Birefringence Signals in Mammalian and Frog Myocardium

E-C Coupling Implications

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ABSTRACT Birefringence signals from mammalian and frog hearts were studied. The period between excitation and the onset of contraction in which optical signals were free of movement artifact was determined by changes in scattered incandescent light and changes in laser diffraction patterns. The birefringence signal preceding contraction was found to behave as a change in retardation and was not contaminated measurably by linear dichroic or isotropic absorption changes. There were two components of the birefringence signal in mammalian heart muscles but only one component in the frog heart. The first component of the birefringence signals in both mammalian and frog hearts had a time course coincident with the action potential upstroke. The second component in mammalian preparations was sensitive to inotropic interventions, such as variation of extracellular Ca\(^{2+}\), stimulation frequency, temperature, and epinephrine, in a manner that correlated with the maximum rate of rise of tension. Caffeine (2–10 mM) not only failed to generate a second component in the frog heart, but also suppressed the second component in the mammalian heart while potentiating twitch tension. The results suggest that the second component of the birefringence signal in the mammalian myocardium is related to Ca\(^{2+}\) release from the sarcoplasmic reticulum.

INTRODUCTION

The development of optical techniques has contributed greatly to our understanding of excitation-contraction (E-C) coupling in cardiac muscle. Surface membrane potentials and the spread of excitation have been measured by voltage-sensitive dyes (Salama and Morad, 1976; Morad and Salama, 1979; Dillon and Morad, 1981), and fluctuations of intracellular...
calcium during a contractile cycle have been examined with the Ca²⁺-sensitive protein aequorin (Allen and Blinks, 1978; Allen and Kurihara, 1980a, b; Weir, 1980). Intrinsic optical changes occurring during (Bourret and Gargouil, 1976) and before the onset of contraction (Weiss and Morad, 1981) have also been studied.

A two-component birefringence signal, occurring before the onset of contraction, was first demonstrated in frog skeletal muscle single fibers (Baylor and Oetliker, 1975, 1977a, b, c). The first component was found to have a time course coincident with the surface membrane action potential. The second component, which began before the onset of the twitch, was dissociated from the contraction artifact when development of tension was suppressed by means of stretch, hypertonic solutions, and D₂O replacement of water. Baylor and Oetliker (1977c) concluded that the second component may be related to the activity of the sarcoplasmic reticulum (SR) associated with calcium release. More recent studies show that the time course of the second component and the rise in free intracellular Ca²⁺, as measured with Ca²⁺ indicator dyes, virtually superimpose through the peaks of the signals (Suarez-Kurtz and Parker, 1977; Baylor et al., 1982a, b, c).

In this report we examine the physical and biological properties of the intrinsic birefringence signal in heart muscle and determine the interval of time between excitation and the onset of contraction in which these optical signals are free of movement artifact. Although in the mammalian myocardium birefringence signals with two components preceding contraction were consistently found, in the frog heart the birefringence signal had only one component. The first component of the birefringence signal in both mammalian and frog heart was related to the surface membrane action potential. The second component, present only in mammalian heart, seems to be associated with an early step in E-C coupling, most likely related to Ca²⁺ release or binding of Ca²⁺ to Ca-ATPase. A preliminary report of this work has already appeared (Weiss and Morad, 1981).

METHODS

Preparation

Mammalian cardiac muscle was obtained from the right ventricles or atria of rats, guinea pigs, cats, and elephant seal pups (Mirounga angustirostris). Rats and guinea pigs were killed by cervical dislocation. Other mammals were anesthetized with 30 mg/kg pentobarbital (elephant seal pups) or ether (cats), and frogs were pithed before removal of the heart. Hearts were removed quickly and perfused by the Langendorff procedure with cold Tyrode's solution with 4.4 mM Ca for several minutes to clear blood from the tissue. Atria or right ventricles were removed for further dissection and small atrial or ventricular trabeculae carnae (40–300 µm in diameter × 0.75–1.2 mm long) were tied with silk onto a tension transducer (Endevco semiconductor strain gauge; Endevco Corp., Becton, Dickinson & Co., San Juan Capistrano, CA).
Experimental Arrangement

All experiments were performed on a Zeiss IM35 inverted microscope (Carl Zeiss, Inc., Thornwood, NY), except for those done in the sucrose-gap apparatus. Fig. 1 shows how the microscope was arranged for applications of incandescent and laser illumination. Muscles were mounted in a delrin (plastic) chamber, the bottom of which was a replaceable glass coverslip. An inflow hole and a hypodermic needle attached to suction at opposite ends of the bath allowed oxygenated solutions to perfuse the chamber. A coverslip was placed on top of the chamber for laser experiments, but not for incandescent light applications when a water-immersion objective was used to condense incident light.

**Figure 1.** Schematics of optical apparatus. (A) For measurement of incandescent light intensities. PC: primary condenser; FD: field diaphragm; KG3: Schott heat filter; IF: interference filter (removed for most measurements of birefringence and scattered-light signals); Pol: polaroid polarizers; O1, 40X: water-immersion condensing objective (N.A. 0.75); O2, 25X: collecting objective (N.A. 0.60); An: analyzer (i.e., polaroid polarizer); IL: intermediate microscope lenses; PD: photodiode. (B) For measurement of laser zero-order meridian intensity. Ap: aperture; M: front surface mirror; PH: pinhole (0.5 mm diam); O, 10X: collecting objective (N.A. 0.22); EP, 10X: eyepiece; PD: photodiode. (C) For measurement of laser first-order meridian positron. Ap: aperture; M: front surface mirror; PH: pinhole (0.5 mm diam); O, 5X: collecting objective (N.A. 0.1). The collecting objective and reticon photodiode array were mounted in a holder that could be pivoted about the intersection of the muscle and laser beam so that the objective was always aimed at the intersection point.
Optical Apparatus Used in Birefringence Experiments

A 100-W tungsten-halogen source was used in a Koehler illumination mode (Fig. 1A). The lamp was powered by a regulated DC power supply (6286A; Hewlett-Packard Co., Palo Alto, CA). Light was projected downwards through the following elements before reaching the muscle: (a) primary condenser, (b) field diaphragm, (c) intermediate lenses (two elements), (d) heat filter (Schott KG-3; Schott Optical Glass Inc., Duryea, PA), (e) filter slot to hold a polarizing filter (polaroid sandwiched in glass; Edmund Scientific Co., Barrington, NJ) and/or interference filters (Corion Corp., Holliston, MA), (f) final condenser (40× water-immersion objective, N.A. 0.75; Carl Zeiss, Inc.). Light was collected from below by a neofluor 25× objective (N.A. 0.60; Carl Zeiss, Inc.), passed through a slider that could hold a second polarizer (analyzer) and intercepted by a photodiode (PV 215; EG&G, Inc., Electro-Optics Div., Salem, MA). The 25× objective was selected over a second 40× water-immersion objective because it was found to collect as much resting light through the preparation and was more convenient to use. The photodiode was mounted on a slider positioned in the microscope body at the focal point of a relay lens. The preparation could be observed during an experiment by moving the photodiode slider out of the light path.

The light intensity was linearly transduced by the photodiode and a high-gain current-to-voltage converter (time constant; 0.35 ms). An analog sample-and-hold circuit was used to zero the resting light level before each stimulus so that signals could be observed at high amplification on an oscilloscope (511C; Tektronix, Inc., Beaverton, OR) or a single-channel signal averager (TN-1505; Tracor Northern, Middleton, WI). Because of the characteristic of analog sample-and-hold circuits known as droop (i.e., current leakage through the storage capacitor), some of the optical recordings in this paper have baselines showing a steady drift. In experiments where drift exceeded 2.5 × 10⁻⁵ ΔI/I (R·ms), the recordings were rejected. Because of the small amplitudes of the signals, averaging was performed routinely. All optical data reported in this paper were originally recorded sequentially under identical conditions. Generally, several control photographs of birefringence and scattered-light signals were taken before and after each experimental intervention. Data were rejected if pre- and post-intervention control parameters varied by more than ~15%. Unless otherwise noted, birefringence and scattered-light signals were obtained with white light. Birefringence traces labeled Os and Op were measured with the muscle positioned between crossed and parallel polarizers at an azimuthal angle ψ = 45°, respectively. Tension traces were also signal-averaged and photographed. In addition, tension was monitored continuously on a chart recorder (1500; MFE Corp., Salem, NH).

Optical Apparatus Used for Laser Diffraction Measurements of Sarcomere Shortening

For laser diffraction experiments, a 3-mW helium-neon laser (632.8-nm wavelength; Spectra-Physics, Inc., Mountain View, CA) was directed through a port in the back of the microscope. A 45° front surface mirror below the chamber reflected the beam up to the preparation. A pinhole placed in the objective turret of the microscope limited the diameter of the laser beam that intersected the muscle.
The diffraction pattern of the laser beam through the muscle was visually inspected and the spacing between meridians of a resting muscle was measured on a mylar screen suspended above the preparation. Zero-order meridian light was collected by a 10× objective (N.A. 0.22; Carl Zeiss, Inc.) and refocused through a 10× eyepiece onto a photodiode (PV 444; EG&G, Inc.) for intensity measurements (Fig. 1B). Movement of a first-order meridian was also used as a measure of the earliest muscle shortening. A 5× objective (N.A. 0.1; Cooke Engineering Co., Alexandria, VA) imaged a central slice of a meridian onto a Reticon photodiode array (64 × 2 elements; EG&G, Inc.). The unit (objective plus Reticon array) was fixed above the microscope stage on a radial track so that the objective was always directed at the intersection of the laser beam and the muscle (Fig. 1C). The detector could be centered over either a zero- or a first-order meridian. Each photodiode in the Reticon array integrated light for 2 ms before discharging into a digitizing circuit and memory bank. A sequential discharge (sweep) of the array could be activated as often as every 2 ms by a logic pulse and 16 sweeps could be stored per experimental trial.

The time limit for detection of the earliest movement of a meridian was the integration time of a photodiode (i.e., 2 ms) before the beginning of the sweep in which the movement occurred. By varying the delay between the stimulus and the beginning of the first sweep, the limit of the earliest movement could be estimated to within at least 2 ms of the event itself.

Optical Measurements in a Single Sucrose-Gap Chamber

A sucrose-gap chamber similar to that developed by Morad and Salama (1979) was used. The incandescent illumination system from the Zeiss IM35 microscope (described above) was mounted horizontally so that light could be focused through a side-window of the chamber onto the muscle. A 32× objective (N.A. 0.60; E. Leitz, Inc., Rockleigh, NJ) was used for the final condenser. Light was collected through the opposite side-window by a second 32× objective, passed through an analyzer, and converted to a voltage signal by a photodiode and current-to-voltage amplifier similar to the one described above. A digital sample-and-hold circuit was used in these experiments.

Solutions

Tyrode’s solution was used for all mammalian preparations. The composition of the solution was (mM): 136 NaCl, 5.4 KCl, 2.4 CaCl₂, 0.8 MgCl₂, 0.42 NaH₂PO₄, 5.6 glucose. All solutions were equilibrated with 95% O₂, 5% CO₂ gas, and the pH was 7.3–7.4. The solution in which dissections were carried out was similar but contained 4.4 mM CaCl₂ and 15 mM glucose and was kept at 7–10°C. When solutions with calcium <1.0 mM were used, MgCl₂ was added so that [Mg] + [Ca] = 1.8 mM. Deuterium oxide (D₂O) solutions were made by dissolving the ionic ingredients in 99.8% D₂O (Sigma Chemical Co., St. Louis, MO). CaCl₂ stock solution in H₂O was added to the final solution, which reduced the final D₂O concentration to not less than 99.6%. Solutions were made hypertonic by addition of NaCl. Activity coefficients for NaCl and KCl were taken to be 0.65 and 0.75, respectively. All other constituents were assumed to have activity coefficients of 1.0. The tonicity values ranged from ~1.25 to ~2.25 times normal. In one experiment sucrose was used to alter the tonicity.

Ringer’s solution for frog heart preparations had the following ionic composition (mM): 116 NaCl, 2.0 NaHCO₃, 3.0 KCl, 1.0 CaCl₂. The pH was 7.4–7.6.
Voltage-sensitive Dyes

WW444 (Waggoner-Wang 444; dye 665 in Gupta et al., 1981; chemical nomenclature: 3-hexylrhodamine-5 [1-γ-sodium sulfopropyl-1,3-dihydroquinone-(4)]-dimethinemerocyanine) and merocyanine oxazolone (NK2367 from Nippon Kankoh-Shikiso Kenkyusho Co., Ltd.; dye XXII in Gupta et al., 1981) were used as optical probes of membrane potential in this study.

A small amount of dye was dissolved in warm Tyrode's or Ringer's to a concentration of 0.05 mg/ml. This mixture was diluted again with well-oxygenated solution just before use to a final dye concentration of 1–2 μg/ml. The dye-containing solution was placed in a drip bottle that was closed except for a small gas outflow in the cork. The 95% O₂, 5% CO₂ gas mixture was maintained in the space above the solution so as to reduce the loss of gas without bubbling. The experimental chamber was perfused with the dye solution for ~20 min. The dye signals shown in this paper were measured with light passed through a 628-nm (37-nm half-amplitude bandwidth) interference filter (Corion Corp.).

Analysis of the Second Component

The rate of development of the second component was quantified by one of two methods. One method obtained the rate of change (slope) of the second component in terms of relative light intensity change per time and is referred to as dSC/dt in the text. The other method obtained the relative light intensity change of the second component at an arbitrary time after the foot of the action potential, and this parameter is referred to as Δ2nd component in the text. Whereas dSC/dt gives a measure of maximum rate of change of the second component (preceding contraction), Δ2nd component also includes information on the delay between the rising phase of the action potential and the apparent onset of the second component.

RESULTS

The intrinsic birefringence of heart muscle could be seen by eye when a mammalian or frog atrial or ventricular muscle was placed between two crossed polarizers such that the first polarizer was +45° and the second polarizer (analyzer) was −45° to the longitudinal axis of the muscle. This arrangement of polarizers defines an azimuthal angle ψ = 45° between the polarizers and the muscle. The preparation appeared bright against a dark background, whereas if the muscle was not in the light path, the specimen field was uniformly dark.

When a mammalian myocardial preparation was electrically stimulated, a two-component decrease in light intensity occurred before the onset of contraction (trace O₀, Fig. 2A). The first component had a time course that coincided with the action potential recorded as the signal from the membrane-impermeant, voltage-sensitive dye WW444. The second component was delayed relative to the action potential upstroke and was larger and slower than the first. In the frog heart, however, electrical stimulation led only to a single-component decrease in the birefringence signal preceding the onset of contraction. Like the first component in mammalian heart, this component had a time course coincident with the
FIGURE 2. Optical signals and tension are compared from mammalian (rat) and frog ventricular trabeculae. (A) Rat ventricle. The birefringence signal (trace O_b, white light) can be seen to have two components (of decreasing intensity) occurring before the onset of contraction as monitored by the scattered-light signal (trace O_s, white light). The voltage-sensitive dye signal (trace WW444) was recorded with 628-nm (half-amplitude bandwidth: 37 nm) light under identical conditions. The first component of trace O_b and the action potential upstroke-related dye signal coincide temporally. The experimental conditions were: 24°C; stimulation rate: 20 min⁻¹; [Ca]o: 2.4 mM. Calibration of optical signals is in units of relative intensity change (Δf/fo). The number of sweeps averaged per trace was: O_b: 25; O_s: 8; T (tension): 10; WW444: 1. Muscle reference number: 22.4.80RV. (B) Frog ventricle. Signal-averaged and single-sweep recordings from a frog ventricular trabecula were obtained as in A. Traces labeled O_b have only a single component preceding contraction. This single component is coincident with the voltage-sensitive dye signal (trace WW444). Single-sweep recordings with a good signal-to-noise ratio were obtained from this muscle as well as from several other frog preparations. Good single-sweep recordings of the first component from mammalian preparations were rarely observed, which may relate to the greater surface membrane area-to-cell volume of frog heart. The experimental conditions were: 19.3°C; stimulation rate: 12 min⁻¹; [Ca]o: 1.0 mM. The signal-averaged traces were 50 sweeps each, except for trace WW444, which was 25 sweeps. Muscle reference number: 30.4.80FV.

rising phase of the action potential, measured as the WW444 dye signal (Fig. 2, B and C).

The large intensity deflection after the second component of the birefringence signal in mammalian heart (or the first component in the frog heart) had the same time course as the scattered-light signal (traces
Os, Fig. 2). The scattered-light signal, measured as the intensity of transmitted unpolarized white light, was used as an indicator of the onset of muscle concentration (Hill, 1953). Fig. 3 shows that this late change of light intensity in the birefringence signal could have different polarity,

\[ \text{Fig. 3. (A) Rat ventricle. The movement-related (scattering) part of the birefringence signal could be either an increase or a decrease in light intensity, depending on the position of the muscle in the light path. The first and second components of the birefringence signal recorded from mammalian preparations were always a decrease in intensity when measured through crossed polarizers. The rat ventricular muscle was in normal Tyrode's solution (2.4 mM [Ca]₀) at 23°C and was stimulated at a rate of 30 min⁻¹. Calibrations: upper trace O₅: 2.23 × 10⁻⁴ (Δl/Iₘₕ), 25 sweeps; lower trace O₅: 2.0 × 10⁻⁴, 25 sweeps; time bar: 4 ms. Muscle reference number: 20.4.80RV. (B) Frog ventricle. The direction of the scattering part of the birefringence signal from a frog cardiac preparation could be an increase or a decrease in light intensity. The first component, on the other hand, was always a decrease in light intensity when measured through crossed polarizers. The deflection of the optical traces at the extreme left of the panel is the settling of the analog sample-and-hold circuit. The muscle was in normal Ringer's solution (1 mM [Ca]₀) at 22°C and was stimulated at a rate of 20 min⁻¹. Calibrations: upper trace O₅: 5.4 × 10⁻⁴, 50 sweeps; lower trace O₅: 7.1 × 10⁻⁴, 50 sweeps; time bar: 8 ms. Muscle reference number: 24.7.80FV.} \]

depending on the position of the light spot on the muscle. An increase in light intensity was generally measured when the spot of light was positioned closer to the edge of the muscle.

Attempts to suppress the muscle movement after excitation in order to monitor the full time course of the second component in mammalian heart muscle were unsuccessful. Stretching of preparations to sarcomere
lengths >2.6 \mu m frequently resulted in development of contracture or automaticity. Other methods to suppress tension, such as D_2O replacement of solution water and increasing the tonicity of solutions, were also not effective. In the experiment illustrated in Fig. 4, D_2O was substituted for 99.6% of the solution water and Ca^{2+} was reduced to 0.5 mM. Although tension decreased markedly and the onset of the scattered-light signal was delayed, the full time course of the birefringence signal could not be monitored because of the movement artifact.

Figure 4. The effects of D_2O replacement of 99.6% of the solution water and 0.5 mM [Ca], on the optical signals and tension from a rat ventricular papillary muscle are shown. The onset of contraction was delayed but not eliminated by D_2O and low [Ca]. Control conditions were: 23°C; [Ca]: 2.4 mM; stimulation rate: 20 min^{-1}. Optical calibrations are in terms of relative intensity change (ΔI/I_b). The number of sweeps averaged to obtain each trace are: O_b.control, O_s.control, and T.control: 10 sweeps; O_b.D_2O and O_s.D_2O: 25 sweeps; T.D_2O: 20 sweeps. Muscle reference number: 13.8.80RV.
Definition of the Interval Preceding Active Shortening

Light-scattering signals and laser diffraction patterns were examined during the interval between stimulation and the onset of development of tension to obtain the earliest indication of muscle contraction. Visual inspection of laser diffraction patterns of resting muscle revealed either three or five meridians. When a muscle contracted, the meridians of order greater than zero were seen to move farther apart.

The angular separation of the zero-order from higher-order diffraction meridians specifies the sarcomere spacing in the muscle according to the grating equation:

\[ d_s = \frac{(m \lambda)}{(\eta \cdot \sin \theta_m)} \]

where \( d_s \) = sarcomere length, \( \lambda \) = wavelength of light, \( \theta_m \) = angle between meridians of order 0 and m, and \( \eta \) = refractive index of the medium in which the pattern is observed. By monitoring the intensity profile of a first-order meridian for outward movement (increasing \( \theta_m \)), a measure of the earliest muscle contraction could be obtained. The parameter measured was the weighted center of the meridian, i.e., the vertical line that divides the intensity profile of the meridian into equal areas. Fig. 5A shows 10 intensity profiles of a first-order meridian recorded every 2 ms on a Reticon photodiode array (see Methods). The time relative to the stimulus is given to the right of each trace. The weighted centers of a similar set of first-order meridian traces from the same muscle determined that the earliest muscle contraction occurred between 7.6 and 9.6 ms after stimulation. A plot of the position of these weighted centers vs. the post-stimulation time is shown in Fig. 5B.

The intensity of the zero-order meridian was also measured in several experiments as a monitor of muscle contraction. The zero-order meridian intensity contains light that is undiffracted and multiply diffracted by the sarcomeres of the muscle (Sandow, 1936; Hill, 1953). Fig. 5C shows the time courses of scattered-light, laser zero-order meridian intensity, and birefringence signals, and tension development from the same rat ventricular trabecula used in Fig. 5, A and B. All optical traces were obtained from the same section of the muscle, although the illuminated regions were not identical. The scattered-light and laser zero-order intensity signals show large changes at nearly the same time (~8 ms post-stimulus), which coincide with the large deflection in the birefringence signal (trace \( O_p \)). The position of the laser first-order meridian shifted outward significantly between 7.6 and 9.6 ms post-stimulus.

In some preparations a slight increase in light intensity (\( \Delta I/I_R = 5 \times 10^{-5} \)) of the scattered-light signal was observed before the large motion-related deflection (Fig. 5C). Such early light-transmission changes were at least an order of magnitude smaller than the birefringence signals and have been already described for skeletal muscle (Hill, 1949; Barry and Carnay, 1969; Carnay and Barry, 1969; Kovacs and Schneider, 1977; Baylor and Oetliker, 1977b).
FIGURE 5. The onset of contraction in a rat ventricular trabecula is shown by the intensity change of a laser zero-order diffraction meridian (trace $O_1$, panel C), the position (intensity profile) of a laser first-order diffraction meridian (panel B), and the intensity change of the incandescent scattered-light signal (trace $O_s$, panel B). The signals are compared with the birefringence signal (trace $O_p$, panel C) from the same preparation. (A) The intensity profile of a laser first-order diffraction meridian recorded every 2 ms is shown. The time ($t_s$) of each sweep relative to the stimulus is listed at the right of the panel. The horizontal calibration gives the (Bragg) angle between the zero- and first-order meridians. (B) The weighted centers of a series of intensity profiles of the laser first-order diffraction meridian (as from A) are plotted against time relative to the stimulus. The series of traces used for B have a delay relative to the stimulus different from that used in A. The values are normalized to the position before contraction. There was significant outward movement of the weighted center of the trace that was stored between 7.6 and 9.6 ms post-stimulus. (C) The birefringence (trace $O_p$), scattered-light (trace $O_s$), and laser zero-order intensity (trace $O_1$) signals are compared. The birefringence signal was measured with the muscle between parallel polarizers. The scattered-light signal was obtained with 6.28 ± 37 nm light in this panel. A slight increase in light transmission ($\Delta I/I_R \leq 5 \times 10^{-5}$) occurred before the movement-related scattering deflection. Tension (trace $T$) and the stimulus artifact (trace $S$) are also shown. Calibrations: $O_p$: $3.7 \times 10^{-4}$, 50 sweeps; $T$: 0.5 mg, 5 sweeps. Muscle reference number: 26.3.80RV.
Resting Birefringence

The resting birefringence was measured visually with a Soleil-Babinet variable compensator by a procedure adapted from Bennett (1950). Measurements were made with 626 ± 10 and 538 ± 10 nm light. In two rat ventricular trabeculae the retardation, $R$, was 0.17 $\lambda$ (mean of six determinations) through a muscle thickness of ~150 $\mu$m and 0.125 $\lambda$ (mean of four determinations) through a thickness of ~75 $\mu$m. These values of retardation combined give a birefringence coefficient $B = (7.4 \pm 0.9) \times 10^{-4}$ (mean ± standard deviation). In one guinea pig ventricular trabecula with an approximate thickness of 150 $\mu$m, $R = 0.115 \lambda$ (mean of four determinations) and $B = (4.9 \pm 1.7) \times 10^{-4}$. The coefficient of birefringence in resting skeletal muscle has been reported to be $2.2 \times 10^{-3}$ (Honcke, 1947) and $2.25 \times 10^{-3}$ (Baylor and Oetliker, 1977b). The lower value in cardiac muscle may result from two factors. (a) The myofibrillar content of rat ventricular muscles is 48% by volume (Page et al., 1971) vs. 83% for frog skeletal muscle (Mobley and Eisenberg, 1975). Since most of the resting birefringence comes from the A bands (Noll and Weber, 1934), heart muscle would be expected to have a lower value of birefringence. (b) Because of the elliptical cross section of trabeculae, the light path through the muscle would not be uniform across the illuminated region, causing an underestimation of the birefringence. It has been shown previously that retardation decreases when measured at the edge as compared with the center of skeletal muscle single fibers (Baylor and Oetliker, 1977b).

Retardation Signal and Fresnel’s Equation

The resting intensity of light passed through a birefringent material placed between crossed polarizers can be described by Fresnel’s equation (Cohen et al., 1970; Baylor and Oetliker, 1977b),

$$I_R = k \cdot \sin^2 2\phi \sin^2(\phi/2),$$

where $\phi$ is the azimuthal angle between the crossed polarizers and the muscle longitudinal axis (also called the angle of incidence), $\phi$ is the phase shift of light and is equal to $2\pi R / \lambda$, and $k$ is a constant proportional to the incident light intensity and the area of tissue illuminated. It can be seen that $I_R$ should vary as $\sin^2 2\phi$. The theoretical curve and experimental points are shown in Fig. 6A for rat and frog ventricular trabeculae.

A small change in retardation, $\Delta R$, can be approximated from Fresnel’s equation as

$$\Delta I = (\pi k / \lambda) \sin^2 2\phi \sin \phi \cdot \Delta R.$$  

Then, assuming that the change in retardation preceding contraction is constant from beat to beat, $\Delta I$ should also vary as $\sin^2 2\phi$ (Fig. 6B). The value on the ordinate, $\Delta I$ (6 ms), is the intensity change of the second component measured 6 ms after the foot of the first component (i.e., the
FIGURE 6. (A) The resting light intensity measured through a muscle between crossed polarizers is plotted as a function of the azimuthal angle \( \psi \). The data from three experiments on rat ventricular trabeculae (filled symbols) and one experiment on a frog ventricular trabecula (open symbols) are shown. Values are normalized to the mean of intensities recorded for \( \psi = 45^\circ \) (for each experiment). The smooth curve is \( \sin^2 \psi \) from Fresnel's equation (see text). (B) The change in light intensity of the second component was measured 6 ms after the foot of the action potential upstroke-related first component and plotted as a function of the azimuthal angle \( \psi \). Two experiments from rat ventricular trabeculae are shown. Values are normalized to the mean of intensities recorded at \( \psi = 45^\circ \) (for each experiment). The smooth curve is \( \sin^2 \psi \).
action potential upstroke). The first-component contribution to the signal was subtracted. Cohen et al. (1970) pointed out that the dependence of $\Delta I$ on $\psi$ in Fresnel's equation is consistent either with retardation or linear dichroism.

**Tests to Distinguish Retardation from Linear Dichroism**

The resting light intensity passed by the analyzer at $\psi = 45^\circ$ can be obtained from Fresnel's equation,

$$I_b = \frac{k}{2}(1 - \cos \phi),$$

where $b$ denotes crossed polarizers. If $\psi = 45^\circ$ but the analyzer and polarizer are parallel to each other, the resting light intensity is described by

$$I_p = \frac{k}{2}(1 + \cos \phi),$$

where $p$ signifies that the analyzer is parallel to the polarizer. The total light intensity that emerges from the muscle is the sum of intensities passed by the analyzer when it is anti-parallel (crossed) and parallel to the polarizer. That is, $I_b + I_p = k$, which is independent of the phase shift $\phi$. An immediate consequence is that if $I_b$ changes because of a change of $\phi$, $I_p$ must change equally and in the opposite direction so that the condition $I_b + I_p = k$ is met (Eberstein and Rosenfalck, 1963).

Fig. 7A shows that, when the analyzer was parallel to the polarizer, a positive signal, trace $O_p$, appeared. When the analyzer and polarizer were crossed, the optical signal, trace $O_b$, was in the negative direction. Trace $O_b$ has been electronically inverted in Fig. 7B to make comparison of the time courses easier. The absolute amplification of the signals is identical. The two traces are nearly superimposable, fulfilling the condition of equal and opposite amplitudes to a close approximation. The resting intensities for the two traces were very different, $I_p = 2.5 \times I_b$, so that the relative intensity changes, $\Delta I / I_R$, are not of equal magnitude. The reason that the resting intensities are different is that the muscle retarded less than half the light. Fig. 7 also demonstrates that the birefringence signal is not significantly distorted by early scattering changes. The large deflections in the signals attributed to contraction-related light scattering are in the same direction (increasing intensity; Fig. 7A), which is expected because both signals are from the same optical field (spot) of the muscle and because isotropic absorption and scattering do not depend on the polarization of light. Second, because the amplitude of the scattered-light signal depends on light intensity, trace $O_p$ should experience an early transmission change more than twice as large as trace $O_b$, but the forms of the two traces ($-O_b$ and $O_p$; Fig. 7B) are approximately superimposable through the first two components.

The experiments reported above do not exclude the possibility that a

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1 The optical traces in the figures, which are calibrated as relative changes, $\Delta I / I_R$, are uniformly labeled "O," which distinguishes them from the resting intensities, $I$, and the absolute intensity changes, $\Delta I$, used in the text equations.
A test specific for a change in retardation is shown for a rat ventricular trabecula. The experimental conditions were: 23°C; \([\text{Ca}]_o: 2.4 \text{ mM}\); stimulation rate: 20 min\(^{-1}\). Each trace shown is averaged from 25 sweeps. (A) The muscle was positioned between crossed polarizers for trace \(O_b\) and between parallel polarizers for trace \(O_p\). The second polarizer (analyzer) was turned to achieve the two orientations so that the illumination of the muscle did not change. The resting intensities for the two traces differed by a factor of three. However, the active-signal absolute-intensity changes \((\Delta I)\) are of the same magnitude. Calibrations: \(O_b: 2.1 \times 10^{-3}, (\Delta I/I_b)\); \(O_p: 6.7 \times 10^{-4}\); time bar: 4 ms. (B) From the same preparation, two traces are compared as in A, but one trace \((-O_b)\) has been electronically inverted. The traces are nearly superimposable until the large scattering change distorts the signals. The resting intensities were different by a factor of 2.5. Calibrations: \(-O_b: 7.4 \times 10^{-4}, O_p: 3 \times 10^{-4}\); time bar: 4 ms. Muscle reference number: 13.8.80RV.

dichroic absorption signal could cause light-intensity changes through crossed polarizers. Linear dichroism was therefore measured directly in two guinea pig ventricular trabeculae. The resting dichroism was taken as the ratio of the absorbances for 0° and 90° polarized light, \(A_{0}\)/\(A_{90}\).
The absorbances were determined by the logarithm of the light intensity without the muscle in the light path, \( I_0 \), divided by the intensity through the muscle, \( I_{0\circ} \) or \( I_{90\circ} \). For example \( A_{90\circ} = \log(\frac{I_{0\circ}}{I_{90\circ}}) \). The measurements were made with white light and the average dichroic ratio for each muscle was: muscle 1 (125 \( \mu \)m diam): \( \frac{A_{90\circ}}{A_{0\circ}} = 1.09 \), where \( A_{0\circ} = 0.166 \pm 0.003 \) for two determinations and \( A_{90\circ} = 0.152 \pm 0.002 \) for four determinations; muscle 2 (110 \( \mu \)m diam): \( \frac{A_{90\circ}}{A_{0\circ}} = 1.11 \), where \( A_{0\circ} = 0.222 \pm 0.004 \) for two determinations and \( A_{90\circ} = 0.201 \pm 0.004 \) for three determinations. The difference between resting absorbances for 0° and 90° polarized light was <10%, which indicates a relatively small intrinsic dichroism. An even smaller value has been reported for frog atrial muscle by Bourret and Gargouil (1976).

Experiments were performed to determine whether a dichroic signal occurred within the same time interval as the birefringence signal. A typical example of the results, obtained from muscle 1, is shown in Fig. 8. No obvious signal was (ever) observed, but the data can be analyzed to determine the magnitude of a signal that might be hidden in the noise. For the purpose of comparison with the birefringence signal, the dichroic signal was calculated as the difference between the relative intensity changes of the 0° and 90° transmission signals, \( (\frac{\Delta I}{I})_{0\circ} - (\frac{\Delta I}{I})_{90\circ} \). The maximum dichroic change in the interval between the stimulus and the onset of movement artifact is not greater than \( 5 \times 10^{-5} (\frac{\Delta I}{I}) \), whereas the second component of the birefringence signal measured from the same spot on the muscle achieved an amplitude of \( \sim 10^{-3} (\frac{\Delta I}{I}) \). These results indicate that dichroic changes do not interfere with the retardation measurements by more than 2.5% because the birefringence signal, measured through crossed polarizers, will not be distorted by more than half the relative intensity change of the dichroic signal measured with 0° and 90° polarized light (see Appendix).

First Component of the Birefringence Signal

The first component began during or immediately after the stimulus pulse and reached its maximum amplitude in 1–2 ms in mammalian preparations and in 2–4 ms in frog myocardium. Fig. 9 compares the time course of the birefringence signal from a mammalian atrial trabecula with that of the action potential measured across a sucrose gap from the same preparation. The action potential trace was measured just after the 50 sweeps averaged to obtain the optical trace. The first component coincides with the upstroke of the action potential. In the sucrose-gap apparatus, it was also observed that hyperpolarization of the cell membranes often caused a small birefringence signal of opposite sign compared with that obtained by cell depolarization.

Fig. 2 shows the birefringence signal measured before staining and the action potential-related dye signal after staining the muscle with the voltage-sensitive dye WW444 in both mammalian and frog hearts. The time courses of the first components coincide with the action potential-
FIGURE 8. The birefringence signal (trace $O_b$) is compared with the linear dichroic signal from a guinea pig ventricular trabecula. The $0^\circ$ and $90^\circ$ polarized light transmission signals (traces $O_{0^\circ}$ and $O_{90^\circ}$) were measured from the same optical field of muscle with white light and no analyzer in the light path. The dichroic signal is the difference between traces $O_{0^\circ}$ and $O_{90^\circ}$. It is shown in the Appendix that this difference will be smaller by a factor of 2 if measured with crossed polarizers (as birefringence is). The deflection of the light traces at the extreme left of the figure is the setting of the analog track-and-hold circuit. The experimental conditions were: 26.9°C; $[\text{Ca}]_o$: 2.4 mM; stimulation rate: 50 min$^{-1}$. Tension (trace $T$) and the stimulus artifact (trace $S$) are also shown. Calibrations: $O_b$: $5.8 \times 10^{-4}$ (Δ$F$/F$_0$), 100 sweeps; $O_{0^\circ}$ and $O_{90^\circ}$: $1.1 \times 10^{-4}$, 50 sweeps; $T$: 0.64 mg, 25 sweeps; time bar: 8 ms. Muscle reference number: 25.5.81PV.

related dye signal. This correlation was maintained under conditions such as varied $[\text{Ca}]_o$ and addition of epinephrine. In each preparation, the signals were recorded from the same optical field of tissue. The dye signals were measured with 628-nm nonpolarized light. At this wavelength, depolarization caused an increase in dye absorption. WW444 is very similar to the dye NK2367, which has been studied in frog ventricular preparations under voltage-clamp conditions (Morad and Salama, 1979)
and has been used to measure t-tubular and surface membrane action potentials in skeletal muscle (Nakajima and Gilai, 1980) and membrane potential in nerve (Ross et al., 1977). NK2367 was also used in this study with results similar to those obtained with WW444. WW444 was preferred because it was less sensitive to photobleaching than NK2367, such that, after ≥1 h of continuous illumination, WW444 signals decreased by <20%. Neither dye had significant pharmacological effects on cardiac preparations. There was often a slight increase in peak twitch height (<5%), but no change in time-to-peak tension or the rate of relaxation was observed.

The first component of the birefringence signal was always a decrease in light intensity when the analyzer and polarizer were crossed. Table I shows that for 11 mammalian heart muscles, the mean relative amplitude of the first component was $-2.4 \times 10^{-4}$. In the two experiments where 20 mM KCl was added to the bathing medium, the first component

![Diagram of birefringence signal and action potential](image)

**Figure 9.** The birefringence signal (trace $O_p$) from an elephant seal pup atrial trabecula is compared with the action potential measured as the trans-gap voltage (trace $V_m$) in a sucrose-gap voltage-clamp apparatus. The stimulus artifact is seen on trace $V_m$ as a downward square pulse just before the action potential upstroke. Trace $V_m$ is a single sweep recorded just after the 50 sweeps averaged to obtain trace $O_p$. The dotted line marks the end of the stimulus artifact and shows the coincidence of the action potential upstroke (trace $V_m$) and the first component of the birefringence signal. Experimental conditions were: $O_p$: $2.5 \times 10^{-4}$ ($\Delta l/I_R$); $V_m$: 55 mV; time bar: 8 ms. Muscle reference number: 18.2.81ESA.
became appreciably smaller. These results are consistent with the assertion that the first component represents the rising phase of the action potential.

If the mean relative amplitude of the first component for the four muscles of 90 and 100 μm thickness in Table 1 is computed, its value is $-2.4 \times 10^{-4}$. The value from seven frog ventricular trabeculae of comparable thickness was found to be $-5.0 \times 10^{-4}$. The larger relative amplitude of the first component from frog heart may be related to the

### Table I

**Average Values of the First and Second Components of the Birefringence Signal in Mammalian Heart**

| Muscle number | Thickness | Temperature | Beat rate | First component $\Delta/\Delta t + SD$ (n) | Second component $-\Delta/\Delta t + SD$ (n) | Interval Post-FC E/D |
|---------------|-----------|-------------|-----------|------------------------------------------|------------------------------------------|-------------------|
| 20.4.80 RV    | 100       | 25.0-25.2   | 2.4 50    | 0.6±0.2 (5)                              | 3.1±1.3 (5)                              | 6                 |
| 22.4.80 RV    | 160       | 24.2-24.4   | 2.8 50    | 2.1±0.4 (6)                              | 10.7±4.1 (6)                             | 6                 |
| 19.7.80 RV    | 100       | 23.8-25.0   | 2.4 50    | 4.2±0.6 (9)                              | 8.0±2.0 (9)                              | 6                 |
| 27.8.80 RV    | 230       | 22.9-24.0   | 2.4 50    | 2.1±0.8 (9)                              | 13.9±1.7 (9)                             | 6                 |
| 30.1.81 RV    | 75        | 23.1-23.9   | 2.4 20    | 3.4±1.0 (8)                              | 5.2±0.7 (8)                              | 6                 |
| 1.2.81 RV     | 150       | 21.1-22.6   | 2.4 50    | 1.8±0.3 (5)                              | 3.2±0.5 (5)                              | 6                 |
| 12.7.80 RV    | 90        | 23.2-24.4   | 2.4 20    | 3.9±1.0 (7)                              | 8.8±2.1 (7)                              | 6                 |
| 23.7.80 RV    | 250       | 23.8-24.2   | 2.4 60    | 3.9±0.5 (7)                              | 4.4±1.0 (7)                              | 6                 |
| 21.7.80 RV    | 180       | 24.0-24.8   | 2.4 20    | 1.4 (1)                                  | 15.4 (1)                                 | 6                 |
| (20 mM KCl)   | 2.4 30    | 1.7±0.1 (2) | 16.3±1.6 (2) | 9.6*                                  | 8.9*                                  | 6                 |
| 16.7.80 RV    | 90        | 24.0-25.2   | 2.4 60    | 0.8* (1)                                 | 2.9* (1)                                 | 6                 |
| (20 mM KCl)   | 2.4 30    | 0.6±0.2 (2) | 1.0±0.9 (2) | 1.7*                                  | 3.8*                                  | 6                 |
| 13.8.80 RV    | 150       | 23.4-24.2   | 2.4 30    | 3.9±1.0 (5)                              | 7.7±2.2 (5)                              | 6                 |
| (99.6% D2O)   | 2.4 30    | 3.1±0.6 (4) | 1.5±0.6 (4) | 0.5*                                  | 2.0                                  | 10                |

* Not included in averages.

* Interval post-FC (first component) was measured from the foot of the first-component upstroke.

smaller cell diameter (2–5 μm; Sommer and Johnson, 1969) and hence the greater sarcolemma area per cell volume than that measured in the mammalian heart (10–15 μm diam; Sommer and Johnson, 1969).

**Second Component of the Birefringence Signal**

**Ca²⁺ Dependence** The amount of Ca²⁺ that is released from the SR in mammalian heart muscle during a twitch seems to be related to the extracellular Ca²⁺ concentration (Morad and Goldman, 1973; Allen et al., 1976; Langer, 1965). In contrast, the amount of Ca²⁺ released from the SR in skeletal muscle is relatively insensitive to the [Ca]o (Edman and Grieve, 1964; Armstrong et al., 1972).

The second component of the birefringence signal in the mammalian myocardium was found to be related to the inotropic state of the muscle as influenced by [Ca], and epinephrine but not by muscle rest length (Weiss and Morad, 1981). Fig. 10 shows the experimental traces of the
birefringence signal in normal Ca\(^{2+}\) (2.4 mM) and 0.18 mM Ca\(^{2+}\) Tyrode's solutions. Low \([Ca]_o\) suppressed the rate of development of the second component. Twitch tension and the maximum rate of rise of tension, \(dP/dt_{max}\), were also suppressed.

**Figure 10.** Birefringence signals (traces \(O_b\)), scattered-light signals (traces \(O_s\)), and tension (traces \(T\)) from a rat ventricular trabecula are compared in 2.4 mM and 0.18 mM \([Ca]_o\). Traces \(S\) are the stimulus artifacts. Experimental conditions were: 23.8°C; stimulation rate: 60 min\(^{-1}\). Optical traces are calibrated in terms of relative intensity change (\(\Delta I/I_R\)) and increasing intensity is upwards. The traces are signal averaged as follows: optical traces: 50 sweeps; tension (panel A): 10 sweeps; tension (panel B): 20 sweeps. All traces were sequentially recorded at steady state tension for each experimental condition. The deflection of the optical traces seen at the extreme left of each panel is the settling of the analog track-and-hold circuit. Muscle reference number: 23.7.8ORV.

Fig. 11A shows a plot of log \([Ca]_o\) vs. the relative intensity change of the second component measured 6 ms after the onset of the first component and the maximum rate of tension development, \(dP/dt_{max}\), for three experiments. Within a Ca\(^{2+}\) concentration range of 0.18–4.4 mM, \(\Delta 2nd\)
component increased with increasing [Ca]o. A correlation between ∆2nd component and dP/\(d t_{\text{max}}\) for the same range of Ca\(^{2+}\) for four experiments is shown in Fig. 11B.

**FREQUENCY DEPENDENCE** In most mammalian cardiac muscle (including guinea pig), the frequency of stimulation has a pronounced influence on the magnitude of twitch tension. Increasing the stimulation frequency generally causes greater twitch tension and a faster rate of rise of tension. This effect is thought to be related to an increase in intracel-

![Graph](image)

**FIGURE 11.** (A) Log [Ca]o is plotted against the relative intensity change of the second component, ∆2nd component, measured 6 ms after the foot of the action potential upstroke-related first component of the birefringence signal (open symbols) and the maximum rate of tension development dP/\(d t_{\text{max}}\) (closed symbols). The data are from three experiments on rat ventricular trabeculae. The graph points are normalized to the mean control value for each experiment in conditions of 2.4 mM [Ca]o and a stimulation rate of 30 min\(^{-1}\). The curve was drawn by hand. (B) ∆2nd component (ordinate) is plotted against dP/\(d t_{\text{max}}\) (abscissa) for a range of [Ca]o from 0.18 to 5.0 mM. Four experiments on rat ventricular trabeculae are shown, two of which were also plotted in A. Graph points are normalized to the mean control value for each experiment in conditions of 2.4 mM [Ca]o and a stimulation rate of 30 min\(^{-1}\).

From this comparative perspective, the effect of the frequency of stimulation on the second component and on development of tension in...
rat and guinea pig ventricular trabeculae was investigated. Fig. 12 shows a graph of frequency vs. Δ2nd component (open symbols) and $dP/dt_{\text{max}}$ (filled symbols) for the two preparations. The data are normalized to the mean values for a stimulation rate of 60 min$^{-1}$. The values were determined from at least two measurements at each frequency except for the point at 84 min$^{-1}$, which was a single measurement and for which $dP/dt_{\text{max}}$ was not determined. The effect of frequency on the second component was markedly different in rat and guinea pig myocardium. In rat heart, a change in frequency from 20 to 84 min$^{-1}$ produced only a small increase in Δ2nd component (≈15%), whereas in guinea pig heart, a frequency change from 30 to 60 min$^{-1}$ increased Δ2nd component significantly (≈100%). Although changes in $dP/dt_{\text{max}}$ in the two preparations were comparable, peak tension (not shown in Fig. 12) increased by 50% in guinea pig preparations and decreased slightly (<10%) in the rat heart over the same frequency interval.

**TEMPERATURE DEPENDENCE** Temperature was found to have pronounced effects on the rate of change of the second component. Fig. 13
is an Arrhenius plot of the rate of change of the second component, $dSC/dt$, measured before contraction and $dP/dt_{\text{max}}$ for a temperature range of 22.4 to 30.8°C. The $Q_{10}$ for temperature differences $>4^\circ\text{C}$ was calculated from the equation

$$Q_{10} = \frac{k_2}{k_1} \left[ \frac{T_2}{T_2 - T_1} \right]^{10(T_2 - T_1) / (T_2 + 10)},$$

where $k_2$ and $k_1$ were taken as the rate of change of the second component, $dSC/dt$, at temperatures $T_2$ and $T_1$, respectively (measured in °K). The mean ± standard deviation for six determinations of the $Q_{10}$ was 2.01 ± 0.33. These data suggest that a process other than simple diffusion may be responsible for the second component of the birefringence signal in mammalian myocardium.

**SPECIES DEPENDENCE: MAMMALIAN MYOCARDIUM** Birefringence signals from heart muscle of several mammalian species, including rat, guinea pig, cat, and elephant seal, were investigated. The second component was seen in all mammalian heart preparations examined. Experiments were carried out primarily on rat ventricular muscle because this preparation is known to have abundant SR (Page et al., 1971).

Atrial preparations from guinea pig and cat hearts were studied because it is known that both of these preparations have little or no t-tubular
system (McNutt and Fawcett, 1965; Sperelakis and Rubio, 1971). Signals from atrial preparations (Fig. 14, left panel) were comparable to the signals from ventricular muscles (Figs. 2A and 5A). Thus, it appears that the second component is not related to the presence or absence of a t-system in mammalian myocardium.

**SPECIES DEPENDENCE: FROG MYOCARDIUM** Birefringence measurements were also made in frog heart because this preparation is known to have less abundant and more rudimentary SR than mammalian heart (Staley and Benson, 1968; Sommer and Johnson, 1969; Page and Niedergerke, 1972). In addition, electromechanical studies have failed to show significant Ca\(^{2+}\) release from intracellular pools. Rather, activator Ca\(^{2+}\) seems to enter the cell during depolarization from an extracellular site (Morad and Goldman, 1973; Fabiato and Fabiato, 1978; Chapman, 1979; Boyett and Jewell, 1980). Consistent with this idea is the fact that the birefringence signal in frog heart had only a single component that was related to the surface membrane action potential (Weiss and Morad, 1981). The absence of the second component in the frog heart was consistently observed in all atrial and ventricular trabeculae examined (12 preparations; see also Fig. 2).

Several manipulations that are known to affect E-C coupling in mammalian myocardium via effects on the SR were applied to frog heart muscles in an attempt to cause some change in the birefringence signal.
and possibly to unmask a second component. Reducing [Ca]₀ prolonged the motion-free interval following the action potential upstroke (8–10 ms under normal conditions) but caused no change in the form of the birefringence signal (Fig. 15A). Epinephrine, though it potentiated and

![Figure 15](image)

**Figure 15.** Optical signals from frog myocardium are shown in the presence of varying [Ca]₀ and epinephrine. Experimental conditions were: 22–24°C; stimulation rate: 20 min⁻¹. Optical traces are calibrated in terms of relative intensity change and increasing intensity is upwards. (A) Birefringence (traces O₀) and scattered-light signals (traces O₁) from a frog atrial trabecula are shown in 1 mM (upper) and 0.2 mM (lower) [Ca]₀. The scattered-light traces show that lowering [Ca]₀ lengthens the delay between the action potential upstroke-related single birefringence component and the onset of contraction. Traces O₀ and O₁ in 0.2 mM [Ca]₀ were averaged from 50 and 20 sweeps, respectively. The traces in 1.0 mM [Ca]₀ were 25 sweeps each. Muscle reference number: 27.4.80FA. (B) Birefringence signals from a frog ventricular trabecula were recorded in different [Ca]₀. A trace in the presence of 10⁻⁸ M epinephrine (from the frog atrial trabecula in A) is also shown for comparison. Both interventions appear to affect the optical signal by changing the delay between the action potential upstroke-related first component and the onset of contraction. Each trace is averaged from 50 sweeps. Muscle reference number: 30.4.80FV.

accelerated the onset of tension (Fig. 15B), also had no effect on the birefringence signal in the frog heart.

Caffeine also potentiates peak tension in the frog heart. In frog atrium, the positive inotropic effect has both a transient and a steady state phase.
The difference between the transient and steady state effect has been interpreted as evidence for the presence of an internally releasable calcium pool (Niedergerke and Page, 1981). Experiments were performed to see whether caffeine would cause a change in the birefringence signal in frog atrial trabeculae. Fig. 16 shows that 5 mM caffeine caused a transient

Figure 16. Birefringence signals (trace $O_b$) and tension from a frog atrial trabecula are shown in control conditions (A) and during the initial transient phase of the positive inotropic effect of 5 mM caffeine (B and C). The scattered-light signal (trace $O_s$) is shown for control conditions (A). Tension (traces $T$) and stimulus artifacts (traces $S$) are shown in both panels. The deflections of the optical traces seen at the extreme left of the panels is the settling of the analog track-and-hold circuit. (A) Control conditions were: 23.5°C; [Ca]$^+$: 1 mM; stimulation rate: 20 min$^{-1}$. Calibrations: $O_b$: $10^{-3}$ ($\Delta I/I_{R}$), 5 sweeps; $O_s$: $5.5 \times 10^{-4}$, 10 sweeps; $T$: 0.12 mg; time bar: 8 ms. (B) The birefringence signal was averaged for 10 sweeps during the transient phase of positive inotropy (i.e., the beats between the arrows in C) after addition of 5 mM caffeine to the bathing medium. Tension (trace $T$) was measured during a subsequent caffeine application (the effect was repeatable). Calibrations: $O_b$: $7.2 \times 10^{-4}$, 10 sweeps; $T$: 0.24 mg, 10 sweeps; time bar: 8 ms. (C) The tension recording from a strip-chart recorder showing the initial transient and later steady effect of 5 mM caffeine on twitch tension. Trace $O_b$ in B was obtained during the interval marked by arrows. The vertical calibration is 9.1 mg and the time bar is 1 min. Muscle reference number: 11.8.80FA.
increase in peak tension that lasted ~10–15 beats before decaying to a new steady level that was greater than control (Fig. 16C). In each of four muscles tried, the birefringence signal measured during this transient potentiated phase showed no changes in form or amplitude (Fig. 16B). Higher caffeine concentrations (up to 10 mM) also caused no pre-contraction changes in the birefringence signals in frog myocardium.

**Pharmacological Interventions on Mammalian Heart**

Several agents that are known to have profound effects on contractile force and are thought to modulate the activity of the SR in cardiac muscle were investigated for their effects on the second component of the birefringence signal.

**Epinephrine** In guinea pig ventricular (Fig. 17) and atrial muscle, epinephrine consistently facilitated the second component, \( \frac{dP}{dt_{\text{max}}} \), and peak tension. Fig. 18 shows a graph of \( \frac{dP}{dt_{\text{max}}} \) vs. \( \Delta 2nd \) component from guinea pig and rat ventricular trabeculae. In rat ventricular preparations, epinephrine had variable effects on peak tension, although \( \Delta 2nd \) component and \( \frac{dP}{dt_{\text{max}}} \) generally increased (Fig. 18 and Table II). Epinephrine also caused the onset of the second component to occur earlier. This was especially evident in several rat myocardial preparations, where the rate of change of the second component, \( dSC/\text{dt} \), did not change significantly, but \( \Delta 2nd \) component did. The maximum amplitude of the second component before the onset of the contraction artifact was frequently smaller in the presence of catecholamine, and this appeared to correlate with the earlier deflection of the scattered-light signal and the rise in measurable twitch tension. Epinephrine consistently potentiated the second component in other mammalian preparations, which included atrial muscles of cats and elephant seals and ventricular muscles of cats.

**Caffeine** In mammalian cardiac preparations, caffeine decreases the rate of twitch relaxation, increases peak tension (De Gubareff and Sleator, 1965), suppresses post-extrasystolic potentiation (Blinks et al., 1972; Henderson et al., 1974), and suppresses post-depolarization potentiation in voltage-clamped preparations (Ohba, 1973). In isolated SR vesicle preparations, caffeine reduces the rate of uptake and the capacity of the SR for Ca\(^{2+} \) (Weber and Herz, 1968; Weber, 1968). The effect of caffeine on the SR provides a possible mechanism by which most of its effects on contraction may be explained (Blinks et al., 1972; Henderson et al., 1974).

Fig. 14 shows that caffeine completely suppressed the second component of the birefringence signal before the onset of contraction in a guinea pig atrial trabecula. The action potential-related first component was not noticeably affected by the presence of caffeine. In this experiment, caffeine increased both peak tension (170% of control) and the rate of tension development, but did not alter the time to peak of twitch tension significantly. The scattered-light signal indicates that caffeine caused the onset of contraction to occur ~1.7 ms earlier than in control conditions, which raised the possibility that an earlier movement artifact might
interfere with the second component. Inspection of the birefringence traces in Fig. 14 shows that the relative intensity change of the second component of the control trace is about twice as large as that in the presence of caffeine for the same interval after the first-component upstroke and before the onset of contraction. This suppressant effect of caffeine was consistently seen in all mammalian preparations tested, including guinea pig and rat ventricular trabeculae and elephant seal atrial trabeculae. The effects of caffeine on tension and second component were reversible.

Figure 17. Optical traces and tension from a guinea pig ventricular trabecula are shown in control conditions and in the presence of $10^{-6}$ M epinephrine. Control conditions were: $21.4^\circ C$; $[Ca]$; $2.4$ mM; stimulation rate: $30$ min$^{-1}$. Optical traces are calibrated in terms of $\Delta I/I_R$ and increasing intensity is upwards. Birefringence signals (traces $O_b$) were averaged from 50 sweeps each and scattered-light signals (traces $O_s$) and tension records (traces $T$) were averaged from 25 sweeps each. Trace $S$ is the stimulus artifact. Muscle reference number: 3.2.81GPV.
Weiss and Morad  Birefringence Signals in Mammalian and Frog Myocardium

Discussion

The major finding of this paper is that an intrinsic birefringence signal can be measured in mammalian and frog myocardium before the onset of contraction. In mammalian heart, the birefringence signal has two components that, on the basis of their physical characteristics, appear to arise from changes in retardation. Other types of optical changes within the muscle, such as light scattering and linear dichroism, do not appear to influence the birefringence signal significantly. The findings suggest that the first component of the birefringence signal in mammalian heart and the single component in frog heart result from the upstroke of the action potential of the surface membrane. The second component of the birefringence signal that was present only in mammalian heart seems to be related to the Ca\(^{2+}\)-releasing activity of the SR. Other possibilities that...
may be responsible for the generation of the second component, such as
a change of membrane potential across the SR membrane (Baylor and
Oetliker, 1977c) or conformational changes of the Ca-ATPase in response
to Ca²⁺ release (Weiss and Morad, 1981; Poledna et al., 1982), will also
be discussed.

Physical Characteristics of the Birefringence Signal

The possibility that linear dichroism might give rise to or contaminate
the birefringence signal was investigated. Attempts to measure a linear
dichroic signal directly showed that such a signal could not be larger than
the background noise levels. In the experiment shown in Fig. 8, the
background noise was of the order of 5 × 10⁻⁵ (ΔI/I₀). To compare the
linear dichroic signal with the birefringence signal (since measurement of
each signal involved a different arrangement of the optical elements of
the microscope), a calculation was made that is presented as an appendix.
The mathematical analysis shows that a linear dichroic signal measured
by the method used in Fig. 8 would be attenuated by a factor of 2 if
observed by the arrangement of crossed polarizers. Therefore, linear
dichroism does not contribute a relative intensity change of >2.5 × 10⁻⁵
to the first or second components in cardiac muscle.

Both the first and second components of the birefringence signal were
found to behave as changes in retardation. The test for retardation was

### Table II

Effect of Frequency, Epinephrine, and Caffeine on the Second Component of the Birefringence Signal

| (A)  | (B)   | (C)  | (D)   | (E) | (F)  | (G)   | (H)     |
|------|-------|------|-------|-----|------|-------|---------|
| Muscle number | Muscle diameter | Temperature | Conditions: beat rate | Drug | Number of measurements | Interval* | ASC ±SD ΔSC/Δt ±SD (Δ²/I₀) | ASC ΔSC | ASC ΔSC | ASC ΔSC |
|      | μm    | °C   | min⁻¹ |     | ms  |           | (-Δ²/I₀) | (-Δ²/I₀) | (-Δ²/I₀) | (-Δ²/I₀) |
| 21.7.81RV | 180   | 23.2-24.8 | 30 ND | 2 6 | 16.3±1.6 | - | 6.3±0.3 | - | - | - |
| 3.2.81GPV | 160   | 21.0-22.0 | 60 ND  | 6 8 | 19.5±0.7 | +18 | 8.0±0.2 | +27 | +27 | +27 |
| 30.1.81RV | 75    | 23.0-24.0 | 120 ND | 2 6 | 22.7±0.2 | +39 | 7.7±0.2 | +22 | +33 | +33 |
| 8.12.81RV | 100   | 21.0-22.0 | 60 ND  | 5 8 | 0.7±0.2 | - | 0.9±0.2 | - | - | - |
| 3.2.81GPV | 160   | 21.3-22.0 | 60 ND  | 5 8 | 1.6±0.1 | +129 | 0.9±0.3 | 0 | +156 | +156 |
| 8.12.81RV | 100   | 21.0-22.0 | 30 ND  | 3 6 | 2.5×10⁻⁴ M epi | 3 | 5 | 6.4±0.9 | +129 | 4.0±1.0 | +38 | +128 |
| 30.1.81RV | 75    | 23.0-24.0 | 20 60 ND | 6 5 | 2.8±1.0 | - | 2.9±0.7 | - | - | - |
| 8.12.81RV | 100   | 21.0-22.0 | 30 ND  | 3 6 | 1.0×10⁻⁴ M epi² | 1 | 6 | 7.4 | +139 | 5.4 | +15 | +100 |
| 30.1.81RV | 75    | 23.0-24.0 | 120 ND | 4 8 | 0.9±0.2 | - | 1.0±0.3 | - | - | - |
| 8.12.81RV | 100   | 21.0-22.0 | 50 ND  | 5 6 | 1.0×10⁻⁴ M epi² | 2 | 8 | 4.6±0.1 | +411 | 1.8±0.1 | +80 | +184 |
| 8.12.81RV | 100   | 21.0-22.0 | 30 ND  | 5 6 | 5 mM caffeine | 1 | 6 | 3.1±1.0 | - | 4.7±0.3 | - | - |
| 8.12.81RV | 100   | 21.0-22.0 | 50 ND  | 5 6 | 5 mM caffeine | 1 | 6 | 0.4 | - | 95 | 0.3 | - | - | - |

ΔSC = ASC (Δ²/I₀) (see Methods).

ΔSC/Δt = maximum rate of change (slope) of the second component measured during the measured time interval specified
for the particular muscle.

RV, rat ventricular muscle.

GPV, guinea pig ventricular muscle.

ND, no drug.

* This experiment was performed in 1.2 mM extracellular Ca²⁺.

ΔSC = ASC (Δ²/I₀) (see Methods).

ΔSC/Δt = maximum rate of change (slope) of the second component measured during the measured time interval specified
for the particular muscle.

RV, rat ventricular muscle.

GPV, guinea pig ventricular muscle.

ND, no drug.

* This experiment was performed in 1.2 mM extracellular Ca²⁺.
that the signal inverts symmetrically when the polarizers are changed from a crossed to a parallel configuration (Fig. 7; Eberstein and Rosenfalck, 1963). This test is valid for a field of nonuniform retardation and for white light and was convenient for our experimental setup.

Relation of the First Component to the Action Potential

The first component of the birefringence signal was found to have the same time course as the upstroke of the action potential recorded with membrane-impermeant, voltage-sensitive dyes (Fig. 2) or across a sucrose gap (Fig. 9). Changes in the retardation have been previously correlated with surface membrane potential changes in nerve (Cohen et al., 1968, 1969, 1970, 1971) and skeletal muscle (Baylor and Oetliker, 1975, 1977c). The extent to which the t-system might contribute to the first component in ventricular muscle could not be determined from our experiments. However, the action potential-related first components were recorded from atrial muscle of guinea pigs and atrial and ventricular preparations of frogs, all of which lack t-systems (Sommer and Johnson, 1969; Sperelakis and Rubio, 1971). In rat ventricular muscle, the t-system increases the surface membrane area by \( \sim 25\% \) (Page et al., 1971) so that, even if the transverse tubules produce a signal, it might be difficult to distinguish the relative contribution to the total signal. The result from studies on skeletal muscle, which may well apply here, is that the t-system does not contribute appreciably to the first component of the birefringence signal (Baylor and Oetliker, 1977c).

Relation of the Second Component to E-C Coupling

Studies of birefringence signals in skeletal muscle led to the suggestion that the second component is related to an early step in E-C coupling (Baylor and Oetliker, 1975, 1977a, b, c). We sought to test this hypothesis in cardiac muscle, where the release of Ca\(^{2+}\) from the SR is sensitive to species differences as well as physiological and pharmacological interventions (Morad and Goldman, 1973). In mammalian species, the magnitude of the second component, measured before the onset of contraction, was compared with the maximum rate of rise of twitch tension, \( dP/dt_{\text{max}} \) (Weiss and Morad, 1981), which was taken as a measure of Ca\(^{2+}\) released into a cell during a contractile cycle.

Stimulation rate, epinephrine, and temperature all affected the second component and \( dP/dt_{\text{max}} \) in a parallel fashion (Figs. 12, 13, and 18). Changing [Ca]\(_{\text{c}}\) or stimulation rate is thought to alter the level of Ca\(^{2+}\) in the SR of mammalian myocardium (Langer, 1965; Morad and Goldman, 1973; Allen et al., 1976; Allen and Kurihara, 1980). These results can be contrasted with the effect of muscle rest length on the second component, which showed that a change in rest length sufficient to alter \( dP/dt_{\text{max}} \) by 60% produced little or no change in the second component (Weiss and Morad, 1981).

Two conclusions can be drawn from these data: (a) the second compo-
nent is highly sensitive to alteration of contractile state induced by Ca\textsuperscript{2+} but not by muscle rest length; (b) the birefringence signal is not associated with an artifact related to contraction (Weiss and Morad, 1981).

Further data supporting an SR-related source for the second component come from the frog heart, where the physiological and ultrastructural studies have shown a sparse SR and an absence of releasable Ca\textsuperscript{2+} stores (Page and Niedergerke, 1972; Chapman, 1979; Morad and Goldman, 1973; Morad and Orkand, 1971; Fabiato and Fabiato, 1978). In the frog heart, the second component was never observed under any physiological or pharmacological conditions (Figs. 2, 3, 15, and 16; Weiss and Morad, 1981). Interventions, such as epinephrine, caffeine, alteration of [Ca\textsubscript{i}], and frequency of stimulation, which had marked effects on the birefringence signal in mammalian heart, failed to affect the single-component birefringence signal in the frog heart. Thus, comparative as well as ionic and pharmacological studies suggest that the second component is related to the Ca\textsuperscript{2+}-releasing activity of the SR.

The suppression of the second component (Fig. 14) and the marked alterations in the kinetics of twitch tension development in the mammalian heart in the presence of caffeine (De Gubareff and Sleator, 1965; Blinks et al., 1972; Henderson et al., 1974; Ohba, 1973) seem to transform the E-C coupling characteristics of the mammalian heart into those of the frog heart.

Possible Mechanisms for the Generation of the Second Component

Two mechanisms were considered as possible sources of the second component of the birefringence signal. One possibility was that the second component arises from a change of potential across the SR membrane. Although we were unable to measure reliable signals from mammalian heart muscle with membrane-permeable, voltage-sensitive dyes (Nile Blue A and several indodicarbocyanine dyes; Weiss and Morad, unpublished observation), evidence from skeletal muscle studies shows similarity between the time courses of the second component and the signals of voltage-sensitive dyes (Oetliker et al., 1975; Baylor et al., 1981).

A second mechanism considered was that the second component arises from Ca\textsuperscript{2+} binding to the Ca\textsuperscript{2+} transport molecules of the SR. In support of this idea is the finding that voltage-sensitive dyes can generate signals related to Ca\textsuperscript{2+} binding to the Ca\textsuperscript{2+}-ATPase in isolated SR vesicles (Russel et al., 1979a, b; Oetliker, 1980) or to Ca\textsuperscript{2+} binding to purified Ca\textsuperscript{2+}-ATPase in isolated SR vesicles (Oetliker, 1980). This possibility is consistent with our findings, particularly regarding the caffeine suppression of the second component in mammalian heart muscle. It can be proposed that caffeine inhibits Ca\textsuperscript{2+} uptake by the SR (Weber and Herz, 1968; Weber, 1968) and the second component of the birefringence signal by affecting the Ca\textsuperscript{2+}-ATPase molecules or other membrane sites (Ca\textsuperscript{2+} binding to low-affinity sites on SR vesicles have been implicated in several voltage-sensitive dye signals; Russel et al., 1979a, b). Because the second
component is absent in frog heart, if the second component in mammalian heart is related to Ca\(^{2+}\) binding to the SR, it can be suggested that Ca\(^{2+}\) uptake processes in the frog heart must be mediated differently than in the mammalian heart. In support of such an idea, a Na-Ca countertransporter has been suggested to mediate the relaxation process in the frog heart (Morad, 1982; Chapman, 1979).

APPENDIX

This appendix offers a treatment for the quantitative comparison of light signals measured as birefringence and linear dichroism. Dichroic changes are measured as the difference between the relative intensity changes of transmitted light with incident polarization 0° and 90° to the muscle longitudinal axis. We wish to calculate how large a dichroic signal would appear if measured by the technique used to detect birefringence signals, i.e., if measured from a muscle placed between two crossed polarizers at an azimuthal angle \(\psi = 45^\circ\). The simplifying assumption is made that resting absorption by the muscle is isotropic, i.e., resting dichroic absorption is nil.

Fig. 19 is a diagram in which the X and Y axes represent, respectively, the 90° (transverse) and 0° (longitudinal) axes of the muscle. Referring to the diagram, the linear dichroic signal, \(\Delta D\), can be described by the equation,

\[
\Delta D = \frac{\Delta I_x}{I_x} = \frac{I'_x - I_x}{I_x} = \frac{I'_y - I_y}{I_y},
\]

(1)

**Figure 19.** The Y axis is the muscle longitudinal axis. For the measurement of the birefringence signal the incident light (\(E\)) is polarized along the \(p\) axis, which forms an angle \(\psi = 45^\circ\) with the Y axis. The second polarizer (analyzer) is oriented along the \(b\) axis (\(\psi = -45^\circ\)). For the measurement of linear dichroism, the incident light is polarized along the X and Y axes and there is no analyzer in the light path.
where $I_x$ and $I_y$ are the resting light intensities through the muscle and $I'_x$ and $I'_y$ are the intensities after the dichroic change. Dichroic signals are treated here as arising from a change in absorption for light polarized along the transverse axis of the muscle (the $X$ axis in Fig. 19) so that $\Delta I_x/I_y = 0$. The result of the following analysis is the same, however, whether the absorption change is taken to occur along the longitudinal axis or along both axes with different magnitudes.

The attenuation of a beam of polarized light passing through a muscle can be described by the electric vector of the light, $\vec{E}$, and an absorption factor, $e^{-d\gamma}$, where $d$ is the light path through the muscle and $\gamma$ is the coefficient of absorption. For incident light, with polarization direction transverse (90°) to the muscle longitudinal axis, the electric vector of light emerging from the muscle will be

$$\vec{E}_x = E_o \cos \omega t \cdot e^{-d\gamma \cdot \hat{x}},$$

(2)

where $E_o$ is the amplitude of the wave and $\hat{x}$ is the unit vector of the $X$ axis (see Fig. 19). Because of the birefringent quality of the muscle, the expression for the emerging electric vector of 0° polarized light includes a term, $\phi$, describing the phase lag,

$$\vec{E}_y = E_o \cos(\omega t - \phi) \cdot e^{-d\gamma \cdot \hat{y}}.$$

(3)

Since light intensity is equal to the square of the electric vector averaged over one period, the resting light intensity through the muscle will be

$$I_x = I_y = \frac{1}{2\pi} \int_0^{2\pi} |\vec{E}_x|^2 d\omega t = \frac{1}{2} E_o^2 e^{-d\gamma}.$$

(4)

If a change in the absorption coefficient, $\Delta \gamma$, occurs only for the 90° polarized light, the emerging electric vector becomes

$$\vec{E}'_x = E_o \cos \omega t \cdot e^{-d(\gamma + \Delta \gamma) \cdot \hat{x}}.$$

(5)

The intensity is then

$$I'_x = \frac{1}{2} E_o^2 e^{-2d(\gamma + \Delta \gamma)}.$$

(6)

Combining Eqs. 4 and 6 into Eq. 1 gives a linear dichroic signal

$$\Delta D = e^{-2d\Delta \gamma} - 1.$$  

(7)

The right side of Eq. 7 may be expanded by the series $e^x = 1 + x + x^2/2! + \ldots$. Terms higher than the first order are eliminated because the relative intensity changes in muscle are small, i.e., $<10^{-3}$. The dichroic signal is therefore

$$\Delta D \approx 1 - 1 - 2d\Delta \gamma \approx -2d\Delta \gamma.$$  

(8)

The next step is to examine how the change in the absorption coefficient, $\Delta \gamma$, for light polarized along the $X$ axis would affect the light measured through crossed polarizers at an azimuthal angle $\psi = 45°$ to the muscle, i.e., in the manner commonly used for birefringence. The incident light, which has a polarization direction parallel to the $P$ axis in Fig. 19, is first separated into $x$ and $y$ components and the electric vectors of the light emerging from the resting muscle are obtained in the same way as Eqs. 2 and 3 so that

$$\vec{E}_x = E_o \cos \omega t \cdot e^{-d\gamma \sin \psi \cdot \hat{x}}$$

(9)

and

$$\vec{E}_y = E_o \cos(\omega t - \phi) \cdot e^{-d\gamma \cos \psi \cdot \hat{y}}.$$  

(10)
The light that reaches the detector passes through the second polarizer (analyzer) with a polarization direction parallel to the B axis in Fig. 19. The electric vector is

$$E_b = E_{b_x} + E_{b_y}$$

$$= E_o \cos \psi \cdot e^{-d\gamma} \sin \psi \cos \psi \cdot \hat{b} - E_o \cos(\omega t - \phi) \cdot e^{-d\gamma} \cos \psi \sin \psi \cdot \hat{b}$$

(11)

where $E_{b_x}$ and $E_{b_y}$ are the projections of $E_x$ and $E_y$ onto the B axis. Note that $E_{b_x}$ and $E_{b_y}$ have different directions and therefore different signs and that $\psi$ is always 45°. The intensity of light passed by the analyzer is then obtained by integration (as Eq. 4).

$$I_b = (E_o/2)^2 e^{-2d\gamma}(1 - \cos \phi).$$

(12)

If a change in the absorption coefficient, $\Delta \gamma$, occurs for light polarized along the X axis, the electric vector $E_{b_x}$ is altered and the emerging electric vector becomes

$$E'_b = \frac{1}{2}E_o e^{-d(\gamma + \Delta \gamma)} \cos \omega t - e^{-d\gamma} \cos(\omega t - \phi) \cdot \hat{b}.$$  

(13)

Integrating to obtain the intensity after the absorption change,

$$I'_b = \frac{1}{2}(E_o/2)^2 e^{-2d\gamma} [1 + e^{-2d\gamma} - 2e^{-d\gamma} \cos \phi].$$

(14)

The relative intensity change can then be written by combining Eqs. 12 and 14:

$$\frac{\Delta I_b}{I_b} = \frac{(I'_b - I_b)}{I_b}$$

$$= \frac{\left(\frac{1}{2}\right) \left[ 1 + e^{-2d\gamma} - 2e^{-d\gamma} \cos \phi + 2\cos \phi \right]}{(1 - \cos \phi)}.$$

(15)

The exponential terms can be expanded by the series $e^x = 1 + x + x^2/2! + \ldots$, to give a first-order approximation as was done with Eq. 8. The result is

$$\frac{\Delta I_b}{I_b} \approx -d\Delta \gamma.$$  

(16)

Comparison of Eqs. 8 and 16 suggests that a dichroic signal measured as the relative intensity change through crossed polarizers at an azimuthal angle $\psi = 45°$ will be half as large as when measured as the difference between the $0°$ and $90°$ transmission signals. Thus, Fig. 8 indicates that, since the dichroic signal measured before the onset of movement is no greater than $5 \times 10^{-5}$, it is unlikely to contribute appreciably with the birefringence, which attains a relative magnitude of $10^{-3}$ within that interval.

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