation of such a complex at extremely low dye concentrations, conditions that we had not previously explored (Palade and Vergara, 1981). However such conditions are not generally met in either biochemical or physiological experiments involving arsenazo III.

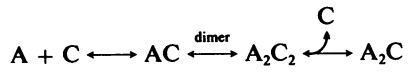
We note that the model presented here cannot distinguish among several distinct kinetic reaction mechanisms. We have presented results evaluating two of these schemes:



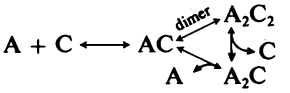
and



both fit the equilibrium data identically. We would contend that two other schemes, not explicitly considered here, must also fit the data:



and



Resolution among these possibilities will have to await analysis of kinetic data (Palade and Vergara, 1981) currently underway. Stopped flow records demonstrating up to three time constants in the complexation reaction at certain dye and calcium concentrations (Palade and Vergara, unpublished results; Gottheiner, 1980) further validate the suggestions made here regarding multiple complex formation and argue against the simpler 1:1 mode of complexation suggested by some others as outlined below.

Comparisons with Other Reports

The results reported in this paper are in agreement with Rios and Schneider (1981), especially regarding the identity of the most commonly observed complex as of the form A_2C_2 despite considerable differences in experimental design (they employed Ca^{++} buffers) and methods of analysis. However, more information regarding the formation of other complexes and additional control experiments is provided in the current report. Our calculated third-order dissociation constant for A_2C_2 formation of 1.46×10^{-14} M^3 falls directly between two estimates made by Rios and Schneider (1981) for the same parameter (1.74 and 1.29×10^{-14} M^3).

While our general conclusion of multiple complex formation between arsenazo III and calcium agrees with reports by Thomas (1979), Ogawa et al. (1980), and Dorogi and Neumann (1981*b*), we disagree with these authors since none incorporated an A_2C_2 complex in their schemes. All three authors did incorporate A_2C formation

[3]The assumption that this form is a 1:1 complex is also further discredited by the necessity of postulating (Ogawa et al., 1980; Brown and Rydqvist, 1981) that the dye's affinity for calcium increases as the dye concentration is raised. Such postulates are not required for schemes involving higher order complexes such as A_2C_2.

[4]The calculated third-order association constant for A_2C_2 formation from our current fittings is 0.69×10^{14} M^{-3}, compared with our earlier estimate (Palade and Vergara, 1981) of 1.21×10^{14} M^{-3}. This difference is partly related to different assumptions made of the total dye concentration and partly to the inclusion of AC in the fitting, since our current analysis using the previous model of A_2C and A_2C_2 still only gives a best fit of 1.28×10^{14} M^{-3}.

at high dye and low calcium concentrations. Thomas (1979) proposed A_2C and AC complex formation, but considered only relatively few data points with his model. He placed a minimum value on a dissociation constant for the AC form, but did not attempt to fit the data with the multiple complex scheme that he proposed. Our data (see Table I) were only poorly fit with a model involving only A_2C and AC. The plotting procedures utilized by Thomas may not be sensitive enough to discriminate well among complex models.[5] Ogawa et al. (1980) also suggested AC and A_2C complex formation, but found that the apparent affinity depended on the dye concentration, a clue that their scheme was internally inconsistent (see footnote 2). More extensive analysis was carried out by Dorogi and Neumann (1981*b*). These authors did not consider as wide a range of dye concentrations as we did, nor did they analyze data at more than one wavelength. Their approach was semi-analytical (Dorogi and Neumann, 1981*a*); after obtaining best fitting parameters for simpler schemes, these had to be modified again for more complex schemes. We believe their choice of AC_2 as a third complex to be unwarranted. Even though they claimed to have ruled out contributions due to an A_2C_2 form, they did not present these results in a manner for others to evaluate. We have tested the Dorogi and Neumann (1981*b*) model as rigorously as possible and found it insufficient to account for our data (Figs. 6, 8).

The majority of the literature references regarding the interaction of arsenazo III and calcium have treated this matter as a simple 1:1 complexation. The methods used by these authors (Ohnishi, 1979; Chiu and Haynes, 1980; Ahmed et al., 1980; Bauer, 1981; Brown and Rydqvist, 1981) are not the most adequate to distinguish AC vs. A_2C_2 complexation or to resolve multiple complex formation. The failure to pursue this matter with more efficient methods has probably led to self-contradicting proposals such as a variable affinity dependent on the dye concentration (Brown and Rydqvist, 1981). We have demonstrated (Figs. 3, 7) the failure of the 1:1 complexation model to account for our data.

Significance

In summary, multiple complex formation at equilibrium between arsenazo III and calcium was carefully reconstructed under conditions of physiological pH and ionic strength. We assert that multiple complex formation must be considered in any serious attempts to calibrate calcium transients recorded in situ in terms of calcium concentration changes. This deconvolution may be difficult because in situ determinations may involve the following (and, perhaps, others not known) complicating factors: (*a*) possible pH differences or transients, (*b*) higher basal Mg^{++} levels and possible Mg^{++} transients, (*c*) reported binding of arsenazo III to subcellular components (Beeler et al. 1980; Rakowski and Sommerer, 1981), some of which may be manifested in the form of a dichroic signal (Baylor et al., 1979*a*, *b*), and finally, (*d*) the fact that multiple complex formation would certainly proceed at different rates that could yield different proportions of the various complexes at different points in time. Thus one certainly could not safely assume that the probe and dye are at equilibrium at all points in time during calcium transients exhibiting time constants of only several milliseconds. Nevertheless, the identification and characterization of the complexes provided in this report should serve as an aid to determining the concentrations of different arsenazo III-calcium complexes formed in transient in situ conditions. Once such determinations are performed it should become more feasible to interpret transient dye signals in terms of calcium concentration changes.

[5]We have calculated that a model involving A_2C and A_2C_2 comes remarkably close to simulating Thomas' data on his plots.

We wish to express our sincerest appreciation to Gretchen Wooden for secretarial help, and to Drs. R. D. Mitchell and P. Volpe for their comments on the manuscript.

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