Dichroic Behavior of the Absorbance Signals from Dyes NK2367 and WW375 in Skeletal Muscle Fibers

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ABSTRACT Absorbance signals were recorded from voltage-clamped single muscle fibers stained with the nonpenetrating potentiometric dyes NK2367 and WW375 and illuminated with quasimonochromatic light from 560 to 800 nm, linearly polarized either parallel (0°) or perpendicular (90°) to the fiber long axis. The signals from both dyes depend strongly on the incident polarization. At any wavelength and/or polarization condition, the total absorbance signal is a superposition of the same two signal components previously identified with unpolarized light (Heiny, J. A., and J. Vergara, 1982, J. Gen. Physiol., 80:203)—namely, a fast step signal from the voltage-clamped surface membrane and a signal reflecting the slower T-system potential changes. The 0° and 90° spectra of both membranes have similar positive and negative absorbance peaks (720 and 670 nm, respectively, for dye NK2367; 740 and 700 nm for dye WW375); in addition, they have the same dichroic maxima (670 for NK2367; 700 for WW375). However, for the surface membrane, the 0° spectra are everywhere more positive than the 90° spectra, whereas the reverse is true for the T-system, which results in a dichroism of opposite sign for the two membranes. These spectral characteristics were analyzed using a general model for the potential-dependent response of an absorbing dye (Tasaki, I., and A. Warashina, 1976, Photochem. Photobiol., 24:191), which takes into account both the dye response and the membrane geometries. They are consistent with the proposal that the dye responds via a common mechanism in both membranes that consists of a dye reorientation and a change in the absorption maxima.

INTRODUCTION

The potentiometric dyes NK2367 and WW375 have been used as fast, linear probes of transmembrane potential in a wide variety of tissues, including squid axon, skeletal and cardiac muscle, and cell culture preparations (cf. Cohen and Salzberg, 1978; Gupta et al., 1981; Morad and Salama, 1979; Nakajima and Gilai, 1980; Heiny and Vergara, 1982a). We have previously shown (Heiny and Vergara, 1982a) that in skeletal muscle the absorbance signals recorded with

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these dyes reflect potential changes across both the surface and T-system membranes. This finding was expected since the dyes are nonpenetrating and should stain all membranes accessible to the extracellular space. An unexpected result was that the relative contribution of these membranes to the total optical signal is strongly wavelength dependent and therefore not weighted in a simple way by the ratio of surface to T-system membrane areas. When a muscle fiber stained with dye NK2367 was illuminated with quasimonochromatic, unpolarized light, the surface membrane signal was shown to be maximal near 720 nm, whereas the T-system maxima occurred near 670 nm. This finding could be exploited to record selectively from either membrane compartment. Its physical basis remained unexplained, but it has important implications for the use of these dyes in skeletal muscle and other membrane systems. Because the spectrum of any dye is closely related to the mechanism of the potential-dependent response, the existence of different spectra for the same dye could mean that the dye senses potential by different mechanisms in different membranes. Therefore, calibrations of the dye response in ΔI/I per millivolt or tests of the response speed or linearity performed in one tissue or membrane compartment may not be directly applicable to another.

We assumed that there were two likely explanations for our previous spectral findings: (a) differences in the membrane compositions of the surface and T-system might influence the resting or potential-dependent dye absorbance spectra; an argument of this kind, based on species-related differences between vertebrates and invertebrates, was proposed by Ross and Reichardt (1979) to account for the different spectra observed with these dyes in neuronal tissues; (b) differences in the geometry of the two membranes could alter the response spectra (in this paper also referred to as potential-dependent or difference spectra), even if the dye responded via a common mechanism in both membranes. This could occur if the dye molecules had a preferred orientation in the membrane, either in the resting or potential-dependent state, and could be readily observed using polarized incident light. Tasaki and Warashina (1976) and Waggoner and Grinvald (1977) have shown that the dichroic spectra of the dye merocyanine 540 in squid axons can be well explained by proposing that the optical signals are due in part to a potential-dependent dye reorientation in a membrane having anisotropic geometry.

The purpose of the present study was to investigate the second hypothesis by further characterizing the spectra of dyes NK2367 and WW375 in the surface and T-system membranes of skeletal muscle using polarized light. The fact that the absorbance signals of these two dyes are highly dichroic in both axon (Ross et al., 1977; Gupta et al., 1981) and skeletal muscle (Baylor et al., 1981) preparations suggested that the spectra characteristics of the surface and T-system signals (Heiny and Vergara, 1982a) might be at least partly explained by orientation effects. These would be expected to be significant in our preparation because of the nearly orthogonal net orientations of the surface and T-system membranes (Peachey, 1965). In addition, it is possible to use kinetic differences in the potential changes occurring in each membrane compartment to identify and separate the optical signals contributed by each membrane. These differences
arise because the potential of the surface membrane is rapidly voltage-clamped in this preparation, whereas that of the T-system is not. Hence, skeletal muscle fibers present a unique physiological system for obtaining further information about the potentiometric response of these dyes.

The 0° and 90° spectra were determined for both membranes and analyzed using a general two-state model (Tasaki and Warashina, 1976) for the optical response of an absorbing dye, which takes into account the orientation effects expected for different dye mechanisms occurring in particular membrane geometries. Preliminary reports of this work have appeared (Heiny and Vergara, 1982a, 1983).

METHODS

Preparation and Protocols

The experiments were performed using cut single muscle fibers of the semitendinosus muscle of *Rana catesbiana* mounted in a specially designed chamber with three vaseline gaps described below. The voltage-clamp methods are essentially the same as reported by Hille and Campbell (1976) and applied by Vergara et al. (1978) and Heiny and Vergara (1982a).

Briefly, single fiber segments of 2–3 cm in length and 100–200 μm in diameter were dissected, keeping one tendon end intact. The segments were stimulated extracellularly and only those able to give all-or-nothing fast twitches were used. These segments were transferred to a small plastic dish in which the fibers were slightly stretched and fixed at both ends. The Ringer's solution in the trough was rapidly exchanged to a K isotonic solution, which induced a spontaneously relaxing K contracture. The relaxed fiber was then transferred to the vaseline-gap chamber and mounted as described previously (Heiny and Vergara, 1982a) but with one addition. Two thin human hairs were placed parallel on either side of the fiber segment in the A pool. They rested on the EA and AB vaseline seals slightly above the top edge of the fiber and served to maintain the level of solution well above the upper edge of the fiber. This improvement prevented inhomogeneities in the extracellular potential caused by an uneven coverage of the muscle fiber with external solution and also resulted in a better preservation of the fiber.

Three-Vaseline-Gap Chamber and Optical Setup

The vaseline-gap chamber used in previous reports from this laboratory (Vergara et al., 1978; Vergara and Bezanilla, 1981; Palade and Vergara, 1982; Heiny and Vergara, 1982a) made use of a tapered fiber optic to illuminate the muscle fiber segment in the A pool.

The fiber optic could not be used in the present experiments because it acted as a randomizer of the incident polarized light. Consequently, a redesigned chamber and illuminating system were used that incorporated the following features: (a) the segment of muscle fiber in pool A was illuminated with a small spot of light focused by a long working distance objective (UMK50, × 32, NA 0.4, E. Leitz, Inc., Rockleigh, NJ); (b) the chamber bottom had a thin glass window below pool A; (c) the plexiglass walls that form the boundaries of pool A were tapered at an angle of 45° to accommodate the cone of light projected by the objective; (d) the total distance between the muscle fiber and the bottom of the chamber was ~2 mm, or shorter than the focal distance of the UMK50 objective. An unscaled diagram is presented in Fig. 1.

The rest of the optical setup was essentially that described by Vergara et al. (1978) and
Heiny and Vergara (1982a). In these experiments, however, a calcite polarizer (Glan-Thompson Prism, Karl Lambrecht Corp., Chicago, IL) on a calibrated, rotatable mount was added in the incident light path. At the start of each experiment, it was aligned so that the 0° axis of polarization was parallel (±5°) to the long axis of the muscle fiber. The light reaching the muscle fiber was linearly polarized, with a wavelength-independent polarization factor \( P \) of 1.0, where \( P = (I_1 - I_2)/(I_1 + I_2) \) and \( I_1 \) and \( I_2 \) are the maximum and minimum light intensities, respectively, detected by a photodiode when an analyzer was rotated. With the polarizer removed from the light path, the polarization factor was 0.1, or slightly less random than with the previous fiber optic chamber \( (P = 0.07; \text{Heiny and Vergara, 1982a}) \).

**Recording Techniques**

Transmitted light from the preparation was collected by a Nikon Fluor 20 (× 20, NA 0.75, Nikon, Inc., Tokyo, Japan) objective. Light not passing through the muscle fiber was blocked by an X-Y slit in the microscope image plane and the resulting image of the fiber segment in the A pool (~100 μm long times the fiber diameter) was formed on a low-noise photodiode (F002E, United Detector Technology, Culver City, CA; or UV100B, EG & G, Inc., Electro-Optics Div., Salem, MA).

**Figure 1.** Schematic diagram of the apparatus used for measurements of absorbance signals in skeletal muscle fibers illuminated with polarized light. The experimental chamber was mounted on the stage of a modified Nikon-Biophot microscope used as a vertical optical bench.

The light signal was converted to voltage and the DC signal component caused by the resting light intensity was displayed on a digital panel meter and recorded for each experimental trace. This DC component was subtracted from the total signal by a sample-
and hold circuit for further amplification of the transient component. Light, current, and voltage data were fed to the multiplexed inputs of a signal averager (Vergara et al., 1978). Data could be acquired as fast as 5 µs per point. In some experiments, both a fast and a slow sampling interval were used to acquire a single trace. Anti-aliasing filters (six-pole low-pass Bessel filters, Frequency Devices, Haverhill, MA) were placed in front of each input and the corner frequencies were selected to be at least two times lower than the Nyquist frequency of the fast sampling interval. The data were stored in digital form on magnetic tape and transferred to a laboratory computer (Nova 3/12, Data General Corp., Westboro, MA) for analysis and graphic display.

**Solutions**

The composition of the external (A pool) solutions was one of the following. (A) Normal Ringer’s (mM): 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 5 Na-MOPS, 10 dextrose. (B) Passive (Na-free) Ringer’s (mM): 55 TEA₂SO₄, 2.5 K₂SO₄, 8 CaSO₄, 10 TEA-MOPS, 2 CsSO₄, 25 dextrose. The internal solution (pools E and C) contained (mM): 80 Cs-aspartate, 20 Cs-MOPS, 20 Cs-EGTA, 5 phosphocreatine, 3 MgSO₄, 3 ATP. The osmolarity of all solutions was adjusted to 240 mosmol by varying the concentration of the major salt, and the pH was adjusted to 7.1. Dyes NK2367 and WW375 were obtained from Nippon-Kankoh Shikiso Kenkyusho Co., Ltd., Okayama, Japan, and Dr. A. Waggoner, Carnegie-Mellon University, Pittsburgh, PA, respectively. They were mixed fresh before each experiment and kept in the dark on ice during to minimize dye bleaching. Dye concentrations of 0.5 mg/ml (≈1 mM) were added to the external solution in pool A to stain the fibers. After 15 min of staining, the external solution was exchanged for one containing a 50-80 times lower dye concentration (≈10 µM).

**Experimental Protocol and Data Analysis**

Although the potentiometric responses of dyes NK2367 and WW375 correspond to changes in absorbance, ∆A, the optical signals were expressed in this paper as -∆I/I, in which ∆I corresponds to the change in intensity measured during the test pulse and I corresponds to the resting (DC) light intensity. For small intensity changes, this fractional transmittance change is linearly related to the absorbance change by the approximate formula ∆A = -0.4301/1. Therefore, the calibrations of the figures, expressed here in terms of -∆I/I, can be considered to represent ∆A except for a scaling factor.

All of the absorbance signals reported in this paper were recorded in response to hyperpolarizing voltage-clamp pulses of 90 mV, from a holding potential of ~100 mV (i.e., to an absolute potential of ~190 mV). As reported previously (Heiny and Vergara, 1982a), both the surface and T-system membranes contribute to the total recorded absorbance signal. Moreover, the time courses of the potential change from each membrane compartment can be easily distinguished in this preparation. The surface membrane is rapidly voltage-clamped to a constant level within the time required to change the surface membrane capacitance. In practice, the speed of the step is determined by the setting time of the voltage-clamp circuit as well as by the extracellular resistance in series with the membrane; it is typically 20-40 µs, depending on how well the latter effects can be electronically compensated (Hille and Campbell, 1978). The T-system potential is not under voltage-clamp control and can differ significantly in speed and amplitude from the potential imposed across the surface membrane. At hyperpolarizing potentials, and for either of the external solutions used (A and B above), no effects of nonlinear conductances on the T-system potential change are expected. Therefore, it changes monotonically to a steady potential approximately equal to the surface membrane, with a typical halftime of 2-4 ms for a 150-µm-diam fiber.

To compare the surface and T-system absorbance changes with respect to the same
transmembrane potential change, the following protocol was used. (a) The surface membrane contribution was measured as the initial absorbance change observed at 20–40 μs; (b) the T-system contribution was measured as the steady state absorbance change recorded at 30 ms, minus the surface membrane (step) component. In some experiments, these were determined from a single, long pulse trace, which was recorded using two sampling rates (e.g., Figs. 3 and 4). However, for experiments in which records were taken over the entire spectrum, this procedure was not feasible for several reasons. The resolution needed for the surface membrane signals (typically $10^{-4}$–$10^{-5}$ fractional transmittance change from a membrane area of $\sim 5 \times 10^{-4}$ cm$^2$, or 1/100 that used in axon recordings with dye WW375; Ross et al., 1977) required extensive signal averaging (64–128 sweeps). Only one fiber survived this large number of square pulses. In addition, dye bleaching was a serious problem with the long exposure times, and the required sampling frequencies allowed only one to two points for the fast signal. Therefore, an alternative protocol was used, in which the surface and T-system spectra were obtained with different pulse protocols. This protocol is illustrated in Fig. 2 and was applied either sequentially to the same fiber (surface spectra followed by T-system spectra) or, in most cases, to separate fibers. The surface membrane signals (right traces) were measured using very short (1-ms) pulses, brief exposure times, extensive signal averaging, and fast data sampling. The surface membrane absorbance change was measured as the difference between the mean baseline value (computed from 25 data points before the pulse start) and the mean signal amplitude 20–40 μs after the pulse start (computed from the next four to eight data points, or by eye from the mean of the peak-to-peak noise at the 20–40-μs transition point). A signal-to-noise ratio of at least 2 for the averaged traces was possible at most wavelengths, and the amplitude measured at the on and off of each pulse agreed within 10%.

![Figure 2](image_url)

**Figure 2.** Experimental procedure to estimate the surface (step) and T-system (steady state) contributions to the total absorbance signals recorded with dye NK2367. Absorbance signals were recorded in response to 30- (left traces) and 1-ms (right traces) voltage-clamp pulses of -90 mV, from a holding potential of -100 mV, and are shown for two wavelengths, 0° polarized light. The 30-ms traces were signal averaged four times and filtered at 5–10 kHz; the first 512 points were acquired at a sampling interval of 20 μs/point and the second at 100 μs/point. The 1-ms traces were averaged 64–128 times and filtered at 25 kHz; all 1,024 points were acquired at 5 μs/point. Separate absorbance calibrations are given for the left and right pairs of traces and are in units of $-\Delta I/I \times 10^4$. Temperature, 12°C; fiber diameter, 140 μm.
T-system signals (left traces) were measured using long pulses, fewer sweeps, and two sampling frequencies. A better signal-to-noise ratio was obtained for this signal, typically 8–10 at 670 nm, 0° (lower left). The T-system absorbance change was measured as the difference between the baseline value and the steady state signals (each computed from the mean of 25 points), after subtraction of the step signal component. The latter was somewhat arbitrarily estimated from the amplitude at 40 μs. Some error was introduced, however, by this step subtraction procedure, since this component was poorly resolved with one to two data points and these traces were more heavily filtered to reduce the number of averages.

These errors are difficult to estimate but would probably not alter the assigned T-system amplitude by >10–20% since the absolute amplitude of the T-system absorbance signals is, on the average, 5–10 times that of the surface membrane for the same transmembrane potential change. Therefore, at wavelengths where the fractional surface signal is small, very little error is introduced (e.g., in Fig. 2, 670 nm, 0°, the amplitude at 40 μs has not changed from baseline), and at wavelengths where it is large, it is also more accurately resolved. In the data of Fig. 2, the ratios of the T-system signals computed from the assigned step value (at 40 μs, on long pulse traces) to those computed using the step values measured from a short pulse trace in the same fiber were 1.01, 1.0, and 1.07, for 720 nm and 0°, 670 nm and 0°, and 690 nm and 0°, respectively. When the data from different fibers were compared, the subtracted surface signal values obtained this way (e.g., obtained from Fig. 6A, T-system data) fell within the mean and standard deviation of the surface signals measured from short pulses (e.g., Fig. 6A and Table 1, surface data), at each of the peak wavelengths.

For either of these protocols, the absorbance signals tended to fade during the course of an experiment, which typically lasted ~2 h and was limited by fiber survivability. To correct for these effects, measurements at reference wavelengths (670, 0° or 720, 90°) were taken after every three to four records. The reference data were well fit to an exponential function and the fitted curve was used to correct all records. This procedure assumes that dye bleaching is probably due to a process such as photolytic breakdown, which would alter the amount of membrane-bound dye (ΔN, Eqs. 7 and 8) but not the wavelength-dependent parameters.

The wavelength dependence of each signal component was determined for several directions of incident polarization. These data were stored in disk files and used to calculate best-fitting parameters for the mathematical model presented in the Appendix, using a nonlinear least-squares fit program based on the Levenberg-Marquardt algorithm (Brown and Dennis, 1972).

RESULTS

Experimental Evidence for a Wavelength-dependent Dichroism in the Surface and T-System Absorbance Signals

Figs. 3 and 4 illustrate the types of absorbance signals recorded from voltage-clamped skeletal muscle fibers stained with dye NK2367 (Fig. 3) or WW375 (Fig. 4) when the polarization of the incident light is varied. The wavelengths shown are those at which either large T-system (670 nm, NK2367; 700 nm, WW375) or surface membrane (720 nm, NK2367; 740 nm, WW375) signals are detected with unpolarized light (Heiny and Vergara, 1982a; Vergara and Bezanilla, 1981). The directions of polarization were chosen to be parallel (0°) or perpendicular (90°) to the radial optic axis (long axis) of the muscle fiber.
Unpolarized or 45° polarized light were also used since these contain equal amounts of the two polarization directions of interest.

It is evident from Fig. 3 that the optical signals recorded with dye NK2367, in addition to being different for different wavelengths, as previously reported (Heiny and Vergara, 1982a), are also dependent on the plane of polarization. At 670 nm, the signal recorded with 0° incident light (top left trace) has a slow monotonic time course that reaches a steady state value with a halftime of ~1.2 ms. At this same wavelength, the optical signal recorded using 90° incident light (center left trace) has both an early, small step component and a much reduced slow component. The optical signal recorded with unpolarized light (bottom left trace) reflects an average of the 0° and 90° traces. The result is an optical signal similar to that previously reported (Heiny and Vergara, 1982a), which has predominantly the slow component.

In contrast to the 670-nm signals, the optical signals recorded at 720 nm are inverted in sign. More importantly, they show significant contributions from both the step and slow waveforms, for each polarization condition. The step
component is large and roughly equal for 0° and 90° light (top and center right traces). The slow component contributes more at 90° than at 0°, which is the opposite of the pattern observed at 670 nm. With unpolarized light (bottom right trace), an optical signal results in which both waveforms contribute significantly and in approximately equal proportions to produce a waveform similar to that previously described (Heiny and Vergara, 1982a).

An analogous, wavelength-dependent dichroic behavior of the absorbance signals was observed in fibers stained with the related merocyanine dye WW375 (Fig. 4). At 700 nm (left traces), the slow signal component is much larger for 0° (top) than for 90° (center) incident light. A small step signal is again recorded for 90° but not for 0° incident light. At 740 nm (right traces), the optical signals at every polarization show significant contributions from both waveforms. The step is significant at both 0° and 90° (top and center traces), and the slow signal is much larger for 90° than for 0° light, in this case being nearly absent at 0°. Each signal is again reversed in sign from the 700-nm signals. At both wavelengths, the 45° signals (bottom traces) reflect an average of the 0° and 90° signals.

It should be noted that in this fiber, the optical signals recorded using 0° incident light (Fig. 4, top traces) showed an almost complete separation of the two waveforms described. As shown previously for dye NK2367 (Heiny and Vergara, 1982a), these waveforms reflect potential changes across either the T-system or surface membranes, respectively.
The signals from these two membrane compartments can be clearly distinguished in this preparation since the surface membrane is rapidly voltage-clamped, whereas the T-system is charged more slowly, which reflects the fact that it behaves electrically as a second, nonisopotential cable in parallel with the surface membrane.

The results shown in Figs. 3 and 4 demonstrate that the plane of polarization, as well as the wavelength of the incident light, influences the relative contribution of the signal from each membrane compartment to the total optical change. They also suggest that the total optical signal recorded at any wavelength or incident polarization is a linear superposition of just these two waveforms. To examine this latter hypothesis more closely, two signal components were separated in the optical signals recorded at several illumination conditions. Figs. 4 and 5A show the un subtracted, unscaled traces used for these comparisons. Fig. 5B shows paired comparisons of the time courses of the slow signals from these same traces after the step, when present, was subtracted (at 40 μs) and after each subtracted trace was scaled to the same final amplitude. The slow signals at 710 nm, 0° (top, solid trace) and 90° (dotted trace), are indistinguishable within the resolution of these recordings. This suggests that a single T-system signal is recorded under each polarization condition. This result is also expected for a membrane network that has a homogenous geometry, on the average, over the fiber cross section, as assumed in the model described below. In this case, changes in incident polarization or wavelength would be expected to alter the amplitude but not the time course of the T-system signal. This time course should then reflect a "weighted average" T-system potential change.

The latter occurs since, optically, absorbance changes occurring along the entire radius are summed, weighted by the T-system membrane area at each radial position. Similar comparisons at other wavelengths (Fig. 5B) also support this assumption, although some small differences in time course are seen, for example, between 710, 0° and 740, 90°.

In some fibers, small differences in time course were also seen at other wavelengths, especially when the signals were very large. Possible reasons for these differences are discussed later (see Discussion), but we feel that they are probably due to another dye-related phenomenon and not, for example, to differences in the weighting of the absorbance changes from different T-system regions. These differences are small, generally less than the noise level of the traces, and would not alter the measurement of the slow signal at 30 ms by >10%. Therefore, for the purposes of this paper, all of the slow signals are treated as the same T-system signal.

Table I summarizes the mean amplitude of the surface and T-system signals recorded with 0° and 90° polarized light, from all experiments and for both dyes. The step signals included in this table were obtained using the more accurate fast pulse protocol (Fig. 2, Methods). The data are given for the same peak wavelengths shown in Figs. 3 and 4. There was a large fiber-to-fiber variability in the magnitude of these signals, as is evident from the deviation in each measurement. This was greatest for the step signal component because of its small size, which at many wavelengths fell below the resolution of the setup.
(\sim 0.2 \times 10^{-4}, \text{for the signal-to-noise ratio obtained under these conditions}). The step signal at 670 nm, 0° (NK2367) was near this limit and its sign could be either positive or negative in different fibers. However, for each signal component, the relative proportion of the 0° and 90° amplitudes at each wavelength is, on the average, the same as shown in Figs. 3 and 4.

To further characterize the wavelength dependence of the dichroism of the

![Figure 5](image)

**Figure 5.** (A) Absorbance signals record with dye WW375 at three wavelengths, for 0° and 90° incident polarizations. Same fiber as Fig. 4. Vertical calibration arrow: \(-1.5 \times 10^{-4}\) for top two traces and \(-3.0 \times 10^{-4}\) for the remainder. 32 sweeps were averaged per trace at 690 nm, and 16 sweeps at 710 and 730. (B) Paired comparison of the time course of the slow components. The records were taken from Figs. 4 and 5A after subtracting a step and scaling each to the same final amplitude. The 710-nm, 0° signal (solid traces) is used for the reference trace in each comparison, and was recorded immediately before the test signal (dotted traces) to avoid small differences in time course caused by changes in the fiber condition.

signals from each membrane, the spectra were determined for 0° and 90° incident light, over the spectral band 560–800 nm. These results are plotted in Fig. 6A for dye NK2367. The surface membrane data used for these spectra were determined exclusively using the short-pulse, fast-sampling protocol described in Methods. The T-system spectra were obtained from different fibers using the long-pulse protocol (Methods, and Figs. 3–5). Data from several
experiments were combined in plotting the spectrum of each membrane. It is evident from these data that the surface and T-system spectra for both polarizations have several common characteristics. Each has a multiphasic pattern with similar peaks. Negative absorbance bands occur near 630 and 670 nm, and positive bands below 600 and near 720 nm. The spectra are dichroic over the entire spectral band, showing the greatest dichroism (0°–90°) at the 670-nm band, and being much less dichroic at the 720-nm band. A striking difference in these spectra, however, is that for the surface membrane, the 0° absorbance change is more positive than the 90° absorbance change at every wavelength, whereas for the T-system, the 90° absorbance change is everywhere more positive than the 0° change, which results in a dichroism of opposite sign for the two membranes.

**TABLE I**

Membrane Responses at Peak Wavelengths Using Polarized Light

|                | 670 nm                      | 720 nm                      |
|----------------|-----------------------------|-----------------------------|
|                | Steady state | Step | Steady state | Step |
| 0°             | 90°          | 90°  | 0°           | 90°  |
| Mean           | –6.44        | –1.14| –0.24        | –0.78| 1.73        | 2.92 | 1.02        | 0.82 |
| SD             | 1.18         | 0.41 | 0.41         | 0.32 | 0.65        | 0.77 | 0.55        | 0.54 |
| N              | 8            | 8    | 10           | 10   | 7           | 7    | 9           | 9    |

(A) Dye NK2367

|                | 700 nm                  | 740 nm                  |
|----------------|--------------------------|--------------------------|
| Mean           | –2.64                    | –0.67                    |
| SD             | 0.45                     | 0.30                     |
| N              | 2                        | 2                        |

(B) Dye WW375

|                | 700 nm                  | 740 nm                  |
|----------------|--------------------------|--------------------------|
| Mean           | –2.64                    | –0.67                    |
| SD             | 0.45                     | 0.30                     |
| N              | 2                        | 2                        |

Mean amplitudes of the surface membrane (step signal component) and T-system (steady state minus step component) optical signals were recorded at 0° and 90°, in response to 90-mV hyperpolarizing voltage-clamp pulses. Amplitudes are given in –ΔI/I x 10^6. Mean fiber diameter from all experiments was 146.5 ± 24.0 μm.

Essentially similar spectral patterns are observed with polarized light for dye WW375 (Fig. 6B), except that the major absorbance bands are shifted 20–30 nm to longer wavelengths. Negative absorbance peaks occur near 650 and 700 nm, and positive bands near 580 and 740 nm. Again, at the longer wavelengths, where the largest potentiometric signals occur, the 700-nm band is more dichroic than the 740-nm band. Similarly, the same reversal of the 0° and 90° spectra of the surface and T-system occurs.

The Spectra Interpreted in Terms of a General Model of the Dye Response in the Surface and T-System

Several characteristics of the observed spectra suggest, at least qualitatively, some general features of any proposed mechanism for the dye response. First, the multiphasic patterns with negative absorbance bands seen in each of these spectra are characteristic of "difference spectra" between some initial and final absorb-
ance states of the dye. This would imply that the optical response in both membranes is associated with a change in the absorbance state of the dye. The similarity in the positive and negative absorbance bands in all of the spectra further suggests that the same absorbance change may occur in both membranes. Second, the fact that the spectra from both membranes are dichroic suggests that the optical response is influenced by the orientation of the dye in these membrane structures. It may also suggest that the dye itself undergoes a potential-dependent reorientation in the membrane when it changes absorbance states.

To test these hypotheses more quantitatively, the observed spectra were analyzed using a general two-state model (Tasaki and Warashina, 1976) for the potential-dependent response of an absorbing dye, which is restricted to particular geometric structures approximating the surface or T-system membranes. It was assumed that at the molecular level, the optical response results from a potential-dependent transition of dye molecules between two states, where each state is characterized by (a) an absorbance function describing the wavelength-dependent molar extinction coefficient, and (b) a geometric factor that describes the net weighting given to each absorbance state by the dye orientation in the membrane and the membrane geometry. In general, both the absorbance function and the geometric factor are different in the two states. If particular assumptions are made about the membrane geometries, and if the absorbance states of the dye are known (either from independent spectroscopic measurements or estimated as described below), then it is possible to calculate 0° and 90° predicted spectra for each membrane, which can be directly compared with the data. The details of this model are outlined in the Appendix, and the relevant conclusions are summarized below.

Fig. 7 shows the general types of 0° and 90° response spectra predicted for the assumed surface and T-system geometries (Fig. A1, Appendix), calculated for three different combinations of a and b. To model these curves, normalized Gaussian functions were assumed for the extinction states [E(λ)] and the geometric factors were chosen for the particular case of a dye reorientation from a "normal" to a "tangent" position in the membrane. It is evident that each of the cases shown produces spectra that are dichroic. Only a purely random distribution of resting dye orientations (A = B = 0.33), without any potential-dependent change in this distribution, would produce spectra that are independent of the incident polarization. Moreover, the dichroism in each case is distinctive. The leftmost traces show the absorbance spectra predicted for an optical response caused by a change in dye orientation alone [i.e., ε₁(λ) = ε₂(λ); Eqs. 7 and 8, Appendix]. For this mechanism, the predicted 0° and 90° spectra are monophasic with a single peak and a wavelength dependence that differs only in amplitude and sign from that of the assumed function. Moreover, the dichroism in the spectra is reversed in sign for the two geometries. For the cylindrical structure, the absorbance spectra are positive for 0° and negative for 90° light. For the T-system geometry, the same reorientation results in a negative absorbance spectra at 0° and a positive spectra at 90°. The center traces show predicted spectra for the case of a dye that undergoes a change in absorbance only, without reorientation (i.e., A = B; Eqs. 7 and 8, Appendix). An important feature of these spectra
is that each is biphasic and crosses the other at a common reversal point, which reflects the fact that the curves are simple difference spectra between initial and final absorbance states. The two geometries again produce a distinct dichroism, with the sign of the $0^\circ$ and $90^\circ$ spectra being opposite for the surface and T-system when compared at any wavelength. The right traces illustrate $0^\circ$ and $90^\circ$ spectra predicted for the case of an optical response caused by both a reorientation and spectral shift, computed for the same initial and final absorbance and

![Spectra Diagram](image)

**Figure 7.** Types of difference spectra predicted for the three general classes of dye mechanisms, computed for the surface and T-system geometries. Circles, $0^\circ$ spectra; crosses, $90^\circ$ spectra. The curves were computed from Eqs. 7 and 8, assuming $\epsilon_1(\lambda) = e^{-|0-720/254|}$, $\epsilon_2(\lambda) = e^{-|0-670/254|}$, $A_F = 0.5$ (tangent 2D), and $B_F = 0$ (normal). The remaining surface and T-system geometric factors were computed from these using the relationships given by Eqs. 4, 11, and 12.

**Figure 6.** (opposite) (A) Parallel (open circles, dotted curves) and perpendicular (crosses, solid curves) absorbance spectra of the surface and T-system optical signals recorded with dye NK2367. The optical data were determined for hyperpolarizing voltage-clamp steps of $-90$ mV, from a holding potential of $-100$ mV, and are plotted in units of $-\Delta I/I \times 10^4$. The smooth lines through the data were drawn by eye. Each point on the surface spectra represents the mean of data from three fibers. The surface data were recorded using short pulses and the T-system spectra were determined from long pulses, as described in Methods. The T-system data are plotted as the mean of data from four fibers. (B) Similar spectra, recorded using dye WW375. All of the data were obtained from one fiber, using the same pulse protocols, applied serially.
The proposed mechanism, consisting of both an absorbance change and a dye reorientation, is responsible for the optical response in both membranes. If the differences in the spectra of the surface and T-system are due to geometric factors alone, and not to differences in the mechanism of the dye response, then Eqs. 4 and 12 (Appendix) predict that when the 0° and 90° spectra of each membrane are added in the ratio 1:2, any differences caused by these geometric factors should cancel. The resulting spectra should be pure difference spectra between the two absorbance states of the dye and should be identical for both membranes, except for the proportionality factor N (Eq. 13, Appendix). The latter should reflect only differences in the relative membrane areas, or possible staining differences.

Fig. 8 shows the results of adding the 0° and 90° spectra in this way. The data obtained from several fibers stained with dye NK2367 are plotted together after each data set was normalized to have the same peak-to-peak difference between the 720- and 670-nm peaks in order to eliminate differences in ΔN. When compared in this way, the spectra of the surface and T-system are remarkably
similar, and these similarities are very consistent in data from different fibers. Within the resolution of the measurements, the absorbance peaks and the reversal points of the difference spectra are the same for both membranes. Each shows a strong positive absorbance peak at 720 nm and strong negative peaks at 670 and 630 nm, with a broader positive band below 600 nm. The spectrum of each membrane has major reversal points near 610 and 690 nm. This contrasts with the separate 0° and 90° spectra of both membranes, which did not show the same reversal wavelengths. Two additional reversal points at 640 and 660 nm are present in the surface spectrum and appear as inflection points at the same wavelengths in the T-system spectrum. These similarities in the two spectra strongly suggest that the dye response is associated with the same underlying absorbance change in both membranes. For the spectral region where the largest absorbance changes occur, this result indicates that when the membrane potential is made more hyperpolarized, the dye changes from an initial absorbance state centered at 670 nm to a final state centered near 720 nm. The resulting
difference spectra of both membranes show a common reversal point, at 690 ± 5 nm.

The ratio of the normalization factors used for the above spectra should equal \( \Delta N^T/\Delta N^S \) (Eq. 13), where the superscripts denote the T-system and the surface, respectively. If the dye stains both membranes equally, this ratio should also reflect the relative areas of each membrane compartment. Since the surface and T-system spectra of Fig. 8 were obtained in different fibers, we performed a few experiments in which the 0° and 90° amplitudes of each membrane signal were measured in the same fiber, at the 670- and 720-nm peaks only. The mean value for this ratio was 4.92 ± 1.69 (N = 8) for fibers having an average diameter of 146.5 ± 24.0 μm and a capacitance of 8.0 ± 0.8 μF/cm².

If we assume that the absorbance states can be approximated with Gaussian functions, it is possible to simultaneously fit the 0° + 2(90°) difference spectra of both membranes to Eq. 13 using the same absorbance states. The fitted parameters for the absorbance states obtained from such fits to the surface and T-system data in several fibers are given in Table II. The smooth curves in Fig. 8 were drawn from Eq. 13, from a fit to the mean of the data sets from both membranes.

| Fiber reference | \( \lambda_1 \) | \( C_1 \) | \( \sigma_1 \) | \( \lambda_2 \) | \( C_2 \) | \( \sigma_2 \) |
|-----------------|----------------|----------|-------------|----------------|----------|-------------|
| 1               | 714.4          | 7.8      | 25.7        | 675.3          | 4.0      | 10.5        |
| 2               | 709.7          | 8.4      | 33.9        | 679.8          | 6.2      | 17.4        |
| 3               | 715.1          | 4.8      | 9.7         | 675.5          | 8.2      | 10.9        |
| Mean            | 713.1          | 7.0      | 23.1        | 676.9          | 6.1      | 12.9        |
| SD              | 2.9            | 1.9      | 12.3        | 2.5            | 2.1      | 3.9         |

**T-system:**

| Fiber reference | \( \lambda_1 \) | \( C_1 \) | \( \sigma_1 \) | \( \lambda_2 \) | \( C_2 \) | \( \sigma_2 \) |
|-----------------|----------------|----------|-------------|----------------|----------|-------------|
| 4               | 718.9          | 3.7      | 28.0        | 674.5          | 6.7      | 16.8        |
| 5               | 718.3          | 4.4      | 21.4        | 674.3          | 5.9      | 17.3        |
| 6               | 705.3          | 6.0      | 31.6        | 678.5          | 7.7      | 22.4        |
| 7               | 713.6          | 5.5      | 24.5        | 672.6          | 5.7      | 13.5        |
| Mean            | 714.0          | 4.9      | 25.1        | 675.0          | 6.5      | 17.5        |
| SD              | 6.2            | 1.0      | 4.5         | 2.5            | 0.9      | 3.7         |

Parameters obtained by fitting the data of Fig. 8 to Eq 13 (Appendix), where \( \epsilon_1(\lambda) \) and \( \epsilon_2(\lambda) \) were assumed to be Gaussian functions of the form \( \epsilon(\lambda) = Ce^{-\frac{\lambda^2}{2\sigma^2}} \).

If the absorbance functions obtained from these fits are assumed, it is then possible to obtain the geometric factors for the initial and final dye orientation states by fitting the 0° and 90° spectra of each membrane to Eqs. 7 and 8. If the same reorientation of dye molecules occurred in both membranes, then the geometric factors obtained from these fits, although numerically different for the two membrane geometries (Appendix, Table A1), should correspond to the same two orientation states. The geometric factors obtained by performing a least-squares fit of the data to Eqs. 7 and 8 are summarized in Table III for all
experiments. Because the surface and T-system spectra were obtained in different fibers, the fitted parameters were $A_{\parallel}$ and $B_{\parallel}$ for the surface spectra, and $A_{\perp}$ and $B_{\perp}$ for the T-system data. In each fit, the assumed absorbance functions were those listed in Table II for the same fiber. The mean values for the parallel geometric factors fitted to the surface spectra were 0.36 and 0.18 for $A_{\parallel}$ and $B_{\parallel}$, respectively. If these values are compared with Table AI (Appendix), they imply a dye reorientation in the surface membrane from an initial orientation state more "normal" to the membrane to a final orientation state more "random." The mean values for these factors, fitted to the T-system spectra, were 0.26 and 0.5 for $A_{\perp}$ and $B_{\perp}$. These correspond to a dye reorientation in the T-system from an initial state normal to the membrane to a final state oriented "tangent 2D" to the membrane.

The results of these fits to the separate surface and T-system spectra therefore suggest that the same dye reorientation occurred in both membranes. Since all of the geometric factors for both membranes can be obtained from the two fitted factors from either membrane (using Eqs. 4, 11, and 12), this hypothesis can be further tested by making the following comparisons. The mean fitted geometric factors obtained from the surface membrane data ($A_{\parallel}$ and $B_{\parallel}$, Table III) predict values of 0.32 and 0.4 for the T-system. For the T-system geometry, these factors imply a dye reorientation from a more normal to a more random position in the membrane (Table AI, Appendix). The direction of this reorientation is the same as that obtained from fitting the T-system data, but the degree of orientation in each state is somewhat smaller. Similarly, the mean fitted geometric factors obtained from the T-system data ($A_{\perp}$ and $B_{\perp}$, Table III) predict values of 0.48
and 0.0 for $A_\parallel$ and $B_\parallel$ of the surface membrane, or a reorientation from normal to tangent 2D. Again, the direction of the predicted reorientation is the same as was obtained from the fitted surface spectra, but the degree of orientation in each state is different, in this case being somewhat greater than observed.

These discrepancies between the average predicted and observed geometric factors of each membrane are probably not in serious conflict with the major conclusions from this model for several reasons. They are only slightly greater than the standard deviation in the values from separate experiments (Table III). More importantly, they deviate in a direction expected if the actual fiber cross section is more flat than round since the $0^\circ + 2(90^\circ)$ spectra of both membranes would be expected to be identical only for the measurements having perfect radial symmetry. The effects of fiber flatness can be estimated (see *Surface Membrane Elliptical Geometry* in the Appendix) and, in general, would produce significant effects on the surface membrane perpendicular geometric factors for the two orientation states of interest (tangent 2D and normal). These can result, therefore, in a large change in the $90^\circ$ difference spectrum of the surface membrane.

To investigate these effects more closely, we performed one experiment in which the $0^\circ$ and $90^\circ$ spectra of both membranes were obtained from the same fiber. To do this, the data were obtained at fewer wavelengths and over a more limited spectral band (660–760). The $0^\circ$ and $90^\circ$ T-system data from this fiber were fit to Eqs. 7 and 8, using the absorbance functions $e_1(\lambda)$ and $e_2(\lambda)$ obtained from the $0^\circ + 2(90^\circ)$ spectrum (not shown) of the T-system only. As shown in the Appendix (*Surface Membrane Elliptical Geometry*), for an elliptical fiber cross section, the 1:2 relationship (Eq. 13) is still valid for the T-system but it is no longer true in general for the surface geometry. The predicted $0^\circ$ and $90^\circ$ curves for the surface membrane obtained from these T-system fits were then compared with the observed surface spectra from the same fiber. These results are shown in Fig. 9. The T-system $0^\circ$ (open circles) and $90^\circ$ (crosses) data were fit (dotted and continuous curves) using initial and final absorbance states centered at 670 and 713 nm, respectively, and corresponding orientation states of normal and tangent 2D. However, the surface $0^\circ$ and $90^\circ$ data were not simultaneously well predicted using the same absorbance and orientation states. In particular, the surface $0^\circ$ spectrum (open circles, dotted curve) is well predicted, whereas the surface $90^\circ$ observed spectrum (crosses) is everywhere more positive than the predicted curve (solid line). This deviation has the direction and magnitude expected if the fiber cross section were more elliptical than circular. For example, if the surface perpendicular geometric factors are corrected for an ellipticity of 0.5 (Table AII, Appendix) with no change in the other geometric factors or absorbance functions, the $90^\circ$ predicted curve (broken line) is everywhere shifted to more positive values and more closely approximates the surface data. This ellipticity is reasonable for our fibers. Measurements of the ellipticity of muscle fibers vary from 0.6 to 0.8 (Blinks, 1965; Hodgkin and Nakajima, 1972) for small fibers, and this number tends to increase with larger fibers and for shorter sarcomere lengths (Blinks, 1965), as was the case in our experiments.
This agreement with the model seems reasonable considering the resolution possible in the measurements, the variability of data from different fibers, and the simplifying assumptions made regarding the geometry of each membrane. A more stringent test of this model could be obtained from many similar fits made simultaneously to the surface and T-system spectra obtained in the same fiber. However, until further independent determinations of the actual shape of the absorbance functions are known, such experiments seem premature and would not be likely to yield more useful information.
DISCUSSION

Interpretation of the Spectra in Terms of a Common Mechanism for the Dye Response in the Surface and T-System

The absorbance signals recorded from both the surface and T-system membranes in skeletal muscle fibers stained with dye NK2367 or dye WW375 are highly dichroic over a wide range of wavelengths. Several qualitative features of the observed 0° and 90° spectra are well predicted by a general model for the potential-dependent response of an absorbing dye (Tasaki and Warashina, 1976), which takes into account the surface and T-system geometries. These include a similar multiphasic pattern with common absorbance peaks and common dichroic maxima. The dichroism of the two membranes differs significantly only in sign, with the relative magnitudes of their 0° and 90° spectra being reversed. These salient features of the spectra are consistent with the proposal that the dye response in both membranes occurs via a common mechanism that includes a change in both the peak absorbance band and in the net dye orientation. When the observed spectra were compared quantitatively with this model, several parameters describing the proposed common mechanism were obtained. These suggest that when the membrane potential is made more negative, the peak absorption band of the dye is shifted ~40 nm to longer wavelengths (from 675 to 715 nm for dye NK2367; from 700 to 740 nm for dye WW375); concomitantly, the dye orientation changes from an alignment more normal to one more tangent to the membrane.

It should be noted that our quantitative studies focused on the two largest absorbance bands in muscle. If we had chosen other absorbance pairs (e.g., 630 and 670, for dye NK2367), the major qualitative conclusions would be the same—that the dichroic behavior of the signals from the surface and T-system can be explained by a common mechanism that includes a dye reorientation in these membrane geometries. The curious differences in the optical signals recorded in skeletal muscle fibers illuminated with polarized or unpolarized light (Baylor et al., 1981; Heiny and Vergara, 1982a, and this paper) can be well explained by orientation effects alone without, for example, invoking differences in membrane composition. This conclusion is further strengthened by the fact that the 0° and 90° spectra of the surface membrane in skeletal muscle follow a pattern similar to that reported for the squid axon, which has a similar geometry (Ross et al., 1977). In both preparations, a positive dichroism was observed at all wavelengths (referred to hyperpolarizing pulses). It is possible that the spectral differences reported for these dyes in other preparations (Ross and Reichardt, 1979) can also be explained by differences in membrane geometries.

Comparison of These Findings with Proposed Physicochemical Models for the Mechanism of Fast-Response Dyes

Both dye NK2367 and WW375 belong to the category of fast potentiometric dyes (Waggoner and Grinvald, 1977; Waggoner, 1979), meaning that they sense potential in tens of microseconds or less (in contrast to slow-response dyes, which work by very different mechanisms). Therefore, it is useful to compare these
findings with the various physicochemical models proposed for the mechanism of this class of dyes (Waggoner and Grinvald, 1977). The most likely of these include the following. (a) A potential-dependent perturbation of the equilibrium between different aggregate forms of the dye (e.g., monomer-dimer), each of which has a different characteristic absorbance band. In this mechanism, the absorbance change may also be associated with a reorientation of the chromophore, if the different aggregate states have different preferred orientations in the membrane. (b) Movement of dye (rotational or translational) between different microenvironments in or near the membrane (e.g., "on-off" the membrane), or into a more or less favorable orientation for absorption (e.g., pure rotation). In this case, the force exerted by the membrane electric field on the charged or dipolar molecule is the immediate cause of the change in orientation. This change might also be accompanied by a change in absorbance, if the absorption transition moment of the dye is influenced by the changed orientation or microenvironment. (c) Electrochromism (or Stark effect), in which the membrane electric field produces a direct effect on the extinction coefficient of the chromophore, unaccompanied by a change in orientation.

Our results with these dyes in skeletal muscle are consistent with a mechanism of either the first or second type (excluding pure rotation) and are incompatible with electrochromism. Detailed physicochemical studies of the behavior of dyes NK2367 and WW375 have not yet been performed. However, our findings can be compared with results obtained with a related dye, merocyanine 540, which has been extensively studied in both model and biological membranes (Tasaki and Warashina, 1976; Rosset al., 1977; Dragsten and Webb, 1978; Smith et al., 1980). It is generally proposed that the response of this dye involves a potential-dependent change in the equilibrium between the monomer and dimer forms of the dye, each having different absorbance maxima and a different net orientation. This mechanism is supported by spectroscopic studies which show that both monomer and dimer forms of merocyananine 540 are present in artificial lipid membranes; the monomer absorbs maximally at 570 nm and is oriented more normal to the membrane, whereas the dimer absorbs maximally at 530 nm and is oriented less normal (Tasaki and Warashina, 1976; Waggoner and Grinvald, 1977; Dragsten and Webb, 1978). The dichroic spectra of this dye in the squid axon showed the same peaks and could be well predicted using orientation states (computed from the same analysis followed in this paper) that corresponded to the monomer-dimer orientations found in model membranes (Tasaki and Warashina, 1976; Waggoner and Grinvald, 1977).

Since dyes NK2367 and WW375 belong to the same class of merocyanine dyes, it is possible that they work by a similar mechanism. Many dyes of this class have large electronic dipole moments that could be moved by the membrane electric field, and many form multiple aggregate states (Dragsten and Webb, 1978; Waggoner and Grinvald, 1977). Preliminary spectroscopic data with dye WW375 at different concentrations (10⁻⁶–10⁻⁴) suggest that this dye readily forms dimers in both aqueous and nonpolar solvents (monomer and dimer peaks near 705 and 630 nm, respectively, in distilled water or Ringer's; peaks near 715 and 660 nm in ethanol, octanol, or 2,6-lutidine; A. S. Waggoner, personal
In uncharged lipid membranes (PC vesicles), these peaks are further shifted to longer wavelengths (near 665 and 715 nm, J. A. Heiny and J. Vergara, unpublished results). Similar results were obtained with dye NK2367 (with all peaks ~20–30 nm to the blue, but the decomposition of this dye made quantitation difficult). Thus, the absorbance peaks found with dyes NK2367 and WW375 in uncharged artificial lipid systems do not directly correspond to those found in nerve and muscle. If aggregate formation is a part of the mechanism of these dyes, then the absorbance must be further red-shifted by the nerve and muscle membrane environment. In addition, if the absorbance peaks in muscle correspond to aggregate forms of the dye, then the net orientation of monomers and dimers in the muscle membrane would have to be the opposite of that reported for merocyanine 540 (Tasaki and Warashina, 1976; Waggoner and Grinvald, 1977; Dragsten and Webb, 1978), since in muscle the short-wavelength peak (670 nm) corresponds to the more perpendicular orientation state. These important differences between the behavior of merocyanine 540 and dyes NK2367 and WW375 will need to be explained in order to further characterize the physicochemical bases of the optical signals reported here.

Relevance of These Findings to Optical Studies of the T-System in Skeletal Muscle

The results presented in this paper have several important implications regarding the use of these dyes in skeletal muscle. First, they give additional confidence in the use of these dyes as probes of T-system potentials since they can explain our previously reported spectral findings. They also support our identification of the slow signal component as originating in the T-system (Heiny and Vergara, 1982a), based on the dichroism expected from the geometry of this membrane compartment.

Second, if our interpretation of the spectral differences is correct, specific conclusions are justified regarding important dye controls that are difficult to perform directly in the T-system. It is likely that the limiting speed of the dye response in the T-system is probably the same as measured in the surface membrane (<20 μs), since this would be expected if the dye works by a similar mechanism. This conclusion is not directly verifiable since the T-system cannot be rapidly voltage-clamped. However, the fact that the T-system optical signal becomes faster in every condition in which the T-system membrane response is deliberately made faster (e.g., by recording in full Na+ and at higher temperature to generate a fast action potential in the T-system; Heiny and Vergara, 1982a, and unpublished results) further supports the idea that the optical recording of these signals is not limited by the response time of the dye. Another conclusion from these studies is that the dye concentration in the T-system membranes is probably not significantly different from that in the surface membrane, since the ratio \( \Delta N^7/\Delta N^5 \) (4.9 ± 1.6) used to scale the 0° + 2(90°) spectra (Fig. 8) has a value close to that expected from the average membrane capacitance measured in the same fibers (8.0 ± 0.8 μF/cm²). If we assume a specific capacitance of 1 μF/cm² and distribute 1–2 μF/cm² on the surface membrane (Hodgkin and Nakajima, 1972), then we would expect a value from 3 to 7 for this ratio. This
also agrees with the ratio of T-system to surface area expected for fibers of our average diameter (146 ± 24 μm) based on morphological data (Peachey, 1965) and would be expected if the dye stained both membranes equally. This conclusion is made tentatively, however, since the fiber-to-fiber deviation in this ratio was large. This deviation is probably due to the errors introduced by dye bleaching (see Methods) and the fact that the surface and T-system do not always bleach at the same rate during an experiment. It may also be due in part to the variable flatness of the fibers, which, as shown, exaggerates the surface membrane 90° signal used in computing this ratio.

Third, the finding that the time courses of the T-system signals recorded for different incident polarizations and wavelengths were very similar (Fig. 5) suggests that the slow optical signal reflects the same average T-system potential change for all illuminating conditions. Optically, potential changes occurring in all regions of the T-system will contribute to the T-system absorbance signal. Assuming that the dye response is rapid and linear, and that the T-system is uniformly stained, the time course of the T-system optical signal will be proportional to the sum of the transmembrane potential transients occurring at each radial position, where each is weighted by the membrane area at that same position. Our results suggest that this "weighted average" time course is not influenced by other possible radial weighting factors. Possible radial inhomogeneities in staining would be expected to distort this time course. However, such differences are unlikely given our estimate of the dye scaling ratio (above) and in view of Endo's results (1966) showing that dyes of similar charge and molecular weight freely penetrate into the T-system. A possible variation in membrane geometry might also introduce a radial dependence to the geometric factors that would affect this time course. However, these would be expected to produce a polarization dependence to the time course, which was not observed. Moreover, there is no morphological evidence for radial geometric gradients of this kind (Mobley and Eisenberg, 1975; Peachey and Eisenberg, 1978). Therefore, we feel that the small differences in time course of the slow optical signals (Fig. 5B) are probably due to a wavelength-dependent slow dye phenomenon similar to that reported for other dyes (Ross et al., 1977). We have sometimes seen a small signal component that has a time course similar to an AC-filtered square pulse with a time constant of ~20 ms. It is especially evident on long pulses (cf. Heiny et al., 1983) and is greater with NK2367 than WW375. No such signal is expected from the T-system potential change under these conditions. A signal component with similar properties has also been reported in axon studies with these same dyes (Salzberg and Bezanilla, 1983, and unpublished results). The origin and spectral dependence of this artificial signal component are not known and will need to be studied further before these dyes are used for quantitative kinetic studies of T-system electrical events.

From a practical viewpoint, it is evident from these results that a better signal-to-noise ratio for recording T-system signals can be obtained with 0° polarized light, since much of the noise in these measurements is contributed by the background light. Therefore, with unpolarized light, most of the T-system signal comes from the 0° component (Figs. 3 and 4) and about half of the incident light (90°) contributes background noise without producing any signal.
Appendix

Surface Membrane Calculation, Assuming Cylindrical Geometry

For the surface membrane, the analysis exactly follows that given by Tasaki and Warashina (1976) for the case of an absorbing dye in a membrane having cylindrical geometry, and is summarized as follows. The unprimed Cartesian coordinate system (Fig. A1), XYZ, describes the orientation of the muscle fiber (modeled as a cylinder) with respect to the incident light. The direction of light propagation is along the Z axis. The axes Y and X are parallel (0°) and perpendicular (90°), respectively, to the long axis of the muscle fiber, and are the directions of plane polarization of interest. The orientation of a dye molecule ($\mu$) at a point on the surface membrane is described by the angles $\theta$ and $\varphi$ relative to the primed coordinate system, $X'Y'Z'$. The position of this point with respect to the unprimed coordinate system is described by the angle $\omega$ about the long axis, which is parallel to $Y'$. A function $f(\theta, \varphi)$ is used to denote the distribution of dye orientations relative to the...
membrane surface and is normalized so that the probability of finding a dye molecule
with $\theta$ between 0 and $\pi$, and $\varphi$ between 0 and $2\pi$ is 1:

$$\int_0^{2\pi} \int_0^{\pi} f(\theta, \varphi) \sin \theta \, d\theta \, d\varphi = 1.$$  (1)

It is assumed that the absorption to light by a dye molecule is given by the $\cos^2$ of the
angle between the dye transition moment and the electric vector of the incident lightwave
(see, e.g., Fredericq and Houssier, 1973).

Geometric weighting factors ($A_f$ and $A_s$, for surface membrane parallel and perpendicular
light) are calculated that represent the average degree of absorbance to parallel or
perpendicular light expected for a dye having an orientation given by $f(\theta, \varphi)$ at the
membrane, and restricted to be in or near a membrane having a particular geometry. For
a cylinder, these are

$$A_f = \int_0^{2\pi} \int_0^{\pi} f(\theta, \varphi) \sin \theta \sin \varphi \, \sin \theta \, d\theta \, d\varphi$$  (2)

and

$$A_s = \frac{1}{2\pi} \int_0^{2\pi} \int_0^{\pi} \int_0^{\pi} f(\theta, \varphi) \cos \theta \sin \omega + \sin \theta \cos \varphi \cos \omega \, \sin \theta \, d\theta \, d\varphi \, d\omega,$$  (3)

**Table AI**

|                | Surface cylinder | T-system isotropic network |
|----------------|------------------|----------------------------|
|                | Parallel | Perpendicular | Parallel | Perpendicular |
| (I) Normal     | 0.0      | 0.5            | 0.5      | 0.2            |
| (II) Random 3D | 0.55     | 0.33           | 0.33     | 0.33           |
| (III) Tangent 2D | 0.5      | 0.25           | 0.25     | 0.58           |
| (IV) Tangent 1D | 1.0      | 0.0            | 0.0      | 0.5            |

Geometric factors for the surface and T-system computed for four orientation states.
The surface membrane (cylinder) factors are taken from Tasaki and Warashina (1976).

where the integrand is the absorption weighted by the angular distribution function at
the membrane surface and the integration is performed over all possible angles. Both
factors are normalized and computed for a unit transition moment. From Eqs. 1–3 above,
the following conservation relationship between the geometric factors can be derived:

$$A_f + 2A_s = 1.$$  (4)

For a particular orientation of dye molecules at the membrane, these factors can be
exactly calculated and are given in Table AI. For a completely random distribution (case
II, "3D random" case), namely, for $f(\theta, \varphi) = 1/4\pi$, $A_f$ is equal to 0.33.

Other cases of interest are: I, "normal," in which all dye molecules are oriented normal
to the membrane surface; III, "tangent 2D," in which all dye molecules are oriented
randomly in the plane parallel to the membrane surface; and IV, "tangent 1D," the case
in which all dye molecules are oriented parallel to the $Y$ axis and in the tangent plane.

To compute the absorbance spectra predicted for parallel or perpendicular incident
light, for a dye that undergoes a change in absorbance in response to a change in
transmembrane potential, we consider that the dye molecules can exist in two distinct states. Each state is described by two parameters: a molar extinction coefficient $\varepsilon(\lambda)$, and an angular distribution function, $f(\theta, \varphi)$. In general, both parameters are different in the two states.

At rest, the intensity of light transmitted through the fiber, for parallel and perpendicular incident light, is given by

$$I_t = I_0 K e^{-2N_{1}A_{1}e_{1}(\lambda)+N_{2}A_{2}e_{2}(\lambda)}}$$

and

$$I_{\perp} = I_0 K e^{-2N_{1}A_{1}e_{1}(\lambda)+N_{2}A_{2}e_{2}(\lambda)}}$$

where $A$ and $B$ denote the geometric factors and $e_{1}(\lambda)$ and $e_{2}(\lambda)$ denote the extinction coefficients for each state; $K$ is a factor that represents the intensity lost because of dye in the extracellular solution (assumed to be isotropic); $N_{1}$ and $N_{2}$ represent the average concentration of dye molecules in each state times the pathlength through the fiber.

A change in light intensity is associated with a small change in the amount of dye in each state. From mass conservation, $\Delta N_{1} = -\Delta N_{2} = \Delta N$. (Note: this relation is also valid for a monomer-dimer conversion if the dimer concentration is given in terms of the number of monomers.) Therefore, the potential-dependent fractional transmittance spectra are given by

$$\frac{\Delta I_{t}}{I_{t}} = [A_{1}e_{1}(\lambda) - B_{1}e_{2}(\lambda)]\Delta N$$

and

$$\frac{\Delta I_{\perp}}{I_{\perp}} = [A_{2}e_{1}(\lambda) - B_{2}e_{2}(\lambda)]\Delta N,$$

where the factor 2.3 has been dropped.

The folds and cavoelae of the surface membranes (Dulhunty and Franzini-Armstrong, 1975; Zampighi et al., 1975) were not considered in this model. Optically and electrically, these membrane areas would be expected to give a fast, surface-membrane-type signal. Since they are approximately spherical in shape, they would behave isotropically and therefore would not significantly contribute to the dichroism of the absorbance signals.

**T-System Calculations**

For the T-system, a radially oriented system of cylinders, confined to the XZ plane and having a homogeneous angular distribution about the fiber long axis, is assumed (Fig. A1). Local variations in the cross section of each tubule from the assumed cylindrical geometry (e.g., junctional vs. nonjunctional regions) are not included. It is assumed that these are homogeneously distributed along the radius and that they will average together with differences in tortuosity and/or membrane folding so as not to change the assumed uniform angular distribution significantly. The same angles $\theta$, $\varphi$, and $\omega$ used above describe the orientation of a dye in each tubule (modeled as a cylinder). A fourth angle (in this case, $\alpha$) is used to describe the orientation of each cylinder about the fiber long axis. In frog muscle, the T-system is an interconnected mesh (Peachey and Eisenberg, 1978), with grid dimensions small with respect to the fiber diameter and without obvious anisotropy in the transverse plane (Mobley and Eisenberg, 1975; Peachey and Eisenberg, 1978). In terms of the distribution of angular orientations, and hence the expected dichroism, the model assumed here and the "mesh" geometry are indistinguishable (although electrically, they would behave differently). For simplicity, longitudinally ori-
ent tubules, undulations in the plane of the T-system, or helicoids are not considered, as they constitute only a small fraction of the total T-system area (Peachey and Eisenberg, 1978). The factors for this geometry are

\[
A_T^I = \frac{1}{2\pi} \int_0^{2\pi} \int_0^{2\pi} \int_0^\pi f(\theta, \varphi) \left( \cos^2 \alpha - \sin \theta \cos \varphi \sin \omega \right)^2 \sin \theta \, d\theta \, d\varphi \, d\omega
\]

and

\[
A_L^I = \frac{1}{4\pi^2} \int_0^{2\pi} \int_0^{2\pi} \int_0^\pi f(\theta, \varphi) \left( \sin \theta \cos \varphi \cos \omega + \cos \sin \omega \cos \alpha + \sin \sin \varphi \sin \alpha \right)^2 \sin \theta \, d\theta \, d\varphi \, d\omega.
\]

It can be readily seen that

\[
A_T^I = A_L^I,
\]

which reflects the fact that the T-system network lies entirely in a plane transverse to the surface membrane. Further, after the integration over \( \alpha \) and \( \omega \) is performed, the same conservation relationship found for the surface membrane, namely,

\[
A_T^I + 2A_L^I = 1,
\]

results, which reflects the fact that both the surface and T-system membranes have azimuthal symmetry about the \( Y \) axis. The results of calculating these factors for the T-system geometry, for the four cases considered above, are given in Table A1.

Finally, it can readily be verified (from Eqs. 4, 7, 8, and 12) that the following relationship holds for both membranes:

\[
\frac{\Delta I_L}{I_L} + 2 \frac{\Delta I_T}{I_T} = [\epsilon_I(\lambda) - \epsilon_D(\lambda)] \Delta N.
\]

**Surface Membrane Elliptical Geometry**

The cross section of isolated muscle fiber is not generally cylindrical (Blinks, 1965). In these experiments, larger fibers were routinely selected, and these tended to have a flatter cross section, which would be better approximated as an ellipse than a circle.

This fiber ellipticity would not be expected to change the T-system geometric factors, since the perpendicular projections would not be significantly affected by changes in the shape of the cross section for a system of small mesh size that is isotropic in the \( XZ \) plane. (Fig. A2). Further, the fiber ellipticity would have no effect on the parallel geometric
factors for either the surface or T-system membranes, since any flattening of the fiber cross section would change the X or Z (cross-sectional components of absorbance), but not the Y projections. However, the fiber ellipticity will influence the perpendicular geometric factors for the surface membrane. To derive a general analytical expression for the perpendicular geometric factors for a dye having general orientation given by \( f(\theta, \varphi) \) in an elliptical surface is tedious and would not result in a general analytical conservation relationship between the parallel and perpendicular geometric factors. However, these factors can easily be obtained numerically for three cases of interest. For a perfectly random distribution of dye orientations in the membrane (case II, Table AI), this factor is independent of the membrane geometry and therefore is the same as for a circular cross section. Two other cases of interest, namely, for a dye oriented normal (case I) or tangent 2-D (case III) to the surface of an ellipse, are calculated below, and the results are expressed as the ratio of the factor for an ellipse to that for a circle.

**CASE I: DYE ORIENTED NORMAL TO THE SURFACE OF AN ELLIPSE**  A unit vector, \( \mathbf{n} \), is defined to be normal to the surface of an ellipse (Fig. A 2); i.e., if the surface of the ellipse is defined by

\[
S(x, z) = \frac{x^2}{a^2} + \frac{z^2}{b^2},
\]

then

\[
\mathbf{n} = \frac{\nabla S}{|\nabla S|} = \frac{1}{\sqrt{\frac{x^2}{a^2} + \frac{z^2}{b^2}}} \cdot \left( \frac{x}{a^2} \hat{x} + \frac{z}{b^2} \hat{z} \right),
\]

where \( \hat{x} \) and \( \hat{z} \) are the cross-sectional unit coordinates of the ellipse (Fig. A 2) and \( a \) and \( b \) are the major and semimajor axes, respectively. The perpendicular projection of a dye having orientation \( \mathbf{n} \) is then the X component of this vector, and the geometric factor is given by

\[
A_\perp = \int \frac{x^2}{a^2} \cdot \frac{1}{\sqrt{\frac{x^2}{a^2} + \frac{z^2}{b^2}}} \cdot ds,
\]

where \( ds \) is an element of perimeter. After making the substitutions \( ds = dx \cdot \sqrt{1 + \frac{x^2 b^4}{z^2 a^4}}, \)

\[
z^2 = b^2 \left( 1 - \frac{x^2}{a^2} \right), \quad X = x/a,
\]

this geometric factor, computed for a quarter ellipse, becomes

\[
A_\perp = \frac{b^2}{a} \int_0^1 \frac{X^2}{(1 - X^2) \left[ 1 + X^2 \left( \frac{b^2}{a^2} - 1 \right) \right]^{1/2}} \cdot dX.
\]

After the substitution \( X = \sin \theta \), this integral has the form

\[
A_\perp = \frac{b^2}{a^2} \int_0^{\pi/2} \frac{\sin^2 \theta}{\left[ 1 + \sin^2 \theta \left( \frac{b^2}{a^2} - 1 \right) \right]^{1/2}} \cdot d\theta,
\]

which can be integrated numerically without singularities.
For a circle \((a = b = 1)\), the value of this integral for the same limits is \(\pi/4\). For ratios of \(b/a\) between 0 and 1, the results of numerically evaluating this integral expressed as a percent difference from the geometric factor derived for a circle are given in Table AII.

**CASE II: DYE ORIENTED RANDOMLY IN THE TANGENT PLANE OF AN ELLIPSE** For this case, a unit vector \(\hat{m}\), where \(\hat{m} = \alpha \hat{x} + \beta \hat{z} + \gamma \hat{y}\), is defined to lie in the plane of the membrane, i.e.,

\[
\hat{m} \cdot \hat{n} = \frac{\alpha x}{a^2} + \frac{\beta z}{b^2} = 0
\]  

(19)

and

\[
\alpha^2 + \beta^2 + \gamma^2 = 1.
\]  

(20)

Eqs. 19 and 20 can be solved to obtain \(\alpha^2\) or the square of the component of \(\hat{m}\) along the \(\hat{x}\) axis. The perpendicular geometric factor for this case is given by

\[
A_\parallel^P = \int \left[ \frac{1 - \gamma^2}{1 + \frac{\beta^2}{\gamma^2} + \frac{\gamma^2}{\alpha^2}} \right] ds.
\]  

(21)

**TABLE AII**

| \(b/a\) | Case I, normal | Case III, tangent 2D |
|---|---|---|
| 0.1 | 0.035 | 1.259 |
| 0.2 | 0.104 | 1.235 |
| 0.3 | 0.193 | 1.205 |
| 0.4 | 0.293 | 1.173 |
| 0.5 | 0.401 | 1.142 |
| 0.6 | 0.515 | 1.112 |
| 0.7 | 0.663 | 1.082 |
| 0.8 | 0.753 | 1.054 |
| 0.9 | 0.876 | 1.027 |
| 1.0 | 1.0 | 1.0 |

Geometric factors for a surface membrane having an elliptical cross section, computed for two orientation states; perpendicular factors are expressed as the ratio of ellipse to circle.

For this case, \(\gamma\), or the \(\gamma\) component of \(\hat{m}\), is the same for a circle and an ellipse, and in computing the correction to this factor caused by fiber ellipticity, the \((1 - \gamma^2)\) terms cancel in the ratio. After making the same substitutions as in case I for \(ds\), \(z\), and \(x/a\), this correction can be computed from the integral

\[
\int_0^{1} \left[ \frac{1 - X^2}{1 + X^2 \left( \frac{b^2}{a^2} - 1 \right) a^2} \right] dX,
\]  

(22)

for a quarter ellipse. For a quarter circle, the value of this integral is \(\pi/4\). The results of numerically evaluating this integral for the same values of \(b/a\) used above and computing the ratios of the values obtained for an ellipse to that for a circle are given in Table AII.
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