

Nile Blue Fluorescence Signals from Cut Single Muscle Fibers under Voltage or Current Clamp Conditions

JULIO VERGARA, FRANCISCO BEZANILLA, and BRIAN M. SALZBERG

From the Department of Physiology and the Brain Research Institute, School of Medicine, and the Molecular Biology Institute, University of California, Los Angeles, California 90024 and the Department of Physiology and Pharmacology, School of Dental Medicine and Institute of Neurological Sciences, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT A method is presented for recording extrinsic optical signals from segments of single skeletal muscle fibers under current or voltage clamp conditions. Such segments, which are cut from intact fibers, are maintained in a relaxed state, while exhibiting otherwise normal physiological properties, including healthy delayed rectifier currents. Extrinsic fluorescence changes are demonstrated, using the permeant potentiometric probe, Nile Blue A. These changes vary nonlinearly with the controlled surface membrane potential, in a manner which suggests that they arise from potential changes in the sarcoplasmic reticulum. According to this interpretation, a simple model based on the gating charge movement implicated in excitation-contraction coupling, provides a self-consistent description of the voltage dependence of the signal that requires no additional parameters.

INTRODUCTION

Potentiometric fluorescent dyes have been used in cell physiology since their introduction as monitors of neuronal membrane potential (Salzberg et al., 1973; Cohen et al., 1974; Waggoner, 1976; Cohen and Salzberg, 1978). In skeletal muscle, these probe molecules may provide information about electrical phenomena occurring in membrane compartments (T system and sarcoplasmic reticulum) that, for reasons of size and topology, are not accessible to direct electrical recording. The T system does, and the sarcoplasmic reticulum (SR) seems likely to, develop transmembrane potential changes whose magnitude and time-courses, if measured experimentally, would contribute to our understanding of excitation-contraction coupling. Morphological studies on frog skeletal muscle fibers (Peachey, 1965) have shown that in contrast to the surface and T tubular membranes, the SR membrane does not contact the extracellular space. Consequently, impermeant dyes can bind only to surface and T-system membranes, whereas permeant dyes will stain SR membrane as well. If the size of the optical signal provided by a potentiometric dye is proportional to the magnitude of the transmembrane potential change and to the stained membrane area monitored, then it may be possible to detect even small potential

changes, if they arise in large membrane compartments. In addition, we can selectively monitor potential changes in different membrane compartments, by using either permeant or impermeant probe molecules. In frog skeletal muscle, the ratio of membrane areas for SR:T system:surface is, for a 145- μm fiber, approximately 196:10:1 (Peachey, 1965) or 73:8:1 (Mobley and Eisenberg, 1975).¹

Merocyanine 540 (dye I of Cohen et al., 1974) is an impermeant fluorescent molecule that is capable of sensing electrical potential changes of the surface and T system membranes (Landowne, 1974; Nakajima et al., 1976; Vergara and Bezanilla, 1976). Muscle fibers stained with permeant dyes such as Nile Blue A (Bezanilla and Horowicz, 1975; Bezanilla and Vergara, 1978) or an indodicarbocyanine dye (dye XIX of Ross et al., 1977; Oetliker et al., 1975) however, exhibit fluorescence changes that are not proportional to either the surface or T system membrane potential. These extrinsic fluorescence changes have been ascribed tentatively to membrane potential changes at the level of the sarcoplasmic reticulum.

We report here experiments performed on single muscle fibers, stained with Nile Blue A, and subjected to conditions of current or voltage clamp (Hille and Campbell, 1976). The results confirm observations made previously on whole muscle or bundles of fibers (Bezanilla and Horowicz, 1975; Bezanilla and Vergara, 1978), and extend these by means of the voltage clamp technique. A simplified model is proposed which relates the presumed sarcoplasmic reticulum membrane potential change, recorded optically, to the charge movement associated with excitation-contraction coupling (Chandler et al., 1976 *b*).

A preliminary report of this work has appeared elsewhere (Vergara and Bezanilla, 1977).

METHODS

The voltage and current clamp techniques that will be described in this paper are modifications of those recently described by Hille and Campbell (1976). Their method employs a potentiometric triple Vaseline gap and an isolated muscle fiber segment whose ends are recut in solutions compatible with the internal ionic environment, and which have low calcium concentration. In general, their procedures were adapted to our purposes by introducing the following two changes. (*a*) Their recording chamber was modified by placing a tapered fiber optic in pool A to illuminate or collect light from a muscle fiber segment that is current or voltage clamped. (*b*) Their soaking procedures and solutions were modified to allow us to work with relaxed fibers that could twitch in the early stages of the experiments but that did not contract after a reasonable waiting period. Fibers were internally equilibrated with solutions having compositions similar to those used in skinned fiber preparations (Ford and Podolsky, 1972).

Differences between Hille's and Campbell's preparation and this preparation are summarized in Table I.

Recording Chamber

Fig. 1 A is a diagram of the recording chamber. The notation used follows Hille and Campbell (1976). Lucite partitions are drawn in black and solution filled pools in white. The optical fiber (of) was an image conduit 38 mm long, tapered from 1 mm to 150 μm (Galileo Electro-Optics Corp., Sturbridge, Mass.). As shown in the side view of the

¹ These ratios do not include the area of caveolae.

chamber, the narrow end of the optical fiber was perfectly level with the partition separating the A and B pools, so that the muscle fiber was actually resting on top of the polished glass surface. The other end of the optical fiber protruded from the bottom of the recording chamber through a hole sealed with sticky wax. The Vaseline seals covering the muscle fiber on partitions EA and AB were made of thick Vaseline, allowing always for a thin layer of the solution in pool A to cover the muscle fiber. This was checked by microscopic observation of the flow of stained solutions in pool A. The resistance between adjacent pools of the recording chamber, in the absence of a short-circuiting muscle fiber, was measured to be higher than 20 M Ω .

Preparation

Fibers were dissected from the dorsal heads of the semitendinosus muscles of large specimens of the bullfrog *Rana catesbiana* or from the ventral heads of the same muscle in the Chilean frog *Calyptocephala gayi*. The muscles were placed in a dissection chamber filled with Ringer's solution in which they could be rotated along their longitudinal axis. The dissection chamber was made of transparent Lucite to permit transillumination of

TABLE I
COMPARISON OF FIBER PROPERTIES IN THIS WORK WITH THE HILLE AND CAMPBELL PREPARATION

	Hille and Campbell (1976)	This work
Fiber diameter, μm	150–180	120–170
Sarcomere length, μm (<i>mean</i> \pm <i>SD</i>)	1.6 ± 0.2	2.23 ± 0.19
Delayed rectifier	Severely decreased	Normal
Contractile state	Contractured	Fiber can twitch
Exposure of fiber interior to Ca containing solutions	During dissection, both ends of 6-mm-length segments were exposed to Ringer's.	During dissection, one end of 10–20-mm-length segments was exposed to Ringer's. The same end was exposed to loading solution during contraction.

the muscles. The muscles were thoroughly cleaned of connective tissue, and a layer of superficial fibers was removed until bundles of loose fibers were found. A bundle of two to five fibers was then pulled away from the rest of the muscle by holding the fibers with a tweezer at a distance of ~10–20 mm from the tendon. Single fiber segments were carefully pulled away from the other fibers of the bundle, peeling them towards the tendon with the aid of needles to cut the connective tissue. This was the first time the interior of the fiber was in contact with Ringer's solution. When an isolated fiber segment was obtained with an undamaged mid-region, it was stimulated with a 0.5-ms current pulse applied between two platinum wires connected to a square pulse generator. Fiber segments (120–170 μm in diameter), giving single twitches when stimulated were completely isolated by cutting the piece of tendon in which they were inserted and removing all the surrounding fibers. Isolated fiber segments were then transferred to a Lucite carrier having a trough along which they could lie and were removed from the dissecting chamber. The solution in the trough of the carrier was then changed from Ringer's solution to the loading solution shown in Table II. This solution change was done within 5 min after the fiber interior was first exposed to Ringer's solution. The fiber segments underwent a contracture in that high K (120 mM) solution, but they

relaxed spontaneously (Hodgkin and Horowicz, 1960). The contractures were usually made isometric, to prevent damage, by fixing the ends of the segments with insect pins. The segments were then transferred to the recording chamber (without passage through an air-water interface) which contained the 1-EGTA (relaxing) solution of Table II, with 0.5–2 $\mu\text{g/ml}$ Nile Blue A (Allied Chemical Corp., Morristown, N.J.) added.

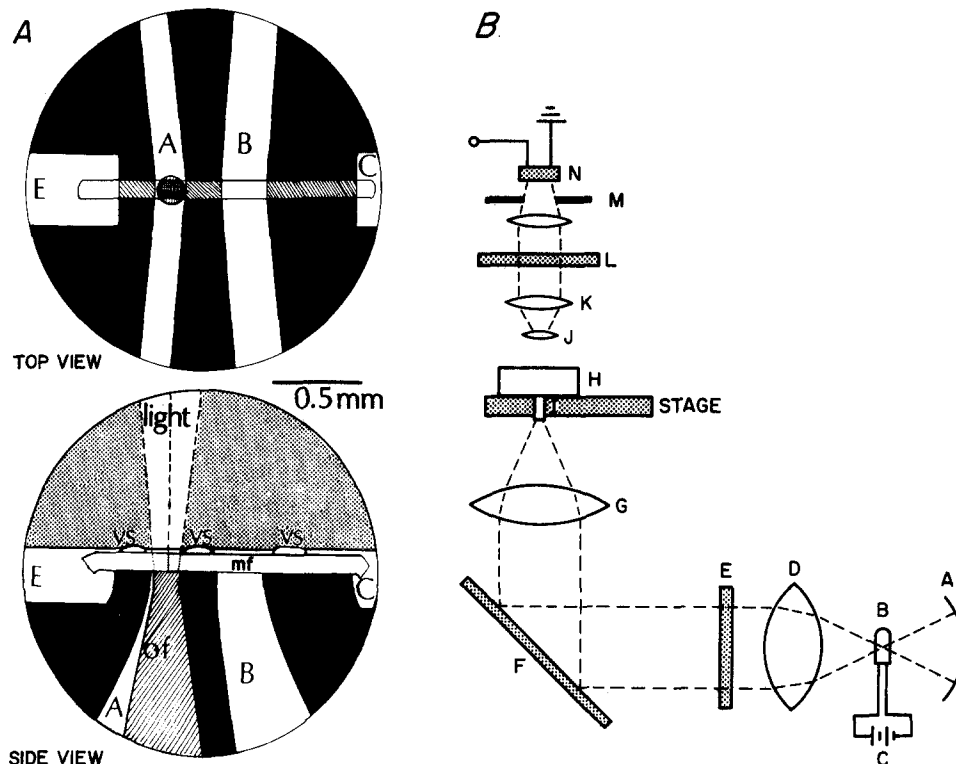


FIGURE 1. (A) Schematic diagram of the experimental chamber. Upper circle is top view, lower circle is a side view. Lucite partitions are represented as solid black areas. A, B, C, E are solution pools named according to the convention of Hille and Campbell (1976). Abbreviations: vs, vaseline seals; mf, muscle fiber; of, image conduit. (B) Schematic diagram of the optical setup built on a microscope. Light produced by a 12-V 100-Watt tungsten-halogen bulb (B) fed by the regulated power supply (C) was collimated by the condenser (D) and mirror (A). The collimated beam was rendered quasi-monochromatic by interference filter (E) and, by means of mirror (R), directed to condensing lens (G), which focused the beam on the image conduit (I) positioned in chamber (H). Light was collected by objective (J, K) and passed through barrier filter (L). The image of the fiber was framed by slit (M) and finally focused on the surface of the photodiode (N).

The concentration of Nile Blue A required to stain whole muscles (20 $\mu\text{g/ml}$) could not be used in the fiber segments because the preparation deteriorated. This effect was observed originally by Oetliker et al. (1975), but the concentrations that we were able to use (0.5–2 $\mu\text{g/ml}$) without obvious deleterious effects on the fibers were somewhat higher than the concentration (0.2 $\mu\text{g/ml}$) that they used successfully. A possible explanation for

this difference, suggested to us by Dr. R. S. Eisenberg, Rush College of Health Sciences, Chicago, is that the presence of EGTA in our staining solution could bind toxic contaminants in the Nile Blue. Although the staining procedure was carried out on the isolated fiber segment, it seems certain that the dye did not diffuse through the sarcoplasm to the region in pool A because they were stained before being mounted and recut, and in these circumstances the diffusion length was too great.

The solution covered all pools and partitions of the recording chamber, and the central portion of the segment was positioned across the three partitions that had been prepared with threads of Vaseline. They were stained for ~10 min, and then three Vaseline seals were applied, without removing the solution, leaving the fiber completely surrounded by Vaseline at the level of the three partitions. The segments were then cut at both ends with microscissors leaving stumps of ~300 μm in pools E and C making the total length ~1.5 mm. The fluid levels were lowered and the following changes in solutions were made immediately. The solution in pool A was changed to Ringer's solution; the solution in pool B was changed to 1-EGTA (relaxing); and the solutions in pools C and E were usually changed to 2-EGTA (relaxing) (Table II). In some

TABLE II
IONIC COMPOSITION OF SOLUTIONS

Solution	Na	K	TMA	Tris	Ca	Mg	Cl	Aspartate	ATP	EGTA	TTX
	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM	mM
Internal solutions											
1-EGTA relaxing	10	120	—	1*	—	1	2	120‡	5	1	—
2-EGTA relaxing	10	120	—	2*	—	1	2	120‡	5	2	—
Loading	10	120	—	1*	1	1	5	120‡	5	—	—
External solutions											
Ringer's	115	2.5	—	5	1.8	—	126.1	—	—	—	—
Ringer's+ TTX	115	2.5	—	5	1.8	—	126.1	—	—	—	0.1
0 Na, TMA	—	2.5	115	5	1.8	—	126.1	—	—	—	—
1/2 Na, 1/2 TMA	57.5	2.5	57.5	5	1.8	—	126.1	—	—	—	—

Abbreviations: EGTA, ethylene glycol-bis-(β -aminoethyl ether)*N,N'*-tetraacetic acid; TMA tetramethylammonium ion; Tris, tris(hydroxymethyl)amino methane; TTX, tetrodotoxin.

* Trizma base was added to buffer the solution at pH = 7.1.

‡ The concentration of aspartate given is only a nominal value. Potassium aspartate was made by neutralization to pH = 7.1 of known amounts of KOH with aspartic acid.

experiments, the solutions in the latter pools were kept without change or changed to 1-EGTA (relaxing) without Nile Blue. The capability of the truncated fiber segment to give action potentials was tested 10 min after the change of solutions. The fiber was then allowed to equilibrate for at least 15 min until no movement could be observed under the microscope when action potentials were elicited.

Immediately after the segment was mounted in the experimental chamber, the action potential produced visible contractions (sometimes dislodging the Vaseline seals and terminating the experiment prematurely). This movement decreased with time, presumably as the EGTA diffused in the sarcoplasm towards the A pool. The time that it took to completely abolish visible movement under the microscope was variable, ranging between 15 and 30 min. This was due, most likely, to the variability in the length of the terminal stumps from preparation to preparation. In a few cases, movement was not eliminated entirely even 45 min–1 h after the ends were cut. It is possible that this occurred because the cut end clotted, preventing the diffusion of EGTA. In general, and as anticipated, 2-EGTA (relaxing) gave more reliable and faster blockage of movement than 1-EGTA (relaxing). All these results suggest that the methods that we used preserved the

excitation-contraction coupling mechanisms in the fiber segments, and that blockage of detectable movement resulted primarily from the presence of EGTA in the sarcoplasm of the fiber. The fluorescence signal, on the other hand, is not blocked by the presence of EGTA, but it deteriorates slowly during the course of an experiment even though the electrical properties of the fiber are maintained.

Voltage Clamp

The electronic arrangement used for voltage and current clamp was the same as that described by Hille and Campbell (1976). In most of our experiments, however, we used sintered Ag-AgCl electrodes (Annex Corp., Santa Ana, Calif.) instead of calomel electrodes.

The holding potential was not measured directly but was estimated in the following manner. The fiber was clamped and a brief depolarizing test pulse was given to elicit an inward sodium current. The holding potential was changed in the hyperpolarizing direction until this sodium current was insensitive to the change in holding potential. Then, a depolarizing pulse of 30 mV amplitude and ~20 ms duration preceded the test pulse, and the decrease in sodium peak current was measured. This decrease was used to estimate the holding potential with the help of the h_{∞} curve for skeletal muscle fibers given by Adrian et al. (1970).

Optical Assembly

Two different optical arrangements were used. One was a vertical optical bench, in which the illumination of the muscle fiber was achieved by focusing the light beam directly on the fiber by means of a long-working distance objective (UMK50, E. Leitz, Inc., Wetzlar, West Germany). The light emerging from the muscle fiber was collected by the fiber optic shown in Fig. 1 *A*, and an image of the wide end of the fiber optic was formed on the active surface of a photovoltaic diode (PV444, EG & G, Inc., Electro-Optics Div., Salem, Mass.). This was attained by placing the photodiode in the optical tube of a Leitz Ortholux microscope. The other configuration used was essentially the inverse of this arrangement and is shown in Fig. 1 *B*; the recording chamber was seated on the stage of a modified C. Reichert Zetopan microscope (American Optical Corp., Buffalo, N. Y.), and the muscle fiber was illuminated through the fiber optic. A bright-field condenser focused the light beam, which had been made quasi-monochromatic with an interference filter, on a point within the wide end of the fiber optic, which, in turn, conducted the concentrated beam to the muscle fiber. The light was collected by a Leitz long-working distance objective (UMK50) focused on the muscle fiber, and a real image of the muscle fiber was formed on a photodiode (PV444) that was placed inside the trinocular tube of the Reichert microscope (Fig. 1 *B*). Some of the optical elements used in the two configurations were different, but the light intensity finally reaching the photodiode was approximately the same. The results obtained with both arrangements were identical, not only in terms of the shape of the signals recorded, but also with respect to the magnitude of the fractional change in measured intensity.

The stained preparation was illuminated with light from a tungsten-halogen lamp (12V, 100 Watt) filtered by an interference filter (no. 35-18-3, Baird Atomic, Bedford, Mass.) with peak transmission at 625 nm and a half band width of 20 nm (full width at half maximum). The collected light was filtered by a barrier filter that was designed to absorb the excitation light (wavelengths shorter than 665 nm), but passed the longer wavelength fluorescence emission (RG 665, Schott, Inc., New York). When both filters were placed between the light source and the preparation, there was some residual intensity (I_r) due to filter transmission overlap, detected by the photodiode. When the barrier filter was afterward placed between the preparation and the photodiode, the

amount of light detected by the photodiode was higher and was called I_t . The effective resting fluorescence (F) of the stained fiber was calculated as the difference $F = I_t - I_r$.

The values obtained for F ranged from 4 to 12 in arbitrary units. In unstained fibers the values of F recorded were significantly smaller, ranging from 0 to 2. Because the background fluorescence in unstained fibers is small, we have chosen to neglect it. This suggests that the F values reflected the degree of staining of the muscle fibers and that they could be considered to be a measure of the extrinsic fluorescence produced by Nile Blue A. The wide range of values of F can be explained in terms of different staining properties of individual fibers, as well as geometrical factors. A temperature effect on the staining properties of each individual fiber is not ruled out.

The results are expressed in this paper in terms of the change of fluorescence ΔF divided by the resting fluorescence F . The magnitude $\Delta F/F$ was approximately constant for different fibers under the same experimental conditions, showing much less variability than ΔF or F separately. The reader is referred to Cohen et al., (1974) and Cohen and Salzberg (1978) for a discussion of $\Delta F/F$ in extrinsic fluorescence measurements.

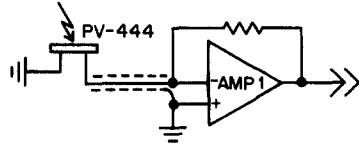
Recording Techniques

Light was transduced into voltage by the PV-444 photodiode and the current-to-voltage converter shown in Fig. 2 A. The output voltage from the converter was passed to the track-and-hold amplifier shown in Fig. 2 B, which allowed us to amplify the light signal several times without saturation of the final amplifier stage (Amp. 4) by the DC component produced by the resting fluorescence. The value of the DC component was subtracted during the "hold" period, which was synchronized to start before the stimulation of the muscle fiber (see legend, Fig. 2 B). Additional amplification and visual monitoring was provided by feeding the output of the track-and-hold amplifier to a 565 or 4103 oscilloscope (Tektronix, Inc., Beaverton, Ore.), as indicated in Fig. 2 C. Independent measurements of the DC background fluorescence were taken frequently during the experiments to calculate the correct $\Delta F/F$, by removing the track-and-hold circuit and reducing the gain of amplifier 4 of Fig. 2 B. The output of the track-and-hold amplifier was finally filtered by a simple RC circuit and fed to one of the inputs of a digital signal averager similar to that described by Bezanilla and Armstrong (1977). The time constant of the filter was set to values ranging between 100 and 500 μs .

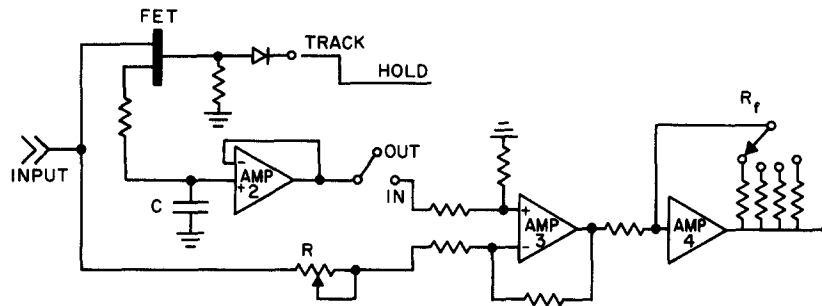
Clamp voltage and current were fed to other inputs of the signal averager as shown in the block diagram of the recording system in Fig. 2 C. Current was recorded as the voltage drop along the internal resistance of the stump of muscle fiber in pool E (I_M) or as the potential drop across an external resistor of 10 k Ω in series with the E electrode (I_E) (i.e., see Fig. 1 B and text page 278, Hille and Campbell, 1976). Whenever the results are given in current units, they were obtained by measuring I_E . Some of the current traces were corrected by subtracting leak and capacity with a circuit similar to that described by Hille and Campbell (1976). A Digitimer 4030 (Devices Instruments, Welwin Garden City, Hertfordshire, England) was used to generate the pulses, to control the signal averager, to program the pulse pattern, and to time the track-and-hold amplifier. All these functions could be performed, whether the experimental conditions required single pulses or trains of pulses, with or without signal averaging. Data could be taken at two different sampling rates during each sweep. The first 512 points were taken at a rate C_1 -100-200 $\mu\text{s}/\text{point}$ and the second 512 being always larger than or equal to C_1 .

The signal averager had four analog input channels and four digital-to-analog output channels. Every sweep was recorded by directly dumping the binary contents of the memory into a stereophonic tape recorder (2300S, Teac Corp. of America, Montebello, Calif.) at a rate compatible with the frequency response of the latter. Records could be

A. LIGHT-TO-VOLTAGE CONVERTER



B. TRACK & HOLD AMPLIFIER



C. BLOCK DIAGRAM OF EXPERIMENTAL SETUP

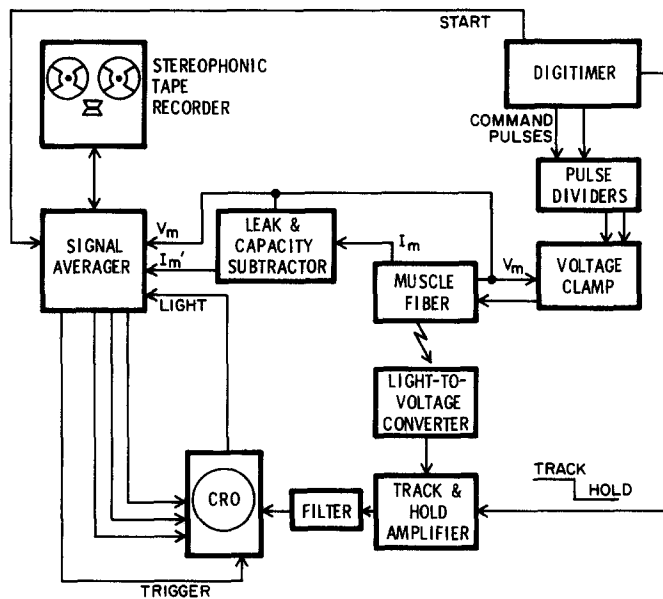


FIGURE 2. (A) Light-to-voltage converter. The photodiode (PV-444) was connected as a current source to amplifier AMP1, wired in a current-to-voltage configuration. (B) Track and hold amplifier. Capacitor C_1 was charged to the voltage of the input when the field effect transistor (FET) was conducting (TRACK mode). During HOLD mode, the FET was not conducting and C_1 maintained the last input voltage before the track-to-hold transition. Amplifier AMP2 read the

retrieved from tape and transferred to memory, thereby recovering the original signals for data analysis. Details of the digital magnetic recording technique will be published elsewhere.

Solutions

The solutions used were isotonic and had the composition listed in Table II. The solutions filling pools E and C of the recording chamber are referred to as internal solutions; these were allowed to diffuse into the sarcoplasm of the muscle fiber for at least 25 min before the records were taken. The superfusion solutions were those filling pool A of the recording chamber.

Temperature

Some experiments were conducted at air-conditioned room temperature. In most cases, a Peltier cooling device (Cambridge Thermionic Corp., Cambridge, Mass.) was used to lower the temperature of the experimental chamber. Temperatures were monitored by introducing a thermistor probe into pool A.

Data Analysis

When the analysis could not be done directly with the signal averager, experimental records were retrieved from the magnetic tape, transferred to a NOVA-3 minicomputer (Data General Corp., Southboro, Mass.), and stored on a magnetic disk. Records could then be analyzed using the computer. To find the maximum rate of rise, the function $A[1 - \exp(-t/\tau_R)]^m \cdot \exp(-t/\tau_D)$ was fitted to the experimental trace according to the least squares criteria using a modified Levenberg-Marquardt method (Brown and Dennis, 1972) and, from the fitted function, the maximum value of the derivative was calculated. The normalized maximum rate of rise was obtained by dividing the maximum rate of rise by the peak amplitude of the signal.²

RESULTS

Fluorescence Signals Accompanying Membrane Action Potentials

Fig. 3 A shows a simultaneous recording of a membrane action potential (upper trace) and the fluorescence change (middle trace) observed when a single fiber stained with Nile Blue was stimulated with a 0.5-ms square current pulse. The signal rises during the decaying phase of the action potential, reaching a peak

² Note that the response time constant of the apparatus was fast compared to the maximum rate of rise of the signal, and was not included in the fits. Also, the fluorescence of Nile Blue A follows the change in membrane potential with a delay of $< 50 \mu\text{s}$ at 15°C . (Cohen, L. B., H. V. Dávila, and B. M. Salzberg. Unpublished observations.)

voltage at C_1 , and a differential amplifier (AMP3) subtracted the input signal from this value when the switch (S) was in the IN position. When S was in OUT position the total light intensity was measured. Amplifier AMP4 was used to further amplify the output of AMP3, and its gain could be varied by changing the feedback resistors (R_f). Resistor (R) adjusted the common mode of amplifier AMP3. (C) Block diagram of the experimental setup. The START signal initiated at the digitimer started the signal averager which, in turn, triggered the four-channel oscilloscope CRO. V_m is membrane potential; I_m is membrane current; I'_m is membrane current after leakage and capacity current have been subtracted. For details see text.

14 ms after the peak of the action potential and then decays smoothly with a half time of 31 ms. A delay is observed between the foot of the action potential and the foot of the signal, and no fluorescence change is detected having a time-course comparable to that of the action potential. The lower trace in Fig. 3 A was obtained by placing the barrier (cut-on) filter between the interference filter and the muscle fiber (Oetliker et al., 1975). No signal is observed. Similarly, when the muscle fibers were not stained, or, when the barrier filter was removed, only flat optical traces were recorded. These results indicate that signals like that shown in the middle trace of Fig. 3 are indeed fluorescence changes originating only from stained muscle fibers and do not correspond to light scattering or absorption changes. The average characteristics of the

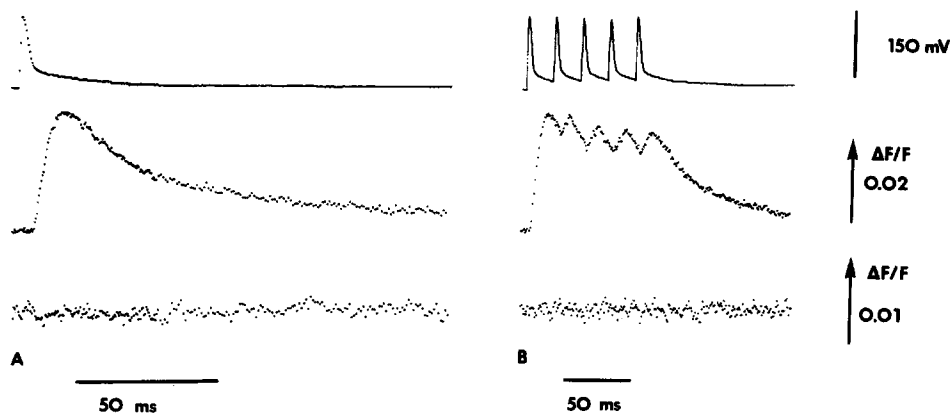


FIGURE 3. Fluorescence signals accompanying membrane action potentials. (A) Upper trace, action potential; middle trace, fluorescence signal; lower trace, fluorescence control (barrier filter between interference filter and muscle fiber). (B) Upper trace, train of action potentials (frequency, 50 Hz); middle trace, fluorescence signal; lower trace, fluorescence control. In this, and in all subsequent figures, single sweeps are shown. The solution in pool A was Ringer's; temperature, 15°C. MU04267-1. Response time constant of light measuring system = 500 μ s.

fluorescence signals recorded from fibers generating action potentials are shown in Table III.

Fig. 3 B shows the relation between a train of action potentials, elicited by repetitive stimulation of the muscle fiber, and the resulting fluorescence signal. One salient feature of the optical change is the absence of any summation during the train. No total fusion is observed even though the frequency of stimulation is 50 Hz at 15°C. The peaks of the fluorescence signals corresponding to the individual action potentials decline during the tetanus, eventually reaching a steady value. Again, as in Fig. 3 A, the bottom trace of Fig. 3 B shows that no optical signal was detected when both filters were placed before the fiber.

Whenever this experiment was repeated on fibers displaying slight movements (detectable by visual observation under the microscope at $\times 400$), the

fluorescence signal seemed to ride on top of a creeping base line. Furthermore, when both filters were placed before the fiber, the light trace was no longer flat, but exhibited a slow and smooth change with a time-course similar to that of the envelope of the creeping fluorescence signal. Fig. 4 shows the results of an experiment that illustrates the influence of a conditioning excitation on the

TABLE III
PARAMETERS OF FLUORESCENCE CHANGES ASSOCIATED TO MEMBRANE ACTION POTENTIALS

(A) Fiber number	(B) $\left(\frac{\Delta F}{F}\right)_{\max}$	(C) $\frac{d}{dt}\left(\frac{\Delta F}{F}\right)_{\max} \cdot 10^3$ ms^{-1}	(D) $\frac{(C)}{(B)}$	(E) Half time to peak ms	(F) Half time of decay ms	(G) Temperature $^{\circ}C$
1	0.019	2.22	0.117	8.0	30	15
2	0.044	5.85	0.133	7.0	26	15
3	0.029	4.26	0.147	7.0	23	15
4	0.027	4.13	0.153	8.0	24	15
5	0.022	3.37	0.153	8.0	36	15
6	0.024	3.58	0.149	7.0	24	15
7	0.024	2.95	0.123	8.0	23	15
8	0.019	2.76	0.145	9.0	26	17
9	0.022	3.83	0.174	8.0	22	17
10	0.025	5.33	0.213	4.4	13	21
11	0.016	3.90	0.244	4.8	15	22

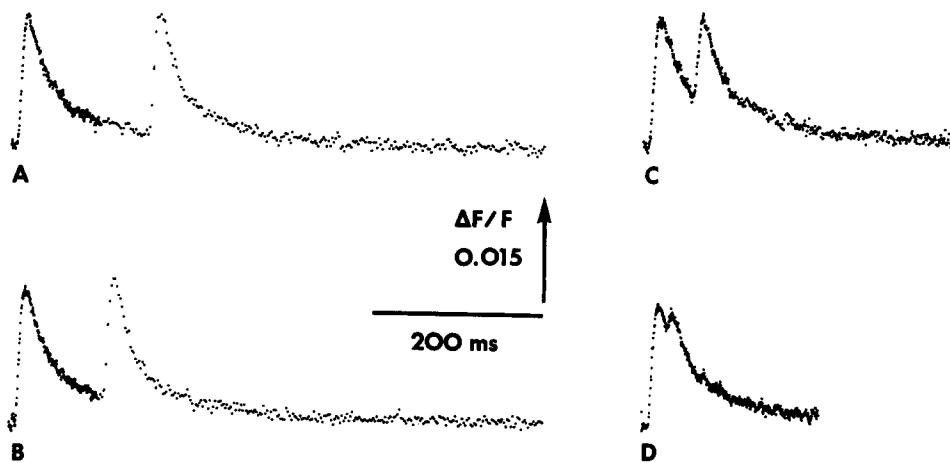


FIGURE 4. Relation between fluorescence signals and action potential separation. Pool A contained Ringer's solution; temperature, 17°C. MU04287-1. Response time constant of light measuring system = 500 μs .

fluorescence signal accompanying a second action potential, as a function of action potential separation. It is observed that, for separations longer than 150 ms (A), the preconditioning action potential has no effect on the peak value of the second signal. When the action potentials are separated by 100 ms (B), there is a small increase in this value, and there is a small decrease when the interval

between action potentials is 20 ms (D). This last case is in agreement with the experiment shown in Fig. 3 B.

Fluorescence Signals Recorded from Voltage-Clamped Muscle Fibers

Fig. 5 shows the fluorescence changes recorded from a voltage-clamped muscle fiber immersed in Ringer's +TTX solution. The fiber was voltage clamped to a holding potential of ~ -105 mV as estimated by the procedure explained in Methods. A depolarizing pulse of 105 mV produced an increase in fluorescence that appears to be maintained during the pulse and declined to its resting level after the end of the pulse. In contrast, a hyperpolarizing pulse of -100 mV is

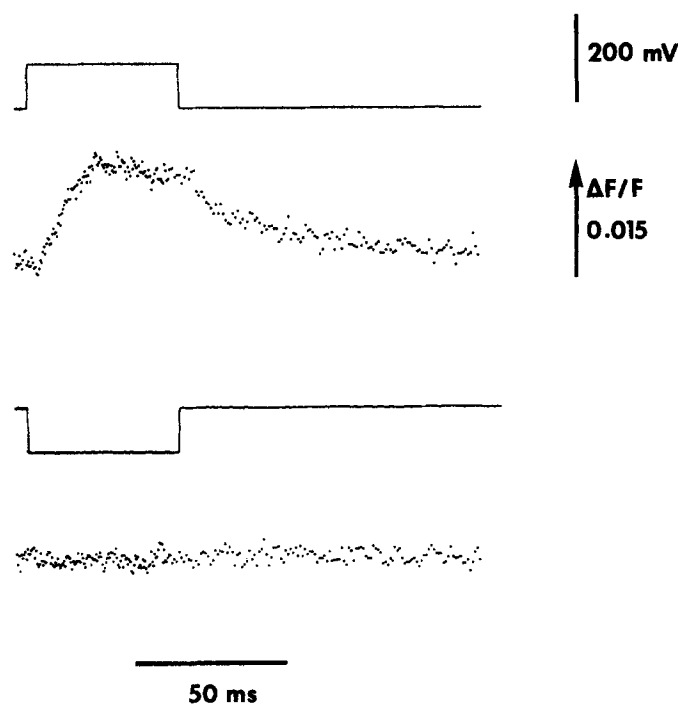


FIGURE 5. Fluorescence signals related to pulse polarity under voltage clamp condition. Holding potential, -105 mV; upper traces, 105 mV depolarizing pulse and fluorescence signal; lower traces, 100 mV hyperpolarizing pulse and fluorescence signal; solution in pool A, Ringer's +TTX; temperature, 21°C . Response time constant of light measuring system = $500 \mu\text{s}$.

not accompanied by any noticeable change in fluorescence. This asymmetry is a strong indication that the fluorescence change is not arising from a membrane system behaving linearly with surface membrane potential changes.³ The normalized maximum rate of rise of the signal associated with the depolarizing

³ Although the potential dependence of the Nile Blue A fluorescence change, in squid giant axons, was best fit, not by a linear relationship, but rather by two straight lines meeting at the resting potential-origin (Cohen et al., 1974), the slope of the dependence for a depolarizing voltage clamp step differed by much less than a factor of two, from that measured with a hyperpolarizing step. (Cohen, L. B., H. V. Dávila, and B. M. Salzberg. Unpublished observations.)

pulse shown in Fig. 5 is 0.073 ms^{-1} , significantly slower than that obtained in experiments performed under current-clamp conditions (see Table III, column D).

A series of optical records obtained with 50-ms depolarizing voltage clamp steps of various amplitudes are shown in Fig. 6. From the top, the upper three

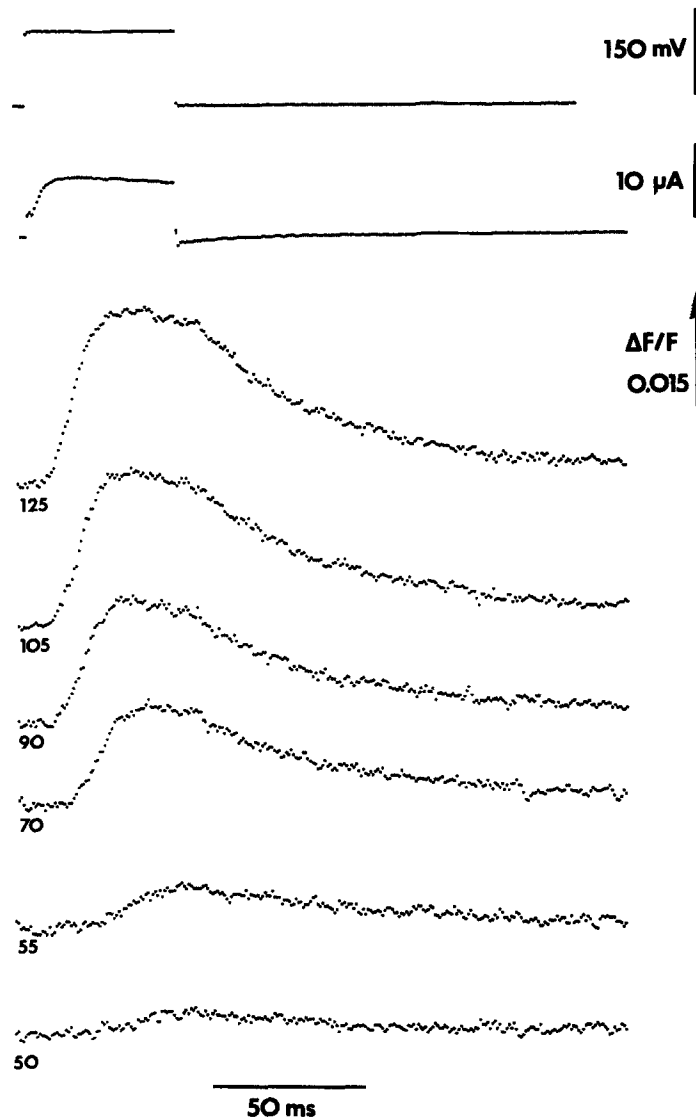


FIGURE 6. Fluorescence signals as a function of pulse amplitude under voltage clamp condition. Upper trace, sample voltage pulse of 125 mV; second trace, sample current for 125 mV pulse; third and succeeding traces, fluorescence signals associated with depolarizing voltage clamp steps of magnitudes indicated; holding potential, -100 mV ; pool A contained 0 Na , TMA solution; temperature, 15°C . MU04277-4. Response time constant of light measuring system = $500 \mu\text{s}$.

traces show voltage, current, and change in fluorescence intensity, respectively, for a 125-mV depolarizing pulse. The fiber was in 0 Na, TMA, and the holding potential was estimated previously to be -100 mV. We note that, in this preparation, in contrast to the one originally reported by Hille and Campbell (1976), delayed potassium currents having a normal appearance are observed. A typical example is shown in the second trace of this figure. The succeeding traces are the optical records obtained for the depolarizations indicated. A clear voltage dependence of both the amplitude and the rate of rise of the fluores-

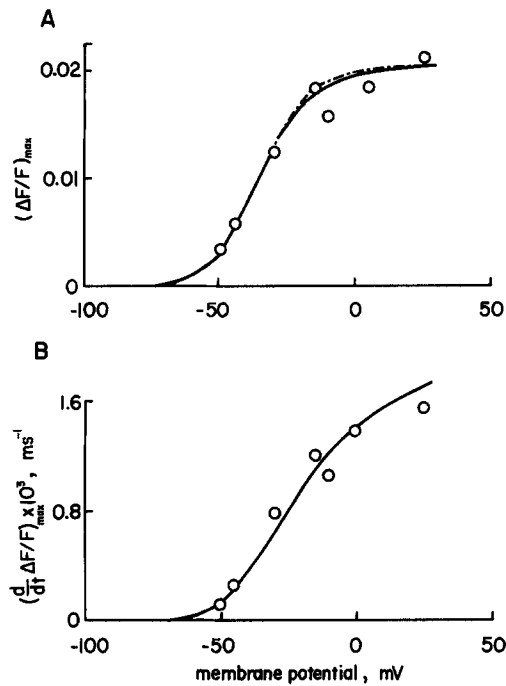


FIGURE 7. Voltage dependence of peak amplitudes and maximum rate of rise of the fluorescence signals. (A) Circles are experimental peak values of $\Delta F/F$ plotted as a function of absolute membrane potential; the dashed line corresponds to $\Delta F/F = 0.0205/\{1 + \exp[-(V + 35)/9]\}$; the continuous curve corresponds to $\Delta F/F = 0.0462/(1 + 1.2\{1 + \exp[-(V + 40)/12]\}^2)$ where V = absolute membrane potential. (B) Circles are experimental values of the maximum rate of rise of $\Delta F/F$, plotted as a function of absolute membrane potential; the continuous curve is the predicted maximum rate of rise of the model presented in the discussion. Experimental values obtained from fluorescence traces shown in Fig. 6.

cence signals is observed, although the time constant of the decaying phase of the signals appears to be independent of the potential maintained during the pulse. For large depolarizations (>90 mV) the fluorescence signal clearly is not maintained during the pulse; instead, a decaying phase is observed that seems to be similar to the decay observed for the peaks of the repetitive stimuli shown in Fig. 3 B (and, of course, in Fig. 5).

The peak amplitude of the fluorescence signals shown in Fig. 6 is plotted as a function of voltage in Fig. 7 A. Depolarizations larger than 50 mV gave

measurable signals and the amplitudes increased monotonically with voltage, saturating at positive membrane potentials. This voltage dependence is less steep than the voltage dependence of tension found in other muscle preparations (Hodgkin and Horowicz, 1960; Heistracher and Hunt, 1969; Bezanilla et al., 1971). On the other hand, it resembles the voltage dependence of the charge movement associated with excitation-contraction coupling (Schneider and Chandler, 1973; Adrian and Almers, 1976; Vergara and Cahalan, 1978).

The maximum rates of rise of the fluorescence signals presented in Fig. 6 have been plotted as a function of voltage in Fig. 7 B. This parameter also depends on membrane potential, and at small depolarizations the fluorescence signals are clearly slower than at large depolarizations. There is also an indication that the maximum rate of rise saturates at very positive potentials.

In another set of experiments we investigated the effect of pulse duration upon the fluorescence signal attendant upon voltage clamp depolarization. Fig. 8 shows the results of an experiment in which a fiber was stimulated with 150-mV depolarizing pulses of 10-, 20-, 40-, and 80-ms durations. The optical record obtained with a very short pulse (10 ms) shows that the signal continues to rise after the pulse is terminated. However, it does not reach the plateau value attained with longer depolarizations, indicating that the surface membrane potential has a delayed influence on the mechanism(s) responsible for the fluorescence change. This figure also demonstrates that the rising phases of the fluorescence signals are superimposable for all pulse durations. The late portion (from ~20 ms after repolarization) of the falling phases of the fluorescence signals also appears to be independent of the pulse duration.

DISCUSSION

Optical signals recorded from whole muscles or bundles of fibers stained with the dye Nile Blue A have been reported previously (Bezanilla and Horowicz, 1975; Bezanilla and Vergara, 1978), and single fibers without voltage clamp control have also been studied using this dye (Oetliker et al., 1975). The present work has, in contrast, employed single muscle fibers where extrinsic fluorescence changes can be studied under voltage clamp conditions. An additional advantage of the preparation described in this paper is that this arrangement permits the modification of the internal environment of the fibers. Although our preparation derives from the technique developed by Hille and Campbell (1976), it differs from theirs in certain respects (see Table I). Our studies require a single muscle fiber with both electrical and mechanical properties intact, and as close to normal physiological conditions as possible, until movement is deliberately blocked. The conditions used by Hille and Campbell, apparently, did not preserve the excitation-contraction coupling, although the electrical properties of the surface and tubular membranes, their main interest, could be studied in detail. Our principal modification of their technique, aside from the introduction of optical methods, involved the initial treatment of the muscle fiber segments. In our work, the fiber segments dissected from the muscle were somewhat longer than theirs, and we took special care to preserve one tendon. These precautions were aimed to minimize diffusion of Ringer's solution to the segment of the fiber that later was going to be in pool A. We also prevented overshooting during the K contractures by maintaining the fiber isometrically

during the change to the high potassium solution (see sarcomere length, Table I). During the contractures, after the fiber was cut, the segments behaved very much like intact isolated single fibers, exhibiting a contraction and a relaxation phase. This procedure was carried out in internal solutions containing normal amounts of Ca^{++} ions (loading solution). In a few experiments, contractures

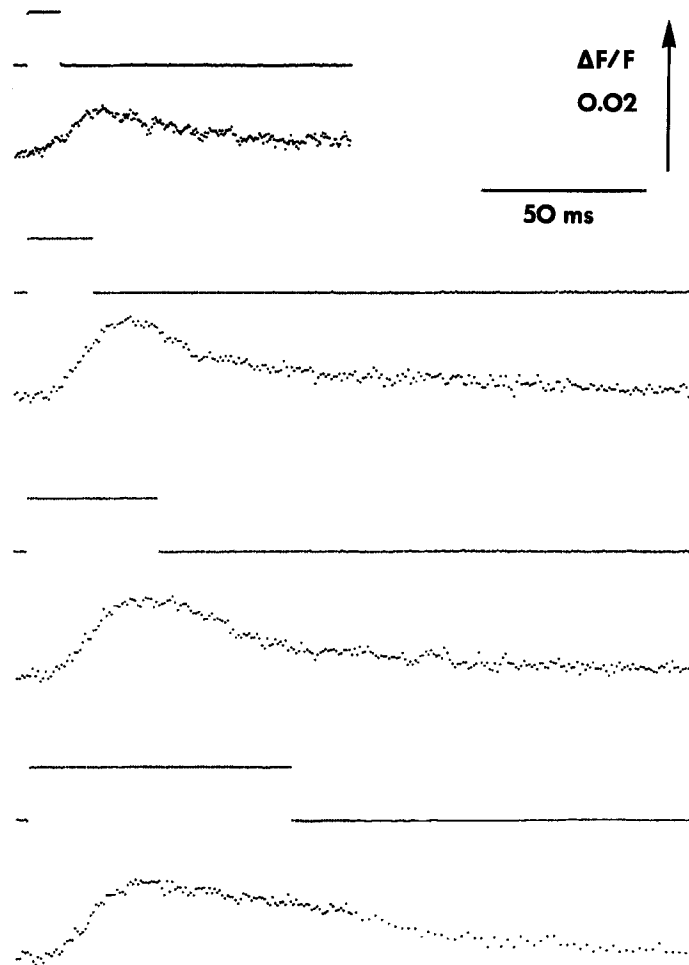


FIGURE 8. Fluorescence signals related to pulse duration under voltage clamp condition. Holding potential, -100 mV; pulse magnitude, 150 mV; pool A contained $1/2$ Na, $1/2$ TMA solution; temperature, 17°C . MU04287-1. Response time constant of light measuring system = $500 \mu\text{s}$.

were induced in the presence of internal solutions lacking Ca^{++} and with EGTA added (relaxing solution); in these cases, the contractures occurred much more rapidly, and the signals recorded later were smaller or sometimes nonexistent. This seems to indicate that the loading solution does serve the purpose of refilling, or preventing, the leak of Ca from internal stores of the fiber during and after contracture.

A final, and significant, difference is that the fiber segments that we used showed large potassium currents (delayed rectifier) having normal appearance (e.g., Fig. 6), in contrast to those observed by Hille and Campbell when they cut their fibers in K-containing solutions. The presence of the delayed rectifier resulted in the production of action potentials displaying a repolarization phase faster than that recorded by Hille and Campbell.

Origins of the Nile Blue Signal

In squid giant axon it has been shown that Nile Blue fluorescence changes are linearly related to membrane potential changes, at least for changes of a given polarity (Cohen et al., 1974;⁴). It is then reasonable to assume that similar mechanisms are operating in muscle. (However, see Cohen and Salzberg [1978] for a discussion of possible complications.) It must be recognized from the outset that the situation in this preparation is much more complicated, because skeletal muscle has a very intricate membrane system, and it has contractile proteins, all of which could be interacting with the dye during the excitation-contraction process.

In muscle one expects to see a signal originating at the surface and tubular membranes with the characteristics of fluorescence changes observed with the impermeant dye merocyanine 540 (Vergara and Bezanilla, 1976). Two of us (Bezanilla and Vergara, 1978) have reported Nile Blue A signals similar to merocyanine fluorescence changes in bundles of intact fibers, in addition to the slower signals reported originally by Bezanilla and Horowicz (1975). This can be taken as good evidence that Nile Blue is also a membrane potential indicator in muscle fibers, at least for surface and tubular membranes. In the experiments reported in this paper, we observed the larger, slower component of the extrinsic fluorescence change reported on whole muscles and bundles (Bezanilla and Horowicz, 1975; Bezanilla and Vergara, 1978), but we were unable to detect the smaller fast component expected from surface and tubular membranes because it is below our experimental resolution.

A simple explanation for the origin of the large fluorescence changes reported in this paper is that Nile Blue is monitoring, in a linear potentiometric fashion, a larger membrane compartment than the surface and T tubule membrane. Bezanilla and Horowicz (1975) proposed that this could be the sarcoplasmic reticulum. Such an explanation is supported by the observation that Nile Blue A, in contrast to merocyanine 540, is thought to be a relatively membrane permeant dye.

The evidence for this is somewhat indirect, but comes from experiments on squid giant axons (Cohen et al., 1974; Ross et al., 1977), where it was found that optical signals obtained with merocyanine 540 and a merocyanine-rhodanine dye (dye XVII), both having localized negative charges, were similar in nature, but opposite in sign, when the dye was added to opposite sides of the membrane. Conversely, signals obtained with four cyanine and oxonol dyes (XIX, XX, XXI and 366) having only delocalized charges, were in the same direction, independent of the side of the membrane to which they were added. These results are consistent if the latter class of dyes are relatively permeant and occupy the same membrane sites regardless of the side from which they are introduced,

⁴ Cohen, L. B., H. V. Dávila, and B. M. Salzberg. Unpublished observations.

whereas the former dyes are impermeant, but occupy equivalent sites on opposite sides of the membrane. Nile Blue A was not itself added to the inside of squid giant axons, but five closely related trinuclear heterocyclic dyes, positively charged like Nile Blue A, exhibited similar fluorescence changes independent of the side to which they were added. Those were Pyronine B, neutral red, neutral violet, acridine orange, and rhodamine B (dyes 29, 13, 17, 1, and 30 of Cohen et al., 1974). In addition, when Nile Blue A was added externally, the internal electrode was generally found to be stained at the end of the experiment and when rhodamine B, Pyronine B, neutral red, and acridine orange were added externally, the axoplasm was found to be stained upon extrusion (Cohen et al., 1974).

In the following, we would like to add to the discussion of Bezanilla and Horowicz, additional experimental evidence that supports the original interpretation of Nile Blue as a potentiometric probe of the sarcoplasmic reticulum membrane and to argue that some of the alternative explanations are now less likely.

In the preparation reported in this paper, movement was monitored visually with a total magnification of $\times 400$. When movement was detected, the time-course of the fluorescence change was clearly distorted, especially during its falling phase. Under these circumstances, the control records obtained with the secondary filter placed between the primary filter and the fiber (Oetliker et al., 1975) showed a deflection with a different time-course than that characterizing the Nile Blue signal. We interpret these changes in light intensities as movement artifacts and in most cases they disappeared later in time.⁵ We can summarize the evidence against Nile Blue signals being gross movement artifacts as follows: (a) We do not detect movement; (b) their time-course is too fast; and (c) when a train of action potentials are elicited, the signal does not fuse (Fig. 3). This evidence does not exclude the possibility that the Nile Blue signal could be associated with phenomena before tension development, such as latency relaxation, or with conformational changes in regulator protein (troponin or tropomyosin) or in heavy meromyosin. Baylor and Oetliker (1977) have discussed the possibility that the birefringence signal could be produced by a local change in sarcomere length during latency relaxation. In comparing the birefringence signal with the fluorescence signal, they conclude that a change of the order of 10^{-2} (which is also our $\Delta F/F$ maximum) is inconsistent with a change of only 7×10^{-5} in sarcomere length (Haugen and Sten-Knudsen, 1976). Furthermore, Gilai has demonstrated that latency relaxation cannot be detected after a tetanic stimulation,⁶ and we have detected normal-looking signals under these conditions (Fig. 3 and footnote 7). The involvement of conformational changes in myoproteins, either contractile or regulatory, in the optical signals reported here, is not ruled out directly. However, if the EGTA-relaxing solutions are preventing contraction by preventing sarcoplasmic free calcium from reaching levels at which troponin is bound, then this would constitute evidence against a protein origin for the Nile Blue fluorescence change.

⁵ It would be desirable, of course, to monitor simultaneously the optical diffraction pattern, as a more sensitive indicator of movement.

⁶ Gilai, A. Personal communication.

⁷ Vergara, J., F. Bezanilla, and B. M. Salzberg. Unpublished observations.

Recently, Suarez-Kurtz and Parker (1977) have recorded birefringence changes while monitoring Ca concentration using the metallochromic indicator dye arsenazo III (Brown et al., 1975). They found that their birefringence and Ca signals can be blocked by microinjection of EGTA. Although the sarcoplasmic EGTA concentration may be substantially higher in their preparation, this result would indicate a difference between their birefringence signal and our Nile Blue signal because the latter is not abolished by EGTA, diffused from the end pools.

Nile Blue as a Sarcoplasmic Reticulum Membrane Potential Probe: A Model

Assuming that the fluorescence changes are in fact proportional to the membrane potential changes of the sarcoplasmic reticulum (SR), and therefore, not linearly related to the surface membrane potential, a number of interesting speculations suggest themselves.

The membrane potential of the T system can change the sarcoplasmic reticulum membrane potential in at least two ways that we would like to discuss. The first possibility is that a temporary electrical connection is established between the T tubule and sarcoplasmic reticulum, at the triad, when the T system membrane is depolarized. This could result in a direct alteration in the membrane potential across the sarcoplasmic reticulum. The current needed to produce this change of membrane potential across the sarcoplasmic reticulum could be carried by any permeant ion moving across the membrane, including calcium. Even though this type of mechanism may explain the time-course of the Nile Blue signal, there is no direct experimental evidence to support it. Furthermore, when Chandler et al. (1976 *a*) discussed this possibility in the context of charge movements related to excitation-contraction (E-C) coupling, they considered a specific model for the connection between the tubule and sarcoplasmic reticulum and predicted currents incompatible with their experimental results.

A second possibility, also proposed by Chandler et al. (1976 *b*), states that the charge movement produced by depolarization of the tubules controls the calcium conductance G_{Ca} of the sarcoplasmic reticulum. We shall consider this model and extend it in the following manner: the SR calcium conductance will be assumed to be controlled directly by the amount of charge, Q , displaced at any particular surface membrane potential. We will discuss, in turn, a single charge-single channel and a double charge-single channel interpretation of this model in order to analyze some of our experimental results.

For simplicity, the calcium conductance of the SR will be assumed to be independent of the SR membrane potential. A leakage conductance will be included. No inactivation of the calcium conductance will be considered, even though it may in fact be present. As a result of the large Ca gradient across the SR membrane, an increase in Ca conductance will produce a Ca ion flow from the SR to the sarcoplasm, changing the SR membrane potential (V_{SR}) towards the Ca equilibrium potential. When the surface and tubular membrane potential (V_m) is repolarized, the charge will move back to its resting position, returning the Ca conductance to its initial value. If the membrane of the SR had only a Ca conductance (G_{Ca}) that is independent of V_{SR} , its membrane potential would

stay at the Ca equilibrium potential (V_{Ca}). This is, of course, unlikely because the repolarization of the SR would then depend only upon the change in Ca concentration in the SR (change in V_{Ca}) and would not depend upon repolarization of the external membrane (V_m). In the model, we have therefore included a leakage pathway, time- and V_m -independent (G_L), in parallel with G_{Ca} to allow the repolarization of V_{SR} when V_m is repolarized. In this case, using a simple lumped parameter model for the SR membrane, V_{SR} is the solution of

$$C \frac{dV_{SR}}{dt} + G_{Ca}(V_{SR} - V_{Ca}) + G_L(V_{SR} - V_L) = 0, \quad (1)$$

where V_L is the equilibrium potential for the leakage conductance and C is the capacitance of the SR membrane.

In the resting state if G_{Ca} is zero, V_{SR} will be V_L . When V_{SR} is at maximum, it will be given by

$$V_{SR} = \frac{G_{Ca}V_{Ca} + G_LV_L}{G_{Ca} + G_L}. \quad (2)$$

This value would correspond to the maximum of the fluorescence signal, at each V_m . The ionic current across the sarcoplasmic reticulum is given by $C dV_{SR}/dt$ which is a quantity we can estimate, in arbitrary units, from the slope of our experimental records, and consequently, the peak current flowing during SR activation is proportional to the maximum rate of rise of the extrinsic fluorescence change.

It would be interesting to predict the functional steady-state dependence of G_{Ca} upon V_m . If we assume for the sake of simplicity that $V_L = 0$, we can rewrite Eq. 2 as:

$$V_{SR_\infty}(V_m) = V_{Ca} \cdot \frac{1}{1 + \frac{G_L}{G_{Ca_\infty}}(V_m)}, \quad (3)$$

where the ∞ sub-index represents steady-state values. According to the model of Chandler et al. (1976 *b*), G_{Ca_∞} would be given by a Boltzmann distribution of V_m :

$$G_{Ca_\infty}(V_m) = \frac{\tilde{G}_{Ca}}{1 + \exp\left[-\frac{V_m - \bar{V}}{k}\right]}, \quad (4)$$

where \tilde{G}_{Ca} is the maximum Ca conductance, \bar{V} , the center voltage of the distribution and k , the steepness. The last two parameters can be determined from charge movement experiments. Substituting Eq. 4 in Eq. 3 we obtain:

$$V_{SR_\infty}(V_m) = \frac{\tilde{G}_{Ca}}{\tilde{G}_{Ca} + G_L} \cdot \frac{V_{Ca}}{1 + \exp\left[-\frac{V_m - \bar{V}}{k}\right]} \quad (5.1)$$

with

$$\bar{V}' = \bar{V} + k \ln \left(\frac{G_L}{G_L + \bar{G}_{Ca}} \right). \quad (5.2)$$

The values obtained by Vergara and Cahalan (1978) from charge movement measurements employing the same preparation used in this paper were $\bar{V} = -40$ mV and $k = 12$ mV. A Boltzmann fit to our data for $\Delta F/F$, as a function of surface membrane potential, as shown in Fig. 7 A, yields values of \bar{V} eq -35 and $k = 9$ mV. In two other experiments, the fitted values for k were 7.5 and 8.3, respectively. Although these k values are smaller than the value obtained by Vergara and Cahalan (1978) and by Adrian and Almers (1976), they are not too different from the values obtained by Chandler et al. (1976 *a*) (viz. 8 mV). The value of \bar{V} , however, is more positive than the \bar{V} obtained from charge displacement measurements. Taking $\bar{V} = -40$ mV (Vergara and Cahalan, 1978), Eq. 5.2 implies that $G_L/\bar{G}_{Ca} = 1.152$. From the fit to the data of Fig. 7 A, the maximum $\Delta F/F$ is 0.0205 and, using this value and the value of G_L/\bar{G}_{Ca} obtained from Eq. 5.1, V_{Ca} is calculated to be 0.0441 in $\Delta F/F$ units.

A tempting way to displace the center point of the distribution, and at the same time to increase its steepness, is to assume that more than one charge is required to move, per site, in order to open a single Ca channel. For example, if two charges are needed, V_{SR} should be related to V_m by:

$$V_{SR_\infty}(V_m) = \frac{V_{Ca}}{1 + \frac{G_L}{\bar{G}_{Ca}} \left[1 + \exp \left(-\frac{V_m - \bar{V}}{k} \right) \right]^2}. \quad (6)$$

Fitting this equation to Fig. 7 A, we obtain $V_{Ca} = 0.0462$ (again, in $\Delta F/F$ units) and $G_L/\bar{G}_{Ca} = 1.2$. These values are not too different from the previous values fitted with Eq. 5 and have the advantage that the k value used was the same as that determined from charge movement measurements (Vergara and Cahalan, 1978).

In both cases, G_L/\bar{G}_{Ca} was ~ 1.2 , indicating that in the SR membrane, G_{Ca} itself does not exceed the resting conductance. This would be quite reasonable if most of the calcium channels were concentrated near the junctional region and the remainder of the SR were just passive membrane (or membrane occupied by calcium pump sites). Then, the calcium conductance might be several times larger than the leakage near the junctional region, but when averaged over the entire SR membrane, G_{Ca} can be smaller than total G_L . Such an arrangement seems likely and would explain the paucity of Ca channels in resealed SR vesicles, since these would be likely to have sheared off without preserving the junctional sites.

The time-course of the fluorescence response can be calculated by raising to an integral power the kinetic equations for charge movement given by Chandler et al. (1976 *a*). The calcium conductance and the probability variables (f_2) of Chandler et al. are correlated by:

$$G_{Ca} = \bar{G}_{Ca} f_2^n, \quad (7)$$

where n is the number of charges required to open the channel. Tentatively, we set $n = 2$, as we did in the steady-state calculations. The factor 0.053 ms^{-1} of Eqs. 11 and 12 of Chandler et al. (1976 *a*) was changed to 0.1 ms^{-1} because this gave a better fit to the experimental results, and the temperature in our experiments was higher.

The solution of Eq. 1 together with Eq. 6 and the kinetic equations of Chandler et al. (1976 *a*) involved functions of the type $\exp(\exp - t)$ and was obtained numerically. The general features of the experimental records were reproduced in this fashion but, although the model predicted a noticeable lag in V_{SR} as a function of time, this was still not as long as the delay observed experimentally. Also, the droop observed in the extrinsic fluorescence signal during the voltage clamp pulse was not reproduced by the model. The model predicts, and we calculated the maximum rate of rise of V_{SR} as a function of V_m , i.e., the peak current during the potential change across the SR, and this is displayed as a continuous curve in Fig. 7 b. In the computation, V_{Ca} was set to 100 mV, $C = 1 \mu\text{F}/\text{cm}^2$, $\tilde{G}_{\text{Ca}} = 60 \mu\text{S}/\text{cm}^2$, and $G_{\text{L}} = 72 \mu\text{S}/\text{cm}^2$. The value of G_{L} is of the same order of magnitude as the value of $28 \mu\text{S}/\text{cm}^2$ obtained by Bezanilla and Horowicz (1975), using an entirely different procedure. It should be pointed out that the theoretical maximum rate of rise increases monotonically with depolarization, saturating at values of V_m beyond our experimental range.

The model that we have discussed is preliminary and incomplete; its main virtue is that it allows us to combine evidence obtained from several experimental sources. As mentioned above, there are some features of the experimental data that are not reproduced by the model. The extra lag observed in the optical records, not well predicted by the model, could be explained, to give an example, by a further increase in the number of charges necessary to open a Ca channel, by introducing a threshold value for the quantity of charge required to open the Ca channels, or by increasing the number of steps between the charge movement and the opening of G_{Ca} . There are many possible mechanisms to account for the decay phase of the Nile Blue signal during a long depolarization: a time dependent G_{L} , a Ca pump dependent on V_{SR} or on calcium concentration, G_{Ca} inactivation, or Ca depletion in the SR, among others. At present, we do not have enough information to decide among these possibilities.

The Effect of Sodium on the Nile Blue Signal

We demonstrated in the Results section that the maximum rate of rise of the Nile Blue signal is larger, when associated with a membrane action potential, than that obtained under comparable voltage clamp depolarization in the presence of TTX. According to the model presented above, this would mean that the presence of sodium increases the peak current across the SR membrane and this would imply a faster kinetics for the charge movement. It is known that in the presence of Na, a tubular regenerative process may be expected to speed up depolarization of the tubular membrane (Adrian and Peachey, 1973), and this may account for the faster kinetics of the charge movement. Bezanilla and Vergara (1978) have studied the voltage dependence of the Nile Blue signal in the presence of half-normal sodium, with and without TTX, and found the

voltage dependence to be less steep in the presence of TTX. This result was explained by a lack of tubular membrane potential control in the absence of TTX. This effect would also contribute to the increased rate of rise of the Nile Blue fluorescence change in the absence of TTX.

Decaying Phase of Nile Blue Signal at Pulse End

The decaying phase of the fluorescence signal was shown to be nearly independent of the driving function at the surface membrane. For example, in trains of action potentials, the decaying phase at the end of the train was almost superimposable on the decaying phase of the signal elicited by a single action potential. Also, when the pulse duration was changed in voltage clamp, the kinetics of the decaying phase was not affected. Finally, the decaying phase remained constant, for different amplitudes of depolarization. The model presented here would predict this behavior, because the time constant of the decay is mainly given by the passive properties of the SR membrane (G_L and C).

Comparison with Other Optical Signals

Oetliker et al. (1975) have made a comparison between the signals produced by Nile Blue A and the fluorescence dye, di-I-C₁ (5); they found that the time-courses are roughly similar, although the sign was reversed. However, they did not compare directly the optical retardation signal and the dye signals. We have found that action potentials separated by 20 ms produce Nile Blue signals of almost the same amplitude, with no summation at 15°C (Fig. 3) and 17°C (Fig. 4). Baylor and Oetliker (1977) found summation in their optical retardation signals when the action potentials were separated by 10 ms at 22°C. Although the two experiments were not carried out under the same conditions, they may indicate certain differences between the two types of signals that would justify further study. Kovacs and Schneider (1977) have recorded transparency changes associated with surface membrane depolarization that have properties similar to birefringence and fluorescence signals, but the transparency changes have not been studied under the same experimental conditions presented in this paper. Without additional information, a comparison at this point seems premature.

Taylor et al. (1975) have recorded the change in Ca concentration in sarcoplasm using the photoluminescent protein aequorin. The Ca transient associated with a single action potential bears some resemblance to the Nile Blue signal. A precise comparison cannot be made, because of experimental differences; however, a tetanus at 50 Hz produced a fused and creeping light signal in contrast to the result shown in Fig. 3. This difference is consistent with the interpretation that Nile Blue is monitoring SR membrane potential because, according to the model, the Ca concentration is proportional to the SR depolarization for a single pulse, but when the SR is repolarized, the Ca concentration is unlikely to decrease with the time-course of repolarization. If, during the repolarization of the SR, a second depolarization is elicited, its time-course may differ from that of the Ca concentration because more Ca will be added to the sarcoplasm. In this way, for a longer train of action potentials, the Ca concentration may grow, out of proportion to the membrane potential.

Although we feel that there is good evidence relating the extrinsic fluorescence changes reported here to alterations in the membrane potential of the sarcoplasmic reticulum, we recognize that this evidence is not yet entirely compelling. In particular, we are troubled by the possibility that several of the optical changes, intrinsic as well as extrinsic, recorded in skeletal muscle may have their origin with contractile (or regulator) protein. Conformational changes in regulator proteins could generate intrinsic optical signals (e.g., birefringence or transparency changes), and they could alter the binding or other response of Nile Blue A, in such a manner as to mimic the optical changes usually associated with variation in membrane potential. In this regard, Scordilis et al. (1975) have indicated that changes in fixed charge on muscle proteins can affect the optical properties of a di-hexyloxacarboyanine dye (dye V of Cohen et al., 1974), when it is associated with these proteins. Also, a number of authors have noted electrostatic effects on the binding of dyes to membranes (Cohen and Salzberg, 1978).

There are a variety of experiments that might help to clarify the issue of whether extrinsic potentiometric probes are monitoring sarcoplasmic reticulum membrane potential or some subsequent process involving myoproteins. Immunological blockade of calcium binding to troponin, or pharmacological, chemical (fixation), or metabolic inhibition of the normal actin-myosin interaction (rigor), during the course of optical studies might help to resolve this point. In addition, it would be most desirable to perform experiments like these under voltage clamp conditions, with simultaneous recording of fluorescence and ionized Ca concentration (using arsenazo III), because such experiments would provide more direct comparison between the presumed SR depolarization and Ca release.

Note Added in Proof Dr. B. M. Salzberg recently showed that in squid giant axon, Nile Blue fluorescent changes always show the same polarity regardless of the site of application of the dye.⁸

We would like to thank Dr. Richard Orkand for his hospitality and for reading the manuscript; Drs. L. B. Cohen and R. S. Eisenberg for reading the manuscript; Dr. M. Cahalan for helpful advice with regard to the vaseline gap techniques; Dr. C. M. Armstrong for support; and Mr. Victor Cordova and Mr. Julio Fernandez for help in designing and building electronic equipment. Supported by Biomedical Research Support Grant RRO-5354, a grant-in-aid from the Muscular Dystrophy Association of America, grant BNS 7705025 from the National Science Foundation, and the Oficina Técnica de Desarrollo of the University of Chile. Additionally, Dr. Vergara is the recipient of a research grant from the University of California, Los Angeles.

Received for publication 10 April 1978.

REFERENCES

ADRIAN, R. H., and W. ALMERS. 1976. Charge movement in the membrane of striated muscle. *J. Physiol. (Lond.)* **254**:339-360.

⁸ Salzberg, B. M. Optical signals from squid giant axons following perfusion or superfusion with potentiometric probes. *Biol. Bull. (Woods Hole)*. In press.

- ADRIAN, R. H., W. K. CHANDLER, and A. L. HODGKIN. 1970. Voltage clamp experiments in striated muscle fibers. *J. Physiol. (Lond.)*. **208**:607-644.
- ADRIAN, R. H., and L. D. PEACHEY. 1973. Reconstruction of the action potential of frog sartorius muscle. *J. Physiol. (Lond.)*. **235**:103-131.
- BAYLOR, S. M., and H. OETLIKER. 1977. A large birefringence signal preceding contraction in single twitch fibers of the frog. *J. Physiol. (Lond.)*. **264**:141-162.
- BEZANILLA, F., and C. M. ARMSTRONG. 1977. A low cost signal averager and data acquisition device. *Am. J. Physiol. Cell Physiol.* **1**:C211-C215.
- BEZANILLA, F., C. CAPUTO, and P. HOROWICZ. 1971. Voltage clamp activation of contraction in short striated muscle fibers of the frog. *Acta Cient. Venez.* **22**(Supl.):72-74.
- BEZANILLA, F., and P. HOROWICZ. 1975. Fluorescence intensity changes associated with contractile activation in frog muscle stained with Nile Blue A. *J. Physiol. (Lond.)*. **246**:709-735.
- BEZANILLA, F., and J. VERGARA. 1978. Fluorescence signals from skeletal muscle fibers. Proceedings from the International Symposium on Cardiac Muscle, Shiraz, Iran. In press.
- BROWN, J. E., L. B. COHEN, P. DE WEER, L. H. PINTO, W. N. ROSS, and B. M. SALZBERG. 1975. Rapid changes of intracellular free calcium concentration. Detection by metallochromic indicator dyes in squid giant axon. *Biophys. J.* **15**:1155-1160.
- BROWN, K. M., and J. E. DENNIS. 1972. Derivative free analogues of the Levenberg-Marquardt and Gauss algorithms for nonlinear least squares approximations. *Numerische Mathematik.* **18**:289-297.
- CHANDLER, W. K., R. F. RAKOWSKI, and M. F. SCHNEIDER. 1976 a. A non-linear voltage dependent charge movement in frog skeletal muscle. *J. Physiol. (Lond.)*. **254**:245-283.
- CHANDLER, W. K., R. F. RAKOWSKI, and M. F. SCHNEIDER. 1976 b. Effect of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol. (Lond.)*. **254**:285-316.
- COHEN, L. B., and B. M. SALZBERG. 1978. Optical measurement of membrane potential. *Rev. Physiol. Biochem. Pharmacol.* In press.
- COHEN, L. B., B. M. SALZBERG, H. V. DÁVILA, W. N. ROSS, D. LANDOWNE, A. S. WAGGONER, and C. H. WANG. 1974. Changes in axon fluorescence during activity: molecular probes of membrane potential. *J. Membr. Biol.* **19**:1-36.
- FORD, L. E., and R. J. PODOLSKY. 1972. Intracellular calcium movements in skinned muscle fibers. *J. Physiol. (Lond.)*. **223**:21-33.
- HAUGEN, P., and O. STEN-KNUDSEN. 1976. Sarcomere lengthening and tension drop in the latent period of isolated frog skeletal muscle fibers. *J. Gen. Physiol.* **68**:247-265.
- HEISTRACHER, P., and C. C. HUNT. 1969. The relation of membrane charges to contraction in twitch muscle fibers. *J. Physiol. (Lond.)*. **201**:589-611.
- HILLE, B., and D. T. CAMPBELL. 1976. An improved vaseline gap voltage clamp for skeletal muscle fibers. *J. Gen. Physiol.* **67**:265-293.
- HODGKIN, A. L., and P. HOROWICZ. 1960. Potassium contractures in single muscle fibers. *J. Physiol. (Lond.)*. **153**:386-403.
- KOVACS, L., and M. F. SCHNEIDER. 1977. Increased optical transparency associated with excitation-contraction coupling in voltage-clamped cut skeletal muscle fibers. *Nature (Lond.)*. **265**:556-560.
- LANDOWNE, D. 1974. Changes in fluorescence of skeletal muscle stained with merocyanine associated with excitation-contraction coupling. *J. Gen. Physiol.* **64**:5a. (Abstr.)

- MOBLEY, B. A., and B. R. EISENBERG. 1975. Sizes of components in frog skeletal muscle measured by methods of stereology. *J. Gen. Physiol.* **66**:31-45.
- NAKAJIMA, S., A. GILAI, and D. DINGEMAN. 1976. Dye absorption changes in single muscle fibers: an application of an automatic balancing circuit. *Pfluegers Arch. Eur. J. Physiol.* **362**:285-287.
- OETLIKER, H., S. M. BAYLOR, and W. K. CHANDLER. 1975. Simultaneous changes in fluorescence and optical retardation in single muscle fibers during activity. *Nature (Lond.)*. **257**:693-696.
- PEACHEY, L. D. 1965. The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. Cell Biol.* **25**:209-231.
- ROSS, W. N., B. M. SALZBERG, L. B. COHEN, A. GRINVALD, H. V. DÁVILA, A. S. WAGGONER, and C. H. WANG. 1977. Changes in absorption, fluorescence, dichroism and birefringence in stained giant axons: optical measurement of membrane potential. *J. Membr. Biol.* **33**:141-183.
- SALZBERG, B. M., H. V. DÁVILA, and L. B. COHEN. 1973. Optical recording of impulses in individual neurons of an invertebrate central nervous system. *Nature (Lond.)*. **246**:508-509.
- SCHNEIDER, M. F., and W. K. CHANDLER. 1973. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature (Lond.)*. **242**:244-246.
- SCORDILIS, S. P., H. TEDESCHI, and C. EDWARDS. 1975. Donnan potential of rabbit skeletal muscle myofibrils. I. Electrofluorometric detection of potential. *Proc. Natl. Acad. Sci. U.S.A.* **72**:1325-1329.
- SUAREZ-KURTZ, G., and I. PARKER. 1977. Birefringence signal and calcium transients in skeletal muscle. *Nature (Lond.)*. **270**:746-748.
- TAYLOR, S. R., R. RÜDEL, and J. R. BLINKS. 1975. Calcium transients in amphibian muscle. *Fed. Proc.* **34**:1379-1381.
- VERGARA, J., and F. BEZANILLA. 1976. Fluorescence changes during electrical activity in frog muscle stained with merocyanine. *Nature (Lond.)*. **259**:684-686.
- VERGARA, J., and F. BEZANILLA. 1977. Nile Blue fluorescence signals in frog single muscle fibers under voltage or current clamp conditions. *Biophys. J.* **17**:5 a. (Abstr.)
- VERGARA, J., and M. CAHALAN. 1978. Charge movements in a cut skeletal muscle fiber. *Biophys. J.* **21**:167 a. (Abstr.)
- WAGGONER, A. 1976. Optical probes of membrane potential. *J. Membr. Biol.* **27**:317-334.