J. Physiol. (1979), **292**, *pp.* 267–295
With 15 *text*-figures
Printed in Great Britain

OPTICAL PROBES OF MEMBRANE POTENTIAL IN HEART MUSCLE

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(*Received 27 July 1978*)

SUMMARY

1. The fluorescent dye Merocyanine-540 and the two weakly fluorescent dyes Merocyanine-rhodanine and Merocyanine-oxazolone are shown to respond as optical probes of membrane potential in heart muscle.

2. In frog hearts stained with Merocyanine-540, the absorption at 540 nm decreases by 0·1–1·0% and increases at 570 nm by 0·01–0·5% during the action potential. With either a 540 or a 570 nm excitation wave-length, the fluorescence increases by 1–2%. The time course of all three optical measurements follows the kinetics of the action potential.

3. Merocyanine-rhodanine exhibits potential-dependent optical responses through a 0·5% decrease in absorption at 750 nm, and Merocyanine-oxazolone has a 1·0% decrease in absorption at 720 nm. Their optical responses have a signal-to-noise ratio of 100/1 and 500/1, respectively.

4. The action spectrum of Merocyanine-rhodanine is triphasic in frog heart with an increase in transmittance from 780 to 700, a decrease from 700 to 600, and an increase from 600 to 450 nm. Merocyanine-oxazolone shows only increases in transmittance during membrane depolarization.

5. The optical responses of these probes are linear with respect to changes in membrane potential.

6. Pharmacological agents or ionic interventions do not alter the membrane potential sensitivity of Merocyanine-540.

7. Rapid spectrophotometric measurements at various phases of the action potential indicate that the potential dependent optical signals of Merocyanine-540 are produced by changes in amplitude of fluorescence and absorption bands. The lack of wave-length displacement as a function of membrane potential, i.e. electrochromism, is not the mechanism governing the voltage sensitivity of Merocyanine-540.

8. The data suggest that these Merocyanine dyes bind to the plasma membrane and serve as linear optical probes of membrane potential in heart muscle.

INTRODUCTION

Squid giant axons stained with a dye having optical properties that are sensitive to the local electrical environment generate potential-dependent optical responses (Cohen, Salzberg, Davila, Ross, Landowne, Waggoner & Wang, 1974). Of the various

0022-3751/79/4170-0643 $01.50

chromophores examined, Merocyanine dyes were found to act as most effective probes of the membrane potential. The action potentials measured with Merocyanine-540 have signal-to-noise ratios which range from 1/1 to 10/1 and $\Delta F/F \simeq 0{\cdot}1\%$ for a single action potential. Fluorescence action potentials were also measured from single neurones of the leech (Salzberg, Davila & Cohen, 1973), frog skeletal muscle fibres (Landowne, 1974; Oetliker, Baylor & Chandler, 1975; Vergara & Bezanilla, 1976), and frog heart (Salama & Morad, 1976). Squid giant axons stained with Merocyanine-540 deteriorate within a few seconds after exposure to intense light (Davila, Salzberg, Cohen & Waggoner, 1973). The photodynamic effect induced by Merocyanine-540 limits the duration of experiments on invertebrate ganglia (Salzberg *et al.* 1973), squid giant axon (Davila *et al.* 1973), and in striated muscle fibres (Oetliker *et al.* 1973), but was not a major factor in the frog heart (Salama & Morad, 1976).

More recently, two weakly fluorescent dyes, Merocyanine-rhodanine (Ross, Salzberg, Cohen, Grinvald, Davila, Waggoner & Wang, 1977) and Merocyanine-oxazolone (Salzberg, Grinvald, Cohen, Davila & Ross, 1977) were shown to act as membrane potential probes which exhibit little or no photodynamic effect in nerve tissue. In axons, these dyes have broad absorption bands and yield large signal-to-noise ratios of 50/1 to 100/1 on a single sweep.

Potential-dependent optical responses were measured not only through the fluorescence emission, but also through changes in absorption of Merocyanine-540-stained axons (Ross, Salzberg, Cohen & Davila, 1974). In the absorption mode action potentials were measured as a decrease in absorption at 520 nm and an increase at 570 nm. From the optical properties of Merocyanine-540 in various solvents and the wave-length dependence of the optical action potentials it has been suggested that membrane-bound dye molecules undergo a transition from a dimer to a monomer state as a function of potential (Ross *et al.* 1974; Tasaki, Warashina & Pant, 1976).

In the present experiments, the three dyes are used to measure optical action potentials in heart muscle. Under current or voltage clamp conditions, the optical signals were found to be a linear function of the membrane potential. The response of the probes to changes in membrane potential was independent of ionic or pharmacological interventions. Rapid spectral changes of Merocyanine-540 during the time course of an action potential showed that the amplitude of fluorescence and absorption band changes with depolarization without any wave-length displacement. The lack of potential-dependent wave-length shifts in the heart muscle suggests that 'electrochromism' is not the primary cause of the voltage sensitive optical response, rather, the data is more consistent with the hypothesis of potential-induced rotations of dye molecules in the electric field of the membrane.

A preliminary report of this work has been presented (Salama & Morad, 1977).

METHODS

Preparation

Hearts of bull frogs *Rana catesbiana* were sliced open, stretched and pinned as a single flap of tissue. Such a flap consisting of atrial and ventricular muscle was then sutured at its periphery to a stainless-steel ring of 2·5 cm in diameter. In single sucrose-gap experiments, thin strips ranging from 0·4 to 0·8 mm in diameter were dissected from the basal region of the frog ventricle (*Rana pipiens*).

In some experiments mammalian hearts from rats and guinea-pigs were used. The whole heart was perfused in a Langendorff-type set-up for 10–20 min before application of the dye. The right ventricular wall was then treated as that described for the frog. For spectral studies with amphibian or mammalian hearts the tissue thickness was kept below 1·5 mm to avoid high optical densities intrinsic to the tissue (optical density $< 0{\cdot}2$ at 580 nm).

Solutions

For mammalian hearts, a modified Tyrode solution equilibrated in 98 % O_2, 2 % CO_2, and at 24 °C was used: NaCl, 118; KCl, 4·7; $CaCl_2$, 2·5; $MgCl_2$, 1·2; KH_2PO_4, 1·2; and $NaHCO_3$, 25 in m-mole/l.; pH $\simeq$ 7·4.

An air equilibrated Ringer solution at room temperature was used for amphibian preparations: NaCl, 116; $NaHCO_3$, 2; KCl, 3; and $CaCl_2$, 1·0, in m-mole/l.; pH 7·6–7·8. In the single sucrose-gap chamber, the gap was perfused with 210 mM-sucrose contaminated with 0·01 mM-$MnSO_4$, and the KCl pool with 120 mM-KCl and 2 mM-$NaHCO_3$. All solutions were prepared with deionized and distilled water. The Ringer solution was allowed to flow intermittently to avoid flow artifacts on the optical traces. In some experiments tetrodotoxin (Sigma) and Adrenaline (Parke-Davis) were added to the normal Ringer solution.

Merocyanine-540 was obtained from Eastman Kodak (Rochester, New York), Merocyanine-oxazolone from Nippon Kankoh-Shikiso Kenkyusho (NK 2367 Okayama, Japan), and Merocyanine-rhodanine (WW 375) was the generous gift of Dr A. Waggoner.

Pluronic F-127 (BASF Wyandotte, Wyandotte, Michigan), a high molecular weight polyol, was occasionally used to increase the solubility of Merocyanine-540.

Staining procedure

Merocyanine-540 was dissolved in water (10^{-3} M) or ethanol (10^{-2} M). In the final staining solution the ethanol concentration did not exceed 0·2 %. The myocardial sheet preparations were then placed in Ringer containing 10^{-4}–10^{-5} M-Merocyanine-540 for 10–20 min. After this period of incubation, the preparation was washed free of dye. The stained tissues were purple, but the colour often appeared localized to the surface layers of the heart.

Bathing the muscle strip in solutions containing Merocyanine-540 produced weaker optical responses compared to the flap preparation. To improve the staining of cells at the centre of the tissue, the dye was added to the Ringer solution during the Langendorff perfusion. This procedure often produced homogenously stained tissue. Staining in the Langendorff-type arrangement was more efficient, requiring 100 times less dye in the perfusate than when the muscle was bathed in dye solutions. Merocyanine-rhodanine and Merocyanine-oxazolone are readily dissolved in Ringer and were also used in concentrations of 10^{-6}–10^{-5} M in Langendorff-type perfusions.

Experimental set-up

(*a*) *The 'cardiac flap' chamber.* The stainless-steel ring retaining the atrio-ventricular flap was held in place at the centre of a Lexan chamber by three glass hooks, which also served as inlets and outlets for the perfusate. Two circular Ag/AgCl electrodes were placed on opposite sides of the muscle for electrical stimulation. The chamber consists of two frames each with a 3·5 cm^2 window made of 0·5 mm thick quartz glass. When sealed, the chamber sandwiches the atrio-ventricular flap between the two windows. The chamber was fastened on an optical bench where the plane of the myocardial flap is vertically oriented permitting the simultaneous measurements of fluorescence and transmittance.

To measure the force generated by the ventricular sheet, a tension transducer was constructed on the retaining steel ring. An arc length of 1 mm was spliced out of the ring and a microstrain gauge (Micro Measurements: Ea 09 015 D.J.; 120 Romulus, Michigan) was directly bonded to the metal opposite the cut segment. The strain gauge varies in resistance by 1 $\mu\Omega$ per micro-inch of displacement. Its resistance forms one branch of a balanced Wheatstone bridge circuit producing a 50 mV deflexion per gram of tension.

(*b*) *Sucrose-gap chamber.* A schematic of the single sucrose-gap chamber adapted for optical measurements is shown in Fig. 1. The view is a top longitudinal section in the horizontal plane. The chamber is divided into three compartments with two 50 μm thick latex diaphragms. Each rubber partition is mounted on a removable fixture and has a 0·4 mm hole at its centre.

The assembly supporting the two diaphragms forms a variable sucrose gap and is inserted in the main body of the chamber between the physiological (*A*) and the KCl pool (*B*).

The ventricular strip is tied to a capacitance tension transducer (Schilling, 1960) in the physiological pool. With the gap set at 2·5 mm, the other end of the muscle was pulled by a thread through the diaphragm assembly. The gap length was then reduced from 2·5 to 1·5 mm to

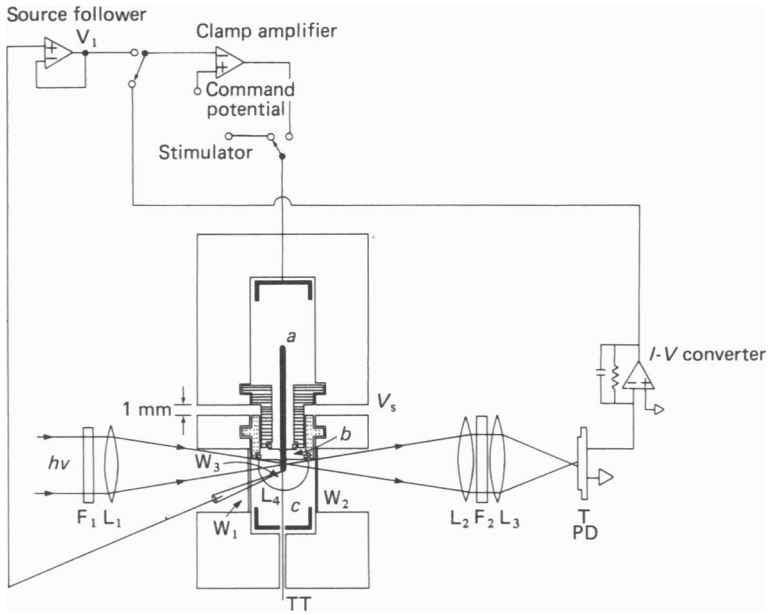

Fig. 1. Schematics of single sucrose-gap chamber and optical apparatus. The chamber consists of three pools perfused with (*a*) KCl, (*b*) sucrose, and (*c*) Ringer solution. The latex partition separating the KCl and sucrose solution is mounted on a Lexan fixture (horizontal hatch lines) which slides inside another fixture (dotted section) holding the sucrose-Ringer latex partition. The length of the sucrose gap can be varied from ½ to 3·5 mm by displacing section *b* from section *a* of the chamber. The incident beam passes through an interference filter F_1 and is focused by lens L_1 on the segment of muscle in the physiological pool (*c*). The light transmitted through the muscle passes through L_2-F_2-L_3 then focuses on a photodiode PD. The scattered light from the muscle is collected at 90° from the incident beam through a bottom window W_3. Intra-cellular potential was recorded with a micro-electrode from cells in the test node. Either the micro-electrode or the optical signal is fed back to the clamp amplifier controlling the current applied across two Ag/AgCl electrodes located in pools *a* and *c*.

slacken the segment of the muscle in the sucrose gap. The two glass windows (W_1 and W_2 on opposite sides) were constructed to permit uniform illumination of the test node (0·5–0·8 mm in length) by the incident beam. The window at the bottom of the chamber (W_3) is within 3 mm of the muscle to achieve efficient collection of the light scattered by the muscle.

The arrangement for the current and potential measuring electrodes and the voltage-clamping instrumentation were essentially similar to those described by Morad & Orkand (1971).

Spectral measurements. The cardiac flap preparations were used to measure spectral properties of a dye bound to the myocardium. Light from a water cooled tungsten-halogen lamp (Q6.6A/T2½/Cl General Electric, Cleveland, Ohio) was passed through a monochromator (Bausch and Lomb, Rochester, N.Y.) adjusted to yield a 10 nm band-width excitation beam. The lamp is current regulated using a DC power supply (Hewlett Packard 6286A). A portion of the emerging beam (4 %) is reflected and measured with a 'compensating' photomultiplier tube while the main portion of the beam is focused on the muscle. Light transmitted through the muscle is detected by a second photomultiplier tube. The signals from the transmitted and compensating

photomultiplier tubes (9524B EMI, Long Island, N.Y.) are subtracted to obtain absorption spectra corrected for relative spectral radiance of the lamp and spectral response of the detectors. The excitation beam is incident on the muscle at an angle of 30°; the forward fluorescence emission is collected at a 45° angle from the incident beam, passed through a monochromator and detected with a third photomultiplier tube (9529B EMI). The high voltage of all three photomultipliers is provided by a Hewlett Packard (6515-A) DC power supply.

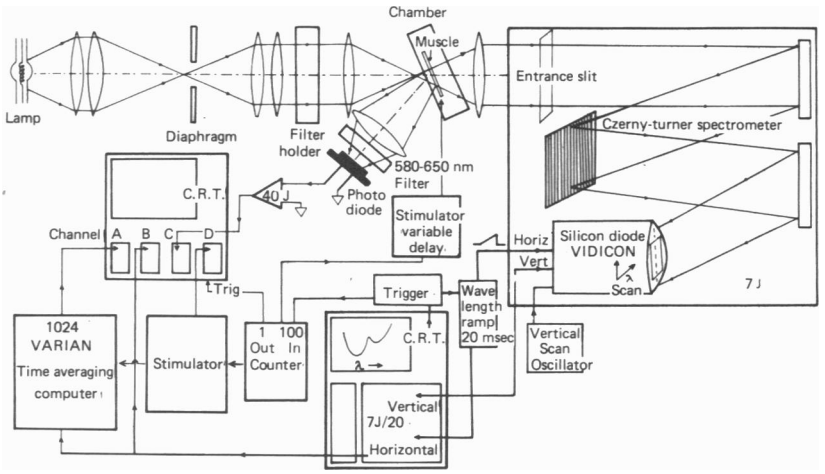

Fig. 2. Potential-dependent spectral measurements with the Rapid Scan Spectrophotometer (RSS). For fluorescence spectra, the incident light is passed through an excitation filter and focused on the muscle. The fluorescence light is detected by the photodiode; the transmitted light is dispersed by the monochromoter and imaged on the Vidicon target. The output of the RSS consists of the transmittance spectrum of the filter plus the fluorescence spectrum of the stained tissue. For transmittance spectra, a heat filter replaces the excitation filter such that white light is focused on the muscle. Spectra are displayed on the CRT of the 7J/20 as light intensity *vs.* λ every 10 msec or can be triggered to display and store a single spectrum recorded at a selected phase of the action potential. To sum spectra measured during the plateau minus spectra taken at resting membrane potential, the output of the RSS is fed to a time averaging computer (TAC) and to a digital counter. The digital counter adds 100 spectra (i.e. waits 1 sec or 100 scans) then stimulates the heart through Ag/AgCl electrodes to elicit an action potential and triggers the TAC to store a single spectrum from the 7J/20. By varying the delay on the electrical stimulus to the heart, the TAC will store a spectrum measured at any chosen phase of the action potential.

For corrected absorption spectra, a control spectrum is taken with the chamber filled with Ringer solution but without a preparation. This signal is then nulled by adjusting the output of the compensating photomultiplier. Thus when the excitation monochromator is scanned for wave-length, the difference of the two photomultipliers yields a base line with less than 5% deviation from zero. After a control scan, the muscle is placed in the chamber to obtain a corrected absorption spectrum of the tissue. The tissue is then stained and the absorption spectrum of the tissue-bound dye is measured.

Measurements of optical signal. To measure small absorption and fluorescence changes the monochromators are substituted by interference filters selected from the spectral data.

For Merocyanine-540 experiments, the absorption and fluorescence signals are optimized with an excitation filter transmitting at 540 ± 10 nm (Baird Atomic Inc., Bedford, Mass.), and a fluorescence interference filter selecting 580–650 nm light. For preparations stained with Merocyanine-rhodanine and Merocyanine-oxazolone the incident beam was passed through interference filters 750 ± 30 nm and 720 ± 20 nm, respectively (Feuer Inc., Montclair, N.J.). The front-scattered light from the muscle was collected and focused on a photodiode (PV 215 or PV 400, EG & G, Salem, Mass.) without a second filter in the light path.

Rapid scan spectroscopy. Potential-dependent spectral changes of Merocyanine-540 bound to frog heart were measured during the course of the action potential with a rapid scan spectrophotometer (7J Tektronix, Beaverton, Oregon), illustrated schematically in Fig. 2. The instrument consists of three components, a spectrophotometer (J 20 Tektronix), an oscilloscope (7313 Tektronix), and an oscilloscope plug-in (7520 Tektronix). The spectrophotometer is a Czerny–Turner monochromator which disperses the light and focuses a selected region of the spectrum on a silicon-diode vidicon detector. The vidicon detector is a linear array of silicon photodiodes which are spaced 15 μm apart. The diodes are reverse-biased such that they are non-conductive and act as capacitors storing a charge. In the dark, they are fully charged, but when a photon strikes, the diodes become partially discharged. On the front surface of the array, each diode receives a 1 nm band-width portion of the dispersed light. Thus the vidicon target integrates and records the spectrum as a pattern of stored electrical charges. The optical information is recovered by repetitively scanning the back of the array with a focused electron beam and monitoring the current necessary to fully recharge each diode. The 'scan time' of the vidicon can be varied from 4 to 20 msec with 6 μsec between scans.

Absorption spectra were measured by passing the incident beam through a heat filter and focusing white light on the muscle. With 20 msec scan times, spectra of tissue-bound Merocyanine-540 were directly displayed on the oscilloscope as intensity of transmitted light versus wave-length.

Fluorescence spectra were obtained by passing the incident beam through an interference filter, transmitting at 540 ± 10 nm. In this case, the diodes measuring the excitation wave-length were saturated, but the fluorescence spectrum was not distorted. Spectra could be continuously displayed or a single spectrum during a chosen phase of the cardiac action potential could be recorded on the oscilloscope. To record a spectrum at the same phase of the cardiac action potential, the electrical stimulation of the heart was synchronized with the internal clock controlling the scanning rate. A 20 msec spectral scan was obtained just before the initiation of the action potential and subtracted from a scan taken at a fixed time during the plateau.

To improve the signal-to-noise ratio of potential-dependent spectral changes, such difference spectra were summed using a time averaging computer (Model C-1024 Varian, Pittsburgh, Pa). By selecting an appropriate scan time during the action potential, a spectrum from any region of the action potential could be recorded.

Tests and precautions. The most serious problem with the measurement of optical signals from contracting tissue is the large signal changes caused by muscle contraction. In frog heart, the onset of contraction occurs approximately 50–100 msec after the upstroke of the action potential. The subsequent motion of the muscle causes large changes in scattered light intensity which are observed in fluorescence, reflectance, and transmittance measurements. In unstained muscles perfused in normal Ringer solution, the mechanical motion causes 0·1–1·0 % changes in scattered light intensity. Once stained with Merocyanine-540, the amplitude of motion-induced fluorescence change increases by a factor of 5. Likewise, contractions produce somewhat larger signal changes of 0·5–5 % in the reflectance and 1–20 % in the transmittance measurements. Staining the muscles often altered the time course as well as the amplitude of the reflectance and transmittance signals that are induced by muscle contractions.

In an attempt to minimize the motion-induced signal, the angle of light collection was varied with respect to the incident beam. The amplitude and time course of the scattered light were found to vary with the angle but the changes were not systematic. The motion-induced signal is found to vary with size, angle and wave-length of the input beam and its location on the muscle. Stretching muscle strips and lowering the $[Ca]_o$ to 0·2 mM decreases the amplitude of the scattered light signals due to muscle motion by a factor of 3–5. In stained muscle strips that are over-stretched and perfused with 0·1 mM-Ca^{2+}, these signals continue to distort the wave form of the optical action potential. The only reliable procedure to suppress the light changes due to muscle contractions from the optical trace was to perfuse the preparation in Ringer solutions containing 10–50 μM-Ca^{2+}. In the frog heart, the duration of the action potential is considerably prolonged in low calcium solution (Harris & Morad, 1971). While perfusion for 15–30 min with low Ca^{2+} solutions often abolishes the motion-induced signal from the fluorescence and reflectance trace, such a signal is often detected in the transmittance mode.

In multicellular preparations, individual cells do not contribute equally to the optical signal

since the optical path is different for cells located at varying distances from either the input beam or the collecting lens.

The heterogeneity of detection of the optical signal depends mostly on the degree of quenching of the incident beam and the homogeneity of dye distribution throughout the extracellular space.

To estimate the extent of penetration of the incident beam, the absorbance of ventricular flaps stained with Merocyanine-rhodanine was measured at 750 nm. For an 0·6 mm thick tissue, the absorbance varied from 0·3 to 0·5 in four experiments such that the intensity of the transmitted light is 30–50 % of the incident light intensity. Assuming that the absorption of the tissue is homogeneous, cells at the illuminated surface are exposed to light intensities 2–3 times greater than cells 0·6 mm deeper. Hearts stained in a Langendorff-type set-up appear uniformly stained. Estimates of dye distribution were made by comparing levels of dye fluorescence in various regions of the heart. In five experiments, the amplitude of the fluorescence level varied by about 10 % in each ventricle, using a 0·5 mm diameter scanning beam. Since the micro-electrode samples surface potentials while the dye signal originates from surface and interior cells, the lag between the two signals may be due to the lag in development of action potential at various depths of the muscle. Thus a comparison between the optical and micro-electrode responses must consider the geometry, conduction velocity, homogeneity of staining, and illumination of the preparation.

RESULTS

Membrane potential measurements with Merocyanine dyes

Merocyanine-540. Frog hearts stained with Merocyanine-540 were washed of excess dye in a low Ca solution. The fluorescence emission from the dye bound to the tissue was monitored simultaneously with the membrane potential recorded with an intracellular micro-electrode placed in the illuminated segment of the tissue (Fig. 3). During the upstroke of the action potential, the fluorescence increased by 1·8 %, and the time course of the optical signal was found to be similar to the micro-electrode trace. In the atrio-ventricular flap preparation, action potentials are triggered by the *sinus venoses*, but additional action potentials may be generated through direct electrical stimulation (Fig. 3, second action potential of the lower left panel). In a stable preparation the base line drift during a single action potential was usually less than 0·03 % of the amplitude of the optical signal. The electrical activity from various regions of the heart could be monitored by displacing the preparation with respect to the exciting light. Although the fluorescence action potentials had a slower upstroke and rate of repolarization than those recorded with the micro-electrode, the characteristic shape and duration of pacemaker, atrial, and ventricular cells could be clearly distinguished (Salama & Morad, 1976). Illuminating the muscle with a 1 mm beam, the fluorescence action potential typically had a signal to noise ratio of 40/1 (3 kHz band width). In order to compare the time course of the action potential obtained from the optical and micro-electrode techniques more precisely, smaller preparations were used to reduce the time delays due to propagation of the action potential. Fig. 4 (top panels) compares the Merocyanine-540 fluorescence signal with the intracellular recordings. A good correlation is observed between the time course of the two action potentials. In the top right panel, the muscle was stimulated by a hyperpolarizing current pulse. The upstroke of the optical action potential was delayed and its rise time was slower by 2 msec. The delay between the two traces was found to depend on the location of the micro-electrode impalement. With the electrode placed near the sucrose-Ringer partition,

the micro-electrode preceded the optical signal and for impalement at the far end of the test node, the optical signal occurred prior to the electrode response. The slight differences in the upstroke velocity may be due to the averaging nature of the fluorescence signal in the multicellular tissue.

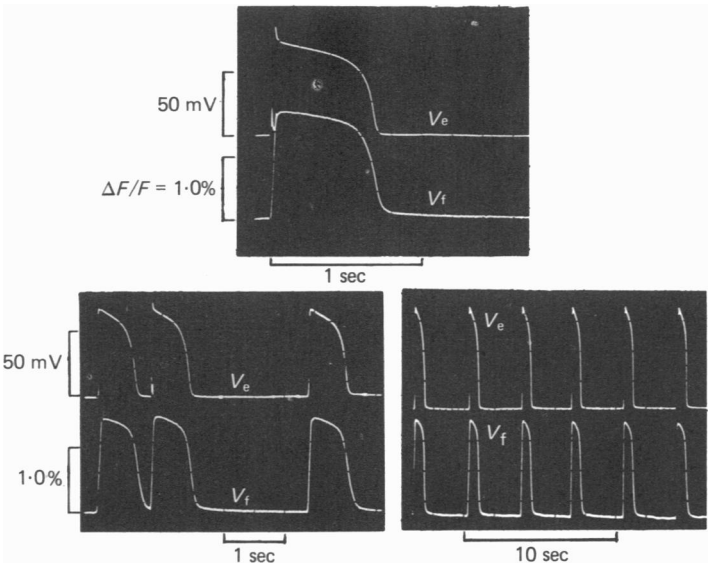

Fig. 3. Comparison of fluorometric response with intracellularly recorded action potentials. A frog ventricular flap is stained with Merocyanine-540 and equilibrated in 50 μM-Ca^{2+} Ringer to suppress contractions. Spontaneous action potentials are recorded with a micro-electrode (trace, V_e) impaled in one of the cells illuminated by a 2 mm diameter light beam from which the fluorescence is recorded (V_f). During the action potential (top panel), the fluorescence increases by 1·8 % and follows the time course of the micro-electrode measurement (V_e). In the lower left panel, three action potentials are recorded; the second one is elicited by electrical stimulation of the muscle. In the lower right panel, six spontaneous action potentials are shown to demonstrate the stability of the fluorescence base line.

The rapid jumps on the electrode trace during the on and off of the current transients were not present in the optical trace. The rapid jump in the intracellular recordings observed during the passage of current is caused by a voltage drop across the extracellular resistance in series with the membrane resistance (Beeler & Reuter, 1970; Tarr & Trank, 1971; Goldman & Morad, 1977). That the optical signal does not detect this extracellular voltage component suggests that the dye molecules respond only to the transmembrane potential rather than to the potential changes in the extracellular clefts.

Merocyanine-rhodanine (WW375) and Merocyanine-oxazolone (NK2367). Two weakly fluorescent dyes, Merocyanine-rhodanine and Merocyanine-oxazolone, also serve as optical probes of membrane potential through changes in absorption. In Ringer solution Merocyanine-rhodanine had absorption maxima at 700 and 605 nm and photobleached in 3–4 hr while Merocyanine-oxazolone absorbed at 560 nm and had a shoulder at 695 nm and photobleached rapidly. Absorption of these dyes in the frog ventricle was shifted towards longer wave-lengths, 720 and 670 for Mero-

cyanine-rhodanine and 700 and 640 for Merocyanine-oxazolone. In Fig. 5*A*, the reflected and transmitted light were monitored at 750 ± 30 nm from an unstained muscle equilibrated in 1 mM-Ca^{2+} Ringer. The optical signals had a time course resembling that of muscle contraction. Staining with Merocyanine-rhodanine produced a decrease in base line transmittance and reflectance. In Fig. 5*B* the reflect-

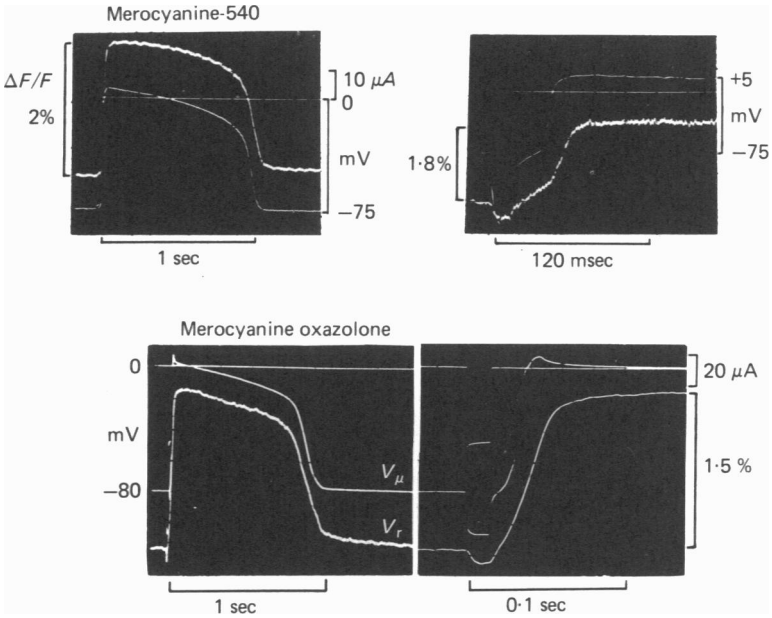

Fig. 4. Optical action potentials from Merocyanine-540 and Merocyanine-oxazolone in single sucrose-gap apparatus. Frog ventricular muscle strip (0·5 mm in diameter, 0·8 mm long) stained with the dye is placed in the sucrose-gap chamber. The optical signals are simultaneously monitored with the applied current and the intracellular potential recorded with a micro-electrode. In the top left panel, the fluorescence of Merocyanine-540 increases by 2 % and has the time course of the action potential; ΔF = 30 nW. In the top right panel, the rise times of the two traces are compared. In the lower panels, the reflectance (V_r) of Merocyanine-oxazolone is simultaneously recorded with intracellular potential and with a micro-electrode (V_μ). During the action potential, the reflectance increases with a $\Delta R/R$ = 1·5 %, ΔR = 240 nW. The micro-electrode trace has a rapid jump during the hyperpolarizing current transient followed by a slower charging of the membrane's capacitance. The rapid jump is not detected by either optical signal.

ance shows an increase in signal synchronous with upstroke of transgap action potential. In the transmittance signal the motion-induced signal change is two orders of magnitude larger and opposite in direction than the reflectance signal. Consequently, potential-dependent responses are masked by the motion-induced changes in the optical signal. For absorption dyes, the reflected light seems to be a better mode of measuring potential-dependent optical signals since it was less sensitive to motion artifacts. In Fig. 5*C* and 5*D* the muscle is perfused with 50 μM-Ca^{2+} Ringer and the reflected light signal was compared with an intracellular microelectrode recording. The increase in reflected light due to the action potential (ΔR) over the change in reflectance due to staining (R = reflectance before staining −

reflectance after staining) was equivalent to a 0·8 % increase in transmittance ($\Delta T/T$) of tissue-bound dye. Note that the optical trace is not entirely free of mechanical artifact in this preparation.

The signal-to-noise ratio of the optical action potential derived from Merocyanine-rhodanine was 2–5 times higher than with Merocyanine-540. Although the optical

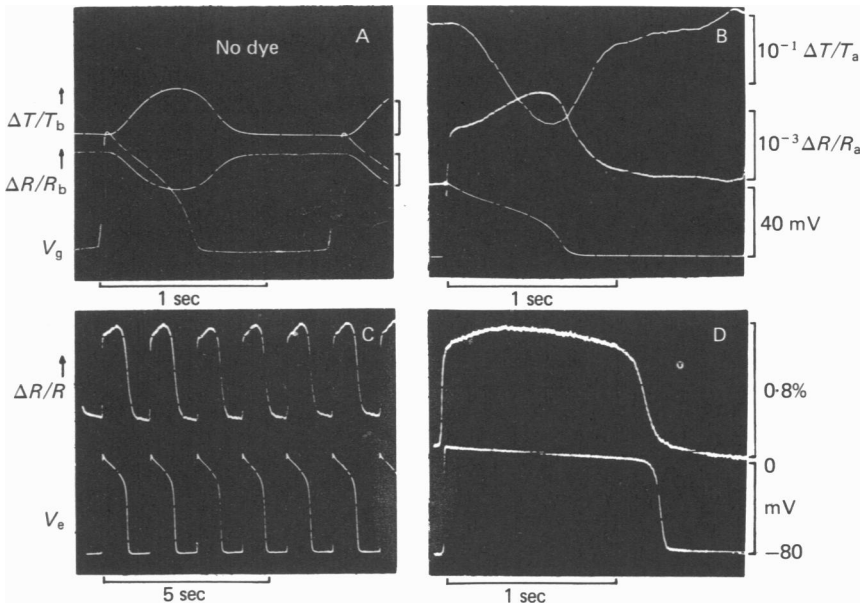

Fig. 5. Optical action potentials from a frog ventricular strip stained with Merocyanine-rhodanine. In *A* and *B*, transmittance (top trace), reflectance (middle trace), and trans-gap action potential (lower trace) are simultaneously recorded. With no dye, both optical signals show a response characteristic of a contraction (*A*). T_b and R_b are the transmittance and reflectance *before* staining. In *B*, the test node is stained and washed of excess dye. The reflectance exhibits a rapid rise synchronous with the upstroke of the trans-gap action potential followed by a slow phasic increase synchronous with the transmittance signal which is mostly caused by muscle contractions. T_a and R_a are the transmittance and reflectance after staining. In *C* and *D*, the muscle is equilibrated in 50 μM-Ca^{2+} Ringer; the reflectance and micro-electrode signals are simultaneously recorded. The plateau of the optical action potential detects some of the mechanical motion of the muscle which is not entirely subdued. $R = R_b - R_a$, ΔR = the change in reflectance during the upstroke of the action potential, $\lambda_{ex} = 750 \pm 30$ nm. The frequency response of the optical system is 3 kHz.

action potentials were largest with incident light of 750 nm, they could be also measured at other wave-lengths. For each interference filter the change in transmittance during the upstroke of the action potential (ΔT) was divided by changes in base line transmittance (T) due to the staining. In hearts stained with Merocyanine-rhodanine the transmittance increased from 780 to 730 nm, decreased from 730 to 660 nm and again increased from 660 to 480 nm (Fig. 6, top graph).

Reflected light measurements from muscles stained with Merocyanine-oxazolone were found to yield the highest signal to noise ratio (about 200/1) from a single action potential (Fig. 4, lower panels). The signal change was due to a decrease in

dye absorption at 720 ± 30 nm. As the experiment proceeded, the signal-to-noise ratio of the optical action potential decreased, the deep blue colour of the stained muscle changed to pink, but the electrical activity monitored with a micro-electrode remained normal. The muscle can be restained to recover the initial amplitude of the optical signal. The amplitude of the Merocyanine-oxazolone optical action potential decreased by 50% in about 1 hr of continuous light exposure. Investigation of the action spectrum of Merocyanine-oxazolone did not reveal a triphasic

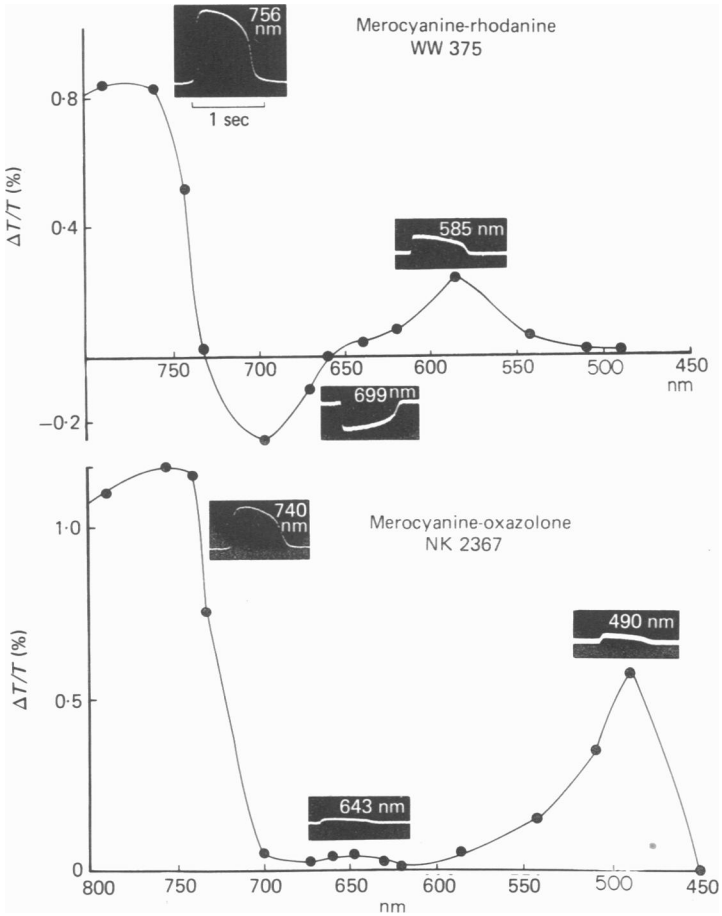

Fig. 6. Action spectra of Merocyanine-rhodanine and Merocyanine-oxazolone. The transmittance T_b of an unstained frog ventricular flap is measured at various wave-lengths with a series of 10 nm half band-width interference filters. The muscle is stained, washed of excess dye, and the transmittance (T_a) is remeasured. The action spectrum of the dye is a plot of $\Delta T/T$ *vs.* wave-length where ΔT = change in transmittance during the upstroke of the action potential and $T = T_b - T_a$. Upper graph: the action spectrum of Merocyanine-rhodanine is triphasic. The insets show the typical time courses of the transmittance action potentials measured at various wave-lengths. Lower graph: the action spectrum of Merocyanine-oxazolone has two bands which are in the same direction. Note that in the insets, the optical action potential measured at 470 nm has a considerably lower signal-to-noise ratio than at 740 nm although $\Delta T/T$ is only half as large. The lower signal-to-noise ratio at 470 than 740 nm is due to the lower efficiency of the optical apparatus at shorter wave-lengths.

wave-length dependence. Instead, the transmittance increased in the range of 780–690 nm and again increased from 600 to 450 nm (Fig. 6, lower graph).

Linearity of the optical signals with membrane potential. The similarity of the optical and micro-electrode traces suggest that the optical signals monitor the membrane potential and not current or conductance changes. To verify that the potential dependence is not coincidental, the optical signal was measured under conditions where the membrane potential was controlled.

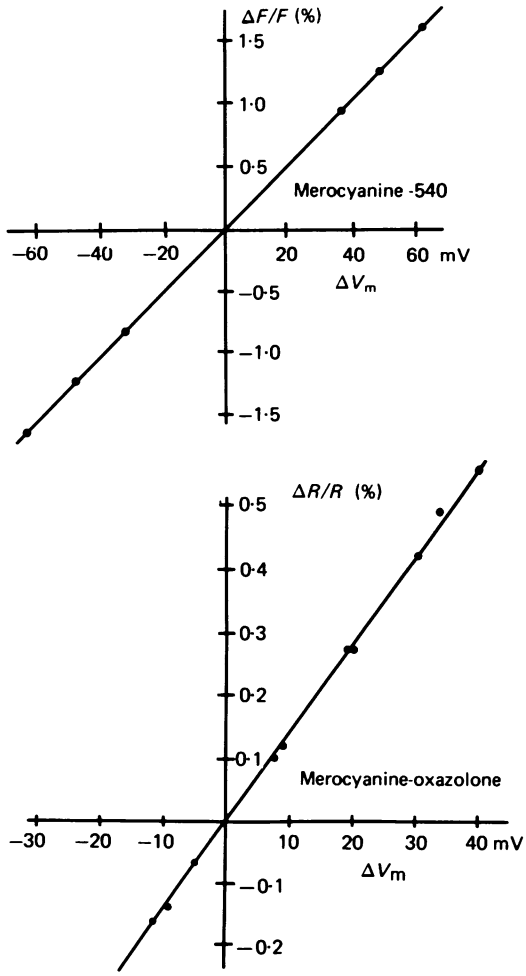

Fig. 7. Linearity of optical responses with membrane potential changes. Hyperpolarizing and depolarizing current pulses were applied to muscle strips equilibrated in 10^{-6} M-TTX and 50 μM-Ca^{2+} Ringer. The micro-electrode and optical signals were compared at the end of the constant current pulse. In top graph, the test node is stained with Merocyanine-540. A plot of the fluorescence change (ordinate) versus intracellular potential changes (abscissa) recorded with the micro-electrode yield a linear relation. Lower graph is from the same muscle strip shown in Fig. 4. A linear relation is obtained when the reflectance change (ordinate) at the end of the current pulse is plotted as a function of the intracellular potential change (abscissa). The micro-electrode signals are corrected for the rapid voltage drop across the extracellular series resistance during the onset of the applied current.

In the presence of 10^{-6} M-tetrodotoxin and in 50 μM-Ca^{2+} Ringer the optical and the micro-electrode signals were simultaneously measured in response to applied current in a single sucrose-gap chamber. By passing depolarizing and hyperpolarizing current pulses of various amplitudes, the optical responses were found to be linearly related to membrane potential (Fig. 7).

In five experiments with frog ventricular muscles stained with Merocyanine-540, an increase of fluorescence over background fluorescence $\Delta F/F$ of 0·5 % was equivalent to an 18 mV membrane depolarization. When the change in the fluorescence signal was compared to the membrane potential and current, linear and N-shaped relations were obtained, respectively.

In similar experiments, reflected light responses of Merocyanine-rhodanine and Merocyanine-oxazolone (Fig. 7, lower graph) were also found to be linearly related to membrane potential changes. During the applied current pulses, the time course of the micro-electrode and optical signals are not always identical. Even after correcting the intracellular recordings for the extracellular series resistance (as described by Goldman & Morad, 1977), there still appears to be some discrepancy between the two traces.

The data points in the experiments of the type described in Fig. 7 were obtained with constant current pulses lasting 20–40 msec in duration. Although such a duration was sufficient to hyperpolarize the membrane to a constant level, depolarizing pulses often failed to produce a constant membrane potential signal. This was particularly the case when the optical signal was compared to that measured with the micro-electrode. It was felt that the depolarizing pulses produce a regenerative response in the central fibres despite the presence of TTX sufficient to elicit 'aberrant' and graded action potentials after the termination of the pulse. Comparison of the optical and micro-electrode signals in a multicellular tissue therefore, must consider the size and the geometry of the tissue, the extracellular resistance, the homogeneity of dye staining, and the adequacy of the light collecting system.

Under voltage-clamping conditions, the optical signal follows the changes in membrane potential and not the membrane current. Fig. 8 (*A* and *B*) shows simultaneous traces of Merocyanine-rhodanine reflectance, membrane current, and transmembrane potential. Note that when the action potential is interrupted during the plateau by a clamp step, the reflectance signal (middle traces) is quite similar to the membrane potential signal (top traces) rather than the membrane current (lower traces). These experiments also suggest that the optical signals follow the change in membrane potential linearly. In some experiments the optical signal was fed back through the clamp amplifier to control the membrane potential. In Fig. 8*C* three sweeps are superimposed. The first shows the upstroke and part of the plateau of a normal optical action potential. The second and the third traces are interrupted by depolarizing and repolarizing clamp steps at low loop gain. In panel *D* the gain of feed-back loop is increased and the clamp step reaches a steady state in about 2 msec. The current, although noisy and faster in reaching steady level, is essentially similar to current records obtained using the micro-electrode in the feed-back loop, Fig. 8*A* and *B* (Morad & Orkand, 1971).

Photodynamic effects of Merocyanine dyes. In ventricular flaps stained with Merocyanine-540, photodynamic damage is small and remains undetected for several

hours of continuous illumination (Fig. 9*A* and *B*). Perfusing the whole heart in Ringer equilibrated with 95 % O_2 and 5 % CO_2, or increasing the diameter of the incident beam from 1 to 20 mm did not increase the susceptibility of the muscle to the phototoxic effects of the dye.

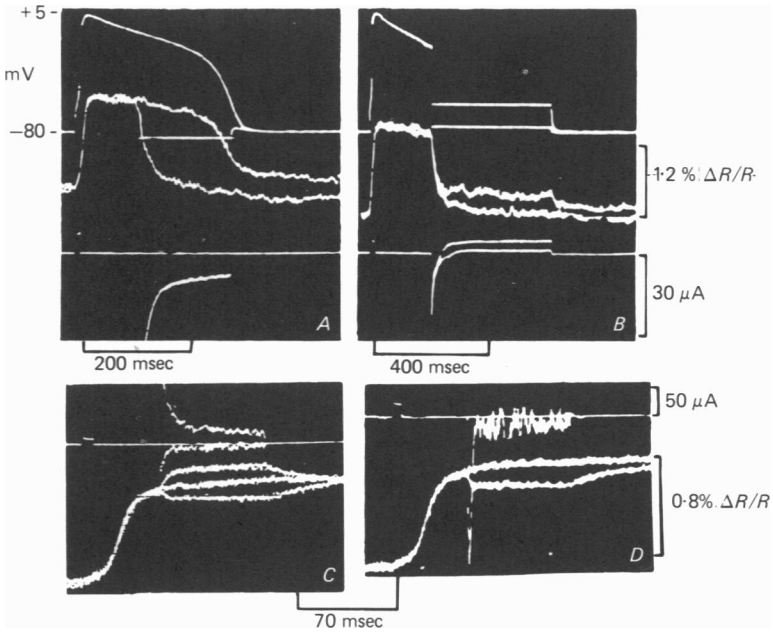

Fig. 8. Micro-electrode and 'optical' voltage clamps. Frog ventricular strips stained with Merocyanine-rhodanine are placed in the sucrose-gap chamber and test nodes (0·5 mm in diameter by 0·6 mm in length) are bathed in 50 μM-Ca^{2+} Ringer. In the top panels, the micro-electrode signal is fed back to the clamp amplifier controlling the applied current. In *A*, two sweeps are superimposed to show the normal course of the action potential and an action potential interrupted by a voltage clamp to the resting membrane potential. The reflectance at 750 nm (middle traces) follows the micro-electrode (top traces) but not the applied current (lower trace). In *B*, two action potentials are interrupted by voltage clamp steps to −60 and −80 mV for 400 msec. The reflectance responds to the clamp step by maintaining levels of light intensity which are proportional to the membrane potential. In the lower panels, the optical signal instead of the micro-electrode controls the applied current. In *C*, depolarizing (top reflectance trace) and repolarizing (lower reflectance trace) optical clamp steps are associated with outward (top current trace) and inward (lower current trace) currents, respectively. With increased gain on the feed-back loop, the current trace reaches a steady-state level in 2 msec (panel *D*).

In muscle strips the fluorescence action potentials consistently had a smaller signal to noise ratio (5/1 or less). By slicing the strip and comparing the colour of the tissue at the centre and at the edges, it was apparent that mostly surface cells were stained.

In muscle strips stained with the Langendorff procedure (to improve staining at the centre of the strip), the phototoxic effect was found to occur more rapidly. Fig. 9*C* shows the time course of fluorescence and the micro-electrode action potential

recorded from a ventricular strip bathed in 1 mM-Ca^{2+} Ringer. With continuous light exposure, the cells depolarized and the amplitude and duration of the action potential decreased rapidly (Fig. 9*D* and *E*). To estimate the rate of phototoxic effect, the amplitude of the optical signal was used as a measure of the viability of the tissue cells. In ten experiments the amplitude of the optical action potential measured from Merocyanine-540 stained muscle decreased by 50 % in 10 min.

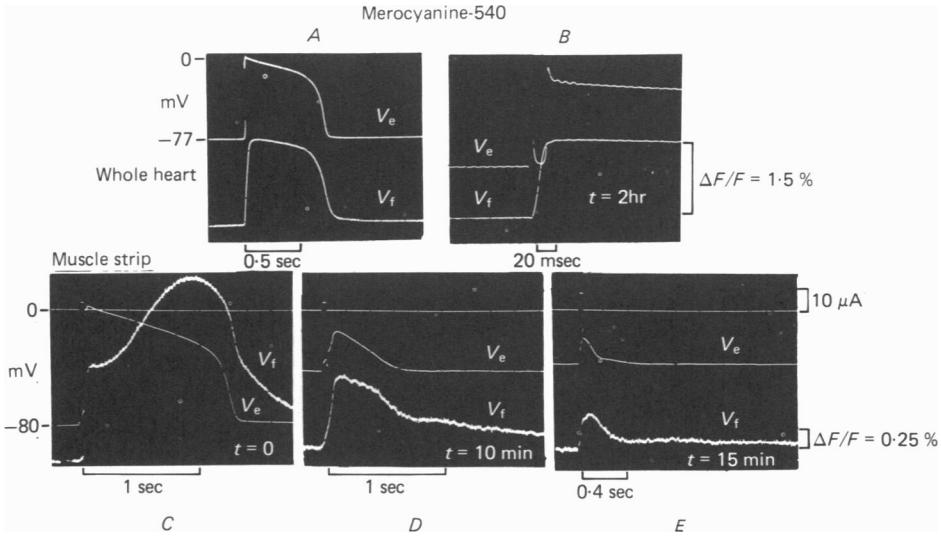

Fig. 9. Phototoxic effects of Merocyanine-540 in whole heart and in muscle strips. A frog ventricular flap is bathed in staining solution then equilibrated in 50 μM-Ca^{2+} Ringer. Action potentials recorded with the fluorescence (V_f) or the micro-electrode (V_e) appear normal following 2 hr of illumination (panel *A*) and their rise time is under 20 msec (panel *B*). In the lower panels, a heart stained in a Langendorff perfusion is dissected into a muscle strip and equilibrated in 1 mM-Ca^{2+} Ringer in the sucrose-gap chamber. The micro-electrode (V_e) and fluorescence (V_f) action potentials are initially normal with rapid upstroke indicative of healthy preparation (panel *C*). The slow phasic responses in the optical traces are due to muscle contractions. After 10 min of illumination, the membrane depolarizes from −80 to −40 mV and the rise time of both action potentials doubles (panel *D*). The amplitude and duration of the action potentials deteriorate further 5 min later (panel *E*). All solutions were equilibrated with air.

The rate of phototoxic effect is not affected by the $[Ca]_o$ but depends on the oxygen concentration in the bathing medium (Fig. 10*A* and *B*). A plot of the fluorescence upstroke as a function of time for preparations bathed in solutions equilibrated with 95 % N_2 plus 5 % CO_2 (1 % O_2) shows that fluorescence decreases by 50 % in 40 min. The phototoxic effect in the heart was often found to be reversible. In Fig. 10*C* a stained muscle was illuminated continuously for 15 min until the optical signal was markedly reduced. The muscle was then kept in the dark for an equal time interval and the optical signal recovered to its initial amplitude.

In frog heart stained with Merocyanine-oxazolone, the optical action potential had a rise time comparable to the micro-electrode. After continuous illumination for 2–4 hr, the amplitude of the optical action potential decreased but its rate of

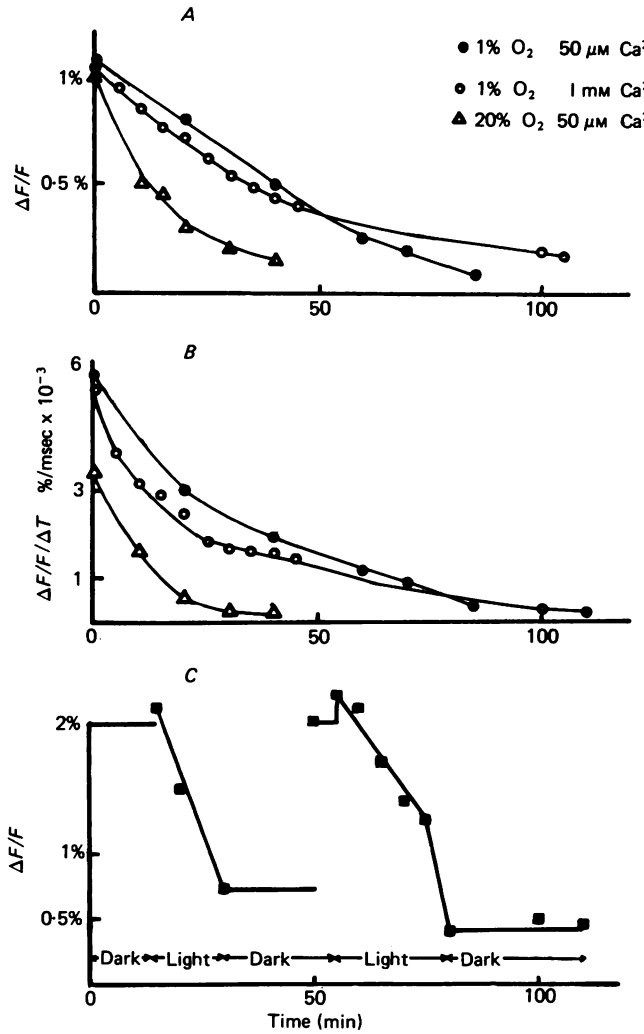

Fig. 10. Effect of Ca, O_2, and light on the rate of photodynamic damage. A muscle stained with Merocyanine-540 is placed in the sucrose-gap chamber and kept in the dark. In graph (*A*), the amplitude of the fluorescence action potential is plotted *vs.* time of illumination. In air equilibrated solutions containing 50 μM-Ca^{2+}, the amplitude of the optical action potential is reduced by 50 % in 10 min. (△), in nitrogenated solutions containing either 50 μM (○) or 1 mM (●) Ca^{2+}, it is reduced by 50 % in 45 min. In graph *B*, the rate of rise of the fluorescence action potential is plotted for the same experiments; it is reduced by 50 % in 10 min for air equilibrated solutions (△) and in 45 min in nitrogenated solutions at high (●) or low (○) Ca concentrations. In graph *C*, the deterioration of the optical action potential is partly reversed by keeping the muscle in the dark. When the muscle strip is kept in the dark for 20 min the amplitude of the optical action potential is somewhat recovered ($\Delta F/F$ = 2·0 %). After another exposure to the light beam, the action potential deteriorates and does not recover after a 30 min dark period.

rise did not change significantly. Continuous illumination in the presence of Merocyanine-oxazolone had no measureable effect on the micro-electrode signal. Merocyanine-oxazolone visibly photobleached, and restaining restored the amplitude of the optical action potential.

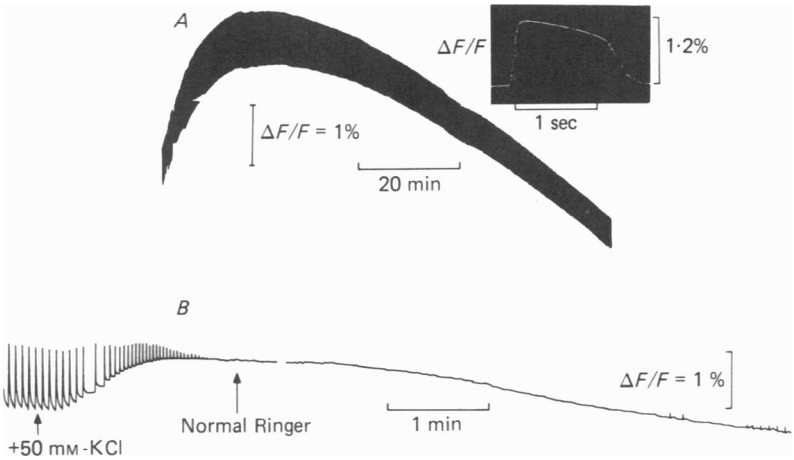

Fig. 11. Base line instability during KCl-induced depolarization. A frog ventricular flap is stimulated at 12 beats/min and bathed in 1 mM-Ca^{2+} Ringer. The muscle is then stained with Merocyanine-540 and washed in 50 μM-Ca^{2+} to suppress contractions. The fluorescence base line (trace *A*) increases during the first 20–30 min of light exposure then decreases at about 0·06 % per min, or about 0·001 % during an action potential (see inset). Upon addition of isotonic 50 mM-KCl Ringer, the fluorescence intensity increases due to KCl-induced depolarization, recovers to its initial level in normal Ringer, then drifts downwards (trace *C*).

Ionic and pharmacological interventions

KCl-induced depolarization. In order to compare fluorescence changes of Merocyanine-540 in response to depolarization caused by high $[K]_o$, the base line drift was required to be smaller than the potential dependent response. The scattered light (580–650 nm) from an unstained ventricular flap decreased at a rate of 6 % per hour. Once the heart was stained, the fluorescence base line increased for 20 min, then gradually decreased (Fig. 11*A*). Even for long action potentials of 1 sec or longer (see inset), the base line drift during a single action potential was under 0·003 %. Compared to a 1 % fluorescence change during the upstroke, the drift is about 3×10^{-3} times smaller. On the other hand, during a 2–3 min KCl depolarization, the base line drift was about 0·4 % and became significant with respect to the potential-dependent response.

In Fig. 11*B*, optical action potentials from a preparation equilibrated in 50 μM-Ca Ringer were continuously recorded prior to a KCl-induced contracture. Exposure of the muscle to 50 mM-KCl solution caused an increase in base line fluorescence and suppressed the action potential. The magnitude of the fluorescence increase was consistent with the estimated membrane depolarization. The base line fluorescence recovered following the reintroduction of normal Ringer. However, the fluorescence base line did not stabilize sufficiently to permit a quantitative analysis of the change in fluorescence in response to variations of $[K]_o$.

Effect of divalent cations and drugs. To examine the possible interactions of the dye molecules in the environment of the membrane with ions in the Ringer solution, the potential-dependent optical response was monitored under various ionic substitutions. Although divalent cations were found to alter the absorption spectrum of Merocyanine-540 in water solution, they did not induce spectral changes in tissue-bound dye. The optical response did not differ from the micro-electrode measurement when Mg^{2+} (20 mM), Mn^{2+} (10 mM), or Ni^{2+} (1 mM) was added to the Ringer solution. Change in the pH of the bathing solution from 6·0 to 8·0 had little or no effect on the optical response. Thus ionic perturbations do not seem to disturb the voltage-dependent dye response.

Pharmacological agents such as epinephrine and tetrodotoxin did not interact with the membrane-bound dye. Addition of 10^{-6} M-tetrodotoxin in a stained preparation produced, as expected, a decrease in the fast inward current and suppressed the upstroke of the action potential. The addition of 10^{-6} M-adrenaline produced an increase in the amplitude and duration of the action potential in the stained preparation. In the atrio-ventricular flap preparations the chromophore did not interfere with the arrhythmogenic properties of adrenaline.

Spectral properties of Merocyanine-540

Solvent properties. Merocyanine-540 is a *solvatochromic* dye in that it exhibits a shift in its maximum absorption when transferred to solvents of lower polarity (Tasaki *et al.* 1976). Dye dissolved in water in 10^{-7} M concentrations has an absorption maximum at 540 nm and a secondary peak at 490 nm. Increasing the concentration to 10^{-4} M shifts the maximum to 490 nm.

The solubility of Merocyanine-540 decreased with increasing salt concentration. 2 mM of the chromophore could be dissolved in water, 10^{-4} M in Ringer solution, and approximately 10^{-5} M in artificial sea water. In 1 mM-Ca^{2+} Ringer or in sea water, the absorption was somewhat altered with a major peak at 510 and a secondary peak at 580 nm. Titrations with inorganic salts showed that these spectral changes were caused by the addition of divalent cations Ca^{2+} and Mg^{2+}. Monovalent cations, Na^+ and K^+, did not alter the absorption spectrum.

In the presence of Pluronic the solubility of the dye in Ringer was increased to 10 mM. The absorption spectrum of Merocyanine-540 in Ringer solution was not affected by the presence of up to 2 % Pluronic.

Spectral characteristics of Merocyanine-540 bound to heart tissue. The atrium of the bull frog was used as the preparation of choice to measure the absorption spectrum of the dye in heart tissue. This preparation was selected for its large surface area (5–10 cm^2), its thinness (0·2–0·5 mm), and its transparency (O.D. = 0·1–0·4 at 540 nm). In Fig. 12, trace *A*, the absorption of the non-stained atrium is measured in the range of 500–580 nm. The muscle is then stained with Merocyanine-540 and the absorption spectrum once again measured. The tissue-bound dye has a major absorption band at 540 nm and a secondary peak at 570 nm (trace *B*). After re-staining the heart with dye solution dissolved in 0·1 % Pluronic, the absorption at 570 nm increases relative to the 540 nm (trace *C*). Prolongation of the staining period with Pluronic enhances the 570 nm peak which becomes the dominant absorption band (trace *D*).

The absorption spectrum of Merocyanine-540 in the ventricle of the frog, rat or guinea-pig is similar to that measured in the frog atrium. This measurement is more difficult in mammalian heart because the intrinsic absorption of the tissue is high in the region of 540–570 nm.

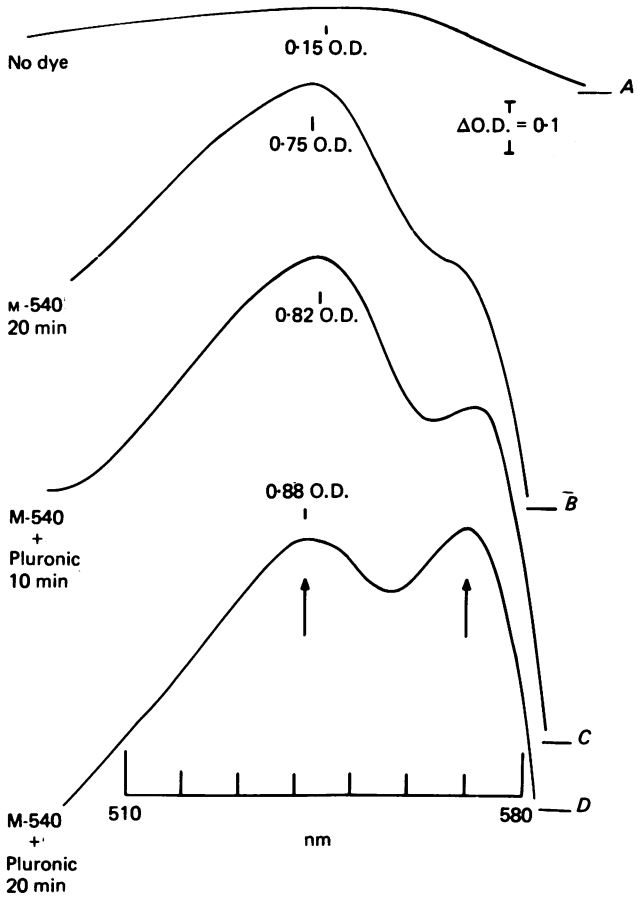

Fig. 12. Effect of Pluronic on the absorption spectrum of Merocyanine-540 in heart muscle. The intrinsic absorption spectrum of frog atrial muscle is shown in spectrum *A*. The muscle is incubated for 20 min in 10^{-5} M-Merocyanine (ethanol < 0·1 %) then washed for another 20 min. The absorption spectrum of the dye bound to the muscle has a maximum at 540 nm and a shoulder at 570 nm (spectrum *B*). The atrial muscle is restained for 10 min in the presence of dye and 0·1 % Pluronic, and the 570 nm absorption is enhanced compared to the 540 nm absorption (spectrum *C*). After another staining period with Pluronic, the 570 nm absorption is further enhanced and becomes larger than the 540 nm absorption (spectrum *D*).

The fluorescence emission in hearts stained without Pluronic had a single peak at 585 nm and the excitation spectrum had a major band at 540 nm and a much smaller band at 570 nm. In the Pluronic stained preparation, the fluorescence spectrum was the same but the excitation spectrum shifted with the major peak at 570 nm and a secondary band at 540 nm.

Potential-dependent spectral changes: rapid scan spectroscopy. Optical action potentials using Merocyanine-540 can be measured by one of four possible modes: a

decrease in absorption at 540 nm, an increase in absorption at 570 nm, or an increase in fluorescence using either 540 or 570 nm excitation (Salama & Morad, 1976). A Rapid Scan Spectrophotometer was used to measure a 400 nm spectrum in 10 msec, either at rest or during the plateau of the cardiac action potential. The fluorescence spectrum of tissue-bound Merocyanine-540 was measured at the resting potential

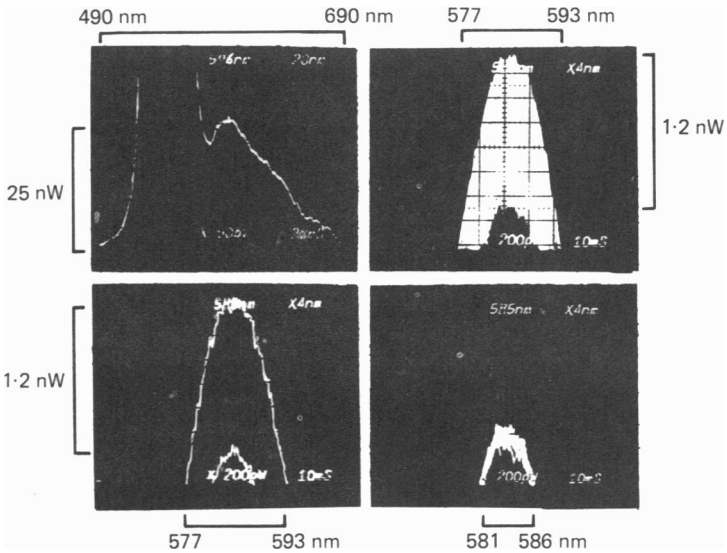

Fig. 13. Potential-dependent fluorescence spectral changes. Fluorescence spectra from a frog ventricular flap stained with Merocyanine-540 and equilibrated in 50 μM-Ca^{2+} Ringer are measured in 10 msec with the 7J/20 Rapid Scan Spectrophotometer. In the top left panel, the light intensity is off scale at the 540 nm excitation wave-length, and the dye fluorescence peaks at 585 nm. For the other panels, the horizontal and vertical scales are expanded by a factor of 4. In the lower left, two spectra are recorded at resting membrane potential (lower trace) and at the plateau of the action potential (top trace). The membrane depolarization produces a 4·8% increase in fluorescence intensity at 585 nm with no shift in peak wave-length. When fluorescence spectra are continuously displayed during an action potential, the envelope of the fluorescence band increases rapidly during the upstroke and recovers more gradually during the repolarization phase of the action potential (top right panel). In the lower right panel, 40 spectra taken at resting membrane potential are superimposed; the signal-to-noise ratio of the fluorescence enhancement is 6/1 for a single action potential.

(Fig. 13, top left panel). The peak of the 540 nm excitation is off-scale, and the fluorescence maximum is at 585 nm. With the horizontal and vertical scales expanded by a factor of 4, the fluorescence band was monitored during the action potential. In the lower left panel, the spectrum during the plateau (top trace) and at rest (bottom trace) are recorded. During depolarization, the peak fluorescence emission is enhanced by 4·8% and no wave-length shift is observed. When spectra were recorded every 10 msec and continuously displayed on the storage oscilloscope, the envelope of the fluorescence emission increased during the upstroke of the action potential and returned to its initial level during repolarization (top right panel). In the bottom right panel, 40 spectra are superimposed with the heart at rest to indicate the noise level and stability of the system.

The transmittance spectrum of the stained muscle was also analysed using the Rapid Scan Spectrophotometer (Fig. 2). Differences in the spectra measured at rest and during the plateau are small but noticeable, and could be magnified using time averaging technique (compare top panel 14, and Fig. 15*C*). In panels *A* to *D*, transmitted and fluorescent light are simultaneously measured by two photodiodes

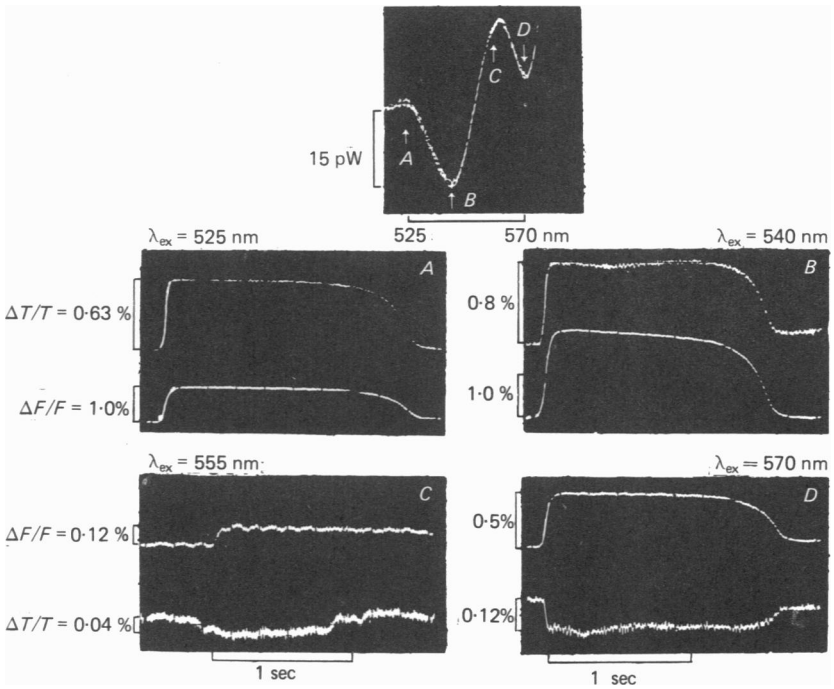

Fig. 14. Wave-length dependence of transmittance action potentials. Transmittance spectra are measured in 20 msec from a frog ventricular muscle stained with Merocyanine-540 with the 7J/20 spectrophotometer. Two spectra are measured (top panel); the spectrum taken during the plateau of the action potential shows a small increase in transmitted light at 540 nm and a small decrease at 570 nm compared to a spectrum taken at resting membrane potential. In panels *A* to *D*, the time course of potential-dependent fluorescence (λ_{em} = 580–650 nm) and transmittance changes are simultaneously recorded with two photodiodes. With a 555 ± 10 nm excitation filter at F_1 (Fig. 2) there are little or no optical responses (panel *C*). With a 570 ± 10 nm filter, the fluorescence increases while the transmittance decreases (panel *D*). With a 525 ± 10 (panel *A*) and 540 ± 10 (panel *B*), both the fluorescence and transmittance increase during the action potential. *T* = transmittance before staining minus transmittance after staining.

respectively located at the back and front of the chamber. In panel *B*, the action potential is recorded as a 2·1 % increase in fluorescence (bottom trace) or an 0·8 % increase in transmittance (top trace) at 540 nm. At 525 nm there is a 1 % fluorescence and a 0·63 % transmittance increase during the action potential (panel *A*) while at 555 nm the optical signal is very small in both modes (panel *C*). With a 570 nm excitation, there is a 0·5 % increase in fluorescence but a 0·12 % decrease in transmittance. Hence a membrane depolarization induces a decrease at 540 nm and an increase at 570 nm absorption band. Note that with 570 nm excitation beam, the

fluorescence and absorption changes are in the same direction, while with 540 nm excitation beam they are in opposite directions. The highest signal-to-noise ratio was obtained by monitoring the fluorescence at 580–650 nm using a 540 nm excitation beam.

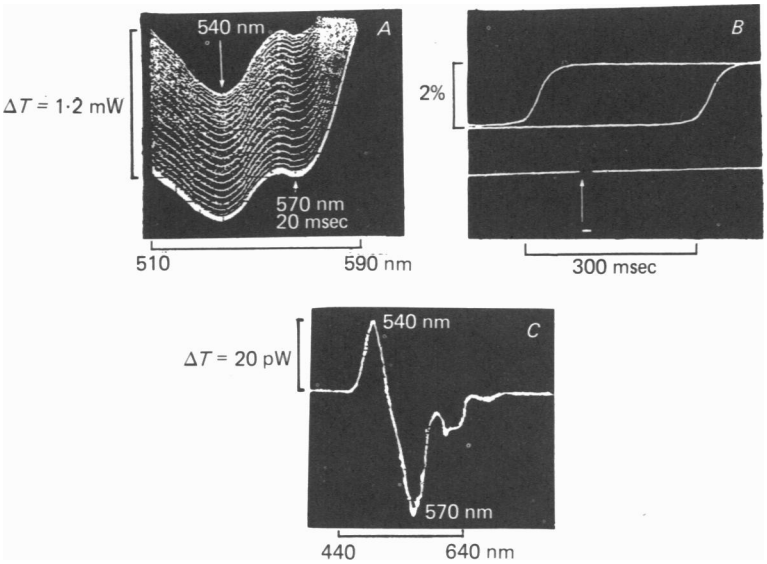

Fig. 15. Potential-dependent difference transmittance spectra. In panel *A*, the transmittance spectra of a frog ventricular muscle stained with Merocyanine-540 is measured in 20 msec during a contraction. Each spectrum has two minima at 540 and 570 nm. The transmittance increases uniformly at peak tension (top spectrum) and recovers to its initial level as the muscle relaxes (lower spectrum). The heart is then equilibrated in 50 μM-Ca^{2+} Ringer to suppress contractions. In panel *B*, two optical action potentials are superimposed. The arrow represents a 6 μsec logic pulse which triggers the time averaging computer (TAC) to record a single spectrum during the subsequent 20 msec time interval. The two optical action potentials are kept out of phase by changing the delay on the electrical stimulus such that the TAC records a spectrum during the plateau of the action potential then subtracts a spectrum taken at rest. With a heat filter at F_1, 100 difference spectra are summed (panel *C*). The potential-dependent transmittance spectrum shows an increase at 540 nm and a decrease at 570 nm with no displacements of the minimum wave-lengths shown in panel *A*.

To detect a possible wavelength displacement of the two absorption peaks, difference spectra were time-averaged to improve the signal-to-noise ratio of the potential-dependent transmittance changes. In Fig. 15, multiple spectra are displayed during a muscle contraction (panel *A*). The lowest trace depicts a spectrum with the heart in a relaxed state while the top trace is the spectrum recorded at the peak of contraction. The preparation is then perfused in low Ca^{2+} Ringer to suppress tension. In panel *B*, two fluorescence action potentials were temporally displaced with respect to the spectrum registered by delaying the electrical stimulus. Thus a spectrum could be recorded before the upstroke or during the plateau of the cardiac action potential. The difference of 100 spectra taken during the plateau and at resting potential is illustrated in Fig. 15*C*. The wave-length dependence of the optical response was biphasic with an increase in transmittance at 540 nm and a decrease at 570 nm. In this experiment, the relative amplitude of the 540 and 570 nm re-

sponses were not corrected for variation in efficiency of the apparatus as a function of the wave-length. Nevertheless, the experiment shows that within the accuracy of ± 2 nm, the peak optical responses occur at 540 and 570 nm and that there are no potential-dependent wave-length shifts.

Measurement of optical signal with Merocyanine-540 in mammalian heart. Absorption and fluorescence action potentials were also measured in rat and guinea-pig hearts stained with Merocyanine-540. The signal-to-noise ratio of such measurements were often smaller than those measured in the frog heart. This may be due to the high intrinsic absorption of the tissue due to the presence of myoglobin (0·2 mM in the rat ventricle; Wittenberg, 1970). The major intrinsic absorption peaks of the tissue appeared at 450, 540, and 582 nm similar to those observed with oxmyoglobin. The measurements of potential-dependent optical signal in mammalian tissue appeared at 450, 540, and 582 nm similar to those observed with oxymyoshift to deoxymyoglobin decreases the 540 and 582 nm peaks, and increases the 556 nm peak.

DISCUSSION

The main results of this study are that Merocyanine dyes serve as effective probes of transmembrane potential in heart muscle. The change in extrinsic fluorescence and/or absorption of the ventricular muscle stained with such dyes are linear with the changes of potential across the membrane. The fluorescence signal follows rapidly and accurately the time course of cardiac action potential. Signal-to-noise ratios are fairly large for various dyes and signal averaging is not required. The high signal-to-noise ratio makes it possible to use the optical signal in the feed-back loop of the voltage clamp. Not all of the Merocyanine dyes used in this study are biologically inert. Merocyanine-540 demonstrates consistently larger phototoxicity in ventricular strips than Merocyanine-rhodanine (WW 375) or Merocyanine-oxazolone (NK 2367). However, this effect is considerably smaller in cardiac muscle than that reported in nerve tissue (Ross *et al.* 1977). The rapid-scan spectrophotometric results provide evidence against an electrochromic (i.e. a potential-dependent spectral shift) mechanism mediating the voltage sensitivity of the Merocyanine-540; rather, the findings support the existence of two populations of membrane-bound dye molecules with different absorption transition moments. Depolarization produces an increase in fluorescence yield for both molecular species although the absorption transition moment of one species increases and the other decreases.

Amplitude of the signal. Merocyanine-540 in the frog heart produces a $\Delta F/F$ of about 1–2 % for 100 mV depolarization, a value 5–10 times greater than the axon. However, when this value is adjusted for the ratios of excitable to non-excitable membrane present in frog ventricular muscle,

$$\frac{\text{excitable membrane}}{\text{endothelial membrane}} = \frac{10}{1}, \text{ Page \& Niedergerke, 1972,}$$

and giant axon

$$\frac{\text{axon membrane}}{\text{Schwann cell membrane}} = \frac{1}{4}, \text{ Cohen } \textit{et al.} \text{ 1974,}$$

values of $\Delta F/F$ become approximately equal (0·5–1 %).

In skeletal muscle, 90% of the fluorescence action potential originates from T-tubular membranes (Vergarra & Bezanilla, 1976). If one assumes a 100 mV depolarization of the T-tubules during the action potential, then a $\Delta F/F$ of 0·7% is similar to values measured in heart muscle. Thus the voltage sensitivity of Merocyanine-540 appears to be independent of the preparation. On the other hand, values of $\Delta T/T$ for Merocyanine-rhodanine and Merocyanine-oxazolone in cardiac muscle are more than an order of magnitude larger than that measured in nerve, which makes it difficult to explain the differences in voltage sensitivity on the basis of uniform dye binding to excitable and non-excitable membrane fractions. This finding suggests that species specificity may play a significant factor in dye-membrane interaction of the later probes.

Although $\Delta F/F$ or $\Delta T/T$ is constant for a particular preparation at a chosen wavelength, the experimental apparatus determines in part the signal-to-noise ratio of the optical signals. The improvement in signal-to-noise ratio (100/1 to 500/1) with Merocyanine-rhodanine and Merocyanine-oxazolone, may be attributed in part to high energy output of the light source and the greater quantum efficiency of the photodiode at longer wave-lengths.

Source of optical signals. Available evidence suggests that the optical signals from Merocyanine dyes originate from membrane-bound dye molecules.

The potential-dependent optical signals can be measured in heart muscle for several hours while the tissue is continuously washed in dye-free solutions, which implies a strong dye-tissue interaction. From a comparison of the absorption spectrum of Merocyanine-540 in Ringer (peak at 510 and shoulder at 580 mm) and in tissue (Fig. 12), the voltage-dependent displacement of dye molecules from a Ringer to a tissue bound environment could not account for the observed potential dependent spectral changes (Figs. 13–15). Thus the optical signals could not be attributed to possible dye movement between the membrane and the nearby aqueous phase (Waggoner & Grinvald, 1977). Merocyanine-rhodanine and Merocyanine-oxazolone also appear to have a strong dye-tissue interaction since their absorption spectra differ in Ringer and in tissue and since they become more stable upon binding to heart muscle but decompose rapidly in solution. These observations suggest that the dye molecules are likely to be located either in the membrane or the cytoplasm rather than the extracellular aqueous phase. The localized charge on Merocyanine dyes (Waggoner, Sirkin, Tolles & Wang, 1975) and the reversal in the direction of the optical signals for axons stained from outside or inside (Ross *et al.* 1977) suggest that the dyes primarily bind to the membrane.

Kinetics of the Optical Signal. In a ventricular flap preparation, there is a difference (10–15 msec) in the rise time of the action potential as measured electrically or optically. The delay in the rise time of the optical signal is an expression of the multicellular nature of the preparation rather than the molecular origins of the signal. This conclusion is consistent with the observation that decreasing the size of tissue exposed to the light beam decreases the delay.

Even in ventricular strips (0·5–1·0 mm in diameter), the optical signal is slower than the electrical signal (Fig. 4). This delay is in part due to the averaging property of the optical signal, especially in the radial direction (see Tests and Precautions). Another contributing factor to the disproportionate detection of the potical signal may be the heretogeneity of dye distribution in the extracellular space.

In uniformly stained axons, a delay of about 30 μsec between the micro-electrode and Merocyanine-540 signal is attributed to a delay of the molecular events producing the optical signal (Ross *et al.* 1977).

Base line stability of the optical signal. In ventricular flaps stained with Merocyanine-540, the rate and direction of fluorescence base line drifts show considerable variation from preparation to preparation. However, in most experiments, after washing the excess dye, there seems to be an initial increase followed by a decrease in fluorescence (Fig. 11*A*). The initial increase in fluorescence may be caused by a reduction of effective dye concentration due to light exposure which decreases the process of self-quenching until the fluorescence reaches a maximum. Thereafter, photobleaching produces a gradual decrease in fluorescence.

The drift on the optical traces tends to be considerably faster with Merocyanine-oxazolone compared to Merocyanine-rhodanine and it seems to be brought about by a decrease in absorption due to dye bleaching.

For the three probes the rate of drift is accelerated by continuous versus intermittent exposure to the incident beam. Thus the loss of tissue-bound dye may be due to a combination of washout and photobleaching. In heart muscle, the optical technique is sufficiently accurate to measure 5 mV changes in membrane potential if the potential changes occur rapidly (0·1–100 msec) compared to the drift (Fig. 11, inset).

Photodynamic damage. Frog ventricles stained with Merocyanine-540 and exposed to light intensities of 10–100 μW/mm^2, did not exhibit noticeable phototoxic effects (Fig. 9*A*, *B*). On the other hand, similar light intensities produced depolarization and gradual suppression of the action potential in ventricular strips (Figs. 9, 10). Unlike the axon (Ross *et al.* 1977) the process in the ventricular strip is slower and partly reversible (Fig. 10). The apparent lack of phototoxic effects in whole ventricular flaps may be partly caused by less efficient staining of the deeper fibres since diffusion of dye through the extracellular spaces is more restricted. The electrical activity of the photo-damaged cells may be sustained through their electrical coupling with the large number of surrounding cells. In deeply stained ventricular strips (using the Langendorff perfusion), the rapid onset of phototoxicity (Fig. 9*C*, *D*, *E*) is apparently due to higher concentrations of tissue-bound dye rather than the smaller dimensions of the preparation.

The slower rate of phototoxic damage in muscle strips versus squid axons, and the partial recovery from such damage following a dark period (Fig. 10*C*) may be in part attributed to the characteristics of a multicellular preparation with electrically coupled cells. Upon illumination, stained cells depolarize at varying rates due to gradients of light intensity and oxygen concentration (Fig. 10*A*, *B*) such that the extent of phototoxic damage differs throughout the preparation. Species variations could also account for differences in phototoxicity in heart and axons as implied by the single cell experiments on *Sepia* (Chalazonitis, 1964) and lobster nerves (Pooler, 1972).

In frog heart, Merocyanine-rhodanine and Merocyanine-oxazolone appear to have little photodynamic damage in agreement with studies on squid axon and barnacle neurones (Ross *et al.* 1977; Salzberg *et al.* 1977). It appears, therefore, that the phototoxic effects observed in heart is phenomenologically similar to that observed in other excitable tissues.

Comparison of spectral characteristics in heart, skeletal muscle, nerve, and black lipid membranes. For Merocyanine-540, absorption and excitation spectra appear to be similar in frog, rat, and guinea-pig hearts but differ slightly in nerve and black lipid membranes. All the spectral evidence reported so far suggests two absorption peaks for membrane-bound Merocyanine-540, one at 570 nm, and the other at a lower wave-length which seems to have a different peak in various preparations. For instance, in axons and black lipid membranes the lower peak is measured at 520 nm (Ross *et al.* 1974; Waggoner & Grinvald, 1978), in crab nerve at 525 nm (Tasaki *et al.* 1976), and in heart and in mitochondria at 540 nm (Chance, Baltscheffsky, Van der Kooi & Cheng, 1974). The relative amplitude of the two absorption peaks are also different: in heart, the maximum absorption is measured at 540 nm rather than 570 nm, while the opposite is true for axons (Ross *et al.* 1974; Tasaki *et al.* 1976) and black lipid membranes (Waggoner & Grinvald, 1978). The Pluronic experiments (Fig. 12) suggest that the relative amplitude of the absorption peaks may depend on the staining medium rather than a real difference in dye–membrane interactions for various preparations.

The wave-length dependence and the directions of the Merocyanine-540 optical signals are essentially the same in heart (Figs. 14, 15) (Salama & Morad, 1976), skeletal muscle (Nakajima, Gilai & Dingman, 1976), nerve (Ross *et al.* 1977), and black lipid membranes (A. S. Waggoner, personal communication). The probe is more sensitive to potential changes through its fluorescence rather than transmittance mode with values of $\Delta F/F$ 5–10 times greater than $\Delta T/T$.

In heart muscle the largest value of $\Delta F/F$ is obtained with a 540 nm excitation beam rather than a 570 nm beam, as in axons (Ross *et al.* 1977). In heart, peak values of $\Delta T/T$ are measured as a decrease at 540 nm and an increase at 570 nm while in axons (Ross *et al.* 1974) and in black lipid membranes (A. S. Waggoner, personal communication) the decrease in $\Delta T/T$ is measured at 520.

Therefore, besides a displacement of the lower wave-length absorption band, the main properties of Merocyanine-540 bound in heart and other preparations are in agreement and the same mechanism(s) is likely to generate the optical responses.

Possible mechanism(s) for generation of Merocyanine-540 signal in heart muscle. Since the maximum absorption of Merocyanine-540 is displaced towards longer wave-length in environments of decreasing polarity (i.e. *solvatochromism*, Tasaki *et al.* 1976), a voltage sensitive displacement of its absorption toward longer wavelengths (i.e. an *electrochromic* effect) could likewise generate the optical signals. Dynamic spectral measurements during the course of the cardiac action potential show no potential-dependent wave lengths-shifts (Figs. 13–15) which rules out electrochromism as a possible mechanism for generation of the optical signal.

From similarities of the spectra in organic solvents and in the membrane, Ross *et al.* (1974) first suggested that membrane bound dye molecules exist either as weakly fluorescent aggregates or highly fluorescent monomers. According to this hypothesis, membrane depolarization causes a shift from the dimer to monomer state which produces a decrease in dimer absorption at 520 nm and an increase in monomer absorption at 570 nm. The potential-dependent fluorescence increase is associated with the increase in monomer population while the weakly fluorescent dimers have little effect on the fluorescence.

In heart, some of the observations described in this report are not entirely compatible with a dimer to monomer transition. For instance, the absorption band at 540 nm which would represent the dimer species has a fluorescence yield of comparable magnitude to the monomer absorbing at 570 nm (unpublished observation, Salama; see also Fig. 7 of Ross *et al.* 1977). This is inconsistent with the dimer to monomer hypothesis since dimers generally have a significantly lower fluorescence yield than monomers.

If the dimer fluoresces in heart, then a decrease in dimer population during the action potential would be expected to produce a potential-dependent decrease in absorption and fluorescence. But in frog heart, the decrease in absorption at 540 nm is associated with an increase in fluorescence (Fig. 14).

In principle, the relative absorbance at 540 and 570 nm should be proportional to the ratio of dimers to monomers at equilibrium which should vary with the total concentration of membrane-bound dye. The larger absorbance at 540 nm compared to 570 nm could not be reduced by lowering either the staining period or the dye concentration in the staining solution. On the other hand, in the presence of Pluronic in the staining solution, the absorbance at 570 nm was greater than at 540 nm (Fig. 12).

The data in heart suggests the existence of two fluorescent species which increase in fluorescence during a membrane depolarization. In fact, such an interpretation is also consistent with the excitation action spectrum measured in axons (Ross *et al.* 1977). Thus the interpretation that the 540 nm absorption band represents a dimer species in heart is incompatible with (1) the Pluronic effect, (2) the high fluorescence yield from a 540 nm excitation, and (3) the increase in fluorescence associated with a decrease in absorption. The data in heart is compatible with polarized light experiments on lipid bilayers which show that dye molecules absorbing at 520 and 570 nm are preferentially oriented parallel and perpendicular to the plane of the membrane (A. S. Waggoner, personal communication). The polarized light experiments do not necessarily require the existence of a dimer, but could be consistent with a dimer to monomer transition if the rotation of the molecules produces a shift between dimer and monomer species. Alternatively, the optical responses of Merocyanine-540 could be explained by molecular rotations in the membrane's electric field (Conti, 1975) without postulating the formation of an aggregate state.

Spectral characteristics of Merocyanine-rhodanine and Merocyanine-oxazolone. For both absorption dyes, the action spectra ($\Delta T/T$ *vs.* wave-length) appear to vary for different excitable cells. In heart muscle, the action spectrum of Merocyanine-rhodanine is triphasic (Fig. 6, top graph) while in axons it is monophasic (Ross *et al.* 1977). Polarized light experiments from the centre and edge of the axon show that the action spectra are triphasic for molecules with transition moments oriented perpendicular to the plane of the membrane and are monophasic for those oriented parallel to the membrane. These experiments suggest that in some situations geometrical factors may influence the action spectrum measurements. For instance, the triphasic action spectrum in frog skeletal muscle could be a consequence of the geometrical arrangement of the T-tubular membranes (Baylor & Chandler, 1978; Nakajima & Gilai, 1978).

The Merocyanine-rhodanine triphasic action spectrum in frog ventricular muscle

(Fig. 6) cannot be attributed to the orientation of its excitable membranes since it lacks a T-tubular system. On the other hand, the spectra in heart and skeletal muscle are consistent with measurements on vertebrate neurones, and support the hypothesis that for invertebrate excitable cells the action spectrum is monophasic while for vertebrate cells the spectrum is triphasic (Ross & Rheichardt, 1979). However, the monophasic action spectrum of Merocyanine-oxazolone in heart muscles (Fig. 6) complicates the situation since it differs from both vertebrate and invertebrate preparations.

We are grateful to Dr B. Chance for support and guidance in the initial stages of this work. We thank Drs A. Waggoner and B. Salzberg for their advice and their generous gifts of Merocyanine-rhodanine and appropriate filters.

This work was supported by N.I.H. grant HL 17702.

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