

# Arsenazo III and Antipyrylazo III Calcium Transients in Single Skeletal Muscle Fibers

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**ABSTRACT** The metallochromic calcium indicators arsenazo III and antipyrylazo III have been introduced individually into cut single frog skeletal muscle fibers from which calcium transients have been elicited either by action potential stimulation or by voltage-clamp pulses of up to 50 ms in duration. Calcium transients recorded with both dyes at selected wavelengths have similar characteristics when elicited by action potentials. Longer voltage-clamp pulse stimulation reveals differences in the late phases of the optical signals obtained with the two dyes. The effects of different tension blocking methods on Ca transients were compared experimentally. Internal application of EGTA at concentrations up to 3 mM was demonstrated to be efficient in blocking movement artifacts without affecting Ca transients. Higher EGTA concentrations affect the Ca signals' characteristics. Differential effects of internally applied EGTA on tension development as opposed to calcium transients suggest that diffusion with binding from Ca<sup>++</sup> release sites to filament overlap sites may be significant. The spectral characteristics of the absorbance transients recorded with arsenazo III suggest that *in situ* recorded signals cannot be easily interpreted in terms of Ca concentration changes. A more exhaustive knowledge of the dye chemistry and/or *in situ* complications in the use of the dye will be necessary.

## INTRODUCTION

The release and resequestration of calcium ions have long been thought to play central roles in muscle excitation-contraction coupling and relaxation, respectively (Ebashi and Endo, 1968). Probes of myoplasmic calcium concentration changes are critical to the investigation of these processes. Several calcium-sensitive substances have been introduced as optical probes in physiological studies. The first probe introduced in muscle physiology was murexide (Jobsis and O'Connor, 1966), but the small size and poor signal-to-noise ratio

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of the murexide signals pose severe problems for investigators intent on extensive experimentation involving calcium turnover processes. The photoprotein aequorin (Shimomura et al., 1962) has been used more extensively (Ridgway and Ashley, 1967; Taylor et al., 1975; Blinks et al., 1978; Taylor et al., 1979). However, the signal-to-noise ratio of aequorin signals in muscle fibers so far reported in the literature is still less than ideal to carry out quantitative physiological experiments. In addition, the chemical reactions leading to light emission with aequorin are acknowledged to be slow, complex, and still not fully agreed upon (Hastings et al., 1969; Loschen and Chance, 1971; Blinks et al., 1976).

Two calcium-sensing chemical agents that have more recently demonstrated potential for investigating intracellular calcium concentration changes are the metallochromic indicators arsenazo III and antipyrylazo III. Arsenazo III has found widespread usage both in biochemical (Scarpa, 1975; Vallieres et al., 1975; Johnson and Scarpa, 1976; Ohnishi, 1979; Scarpa, 1979), as well as physiological (e.g., Brown et al., 1975; DiPolo et al., 1976; Brinley et al., 1977; Brown et al., 1977; Gorman and Thomas, 1978; Yingst and Hoffman, 1978; Brinley et al., 1978) systems. These dyes give large absorbance changes with improved signal-to-noise ratio in response to calcium concentration changes. In frog skeletal muscle the first work with arsenazo III was performed by Miledi et al. (1977*a*, *b*, and *c*, 1979, and 1980), and Suarez-Kurtz and Parker (1977). Their early work (Miledi et al., 1977*b*) showed that all the calcium changes detected were caused by release of calcium from within the muscle fiber, not dependent upon entry of calcium from the extracellular medium (unless endplate channels were activated [Miledi et al., 1980]), and could not be detected in detubulated fibers. There was, however, a puzzling technical observation that was not critically addressed in these studies: the signals elicited at the two wavelengths used (532 and 602 nm) had different time courses. Subsequently, Baylor et al. (1979*a* and *b*) have used arsenazo III in isolated single muscle fibers and discovered a dichroic component in the optical signals that has a spectrum similar to that of the free dye and which might be responsible for some distortion in the time course of the dye signals at certain wavelengths.

Antipyrylazo III was introduced as a calcium-sensing indicator more recently than arsenazo III. On the basis of *in vitro* experiments it was concluded that this substance has a relative affinity for calcium in between that of arsenazo III and murexide (Scarpa et al., 1978). The authors suggested that this dye might be sensitive enough to detect calcium transients with good signal-to-noise ratio without buffering free calcium levels as much as arsenazo III. Calcium transients have recently been detected with this indicator inside a cut muscle fiber preparation (Kovacs et al., 1979). On the basis of the *in vitro* experiments performed by others (Scarpa et al., 1978*a* and *b*) and an apparently simple exponential behavior of their physiological signals, Kovacs et al. (1979) have presented a three-compartment model of calcium metabolism inside muscle fibers based in part on the observation that antipyrylazo III calcium transients always declined toward the end of voltage-clamp pulses.

However, Miledi et al. (1977b) observed a continuous increase ("creep") in arsenazo III transients during voltage-clamp pulses, at least for several hundred milliseconds (Miledi et al., 1980). Because the method of fiber preparation was different in arsenazo III and antipyrylazo III studies, it had not yet been possible to determine whether this dissimilarity between the signals reflected a difference between the two dyes or the two modes of fiber preparation.

We report here a more strict comparison of the signals obtained with arsenazo III and antipyrylazo III inside cut skeletal muscle fibers under current and voltage-clamp conditions. Some preliminary results have been previously reported (Palade, 1979; Palade and Vergara, 1981).

#### METHODS

##### *Optical Measurements*

Cut frog single muscle fibers were mounted in a triple vaseline gap voltage-clamp chamber similar to that originally described by Hille and Campbell (1976). The chamber was modified according to Vergara et al. (1978) for optical determinations. The modification consisted of the mounting of a tapered fiber optic (Galileo Electro-Optics Corp., Sturbridge, Mass.) in the A pool part of this chamber (Hille and Campbell, 1976; Vergara et al., 1978) with the tapered end (150  $\mu\text{m}$  Diam) in close contact under the muscle fiber. The untapered portion of the fiber optic protruded underneath the chamber. This assembly was mounted on the stage of a compound microscope (Nikon Biophot; Nikon Inc., Garden City, N. Y.). Quasi-monochromatic light generated by a 12-V, 100-W quartz-halogen bulb driven by a regulated DC power supply (Kepco JQE-25-20-VP; Kepco, Inc., Flushing, N. Y.) was filtered by narrow band interference filters ( $\frac{1}{2}$  bandwidth 10 nm; Ditric Optics, Inc., Duryea, Pa.) and focused through the microscope condenser onto the untapered portion of the fiber optic. The light was passed through the fiber optic and through the A pool portion of the fiber, and was collected by a long working distance objective (Leitz UMK-50, magnification  $\times 32$ , numerical aperture 0.6; E. Leitz, Inc., Rockleigh, N. Y.). The chamber was positioned by moving the microscope stage until the light through the fiber optic was clearly visible and the fiber striations were well delineated through the eyepieces of the trinocular head. Fiber movement during an experiment was monitored visually in this manner, and even the slightest local movement in the A pool was easily detected. Sarcomere spacing and fiber diameter were measured by means of an eyepiece micrometer.

For measurement of optical transients the light was instead directed to a photodiode (PV-444A; EG&G, Inc., Salem, Mass.) seated in a light-free casing with adjustable slits that allowed only the fiber portion of the A pool to be focused on the photosensitive surface. The slits were adjusted to record from  $\sim 80\%$  of the fiber length in the A pool and  $\sim 90\%$  of the fiber width. The current output of the photodiode was connected to an FET input current-to-voltage converter that allowed measurement of resting and fast transients of light intensities. Amplification of the small transients in light intensity was made through an additional amplification stage with subtraction of the basal light intensity by a track and hold circuit (Vergara et al., 1978). The resulting optical transients were then filtered with a 100- $\mu\text{s}$  time constant and sampled and stored by the same data acquisition device described elsewhere (Vergara et al., 1978).

### *Electrical Measurements and Voltage Clamp*

Electrodes were made with Ag/AgCl pellets and mounted in 1% agar, 1 M KCl. These were kept short circuited in 1 M KCl in between experiments. The voltage-clamp circuit used was the same as that described by Hille and Campbell (1976) with the modification that membrane currents were measured as the voltage drop across a 10-k $\Omega$  resistor as suggested by the authors. Other details of the circuitry, including the leak, capacity, and series resistance compensation, were as described by Hille and Campbell (1976). To accompany optical records, either the  $V_m$  or  $I_m$  output of the clamp was also sampled for the purposes of aligning the optical signal in time with respect to the stimulus and checking the adequacy of the clamp and the electrical properties of the fiber. Since the fiber optic was  $\sim 150 \mu\text{m}$  in diameter, the A pool gap was generally set to 160–180  $\mu\text{m}$ , a length not ideally suited for perfect potential control in the presence of large sodium currents in fibers smaller than 180  $\mu\text{m}$  in diameter. In a few cases this resulted in small ( $< 5 \text{ mV}$ ) transient perturbations in the  $V_m$  trace that could be ascribed to inadequacy of the clamp. Otherwise, normal electrical behavior was seen in these fibers, as evidenced by sodium currents of up to 5 mA/cm<sup>2</sup> and delayed potassium currents of up to 9 mA/cm<sup>2</sup>, depending on the test potential. Current stimulation elicited action potentials in current clamp mode, and both  $V_m$  and optical signals were recorded.

### *Ancillary Equipment*

For arsenazo III experiments it was necessary to micro-iontophorese the dye into the A pool portion of fiber closest to the E pool. This was accomplished by passing current through a microelectrode into the muscle fiber once it was mounted in the chamber with the vaseline seals in place. The circuit was closed through an external electrode in the E pool. Microelectrodes were fabricated of thin walled capillary tubing (TW 150F; WP Instruments, Inc., New Haven, Conn.) with filament that allowed back-filling with a minimal volume of solution consisting of concentrated arsenazo III (98% pure, Sigma Chemical Co., St. Louis, Mo.) in de-ionized water. Such electrodes had tips of  $< 1 \mu\text{m}$  Diam and did not appear to damage the fiber upon impalement. Microelectrodes fabricated in the same manner filled with 3 M KCl had resistances on the order of 1–2 M $\Omega$ ; when filled with the dye solution (and immersed in Ringer's solution), their resistances were generally on the order of 10–20 M $\Omega$ . Dye iontophoresis generally required 5–20 min of current passing, which hyperpolarized the fibers 20–40 mV during this period to achieve uniform heavy staining of the fiber in the A pool. Since arsenazo III was found to diffuse away readily from the injection site, the microelectrode was generally maintained in the fiber during the course of the experiment. The microelectrode tip was kept just outside the area from which optical signals were measured and the current passing circuit was disconnected while experimental records were taken. It was occasionally necessary to reinject the fiber by reconnecting the current passing circuit in order to maintain heavy staining (0.2–1.6 mM arsenazo III inside the fiber). Frequently, the injection procedure caused swelling at the injection site, usually accompanied by an increased resting membrane conductance. Results obtained from those fibers were not analyzed, and experiments were generally terminated when such changes were detected. The preparation was cooled by means of Peltier elements (Borg-Werner Thermoelectrics, Des Plaines, Ill.) mounted underneath most of the fiber chamber.

### *Fiber Preparation*

**DISSECTION** Cut twitch fibers from the dorsal heads of the semitendinosus muscles of *Rana temporaria* or *Rana catesbiana* were used. These were prepared in one of the following ways.

(a) Extracellular stimulation with brief (1 ms) pulses allowed the selection of fast-twitch fibers from muscles or bundles of muscle fibers in Ringer's solution. Several such fibers could then be dissected partly away from the muscle by pulling the tendon ends in a direction perpendicular to the muscle until nearly at the first endplate. The fibers were then carefully teased away from one another over that region, and the fiber exhibiting the strongest twitch and most rapid relaxation was selected. Holding this fiber taut, the Ringer's solution was then removed and replaced by a solution containing 80 mM  $K_2SO_4$ , 3 mM KMOPS, and 0.01 mM  $K_2EGTA$ , pH 7.1. After completion of the resultant  $K^+$  contracture and ensuing relaxation, the fiber was cut near the endplate and quickly transferred to the experimental chamber previously flooded with one of the internal solutions listed in Table I.

(b) The muscle was stretched slightly (~10%) beyond resting length in Ringer's solution. The solution was then removed and replaced by a solution containing 80 mM  $K_2EGTA$ , 2 mM  $Na_2ATP$ , pH 7.1. The muscle was rinsed in the solution twice and then placed in the cold (5°C) for 15–30 h before dissection (Almers and Palade, 1981) of a single fiber in a solution of much lower EGTA concentration (0.02–3.0 mM) and transferral to the experimental chamber.

(c) The muscle was stretched slightly beyond resting length in Ringer's solution. The solution was then replaced by a solution containing 80 mM  $K_2SO_4$ , 3 mM KMOPS, and 0.1 mM EGTA, pH 7.1. Dissection from this muscle could begin immediately after completion of the relaxation after the contracture. Viable fibers could be obtained from such muscles for up to 36 h if stored at 5°C.

The first of these procedures could assure successful fiber selection on the basis of viability until just before transferral, but tended to be more time-consuming than the other two dissections of depolarized fibers. Of the latter, the lengthy presoak in high EGTA clearly yielded a lower proportion of viable fibers, although such fibers could generally be distinguished on the basis of their appearance. Fibers from muscles presoaked in high EGTA generally exhibited greatly reduced mechanical activity as well. The third procedure was formulated as an intermediate approach, being less lengthy in fiber preparation, but still resulting in a high proportion of viable fibers.

**MOUNTING** Single fibers transferred to the flooded experimental chamber were mounted across the partitions in the same way described for the Hille and Campbell (1976) technique (Vergara et al., 1978). In most of the experiments the cut ends of the fibers protruded 250–300  $\mu m$  into the E and C pools. In some experiments the fibers were highly stretched before seal placement by pinning the ends into a thin layer of plastic embedding material in the bottom of the E and C pools. After seal placement the solution level was lowered to isolate the pools, the electrodes were introduced, and then the solution in pool A was exchanged for Ringer's solution or another external solution.

### *Solutions*

The compositions of solutions used are shown in Table I. All external solutions were adjusted to pH 7.25; internal solutions were adjusted to pH 7.1. To the internal solutions variable concentrations of  $K_2EGTA$  or  $Cs_2EGTA$  were substituted in place of some of the K-aspartate or Cs-aspartate..

In the case of dye experiments not involving arsenazo III, 1–5 mM dye was added to the internal solution. Diffusion of dyes into cut fibers was successful with all dyes except arsenazo III. Phenol red was the fastest dye to diffuse and experiments could start after a waiting period of 5–10 min. Antipyrylazo III was the slowest diffusible dye and experiments required a wait of at least 30–45 min after cutting the ends of the fibers in pools E and C with dye-containing internal solutions. All dyes were obtained from Sigma Chemical Co. in the highest purity offered, with the exception of chlorophosphonazo III, which was obtained from K & K Laboratories, Plainview, N. Y.

#### Optical Calibration Procedures

To estimate resting pH and Mg levels in muscle fibers, a capillary (cf. Miledi et al., 1977b) of 137  $\mu\text{m}$  inner diameter, approximately equal to the average fiber diameter used for this study, was positioned on the experimental chamber instead of a fiber, and solutions were internally applied and removed by suction. Light intensities through different solutions were measured with the same apparatus used for muscle fibers except that the output from the light amplifier was recorded with a digital voltmeter for additional accuracy. Solutions used were in general 100 mM KCl plus

TABLE I

External solutions							
(mM)	NaCl	CaCl <sub>2</sub>	KMOPS	TMACl	Urea	TTX	
Normal Ringer's	118	1.8	2.5	—	—	—	
Hypertonic Ringer's	118	1.8	2.5	—	200–300	—	
TTX Ringer's	118	1.8	2.5	—	—	10 <sup>-7</sup> M	
TMA Ringer's	—	1.8	2.5	118	—	—	

Internal solutions	Aspartate		PIPES		Creatine PO <sub>4</sub>	MgSO <sub>4</sub>	Na <sub>2</sub> ATP
	K <sup>+</sup>	Cs <sup>+</sup>	K <sup>+</sup>	Cs <sup>+</sup>			
K <sup>+</sup>	100	—	5.0	—	5.0	1.0	3.0
Cs <sup>+</sup>	—	100	—	5.0	5.0	1.0	3.0

20 mM KMOPS, pH 6.9, which were partly decalcified with the cation exchange resin AG50W-X8 (Bio-Rad Laboratories, Richmond, Calif.) by a batch procedure to remove divalent ions before the addition of various concentrations of magnesium or alteration of pH.

For determinations of resting pH and magnesium levels, resting absorbances at two wavelengths were expressed as  $A_{\lambda_1}/A_{\lambda_2}$ . Light intensity determinations from the capillary calibrations were converted into absorbance units according to the definition  $A = \log_{10} I_0/I$ , where  $I_0$  is the light intensity in the absence of dye, and  $I$  is the light intensity in the presence of dye measured at the same wavelength.

Calibrations in certain experiments involving physiological transients, are expressed in terms of  $\Delta A_{\lambda_1}/A_{\lambda_2}$ , with the numerator calculated according to  $\Delta A = \log_{10} I/I + \Delta I$ , where  $I$  represents light intensity through the resting fiber or capillary in the presence of dye and  $\Delta I$  is the change in light intensity upon stimulation of the fiber. For other muscle fiber experiments in which a small movement artifact was recorded along with the transients, cancellation of the movement artifact was implemented by

subtraction of  $\Delta A$  at a third wavelength ( $\lambda_3$ ) resulting in a final expression of  $(\Delta A_{\lambda_1} - \Delta A_{\lambda_3})/A_{\lambda_2}$ .

In certain arsenazo III experiments,  $\lambda_3$  was the same as  $\lambda_2$  (532 nm, cf. Miledi et al., 1977b). In most experiments, however, a wavelength at which no dye species or Ca-dye complex absorbed (i.e., 740 nm for arsenazo III, and 790 nm for antipyrylazo III) was chosen in order to use  $\Delta A_{\lambda_3}$  as an indication of movement alone. In these cases  $\Delta A_{\lambda_3}$  was linearly approximated by  $-0.434\Delta I_{\lambda_3}/I_{\lambda_3}$  (cf. Kovacs et al., 1979) since these absorbance changes were very small.

Dye concentrations inside fibers were roughly estimated according to:

$$[\text{dye}] = (\log_{10} (I_o/I))/\epsilon l$$

where  $\epsilon$  represents the molar extinction coefficient of the calcium-free dye at the wavelength chosen (532 nm for arsenazo III, 550 nm for antipyrylazo III). The mean path length  $l$  was assigned a value of  $\pi/4$  times the fiber diameter, assuming a cylindrical fiber as a first approximation.

To check the adequacy of the optical set-up to faithfully measure absorbances despite limitations in the spectral characteristics of the photodiode and light source, quasi-monochromatic light of all the wavelengths used in this study was shone through neutral density filters, nominally of 0.1 and 0.5 absorbance units (Kodak N.D. 0.1 and 0.50 Wratten gelatin filters; Eastman Kodak Co., Rochester, N. Y.). Intensity readings were taken at several wavelengths, and absorbances were calculated and compared with those obtained with the same filters using a calibrated Hitachi model 100-60 spectrophotometer. The accuracy of the absorbance measurements in the experimental set up was found to be better than 6% in most of the wavelengths used with an upper limit of 10% error in very long (790 nm) or very short (470 nm) wavelengths.

In our experiments we recorded light intensity with a linear amplifier rather than absorbance with a logarithmic amplifier. This introduced an error that slightly distorts the shape of the actual transients. Nevertheless, calibration in terms of  $\Delta A/A$  of traces that were originally recorded as light intensity changes was preferred for reasons of convention and ease of comparison with *in vitro* determinations. The largest changes reported here would theoretically deviate from the linearity of their  $\Delta A/A$  calibrations by ~5%, with cases where peak  $\Delta A/A$  was  $\leq 0.1$ , being in error by <2%.

## RESULTS

### *Arsenazo III Experiments under Current Clamp Conditions*

Muscle fibers microinjected with arsenazo III displayed transient changes in absorbance in response to electrically recorded action potentials elicited by suprathreshold current pulses under current clamp conditions. Fig. 1 demonstrates the temporal relationship between an action potential, arsenazo III absorbance change and an optical indication of fiber movement. The movement trace was recorded before arsenazo III injection and at a gain five times higher than the subsequently recorded arsenazo III signal. The dye signal lags behind the action potential but starts well before fiber movement and begins to decay well before fiber movement has reached its maximum.

The temporal overlap between the arsenazo III absorbance change and fiber movement creates severe complications in measuring calcium transients

inside muscle fibers if the fiber movement is too large. One of the first problems addressed in the present study was the exploration of means to block fiber movement without distortion of the calcium transients themselves. Two traditional methods for reducing tension development have been used: severe stretch (Gordon et al., 1966; Miledi et al., 1977a) and treatment with hypertonic solutions (Hodgkin and Horowicz, 1957; Cleworth, 1967; Caputo, 1968; Gordon and Godt, 1970). Arsenazo III calcium transients have been recorded

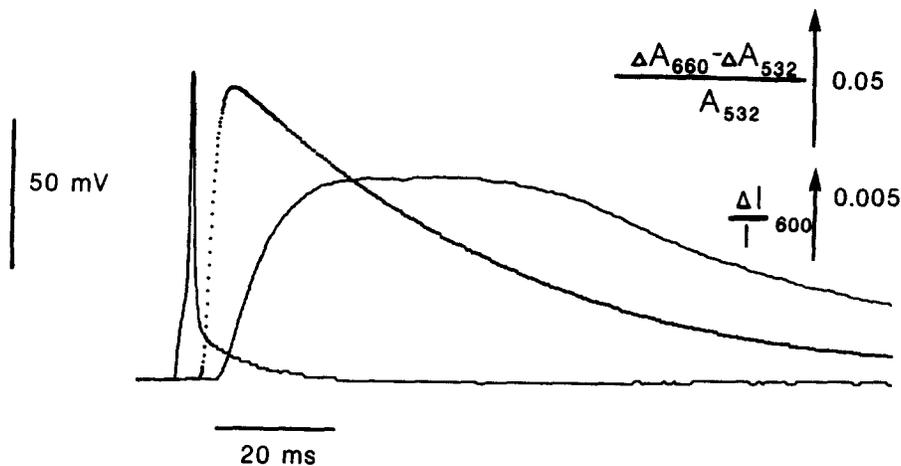


FIGURE 1. Temporal relations between action potential, arsenazo III transient, and optical movement artifact. Optical traces in response to an action potential elicited by current stimulation in current clamp mode to give an action potential. The fiber was dissected from a muscle soaked overnight in 80 mM  $K_2EGTA$  and cut in a solution containing  $10^{-4}$  M EGTA. The movement artifact (continuous trace) was obtained before dye injection and is compared with the arsenazo III transient (dotted trace) subsequently recorded by subtracting the 532-nm signal from the 660-nm signal after dye injection to correct for the <15% contribution due to movement. Absorbance calibration corresponds to arsenazo III signal and intensity calibration corresponds to movement artifact measured at 600 nm. Temperature: 21°C. Action potential from  $-105$  mV to  $+20$  mV. Estimated internal dye concentration: 1.61 mM. In this and subsequent figures the direction of the arrow on the absorbance calibration bar refers to the direction of increased absorbance.

from cut fibers treated both ways with  $<2 \times 10^{-5}$  M EGTA present in the solution bathing the cut ends. In addition, another treatment involving presoaking the muscle 15–30 h in 80 mM  $K_2EGTA$  before fiber dissection and mounting with  $10^{-4}$  M EGTA present in the cut end solution was used. The rationale for this latter procedure was that the EGTA presoak, for reasons that remain to be investigated, significantly reduced and sometimes completely abolished fiber movement associated with an action potential without interference in the calcium release process as shown below.

Calcium transients elicited by action potential stimulation in arsenazo III-injected fibers displayed basically similar characteristics regardless of the method of fiber preparation. Table II summarizes certain parameters of these transients. Some of the variability may have been due to variations in the action potential duration that were noted from fiber to fiber. However, stretched fibers in this study generally yielded smaller (column A) and slightly slower (columns B–E) transients. Calcium transients from presoaked fibers appeared not to be adversely affected by increasing concentrations of EGTA in the cut end (internal) solution.

*Experiments under Voltage-Clamp Conditions*

A comparison of the signals obtained under voltage-clamp conditions with the three previously described movement blocking methods is made in Fig. 2.

TABLE II  
SOME PARAMETERS RELATING TO CALCIUM TRANSIENTS IN RESPONSE TO  
ELICITED ACTION POTENTIALS

Procedure	A	B	C	D	E	F	G
	Peak $\Delta A_{680}/A_{832}$	Time to start (delay) <i>ms</i>	Time to $\frac{1}{2}$ rise <i>ms</i>	Time to peak <i>ms</i>	Time to $\frac{1}{2}$ decay <i>ms</i>	Estimated [arsenazo III] <i>mM</i>	<i>n</i>
80 mM EGTA presoak; 0.02 mM EGTA in end pools	0.035±0.026	1.3±0.3	3.9±0.8	7.0± 1.8	18.6±7.9	0.90±0.51	10
80 mM EGTA presoak; 0.1 mM EGTA in end pools	0.065±0.038	1.3±0.3	3.6±0.5	6.8±0.7	25.0±11.1	1.06±0.43	16
80 mM EGTA presoak; 1 mM EGTA in end pools	0.072±0.038	1.1±0.3	3.1±0.5	5.7±0.4	21.9±9.5	1.06±0.03	3
Stretched 3.4–4.0 $\mu$ m; 0.02 mM EGTA in end pools	0.015±0.005	1.5±0.2	4.8±0.6	10.0±0.6	34.6±14.2	0.38±0.24	4
Hypertonic urea; 0.02 mM EGTA in end pools	0.043±0.011	1.8±1.1	4.1±0.5	10.3±6.0	29.5±23.3	0.76±0.04	2

All parameters relating to the timing of the calcium transients were measured from the time at which the action potential peaked.

All experiments were performed at room temperature.

Values correspond to mean  $\pm$ SD.

Under these conditions of more prolonged and controlled depolarization there are some quantitative differences in the amplitudes of the signals (again, the stretched fiber yielded smaller transients), but in general their qualitative characteristics and time courses are remarkably similar.

These voltage-clamp results demonstrate certain notable features of the arsenazo III signals during prolonged (50 ms) depolarizations. Three distinct phases in the signals (rising, intermediate, and decaying) are evident in all arsenazo III-injected fibers regardless of mode of preparation. With small depolarizations the calcium transient reaches a maximum and then decays during the pulse. As the pulse potential is increased, an intermediate phase of

## ARSENAZO III

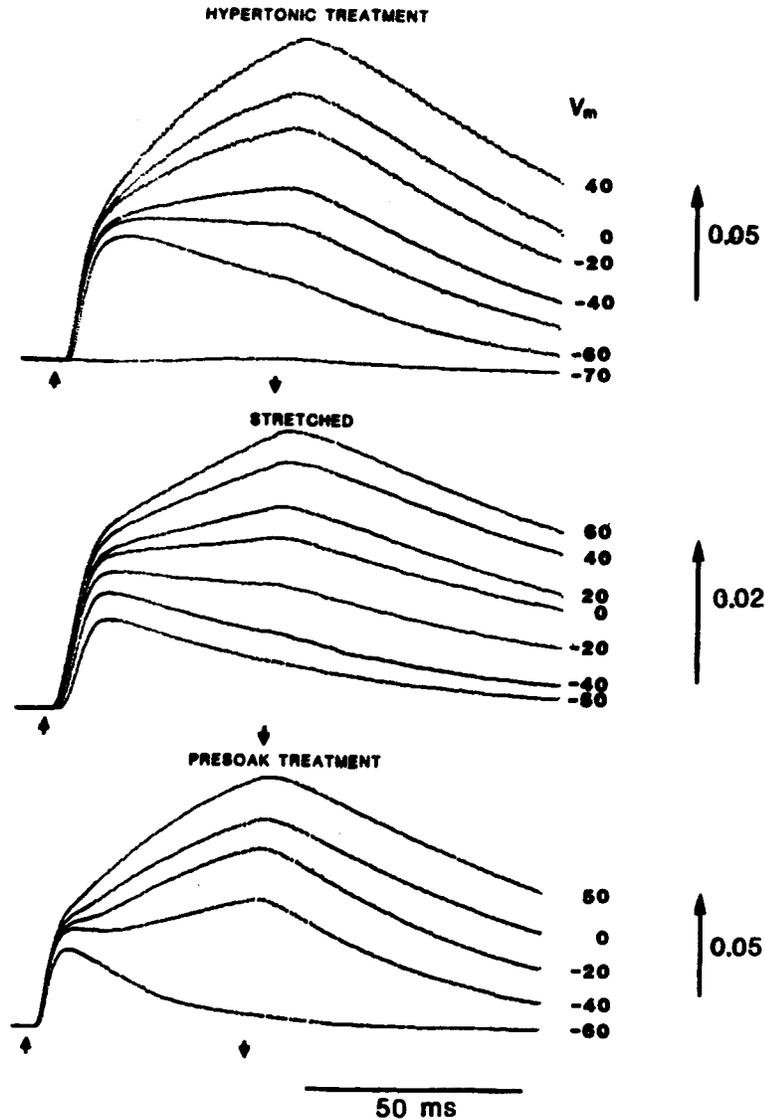


FIGURE 2. Arsenazo III calcium transients elicited from cut muscle fibers in response to 50-ms depolarizing pulses from a holding potential of  $-100$  mV to the potentials indicated. Upward arrows indicate the beginning of the pulse; downward arrows indicate the end. Signal amplitudes were calibrated according to the difference between the absorbance changes at 660 and 532 nm divided by the resting fiber absorbance at 532 nm. Three different modes of fiber preparation are compared: hypertonic treatment with 200 mM urea added to Ringer (top fiber) stretching to  $3.7$   $\mu\text{m}$  sarcomere spacing (middle fiber), and a fiber prepared from a muscle soaked overnight in 80 mM  $\text{K}_2\text{EGTA}$  (bottom fiber). Temperature:  $22^\circ\text{C}$ . Voltage clamp experiment. Estimated internal dye concentrations: 1.37 mM (top), 0.90 mM (middle), 1.17 mM (bottom). Internal solutions contain 0.1 mM  $\text{K}_2\text{EGTA}$ . Calibrations are in terms of  $(\Delta A_{660} - \Delta A_{532})/A_{532}$ .

the signal becomes evident. By comparing successive sweeps at different potentials, it can be seen that the intermediate phase decays less rapidly, plateaus, and eventually rises throughout the pulse, reaching greater and greater amplitudes at larger and larger depolarizations. The potential at which a plateau is reached in the intermediate phase varies from fiber to fiber, but the basic observation is common to all fibers studied. As a consequence of this intermediate phase, the amplitude of the arsenazo III signal may attain values several times larger than those elicited in the same fiber with action potentials. Upon termination of the pulse the signals decay after a short lag and return to baseline (not shown).

#### *Effects of Higher Internal EGTA Concentrations*

Although the three aforementioned procedures for fiber preparation have yielded a fairly consistent picture of calcium transients obtained with arsenazo III, the experiments are technically difficult to perform. For the purposes of easily obtaining a more viable preparation, millimolar concentrations of EGTA were added to the cut end solutions after a depolarized dissection (Methods, dissection procedure *c*) in lower EGTA. The precedent for such a protocol to eliminate movement artifacts in muscle fiber optical studies was set in Nile Blue fluorescence studies by Vergara et al. (1978). We felt it necessary to demonstrate that application of EGTA internally at moderate concentrations did not interfere significantly with the transients and to determine at what EGTA concentrations deleterious effects did occur.

Fig. 3 demonstrates arsenazo III calcium transients obtained at two different EGTA concentrations and the extent of residual movement contributions to the uncorrected records. Fig. 3A shows two traces recorded at 660 and 740 nm, respectively, from a fiber in which 3 mM EGTA was added to the cut end solution. Subtraction of the 740-nm trace (containing no dye-related absorbance changes) from the 660-nm trace yields the uppermost trace in Fig. 3B, thus correcting the 660-nm record for a small residual movement artifact under these conditions. Fig. 3B demonstrates a family of such corrected traces from the same fiber in response to 50-ms depolarizing pulses to the potentials indicated. Despite the large effect of EGTA on movement, the calcium transients shown here are qualitatively the same as those seen with EGTA levels 30-fold lower using other immobilization procedures (Fig. 2). As shown, even exceptionally large calcium transients may be recorded in the presence of 3 mM EGTA. Fibers exhibiting such large absorbance changes are only occasionally encountered, but have been reported in fibers not treated with EGTA (cf. Miledi et al., 1977*b*, Fig. 3). Generally our results with 3 mM EGTA were fully within the normal range of results obtained with other immobilization procedures.

Fig. 3C demonstrates the effects of application of 9 mM EGTA to the cut ends of another fiber. At these higher EGTA concentrations no residual movement contributions were recorded but the signals are indeed modified in several ways. First, the prominent upward creep of the intermediate phase characteristically seen at lower EGTA concentrations (e.g., Fig. 3B, 0 mV

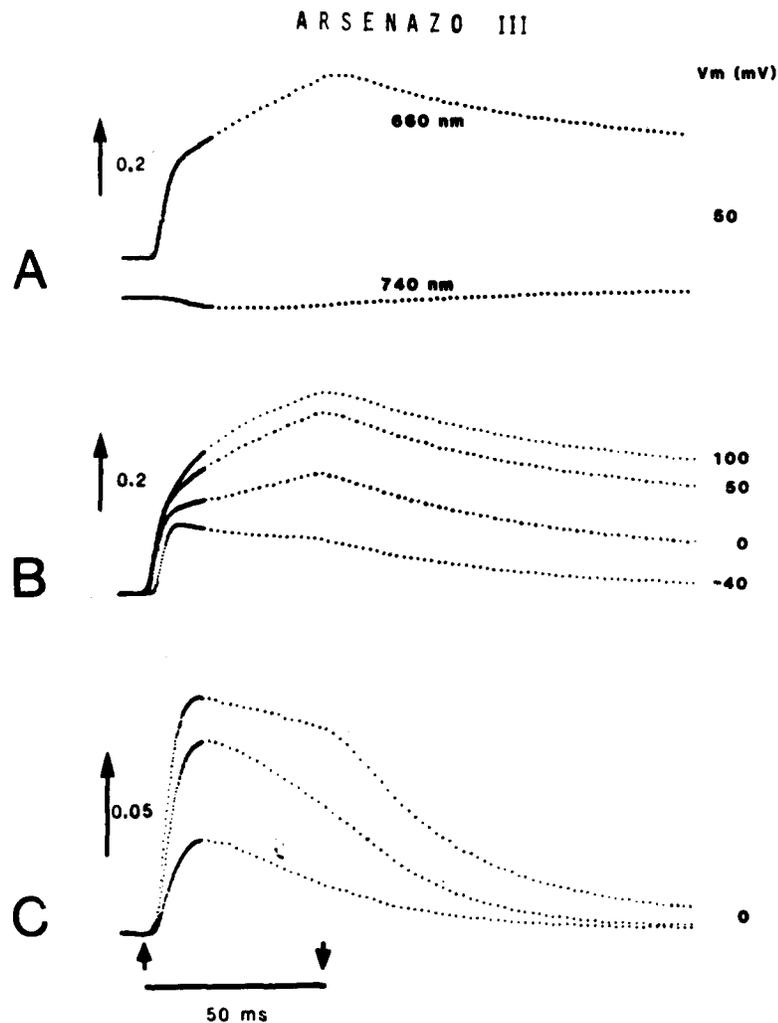


FIGURE 3. (A) Arsenazo III calcium transients elicited in response to 50-ms depolarizing pulses from a holding potential of  $-100$  mV to  $+50$  mV from a muscle fiber whose ends were cut in  $3$  mM  $K_2EGTA$  internal solution. The upper trace was recorded at  $660$  nm, and the lower trace was recorded at  $740$  nm. The  $740$ -nm trace should strictly reflect the contribution of movement with no dye-related absorbance changes. Temperature:  $10^\circ\text{C}$ . Voltage clamp experiment. Estimated internal dye concentration:  $0.79$  mM. Calibrations:  $\Delta A_{660}/A_{532} = 0.2$  for the upper trace and  $\Delta A_{740}/A_{532} = 0.2$  for the lower trace. (B) Calcium transients from the same fiber in response to 50-ms pulses as above to the potentials indicated to the right of each trace. Movement artifacts were subtracted at  $740$  nm. Calibration:  $(\Delta A_{660} - \Delta A_{740})/A_{532} = 0.2$ . (C) Arsenazo III calcium transients elicited from a fiber whose ends were cut in  $9$  mM  $K_2EGTA$  internal solution. All three transients were elicited in response to 50-ms depolarizing pulses to  $0$  mV. The top trace is a control after several minutes of rest, the lowest was obtained after several stimuli had been applied at 20-s intervals, and the intermediate trace after period of rest for 2 min after the last pulse. Temperature:  $10^\circ\text{C}$ . Voltage clamp experiment. Estimated internal dye concentration:  $0.66$  mM. Movement was completely eliminated by the high EGTA internally. Calibration:  $\Delta A_{660}/A_{532} = 0.05$ .

trace) or with other immobilization procedures (Fig. 2, 0 mV traces) is not evident. Secondly, the transients decay much more rapidly upon termination of the pulse. Finally, stimulation at the usual rate of 3/min results in a type of use-dependent inhibition of the transient. A period of rest of 3–5 min between pulses at the holding potential of  $-100$  mV is required to successfully elicit a second calcium transient of comparable magnitude to that elicited by the first pulse. This effect is not seen with 3 mM EGTA at the normal 3/min stimulation rate.

In conclusion, there appears to be a window of EGTA concentrations that can be used to measure unaffected arsenazo III calcium transients without interference from movement artifacts.

#### *Effects of Blocking Membrane Conductances*

We have thus far shown calcium transients elicited in voltage-clamped muscle fibers in which neither of the major depolarization-activated membrane conductances (Na or K) has been blocked. In Fig. 4A we present records

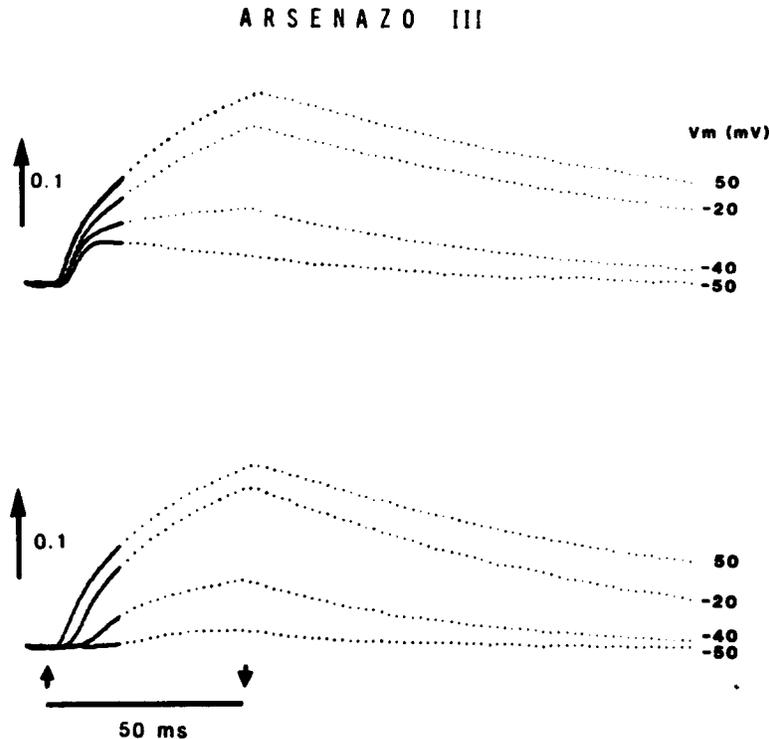


FIGURE 4. Arsenazo III calcium transients elicited in response to 50-ms depolarizing pulses to the potentials indicated from a fiber whose ends were cut in 3 mM EGTA,  $\text{Cs}^+$ -containing internal solution. The top set of traces was elicited in normal Ringer, the lower set in Ringer plus  $10^{-7}$  M added TTX. Both sets of transients were obtained from the same fiber after a solution change. Temperature:  $10^\circ\text{C}$ . Voltage clamp experiment. Estimated internal dye concentration: 0.80 mM. Calibration:  $(\Delta A_{660} - \Delta A_{740})/A_{532} = 0.1$ .

obtained from a fiber with  $\text{Cs}^+$  present in the cut end solution (as in the antipyrylazo III experiments of Kovacs et al., 1979) rather than  $\text{K}^+$ . This substitution blocked delayed rectification (see also Hille and Campbell, 1976; Vergara and Bezanilla, 1981), but the calcium transients obtained were not significantly different from those previously shown in Figs. 2 and 3A. External application of  $10^{-7}$  M tetrodotoxin (TTX) to the same fiber additionally blocked the sodium conductance. Sodium channel blockage slowed down the early rising phase of the signals and revealed a gradation of the initial rise with respect to potential (Fig. 4B). In contrast, the intermediate and decaying phases of the signal appeared scarcely affected by TTX.

*Some Problems Regarding the Use of Arsenazo III*

Results have been presented without particular emphasis on how to relate the measured absorbance changes to calcium concentration changes. We describe below certain anomalies of the arsenazo III signals that are difficult to explain on the basis of previously reported data in the literature about the equilibrium chemistry of the dye (e.g., Scarpa, 1979).

In Fig. 5 action potential stimulation of a fiber is used to elicit optical traces at three selected wavelengths in the absence of detectable fiber movement. In

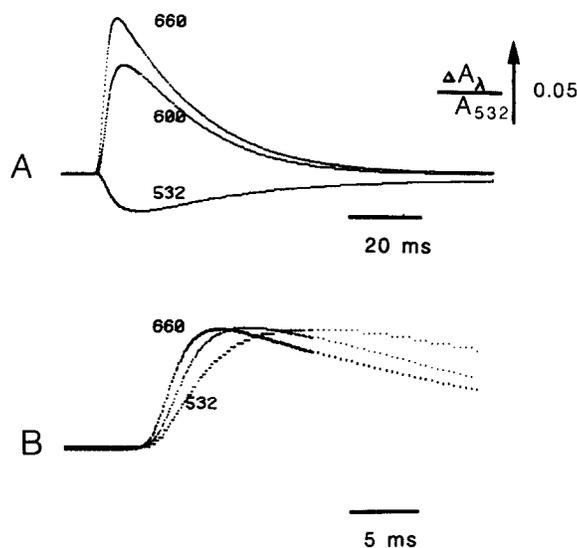


FIGURE 5. Temporal comparison of calcium transients at three different wavelengths in a fiber presoaked overnight in high EGTA, cut in a solution containing  $10^{-4}$  EGTA and exhibiting no visible movement. The transients are displayed at two different time bases and are scaled to the same size in B to demonstrate that even the rising phases show dissimilar kinetics. The action potential stimuli were from a hyperpolarized resting potential of  $-105$  mV in each case to an overshooting value of  $+15$  mV. Temperature:  $21^\circ\text{C}$ . Estimated internal dye concentration:  $1.20$  mM.

Fig. 5A the traces shown are unscaled records with increased absorbance indicated by upward deflections. The signals at 660 and 600 nm show absorbance increases and the 532-nm trace shows an absorbance decrease. Qualitatively this fulfills the expectations of equilibrium calibration determinations of calcium added to arsenazo III. However, the 660-nm trace is clearly larger than the 600-nm one. This finding was consistently observed in all our experiments and agrees with other similar calcium transient determinations in muscle (Baylor et al., 1979a). This greater absorbance increase at 660 nm is not mirrored in equilibrium calibration determinations (Scarpa, 1979; Palade and Vergara, 1981), where 600 nm absorbance increases are larger than 660-nm ones.

In Fig. 5B the first 25 ms of the traces in Fig. 5A have been expanded and scaled to the same size and direction to emphasize that there are clear differences in the time courses of the signals obtained at the different wavelengths, with the 660-nm trace fastest and the 532-nm trace slowest.

In Fig. 6 traces from another fiber are compared at 660 and 600 nm in response to an action potential elicited in current clamp (top) and two different 50-ms depolarizations under voltage-clamp conditions (middle and bottom). The larger of each pair of traces is the one obtained at 660 nm. Under voltage-clamp conditions, the longer stimuli cause 600-nm traces to be more nearly equal in amplitude to the 660-nm traces. Comparison of the bottom two traces suggests additionally that larger depolarizations further reduce the difference in amplitudes between 600- and 660-nm records. Finally, as most apparent in the bottom trace, the signals at the two wavelengths are most nearly equal in amplitude at the end of a long depolarization; termination of the pulse then increases the differences in amplitudes again.

A simple model for dye-calcium complexation inside muscle fibers would predict that amplitudes of signals obtained at different wavelengths be comparable to those amplitude differences seen with *in vitro* equilibrium calibrations. Furthermore, the time courses of the signals at the different wavelengths should be the same if the signals only reflected a simple complexation process. Clearly, these expectations are not fulfilled. We have addressed two possible additional contributions to the observed absorbance changes, myoplasmic pH and Mg concentration changes, in order to assess their role in the observed time course and amplitude differences. Both pH and Mg changes are known to affect arsenazo III absorbance (Scarpa, 1979), and if the changes were large enough in our cut fibers, they could in principle contribute to the observed discrepancies.

Measurements of resting pH and transient pH changes were performed by applying 1–5 mM phenol red (Herbst, 1970; Baylor et al., 1979a and b) or bromocresol purple (MacDonald and Jobsis, 1976; MacDonald et al., 1977) to the cut ends of the preparation. Resting pH determinations were performed by comparing the ratios of absorbances in cut fibers at two different wavelengths with values generated as a function of pH using the capillary calibration procedure described in Methods. Results from six fibers are shown in Table IIIA, and indicate a resting pH of 6.7–6.9 in our cut fiber preparation.

Fig. 7 demonstrates absorbance changes in a phenol red-containing fiber in response to a 50-ms depolarizing pulse to 0 mV from a holding potential of  $-100$  mV. The upper trace was recorded at 550 nm, where acidification produces a decrease in phenol red absorbance. The second trace was recorded at 620 nm, where little or no dye contribution should be seen, and should reflect movement contributions alone. The third trace represents the difference between the first two, still corresponding to a decrease in dye absorbance at

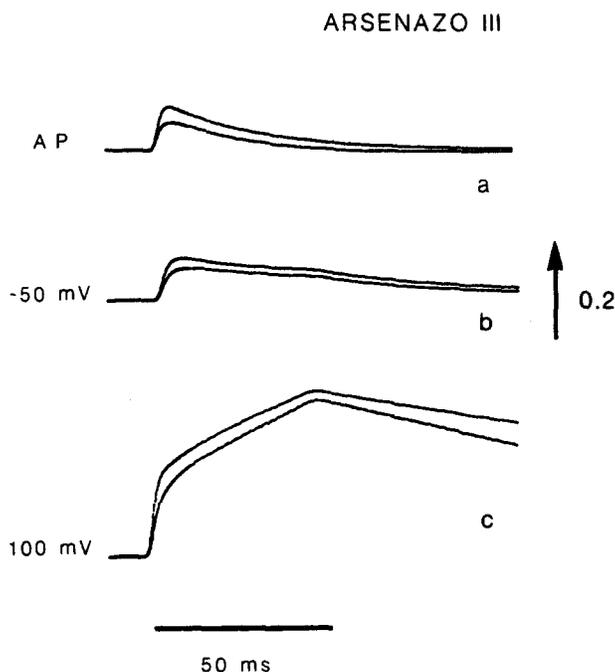


FIGURE 6. Arsenazo III calcium transients recorded at 660 and 600 nm elicited in response to action potential stimulation (*a*), or to voltage clamp pulses of 50-ms duration to the potentials indicated ( $-50$  mV [*b*] and 100 mV, [*c*]). As in Fig. 5 this fiber showed no visible movement and no subtraction was necessary. The fiber was prepared in the same manner as the one in Fig. 5. The vertical calibration bar refers to  $\Delta A_{660}/A_{532}$  and  $\Delta A_{600}/A_{532}$ . In each pair of traces the 660-nm signal is the largest. Temperature:  $21^{\circ}\text{C}$ . Estimated internal dye concentration: 1.20 mM.

550 nm. The extent of acidification in this record corresponds to 0.01 pH units. Action potential studies with other fibers generally revealed slightly smaller pH changes that tended to decay more rapidly. The largest pH change recorded from voltage-clamp experiments was 0.015 pH units.

In vitro experiments have been performed to investigate the effect of pH on arsenazo III absorbance. These determinations, using 0.45 mM arsenazo III, in the presence of 0.1 mM added  $\text{Mg}^{++}$  (see below) suggested a maximal  $\Delta A_{660}/A_{532}$  or  $\Delta A_{600}/A_{532}$  of +0.002 in response to a 0.015-pH unit acidification

in the presence of up to 0.1 mM added  $\text{Ca}^{++}$ , or roughly only 1–2% of an average arsenazo III voltage-clamp transient.

We have attempted similarly to assess magnesium contributions with the metallochromic indicator chlorophosphonazo III, which, according to our *in vitro* calibrations (in accord with results of Ferguson et al., 1964, but in disagreement with Yoshikami and Hagens, 1978) has high affinity for both calcium and magnesium at physiological pH. This feature enables measurements of free magnesium when present in vast excess over free calcium, as in

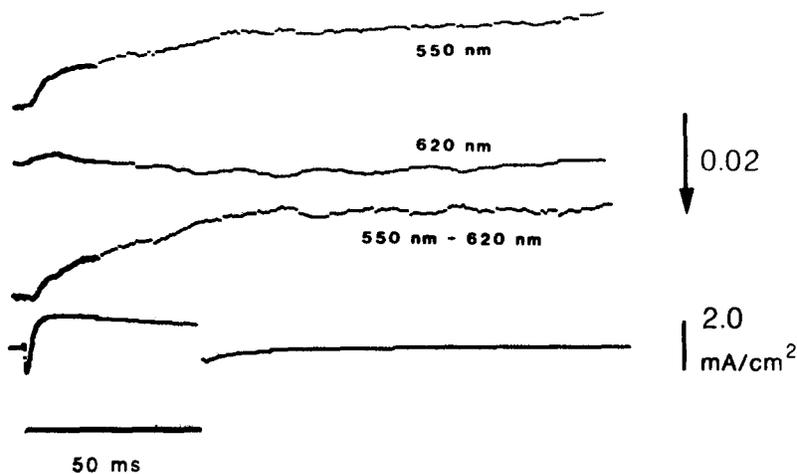


FIGURE 7. Absorbance changes in response to 50-ms pulses from a holding potential of  $-100$  to  $0$  mV in a phenol red-containing fiber. The upper trace, recorded at  $550$  nm, includes both pH and movement contributions. The second trace, recorded at  $620$  nm, includes only movement contributions. The third trace is a subtraction of the second trace from the first to represent pH contributions only. The bottom trace represents the accompanying current record. Experiment performed at room temperature ( $21^\circ\text{C}$ ) with  $3$  mM  $\text{K}_2\text{EGTA}$  and  $5$  mM phenol red applied to the cut fiber ends. Calibrations:  $\Delta A_{550}/A_{550} = 0.02$  (upper),  $\Delta A_{620}/A_{550} = 0.02$  (second),  $(\Delta A_{550} - \Delta A_{620})/A_{550} = 0.02$  (third). The downward arrow signifies the direction of increased absorbance.  $I = 2$   $\text{mA}/\text{cm}^2$  (fourth). *In vitro* calibrations have suggested that  $(\Delta A_{550} - \Delta A_{620})/A_{550}$  should decrease by  $0.193$  for a pH change from  $6.8$  to  $6.7$ , allowing us to estimate the acidification in the fiber above as  $0.01$  pH units.

resting muscle fibers. Measurements with chlorophosphonazo II gave an upper limit for free magnesium concentration of  $\sim 10^{-4}$  M when assessed by the capillary calibration procedure at pH  $6.9$  with no added  $\text{Ca}^{++}$  (Table IIIB). These low values probably result from strong fiber buffering with an excess of ATP (a strong  $\text{Mg}^{++}$  buffer) and  $100$  mM aspartate (a weak  $\text{Mg}^{++}$  buffer) in the end pool solutions, in addition to the EGTA. Attempts in the present study to assess the time course and amplitude of possible magnesium transients in these fibers have yielded inconclusive results and the influence of possible  $\text{Mg}^{++}$  concentration changes on arsenazo III signals remains to be studied.

*Antipyrylazo III Experiments*

In view of some of the problems discussed above for arsenazo III experiments, we have attempted to gain additional experimental evidence with another calcium-sensing dye. Antipyrylazo III (Scarpa et al., 1978) has previously been used to study calcium transients in a cut muscle fiber preparation

TABLE IIIA  
ESTIMATION OF RESTING pH LEVELS IN CUT FIBERS

	Capillary calibrations	0.1 mM EGTA		3 mM EGTA	
	$A_{550}/A_{450}$	Fiber 06309A	Fiber 06309B	Fiber 06309C	Fiber 07039A
Phenol red (2 mM) pH 6.4	0.136				
6.6	0.240			0.363	0.308
6.8	0.373	0.464	0.387		
7.0	0.569				
7.2	0.781				
	$A_{600}/A_{498}$	Fiber 07029A	Fiber 07039B		
Bromocresol purple (1 mM) pH 6.4	3.19				
6.6	3.32		3.69		
6.8	3.73	4.05			
7.0	4.32				
7.2	4.29				

TABLE IIIB  
ESTIMATION OF RESTING  $Mg^{++}$  CONCENTRATION

	Capillary calibrations	0.1 mM EGTA		3 mM EGTA	
	$A_{650}/A_{550}$	Fiber 10039B	Fiber 10049B	Fiber 10049A	Fiber 10049C
Chlorophosphonazo III (1 mM)					
0	0.66			0.70	
+5×10 <sup>-5</sup> Mg	0.71	0.75			0.79
+10 <sup>-4</sup> Mg	0.80		0.83		
+2×10 <sup>-4</sup> Mg	0.90				
+5×10 <sup>-4</sup> Mg	1.24				
+10 <sup>-3</sup> Mg	1.52				
Overall mean $Mg^{++} \pm$ SD:		84±38 $\mu$ M			

(Kovacs et al., 1979). Certain features of the transients reported by these authors differed from those described for arsenazo III in the current paper. A strict comparison of the transients in the same preparation seemed necessary.

As with arsenazo III measurements, the signals needed to be collected in the relative absence of fiber movement. Experiments with antipyrylazo III

were mostly performed using the EGTA presoak or high internal EGTA (e.g., 3 mM) procedures of movement blocking. Fig. 8 documents an example of antipyrylazo III calcium transients associated with an action potential elicited by current pulse stimulation. Traces are shown at three wavelengths that yield large dye absorbance changes. Some of the signal parameters of such antipyrylazo III transients associated with action potentials are tabulated in Table IV along with arsenazo III data for comparative analysis. The antipyrylazo III transients obtained at 710 nm may be slightly smaller and faster than arsenazo III transients at 660 nm under comparable conditions, but in general

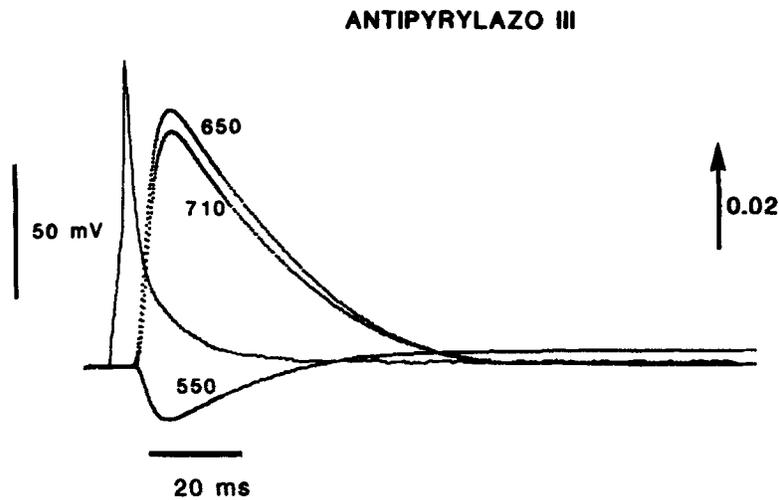


FIGURE 8. Antipyrylazo III calcium transients elicited at three wavelengths in response to an action potential from a fiber prepared as in Fig. 5, but with antipyrylazo III diffused in through the cut ends. Action potential from  $-100$  to  $+20$  mV. In this fiber subtraction of the movement artifact was performed at 790 nm, and the vertical calibration of 0.02 refers to:  $(\Delta A_{710} - \Delta A_{790})/A_{550}$ ,  $(\Delta A_{650} - \Delta A_{790})/A_{550}$ , and  $(\Delta A_{550} - \Delta A_{790})/A_{550}$ . Temperature:  $21^{\circ}\text{C}$ . Estimated internal dye concentration: 1.19 mM.

they are remarkably similar. The time courses of the antipyrylazo III signals at the different wavelengths seen in Fig. 8 were quite similar in the experiment shown, but other fibers have shown very large overshoots in the transients recorded at 550 nm.

In voltage-clamp experiments striking differences are found between antipyrylazo III and arsenazo III transients. An example is provided in Fig. 9B in an experiment carried out under nearly identical conditions to those shown in Fig. 3B for an arsenazo III-injected fiber. Although the initial rising phases of the two dye signals are similar, it can be seen that the upward-creeping intermediate phase of the arsenazo III signal is not detected with antipyrylazo III. Instead, the intermediate phase of the antipyrylazo III signals decays, after a peak, even while the depolarization is maintained. Because of this decay, antipyrylazo III signals elicited in voltage-clamp mode do not attain

amplitudes much greater than those elicited by action potentials in the same fiber. Additionally, the relaxation phase of the antipyrylazo III signal after the end of the pulse is more rapid than that seen with arsenazo III. In summary, although the two dyes appear to give similar signals with stimulation with action potentials, their signals differ significantly when longer stimuli such as the 50-ms voltage-clamp pulses are used.

The arsenazo III and antipyrylazo III signals shown in Figs. 3B and 9B, respectively, were obtained with 3 mM EGTA added to the internal solution, bathing the cut fiber ends. It might be argued that the 3 mM EGTA may affect the antipyrylazo III signals more than the arsenazo III signals. However, there are two pieces of evidence to suggest that this is not the case. First, some

TABLE IV  
COMPARISON BETWEEN ARSENAZO III AND ANTIPYRYLAZO III TRANSIENTS  
IN RESPONSE TO ELICITED ACTION POTENTIAL UNDER IDENTICAL  
CONDITIONS

Procedure	A	B	C	D	E	F	G
	Peak	Time to start (delay) <i>ms</i>	Time to ½ rise <i>ms</i>	Time to peak <i>ms</i>	Time to ½ decay <i>ms</i>	Estimated [dye] <i>mM</i>	<i>n</i>
Arsenazo III 80 mM EGTA presoak; 0.02-0.1 mM EGTA in cut end pools	$\Delta A_{680}/A_{532}^*$ 0.054±0.037	1.3±0.3	3.7±0.6	6.9±1.2	22.5±10.3	0.99±0.47	26
Antipyrylazo III 80 mM EGTA presoak; 0.02-0.1 mM EGTA in cut end pools	$\Delta A_{710}/A_{550}^*$ 0.043±0.011	1.0±0.1	2.8±0.4	5.6±1.3	18.3±3.8	0.58±0.29	6

All parameters relating to the timing of the calcium transients were measured from the time at which the action potential peaked.

All experiments were performed at room temperature.

Values correspond to mean ± SD.

\* In some cases movement artifacts were subtracted using a third wavelength (see Methods).

experiments were performed at significantly lower EGTA levels ( $10^{-4}$  M) without blocking movement by other procedures. When the signals were corrected for the movement contribution to the traces (by subtraction of the movement artifact recorded alone at 790 nm) the antipyrylazo III transients still reached a maximum early in the pulse and evidenced a declining intermediate phase. Second, as shown in Fig. 9C, with 9 mM EGTA present internally, the antipyrylazo III signals did become attenuated in a use-dependent manner similar to that previously described for arsenazo III in Fig. 3C. Such use-dependent inhibition was not observed at the normal stimulation rates used for these experiments with 3 mM EGTA present in the cut end solutions. Thus, as with arsenazo III transients, there appears to be a narrow window of EGTA concentrations that aids in blocking movement without distortion of the antipyrylazo III calcium transients.

We have also examined the effects of a 0.015 pH unit acidification on antipyrylazo III absorbance in vitro with 600  $\mu$ M dye in a manner similar to

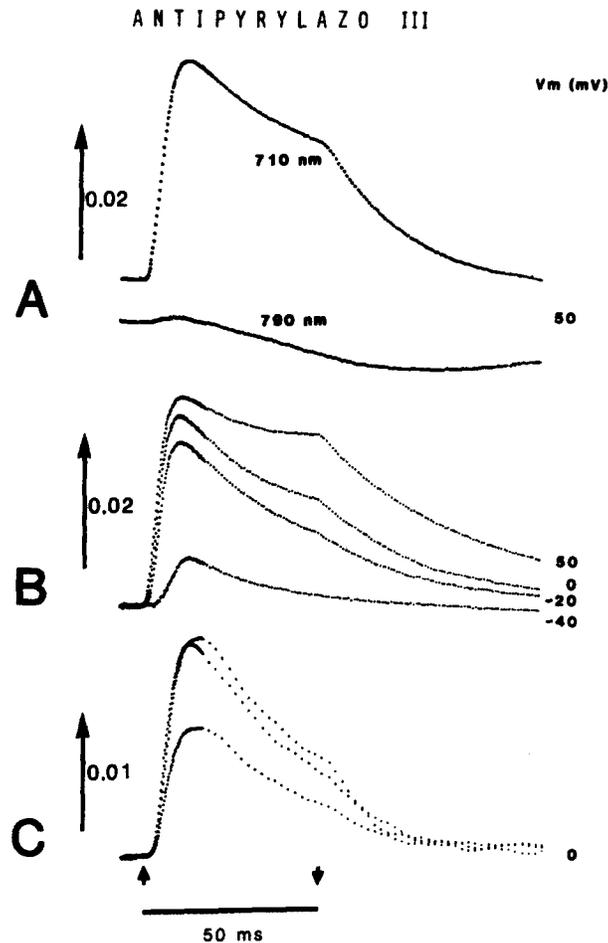


FIGURE 9. (A) Antipyrylazo III calcium transients from a fiber prepared identically to the arsenazo III injected fiber of Fig. 3A with ends cut in 3 mM EGTA-containing internal solution. 50-ms pulses to +50 mV with absorbance records at 710 nm (upper) and 790 nm (lower, reflecting only movement). Calibration:  $\Delta A_{710}/A_{550} = 0.02$  (upper);  $\Delta A_{790}/A_{550} = 0.02$  (lower). (B) To the same fiber 50-ms pulses to the potentials indicated were applied. Movement artifact subtraction was performed at 790 nm. Holding potential was maintained at -100 mV. Temperature: 10°C. Voltage clamp experiment. Estimated internal dye concentration: 3.0 mM. Calibration:  $(\Delta A_{710} - \Delta A_{790})/A_{550} = 0.02$  (C) Antipyrylazo III calcium transients elicited in response to 50-ms depolarizing pulses to 0 mV from a fiber whose ends were cut in 9 mM EGTA-containing internal solutions. The protocol was the same as that described for Fig. 3C. Temperature: 10°C. Voltage clamp experiment. Estimated internal dye concentration: 0.61 mM. No movement artifact subtraction was necessary since the high EGTA concentration had eliminated movement. Calibration:  $\Delta A_{710}/A_{550} = 0.01$ .

our determinations with arsenazo III. Such acidification resulted in a slight decrease in absorbance at 710 nm in the presence of 100  $\mu$ M added Ca, corresponding to only a  $-0.0006$  increment in  $\Delta A_{710}/A_{550}$  terms, or  $\sim 2\%$  of a typical antipyrylazo III transient.

#### DISCUSSION

The measurements of calcium transients in muscle fibers with arsenazo III and antipyrylazo III described in this article were designed to assess the capabilities and limitations of these dyes as calcium sensors in intracellular studies. Calcium transient determinations in muscle fibers demand probes of high sensitivity and fast response time that can follow extremely rapid and very large swings in myoplasmic calcium levels.

The signal-to-noise characteristics of the arsenazo III calcium transients recorded here show great improvement over calcium transients recorded with murexide (Jobis and O'Connor, 1966) or aequorin (Blinks et al., 1978). Compared with arsenazo III records obtained from fibers not singly isolated (Miledi et al., 1977*b*), significant improvement is also seen. The arsenazo III transients elicited by either action potential stimulation or by voltage-clamp pulse display similar characteristics in these cut muscle fibers as those reported by Miledi et al., (1977*b*) in their stretched intact fibers. Results presented here include more extensive voltage-clamp results, particularly in the presence of external sodium.

We have found no significant points of disagreement with the results of Miledi et al. (1977*b*), although our examination of the time courses of transients obtained at different wavelengths suggests more cautious interpretations. We have found it feasible to make use of a window of EGTA concentrations inside muscle fibers to record arsenazo III transients, a result in apparent contradiction with the report of Suarez-Kurtz and Parker (1978). However, it is not clear whether the actual concentrations of EGTA inside the fibers were at all similar in the two studies (see later). Finally, our results show no significant discrepancies with those results reported by Baylor et al., 1979*a* and *b*. Although we have not attempted to dissect arsenazo III transients into calcium and dichroic components, we present here more extensive voltage-clamp results over a far wider range of potentials, mostly at 660 nm, which should reflect minimal dichroic contributions (Baylor et al., 1979*b*). Antipyrylazo III transients reported here also appear qualitatively similar to those of Kovacs et al., (1979). The slightly smaller signals we recorded may reflect differences in temperature between the two studies.

One aspect of calcium transients that we have preliminarily explored involved the effects on signal characteristics of blockers of muscle membrane conductances that are activated upon depolarization. Comparison of Figs. 3B ( $K^+$  in the cut end solution) and 4A ( $Cs^+$  in the cut end solution as in the antipyrylazo III experiments of Kovacs et al., 1979) reveals no dramatic effect in the characteristics of the arsenazo III signals when delayed rectifier  $K^+$  currents are blocked. The replacement of internal  $K^+$  by  $Cs^+$  in principle

should also affect that part of the resting sarcoplasmic reticulum (SR)  $K^+$  conductance that is  $Cs^+$  sensitive (Coronado and Miller, 1979). The absence of any dramatic effect of internally applied Cs suggests that this SR conductance may not play a crucial role in the release and re trapping of calcium by the SR. In contrast, a clear effect on the calcium transients is seen when sodium conductance is blocked by TTX, as shown by comparison of Figs. 4A and B. Similar results were obtained in antipyrylazo III experiments (not shown) in which TTX was added or tetramethylammonium (TMA) was substituted for Na ions in the external solution. Both the voltage dependence and time course of the signals were altered in a manner compatible with the presence of a tubular membrane sodium conductance (Adrian et al., 1969; Costantin, 1970; Bezanilla et al., 1972; Adrian and Peachey, 1973; Bastian and Nakajima, 1974). Such results are in agreement with findings that the tubular sodium conductance acts to speed the rate of transverse-tubular depolarization (Vergara and Bezanilla, 1981) and hence the initial rate of calcium release from the SR. In certain experiments, e.g., Figs. 2, 6, and other experiments not shown, pulses to beyond the  $Na^+$  equilibrium potential revealed no diminution in either the rate of rise or amplitude of the signals, which suggests no major direct role of sodium influx per se.

The successful use of 2–3 mM EGTA applied to the cut ends of the fibers to effectively block tension development without apparent distortion of either arsenazo III or antipyrylazo III optical calcium signals is certainly a significant finding. A precedent for such application of EGTA was earlier set in Nile Blue A optical experiments in cut muscle fibers (Vergara et al., 1978). We cannot be certain that the concentration of EGTA in the portion of the fiber recorded from is the same as that applied to the cut ends. If it is, it appears surprising that the presence of a calcium-buffering agent of reported higher affinity for calcium than either dye does not seriously deteriorate either signal. However, despite its high affinity for calcium, EGTA does not complex calcium particularly rapidly (Hellam and Podolsky, 1969; Smith et al., 1977; Harafuji and Ogawa, 1980), and several reports suggest that the dyes may be significantly faster in their rates of calcium complexation (Brown et al., 1975; Scarpa, 1979; Palade and Vergara, 1981). Thus EGTA might only slowly buffer a calcium transient (Tsien, 1980). When the EGTA concentration was raised high enough in the present study, distortions were noted in the calcium transients (Figs. 3C and 9C). These distortions did not seriously affect the initial rising phases of the dye signals, but did significantly affect the intermediate and decaying phases, particularly in the arsenazo III traces. Additionally, a use-dependent inhibition of the transients was observed, which suggests that when the EGTA does finally chelate large quantities of calcium, it lowers the free myoplasmic calcium levels to the point (cf. Duggan, 1977) where the calcium pump is no longer maximally activated. Then, under conditions of repetitive stimulation, the SR may become temporarily depleted of calcium, as reflected by the small calcium signals obtained under these conditions.

Another consideration in the EGTA-related findings is a means of inter-

preting how EGTA, if it binds calcium more slowly than the dyes, is able to block tension generation, in the face of evidence demonstrating that the rate of calcium binding by troponin is extremely rapid (Johnson et al., 1979). We have not investigated this problem sufficiently to provide a definitive answer, but we suggest that the solution to this problem may be related to the supposition that the dyes, other myoplasmic chelators, and EGTA itself bind calcium in the 0.5- $\mu\text{m}$  (Peachey, 1965; Mobley and Eisenberg, 1975) diffusional space that separates the terminal cisternae release sites from the region of myofibrillar overlap where the cross-bridge-generated tensions develop. Under these conditions, the extra buffering capacity due to the added EGTA would slow and reduce the  $\text{Ca}^{++}$  profile approaching the troponin, thereby blocking tension development but still allowing Ca-dye complex formation (as outlined above) in the diffusional space. That diffusion with binding might be slow enough to permit such a possibility is given some credibility by the observation that when movement is observed under our experimental conditions, it invariably lags behind the onset of the calcium transient by several milliseconds (Fig. 1A). The differential effect of a narrow range of EGTA concentrations on tension development as opposed to calcium transients is unlikely to be due to a direct effect of EGTA on the contractile apparatus in view of the abundant number of quantitative skinned fiber studies (reviewed in Endo, 1977) involving tension development determinations in the presence of high concentrations of EGTA as a calcium buffer. Thus we suggest that calcium diffusion with binding may need to be given more careful scrutiny in models that attempt to relate calcium movements to tension production (e.g., Ashley and Moiescu, 1972). Such diffusion with binding has indeed been given consideration in other preparations in which calcium entry is believed to take place nearly exclusively across surface membranes (Smith and Zucker, 1980). The consequences of such diffusion with binding on interpreting signals in terms of calcium concentration changes will be discussed later.

#### *Differences in Results Obtained with the Two Dyes*

A number of results are not held in common with both probes. The most significant of these results concerns the disagreement in the signals during the longer voltage-clamp stimulation, since these differences are not only quantitative but qualitative.

We can rule out some possibilities that could be contributory toward the secondary rise (intermediate phase) of arsenazo III signals, which is not mirrored by the antipyrylazo III transients. A differential sensitivity of the indicators to possible changes in myoplasmic pH levels can be excluded because these have been demonstrated to be very small under our experimental conditions. Differential dye compartmentalization seems unlikely because both dyes are observed to diffuse easily once inside the fiber, and resting absorbance values do not indicate either dye being appreciably in a calcium-dye complex form, as would be the case if one had penetrated into the SR. With regard to differential sensitivity of the dyes to  $\text{Mg}^{++}$ , at the wavelengths used arsenazo III is more sensitive to changes in  $\text{Mg}^{++}$  levels than antipyrylazo

III. However, the 0.1-mM upper limit given by Baylor et al. (1979*b*) for a magnesium transient associated with a twitch would result, according to our *in vitro* calibrations, in a  $\Delta A_{660}/A_{532}$  of only 0.007, or ~10% of the values we find for arsenazo III twitch transients. Proportionately, antipyrylazo III signals would be even less affected at the wavelengths used. In summary,  $Mg^{++}$  transients associated with long voltage-clamp pulses would have to be considerably larger than 0.1 mM to explain the differences seen in transients with the two dyes under voltage-clamp conditions.

Possible chemical peculiarities of the dyes need to be explored further to resolve this matter. In this regard, one of the more obvious potential sources of concern involves the recognition that these probes, by virtue of their high affinity for calcium, cannot be presumed to be truly inert. At the levels of intracellular dye concentrations used in this work, both dyes probably reduce free calcium to levels that may interfere with SR calcium resequestration. Antipyrylazo III, having a lower affinity for calcium than arsenazo III, should produce less interference and may therefore provide a more faithful picture of the myoplasmic free calcium concentration change. The arsenazo III signals described here, on the other hand, may more closely monitor the calcium release process from the SR. Further experimentation at low intracellular dye concentrations with both dyes will be necessary to verify these possibilities.

With respect to arsenazo III calcium transients, it is disturbing to note that the time course of the signals is different at different wavelengths. An explanation for this finding has recently been suggested by Baylor et al. (1979*a* and *b*), involving a dichroic component to the signals that may reflect a population of dye molecules bound to oriented subcellular structures. This hypothesis has been further reinforced by recent biochemical findings regarding arsenazo III binding to myoplasmic constituents *in vitro* by Beeler et al. (1980). Baylor et al. (1979*a*), however, have demonstrated minimal dichroic contribution to arsenazo III transients at 660 nm in heavily injected fibers, and we have attempted to adhere to those conditions in the present study. Nevertheless, we are still reluctant to calibrate our signals in terms of calcium concentration changes even under these conditions because we find that arsenazo III transients are inevitably larger at 660 nm than at 600 nm, even in heavily injected fibers. Such findings have also been reported by Baylor et al. (1979*a*) even when corrections are made to remove dichroic contributions, and similar findings have been documented for arsenazo III transients in other nonmuscle preparations (e.g., Ahmed and Connor, 1979). Our *in vitro* calibrations suggest that larger absorbance signals should be obtained at 600 nm but also that arsenazo III forms more than one calcium-dye complex (Thomas, 1979; Ogawa et al., 1980; Palade and Vergara, 1981). The complexes almost assuredly have different absorbance characteristics and may be formed in different ratios in the millisecond time domain than at equilibrium.

Recently reports have also surfaced regarding the formation of more than one calcium-antipyrylazo III complex (Ogawa et al., 1980; Palade and Vergara, 1981). Thus for this indicator as well, calibrations with the aid of equilibrium measurements alone should not be considered safe. For both dyes

the possible need to take into account calcium diffusion with binding suggests additionally that only a portion of the dye molecules (those closest to the SR terminal cisternae) contribute strongly to the transients observed, especially at short times. This would certainly invalidate calibrations in which absorbance changes were related to calcium concentration changes assuming homogenous distributions of both calcium and dye molecules.

Although certain of the conclusions drawn here may represent a setback to experimenters intent on using these dyes for quantitative analysis of myoplasmic calcium changes, some useful qualitative information may nevertheless be gathered. Further detailed chemical analysis may eventually allow calibration of such transients, provided kinetic schemes for the interactions of the probes with calcium are examined rather than merely equilibrium ones, as in the past. Finally, the fact that the two dye signals do differ qualitatively in certain aspects holds forth the possibility that the competition between the dyes and myoplasmic calcium buffering and sequestration mechanisms may be further explored by the comparative use of both dyes in a manner that could yield useful, complementary information.

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