Changes in Phenol Red Absorbance in Response to Electrical Stimulation of Frog Skeletal Muscle Fibers

S. HOLLINGWORTH and S. M. BAYLOR

From the Department of Physiology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104-6085

ABSTRACT Intact single twitch fibers from frog muscle were stretched to long sarcomere length, micro-injected with the pH indicator dye phenol red, and activated by action potential stimulation. Indicator-related absorbance changes (denoted by \( \Delta A_0 \) and \( \Delta A_{90} \)) were measured with 0° and 90° polarized light (oriented, respectively, parallel and perpendicular to the fiber axis). Two components of \( \Delta A \) were detected that had generally similar time courses. The "isotropic" component, calculated as the weighted average \( (\Delta A_0 + 2 \Delta A_{90})/3 \), had the wavelength dependence expected for a change in myoplasmic pH. If calibrated in pH units, this signal's peak amplitude, which occurred 15-20 ms after stimulation, corresponded to a myoplasmic alkalization of average value \( 0.0025 \pm 0.0002 \) (±SEM; \( n = 9 \)). The time course of this change, as judged from a comparison with that of the fibers' intrinsic birefringence signal, was delayed slightly with respect to that of the myoplasmic free \([Ca^{2+}]\) transient. On average, the times to half-peak and peak of the phenol red isotropic signal lagged those of the birefringence signal by \( 2.4 \pm 0.2 \) ms (±SEM; \( n = 8 \)) and \( 8.4 \pm 0.5 \) ms (±SEM; \( n = 4 \)), respectively. The other component of the phenol red signal was "dichroic," i.e., detected as a difference \( (\Delta A_0 - \Delta A_{90} > 0) \) between the two polarized absorbance changes. The wavelength dependence of this signal was similar to that of the phenol red resting dichroic signal (Baylor and Hollingworth, 1990. J. Gen. Physiol. 96:449-471). Because of the presence of the active dichroic signal, and because \(~80\%\) of the phenol red molecules appear to be bound in the resting state to either soluble or structural sites, the possibility exists that myoplasmic events other than a change in pH underlie the phenol red isotropic signal.

INTRODUCTION

The experiments described in this and the accompanying papers (Baylor and Hollingworth, 1990; Pape et al., 1990) were undertaken to study possible rapid
changes in cytoplasmic pH ($\Delta p$H) during excitation–contraction (E-C) coupling in frog skeletal muscle fibers. In an earlier publication (Baylor et al., 1982b), the possible existence of a small and transient alkalization of myoplasm ($\Delta p$H = +0.004; time-to-peak, 20–30 ms following a single stimulated action potential) was suggested from one experiment in which a fiber had been injected with the pH indicator, phenol red. The principal aim of the experiments of this article was to study the properties of the myoplasmic absorbance change(s) detectable from phenol red, to see if a reproducible component of the dye signal could be identified as a myoplasmic pH transient.

This paper confirms the existence of a possible pH transient reported by phenol red. In response to a single action potential, the peak amplitude of the “isotropic” component of the absorbance change had the wavelength dependence of a pH difference spectrum and, if calibrated in pH units, had an average value of $+0.0025 \pm 0.0002$ (±SEM; $n = 9$). The relatively fast time course of this component, the rising phase of which followed closely after that of the myoplasmic free $[Ca^{2+}]$ transient ($\Delta [Ca^{2+}]$), suggests that this signal reflects an event closely related to the E-C coupling process.

The experiments also revealed a “dichroic” component of the phenol red absorbance change, detectable as the difference between absorbance changes measured with $0^\circ$ and $90^\circ$ polarized light. Although the time course of this component was similar to that of the possible pH transient from myoplasm, its spectral and polarization properties indicate that other (non-pH) mechanisms are involved in its generation. It is possible, however, that the events that underlie the dichroic signal also contribute optically to the apparent pH change reported by the isotropic signal. For this and other reasons, the attractive interpretation that the isotropic signal simply reflects a myoplasmic alkalization must be considered tentative. Other experiments, designed to discriminate between the event(s) that might give rise to these phenol red signals from myoplasm, are described in the following article (Pape et al., 1990).

A summary of some of the conclusions has been published previously in abstract form (Baylor et al., 1987).

**METHODS**

The preceding paper (Baylor and Hollingworth, 1990) described the procedures for fiber preparation, phenol red injection, and measurement of dye absorbance from single frog twitch fibers in the resting state. The general methods for data collection and analysis of signals that result from action potential stimulation have also been described (Baylor et al., 1986; Baylor and Hollingworth, 1988). Briefly, the fibers, which were stretched to long sarcomere length (3.6–4.2 μm) and bathed in a Ringer's solution maintained at 16–17°C, were stimulated by supra-threshold shocks from a pair of extracellular electrodes positioned near the site of dye injection. Changes in fiber optical properties (absorbance or birefringence; see below) and fiber tension were recorded simultaneously by a PDP 11 computer (Digital Equipment Corp., Marlboro, MA) through an analogue-to-digital converter board (model DT1711; Data Translation Inc., Marlboro, MA).
Types of Signals Measured

Absorbance changes. For these measurements, a small region of fiber near the site of dye injection was transilluminated with unpolarized, quasi-monochromatic light that had been focused to a small spot (diameter, 30-73 μm) or slit (50-90 μm wide, 300 μm long) contained within the fiber width. The wavelength was selected by interference filter from a set of either “narrow band” (10-nm band pass) or “wide band” (30-nm band pass) filters. The following equations were used to relate fractional changes in transmitted light intensity (ΔI/I) to dye-related absorbance changes:

\[ \Delta A_T(\lambda) = -\frac{\Delta I(\lambda)/I(\lambda)}{\log_{10}} \]  
\[ \Delta A_o(\lambda) = (\lambda_{ref}/\lambda)^X \Delta A(\lambda_{ref}) \]  

In Eq. 1, \( \Delta A_T \) denotes the total absorbance change of the fiber at the wavelength \( \lambda \) of peak transmittance by the filter; this change includes contributions from both fiber intrinsic absorbance (denoted \( \Delta A \)) and dye-related changes (denoted \( \Delta A \)). Eq. 2 gives an empirical method for estimation of \( \Delta A \), at the dye-related wavelength, \( \lambda \), by means of \( \Delta A \), measured at a reference wavelength, \( \lambda_{ref} \) where dye-related changes are negligible. In the case of phenol red, \( \lambda \) was selected to lie between 450 and 600 nm and \( \lambda_{ref} \) was chosen to be either 630 or 640 nm. \( \Delta A(\lambda) \) was then obtained by subtraction of \( \Delta A_o(\lambda) \) from \( \Delta A_T(\lambda) \). The exponent \( X \) in Eq. 2 must be specified and might vary with experimental circumstances. The first section of Results explains the choice of the value of 1.6 for \( X \), the value used subsequently throughout Results. Since the absorbance measurements were made with a polarizing beam-splitting prism positioned in the light path between the muscle fiber and two identical photo-detectors, changes in the absorbance of light polarized parallel and perpendicular to the fiber axis (denoted by \( \Delta A_o \) and \( \Delta A_{op} \), respectively) were separately detected and analyzed.

Birefringence changes. To measure changes in the intrinsic optical retardation of the fiber (referred to as the intrinsic birefringence signal and reported as \( \Delta I/I; \) Baylor and Oetliker, 1977), a 300-μm length of fiber, positioned in the light path between two linear polarizers oriented at +45 and −45 degrees with respect to the fiber axis, was illuminated with light of long wavelength, between 700 and 850 nm, where phenol red’s absorbance is negligible. In a highly stretched fiber at 16°C, the early component of the birefringence signal (cf. Fig. 1) normally has a peak value of \(-1\) to \(-3 \times 10^{-3} (\Delta I/I)\) and a time-to-peak of 8-10 ms after stimulation. The time course of this component of the birefringence signal is closely similar to that of \( \Delta[Ca^{2+}] \) recorded with \( Ca^{2+} \) indicator dyes (Suarez-Kurtz and Parker, 1977; Baylor et al., 1982; Kovacs et al., 1983; Maylie et al., 1987). For example, in cut fibers at 17°C and stimulated by a single action potential, the rising phase of the birefringence signal lags that of \( \Delta[Ca^{2+}] \) recorded by antipyrilazo III by ~0.5 ms, whereas the half-width of the birefringence signal exceeds that of \( \Delta[Ca^{2+}] \) by ~1.5 ms (Irving et al., 1987; Maylie et al., 1987). Thus, a comparison of the time course of the phenol red absorbance change with the time course of the intrinsic birefringence signal permits an indirect comparison of the phenol red time course with that of \( \Delta[Ca^{2+}] \). Additionally, the intrinsic birefringence signal was routinely monitored throughout each experiment to assess fiber viability. An experiment was discontinued whenever the amplitude or time course of the birefringence signal changed suddenly or fell outside the range of normal values given above.

Tension transients. Changes in fiber tension during activity were recorded in most experiments as a second means of assessing fiber condition; for this purpose, a tension transducer (model AE801; Aksjeselskapet Microelektronikk, Horten, Norway) was attached to one tendon end of the fiber. Since the fibers were highly stretched and lowered onto pedestal supports to
minimize movement artifacts in the optical records, the amplitudes of the tension transients were in general only a few percent of those of normal tension responses.

In Results, reference is occasionally made to the "simultaneous" measurement of more than one type of optical signal, e.g., absorbance and birefringence changes, or absorbance changes measured at different wavelengths. For these comparisons, the different signals were measured sequentially in time, interleaved by bracketing measurements of a particular signal. Since the latter measurements typically differed by no more than a few percent, it was possible, with appropriate small scaling of signal amplitudes, to refer all measurements to a single time. Because the ΔA signals from phenol red in myoplasm were small, comparable in magnitude to the intrinsic absorbance changes of the fiber, two to eight sweeps taken at the wavelengths of interest were averaged to increase the signal-to-noise ratio.

**Calibration of Small Changes in Phenol Red Absorbance in ΔpH Units**

The preceding paper (Baylor and Hollingworth, 1990) demonstrated that changes in myoplasmic pH driven by acid and alkaline loads are accompanied by phenol red-related absorbance changes. Moreover, in spite of the demonstration that myoplasmic ΔpH appears to be sensed by at least three subpopulations of dye molecules, each with a different pK (−log10 dissociation constant) for protons, the amplitudes of ΔA(λ) were approximately as expected from the use of a single pK equal to that determined for the indicator in the in vitro calibrations, namely, 7.73. Because the phenol red absorbance signals are linearly dependent on dye concentration, the normalized absorbance change ΔA(λ)/A(λin), denoted ΔA(λ), should, for small changes, be proportional to ΔpH. A(λin) denotes resting absorbance measured at the isosbestic wavelength for the pH response.

The proportionality between ΔA(λ) and ΔpH is given by:

$$\frac{\Delta A(\lambda)}{\Delta \text{pH}} = -\frac{\Delta A_{\text{max}}(\lambda) \cdot \log_{10} 10 \cdot \frac{10^{\text{pH}-\text{pK}}}{(1 + 10^{\text{pH}-\text{pK}})^2}},$$

which can be derived from the differential forms of Eqs. 1 and 2 in Baylor and Hollingworth (1990). (In Eq. 3, ΔA_{\text{max}}(\lambda) denotes the change in normalized absorbance of deprotonated dye on protonation.) Eq. 3 was routinely used, as described below, to calibrate ΔA(λ) signals. Substitution in Eq. 3 from Eqs. 1 and 2 of Baylor and Hollingworth (1990) gives:

$$\frac{\Delta A(\lambda)}{\Delta \text{pH}} = -\log_{10} 10 \cdot \frac{[\bar{A}(\lambda) - \bar{A}_{\text{max}}(\lambda)] \cdot [\bar{A}_{\text{acid}}(\lambda) - \bar{A}(\lambda)]}{\Delta A_{\text{max}}(\lambda)},$$

where $\bar{A}_{\text{acid}}(\lambda)$ denotes the normalized absorbance of deprotonated dye, $\bar{A}_{\text{acid}}(\lambda)$ the normalized absorbance of protonated dye, and $\bar{A}(\lambda)$ the normalized absorbance of phenol red measured in the fiber. Eq. 4 shows that the calibration of ΔpH from ΔA(λ) depends on the in vivo measurement of $\bar{A}(\lambda)$ but does not depend on the particular value assumed for pK. In contrast, since $\bar{A}(\lambda)$ is dependent on the difference between pH and pK (Eqs. 1 and 2 of Baylor and Hollingworth, 1990), the calibration of resting pH does depend on the assumed value of pK.

Although 480 nm is an isosbestic wavelength for pH in the in vitro measurements, the data in the preceding paper suggest that, in the myoplasmic environment, λ_{iso} = 490 nm rather than 480 nm. Additionally, the data of the present paper indicate that in myoplasm ΔA_{\text{max}} is largest at λ = 570 nm, rather than at λ = 560 nm as expected from the in vitro calibrations. Hence, Eq. 3 was applied with λ_{iso} = 490 nm and λ = 570 nm. The values of ΔA_{\text{max}}(570) used in Eq. 3 were −5.71 and −5.02, corresponding, respectively, to ΔA measurements made with the narrow and wide band 570-nm interference filters. These values were obtained by an appropriate smoothing of the in vitro calibration curves red-shifted by 10 nm (cf. Baylor and
Hollingworth, 1990). For each application of Eq. 3, myoplasmic pH was taken as the value of $pH_{\text{app}}$ (apparent pH) obtained from the curve fitted to the measured values of $A(\lambda)$ (cf. Fig. 3 of Baylor and Hollingworth, 1990) and $A(490)$ was also obtained from this fitted curve. The effective pK of phenol red in myoplasm was, as mentioned above, assumed to be 7.73.

**RESULTS**

**Wavelength Dependence of the Intrinsic Absorbance Change Detected during Fiber Activity**

An accurate resolution of a dye-related absorbance change ($\Delta A$) requires a correction for the fiber's intrinsic ($\Delta A_i$). This correction was particularly important for the high signal-to-noise ratio. Separate measurements were therefore made on fibers not injected with dye in order to characterize the wavelength dependence of $\Delta A_i(\lambda)$.

The upper four pairs of traces in Fig. 1A show intrinsic transmission changes (proportional to $\Delta A_i$; cf. Eq. 1) recorded from a highly stretched fiber in response to single action potentials. At each $\lambda$ (indicated in nanometers to the left of the records), 0° and 90° polarized changes are shown. All records have a closely similar
waveform, characterized by a very small, early decrease in transmitted light, followed by a reversal to a maintained transmittance increase. These changes are typical of those seen in highly stretched fibers, in which contamination by movement artifacts has been essentially eliminated. The lowermost trace in Fig. 1 A shows the intrinsic birefringence signal recorded simultaneously with 810-nm light.

In general, the amplitude of intrinsic transmission changes of the type shown in Fig. 1 A increases as the wavelength decreases. In a previous article, the amplitude was assumed to follow a $\lambda^{-1}$ dependence (Baylor et al., 1982b). To obtain a more accurate description of this wavelength dependence, records of the type shown in Fig. 1 A were analyzed to determine the exponent X that yielded a least-squares fit of the traces by the relationship given in Eq. 2. In four fibers the 0° and 90° transmission traces, measured at wavelengths between 450 and 810 nm, were separately analyzed. On average, the best-fit value of X was 1.55 (±0.24 SEM) for 0° light and 1.69 (±0.36 SEM) for 90° light. Because of the similarity of these values, a value of $X = 1.6$ was chosen for use in Eq. 2 with both forms of polarized light for the remainder of the paper.

Fig. 1 B shows, for the 450-, 510-, and 570-nm traces in Fig. 1 A, the residual error associated with estimation of $\Delta A_i(\lambda)$ if Eq. 2 is used with $X = 1.6$ and $\lambda_{ref} = 630$ nm. For this experiment, the estimation worked well for both 0° and 90° absorbances, as the traces in Fig. 1 B are nearly flat (maximum error ~0.0002 $\Delta A$ units). This estimation procedure cannot, however, be assumed to work with such small error in all experiments. First, even in highly stretched fibers in which movement artifacts have been essentially eliminated, there is variability in the intrinsic waveforms, with the result that errors in the residual traces, particularly for wavelengths considerably shorter than $\lambda_{ref}$, can occasionally be as large as 0.0005 $\Delta A$ units or more. Second, highly stretched fibers sometimes showed atypical intrinsic components that had a time course similar to that of the remaining twitch tension and therefore were probably related to residual fiber movement. Third, additional movement-related components were sometimes observed in dye-injected fibers; the wavelength dependence of these signals often appeared similar to that of resting dye absorbance but has not been characterized in detail. Usually, the intrinsic and dye-related movement artifacts could be reduced by further stretch of the fiber; however, additional stretch also increased the possibility of fiber damage and was therefore used with caution. The early time course of the intrinsic-corrected records, i.e., before fiber movement becomes appreciable, is less subject to the uncertainties introduced by such movement artifacts.

**Absorbance Changes from Phenol Red during a Twitch**

Fig. 2 A shows original records of twitch tension (lowermost trace) and absorbance changes recorded at six different wavelengths and two planes of polarization, from a fiber region containing ~1 mM phenol red. The changes recorded with 630-nm light are similar to those seen in Fig. 1 A and reflect $\Delta A_i(\lambda)$ alone. The other records, particularly at 570, 540, and 510 nm, show clear evidence of an absorbance change in addition to $\Delta A_i(\lambda)$. To view the dye-related change directly, the traces at $\lambda < 630$ nm were corrected according to Eq. 2; further, a 1:2 weighted average (i.e., the
“isotropic” signal; Baylor et al., 1982a) of $\Delta A_0$ and $\Delta A_{90}$, respectively, at each wavelength was calculated. These changes are shown in Fig. 2 B, where an increase in absorbance has been plotted as an upward deflection. A phenol red isotropic signal appears well resolved at 570 and 540 nm and reasonably well resolved at 510 nm. However, the changes at 480 and 450 nm, particularly at later times, are close to the uncertainty level expected for the intrinsic correction and therefore may not entirely reflect a dye-related component. Nevertheless, the pattern of the changes in Fig. 2 B clearly indicates the existence of a phenol red-related $\Delta A$. As resolved in the 570-nm trace, this signal starts shortly after stimulation, reaches a peak 15–20 ms after stimulation, and appears to return to a smaller maintained level at times longer than 100 ms. The wavelength dependence of the early absorbance change in Fig. 2 B was qualitatively that expected for an underlying $\Delta pH$ mechanism, namely, a nearly maximal change at $\lambda = 570$ nm, a $\Delta A$ close to zero for $\lambda = 480$ nm, and a reversal in polarity for $\lambda < 480$ nm (cf. Fig. 1 C of Baylor and Hollingworth, 1990). If
calibrated by the procedure given in Methods, the $\Delta A(570)$ signal at peak corresponds to an alkalization of 0.0034 $\Delta pH$ units.

The symbols in Fig. 3 plot the amplitude of the isotropic $\Delta A$ as a function of wavelength, both for the experiment of Fig. 2 and for a second experiment. Two spectral curves are also shown in Fig. 3. The dotted curve is the unshifted $\Delta pH$ difference spectrum given in the preceding paper (Baylor and Hollingworth, 1990), whereas the continuous curve is this curve red-shifted by 10 nm. The muscle data are approximately fitted by either curve; however, the 10-nm shifted curve appears to give a better fit to the longer wavelength data, which are likely to be the most reliable. The finding that the 10-nm shifted spectrum more accurately describes the muscle data is expected for a $\Delta pH$ mechanism, since a 10-nm red-shift also

![Figure 3. Wavelength dependence of the phenol red isotropic $\Delta A$ measured in response to a single action potential. Muscle data (symbols) represent the relative amplitudes of absorbance waveforms of the type shown in Fig. 2B, least-squares fitted from the beginning of the trace through time-to-peak by the waveform measured at 570 nm. The dotted curve is an in vitro pH difference spectrum (cf. Fig. 1C of Baylor and Hollingworth, 1990) smoothed appropriately for comparison with the muscle measurements, which were made with wide band (±15 nm) filters. The continuous curve has the same shape as the dotted curve but has been red-shifted by 10 nm. The amplitude of each curve was separately set by a least-squares fit to the muscle data. Solid circles, Fiber 110785.2, same run as in Fig. 2. Crosses, Fiber 111385.2; 16° fiber diameter, 90 μm; striation spacing, 3.8 μm; $pH_{app}$, 7.26; phenol red concentration, 1.5 mM.](image)

characterized phenol red's resting isotropic signal, which was reflective of myoplasmic resting pH (Baylor and Hollingworth, 1990). The isotropic absorbance change measured at 570 nm and converted to pH units by the procedure given in Methods will be referred to below as the $\Delta pH_{app}$ signal.

**Fiber-to-Fiber Variation in $\Delta pH_{app}$**

Fig. 4 shows examples of $\Delta pH_{app}$ (dotted traces) recorded in four different experiments. Also shown in Fig. 4 are the intrinsic birefringence signals measured simultaneously (continuous traces, plotted as $-\Delta I/I$). The general pattern seen in these and other experiments was that the birefringence and $\Delta pH_{app}$ signals appeared to start at approximately the same time, although the times to half-peak and peak of the birefringence signal preceded those of the absorbance change by, on average,
2.4 ± 0.2 ms (±SEM; n = 8) and 8.4 ± 0.5 ms (±SEM; n = 4), respectively. The waveforms shown in Fig. 4 indicate that in highly stretched fibers that contain near millimolar quantities of phenol red, a reasonably reproducible ΔpH_{app} signal can be measured. (Note that in Fig. 4 the variability in the falling phases of the birefringence signals reflects variable movement artifacts, which occur about the time of tension development [not shown]. Small movement artifacts may also contaminate the falling phases of the ΔpH_{app} signals.)

**FIGURE 4.** Comparison of time courses of the intrinsic birefringence signal (solid traces) and the phenol red isotropic ΔA(570) signal (dotted traces) in four different fibers. Variable movement artifacts are apparent in the birefringence signals beginning 10–15 ms after stimulation. The traces have been normalized to the same peak height, given by the calibration arrow. The fiber numbers and the peak values of the birefringence signals (in units of 10^{-5} ΔI/I) and the ΔA(570) signals (in units of 10^{-5} ΔA) were: (A) 110785.2, -1.76, 0.59; (B) 111385.1, -1.32, 0.31; (C) 111385.2, -1.44, 0.19; and (D) 010386.3, -2.70, 0.30. The fiber diameters, number of signal-averaged sweeps at 570 nm, and resting absorbance values measured at 480 nm were as follows: (A) 120 μm, five sweeps, A(480) = 0.097. (B) 115 μm, five sweeps, A(480) = 0.144. (C) 90 μm, three sweeps, A(480) = 0.065. (D) 83 μm, five sweeps, A(480) = 0.058. Temperature, 16–17°C; striation spacing, 3.8–4.1 μm. Slit illumination was used for all absorbance measurements. See Table I for calibration of ΔA in ΔpH_{app} units and for other experimental details.

**Concentration Dependence of the Phenol Red ΔpH_{app}**

In several experiments it was possible to measure ΔpH_{app} over a range of myoplasmic phenol red concentrations in the same fiber. This was possible because phenol red concentrations varied as a function of time after injection as well as distance from the injection site (cf. Baylor and Hollingworth, 1990). Fig. 5 A summarizes results from three fibers. An additional constraint applied to the selection of the data for Fig. 5 A was that the value of resting pH_{app} be similar for all ΔpH_{app} measurements from the same fiber. Under this constraint it is expected that ΔpH_{app} will be independent of phenol red concentration if: (a) the ΔA(570) from myoplasm is
linearly proportional to dye concentration, and \(b\) the underlying mechanism responsible for the signal (e.g., a change in myoplasmic pH) is not itself modified by the presence of phenol red. Fig. 5A shows that, within experimental error, this expectation is fulfilled. Thus, for similar values of \(pH_{app}\), meaningful estimates of \(\Delta pH_{app}\) can be made, independent of the indicator concentration in the fiber.

**Peak \(\Delta pH_{app}\) vs. \(pH_{app}\) and Birefringence Amplitude vs. \(pH_{app}\)**

A basic physiological property of the phenol red signal concerns the possible dependence of the amplitude of \(\Delta pH_{app}\) on \(pH_{app}\). Fig. 5B summarizes data from nine different experiments related to this point. The data indicate that there is no marked dependence of \(\Delta pH_{app}\) on \(pH_{app}\) (correlation coefficient, \(-0.160\)). (The possible decrement in \(\Delta pH_{app}\) at a \(pH_{app}\) value near 7.5 suggested by Fig. 5B must be considered a preliminary observation, since it depends on the results from one experiment only.)

Similarly, a comparison (not shown) of the amplitude of the intrinsic birefringence signal versus \(pH_{app}\) revealed no correlation between the two variables (range of \(pH_{app}\)'s, 6.81–7.51; range of birefringence amplitudes, \(1.1–2.8 \times 10^{-3} (-\Delta I/I)\); correlation coefficient, \(-0.190\); \(n = 9\)). Since the birefringence signal is closely related to the myoplasmic free [\(Ca^{2+}\)] transient (see Methods), this result suggests that in the intact fiber the myoplasmic [\(Ca^{2+}\)] transient, and hence \(Ca^{2+}\) release from the sarcoplasmic reticulum (SR), is not strongly dependent on the resting level of \(pH_{i}\) in the physiological range. This conclusion contrasts with a recent study of the putative SR \(Ca^{2+}\) release channel after its reincorporation into lipid bilayers, which
showed that the channel conductance (open probability) was extremely pH sensitive over a similar pH range (Ma et al., 1988).

Characteristics of ΔpH_
app during Repetitive Stimulation

Fig. 6 A shows results from a phenol red-injected fiber stimulated by a train of five external shocks separated by 15 ms. At 630 nm the original ΔI/I records are shown, whereas at 570 and 480 nm the intrinsic-corrected records (proportional to dye-related ΔA) are shown. As expected for a ΔpH signal, the dye-related change observed at 480 nm is small in comparison with the change observed at 570 nm. Fig. 6 B (top) shows the corresponding ΔpH_
app trace, calibrated from the isotropic ΔA(570) record by the usual procedure; also shown is a ΔpH_
app trace obtained close in time in response to a single stimulated action potential. The lower records in Fig. 6 B are the tension transients simultaneously measured in response to the single and five-shock stimulations. The ΔpH_
app records indicate that each additional action potential produced a further transient increment in ΔpH_
app, although the amplitude of these increments appeared to decrease with subsequent shocks in the train. For example, the increment in ΔpH_
app produced by the second action potential was only 30% of the change produced by the first, while the third through fifth action potentials produced increments that were ~10–20% of that produced by the first.

Evidence for a Phenol Red Active Dichroic Signal during Fiber Activity

In resting fibers, the phenol red absorbance signal, A(λ), was characterized by the presence of a small dichroic component, with A₀ - A₉₀ > 0 (Baylor and Hollingworth, 1990). The wavelength dependence of this dichroic component was characteristic of dye in an apparently acidic environment, with the value of dichroic pH_
app being, on average, 6.24. Because of this indication of the existence of oriented
phenol red molecules in the resting state and because some Ca\textsuperscript{2+}-indicator dyes have revealed prominent dichroic signals in response to electrical stimulation (Baylor et al., 1982a), it was of interest to determine if there was also a phenol red dichroic signal during fiber activity.

Fig. 7A shows the relevant data from the fiber of Fig. 2A. The second trace from the bottom is the intrinsic dichroic signal recorded at 630 nm. Also shown are the dye-related dichroic signals (upper five records). These latter traces were obtained by subtraction of the intrinsic component, estimated from the 630-nm dichroic signal by the usual procedure, from the total dichroic signal recorded at each \( \lambda \). In Fig. 7A, the signals at \( \lambda = 570, 540, \) and 510 nm are small and close to the variation expected for the uncertainties of the intrinsic correction procedure (cf. Fig. 1B); however, the changes at 480 and 450 nm appear to be well outside this range. Records similar to those of Fig. 7A were seen in all fibers injected with a reasonably large concentration (>0.5 mM) of dye (cf. column 9 of Table I, which indicates the amplitude of the active dichroic signal at 480 nm). The time course of this dichroic signal (cf. columns 10 and 11 of Table I) was generally similar to, although probably...
slightly slower than that observed for the isotropic $\Delta A(570)$ signal (column 6 vs. 10, and 7 vs. 11 of Table I). Interestingly, the wavelength dependence of the dichroic component (Fig. 7B and all other fibers examined) was generally similar to that of the phenol red resting dichroic signal (cf. Fig. 2D of Baylor and Hollingworth, 1990). This spectrum is, in turn, quite different from that of phenol red's resting isotropic signal (cf. Fig. 3 of Baylor and Hollingworth, 1990) and that of the isotropic absorbance change (Fig. 3 of this paper). This unique wavelength dependence, as well as the similarity of the time course of the phenol red dichroic change to that of the arsenazo III and dichlorophosphonazo III dichroic signals (Baylor et al., 1982a), argue that the phenol red dichroic signal reflects some specific change in the properties of individual dye molecules and is not, for example, simply an artifact of fiber movement or the result of a systematic error in the correction for the intrinsic absorbance change. The presence of an active phenol red dichroic signal with a time course similar to that of the active isotropic signal raises concerns about the interpretation that the $\Delta pH_{app}$ signal simply reflects a myoplasmic $\Delta pH$.

### Table I

**Characteristics of Myoplasmic Phenol Red Absorbance Changes during a Twitch**

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<th>Fiber</th>
<th>$A(490)$</th>
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<th>$\Delta A(570)$</th>
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<td></td>
<td>$\Delta A_{app}(480)$</td>
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<td>(±SEM)</td>
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<td>±0.0002</td>
<td>±0.3</td>
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<td>±0.0002</td>
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<td>±2</td>
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</table>

Column 1 gives the fiber identification, column 2 the estimated value of dye-related absorbance at 490 nm, the probable isosbestic wavelength for pH changes in myoplasm, and column 3 the corresponding estimate of total dye concentration. Column 4 gives the apparent pH of myoplasm estimated from the resting spectrum (cf. Baylor and Hollingworth, 1990) and column 5 the peak change in this variable as estimated from the isotropic $\Delta A(570)$ signal and the calibration procedure given in Methods. Columns 6 and 7 give the time after stimulation for $\Delta pH_{app}$ to reach half its peak value and its peak value, respectively. Column 8 gives the relatively steady average level of $\Delta pH_{app}$ observed 130-170 ms after stimulation. Columns 9-10 give information analogous to columns 5-7, but for the dichroic component of the phenol red $\Delta A$ measured at 480 nm. The information in the table was obtained from the run in each fiber having the highest dye concentration and least interference from movement artifacts. A dashed entry indicates that movement artifacts prevented reliable determination of the signal characteristic.
DISCUSSION

The experiments described in this paper indicate that, in response to action potential stimulation, a small but reproducible isotropic absorbance change, \((\Delta A_0 + 2\Delta A_{90})/3\), can be detected from phenol red in the myoplasm of frog skeletal muscle fibers. This signal has the obvious features expected if it reflects a rapid change in myoplasmic pH accompanying the excitation–contraction coupling process. First, the amplitude of the signal detected at 570 nm was essentially identical when measured with 0° and 90° polarized light, as expected if this absorbance change primarily reflects the activity of dye molecules in the myoplasmic solution (i.e., not bound to oriented structures). Second, the wavelength dependence of the signal (Fig. 3) was similar to that observed in vitro for a change in pH, and close inspection suggested a red-shift similar to the 10-nm red-shift observed for phenol red’s resting absorbance spectrum in myoplasm (Baylor and Hollingworth, 1990). Third, the time to peak of the signal was earlier than that of tension development, although somewhat slower than that of the myoplasmic free \([Ca^{2+}]\) transient. This latter conclusion follows from: (a) the earlier time course of the intrinsic birefringence signal when compared with the phenol red isotropic signal (Fig. 4), and (b) the slightly earlier time course of the free \([Ca^{2+}]\) transient, as previously measured with absorbance indicator dyes, when compared with that of the intrinsic birefringence signal (see Methods). It is unlikely that there is any significant kinetic delay between the myoplasmic pH change (if real) and the observed isotropic absorbance change of phenol red, since in in vitro measurements the indicator appears to respond to changes in proton concentration in a small fraction of a millisecond (Hammes, 1974; see also Cogdell et al., 1973).

If the phenol red isotropic signal is driven by a myoplasmic pH change and if the relationship given in Methods applies to its calibration, the average amplitude of \(\Delta p\text{H}\) in response to a single action potential was +0.0025 (column 5 of Table I). This amplitude corresponds to a change of \(\sim 0.001\) of the indicator from its proton-bound to its proton-free form. As shown in column 7 of Table I, the peak change occurred 15–20 ms after stimulation; thereafter, the dye signal returned toward baseline with a time course that varied somewhat from fiber to fiber. In most fibers, however, there appeared to be significant recovery in \(\Delta p\text{H}_{\text{app}}\) that began soon after the peak of the signal (cf. Fig. 4). Some events possibly related to this early recovery will be considered in the following paper (Pape et al., 1990), which also further investigates what events may underlie the main rising phase of the signal. At relatively late times, e.g., 150 ms after stimulation, the \(\Delta p\text{H}_{\text{app}}\) signal appeared to reach a maintained elevation that was \(\sim 30\%\) of the peak change (cf. columns 5 and 8 of Table I). Some possible origins of a maintained \(\Delta p\text{H}\) signal were discussed previously (Baylor et al., 1982b) and have not been considered further in the present work.

Comparisons with Results from Cut Muscle Fibers

There are two reports in the literature (Palade and Vergara, 1982; Irving et al., 1989) that describe myoplasmic absorbance changes measured with phenol red in response to electrical stimulation of frog “cut” twitch fibers (Hille and Campbell,
Irving et al. (1989) measured the isotropic ΔΛ(570) signal at 18°C in response to a single action potential. A transient alkalization of peak amplitude 0.004-0.008 pH units was detected early in the experiments (10-25 min after addition of dye to the end pools), at which time dye concentration at the fiber center was 1-3 mM and the condition of the cut fiber was probably relatively close to that of an intact fiber. With time, however, the amplitude of the apparent pH change decreased considerably, without a significant change in the time course. This alteration of signal amplitude may be related to a progressive change in the physiological state of a cut fiber during the course of an experiment (Maylie et al., 1987). Interestingly, in the cut fiber experiments the average times to half-peak and peak of the apparent alkalization were 20 and 50 ms, respectively (Fig. 4 of Irving et al., 1989), values that did not change significantly with time. Thus, the apparent pH change measured early in a cut fiber experiment appears to be both larger and slower than that observed by us in intact fibers (cf. columns 5-7 of Table I). It is possible (M. Irving et al., personal communication) that these differences reflect a reduction in the myoplasmic buffering power of the cut fibers, due to a diffusional exchange with the end-pool solutions that was well underway before the first phenol red transients could be measured. Nevertheless, the phenol red signal in these cut fiber experiments was qualitatively similar to that reported here for intact fibers.

In contrast, Palade and Vergara (1982) reported an early myoplasmic acidification in cut fibers that contained 2 mM phenol red and that were stimulated by either action potential or voltage-clamp depolarizations (21°C). For example, in response to a 50-ms voltage pulse to 0 mV from a holding potential of −100 mV, an apparent pH change of ~−0.003 pH units was recorded by 10 ms after the onset of the pulse, and this signal continued to increase in amplitude to a peak value of ~−0.01 units at 100–150 ms after the pulse onset. “Slightly smaller pH changes that tended to decay more rapidly” were observed in response to action potential stimulations. The reason for the apparent acidification observed in these fibers is not clear. It might, however, be related to the use by Palade and Vergara of 3 mM EGTA in the internal solution. Since EGTA in myoplasm is expected to release a significant quantity of protons as it binds Ca²⁺ in response to the myoplasmic Ca²⁺ transient, a net increase in myoplasmic proton concentration during activity may occur in fibers that contain millimolar concentrations of EGTA (P. C. Pape, personal communication).

Possible Early Alkalizations of Myoplasm Detected with Other Indicator Dyes

In the cut-fiber experiments of Irving et al. (1989), a fluorescein-based pH indicator, dimethyl-carboxyfluorescein (Me₂CF), was also used to study possible myoplasmic pH changes in response to action potential stimulation. Unfortunately, a strong pharmacological action of the dye was observed within 15–30 min after addition of the indicator to the end-pool solution, namely, the dye progressively reduced and finally abolished the intrinsic birefringence signal. Since the electrical properties of the fibers remained normal during this 30-min period, Me₂CF in myoplasm appears to have specifically blocked Ca²⁺ release from the sarcoplasmic reticulum. Nevertheless, before block of Ca²⁺ release (i.e., while the amplitude of the birefringence signal was still reasonably normal), an absorbance change was detected from the indicator, consistent with an apparent alkalization of myoplasm of peak value 0.03–0.04 pH
units and a time to peak of ~20 ms after stimulation (17–18°C). (Within a few
minutes the amplitude of the indicator signal decreased, concurrent with the
pharmacological block of the birefringence signal.) Although the 0.03–0.04 ampli-
tude of the apparent pH change seems very large, there are several reasons to
question the validity of the calibration of the Me\textsubscript{2}CF signal: (a) the indicator had a
strong pharmacological effect on E-C coupling; (b) a large bound fraction of ~0.8
was detected for the dye, which raises the possibility that the pH dependence of its
optical signal in vivo was significantly different than in the in vitro calibrations; (c) the
resting pH signal from the indicator was quite acidic, 6.2–6.4 units (M. Irving et al.,
personal communication), which again indicates either a strong pharmacological
action of the dye on fiber properties or a problem with the calibration of the pH
signal in the myoplasmic environment.

An apparent alkalization of myoplasm has also been reported (Konishi et al., 1989)
from intact frog fibers injected with arsenazo I, an absorbance dye that is sensitive to
changes in both Mg\textsuperscript{2+} and H\textsuperscript{+} (DeWeer et al., 1981; Baylor et al., 1982b). In
response to a single action potential, the early isotropic absorbance change measured
with this indicator had a time to peak of ~15 ms after stimulation and an amplitude,
if calibrated in pH units, of +0.0035 ± 0.0009 (±SEM). Although possible
contributions to the signal from a change in myoplasmic [Mg\textsuperscript{2+}] were not ruled out,
the properties of the apparent alkalization detected at early times with arsenazo I are
in close agreement with those seen in intact fibers with phenol red (Table I).

Thus, results from three pH indicators (phenol red, Me\textsubscript{2}CF, and arsenazo I)
introduced intracellularly into frog skeletal muscle fibers point to the existence of an
early alkalization of myoplasm, the rising phase of which appears to lag that of the
intrinsic birefringence signal and therefore that of the free [Ca\textsuperscript{2+}] transient. Yet,
since all three of the indicators have two or more methodological drawbacks (e.g.,
existence of a large bound fraction in myoplasm, a strong pharmacological effect on
E-C coupling, interference from divalent cations, or presence of a dichroic signal
during activity), further study, with less complicated indicators, of the probable
alkalization would clearly be desirable.

Since the SR Ca\textsuperscript{2+} release waveform itself precedes rather than follows the [Ca\textsuperscript{2+}]
transient (see, for example, Baylor and Hollingworth, 1988), none of the pH
indicator dye experiments provides support for the suggestion of Shoshan et al.
(1981) that a sudden myoplasmic alkalization serves as the normal physiological
tigger for SR Ca\textsuperscript{2+} release during E-C coupling.

**Alternative Explanations for the Apparent Early Alkalization of Myoplasm Observed in Intact Fibers**

The possibility should be considered that the apparent early alkalization detected
with phenol red (Fig. 4 and Table I) and the other indicators might reflect
myoplasmic events other than a bulk pH change. The most obvious alternative
possibility is that the optical signals reflect some myoplasmic change driven by the
rise in free [Ca\textsuperscript{2+}]. Although, in the case of phenol red, in vitro calibrations show
that the indicator does not itself respond to even millimolar changes in free [Ca\textsuperscript{2+}]
(S. M. Baylor and S. Hollingworth, unpublished observations), a number of myoplasmic
changes are directly caused by the rise in [Ca\textsuperscript{2+}] during a twitch. For example, a
relatively large total amount of Ca\textsuperscript{2+}, \(-0.2-0.3\) mM if referred to the myoplasmic water space (Baylor et al., 1983; Maylie et al., 1987; Baylor and Hollingworth, 1988), is released from the sarcoplasmic reticulum into the myoplasm. One physical change accompanying normal fiber activity is a small increase in the temperature of the fiber (cf. Curtin et al., 1984). A rise in temperature might change dye properties, for example, the pK of the indicator, which in turn could generate an optical change indistinguishable from that caused by a true \(\Delta pH\). The magnitude of the myoplasmic temperature increase, however, appears to be sufficiently small that a temperature-driven change in pK can be ruled out as the source of the \(\Delta pH_{\text{app}}\) measured with phenol red. According to Curtin et al. (1984), at \(16^\circ C\) the peak change in fiber temperature in response to a single action potential is unlikely to exceed \(5 \times 10^{-5}^\circ C\) during the rising phase of force development, whereas Hastings and Sendroy (1924) (see also Van Slyke et al., 1949) report a change in pK of phenol red of \(-0.125\) for a temperature increase from 20 to 38\(^\circ C\). Thus, the change in indicator pK due to the temperature increase of the fiber is not expected to exceed \(-2 \times 10^{-5}\). This change, at a constant pH of 7.0, would produce a change in the fraction of the indicator in the proton-bound form of \(-6 \times 10^{-6}\) or, equivalently, just under 1% of the change produced by a true alkalinization of 0.003 units (the magnitude of the isotropic signal in Table I).

Another more likely possibility is related to the fact that most of the Ca\textsuperscript{2+} released into the myoplasm binds to metal sites available both on structural elements (e.g., thin filament troponin sites, sarcoplasmic reticulum Ca\textsuperscript{2+} pump sites) as well as on soluble proteins (e.g., parvalbumin, calmodulin, phosphorylase kinase). Because \(~80\%\) of the phenol red in myoplasm appears to be bound, both to structural and soluble sites (Irving et al., 1989; Baylor and Hollingworth, 1990), there is a real possibility that Ca\textsuperscript{2+} binding to receptor sites on proteins might (a) affect the concentration of protons in the local environment of the dye (but not the bulk pH), for example, by neutralization of fixed negative charges on the proteins; or (b) displace or otherwise alter the properties of the indicator molecules bound to these sites or adjacent structures. In either case, an indicator-related absorbance change with properties indistinguishable from that caused by a true bulk \(\Delta pH\) could arise.

The following calculation estimates the amount of phenol red required to participate in one such change in order that the \(\Delta pH_{\text{app}}\) observed in intact fibers might be explained by a non-pH mechanism of this type. As estimated in the previous paper (Baylor and Hollingworth, 1990), a large fraction of dye, perhaps as much as 0.77 of the total, might be bound to sites on soluble proteins, with the effective pK of this dye being perhaps 0.2 units less than that of the dye free to diffuse in the myoplasmic solution. (The estimated fraction of freely diffusible dye was \(~0.2\) of the total phenol red, for which we presume the pK is 7.73.) If 7% of the dye molecules in the free pool redistributed to the pool of dye bound to soluble proteins, the fraction of the total dye in the proton-bound form would decrease by 0.001, a change identical to that produced by a 0.003 increase in pH relative to a resting pH of 7.0. A redistribution of this sort cannot be ruled out by the experiments of this paper. Similarly small redistributions of dye from the oriented to the soluble pools might also underlie the isotropic and/or dichroic signals.
Thus, changes in apparent protonation of bound phenol red molecules as a result of the myoplasmic free $[\text{Ca}^{2+}]$ transient (unrelated to an actual $\Delta \text{pH}$) cannot be ruled out as a mechanism contributing to the small signals actually detected. In the case of the isotropic signal, the summation observed during repetitive stimulation (Fig. 6B) might seem to be inconsistent with a signal primarily driven by the free $[\text{Ca}^{2+}]$ transient, since it is known that the $[\text{Ca}^{2+}]$ transient under the conditions of a brief, high frequency tetanus does not summate significantly (cf. Quinta-Ferreira et al., 1984; Maylie et al., 1987). However, since $\Delta \text{pH}_{\text{app}}$ has a slower time course than $\Delta[\text{Ca}^{2+}]$, summation is to be expected for a response driven by $[\text{Ca}^{2+}]$ (cf. the delayed and summated $\text{Ca}^{2+}$ dye responses observed during repetitive stimulation with arsenazo III [Baylor et al., 1982a] and fura-2 [Baylor and Hollingworth, 1988]).

Thus, the attractive interpretation that the phenol red isotropic signal directly and simply reflects a myoplasmic alkalization is one of several hypotheses consistent with the experiments of this paper. For this reason, further discussion of this signal, including consideration of the possible physiological significance of a myoplasmic alkalization associated with E-C coupling, will be considered after presentation of the results contained in the following paper (Pape et al., 1990).

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