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USE OF METALLOCHROMIC DYES TO MEASURE CHANGES IN MYOPLASMIC CALCIUM DURING ACTIVITY IN FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. Changes in transmission of quasi-monochromatic light were measured in singly dissected, dye-injected twitch fibres following a single propagated action potential. The records, after correction for the intrinsic transmission signal, indicate changes in dye-related absorbance, ΔA. This paper describes the different components of dye-related signals in fibres injected with either Arsenazo III, Antipyrylazo III or Dichlorophosphonazo III.

2. Fibres injected with Arsenazo III can show two kinds of changes in dye-related absorbance, an early isotropic change and a later dichroic change. The isotropic signal, which is the main subject of this paper, is transient in nature; it starts to develop before tension, reaches a peak in about 10 msec and is nearly over by 0·1 sec (16 °C). This signal is largest at 650–660 nm and measurements in this range indicate that the peak ΔA varies approximately linearly with dye concentration between 0·2 and 0·7 mM. The wavelength dependence of the peak amplitude can be qualitatively fitted by the Ca^{2+}-difference spectrum determined from cuvette calibration measurements. There may be a small maintained (0·4–0·5 sec) absorbance change of a few percent of the peak value at 650–660 nm, possibly reflecting a maintained increase in myoplasmic pH or free [Mg^{2+}].

3. In a fibre injected with approximately 0·5 mM-Antipyrylazo III, there were two kinds of dye-related absorbance signals, both of which were isotropic. There was no signal that was obviously dichroic. The earlier signal was similar in time course to the early isotropic Ca^{2+} signal which was measured with Arsenazo III, and its magnitude followed the wavelength dependence of the Ca^{2+}-difference spectrum determined from cuvette calibration measurements. By contrast, the wavelength dependence of the later absorbance change was similar to either the H^+ or Mg^{2+}-difference spectrum. The direction of this late signal (0·2 sec after stimulus) would correspond to an increase in either myoplasmic pH or free [Mg^{2+}]. Records of the absorbance change at all wavelengths can be fitted by a linear combination of the Ca^{2+} waveform and the H^+/Mg^{2+} waveform.

4. Fibres injected with Dichlorophosphonazo III showed three dye-related absorbance changes. There was an early isotropic signal, a later dichroic signal and a second

isotropic signal. The wavelength dependence of the first part of the early signal is similar to the Ca^{2+}-difference spectrum whereas the wavelength dependence of the second isotropic signal is similar to the H^+- or Mg^{2+}-difference spectrum. As was the case with Arsenazo III and Antipyrylazo III, the direction of the second signal at late times would correspond to an increase in either pH or free [Mg^{2+}]. Replacing H_2O with D_2O resulted in a marked diminution of the dichroic signal. In D_2O, linear combinations of two basic isotropic waveforms were sufficient to account for the absorbance changes measured at all wavelengths.

5. With all three metallochromic dyes, the time course of the early isotropic signal is similar to that of the second component of the intrinsic birefringence signal, at least to time of peak. On the assumption that this birefringence signal bears a unique temporal relationship to the myoplasmic free [Ca^{2+}] waveform, at least to time of peak, the similarity suggests that all three dyes track free [Ca^{2+}] with similar speed.

6. The conclusion from the experiments is that there are, in general, two dye-related isotropic absorbance signals seen with Arsenazo III, Antipyrylazo III and Dichlorophosphonazo III. One has an early, transient time course and appears to be due to the formation of Ca^{2+}:dye complex in response to a transient increase in myoplasmic free [Ca^{2+}]. The other signal persists after the free [Ca^{2+}] transient has decayed. This appears to be due to a change in H^+:dye or Mg^{2+}:dye complex, such as would occur if there were a small maintained increase in myoplasmic pH or free [Mg^{2+}].

INTRODUCTION

The experiments described in this paper are primarily concerned with comparing optical signals in stimulated single muscle fibres injected with one of the metallochromic indicator dyes Arsenazo III, Antipyrylazo III or Dichlorophosphonazo III. Since pH and free [Mg^{2+}] remain almost constant following an action potential (Baylor, Chandler & Marshall, 1979*a*, 1982*a*), the general expectation was that any changes observed in dye-related absorbance would be due solely to formation of Ca^{2+}:dye complex in response to an increase in myoplasmic free [Ca^{2+}]. It was therefore surprising in the early stages of the investigation to find that the waveform of the dye signal sometimes varied with the wavelength of the incident light. The differences were far too large to be explained by changes in the optical properties of uninjected fibres, so it was necessary to consider the possibility that the dye molecules could give rise to signals other than those associated with the binding of Ca^{2+}.

An important clue about the nature of one of the unexpected signals came from the observation that with either Arsenazo III or Dichlorophosphonazo III different temporal waveforms could be obtained with light plane polarized along and at right angles to the fibre axis. The properties of this 'dichroic' signal will be described in the following paper (Baylor, Chandler & Marshall, 1982*b*).

The present paper is concerned with the remaining components of the dye signals. These changes in dye absorbance are isotropic in nature and may arise from dye molecules dissolved in the myoplasmic solution. With all three dyes the earliest absorbance change varies with wavelength approximately as a Ca^{2+}-difference spectrum measured in cuvette calibrations. This signal, then, appears to reflect the

expected formation of Ca^{2+}:dye complex which occurs in response to the rise in myoplasmic free [Ca^{2+}]. Furthermore, the early time course of this Ca^{2+} signal is similar to the time course of the intrinsic birefringence signal which was used as a temporal bench mark. This indicates that all three dyes may track the myoplasmic free [Ca^{2+}] transient with similar and possibly rapid speed.

After the early Ca^{2+} and dichroic signals have returned to base line, about 0·2 sec at 16 °C, a small isotropic signal persists, best seen with Antipyrylazo III and Dichlorophosphonazo III. The wavelength dependence of this signal is qualitatively consistent with a small increase in myoplasmic pH and/or free [Mg^{2+}] such as might accompany the breakdown of phosphocreatine.

The general conclusion of the paper is that Arsenazo III and Antipyrylazo III are the best of the three dyes to use for accurately reporting rapid intracellular changes of free [Ca^{2+}] in skeletal muscle. With each dye it is possible to select a range of wavelengths where the Ca^{2+} signal is large and any dichroic and/or pH/[Mg^{2+}] signal is small. With Arsenazo III this range is 650–660 nm whereas with Antipyrylazo III it is 650–720 nm. Some of these results may be useful in future studies concerned with cell types other than muscle.

Preliminary reports of some of the results have appeared (Baylor, Chandler & Marshall, 1979*a*, *b*, *c*, 1981).

METHODS

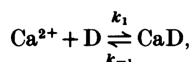
The experiments were carried out at Yale University using the same procedures described in the preceding paper (Baylor *et al.* 1982*a*). Fibre activity in all cases was initiated by an external shock which caused a single propagated action potential. Computer programs were sometimes used to fit a particular experimental record either by scaling another waveform or by scaling and adding two other waveforms. The value(s) of the scaling constants(s) was(were) adjusted to minimize the sum of the differences squared between the particular experimental record and the scaled record(s). An indication of goodness of fit is sometimes illustrated by plotting the residual record, given by the difference between the experimental record and the scaled record(s).

Dye calibration measurements were made using either the same 10 nm interference filters and photodiode that were used in the experiments (Figs. 1 and 6) or a rapid-scan spectrometer (OMA system, Princeton Applied Research, Figs. 7, 8, 10, 11, 14, 15 and 17). Measurements with both methods when carried out on the same dye solution gave closely similar absorbance curves.

Theoretical considerations

Before describing the experimental results it may first be helpful to discuss some of the principles involved in making measurements with metallochromic dyes. These considerations can influence the interpretation of dye-related optical signals recorded from cells during activity. To make the discussion as simple as possible, only 1:1 stoichiometry of cations with dye will be considered.

Speed of ion:dye reactions. When interpreting dye absorbance changes it is tempting to assume that the optical signal provides an instantaneous assessment of the underlying cause. For example, in the case of the Ca^{2+} signal it is simplest to suppose that the optical waveform, after suitable (possibly non-linear) scaling, gives the time course of the myoplasmic free [Ca^{2+}] transient. This would not be the case, however, if reaction rates between Ca^{2+} and dye were slow. For simple 1:1 stoichiometry, i.e.



$$Ca^{2+} + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CaD,$$

changes in [CaD] should follow

$$d[CaD]/dt = k_1[Ca^{2+}][D] - k_{-1}[CaD] \quad (1)$$

where Ca^{2+}, D and CaD refer to free Ca^{2+}, free dye and Ca^{2+}:dye complex and k_1, k_{-1} refer to forward, backward rate constants; [ ] denotes concentration. Here and elsewhere valence signs have been

omitted from dye and dye complexes; CaD and D may include different protonated forms as was considered for Mg^{2+}: dye complexes in the previous paper (Appendix of Baylor *et al.* 1982*a*). The associated equilibrium relationship is

$$[Ca^{2+}][D]/[CaD] = K_{CaD} \quad (2)$$

in which K_{CaD}, the dissociation constant, is given by $K_{CaD} = k_{-1}/k_1$.

If free Ca^{2+} concentration undergoes a step change from a resting level of zero to a new level indicated by $[Ca^{2+}]$, [CaD] increases from zero to a new level according to eqn. (1). Since [D] + [CaD] is constant (neglecting the possible presence of MgD for the moment), it is easy to show that the increase in [CaD] follows an exponential time course with a rate constant k given by

$$k = k_1[Ca^{2+}] + k_{-1} \quad (3)$$

$$= k_1\{[Ca^{2+}] + K_{CaD}\}. \quad (4)$$

If $[Ca^{2+}] \ll K_{CaD}$ (which appears to apply to Arsenazo III and Antipyrylazo III but possibly not to Dichlorophosphonazo III), the first term on the right hand side can be neglected and $k \simeq k_{-1}$. In this case, for any Ca^{2+} waveform, changes in [CaD] should follow changes in free $[Ca^{2+}]$ with a time constant given by $1/k_{-1}$. In other words, the temporal waveform of [CaD] would be proportional to the free $[Ca^{2+}]$ waveform put through a first order filter which has a time constant $1/k_{-1}$.

On the other hand, experimentally measured rate constants for dye solutions in cuvettes are usually not carried out using fixed waveforms for free $[Ca^{2+}]$. Rather, the techniques which are used, such as temperature jump or stopped flow, frequently rely on changes which occur under the condition of constant total $[Ca^{2+}]$ (i.e. free $[Ca^{2+}]$ + [CaD] is constant) as well as constant total dye concentration (i.e. [D] + [CaD] is constant). In this case (Gutfreund, 1972) small perturbations decay exponentially with a rate constant k' given by

$$k' = k_1\{[Ca^{2+}] + [D] + K_{CaD}\}. \quad (5)$$

In this equation $[Ca^{2+}]$ and [D] are used to designate the steady concentrations of free $[Ca^{2+}]$ and free dye concentration on which the perturbations are imposed. Eqn. (5) applies equally well to the rate constant associated with the change in [CaD] which would follow a small step change in total (rather than free) $[Ca^{2+}]$ under the condition of constant total dye concentration. If $[Ca^{2+}] + [D] \ll K_{CaD}$, the rate constant which is usually measured with temperature jump or stopped flow, k', will be approximately equal to k_{-1}.

The question, then, is what are the values of k_{-1} for the metallochromic dyes used in the experiments? Scarpa, Brinley & Dubyak (1978) report that the relaxation time for Arsenazo III is approximately 2·8 msec (20 °C) following a temperature jump; this means $k' = 357$ sec^{-1} and therefore $k_{-1} < 357$ sec^{-1}. If the temperature jump was done with micromolar amounts of $[Ca^{2+}]$ and [D], Arsenazo III should track a small free $[Ca^{2+}]$ transient with a time constant somewhat greater than 2·8 msec.

A direct estimate of k_{-1} for Arsenazo III is given by Ogawa, Harafuji & Kurebayashi (1980). Using stopped flow, they mixed one solution containing CaD with another solution containing an excess of the Ca^{2+}-chelating agent GEDTA. Using this method they were able to obtain a value for k_{-1} of 85 sec^{-1} at 20 °C. According to this value, the dye should track a small free $[Ca^{2+}]$ transient with a time constant of about 12 msec.

The two measurements on Arsenazo III indicating $2{\cdot}8 < k_{-1} \leqslant 12$ msec seem inconsistent with the finding that changes in Arsenazo III absorbance in squid axons following Ca^{2+} entry during a brief depolarization occur with a delay of no more than 0·4 msec (Brown, Cohen, De Weer, Pinto, Ross & Salzberg, 1975). However, if the effect of stimulation is to produce a step change in total $[Ca^{2+}]$, rather than free $[Ca^{2+}]$, the rate constant associated with subsequent changes in [CaD] would be given by eqn. (5) rather than eqn. (4). Since a rather large concentration of Arsenazo III, 0·3 mM, was used in the squid experiments, the observed rate constant k' would be determined predominantly by the relatively large second term in eqn. (5), k_1[D]. A value of 0·4 msec for $1/k'$ and 0·3 mM for [D] would require k_1 to be slightly less than $8{\cdot}3 \times 10^6$ M^{-1} sec^{-1}, a value easily within the range of possibility for reactions involving Ca^{2+} complexation with various ligands (Diebler, Eigen, Ilgenfritz, Maas & Winkler, 1969) and close to the value of $4{\cdot}4 \times 10^6$ M^{-1} sec^{-1} given as a rough estimate for k_1 by Ogawa *et al.* (1980).

The relaxation time for Antipyrylazo III, determined with temperature jump, is considerably less than that for Arsenazo III, being given as 0·18 msec at 20 °C (Scarpa *et al.* 1978); this indicates that $k' = 5{\cdot}6 \times 10^3$ sec^{-1} and $k_{-1} < 5{\cdot}6 \times 10^3$ sec^{-1}. If free [Ca^{2+}] and free [D] were considerably smaller than K_{CaD} during the temperature jump measurement, k' would be determined mainly by k_{-1}, according to eqn. (5). This estimate for k_{-1}($5{\cdot}6 \times 10^3$ sec^{-1}) is consistent with Ogawa *et al.* (1980) who report $k_{-1} > 10^3$ sec^{-1}. Thus, in muscle experiments Antipyrylazo III should track a small free [Ca^{2+}] transient with a time constant certainly less than 1 msec and possibly as small as 0·18 msec.

Dichlorophosphonazo III also appears to react rapidly with Ca^{2+}. P. Smith (quoted in Yoshikami & Hagins, 1978) found that reactions of this dye with Ca^{2+} were too rapid to allow temporal resolution with an apparatus whose mixing time was less than 2 msec, although no additional detail is given. A theoretical range of upper limits of 155–1550 sec^{-1} for k_{-1} would be set by using 10^8–10^9 M^{-1} sec^{-1} as a maximum value for k_1 (Diebler *et al.* 1969) and $K_{CaD} = 1{\cdot}55$ μM (Fig. 4*b* in Yoshikami & Hagins, 1978).

The general expectation, then, is that all three indicator dyes (Arsenazo III, Antipyrylazo III and Dichlorophosphonazo III) should track free [Ca^{2+}] transients in a muscle fibre reasonably rapidly but with somewhat different time constants.

Effect of free [Mg^{2+}] on the apparent dissociation constant of CaD. All three of the metallochromic dyes which we used to measure free [Ca^{2+}] transients can also bind Mg^{2+}. It is therefore important to understand how the concentration of Mg^{2+}, and therefore of MgD, can affect the concentration of CaD. The first effect to consider is how K^{app}_{CaD}, the apparent dissociation constant of the dye for Ca^{2+}, depends on free [Mg^{2+}].

At equilibrium and at constant pH the concentrations of free dye [D], Mg^{2+}-complexed dye [MgD], Ca^{2+}-complexed dye [CaD] and total dye [D_T] obey

$$[Mg^{2+}][D]/[MgD] = K_{MgD}, \tag{6}$$

$$[D_T] = [D]+[MgD]+[CaD] \tag{7}$$

and eqn. (2). The apparent dissociation constant of the dye for Ca^{2+} is defined according to

$$[Ca^{2+}]\{[D_T]-[CaD]\}/[CaD] = K^{app}_{CaD} \tag{8}$$

from which the well known relation

$$K^{app}_{CaD} = K_{CaD}\{1+[Mg^{2+}]/K_{MgD}\} \tag{9}$$

can be derived. K_{CaD} and K_{MgD} are the dissociation constants which are measured when [Mg^{2+}] and [Ca^{2+}] are zero, respectively (see Baylor *et al.* 1982*a*).

Effect of free [Mg^{2+}] on the Ca^{2+}-difference spectrum. Since an increase in [CaD] reduces [D], an increase in [CaD] will also tend to reduce [MgD] according to eqn. (6). If free [Mg^{2+}], [D] and [MgD] are in equilibrium, changes in [MgD] following a change in [CaD] can be evaluated by combining eqn. (6) and (7). Under the condition that free [Mg^{2+}] remains constant, as appears to be the case following a single action potential (Baylor *et al.* 1982*a*), changes in [MgD] and [CaD] (denoted by Δ) are related according to

$$\Delta[MgD] = -\left\{\frac{[Mg^{2+}]}{[Mg^{2+}]+K_{MgD}}\right\}\Delta[CaD]. \tag{10}$$

Eqn. (10) shows that the equilibrium change in [MgD] occurs in constant proportion to the change in [CaD]. It should be noted that this relation is independent of whether free [Ca^{2+}], [D] and [CaD] are in equilibrium but only requires that free [Mg^{2+}], [D] and [MgD] be in equilibrium.

In general, the change in absorbance ΔA, per pathlength l, which would accompany a change in [CaD] and [MgD], under the restriction imposed by eqn. (7), is given by

$$\Delta A/l = (\epsilon_{CaD}-\epsilon_D)\Delta[CaD]+(\epsilon_{MgD}-\epsilon_D)\Delta[MgD]. \tag{11}$$

ϵ_D, ϵ_{MgD} and ϵ_{CaD} refer to molar extinction coefficients for D, MgD and CaD. If Mg^{2+}:dye equilibrium has been reached, eqns. (10) and (11) can be combined. Defining $\Delta\epsilon_{CaD} = \epsilon_{CaD}-\epsilon_D$ and $\Delta\epsilon_{MgD} = \epsilon_{MgD}-\epsilon_D$ one obtains

$$\Delta A/l = \left\{\Delta\epsilon_{CaD}-\frac{[Mg^{2+}]}{[Mg^{2+}]+K_{MgD}}\Delta\epsilon_{MgD}\right\}\Delta[CaD]. \tag{12}$$

The term in brackets on the right side of eqn. (12) determines the wavelength dependence of the absorbance change and shows how the dependence varies with $[Mg^{2+}]$. If $[Mg^{2+}] = 0$ or K_{MgD} is very large (so that [MgD] is negligible), the observed spectrum is simply proportional to $\Delta\epsilon_{CaD}$. At the opposite extreme, if $[Mg^{2+}] \gg K_{MgD}$ (so that [D] is negligible) the observed spectrum is proportional to the difference $\Delta\epsilon_{CaD} - \Delta\epsilon_{MgD} = \epsilon_{CaD} - \epsilon_{MgD}$.

If the stoichiometry of Ca^{2+}:dye is 1:2 and that for Mg^{2+}:dye is 1:1, as appears to apply for Antipyrylazo III under the conditions of our measurements, the $[Mg^{2+}]/([Mg^{2+}] + K_{MgD})$ terms on the right hand sides of eqns. (10) and (12), as well as (19) below, should be multiplied by 2 and CaD should be replaced by CaD_2.

Effect of the kinetics of Mg^{2+}:dye complexation on the Ca^{2+}-difference spectrum. If Mg^{2+}:dye reactions are rapid, so that eqn. (12) holds, ΔA would always be proportional to Δ[CaD] with a constant proportionality factor. Thus, absorbance changes measured at different wavelengths would follow the same waveform and could therefore be made to superimpose by appropriate scaling.

On the other hand, if Mg^{2+}:dye reactions were slow, a constant proportionality between Δ[MgD] and Δ[CaD], eqn. (10), would not necessarily hold. In this case changes in [MgD] can be calculated from the differential equation

$$d[MgD]/dt = k_2[Mg^{2+}][D] - k_{-2}[MgD], \tag{13}$$

similar to eqn. (1). Thus, changes in [MgD], [CaD] and [D] would be described by eqns. (1), (7) and (13). In general Δ[MgD] and Δ[CaD] could follow different temporal waveforms so that the measured change in dye absorbance would reflect a time-dependent linear combination of the two underlying difference spectra, $\Delta\epsilon_{CaD}$ and $\Delta\epsilon_{MgD}$. In this case, absorbance changes measured at different wavelengths could follow different waveforms.

Even if the kinetics of Mg^{2+}:dye reaction are slow, however, an interesting special case, in which Δ[MgD] and Δ[CaD] vary in constant proportion, occurs if [CaD] increases exponentially with time from a very low level (see below). In muscle such an exponential increase approximately holds during the first part of the Ca^{2+} transient since the Ca^{2+}-related absorbance change increases approximately exponentially during this time (see Fig. 18 for example). Therefore, during the early phase of the Ca^{2+} transient changes in dye absorbance should follow the same (exponential) waveform at all wavelengths.

The theoretical basis for the constant proportion between Δ[MgD] and Δ[CaD] can be obtained in the following way. Consider

$$[CaD] = [CaD]_0 \exp(\alpha t) \tag{14}$$

where $[CaD]_0$ refers to CaD concentration at some arbitrarily chosen zero time and α gives the rate constant for the rising phase. Eqn. (13) can be combined with eqns. (7) and (14) to give

$$d[MgD]/dt = k_2[Mg^{2+}]\{[D_T] - [CaD]_0 \exp(\alpha t)\} - \{k_2[Mg^{2+}] + k_{-2}\}[MgD]. \tag{15}$$

Since $[Mg^{2+}]$ stays approximately constant during the Ca^{2+} transient, the concentration [MgD] is the only unknown variable. Using the initial condition

$$[MgD]_0 = \left\{\frac{k_2[Mg^{2+}]}{k_2[Mg^{2+}] + k_{-2}}\right\}[D_T], \tag{16}$$

which is appropriate for resting $[Ca^{2+}] \simeq 0$, eqn. (15) can be solved to give

$$[MgD] = [MgD]_0 - [CaD]_0\left\{\frac{k_2[Mg^{2+}]}{k_2[Mg^{2+}] + k_{-2} + \alpha}\right\}\{e^{\alpha t} - e^{-(k_2[Mg^{2+}] + k_{-2})t}\}. \tag{17}$$

Since the initial displacement $[CaD]_0$ should be made vanishingly small, the second (decaying) exponential on the right hand side can be neglected once the Ca^{2+} transient is underway. In this case Δ[MgD] and Δ[CaD] would change in constant proportion according to

$$\Delta[MgD] = -\frac{k_2[Mg^{2+}]}{k_2[Mg^{2+}] + k_{-2} + \alpha}\Delta[CaD] \tag{18}$$

$$= -\left\{\frac{1}{1 + \frac{\alpha}{k_2([Mg^{2+}] + K_{MgD})}}\right\}\left\{\frac{[Mg^{2+}]}{[Mg^{2+}] + K_{MgD}}\right\}\Delta[CaD]. \tag{19}$$

If the rate constant for the exponential rise in [CaD] is much smaller than the rate constant associated with Mg^{2+}:dye complexation, i.e. if $\alpha \ll k_2([Mg^{2+}]+K_{MgD})$, eqn. (19) reduces to the equilibrium relationship, eqn. (10). The experimental values for α are of the order of 1 msec^{-1} so that the rate constant for changes in Mg^{2+}:dye should be at least 10 msec^{-1} for eqn. (10) to apply reasonably well. This requirement is met if $k_2 \geq (10^4\ sec^{-1})/([Mg^{2+}]+K_{MgD})$. The values of K_{MgD} at pH = 6·9 are 6·2 mM for Arsenazo III (Baylor *et al.* 1982*a*), 6·7 mM for Antipyrylazo III (Ríos & Schneider, 1981) and 0·21 mM for Dichlorophosphonazo III (Baylor *et al.* 1982*a*). Using a value of 2 mM for free [Mg^{2+}], the lower limits required for k_2 can be calculated to be $1{\cdot}2 \times 10^6$ M^{-1} sec^{-1} for Arsenazo III, $1{\cdot}1 \times 10^6$ M^{-1} sec^{-1} for Antipyrylazo III and $4{\cdot}5 \times 10^6$ M^{-1} sec^{-1} for Dichlorophosphonazo III. Values as high as this are possible since they lie within the range 10^4–10^7 M^{-1} sec^{-1} reported in Table 3 of Diebler *et al.* (1969) for reactions involving Mg^{2+} complexation with various ligands.

As far as we know, kinetic data are not available for the reactions between the above dyes and Mg^{2+}. If the reaction rates are slower than the limits calculated above, the wavelength dependence of the rising phase of the calcium transient will fit a difference spectrum based on eqns. (19) and (11) rather than (10) and (11). This corresponds to a difference spectrum which could be obtained in cuvette calibrations using an artificially low value of free [Mg^{2+}]. This point will be discussed later in connexion with the experiments using Dichlorophosphonzao III. Again, it should be pointed out that the use of eqn. (19) does not involve the speed of the Ca^{2+}:dye reaction, only the speed of the Mg^{2+}:dye reaction.

Effect of intracellular Ca^{2+}-buffering capacity of metallochromic dyes. One of the factors to consider is whether myoplasmic dye might act as a substantial Ca^{2+} buffer and thereby modify the free [Ca^{2+}] transient. The exact value of peak free [Ca^{2+}] following an action potential is not known with certainty but is probably in the range 2–10 μM (see Discussion). For comparison, measurements of Ca^{2+} transients with the metallochromic dyes used in this paper were usually made with 0·1–1 mM-dye of which perhaps 5–10% might be complexed with Ca^{2+} at the peak, requiring 5–100 μM-Ca^{2+} to be complexed with dye. In practice, the lower limit of 5 μM-Ca^{2+}:dye complex was required to give a reliable Ca^{2+} signal. In nearly all our dye experiments, then, there is more Ca^{2+} complexed with dye than there is free.

The above values for concentrations of free Ca^{2+} and of Ca^{2+} complexed with dye should be considered in relation to the amount of Ca^{2+} which is bound to other intracellular buffers. For example, there is about 0·07 m-mole troponin present in 1 kg of whole muscle (Ebashi, Endo & Ohtsuki, 1969). Using the factor 0·58 to convert whole muscle weight to myoplasmic H_2O exclusive of s.r. (sarcoplasmic reticulum) and mitochondria (see Baylor *et al.* 1982*a*), one obtains 0·07/0·58 = 0·12 mM for troponin concentration. Each troponin has two Ca^{2+}-regulatory sites (Potter & Gergely, 1975; Johnson, Robinson, Robertson, Schwartz & Potter, 1981) so that troponin provides $0{\cdot}12 \times 2 = 0{\cdot}24$ mM concentration of Ca^{2+}-buffer sites. Calculations by Robertson, Johnson & Potter (1981) indicate that most of the Ca^{2+}-regulatory sites become transiently occupied during a twitch, so that 0·15–0·2 mM-Ca^{2+} would be bound. This amount of Ca^{2+}, plus the smaller amounts which are bound to other Ca^{2+}-buffer systems (Robertson *et al.* 1981), would require that approximately 0·2 mM-Ca^{2+} be released following a single action potential.

Although these numbers are somewhat approximate, they appear to indicate that the concentration of Ca^{2+}:dye complex which is formed following an action potential is a small fraction of the total [Ca^{2+}] which is released, at least with dye concentrations of a few tenths mM. Thus, in this range of dye concentrations the amount of myoplasmic Ca^{2+} which is bound to dye is considerably smaller than that which is bound to other myoplasmic buffers such as troponin.

RESULTS

Arsenazo III

Effect of free [Ca^{2+}] on the Arsenazo III absorbance spectrum. Fig. 1 *A* shows Arsenazo III absorbance spectra obtained from cuvette calibrations, one for [Ca^{2+}] = 0 and the other for a nearly saturating level of [Ca^{2+}]. The difference between these two curves is plotted in part *B*. All curves have been normalized for dye concentration by dividing by *A*(570); this procedure forces the difference spectrum in part *B* to be equal to zero at 570 nm or, more precisely, at the 'average' wavelength passed by our

570 nm filter. Separate measurements have shown that this wavelength is close to one of the isosbestic wavelengths of the dye for Ca^{2+}.

It was previously shown that the Arsenazo III resting spectrum in muscle fibres closely resembles the $[Ca^{2+}] = 0$ curve in Fig. 1*A* (Fig. 13 of Baylor *et al.* 1982*a*). During fibre activity, changes in dye absorbance which accompany formation of Ca^{2+}:dye complex would therefore be expected to have a wavelength dependence similar to that given in Fig. 1*B* if the reaction between Mg^{2+} and dye is rapid. Even if the reaction were slow, the curve should provide a reasonable approximation since

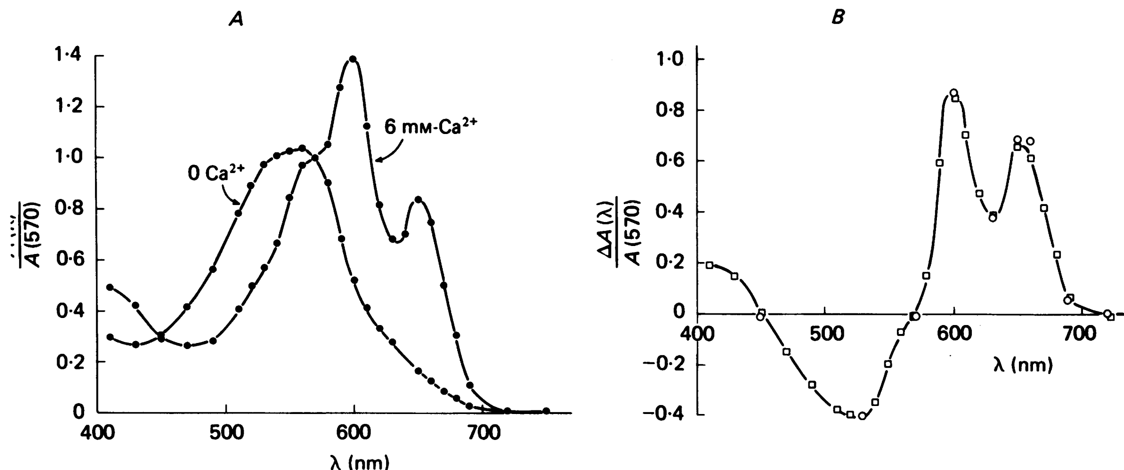


Fig. 1. Effect of Ca^{2+} on the absorbance spectrum of Arsenazo III. *A*, the normalized absorbance $A(\lambda)/A(570)$ is plotted against λ for solutions containing 0 and 6 mM-Ca^{2+}. *B*, □, difference between the two curves in *A*; ○, similar difference obtained using 1 mM-Arsenazo III rather than 30 μM. The calibrating solutions in *A* contained 150 mM-KCl, 1·08 mM-$MgCl_2$, 30 μM-Arsenazo III and 10 mM-PIPES buffered to pH = 6·9; the $[Ca^{2+}] = 0$ solution contained 0·5 mM-K_2 EGTA and the $[Ca^{2+}]$ = 6 mM solution contained 6 mM-$CaCl_2$. The calibrations were done in a 1 cm cuvette placed in the optical bench arrangement which was used for the experiments. The measurements with 1 mM-Arsenazo III (*B*) were carried out in a similar way using a 100 μm cuvette.

the wavelength dependence of the absorbance change observed with Ca^{2+} addition is relatively insensitive to mM amounts of $[Mg^{2+}]$ (Kendrick, Ratzlaff & Blaustein, 1977). The main features of the difference spectrum are that there are two isosbestic wavelengths, near 450 and 570 nm; a decrease in absorbance between 450 and 570 nm; two positive peaks in absorbance, at 600 and 650 nm, separated by a valley at 630 nm; and little change in absorbance for wavelengths greater than 700 nm. The two peaks at 600 and 650 nm, separated by a valley, are characteristic of the formation of Ca^{2+}:dye complex and are not seen with changes in H^+:dye or Mg^{2+}:dye complex (Kendrick *et al.* 1977).

Transmission changes with low and high concentrations of Arsenazo III. In the early stages of the work we noticed that the dye-related absorbance changes following stimulation sometimes showed different waveforms at different wavelengths. With Arsenazo III these differences were best seen with a low concentration of dye and were invariably associated with different signals being observed with incident light

plane polarized along (0°) and perpendicular to (90°) the fibre axis. At high dye concentration such differences were much less marked.

Fig. 2 illustrates some of these general features in two fibres that had, as usual, been highly stretched and pressed on a pedestal to eliminate movement artifacts. The records in part *B* were taken from a fibre that had been injected with a relatively high concentration of dye, about 0·6 mM. Optical records were taken at four wavelengths, as indicated, using 0° and 90° polarized light. The 0° and 90° records virtually superimpose, showing that the optical changes have almost no polarization dependence and may therefore be considered to be isotropic. The reasons for using the 0° and 90° orientations, rather than other ones, will become apparent later (Baylor *et al.* 1982*b*).

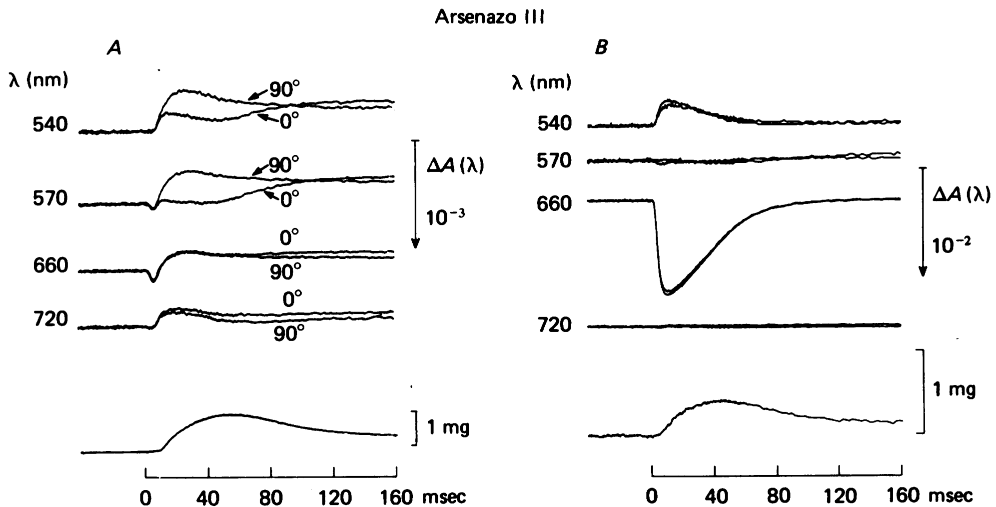


Fig. 2. Absorbance changes in fibres injected with low and high concentrations of Arsenazo III. *A*, optical records (average of two sweeps each) at four different wavelengths, 0° and 90°, as indicated. In this experiment quasi-monochromatic light was produced with a set of filters having a half band of approximately 30 nm. Dye concentration was estimated to be about 0·06 mM. Vertical diameter, 87 μm; horizontal diameter, 92 μm; sarcomere spacing, 4·3 μm; Ringer, 15·6 °C; fibre 062678·2. *B*, similar to *A* except that 0·6 mM dye and 10 nm filters were used. Each record was signal averaged two to six times. Vertical and horizontal diameter, 69 μm; sarcomere spacing, 4·3 μm; Ringer, 16 °C; fibre 010579·2. In this and subsequent Figures the fibre was stimulated at $t = 0$. An upward deflexion, unless other indicated, denotes an increase in intensity of transmitted light or a decrease in fibre absorbance. A tension record is usually included for comparison, placed at the bottom of the Figure.

The signals in Fig. 2*B* are very different from those obtained from uninjected fibres with movement artifacts eliminated (see, for example, Fig. 7*C* and *D* of Baylor *et al.* 1982*a*). The amplitude and wavelength dependence are qualitatively consistent with what one expects for a transient increase in Ca^{2+}:dye complex. The records at $\lambda = 720$ nm, where the dye does not absorb light, and at $\lambda = 570$ nm, the isosbestic wavelength, are flat. The records at $\lambda = 540$ nm show an increase in transmitted light, corresponding to a decrease in absorbance, whereas the records at 660 nm show the opposite change. These transient changes occur rapidly, preceding tension. They

reach a peak at about 10 msec and are almost over by 0·1 sec. Both the 540 and 660 nm waveforms are temporally similar, indicating that perhaps only one underlying process or spectral change is involved.

The relatively simple situation seen with heavily injected fibres, Fig. 2*B*, is not observed following weak or moderate dye injection. This is best illustrated with a weak injection, Fig. 2*A*. In this experiment the concentration of dye was estimated to be only 0·06 mM. As in part *B*, the optical changes at 720 nm should not be influenced by the presence of dye and should therefore reflect changes in the intrinsic properties of the fibre itself. This appears to be the case since the records at the gain shown bear a close resemblance to those obtained from uninjected fibres (Baylor *et al.* 1982*a*). Similar changes in the 720 nm records are also present in part *B* but are not so obvious because only one tenth the vertical gain was used.

The records at $\lambda = 660$ nm, part *A*, show an early downward component which, as suggested for the corresponding isotropic signal in part *B*, may be due to the formation of Ca^{2+} : dye complex. The later part of the 660 nm signal in part *A* is similar to the 720 nm signal and therefore most likely reflects changes in the intrinsic optical properties of the fibre rather than changes associated with dye.

The two traces taken at $\lambda = 570$ nm are considerably different from one another, a somewhat surprising finding since one expects no dye-related absorbance change to accompany the formation of Ca^{2+}:dye complex at the isosbestic wavelength. The difference between the 90° and 0° signals is substantially greater than the difference observed at 720 nm in this fibre or at 570 nm in uninjected fibres (Baylor *et al.* 1982*a*). Furthermore, both the direction and the time course of the 570 nm difference signal are different from the intrinsic difference signal. Thus, the 570 nm records in Fig. 2*A* indicate the presence of a dichroic signal which depends on the presence of dye. There is also an indication of an early downward isotropic signal at 570 nm, possibly because in this experiment 30 nm filters were used to pass quasi-monochromatic light and, consequently, the 570 nm light contained wavelengths away from the isosbestic point. The traces at 540 nm also show a substantial dichroic signal. The dichroic signal at both wavelengths appears to develop after the isotropic signal and to decay to nearly zero by 160 msec. The latter point is evident from the similar final differences between $\Delta A(\lambda)$ at 0° and 90° at the different wavelengths.

The two experiments in Fig. 2 show that Arsenazo III can give different types of signals depending on concentration. At very low concentration the signals are predominantly dichroic whereas at high concentration they tend to be larger and predominantly isotropic. Both signals are transient in nature. In the following sections some of the properties of the isotropic signal which is seen in relatively heavily injected fibres will be described. The properties of the dichroic signal will be described in the following paper (Baylor *et al.* 1982*b*).

Effect of Arsenazo III concentration on the isotropic signal. Following injection, dye molecules diffuse along the fibre away from the injection site. It is therefore necessary in analysing long duration experiments to know how the active absorbance change varies with dye concentration.

Fig. 3*A* shows changes in 650 nm absorbance which have been recorded at different times, as indicated, following completion of Arsenazo III injection. This particular wavelength was selected so that the isotropic Ca^{2+} signal would be large and would

contain little contribution from the dichroic waveform (Fig. 2 of this paper and Baylor *et al.* 1982*b*). In this experiment the intrinsic transmission change was sufficiently small that practically all the observed signal was dye-related (see 750 nm trace in Fig. 5*A*). All the absorbance records in Fig. 3*A* show a rapid, transient change which precedes tension. During the course of the experiment, from 7 to 62 min following injection, the magnitude of the $\Delta A(650)$ signal progressively decreased as the dye concentration, as indicated by dye-related $A(570)$, decreased.

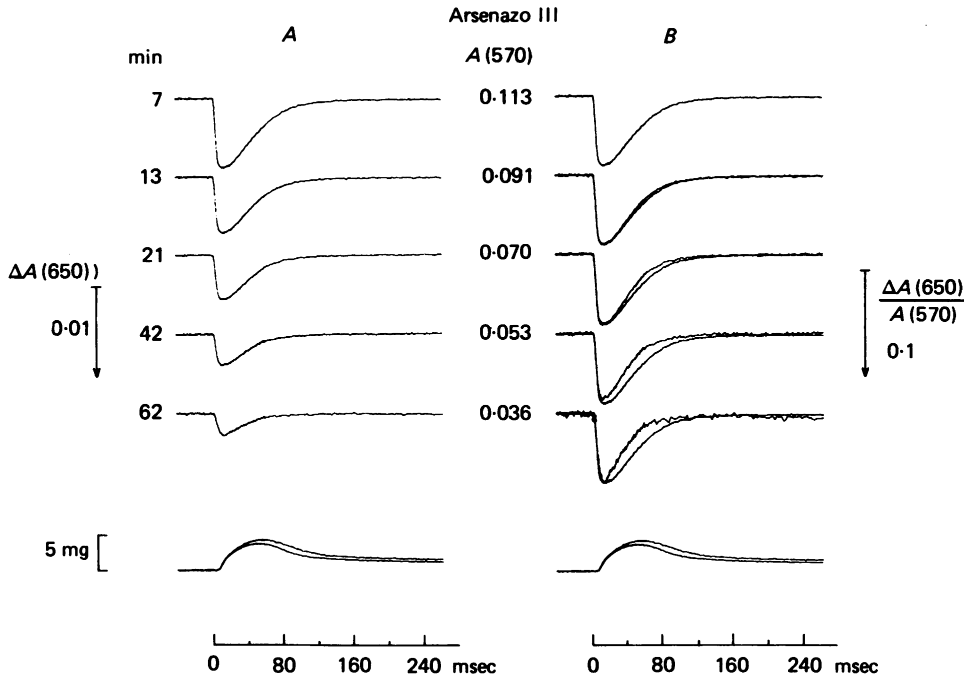


Fig. 3. Absorbance changes at different times following Arsenazo III injection. *A*, records of $\Delta A(650)$ and tension. Each record was signal averaged one to two times. The time following injection and the value of dye-related $A(570)$ is indicated beside each $\Delta A(650)$ record. The bottom trace shows tension at 7 and at 62 min. *B*, records of $\Delta A(650)/A(570)$ and tension with the longer lasting 7 min record being superimposed on all optical traces. In this experiment the intrinsic transmission change (see 750 nm trace in Fig. 5*A*) is sufficiently small that it can be neglected. Vertical diameter, 56 μm; horizontal diameter, 53 μm; sarcomere spacing, 3·9 μm; light polarized 90° with respect to fibre axis; 15·6 °C; fibre 062878·1.

To a good approximation, the decrease in the peak value of $\Delta A(650)$ paralleled the decrease in dye-related $A(570)$. This is best shown by scaling the $\Delta A(650)$ traces according to $A(570)$, as is done in Fig. 3*B*. For purposes of comparison the absorbance change at 7 min has been superimposed on all traces. The superimposed traces are very similar through time-to-peak. The return to base line, however, became somewhat more rapid at later times as the Arsenazo III concentration decreased. The reason for this effect is not known but may be related to one of the following. Firstly, the Ca^{2+}-buffering capacity of the dye, though not so great as that of troponin as discussed earlier, might prolong the duration of the free $[Ca^{2+}]$ waveform. Secondly,

Arsenazo III might slow the operation of the s.r. Ca^{2+} pump (e.g. it has been shown to reduce the amount of Ca^{2+} which is taken up by fragmented bullfrog s.r. (Ogawa *et al.* 1980)). Thirdly, the free $[Ca^{2+}]$ waveform may be prolonged by some transient non-specific fibre damage which is present immediately following injection (when the dye concentration is largest).

Although the records in Fig. 3 were made with 90° polarized light, the results should be independent of the form of polarization; this follows from the fact that the $\Delta A(650)$ signal is isotropic (Fig. 2*B* and Baylor *et al.* 1982*b*) and $A(570)$ is essentially the same with 0° and 90° light (Baylor *et al.* 1982*a*).

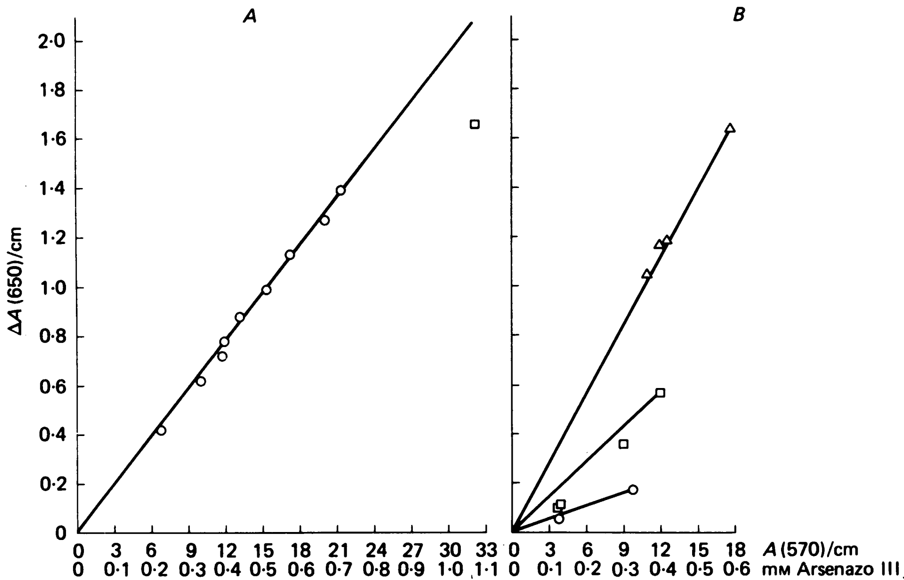


Fig. 4. Peak values of $\Delta A(650)$/cm plotted against dye-related $A(570)$/cm or Arsenazo III concentration. *A*, data from the experiment in Fig. 3. ○, from the 650 nm records in Fig. 3*A* and others not shown. □, from an early 660 nm record, multiplied by the factor 0·95 to give the corresponding value for 650 nm (see Fig. 6). The waveform of the 660 nm signal, suitably scaled, virtually superimposed the first 650 nm signal. *B*, data from three other fibres. Δ, vertical diameter, 69 μm; horizontal diameter, 77 μm; sacromere spacing, 3·9 μm; 90° polarized light; temperature 15·5 °C; fibre 071378·1 following second dye injection. □, vertical and horizontal diameters, 82 μm; sarcomere spacing, 4·3 μm; unpolarized light; 15·9–16·4 °C; fibre 061578·1. The measurements were made with a 660 nm filter, half band 30 nm; consequently, peak values have been multiplied by 1·21 to give values corresponding to the usual 650 nm filter, half band 10 nm. ○, vertical diameter, 74 μm; horizontal diameter, 88 μm; sarcomere spacing, 3·9 μm; unpolarized light; 15·0–15·7 °C; fibre 061478·1.

Fig. 4*A* shows peak values of $\Delta A(650)$/cm plotted against either dye-related $A(570)$/cm or concentration of Arsenazo III. The circles were determined from the 650 nm records, some of which are shown in Fig. 3*A*. The square is from a record taken at 660 nm, appropriately scaled to correspond to the magnitude at 650 nm. The Figure shows that the peak value of the $\Delta A(650)$ signal varied linearly with dye concentration between 0·2 and 0·7 mM and was below the linear extrapolation at a

concentration exceeding 1 mM. Since the square data point was obtained immediately following completion of the injection and withdrawal of the electrode, it is difficult to be certain that temporary fibre damage did not contribute to the relatively smaller size of the signal at this dye concentration.

In three other experiments it was possible to follow absorbance changes over a fairly wide range of dye concentration following injection. Values of peak $\Delta A(650)$/cm are plotted against $A(570)$/cm in Fig. 4*B*. Data from two fibres (triangles and circles, plus the origin) follow a linear relationship between $\Delta A(650)$ and $A(570)$ whereas data from a third fibre (squares) follow a slightly curvilinear relationship. In all three fibres (not shown) the active signal became somewhat shorter in duration as the dye concentration decreased, similar to the results shown in Fig. 3*B*.

The conclusion from these experiments is that the peak amplitude of the active $\Delta A(650)$ signal varies approximately linearly with dye concentration between 0·2 and 0·7 mM, although the proportionality factor appears to vary from fibre to fibre. Thus, results obtained at different dye concentrations in the same fibre can be compared by scaling by dye-related $A(570)$.

Wavelength dependence of the early isotropic Arsenazo III signal. The experiment illustrated in Figs. 3 and 4*A* was somewhat atypical in that there was little intrinsic transmission change and practically no dichroic signal early in the experiment (at 3 min and at 22 min following injection, although a dichroic signal appeared later). Furthermore, the $\Delta A(650)/A(570)$ signals recorded at 7, 13 and 21 min following injection were very similar in their entire time course (Fig. 3*B*). It was therefore possible in the first 21 min to measure the active isotropic signal at different wavelengths during a period in which the $\Delta A(650)/A(570)$ signal, and presumably other $\Delta A(\lambda)/A(570)$ signals, changed very little.

Fig. 5 *A* shows some of the records obtained in the first 21 min following injection. The signals at 570 and at 750 nm are essentially flat as is expected from the Ca^{2+}-difference spectrum shown in Fig. 1*B*. The waveforms at the other wavelengths are similar to one another and in a direction consistent with a transient formation of Ca^{2+}:dye complex.

In order to see whether a single waveform is sufficient to fit the records at all wavelengths, a 650 nm record obtained close in time was scaled to fit each of the other records using a least squares computer program. Fig. 5*B* shows the residuals, i.e. the difference between each experimental record and the scaled 650 nm record. The flat appearance of the residuals shows that at these and other wavelengths (not shown) the time course of the optical change is similar to the time course of the 650 nm record. The residual at 600 nm shows the greatest departure from a perfect fit but it is difficult to tell whether the deviation is real or within experimental error.

Fig. 6 shows peak values of $\Delta A(\lambda)/A(570)$ plotted against wavelength. The circles are from the experiment in Fig. 5 and the smooth curve is the Ca^{2+}-difference curve in Fig. 1*B*, multiplied by 0·094 to fit the average value at 650 nm. The curve provides a rough fit to the experimental points. There are two isosbestic points at 450 and 570 nm and, in particular, there are two peaks at 600 and 650–660 nm which are typical of changes in Ca^{2+}:dye complexation and are not seen with changes in either H^{+}:dye or Mg^{2+}:dye complexation. However, there are clear discrepancies between the shape of the cuvette Ca^{2+}-difference spectrum and the muscle data points. For

example, in all muscle fibres examined the isotropic 650–660 nm signal was larger than the isotropic 600 nm signal, with the 660 nm signal being slightly larger than the one at 650 nm.

If the formation of Ca^{2+}:dye complex follows simple 1:1 stoichiometry and if the kinetics of cation complexation are sufficiently rapid, the scaling factor used for the curve in Fig. 6 would suggest that approximately 0·094 of the dye was complexed with Ca^{2+}, implying that the peak free $[Ca^{2+}]$ is equal to 0·104 times the dissociation constant of the Ca^{2+}:dye complex as required by eqn. (2). However, the fact that the curve does not provide a good fit raises doubts about the adequacy of using the cuvette calibrations shown in Fig. 1 plus the assumption of simple 1:1 stoichiometry.

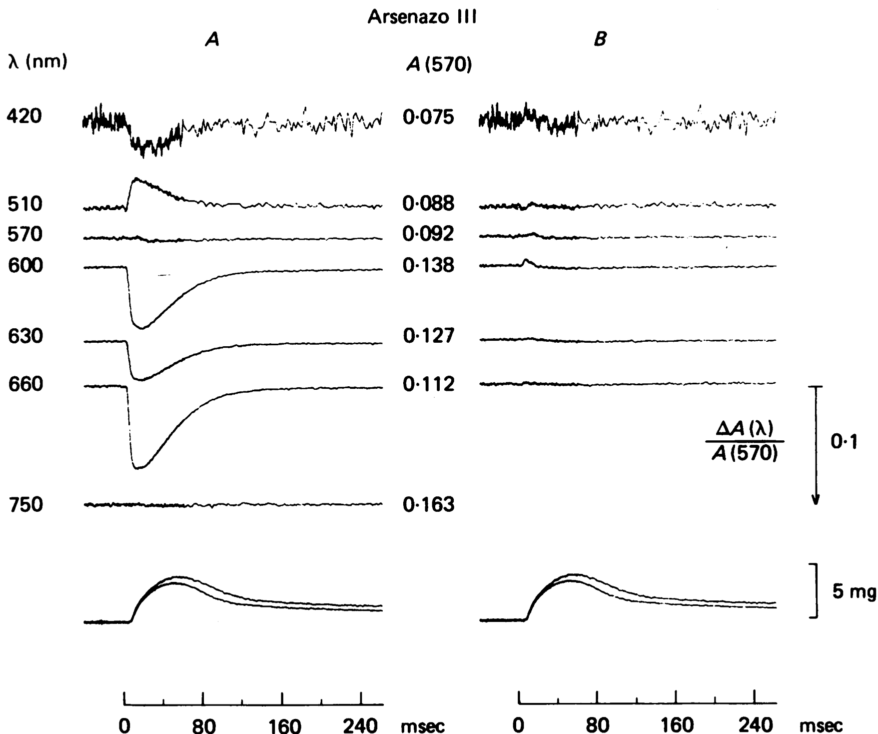


Fig. 5. Changes in Arsenazo III absorbance recorded at different wavelengths. *A*, records of $\Delta A(\lambda)/A(570)$ and of tension taken 3–20 min following injection. Wavelength and dye-related $A(570)$ are indicated beside each optical record. All optical records are single sweeps, obtained using 90° polarized light. The two tension records are the same as in Fig. 3. *B*, residual records obtained by taking the difference between the records in *A* and records at 650 nm (such as those shown in Fig. 3), scaled to give a least squares fit. The 650 nm record taken at the nearest time was selected for the fit.

These doubts are strengthened by recent reports indicating that Ca^{2+}:dye complexes other than 1:1 can occur (Thomas, 1979; R. Y. Tsien, personal communication; Palade & Vergara, 1981; Ríos & Schneider, 1981) and that most of the Arsenazo III injected into muscle fibres may be bound to intracellular constituents (Beeler, Schibeci & Martonosi, 1980). Since binding may change the interactions of dye with Ca^{2+} (cf. Beeler *et al.* 1980) accurate calibrations would require taking into account

the properties of the bound form(s) of Arsenazo III. Until this is done, quantitative conclusions about the absolute level of free [Ca^{2+}] in muscle, determined from Arsenazo III experiments, should be made with caution.

The Ca^{2+}-difference spectrum in Fig. 6, based on the results in Fig. 1*B*, was determined using either 30 μM or 1 mM-Arsenazo III, concentrations which span the range usually used in the muscle experiments. The level of [Ca^{2+}], on the other hand, was saturating. It is natural to ask whether a muscle-like difference spectrum can be obtained in cuvette measurements using the same range of dye concentrations, but with levels of free [Ca^{2+}] which match those measured in muscle fibres, i.e. which give values of $\Delta A(650)/A(570)$ ranging from 0·02 to 0·09. The results shown in Fig. 4 of Palade & Vergara (1981) and our own unpublished calibrations indicate that this cannot be done.

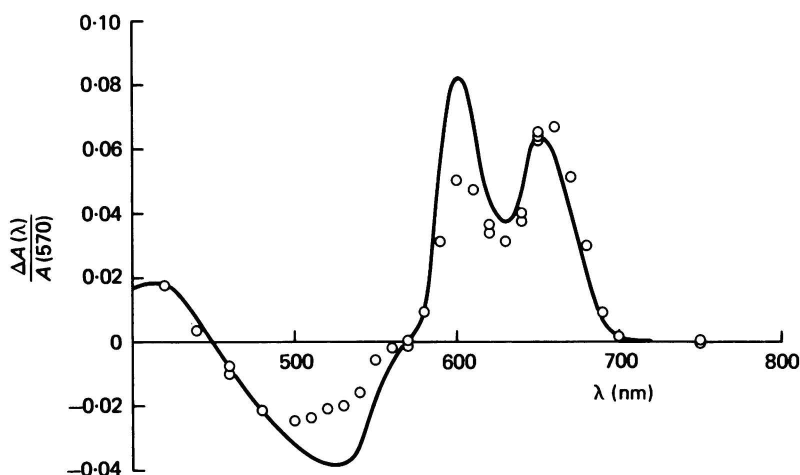


Fig. 6. Wavelength dependence of the early Arsenazo III signal. ○, peak values of $\Delta A(\lambda)/A(570)$ from the experiment in Fig. 5. The curve is the same as in Fig. 1*B*, multiplied by the scaling factor 0·094 so it would fit the average of the 650 nm points.

However, a significantly better fit to the experimental points can be obtained using calibration curves measured at very low dye concentration. Fig. 7 shows the same experimental points as Fig. 6. The curve is based on the difference in absorbance of 40 nM-Arsenazo III measured in a saturating level of free [Ca^{2+}] and in a zero level, [Mg^{2+}] = 1·08 mM and pH = 6·9. The calibration curve shows a larger change at 650 nm than at 600 nm (see also fig. 3*A* of Brown, Brown & Pinto, 1977), in agreement with the experimental points. The valley of the calibration curve, however, is shifted slightly to the left of the minimum shown by the data points. The scaling to fit the 650 nm points also results in the curve lying to the left of the data points at wavelengths greater than 650 nm. Although the over-all fit is improved by using the calibration curve determined at low dye concentrations, deviations are still present which are probably outside the range of experimental error. The qualitative improvement of the fit, and especially the agreement at the 600 and 650 nm peaks, raises the interesting possibility that the stoichiometry of the Ca^{2+} : dye reaction inside the fibre, following a single action potential, may be the same as that observed in

a cuvette at extremely low dye concentration, possibly 1:1 (since cation complexes involving only one dye molecule are favoured at low dye concentration). Such a possibility would be consistent with the linear relationship found between $\Delta A(650)$ and $A(570)$ (Fig. 4). If 1:1 stoichiometry is correct, the scaling factor 0·062 indicates that 0·062 of the dye was bound to Ca^{2+} at the peak of the Ca^{2+} signal and that peak free [Ca^{2+}] was equal to 0·066 times the dissociation constant of the dye for Ca^{2+} (eqn. (2)).

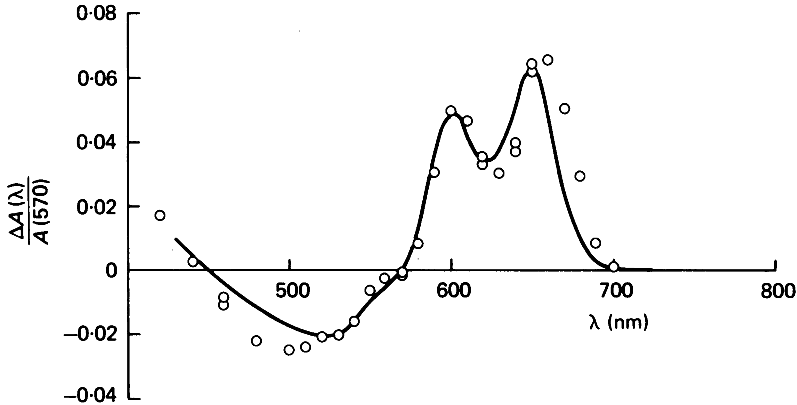


Fig. 7. Wavelength dependence of the early Arsenazo III signal, compared with a Ca^{2+}-difference curve obtained at low dye concentration. Same data points as in Fig. 6. The difference curve was obtained under similar conditions as the curve in Fig. 1*B* (i.e. saturating [Ca^{2+}] minus 0 [Ca^{2+}]) except that the dye concentration was 40 nM, a 10 cm cuvette was used, and a scaling factor of 0·062 was applied to fit the 650 nm points. Preliminary measurements indicate that the dissociation constant of Arsenazo III for Ca^{2+} is 150–200 μM at dye concentrations below 0·1 μM.

Late changes in Arsenazo III absorbance. In the preceding paper (Baylor *et al.* 1982*a*) the possibility of a late change in pH and/or free [Mg^{2+}] was discussed. Since a late signal is seen with two other metallochromic indicators, Antipyrylazo III (see Fig. 11) and Dichlorophosphonazo III (see Figs. 15 and 17*B*), it was of interest to examine carefully the final portions of optical records from fibres injected with Arsenazo III.

Fig. 8 shows the final level of $\Delta A(\lambda)/A(570)$ plotted against wavelength from the experiment illustrated in Fig. 5. The data clearly show that any maintained signal is small and difficult to resolve. Two difference spectra obtained in cuvettes are included for comparison, scaled to match the experimental points at 650 nm. The continuous curve shows the change expected for an increase in pH of 0·01 unit and the dashed curve shows that expected for a 3·4 % increase in free [Mg^{2+}] (both curves relative to resting [Mg^{2+}] = 1·08 mM, pH = 6·9, and [Ca^{2+}] = 0). The evidence to date indicates that under the conditions of our experiments and calibrations, only 1:1 complexes of Mg^{2+}:Arsenazo III are formed (Thomas, 1979; R. Y. Tsien, personal communication; Palade & Vergara, 1981; Ríos & Schneider, 1981; Baylor *et al.* 1982*a*). The data points in the Figure would be consistent with a small increase in either pH or free [Mg^{2+}], or with a small maintained elevation in free [Ca^{2+}] amounting to a few percent of the peak change (compare Fig. 8 with Figs. 6 and 7).

Average values of peak and steady $\Delta A(650)/A(570)$ Arsenazo III signals. Peak and steady values of $\Delta A(650)/A(570)$ from ten fibres having dye concentrations

0·16–0·58 mM are tabulated in Table 1. The peak signal varied considerably from fibre to fibre, ranging from 0·018 to 0·094, and had an average value of 0·05. Although there is an over-all trend in the direction of larger values of $\Delta A(650)/A(570)$ being associated with higher dye concentrations (compare columns 4 and 5), the trend appears to be fortuitous since it is usually not present in individual fibres as shown in Fig. 4.

TABLE 1. Peak and steady values of dye-related $\Delta A(650)/A(570)$ during a twitch in Arsenazo III injected fibres

Fibre reference (1)	Diameter (μm) (2)	$A(570)$ (3)	Arsenazo (mM) (4)	$\Delta A(650)/A(570)$ Peak (5)	$\Delta A(650)/A(570)$ Steady (6)	Steady ÷ Peak (7)
061478·1	88	0·086	0·33	0·018	0·0003	0·017
061578·1	82	0·098	0·40	0·048*	0·0009	0·019
062878·1	53	0·093	0·58	0·065	0·0021	0·032
071378·1	77	0·135	0·58	0·094	0·0033	0·035
010579·1	64	0·045	0·23	0·029†	0·0017	0·059
010579·2	69	0·114	0·55	0·071†	n.r.	—
011979·1	105	0·085	0·27	0·056†	0·0009	0·016
012279·1	90	0·077	0·29	0·025†	0·0013	0·052
012279·2	126	0·061	0·16	0·033†	0·0041	0·124
012379·1	73	0·122	0·56	0·059†	0·0015	0·025
Mean ± S.E.M.				0·050 ± 0·008	0·0018 ± 0·0004	0·042 ± 0·011

* (660, 30) × 1·21. † (660, 10) × 0·95. n.r. = steady level not resolvable.

Column (1) gives the fibre reference; column (2) gives diameter; column (3) gives dye-related $A(570)$ and column (4) gives Arsenazo III concentration based on columns (2)–(3) and a molar extinction coefficient of 3×10^4 M^{-1} cm^{-1} (see Baylor *et al.* 1982*a*). Columns (5) and (6) give peak and steady (0·4–0·5 sec) values of absorbance changes and column (7) gives the ratio. The steady values were taken as the difference between the actual 650 nm measurement and the intrinsic value estimated by linear interpolation between the 570 and 720 or 750 nm measurements. The values of $\Delta A(650)$ are based on our standard 650 nm interference filter, half band 10 nm. Values from fibre 061578·1 where obtained using a 660 nm filter, half band 30 nm; they were multiplied by 1·21 to allow comparison. Similarly, the values from the last six fibres were obtained with a 660 nm filter, half band 10 nm, and they were multiplied by 0·95 to allow comparison.

The steady levels $\Delta A(650)/A(570)$ were so small that the values in columns (6) and (7) of Table 1 represent a rough estimate, at best. Taken literally, the average value in column (6), 0·0018, could be explained by an increase in pH of 0·008 units or an increase in free $[Mg^{2+}]$ of 2·7 % (both changes relative to $[Mg^{2+}] = 1·08$ mM, pH = 6·9, $[Ca^{2+}] = 0$); it could also be explained by a maintained increase in free $[Ca^{2+}]$ of a few percent of the peak value, column (7). There was no indication of a dichroic signal at late times.

Antipyrylazo III

Early changes in absorbance in a fibre injected with Antipyrylazo III. One of the aims of the investigation was to compare optical signals obtained with different Ca^{2+}-sensitive indicator dyes. An obvious candidate to try was the dye Antipyrylazo III, first used in biological systems by Scarpa *et al.* (1978) and later in cut muscle fibres by Kovács, Ríos & Schneider (1979), Schneider, Ríos & Kovács (1981) and Palade & Vergara (1981). The absorbance spectrum of Antipyrylazo III, like Arsenazo III,

is influenced by pH and Mg^{2+}, as well as Ca^{2+}, and therefore it may be necessary to consider changes in ions other than Ca^{2+} in interpreting the results. The stoichiometry of the Mg^{2+}: dye complex is probably different from the Ca^{2+}: dye complex under the conditions of our experiments; Ca^{2+}: Antipyrylazo III appears to be primarily 1:2 in the range of pH, free [Mg^{2+}] and free [Ca^{2+}] likely to be encountered in a muscle fibre (Palade & Vergara, 1981; Ríos & Schneider, 1981) whereas Mg^{2+}: Antipyrylazo III appears to be 1:1 (Ríos & Schneider, 1981).

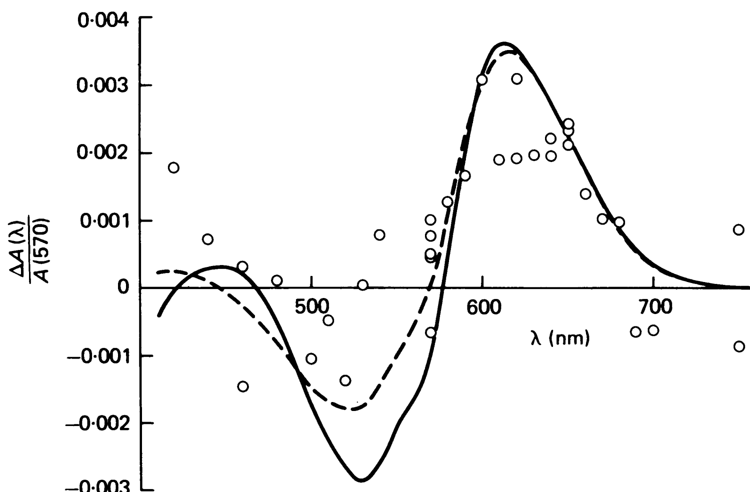


Fig. 8. Wavelength dependence of the maintained Arsenazo III signal. ○, $\Delta A(\lambda)/A(570)$ recorded 0·4–0·5 sec following stimulation, without correction for intrinsic changes. The curves are taken from cuvette calibrations using 150 mM-KCl, 1·08 mM-$MgCl_2$, pH = 6·9 23 μM-Arsenazo III. The continuous curve shows the optical change expected for an increase in pH of 0·01 units, constant [Mg^{2+}] of 1·08 mM. The dashed curve shows the change expected for an increase in [Mg^{2+}] of 3·4% (with respect to 1·08 mM), constant pH 6·9.

Fig. 9*A* shows original optical records obtained from a fibre injected with Antipyrylazo III. Both 0° and 90° plane polarized incident light were used at all wavelengths except 720 nm (where 0° light was used) and 800 nm (where 90° light was used). The 0° and 90° records taken at the same wavelength virtually superimpose indicating that little dichroic signal was present. For further analysis the records at 0° and 90° were averaged. In addition, the records were corrected for intrinsic transmission changes by subtracting the 800 nm record, smoothed and scaled according to $1/\lambda$.

The corrected records, shown in Fig. 9*B*, are therefore the estimated dye-related signals. The first record to consider is the one obtained at 720 nm. At this wavelength changes in pH or Mg^{2+} have little effect on absorbance (although any such change would alter the dissociation constant of dye for Ca^{2+}) whereas the effect of Ca^{2+} on absorbance is large (Scarpa *et al.* 1978; also see Fig. 10 of this paper). This record, then, is a good candidate for a pure Ca^{2+} signal. It resembles the 650–660 nm Arsenazo III signal in Figs. 2*B*, 3 and 5, although it is somewhat briefer in duration as would be expected from the difference in temperature.

For the same reasons given above for the 720 nm signal, the 650 nm record in Fig. 9*B* is also a good candidate for a Ca^{2+} signal. The 650 nm record can be scaled to approximately superimpose the 720 nm record. The other records fail in this respect as they all exhibit maintained changes at late times following stimulation. Thus, it is clear that a single underlying process, namely the formation of a single Ca^{2+}:dye complex, cannot explain all the absorbance changes seen at different wavelengths.

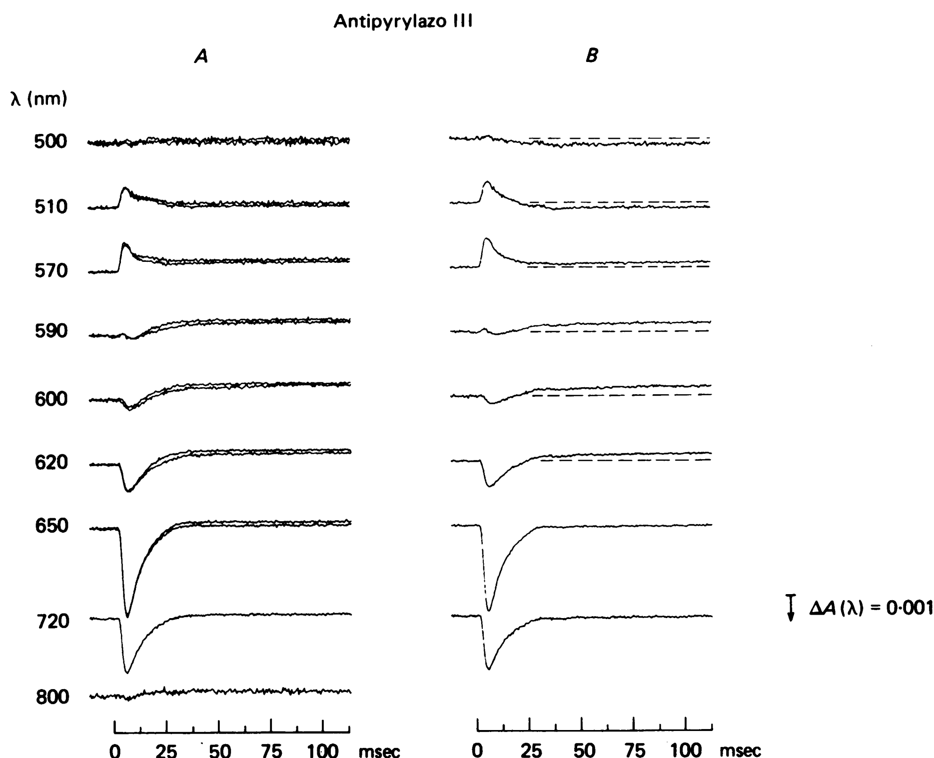


Fig. 9. Absorbance changes at different wavelengths following Antipyrylazo III injection. *A*, single-sweep changes in absorbance recorded at the indicated wavelengths, 0° and 90° polarized light. *B*, records from *A* averaged for 0° and 90° light and corrected for intrinsic transmission changes, estimated by scaling the 800 nm record according to a $1/\lambda$ wavelength dependence. The 800 nm record was digitally smoothed to reduce noise before making the $1/\lambda$ correction. Vertical diameter, 111 μm; sarcomere spacing, 3·9 μm; temperature, 20 °C; fibre 012480·1. In this experiment the dye was pressure injected, rather than ionophoresed into the fibre. Dye-related $A(610)$ varied from 0·10 to 0·08, corresponding to an average dye concentration of approximately 0·53 mM. Tension was not recorded.

Of particular interest is the record taken at 600 nm, a wavelength close to the isosbestic wavelength for formation of Ca^{2+}:dye complex. The trace shows first a transient increase in absorbance (decrease in transmitted light) followed by a maintained decrease in absorbance. Close inspection, however, reveals that the first change does not begin until the earliest signals, such as those at 650 and 720 nm, are about halfway to peak. This suggests that it may be possible to determine the spectral properties of the Ca^{2+} signal by analysing only the first few milliseconds of the optical transients.

The Ca^{2+}-difference spectrum was therefore estimated by scaling the first part of the 720 nm trace, halfway to peak, to give a least squares fit to each of the other traces over the same time period. The scaling constants are plotted as circles in Fig. 10. The curve in the Figure is the difference spectrum obtained in a cuvette when Ca^{2+} was added to a Ca^{2+}-free calibration solution; the curve is also scaled to give unity at 720 nm. The close agreement between the calibration curve and the data points provides strong evidence in support of the idea that the earliest absorbance change is due to the formation of Ca^{2+}:dye complex. The increase in free $[Ca^{2+}]$ necessary to produce the peak concentration of complex is estimated as 2·9 μM, as calculated below.

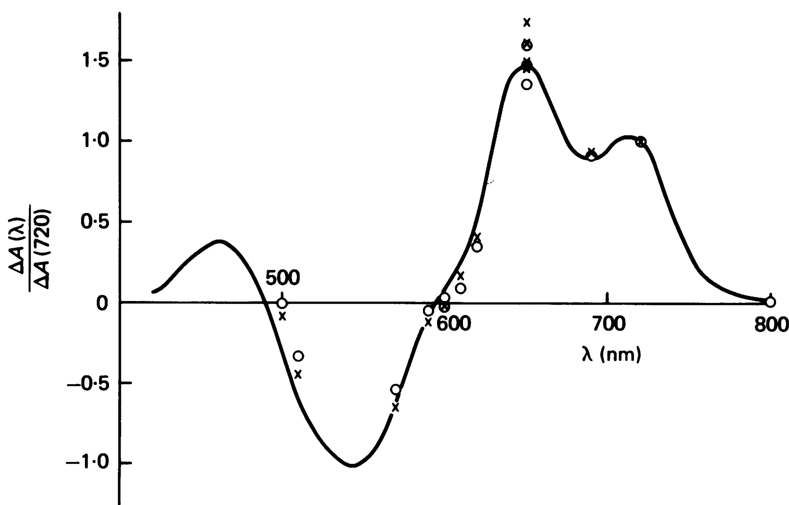


Fig. 10. Wavelength dependence of the earliest change in Antipyrylazo III absorbance. ○, scaling constants determined by fitting the early part of the 720 nm record, half way to peak, to records at different wavelengths. ×, scaling constants for 720 nm obtained by fitting linear combinations of the 600 and 720 nm records to records at different wavelengths (see Fig. 12). The fitting procedures force ○ and × to be unity at 720 nm and × to be zero at 600 nm. From the experiment in Fig. 9. The curve is a scaled cuvette Ca^{2+}-difference spectrum using pH = 6·9, $[Mg^{2+}]$ = 2 mM, Antipyrylazo III = 70 μM, $[Ca^{2+}]$ = 0·25 and 0 mM.

The estimate of peak myoplasmic free $[Ca^{2+}]$ is calculated from the peak value of the 720 nm signal in Fig. 9*B*, ΔA = 0·0022. For the calculation we have used the calibration constants of Ríos & Schneider (1981) who give information about Mg^{2+} as well as Ca^{2+} complexes. The value 0·0022 corresponds to the formation of 12 μM 1:2 complex involving Ca^{2+} and Antipyrylazo III (based on a value of $1{\cdot}64 \times 10^4\ \mathrm{M^{-1}\,cm^{-1}}$ for the change in molar extinction coefficient at 720 nm and a value of 111 μm for fibre diameter). The dissociation constant for the 1:2 complex is $3{\cdot}69 \times 10^{-8}\ \mathrm{M}^2$ in the absence of Mg^{2+}, pH = 6·9. In the presence of 2 mM-Mg^{2+}, which is taken as a rough estimate of internal free $[Mg^{2+}]$ (Cohen & Burt, 1977; Hess & Weingart, 1981; Baylor *et al.* 1982*a*), the apparent dissociation constant increases to $6{\cdot}22 \times 10^{-8}\ \mathrm{M}^2$ (based on a value of 6·7 mM for K_{MgD}, the dissociation constant for Mg^{2+}, and the scaling factor $[1+[Mg^{2+}]/K_{\mathrm{MgD}}]^2$, similar to the factor in eqn. (9) but squared to allow for the 1:2 Ca^{2+}:dye stoichiometry). This value of the apparent dissociation constant, 12 μM 1:2 Ca^{2+}:dye complex, and 0·506 mM-dye not complexed with Ca^{2+} (0·53 mM-total dye minus 2 × 12 μM-complex) give 2·9 μM for the value of peak free $[Ca^{2+}]$.

Late changes in Antipyrylazo III absorbance. It is also possible to determine the spectral shape of the maintained change in Antipyrylazo III absorbance. Fig. 11*A* shows the late change in absorbance, measured approximately 200 msec following stimulation, from the records in Fig. 9*A* and others (not shown). These points have not been corrected for the intrinsic change in fibre absorbance, which can be estimated by scaling the 800 nm value according to $1/\lambda$ as shown by the curve.

The circles in part *B* of Fig. 11 were obtained by first subtracting the $1/\lambda$ curve in part *A* from the points and then scaling by $-1/\Delta A(600)$. Thus, points above the abscissa correspond to an increase in dye-related absorbance. The two curves, based on cuvette calibrations, show the spectral shapes expected for changes in absorbance due to an increase in either pH (continuous curve) or free [Mg^{2+}] (dashed curve). The shape of these curves is markedly different from that of the Ca^{2+} curve in Fig. 10 so that the general agreement between the experimental points and the calibration curves is good evidence that the late Antipyrylazo III absorbance change is not due to a maintained change in Ca^{2+}:dye complex but may be due to changes in H^+:dye and/or Mg^{2+}:dye complex.

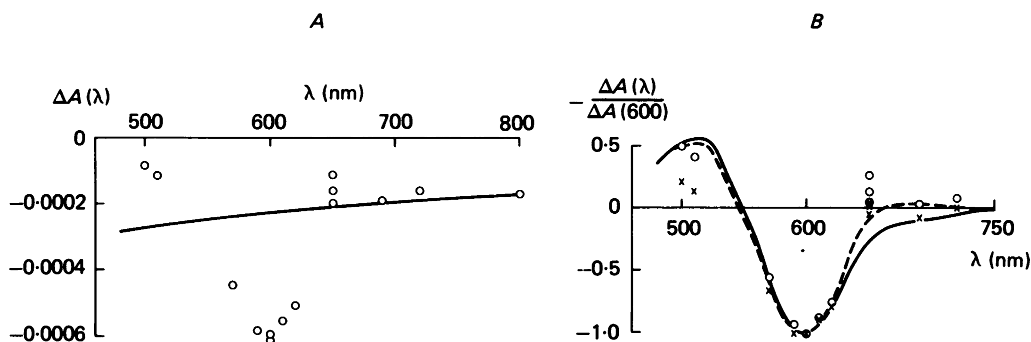


Fig. 11. Wavelength dependence of the maintained change in Antipyrylazo III absorbance. *A*, ○, steady level of $\Delta A(\lambda)$ measured at 200 msec, from the experiment in Fig. 9*A*, uncorrected for the change in intrinsic transmission. The curve shows an estimate of this change, obtained by scaling the 800 nm value according to $1/\lambda$. *B*, ○, steady level of $\Delta A(\lambda)$ from part *A*, corrected for intrinsic changes and normalized by $-\Delta A(600)$. ×, scaling constants for 600 nm obtained by fitting linear combinations of the 600 and 720 nm records to records at different wavelengths. The scaling and fitting procedures force ○ and × to be -1 at 600 nm and × to be zero at 720 nm. The two curves are difference spectra obtained from calibration measurements. They represent the absorbance changes which should accompany an increase in either pH (continuous) or [Mg^{2+}] (dashed) using a base level pH = 6·9 and [Mg^{2+}] = 2 mM, 30 μM-dye, [Ca^{2+}] = 0. The scaling constant used for converting the data in part *A* to *B* makes the pH curve represent an increase of 0·0061 units and the Mg^{2+} curve represent an increase of 1·7 %.

The magnitude of the increase, estimated from our Ca^{2+}-free calibrations and the scaling factors used in Fig. 11*B*, would be (1) 0·0061 pH units (relative to pH 6·9) if free [Mg^{2+}] were constant at 2 mM or (2) a 1·7 % increase in free [Mg^{2+}] (relative to 2 mM) if pH were constant at 6·9.

Reconstruction of Antipyrylazo III absorbance changes in terms of two basic waveforms. The results in Figs. 9–11 show that the wavelength dependence of the earliest change in Antipyrylazo III absorbance resembles a Ca^{2+}-difference spectrum whereas the late

change resembles a H^+- and/or Mg^{2+}-difference spectrum. This section shows that the entire time course of the absorbance change at all wavelengths can be explained in terms of two basic waveforms, one having the wavelength dependence of the Ca^{2+}-difference spectrum and the other having the wavelength dependence of the H^+- and/or Mg^{2+}-difference spectrum.

The approach was to use the 720 nm record in Fig. 9*B* to represent the Ca^{2+}:dye waveform and the 600 nm record to represent the H^+:dye/Mg^{2+}:dye waveform. Each of these wavelengths was selected to make the desired signal large and the undesired signal small. A least squares computer program selected the scaling constants.

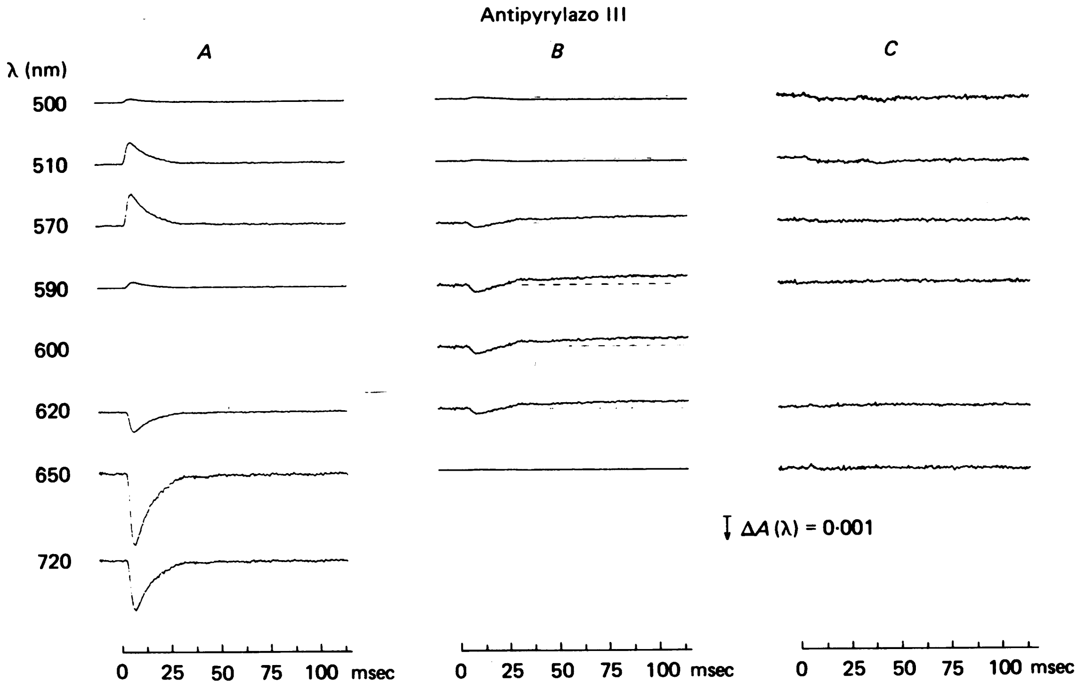


Fig. 12. Reconstruction of Antipyrylazo III signals in terms of two basic waveforms. A least squares computer program was used to fit a linear combination of the 720 and 600 nm records to each of the other records in Fig. 9*B*. The assignments of the 720 and 600 nm records are shown in *A* and *B*, respectively. *C* shows the residuals, i.e. the difference between the experimental record (Fig. 9*B*) and the fitted waveform (sum of *A* plus *B*).

Fig. 12*A* shows the amount of 720 nm (Ca^{2+}:dye) waveform assigned to each of the records in Fig. 9*B*. Fig. 12*B* shows the corresponding assignment of the 600 nm (H^+:dye/Mg^{2+}:dye) waveform. The difference between the actual records in Fig. 9*B* and the sum of the two assignments in parts *A* and *B* is shown in part *C*. These residual records are sufficiently flat to be consistent with the idea that only two underlying waveforms are required to explain the time course of the Antipyrylazo III signals at all wavelengths.

The scaling constants from Fig. 12, parts *A* and *B*, are plotted as crosses in Figs. 10 and 11*B*. They agree closely with the previously obtained circles and with the calibration curves. This agreement provides additional support for the notion that

one of the basic waveforms is due to the transient appearance of Ca^{2+}:dye complex whereas the other basic waveform is produced by changes in H^+:dye and/or Mg^{2+}:dye complex. Unfortunately, the small amplitude of the maintained signal and the similarity of the H^+- and Mg^{2+}-difference spectra prevents resolution of the second waveform into the individual changes associated with H^+:dye and Mg^{2+}:dye complexation.

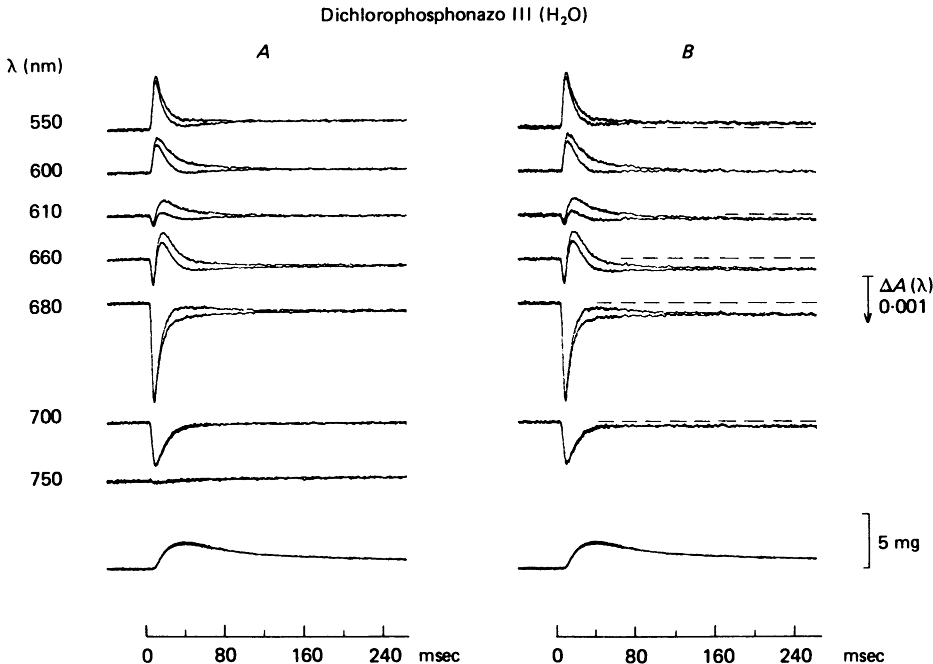


Fig. 13. Absorbance changes at different wavelengths following Dichlorophosphonazo III injection. *A*, changes in absorbance recorded at different wavelengths using 0° and 90° plane polarized light. Each trace is the average of two sweeps. For $\lambda \leqslant 680$ nm the 90° record is on top. The tension traces show two records, superimposed, taken at the beginning and end of the run. Dye-related $A(600)$ decreased from 0·175 to 0·158 during the run, corresponding to dye concentrations 0·359–0·324 mM. *B*, same records as in *A* after correction for the intrinsic transmission change, made by subtracting the 750 nm trace scaled by $1/\lambda$. Vertical diameter, 80 μm; horizontal diameter, 116 μm; sarcomere spacing, 3·9 μm; temperature, 15·9 °C; fibre 070678·2.

Dichlorophosphonazo III

Changes in absorbance in fibres injected with Dichlorophosphonazo III. The third Ca^{2+}-sensitive indicator which was injected was Dichlorophosphonazo III, a high affinity dye used in squid axons by Brown *et al.* (1975) and in photoreceptor experiments by Yoshikami & Hagins (1978). According to the latter authors, the stoichiometry of Ca^{2+}:dye is 1:1 with $K_{CaD} = 1{\cdot}55$ μM at $[Mg^{2+}] = 0$ and pH = 6·8. The absorbance of the dye is also sensitive to pH and Mg^{2+}, as well as to Ca^{2+} (Yoshikami & Hagins, 1978).

Fig. 13*A* shows original records from a muscle fibre taken at different wavelengths, using 0° and 90° light, and Fig. 13*B* shows the records after correction for the intrinsic transmission change, made by subtracting the 750 nm records scaled according to

$1/\lambda$. The records in Fig. 13*B*, which are considered to be dye-related, are rather complicated and are composed of at least three different waveforms: (1) an early isotropic waveform which is clearly upward at 550 and 600 nm and downward at 660, 680 and 700 nm; (2) a later dichroic waveform, indicated by a non-zero difference in the 0° and 90° traces for wavelengths between 550 and 680 nm; (3) a maintained, isotropic waveform. The isotropic signals, (1) and (3), will be described below and the dichroic signal (2) will be described in the following paper (Baylor *et al.* 1982*b*).

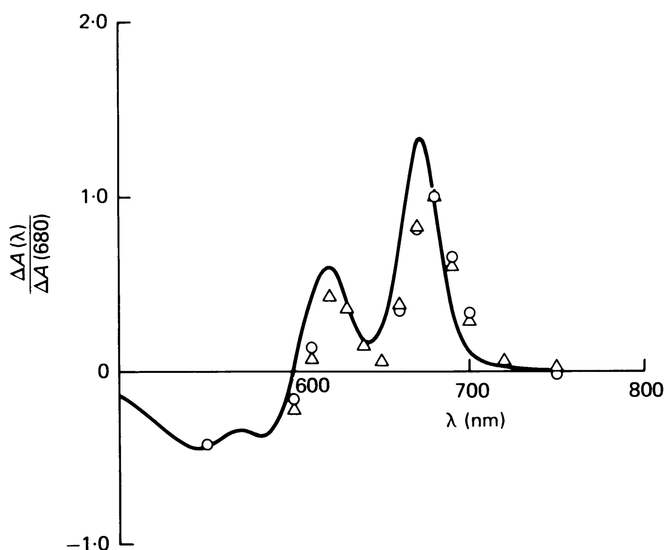


Fig. 14. Wavelength dependence of the earliest change in Dichlorophosphonazo III absorbance. ○, scaling constants determined by fitting the early part of the 680 nm absorbance change, halfway to peak, to absorbance changes at different wavelengths. Averaged 0° and 90° records in Fig. 13 and other records not shown were used for the fits. △, similar data from another fibre. Dye-related $A(600)$ varied from 0·071 to 0·064 corresponding to 139–125 μM dye. Vertical diameter, 98 μm; horizontal diameter, 122 μm; sarcomere spacing, 3·9 μm; temperature 15·6 °C; fibre 070678·1. The curve represents the Ca^{2+}-difference spectrum for 0·3 μM-dye, 0·3 mM-Mg^{2+}, and pH = 6·9. It was obtained by subtracting a $[Ca^{2+}] = 0$ curve (containing 0·5 mM-EGTA) from a saturating $[Ca^{2+}]$ curve; the curve was then scaled by dividing by $\Delta A(680)/A(600) = 0{\cdot}305$.

Early changes in Dichlorophosphonazo III absorbance. The complex shape of the records in Fig. 13*B* makes it difficult to separate unambiguously the various underlying waveforms. Since the earliest absorbance change observed with Dichlorophosphonazo III is isotropic and occurs at about the same time as the early Ca^{2+} signals obtained with Arsenazo III and Antipyrylazo III, it seems likely that this signal also reflects the formation of Ca^{2+}:dye complex. If this idea is correct, the wavelength dependence of the signal should match a Ca^{2+}-difference spectrum obtained in a cuvette.

Fig. 14 shows data points obtained by scaling the average 680 nm signal, halfway to peak, to the signals at other wavelengths. The circles are from the experiment in Fig. 13 and the triangles are from another experiment. The curve is the Ca^{2+}-difference spectrum determined at a low dye concentration (0·3 μM), pH = 6·9, $[Mg^{2+}]$ = 0·3 mM, and scaled to give unity at 680 nm. The value for $[Mg^{2+}]$ is probably less than the

correct myoplasmic value for resting free $[M^{2+}]$ but was selected to be consistent with the resting level indicated by this dye (Baylor *et al.* 1982*a*).

The curve in Fig. 14 shows reasonable agreement with the data points although the curve lies slightly to the left of the points. The shift is most apparent at 600–610 nm, around the isosbestic wavelength, and at 670–680 nm where the curve peaks. The maximum in the cuvette curve is near 670 nm whereas the muscle values from both experiments were largest using the 680 nm filter.

Although the wavelength dependence of the early absorbance change, Fig. 14, agrees qualitatively with the Ca^{2+}-difference spectrum, a quantitative problem arises with respect to the magnitude of the signal. The peak of the 680 nm signal in Fig. 13*B* is 0·0021 absorbance units and dye-related $A(600)$ was 0·165 at the time that the records were taken. The experimental value of $\Delta A(680)/A(600)$, 0·0127, is only 0·042 times the value 0·305 which was obtained for the Ca^{2+}-difference calibration in Fig. 14 using a saturating level of $[Ca^{2+}]$. This indicates that only 4·2% of the dye was complexed with Ca^{2+} at the peak of the signal. On the basis of 1:1 stoichiometry, $K_{CaD} = 1{\cdot}55\ \mu M$ (at pH = 6·8, Yoshikami & Hagins, 1978; our preliminary calibrations at pH = 6·9, 0·3 μM dye give approximately 1·5 μM), $K_{MgD} = 0{\cdot}21$ mM (Baylor *et al.* 1982*a*), $[Mg^{2+}] = 0{\cdot}3$ mM and eqns. (8) and (9), the peak value of free $[Ca^{2+}]$ would be only 0·165 μM, a value altogether too low to be responsible for contractile activation during a twitch.

There are several possible explanations for the small size of the early Dichlorophosphonazo III signal. Five specific examples are listed below, of which (3) and (5) seem to be the most reasonable.

(1) The speed of reaction of free dye with Ca^{2+} is too slow to follow the Ca^{2+} transient. This seems unlikely since reactions of this dye with Ca^{2+} are reported to be rapid (P. Smith, quoted in Yoshikami & Hagins, 1978) and the Dichlorophosphonazo III Ca^{2+} signal appears to have a similar time course to peak as the Ca^{2+} signals obtained with the other two dyes (Fig. 18).

(2) The earliest Ca^{2+} signal is severely contaminated by other signals by the time of peak. For example, in Fig. 13 the peak of the 700 nm trace may be a better indication of free $[Ca^{2+}]$ than the 680 nm trace which contains a significant dichroic component as well as a faint suggestion of an upward component (clearly seen at 660 nm). The calibration curve in Fig. 14 would need to be multiplied by 2·75 to match the 700 nm point, giving $2{\cdot}75 \times (0{\cdot}042) = 0{\cdot}116$ for the fraction of dye bound to Ca^{2+}. This raises the estimate of free $[Ca^{2+}]$ from 0·165 to 0·49 μM. Even this value seems almost an order of magnitude too small (for example, the Antipyrylazo III estimate was 2·9 μM). Although this explanation cannot be ruled out, it seems unlikely that the true Ca^{2+} signal in Fig. 13 could be so heavily masked.

(3) Most of the Dichlorophosphonazo III is bound to intracellular constituents and this binding decreases the affinity for Ca^{2+}. This explanation is a reasonable possibility. If binding also decreased the affinity for Mg^{2+} by an order of magnitude, the estimate for resting free $[Mg^{2+}]$, 0·3 mM (Baylor *et al.* 1982*a*), would need to be increased to 3 mM, which is more consistent with estimates based on other methods (Cohen & Burt, 1977; Hess & Weingart, 1981).

(4) Some of the Dichlorophosphonazo III is bound to intracellular constituents and cannot react with Ca^{2+} or Mg^{2+}. This explanation, like (3) above, could also account for the low estimate of myoplasmic free $[Mg^{2+}]$. The simplest case to consider is that the bound dye does not react with Mg^{2+} or Ca^{2+} and that the remaining dye behaves as though it were in free solution, with the ability to form 1:1 complexes with Mg^{2+} and Ca^{2+} rapidly. Using 2 mM for free $[Mg^{2+}]$ and 0·21 mM for K_{MgD} (Baylor *et al.* 1982*a*), eqn. (9) indicates that the apparent dissociation constant of the unbound dye for Ca^{2+} should be $1{\cdot}55 \times (1 + 2/0{\cdot}21) = 16{\cdot}3\ \mu M$, possibly resulting in an increase in the previous estimate of peak free $[Ca^{2+}]$. Using the same values of free $[Mg^{2+}]$ and K_{MgD}, eqn. (6) indicates that 90% of the dye which is not bound to cellular constituents inside the resting fibre would be complexed with Mg^{2+}. According to eqn. (12), then, the observed difference spectrum should be close to the difference between the Ca^{2+}- and Mg^{2+}-difference spectra, $\Delta\epsilon_{CaD} - \Delta\epsilon_{MgD} = \epsilon_{CaD} - \epsilon_{MgD}$.

Measurements made by Yoshikami & Hagins (1978) and confirmed by us indicate that the molar extinction coefficient of Mg^{2+}:dye is greater than that for Ca^{2+}:dye for λ between 600 and 700 nm. This means that an increase in [CaD] should give rise to a decrease in dye absorbance rather than the increase which was observed, Fig. 14. Thus, explanation (4) fails to work if the speed of reaction between Mg^{2+} and dye is sufficiently rapid that at any moment during the Ca^{2+} transient, equilibrium conditions (i.e. eqn. (6)) hold for Mg^{2+}:dye complexation.

(5) Same as explanation (4) with the additional assumption that the reaction rates associated with Mg^{2+}:dye complexation (eqn. (13)) are limiting. In this case, eqn. (19) should determine the ratio $\Delta[MgD]/\Delta[CaD]$ during the early rising phase of the Ca^{2+} transient. The agreement shown between the data points and the curve in Fig. 14 indicates that the ratio is approximately the one which is determined from eqn. (10) using $[Mg^{2+}] = 0{\cdot}3$ mM. Since $K_{MgD} = 0{\cdot}21$ mM (Baylor *et al.* 1982*a*), eqn. (10) gives $\Delta[MgD]/\Delta[CaD] = -0{\cdot}6$. If the true value of resting free $[Mg^{2+}]$ is taken to be 2 mM, the ratio $\alpha/k_2([Mg^{2+}]+K_{MgD})$ according to eqn. (19) is approximately 0·5. The values of α for the two fibres in Fig. 14 were 1–2 $msec^{-1}$ so that $k_2([Mg^{2+}]+K_{MgD})$ would need to be 2–4 $msec^{-1}$, implying that $k_2 = 1\text{–}2 \times 10^6\ M^{-1}\ sec^{-1}$ (using $[Mg^{2+}] = 2$ mM, $K_{MgD} = 0{\cdot}21$ mM). Although measurements of k_2 have not been carried out, as far as we know, the above values fall within the range of rate constants reported by Diebler *et al.* (1969) for reactions between Mg^{2+} and a variety of ligands.

With this explanation, the shape of the observed difference spectrum during the quasi-exponential rising phase would follow eqns. (19) and (11). After the exponential phase, the observed difference spectrum at any given time would depend on the exact shape of the [CaD] waveform as well as on the rate constants governing the complexation of Mg^{2+} with dye. In general, the wavelength dependence of the absorbance change would correspond to a linear combination of the Mg^{2+}- and Ca^{2+}-difference spectra and the relative proportion of one to the other would vary during the transient. At times somewhat after the peak in the 700 nm signal in Fig. 13, there is an indication at 610 and 660 nm that the isotropic absorbance change might actually reverse, in keeping with the idea that there is a greater contribution from the Mg^{2+}-difference spectrum as Mg^{2+} slowly dissociates from Mg^{2+}:dye complex. Unfortunately, the presence of the dichroic signal makes an exact analysis difficult. To pursue this explanation any further, it would be necessary to eliminate the dichroic signal and to know the Dichlorophosphonazo III rate constants for Mg^{2+} complexation.

Late changes in Dichlorophosphonazo III absorbance. After about 200 msec the absorbance changes shown in Fig. 13 reach an apparent steady level and this maintained change appears to be isotropic. The circles in Fig. 15*A* show the spectrum of the late change from the experiment in Fig. 13*A*, uncorrected for intrinsic changes. The curve shows the estimated intrinsic change, using the 750 nm value and a $1/\lambda$ scaling. The circles in Fig. 15*B* show the dye-related maintained change, obtained by subtracting the curve in Fig. 15*A* from the data points. The two curves in Fig. 15*B*, from cuvette calibrations, show the changes in absorbance which should accompany an increase in pH of 0·0043 units (continuous curve) or an increase in free $[Mg^{2+}]$ of 0·8% (dashed curve). A reasonable fit could also be made using a Ca^{2+}-difference spectrum, Fig. 14. Since the wavelength dependence of Antipyrylazo III late changes is consistent with an increase in pH and/or free $[Mg^{2+}]$ (Fig. 11*B*), and not free $[Ca^{2+}]$ (see Fig. 10), we have tentatively ruled out a maintained elevation in free $[Ca^{2+}]$ as the explanation for the Dichlorophosphonazo III late signal.

Effect of D_2O on the Dichlorophosphonazo III signal. A somewhat simpler picture emerges if a fibre containing Dichlorophosphonazo III is placed in a Ringer solution in which H_2O has been replaced with D_2O. Fig. 16*A* shows records taken with 0° and 90° polarized light. The main difference between these records and those taken in H_2O, Fig. 13*A*, is the virtual absence of the dichroic signal.

Fig. 16*B* shows the isotropic waveforms, 0° and 90° records averaged, corrected for the intrinsic transmission change. Since the individual waveforms at different

wavelengths will not scale one into the other, it is again clear that more than one underlying dye signal is involved. Fig. 17 shows the wavelength dependence of the rising phase (*A*) and of the maintained signal (*B*). The curves are taken from the same [Ca^{2+}], pH and [Mg^{2+}] calibrations that were shown in Figs. 14 and 15*B* since separate calibration measurements were not carried out in D_2O. As was the case for the isotropic signals in H_2O Ringer (Figs. 14 and 15*B*), the rising phase is qualitatively consistent with an increase in Ca^{2+}:dye complex (Fig. 17*A*) whereas the maintained change is consistent with a change in H^+:dye or Mg^{2+}:dye complex (Fig. 17*B*). In addition, the traces at all wavelengths could be approximated by linear combinations of two basic waveforms (not shown), similar to the results shown in Fig. 12 for Antipyrylazo III. This supports the idea that at all times the Dichlorophosphonazo III isotropic signal can be explained by a combination of a Ca^{2+} signal and a H^+/Mg^{2+} signal.

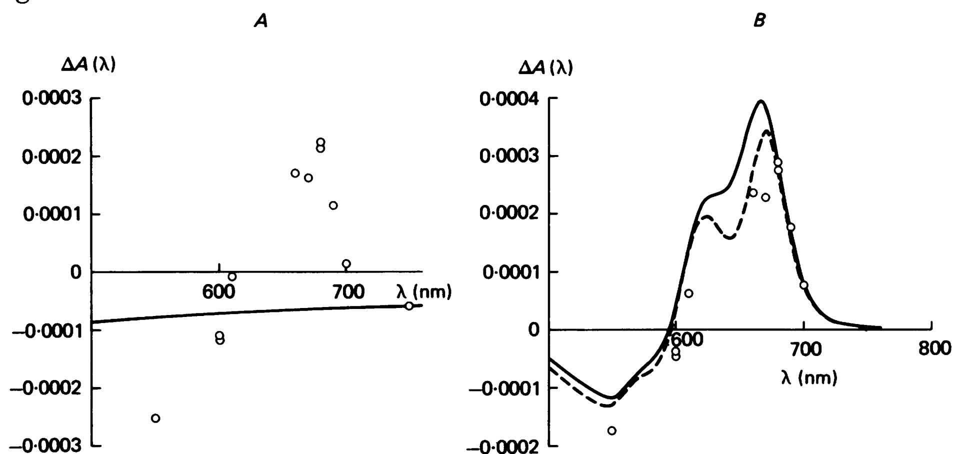


Fig. 15. Wavelength dependence of the maintained change in Dichlorophosphonazo III absorbance. *A*, ○, steady level of $\Delta A(\lambda)$, measured at 250 msec, from the experiment in Fig. 13*A*, uncorrected for the change in intrinsic transmission. The curve shows an estimate of this change, obtained by scaling the value at 750 nm according to $1/\lambda$. *B*, ○, steady level of $\Delta A(\lambda)$ from part *A*, with intrinsic transmission values subtracted. The two curves, obtained from calibration measurements, show the change expected for an increase in either pH (continuous curve, $\Delta pH = 0{\cdot}0043$, fixed [Mg^{2+}]) or [Mg^{2+}] (dashed curve, $\Delta[Mg^{2+}]/[Mg^{2+}] = 0{\cdot}008$, fixed pH). The calibration solution contained 30 μM-dye, 0-Ca^{2+}; [Mg^{2+}] = 0·3 mM and pH = 6·9 were used for base levels.

In another experiment in D_2O (Fig. 19*B*) a very small amount of dye was injected, estimated to be no more than 23 μM. Dye-related signals were isotropic and appeared to follow predominantly one waveform, similar in time course to the Ca^{2+} waveform seen with Arsenazo III and Antipyrylazo III. The wavelength dependence of the amplitude of the signal was qualitatively similar to that shown by the data points in Fig. 14 for an increase in Ca:dye complex.

In summary, the results indicate that Dichlorophosphonazo III signals in D_2O Ringer are primarily isotropic, with an early Ca^{2+} waveform and a late H^+/Mg^{2+} waveform. The two waveforms may be present in different relative amounts at different dye concentrations; for example, with very little dye inside a fibre, the Ca^{2+} waveform appears to predominate (Fig. 19*B*). The reason for such a concentration

dependence is unclear. Perhaps the first small amount of dye that is injected is complexed or bound in a fashion that would still allow reaction with Ca^{2+} but would not allow the changes associated with the H^+/Mg^{2+} signal.

Comparison of Arsenazo III, Antipyrylazo III and Dichlorophosphonazo III signals

Temporal comparison of the earliest absorbance changes. Since the three indicator dyes may react with Ca^{2+} ions with somewhat different speeds (see pp. 142–143) it was of interest to compare the early time course of the Ca^{2+} signals which are observed with the different dyes. Because all the dyes absorb light in the same range of wavelengths, it did not seem practicable to make direct comparisons by injecting two dyes into the same fibre with hopes of reliably measuring individual Ca^{2+} signals from each of the two dyes. Rather, different fibres were injected with different dyes and the intrinsic birefringence (strictly speaking, optical retardation) signal (Baylor & Oetliker, 1977*a*) of each fibre was used as a temporal bench mark.

Fig. 18 shows results from three fibres using the three dyes in question. Part *A* shows superimposed records of 660 nm Arsenazo III absorbance and 750 nm

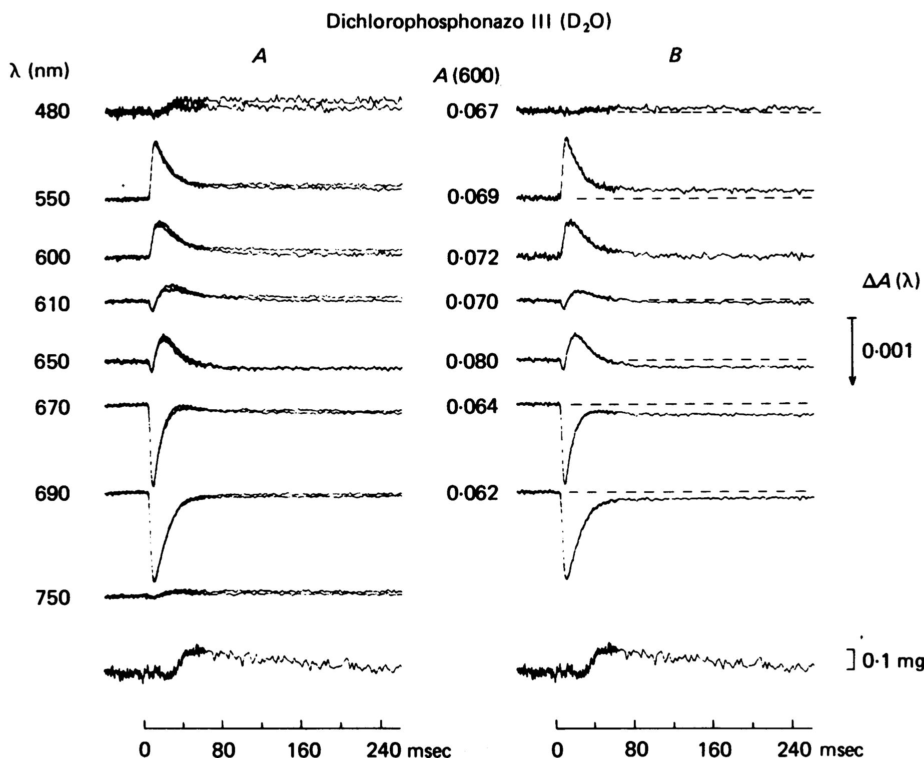


Fig. 16. Absorbance changes following Dichlorophosphonazo III injection with fibre in D_2O Ringer. *A*, changes in absorbance using 0° and 90° polarized light. Wavelength and dye-related *A*(600) are indicated beside each pair of records. Each trace is the average of two sweeps. *B*, records at each wavelength in *A* were averaged and then corrected for intrinsic transmission changes by subtracting the 750 nm record scaled according to $1/\lambda$. Vertical diameter, 80 μm; horizontal diameter, 98 μm; temperature, 15·5 °C; fibre 071178·2.

birefringence, scaled so that the peaks match. The rising phases superimpose almost exactly, in agreement with Suarez-Kurtz & Parker (1977); the times-to-half-peak are within 0·1 msec of each other. After the peak the traces diverge and at later times a movement artifact is apparent on the birefringence record. Similar comparisons were carried out in a total of eleven fibres injected with Arsenazo III. On average the time-to-half-peak for birefringence lagged that for Arsenazo III absorbance by 0·4 msec (standard deviation $\pm$0·5 msec range, $-$0·4 to $+$1·4 msec).

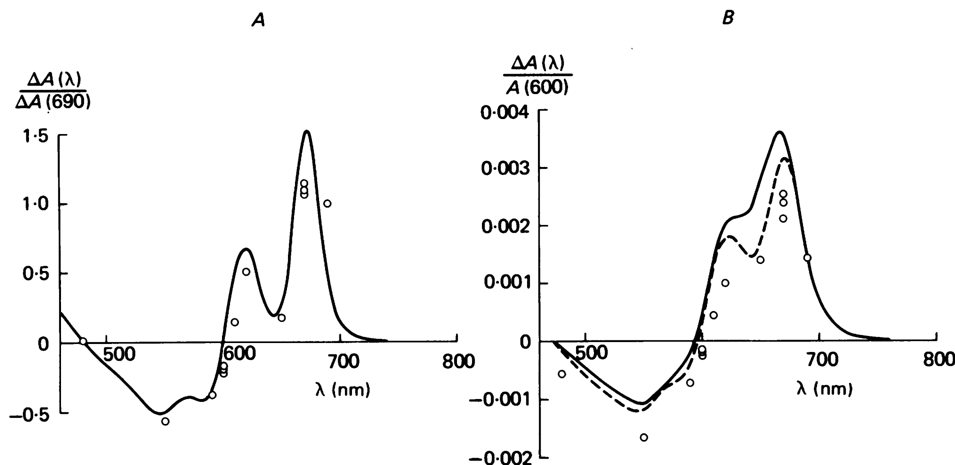


Fig. 17. Wavelength dependence of the rising phase and the maintained level of the Dichlorophosphonazo III signal recorded in D_2O Ringer. From the experiment in Fig. 16*B*. *A*, ○, scaling constants, adjusted for changes in *A*(600), obtained by fitting the 690 nm signal, halfway to peak, to the other signals. The curve is taken from Fig. 14 and arbitrarily scaled. *B*, ○, steady levels of absorbance (200–250 msec) normalized by *A*(600) to allow for decreasing dye concentration during the experiment. The continuous and dashed curves are scaled from those in Fig. 15*B* and represent the absorbance changes expected in H_2O for a 0·0065 unit increase in pH (continuous curve) or a 1·2 % increase in $[Mg^{2+}]$ (dashed curve).

Fig. 18*B* shows a similar comparison with Antipyrylazo III. The rising phases of the two signals virtually superimpose, with birefringence lagging dye absorbance by 0·2 msec halfway to peak.

Fig. 18*C* shows the Dichlorophosphonazo III comparison, with birefringence lagging dye absorbance by 0·2 msec halfway to peak. This experiment was done using D_2O to avoid complications due to the presence of any dichroic component. This solution reduces the magnitude of the birefringence signal somewhat (Baylor & Oetliker, 1977*a*). The Ca^{2+} waveform was estimated by taking the difference between the 690 and 650 nm records, since the late (H^+/Mg^{2+}) signals at these wavelengths are within a factor of 1·03 of each other (Fig. 17*B*) whereas the early (Ca^{2+}) signals are clearly different (Fig. 17*A*).

In carrying out these comparisons it is important to make certain that the birefringence signal which is measured reflects only changes in the intrinsic optical properties of the fibre and is not contaminated by dye-related signals. Contamination by dye-related isotropic absorbance changes was avoided by making birefringence measurements at long wavelengths where the dyes do not absorb light. Contamination

at long wavelengths, however, would still accompany the dye-related dichroic absorbance change at shorter wavelengths, due to the Kramers–Kronig relationship (e.g. see Ross, Salzberg, Cohen, Grinvald, Davilla, Waggoner & Wang, 1977). However, the magnitude of such a dye-related component of birefringence is estimated to be small (unpublished calculation) and its main contribution should occur after the peak of birefringence since the dichroic signal develops after the Ca^{2+} signal (Baylor *et al.* 1982*b*).

The finding that the Ca^{2+} transients which are measured with all three dyes virtually superimpose the intrinsic birefringence signal through time-to-peak is rather surprising in view of the different reaction speeds which have been reported for the

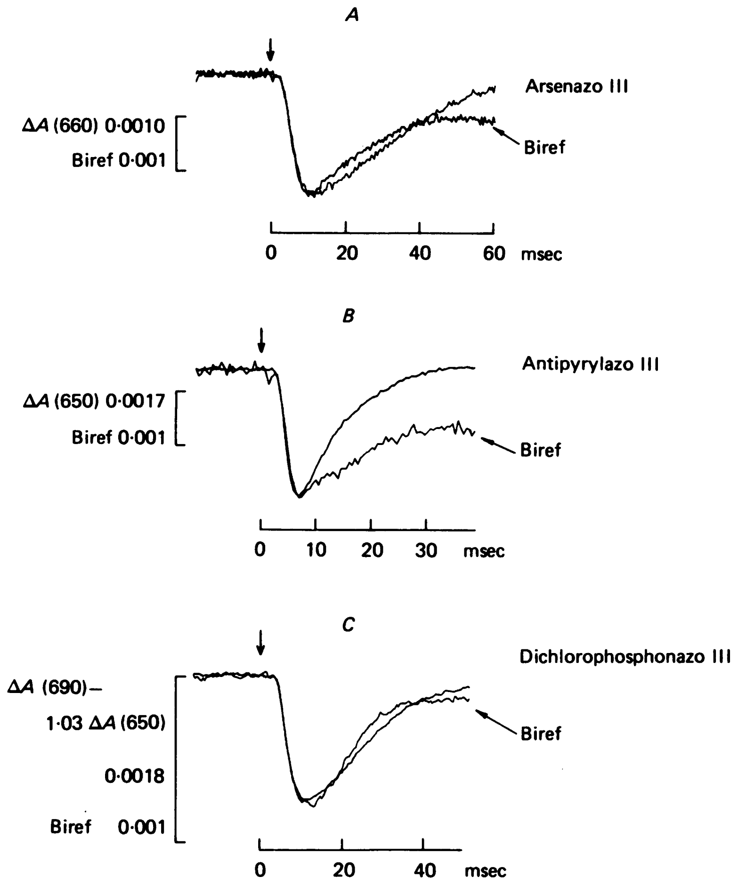


Fig. 18. Temporal comparison of early isotropic signals and birefringence. *A*, single sweep $\Delta A(660)$ and 750 nm birefringence signals following Arsenazo III injection. Dye-related $A(570) = 0{\cdot}077$ corresponding to 0·29 mM-dye. Vertical diameter, 95 μm; horizontal diameter, 90 μm; sarcomere spacing, 3·7 μm; 15·3 °C; fibre 012279·1. *B*, single sweep $\Delta A(650)$ and 800 nm birefringence signal following Antipyrylazo III injection. Same experiment as shown in Fig. 9. *C*, composite $\Delta A(690) - 1{\cdot}03\Delta A(650)$ signal and single sweep 750 nm birefringence signal following Dichlorophosphonazo III injection, D_2O Ringer. Same experiment as shown in Fig. 16. The calibration for the birefringence signals is given in units $\Delta I/I$. In all cases, the birefringence signals at late times may be contaminated by movement-related artifacts.

dyes (see pp. 142–143). If the birefringence signal bears a unique temporal relation to the free [Ca^{2+}] transient, at least to time-to-peak, the results imply that all three dyes track myoplasmic free [Ca^{2+}] with similar delays. Since the kinetics of Ca^{2+}:Antipyrylazo III complexation are reckoned to be rapid (Scarpa *et al.* 1978; Ogawa *et al.* 1980), it is tempting to suppose that all three dyes track free [Ca^{2+}] with little detectable delay during a twitch and that this is the reason that the optical signals always superimpose the birefringence signal during the rising phase. Furthermore, if the birefringence signal also reflects binding of Ca^{2+} to a myoplasmic site (Suarez-Kurtz & Parker, 1977), then the delay associated with Ca^{2+}: site complexation must be comparable to the delays of Ca^{2+}:dye complexation.

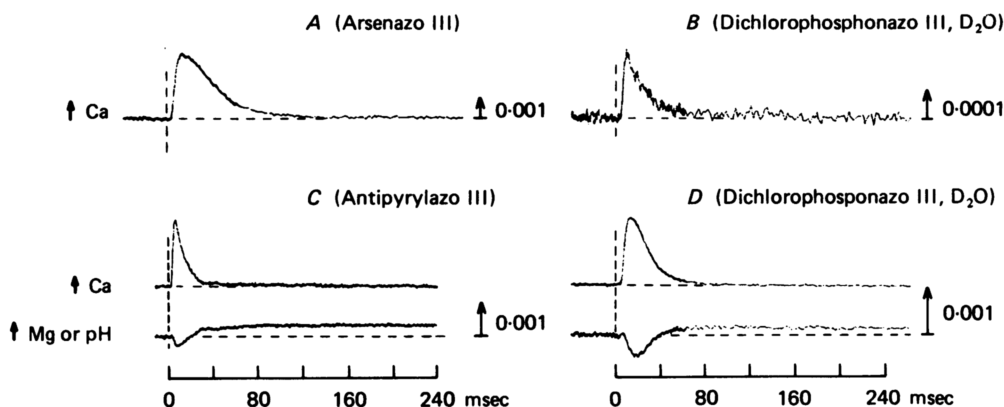


Fig. 19. Comparison of isotropic waveforms recorded with Arsenazo III, Antipyrylazo III and Dichlorophosphonazo III. The polarity of the signals has been adjusted so that an increase in [Ca^{2+}] or pH/[Mg^{2+}] is an upward deflexion. No calibration is given for [Ca^{2+}] or pH/[Mg^{2+}]; the arrows at the right show the magnitude of the absorbance change. *A*, Arsenazo III signal recorded at 650 nm. Same as the record taken at 42 min in Fig. 3*A*. *B*, Dichlorophosphonazo III signal following a weak injection, recorded at 670 nm (corrected for the intrinsic transmission signal by subtracting the 750 nm signal scaled according to $1/\lambda$). Dye-related *A*(600) was too small to measure reliably, $\leqslant 0{\cdot}01$ corresponding to $\leqslant 23$ μM dye. Vertical diameter, 97 μm; horizontal diameter, 102 μm; sarcomere spacing, 3·9 μm; D_2O Ringer, 15·3 °C; fibre 071278·1. *C*, Antipyrylazo III signals recorded at 720 nm (upper trace) and at 600 nm (lower trace). The absorbance change in the lower trace is in the opposite direction to that indicated by the arrow. Same as the corresponding records in Fig. 9*B*. *D*, Dichlorophosphonazo III signals from the experiment in Fig. 16*B*. Upper record, 690 nm record minus 1·03 times 650 nm record. Lower record, 650 nm record minus 1·19 times 610 nm record. See text for additional details.

Comparison of Ca^{2+} waveforms obtained with the three dyes. Fig. 19 shows four Ca^{2+} waveforms from four fibres, plotted on the same time base and scaled to give similar peak amplitudes to facilitate comparison. Record *A* shows an Arsenazo III signal, recorded at 650 nm. Record *B* shows a Dichlorophosphonazo III signal, recorded at 670 nm, from a weakly injected fibre placed in D_2O Ringer. The top record in *C* shows an Antipyrylazo III signal, recorded at 720 nm to avoid contributions from H^+/Mg^{2+} absorbance changes.

The above three waveforms were obtained in a relatively straightforward fashion, using a single wavelength to measure the desired absorbance change. The upper record in part *D* is from the Dichlorophosphonazo III experiment in Fig. 16 in which the

relative amplitudes of both Ca^{2+} and H^+/Mg^{2+} waveforms were similar at all wavelengths. In this case the Ca^{2+} signal was estimated by subtracting the 650 and 690 nm signals as was done in Fig. 18*C*.

The four Ca^{2+} signals in Fig. 19 are clearly similar although there are variations in the degree of peakedness and in the width of the waveforms. The briefer duration of the Antipyrylazo III waveform may be due to the fact that the experiment was done at 20 °C whereas the others were carried out at 15–16 °C. The shape of the falling phase of the Ca^{2+} waveform, in general, may also be sensitive to the concentration of dye (e.g. see Fig. 3*B*). Taking these factors into account, it seems that the differences in Ca^{2+} transients shown in Fig. 19 may be accounted for reasonably well without having to introduce different delays for the reaction between Ca^{2+} and the different dyes.

Comparison of H^+/Mg^{2+} waveforms obtained with the three dyes. It was not possible to obtain a H^+/Mg^{2+} waveform with Arsenazo III. This is partly because the H^+- and Mg^{2+}-difference spectra are very similar to that for Ca^{2+} (e.g. the curves have closely similar isosbestic points) and partly because the absorbance changes which one might expect are very small compared with the measured Ca^{2+} absorbance change. The latter point can be illustrated by comparing the solid curve in Fig. 8, which represents the absorbance change expected for $\Delta pH = 0{\cdot}01$, with the measured Ca^{2+} absorbance change in Fig. 6 or 7. The peak of the pH curve at 610–620 nm is only 6 % of the experimental peak of the Ca^{2+} points at 650–660 nm.

The situation with Antipyrylazo III is very different. The Ca^{2+}-difference spectrum shows an isosbestic point near 600 nm (Fig. 10) whereas the H^+-/Mg^{2+}-difference spectrum is nearly maximal at 600 nm (Fig. 11*B*). This makes determination of a H^+/Mg^{2+} waveform straightforward. H^+-/Mg^{2+}-related absorbance changes, recorded at 600 nm, are shown in the lower trace in Fig. 19*C*. The amplitude of the maintained $\Delta A(600)$ signal is 15–20 % of the peak amplitude of $\Delta A(720)$.

The lower trace in Fig. 19*D* shows a H^+/Mg^{2+} waveform for a Dichlorophosphonazo III experiment in D_2O. The 610 nm record in Fig. 16*B* was subtracted from the 650 nm record. According to Fig. 17*A* and *B* this should eliminate the Ca^{2+} waveform and still leave a substantial H^+/Mg^{2+} waveform.

The two H^+/Mg^{2+} curves in Fig. 19*C*–*D* are similar in that they show an early decrease followed by a maintained increase. In addition, in the experiments from which these traces were obtained it was possible to fit records at different wavelengths by linear combinations of two basic waveforms, as shown in Fig. 12 for the Antipyrylazo III experiment. This indicates that H^+/Mg^{2+} waveforms, such as those shown in Fig. 19*C*–*D*, may account for all the isotropic signals apart from the Ca^{2+} transient itself. Furthermore, the maintained changes appear to be consistent with the changes in pH and free $[Mg^{2+}]$ which accompany phosphocreatine hydrolysis.

It would be of obvious interest to determine the underlying processes which produce the H^+/Mg^{2+} waveform. These processes may involve true myoplasmic changes in pH or free $[Mg^{2+}]$. They may also involve factors which can influence either H^+ or Mg^{2+} binding to dye (without requiring changes in pH or free $[Mg^{2+}]$) or the spectral changes which such binding can produce.

One example of the latter situation, in which true changes in myoplasmic pH or free $[Mg^{2+}]$ are not involved, would be the situation in which the dissociation constant of the dye for cations might be altered by some intracellular event. Another example is the possibility that the reaction between Mg^{2+} and dye is sufficiently slow that during the Ca^{2+} transient the concentration of Mg^{2+}:dye

complex is not always in equilibrium with free [Mg^{2+}] and free dye concentration. This possibility, which was discussed in connexion with the Dichlorophosphonazo III experiments, could explain the early downward signal observed with both Antipyrylazo III and Dichlorophosphonazo III. In both cases the explanation is the same. During the peak of the Ca^{2+} transient, after the quasi-exponentially rising phase is over, the formation of Ca^{2+}:dye complex would have lowered the concentration of free dye. Mg^{2+}:dye complex would continue to slowly dissociate according to the law of mass action and the optical change produced by the decrease in Mg^{2+}:dye would be detected and might be incorrectly interpreted to reflect a decrease in free [Mg^{2+}]. Since this explanation does not require free [Mg^{2+}] to actually change, it would also be consistent with the failure to find an early decrease in free [Mg^{2+}] with Arsenazo I (Baylor *et al.* 1982*a*). Although the explanation is tentative, at best, it would seem important to rule it out before attaching physiological significance to the early deflexion in the H^+/Mg^{2+} signal.

The above explanation is a kinetic one and would not work if the Mg^{2+}:dye reactions were rapid. In that case the effect of Mg^{2+}:dye dissociation would be incorporated into the Ca^{2+}-difference spectrum according to eqn. (12). In the case of Antipyrylazo III, and under the conditions used to obtain the calibration curve in Fig. 10, the isosbestic point for the Ca^{2+}-difference spectrum is somewhat greater than 600 nm at [Mg^{2+}] = 0, pH = 6·9, and about 594 nm at [Mg^{2+}] = 2 mM. If the kinetics of Mg^{2+}:dye complexation were rapid, the experimental isosbestic point would be 594 nm. If there were kinetic delays in Mg^{2+}:dye complexation, the experimental isosbestic point would be greater than 594 nm at the very beginning of the Ca^{2+} transient, according to eqns. (19) and (11), and at later times would appear to shift towards 594 nm. A comparison of the 590 and 600 nm records in Fig. 9*B* indicates that this possibility may occur.

The maintained signal should not be affected by kinetic delays so that it is more likely to reflect true myoplasmic changes in pH or free [Mg^{2+}]. As pointed out previously (Baylor *et al.* 1982*a*), the change is in the direction expected for the increases in pH and free [Mg^{2+}] which are associated with phosphocreatine breakdown. Although calibration of these signals must be treated with caution, it is nonetheless of interest to note that in terms of ΔpH alone the three dyes give similar values: 0·008 for Arsenazo III (based on the average in Table 1), 0·0061 for Antipyrylazo III, 0·0043 for Dichlorophosphonazo III in H_2O and 0·0065 for Dichlorophosphonazo in D_2O. For comparison, the value 0·0044 was obtained with Arsenazo I (Baylor *et al.* 1982*a*). These numbers are somewhat larger than the value measured with Phenol Red, 0·002, or that expected from phosphocreatine hydrolysis in highly stretched fibres, 0·0024 (Baylor *et al.* 1982*a*).

Any increase in free [Mg^{2+}] associated with phosphocreatine hydrolysis would also contribute to the amplitude of the late H^+/Mg^{2+} signal. According to the calculations in Baylor *et al.* (1982*a*), an upper limit to the fractional change in free [Mg^{2+}] is given (coincidentally) by the change in pH, i.e. $\Delta[Mg^{2+}]/[Mg^{2+}] \leqslant \Delta pH$. With this relation it is easy to show that the effect of pH change on dye absorbance is greater than that of Mg^{2+} with all the pH/Mg^{2+}-sensitive dyes which we tested. Consider Arsenazo III, for example. The calibration curves in Fig. 8 show that the changes $\Delta pH = 0{\cdot}01$ and $\Delta[Mg^{2+}]/[Mg^{2+}] = 0{\cdot}034$ produce similar effects on dye absorbance. If, following phosphocreatine hydrolysis, $\Delta[Mg^{2+}]/[Mg^{2+}] \leqslant \Delta pH$, the absorbance change produced by $\Delta[Mg^{2+}]/[Mg^{2+}]$ would be no more than 0·29 times that produced by ΔpH. Similar calculations with Antipyrylazo III (calibration curves in Fig. 11), Dichlorophosphonazo III (curves in Fig. 15) and Arsenazo I (Baylor *et al.* 1982*a*) give factors 0·36, 0·54 and 0·26 respectively.

Thus, the effect of the anticipated changes in pH and free [Mg^{2+}], which result from phosphocreatine hydrolysis, on dye absorbance seems to be of the right order of magnitude to account for the maintained changes in absorbance that are actually observed. The spectral evidence, such as that shown in Figs. 11*B*, 15*B* and 17*B* in support of an increase in pH and/or free [Mg^{2+}], is clearly more complete than that obtained with Phenol Red and Arsenazo I (Baylor *et al.* 1982*a*). However, it is also possible that there might be contributions from other processes which could produce the measured spectral changes without requiring true changes in myoplasmic pH or free [Mg^{2+}].

DISCUSSION

The experiments described in this paper show that changes in dye-related absorbance can be reliably measured in immobilized, intact single muscle fibres which have been injected with one of the Ca^{2+}-indicator dyes Arsenazo III, Antipyrylazo

III or Dichlorophosphonazo III. With all three dyes, the earliest absorbance change can be explained in terms of formation of Ca^{2+}:dye complex. However, other dye-related signals are usually present and these appear to reflect two other types of processes. This pattern may be somewhat general since each of the other two signals has been seen with at least two different dyes. Thus, Arsenazo III and Dichlorophosphonazo III can show dichroic signals, and Antipyrylazo III and Dichlorophosphonazo III can give late changes in absorbance which have the wavelength dependence which is expected to accompany an increase in pH and/or free [Mg^{2+}].

The separation of the optical signals into the different components, where possible, has relied on the use of light of different wavelengths and different planes of polarization. Although an approach of this sort is experimentally more complicated than the use of unpolarized light and only one or two wavelengths, it does seem necessary if one is to separate the various components of the dye-related signals into the basic, underlying waveforms.

The only metallochromic dye of the series which has been used in intact (as opposed to cut) fibres in another laboratory is Arsenazo III (Miledi, Parker & Schalow, 1977; Parker, 1979). In their experiments the Ca^{2+} waveform was estimated by taking the difference $\Delta A(602)$–$\Delta A(532)$, whereas in our experiments the waveform was estimated by correcting the $\Delta A(650)$ or $\Delta A(660)$ signal for the intrinsic transmission change. The time courses of the two estimates following a single action potential appear to be in reasonable agreement. One clear difference, however, is the finding that the peak amplitude of the Arsenazo III Ca^{2+} signal appears to be a linear function of dye concentration up to at least 0·7 mM in our experiments whereas saturation above 0·3 mM is present in their experiments (see Fig. 3 of Parker, 1979). The reason for the difference is not known.

Use of metallochromic dyes as Ca^{2+} indicators

Of the three dyes which we tried, Arsenazo III and Antipyrylazo III seem to be better than Dichlorophosphonazo III for measuring Ca^{2+} transients in muscle fibres. With Arsenazo III the optical measurements should be made with 650–660 nm light. This is because the isotropic absorbance change is maximal at these wavelengths (Figs. 6 and 7) and the dichroic signal is nearly absent (Fig. 2 of this paper and fig. 1 of Baylor *et al.* 1982*b*). Furthermore, any maintained signal following a twitch, such as might accompany an increase in pH or free [Mg^{2+}], is at most a few percent of the peak value (Fig. 8 and column 7 in Table 1). With Antipyrylazo III the Ca^{2+} signal is best measured in the range 650–720 nm where a change in free [Ca^{2+}] has a large effect on absorbance (Fig. 10) and where changes in pH or free [Mg^{2+}] have little effect (Fig. 11*B*). Dichlorophosphonazo III does not seem well suited for measuring Ca^{2+} transients in muscle, since under most circumstances it is not possible to separate unambiguously the Ca^{2+} component of the absorbance change from the other components.

To accurately estimate the change in dye absorbance from the measured optical signal it is usually necessary to correct for the fibre's intrinsic signal. In the highly stretched fibres which we used, in which gross movement artifacts during a twitch appear to be eliminated, this can be done by scaling (according to $1/\lambda$) and

subtracting a signal recorded at a long wavelength, where the dye does not absorb light (720–750 nm for Arsenazo III, 800 nm for Antipyrylazo III). However, if movement is a serious problem this procedure may well not suffice and other corrections may be required.

The relative importance of the correction for the intrinsic signal (in highly stretched fibres) becomes greater at lower dye concentration. For reliable measurements, the Ca^{2+} signal should probably be several times larger than the intrinsic signal (0.5×10^{-4} to 3×10^{-4} absorbance units, Baylor *et al.* 1982*a*). This condition was met for all the experiments tabulated in Table 1 in which the lowest Arsenazo III concentration was 0·16 mM. It was not adequately met for the experiment in Fig. 2*A* in which 0·06 mM-dye was used. For Arsenazo III, then, it seems desirable to use at least 0·1–0·2 mM-dye to record reliable Ca^{2+} transients. For Antipyrylazo III, 0·5 mM-dye is certainly adequate (Fig. 9) and smaller concentrations were not explored.

Similar considerations may apply to the use of these dyes to measure Ca^{2+} signals in other types of cells. Firstly, any changes in intrinsic absorbance (not related to dye) must be determined so that records obtained from dye-containing cells may be appropriately corrected. Secondly, any changes in dye absorbance due to changes in pH or free [Mg^{2+}] need to be assessed. Finally, it is important to be aware of the possibility that other signals, not directly due to cation complexation, may also be present (for example, the dichroic signal seen in muscle fibres).

Comparison of metallochromic dyes with bioluminescent indicators

The metallochromic dyes Arsenazo III and Antipyrylazo III appear to have certain advantages and disadvantages for measuring muscle Ca^{2+} transients when compared with bioluminescent indicators such as Aequorin. Among the advantages are: (1) they are readily available; (2) they are easy to inject (although Antipyrylazo III is somewhat difficult to ionophorese); (3) they give absorbance changes which can be measured with a high signal/noise ratio; (4) they give absorbance changes which vary approximately linearly with free [Ca^{2+}] (light emission from Aequorin appears to vary as $[Ca^{2+}]^{2.5}$, Allen & Blinks, 1979) and (5) they appear to react rapidly with myoplasmic Ca^{2+} (the emission of light by Aequorin follows changes in free [Ca^{2+}] with a time constant of about 10 msec at 20 °C (Hastings, Mitchell, Mattingly, Blinks & van Leeuwen, 1969)). The last statement, concerning the metallochromic dyes, is based on indirect evidence, namely the comparison of the time course of the dye signals with the time course of the intrinsic birefringence signal, and must therefore be considered as somewhat tentative. In this connexion, it would be of interest to compare the time course of Aequorin luminescence with that of birefringence.

The major disadvantages are: (1) the metallochromic dyes give optical signals which are easily contaminated by movement artifacts (Aequorin appears to be relatively insensitive to such artifacts); (2) the dyes are sensitive to pH (Aequorin is not, Allen & Blinks, 1979) and (3) at least one of the dyes, Arsenazo III, is difficult to calibrate for *in vivo* measurements. The last point is illustrated by the finding that the wavelength dependence of the active muscle signal does not match a cuvette Ca^{2+}-difference spectrum which is obtained at comparable concentrations of dye and free [Ca^{2+}]. This kind of discrepancy can only be revealed by making several measurements, each using a different wavelength of light, and comparing the *in vivo*

results with the cuvette calibrations. Since only one optical measurement is made with Aequorin, namely total photon collection, it is not clear at the present time whether a similar kind of calibration problem exists with Aequorin.

A disadvantage which Arsenazo III, Antipyrylazo III and Aequorin share is a significant sensitivity to Mg^{2+}. Mg^{2+} can have two effects on metallochromic dyes; it can change the absorbance of the dye and it can change the apparent dissociation constant for Ca^{2+}. Both effects occur with Arsenazo III but the main effect with Antipyrylazo III is the second one if light of 650–720 nm is used (Scarpa *et al.* 1978; Fig. 11*B* of this paper). In this case, an increase in free [Mg^{2+}] produces an increase in the apparent dissociation constant of Antipyrylazo III for Ca^{2+}. For example, at pH = 6·9 a change in free [Mg^{2+}] from 0 to 2 mM increases the dissociation constant 1·7 times (p. 158). Aequorin also becomes less sensitive to Ca^{2+} in the presence of Mg^{2+}, and the effect is more pronounced than that with Antipyrylazo III. A change in free [Mg^{2+}] from 0 to 1 mM produces a shift in the light emission curve corresponding to an almost three-fold change in free [Ca^{2+}] (Allen & Blinks, 1979).

Calibration of the Ca^{2+} transient

Calibration of the muscle Arsenazo III Ca^{2+} signal by means of cuvette difference spectra is difficult for several reasons. One, as mentioned above, is that the spectrum of the muscle signal is clearly different from the Ca^{2+}-difference spectrum which is measured in a cuvette using the same dye concentration (Fig. 1*B* and other difference spectra, not shown, in which the same range of dye concentration and small concentrations of free [Ca^{2+}] were used). This discrepancy is reduced if very low concentrations of dye are used in the cuvette (Fig. 7), although the significance of this observation is not clear at present. Another reason is that the dye is probably bound to cellular constituents inside the fibre (Beeler *et al.* 1980) so that accurate calibrations would require simulating the myoplasmic environment. A third reason is that at micromolar levels of free [Ca^{2+}] and at the concentration of dye used inside fibres, a large part of the steady level of Ca^{2+}:dye complex should be 1:2 (Thomas, 1979; R. Y. Tsien, personal communication; Palade & Vergara, 1981; Ríos & Schneider, 1981) which would give a dye-squared relationship for $\Delta A(650)$ *vs.* $A(570)$ rather than the linear one which we usually observed (Fig. 4).

Similar difficulties with calibrations have not yet been reported for either the bioluminescent protein Aequorin or Antipyrylazo III. Allen & Blinks (1979), using Aequorin, estimate myoplasmic free [Ca^{2+}] to be 10 μM during a fused tetanus at 21 °C at normal sarcomere spacing. At long sarcomere spacing this value would be somewhat smaller, perhaps 7 μM (see Fig. 11 in Blinks, Rüdel & Taylor, 1978). Using a value of 3 for the tetanus:twitch ratio of myoplasmic free [Ca^{2+}], based on summation of the Arsenazo III Ca^{2+} signal (see Fig. 10 in Baylor *et al.* 1982*b*), the peak free [Ca^{2+}] during a twitch at long sarcomere spacing would be indirectly estimated as 2·3 μM. This is similar to the value 2·9 μM which was directly estimated from the Antipyrylazo III experiment in Fig. 9, 20 °C, using cuvette calibrations.

For comparison, Miledi *et al.* (1977) and Parker (1979) give an average value of 5·25 μM from their Arsenazo III experiments, temperature 7–8 °C. This estimate is based on *in vitro* Arsenazo III calibrations in which the concentration of dye was

similar to that used inside muscle fibres. A similar value for peak free [Ca^{2+}] can be calculated from the Arsenazo III signal using a different rationale for calibration. If Arsenazo III behaves in the same manner inside muscle fibres as it does in calibrating solutions at very low dye concentration, as may be suggested by the approximate agreement between the data points and the curve in Fig. 7, an estimate of peak free [Ca^{2+}] can be obtained using the low dye calibration data and the assumption of 1:1 stoichiometry. The average peak value of $\Delta A(650)/A(570)$ in Table 1 is 0·05, which in the low dye calibrations corresponds to 0·05 of the dye being complexed with Ca^{2+}. Our preliminary calibration measurements indicate that K_{CaD} = 150–200 μM for Arsenazo III concentrations less than 0·1 μM (pH = 6·9, free [Mg^{2+}] = 1 mM). This would give a range 7·9–10·5 μM for peak free [Ca^{2+}] during a twitch. However, it seems clear that any estimate of myoplasmic free [Ca^{2+}] which is based directly on Arsenazo III cuvette calibrations should be interpreted with caution.

Although several uncertainties arise concerning the exact determination of the peak level of myoplasmic free [Ca^{2+}] during a twitch in highly stretched fibres, it is reassuring that all the estimates based on Aequorin, Arsenazo III and Antipyrylazo III fall within a fairly narrow range, 2–10 μM.

These estimates of myoplasmic free [Ca^{2+}] represent a spatial average which is based on the amount of Ca^{2+}:dye complex which is formed in the space accessible to dye, probably myoplasm excluding s.r. and mitochondria. At low free [Ca^{2+}], mainly 1:1 and 1:2 Ca^{2+}:dye complexes appear to be present with the dyes which we used. This means that the extent of Ca^{2+} complexation should depend approximately linearly on free [Ca^{2+}] and, therefore, that the spatially averaged value of concentration of Ca^{2+}:dye complex should be directly proportional to the spatially averaged value of free [Ca^{2+}]. The assumption of a linear relationship between Ca^{2+}:dye concentration and free [Ca^{2+}] relies on having no more than 0·1–0·2 of the total dye complexed with Ca^{2+}. On average, this is the case but it may not prevail locally everywhere in the myoplasm, especially near the sites of Ca^{2+} release. Thus, it is important to bear in mind that free [Ca^{2+}] during the Ca^{2+} transient may be spatially heterogeneous and may vary significantly along each sarcomere. In addition to longitudinal heterogeneities, it is also possible that during the rising phase of the Ca^{2+} transient there may be radial heterogeneities which accompany the inward spread of excitation from the surface (Gonzalez-Serratos, 1966).

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