Absorbance Signals from Resting Frog Skeletal Muscle Fibers Injected with the pH Indicator Dye, Phenol Red

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ABSTRACT Singly dissected twitch fibers from frog muscle were studied on an optical bench apparatus after micro-injection with the pH indicator dye, phenol red. Dye-related absorbances in myoplasm, denoted by $A_0(\lambda)$ and $A_{90}(\lambda)$, were estimated as a function of wavelength $\lambda$ (450 nm ≤ $\lambda$ ≤ 640 nm) with light polarized parallel (0°) and perpendicular (90°) to the fiber axis, respectively. At all $\lambda$, $A_0(\lambda)$ was slightly greater than $A_{90}(\lambda)$, indicating that some of the phenol red molecules were bound to oriented structures accessible to myoplasm. The phenol red “isotropic” signal, $[A_0(\lambda) + 2A_{99}(\lambda)]/3$, a quantity equal to the average absorbance of all the dye molecules independent of their orientation, had a spectral shape that was red-shifted by ~10 nm in comparison with in vitro dye calibration curves measured in 140 mM KCl. The red-shifted spectrum also indicates that some phenol red molecules were bound in myoplasm. A quantitative estimate of indicator binding was obtained from measurements of the dye’s apparent diffusion constant in myoplasm, denoted by $D_{app}$. The small value of $D_{app}$, $0.37 \times 10^{-6}$ cm$^2$ s$^{-1}$ (at 16°C), can be explained if ~80% of the dye was bound to myoplasmic sites of low mobility. To estimate the apparent myoplasmic pH, denoted by $pH_{app}$, the isotropic absorbance of phenol red was fitted by in vitro calibration spectra. $pH_{app}$ was found to be independent of dye concentration (0.2–2 mM), but varied widely (range, 6.8–7.5; mean value, 7.17) among fibers judged from functional characteristics to be normal. When fibers were subjected to acid or alkaline loads by exposure to Ringer’s solution containing, respectively, dissolved CO$_2$ or NH$_3$, the changes in $pH_{app}$ were in agreement with those expected from pH micro-electrode studies. It is concluded that in spite of the several indications for the presence of bound phenol red inside muscle cells, the $pH_{app}$ signal from the indicator is useful for monitoring changes in myoplasmic pH in response to physiological and pharmacological manipulations.

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INTRODUCTION

In vertebrate skeletal muscle, a change in the cytoplasmic proton concentration may accompany the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) (Somlyo et al., 1981). Additionally, the value of resting pH may have an important modulatory effect on the SR Ca\(^{2+}\) release channel (Ma et al., 1988). To evaluate such possibilities, one would like to be able to monitor resting pH and track rapid changes in pH (ΔpH), either in the myoplasm or within the SR.

The absorbance indicator dye, phenol red (phenolsulphonephthalein), was first used to study myoplasmic pH (pHi) in frog skeletal muscle by Baylor et al. (1982b). The estimated pH\(_i\) was 6.9, approximately the value expected under the conditions of the experiments (external pH = 7.1; 16°C). In one experiment (Fig. 15 of Baylor et al., 1982b) a small absorbance change, possibly indicative of a myoplasmic alkalization during muscle activation, was observed. However, neither the resting nor active phenol red signal was studied in detail, as the main concern of Baylor et al. was to rule out the possibility that changes in pH\(_i\) after muscle stimulation might interfere with Ca\(^{2+}\)-related signals measured with the metallochromic dyes, arsenazo III, antipyrylazo III, and dichlorophosphonazo III. To evaluate whether protons play a role in excitation-contraction coupling, a more detailed investigation of myoplasmic signals from pH indicators such as phenol red is clearly necessary. The experiments described in this and the following papers (Hollingworth and Baylor, 1990; Pape et al., 1990) were carried out for this purpose.

One increasingly recognized problem in the use of indicator dyes under intracellular conditions is that most such compounds bind to cytoplasmic constituents; as a consequence, both spectral and kinetic properties of the indicators may differ in the cytoplasm compared with the usual in vitro calibrations (Thomas et al., 1979; Beeler et al., 1980; Baylor et al., 1982a, 1986; Chaillet and Boron, 1985; Maylie et al., 1987a, b, c; Baylor and Hollingworth, 1988; Konishi et al., 1988). This binding problem raises the possibility that an indicator's signal reflects events not directly related to ionic concentrations. The principal aim of the experiments reported in this paper was to evaluate the reliability with which phenol red can report pH\(_i\) in resting muscle fibers and, ultimately, the reliability with which changes in pH\(_i\) during fiber activity might be monitored.

The results indicate that most of the phenol red in myoplasm is bound, and that dye properties are altered because of the binding. Thus, the accuracy of the value of resting pH\(_i\) reported by the indicator (average value, 7.17; n = 8) is uncertain. Nevertheless, the changes in pH\(_i\) reported by phenol red when fibers were subjected to acid or alkaline loads were in reasonable quantitative agreement with those expected from pH micro-electrode measurements. Thus, the dye is useful for the measurement of slow changes in pH\(_i\) in resting muscle fibers and may be useful for the monitoring of rapid changes in myoplasmic pH that possibly occur during excitation-contraction coupling (Baylor et al., 1982b, 1987; Irving et al., 1989).

The main new finding concerning pH in resting fibers is that pH\(_i\) can vary widely, by as much as 0.7 pH units, among fibers that functionally appear normal. This variability may prove important in elucidation of a possible role for protons during Ca\(^{2+}\) release from the sarcoplasmic reticulum and in the calibration of absorbance
signals measured with other indicator dyes. For instance, a quantitative interpretation of Mg$^{2+}$-related signals measured in muscle with arsenazo I, arsenazo III, dichlorophosphonazo III, and antipyrylazo III (Baylor et al., 1982b, c, 1986; Irving et al., 1985, 1989) depends strongly on the value assumed for pH$_i$.

An account of some of the results has appeared in abstract form (Baylor et al., 1987).

METHODS

The experiments and analyses were carried out as previously described (Baylor et al., 1986). Briefly, single-twitch fibers were dissected from leg muscles (semitendinosus or iliofibularis) of English frogs (Rana temporaria), transferred to a chamber on an optical bench apparatus equipped with a 100-W tungsten-halogen light source, and stretched to long sarcomere length (3.6–4.1 μm). The temperature of the Ringer’s solution bathing the fiber was maintained at 16 ± 1°C. Phenol red was pressure-injected into the myoplasm from a micropipette containing 15–30 mM dye dissolved in distilled water. For most experiments the dye solution was titrated with HCl to a pH of 7.0; in one experiment the dye solution was not titrated and had a pH of 8.7 (see legend of Table I). After the injection, the fiber was illuminated with a small spot or slit of light to measure absorbance. The absorbance of light polarized parallel and perpendicular to the fiber axis (denoted by $A_0$ and $A_90$, respectively) was measured simultaneously by means of a beam-splitting polarizer and two separate photodetectors. Absorbance data at different wavelengths $\lambda$ (denoted by $A(\lambda)$) were collected sequentially by insertion of one of a series of interference filters (Omega Optical, Inc., Brattleborough, VT) in the light path. The filters had half-band widths of either 10 nm (“narrow band” series) or 30 nm (“wide band” series). All data were sampled, stored, and analyzed with a PDP 11 computer (Digital Equipment Corp., Marlboro, MA). Total fiber absorbance, dye-related absorbance, and myoplasmic dye concentrations were calculated from the raw measurements of transmitted intensities made in the absence and the presence of the fiber (Baylor et al., 1986). For the conversion of indicator absorbance to concentration units, $\epsilon(480)$, the in vitro molar extinction coefficient for phenol red at 480 nm was taken to be $1.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ (Lisman and Strong, 1979; our own measurements).

Solutions

The normal Ringer’s solution contained (in mM): 120 NaCl, 2.5 KCl, 1.8 CaCl$_2$, and 5 Na$_2$Pipes (sodium salt of piperazine-N,N-bis[2-ethane-sulfonic acid]), pH 7.10. However, a number of experiments (see legend of Table I) were carried out in a “high Ca$^{2+}$” Ringer’s, made with 11.8 mM rather than 1.8 mM CaCl$_2$. Fibers in the high Ca$^{2+}$ Ringer’s survived the dye injection procedure better than fibers in the normal Ringer’s solution, an effect probably attributable to improved sealing of membrane leaks at the injection site (DeMello, 1973). There were no resolvable differences between the phenol red signals measured in the normal Ringer’s and in the high Ca$^{2+}$ Ringer’s; a similar conclusion was reached for myoplasmic Ca$^{2+}$ signals measured with the Ca$^{2+}$ indicator dyes antipyrylazo III and fura-2 (Baylor and Hollingworth, 1988).

For the experiments in which pH$_i$ was manipulated by exposure to acid or alkaline loads, the Ringer’s solution was modified as follows:

(a) Acid load. Normal Ringer’s, titrated to pH 7.40 and bubbled with a mixture of 5% CO$_2$/95% room air for a period of at least 15 min, replaced a high Ca$^{2+}$, CO$_2$-free Ringer’s titrated to pH 7.40.

(b) Alkaline load. Normal Ringer’s, with 5 mM (NH$_4$)$_2$SO$_4$ and titrated to pH 7.9, replaced a
high Ca$^{2+}$, ammonium-free Ringer’s at pH 7.1. In these experiments, the Ringer’s solution was changed by three or four manual pipettings, each of which exchanged about three-fourths of the total bath volume. Because each exchange required ~30 s, the earliest information about changes in pH was not obtained until 2–3 min after the initial exchange.

Source of Phenol Red

Most commercially available phenol red is toxic to muscle fibers. To test dye toxicity, 3 mM phenol red was added to the Ringer’s solution and the ability of the fiber to maintain a normal twitch response and “second component” birefringence signal (Baylor and Oetiker, 1975) was monitored. Phenol red obtained from both Sigma Chemical Co. (St. Louis, MO) and ICN K and K Laboratories Inc. (Plainview, NY) rapidly (<2 min) caused fibers to lose their all-or-none responses to electrical stimulation. Phenol red from Flow Laboratories, Inc. (Rockville, MD), however, showed no detectable toxicity and was therefore used in the experiments. This dye was from the same batch used in the previous experiments (Baylor et al., 1982b) and was generously supplied by Dr. W. K. Chandler.

In Vitro Calibrations of Phenol Red

Absorbance spectra of phenol red at different pH’s were measured in 2-nm wavelength increments on a UV/visible spectrophotometer (cf. Hollingworth and Baylor, 1986). The digitized values of absorbance were later interpolated to 1 nm resolution. The standard calibration solutions contained (in mM) 140 KCl and 10 K$_2$Pipes at pH’s of 6.80, 7.10, or 7.40. At each of these pH’s, absorbance curves measured at dye concentrations of 0.08 mM (in a 1-cm cuvette) and 0.8 mM (in a 1-mm cuvette) were identical.

Fig. 1 A shows absorbance spectra of 0.8 mM phenol red at the three values of pH. Each spectrum is divided by $A(480)$, the absorbance at 480 nm. This wavelength is very close to an isosbestic point, since the values of $A(480)$ at the three pH’s differed from their average value by <1%. Fig. 1 C shows the difference spectra, $\Delta A(\lambda)/A(480)$, obtained by subtraction of the pH 6.8 curve from the other two curves in Fig. 1 A. The two difference spectra have identical shapes, but differ in amplitude by a factor of 2.49. These results are consistent with the calibrations of Lisman and Strong (1979), made over a much wider pH range (5.5–10.0). The spectral changes of Lisman and Strong, and our own, are well fitted by the assumption that they arise from a single protonation reaction having a $pK$ ($-\log_10$ of the proton dissociation constant) of 7.73. In the visible region of the spectrum, the maximum absorbance change occurs at 560 nm, where the ratio $\Delta A(560)/A(480)$ differs by ~5.86 for the proton-bound vs. proton-free forms of the dye (cf. Lisman and Strong, 1979). This change corresponds to a change in molar extinction coefficient with protonation, $\Delta \epsilon(560)$, of $-6.45 \times 10^3$ M$^{-1}$ cm$^{-1}$.

To test for the existence of a systematic bias between absorbance measured in the spectrophotometer and on the optical bench apparatus used for the muscle experiments, the comparisons shown in Fig. 1 B were carried out. A thin-walled glass capillary (inner diameter, 108 $\mu$m) was filled with a calibration solution containing 1.06 mM phenol red at a pH of 7.10 and mounted on the optical bench. Polarized absorbance measurements were made in 10-nm increments between 440 and 640 nm, with the same narrow-band interference filters and procedures used for measurement of dye absorbance in muscle fibers. The values of $A(\lambda)$ are plotted without normalization in Fig. 1 B, where the different symbols denote measurements obtained with the two forms of polarized light.

As expected for dye molecules in solution, the absorbance measurements in Fig. 1 B are isotropic, i.e., show no polarization preference. This can be seen more clearly in Fig. 1 D, where the dichroic absorbance, defined as $A_p(\lambda) - A_q(\lambda)$, has been plotted at higher gain. For 480 $\leq \lambda \leq$ 640 nm the mean value ($\pm$SEM) of the dichroic absorbance was 0.0000 ($\pm$0.0002.
SEM) absorbance units and showed no systematic dependence on wavelength. For $\lambda < 480$ nm, the measurements became somewhat noisy, because the intensity of light from the tungsten–halogen bulb decreases at shorter wavelengths.

For comparison with the spectrophotometer calibrations, the capillary values of $A_0(\lambda)$ and $A_m(\lambda)$ for $460 \leq \lambda \leq 640$ nm were averaged and least-squares fitted with spectral curves derived from the normalized curves shown in Fig. 1 A. Since the optical bench measurements in Fig. 1 B were made with interference filters having a 10-nm band pass, each derived spectrophotometer curve (estimated in 1-nm increments) was obtained as the average of $A(\lambda)$

![Figure 1](image_url)

**Figure 1.** In vitro calibration of phenol red. A, Absorbance versus wavelength for a 0.8-mM phenol red solution at pH 6.80, 7.10, and 7.40. Spectra show, respectively, small, intermediate, and large absorbances at 560 nm, as indicated. In addition to the dye, the calibration solution contained 140 mM KCl and 10 mM of the pH buffer K$_2$Pipes. For each curve the raw absorbance measurements at wavelength $\lambda$, $A(\lambda)$, were normalized by the value of $A(480)$ nm. B, Comparison of polarized absorbance measurements made from a dye-filled capillary on the optical bench (circles, $A_0(\lambda)$; crosses, $A_m(\lambda)$) with a least-squares fitted calibration curve based on the spectral shapes in A. The solution contained 1.06 mM phenol red at a pH of 7.10. The fitting procedure (see Methods) produced estimates of 1.07 mM for dye concentration and 7.08 for pH. C, Difference spectra from A, obtained by subtraction of the pH 6.80 curve from the other two curves, as indicated. D, Difference spectrum, $A_0(\lambda) - A_m(\lambda)$, for the polarized measurements in B.

for the 11 points on the original curve within ±5 nm. Fig. 1 B shows the fit. The only adjustable parameters were the value of pH, which determines the shape of the curve, and the total dye concentration ($[D]$), which determines the amplitude of the curve. The fitted values, which were obtained by the relationships given in the next paragraph, were 7.08 for pH (vs. 7.10 expected) and 1.07 mM for $[D]$ (vs. 1.06 mM expected). The overall good agreement indicates that there is negligible difference between dye absorbances measured on the optical bench and in the spectrophotometer.
To estimate pH and \([D_T]\), the optical bench absorbance data were fitted with a linear combination of two spectrophotometer curves, an absolute spectrum obtained at pH 7.1 and a difference spectrum obtained between pH's 6.8 and 7.4, as follows. Let \(f(pH)\) denote the fraction of the indicator in the proton-bound form at any particular value of pH. Then

\[
f(pH) = \frac{10^{-pH}}{10^{-pH} + 10^{-pK}}.
\]  

Moreover, let \(\tilde{A}_{ph}(\lambda)\) denote the absorbance curve of phenol red measured at a particular pH and normalized by its value at 480 nm (e.g., \(\tilde{A}_{7.1}(\lambda)\) denotes the middle curve in Fig. 1 A). Under the assumption of a single protonation site for the indicator, it follows that

\[
\Delta \tilde{A}_{7.4-6.8}(X) = \tilde{A}_{\text{alk}}(X) + f(pH) \Delta \tilde{A}_{\text{max}}(\lambda).
\]  

Since the (unnormalized) spectrum \(A_r(\lambda)\) measured with the indicator at an unknown pH equal to \(x\) (e.g., the average of the 0° and 90° absorbances in Fig. 1 B) satisfies

\[
A_r(\lambda) = a \tilde{A}_{7.1}(\lambda) + b \Delta \tilde{A}_{7.4-6.8}(\lambda)
\]  

for particular values of \(a\) and \(b\) (cf. Baylor et al., 1982b), it follows that

\[
f(x) = f(7.1) + (b/a)[f(7.4) - f(6.8)].
\]  

Hence,

\[
f(x) = f(7.1) + (b/a)[f(7.4) - f(6.8)].
\]  

Once the right hand side of Eq. 6 has been evaluated from the ratio \(b/a\), the unknown pH may be solved for by means of Eq. 1. The underlying dye concentration \([D_T]\) is, according to Beer's law, equal to \(a/(\varepsilon(480)\ell)\), where \(\ell\) is optical path length.

To apply these relationships, either to the optical bench measurements given in Fig. 1 B, or to the absorbance data from a dye-injected muscle fiber, a least-squares fitting procedure selected the values of \(a\) and \(b\) that minimized the quantity

\[
\sum_\lambda [A_r(\lambda) - a \tilde{A}_{7.1}(\lambda) - b \Delta \tilde{A}_{7.4-6.8}(\lambda)]^2.
\]

For the summation, the absorbance data collected in the range 460 < \(\lambda\) < 640 nm were used.

An analogous procedure was applied to the muscle measurements made with the wide-band interference filters. In this case the standard pH curves measured with the spectrophotometer (Fig. 1, A and C) were averaged over a 30-nm band. The estimated value of myoplasmic pH is not expected to depend significantly on whether the wide or narrow-band interference filters are used for the measurements. This expectation was tested in one experiment (fiber 010386.3), where myoplasmic pH estimated with the two filter sets was indeed found to be essentially identical (to within 0.02 pH units).
Fiber Viability

In 15 experiments phenol red was successfully micro-injected into a single fiber without producing obvious structural damage as a result of the injection. Eight of the fibers survived the injection well, in that they continued to give normal twitch responses and second component birefringence signals (amplitude, $-1 \text{ to } -3 \times 10^{-3}$ fractional change in light intensity; time to peak, 8–10 ms after the stimulating shock) for long periods (0.3–3 h) after injection. Results from these fibers constitute the main subject matter of this paper. A few observations from the other seven fibers are also mentioned, because a correlation was observed between loss of fiber viability and a reduced value of myoplasmic pH as estimated from phenol red.

RESULTS

To determine the indicator-related absorbance of an injected fiber, it is necessary to measure the total fiber absorbance and subtract the contribution of fiber intrinsic absorbance. Fiber intrinsic absorbance at wavelength $\lambda$, $A_i(\lambda)$, may be estimated from total fiber absorbance measured at a longer (reference) wavelength, $A(\lambda_{\text{long}})$. Separate equations were used to estimate $0^\circ$ and $90^\circ$ intrinsic absorbances, denoted by $A_{i,0}$ and $A_{i,90}$, as follows:

$$A_{i,0}(\lambda) = A_0(\lambda_{\text{long}})(\lambda_{\text{long}}/\lambda)^{1.1} \tag{7}$$
$$A_{i,90}(\lambda) = A_{90}(\lambda_{\text{long}})(\lambda_{\text{long}}/\lambda)^{1.5} \tag{8}$$

(Baylor et al., 1986). For our phenol red measurements, $\lambda_{\text{long}}$ was selected to be either 630 or 640 nm.

An example of the analysis applied to a fiber that contained a relatively large concentration of dye, ~2 mM, is illustrated in Fig. 2. Fig. 2 A shows the original measurements of total fiber absorbance obtained at wavelengths between 450 and 640 nm. The $0^\circ$ absorbances (circles) are significantly larger than the $90^\circ$ absorbances (crosses). Fig. 2 B plots the dye-related values, obtained by subtraction of the intrinsic absorbances estimated by means of Eqs. 7 and 8. Additionally, the data in part B have been corrected for a small but steady decrease in dye concentration during the measurements; this decrease was evaluated from interleaved absorbance measurements at 480 nm and is explained by diffusion of dye away from the site of injection (cf. Baylor et al., 1986). The difference between the $0^\circ$ and $90^\circ$ absorbances in part B is smaller than that in part A because $A_i(\lambda)$ is dichroic, with $A_{i,0}(\lambda) > A_{i,90}(\lambda)$ (Baylor et al., 1986). There is still, however, a detectable dichroism in part B (again with $A_0 > A_{90}$) that apparently increases at shorter wavelengths. When compared with the capillary measurements of Fig. 1 B, which were made at similar absolute absorbance levels, the dye-related dichroic signal in Fig. 2 B, while small, appears to be real.

The indicator-related dichroic signal may be seen more clearly by subtraction of $A_{90}$ from $A_0$ and expansion of the gain (Fig. 2 D, circles). An interesting question is whether the spectrum of the dichroic signal is characteristic of any particular pH. The data in Fig. 2 D were therefore least-squares fitted (curve in Fig. 2 D) by the procedure described in Methods. (For this and subsequent fits in the paper, the
Figure 2. Absorbance spectra from a muscle fiber injected with phenol red. A, Raw measurements of total fiber absorbance (intrinsic plus dye-related) obtained with 0° (circles) and 90° (crosses) polarized light. B, Dye-related absorbance, obtained from A by subtraction of the fiber intrinsic absorbance, estimated by Eqs. 7 and 8 in text. The correction requires \( A(640 \text{ nm}) \) to be 0 in B. C, Isotropic dye absorbance (circles), obtained from the measurements in B as the weighted average \( (A_0 + 2A_{90})/3 \). The curve is a cuvette calibration which gives the best fit to the data points at \( \lambda \geq 480 \text{ nm} \) (solid circles). The fitted value of pH was 7.17, as indicated. D, Dichroic dye absorbance (circles), \( A_0 - A_{90} \), obtained from the measurements in B and shown with expanded vertical gain. The curve is a best-fit cuvette calibration to the data points at \( \lambda \approx 480 \text{ nm} \) (solid circles) and indicates a pH of 6.26. The run was taken 10–12 min after dye injection; narrow-band (10-nm) filters were used throughout. In this 84-μm-diam fiber the average isotropic absorbance at 480 nm, 0.124, corresponds to a total dye concentration of 2.00 mM if \( \epsilon(480 \text{ nm}) \) is taken to be \( 1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \), if the myoplasmic water volume is taken to be 0.7 times fiber volume, and if \( p_g \), the geometric correction factor defined in Baylor et al. (1986), is taken to be 0.96. For this experiment, the pH of the normal Ringer’s solution was atypical (7.40 rather than 7.10; see Methods). Temperature, 16°C; fiber 010386.3. See Table I and text for additional details.

Muscle data points for \( \lambda < 480 \text{ nm} \) have not been included in the fit and are usually shown as open circles. As mentioned in Methods, these points are generally less reliable because of low light intensities from the tungsten–halogen bulb.) The data points are well fitted by a calibration curve with a pH of 6.26, a value considerably lower than that expected for the myoplasmic pH (next section).

A similar, small dichroic signal was found consistently in all phenol red injected fibers. Its presence indicates that a fraction of the phenol red molecules are bound to oriented structures accessible to myoplasm (cf. Baylor et al., 1986; Maylie et al.,
1987b, c). Some additional properties of the phenol red dichroic signal are described below.

**General Features of the Phenol Red Isotropic Signal**

Although the dye-related values of $A_0$ and $A_{90}$ in Fig. 2 B are not identical, they are closely similar, with a spectral shape that is quite different from the dichroic signal (Fig. 2 D). This similarity indicates that a major fraction of the phenol red molecules in myoplasm may not be bound to oriented structures but rather may be randomly disposed in the myoplasmic solution (although possibly bound to soluble constituents). The phenol red "isotropic" signal, defined as $(A_0 + 2A_{90})/3$ (Baylor et al., 1982c), is shown in Fig. 2 C (circles). This signal estimates the average value of the absorbance of all the dye molecules, independent of orientation, and therefore reflects contributions from at least two subpopulations of dye, the bound and oriented molecules and the nonoriented molecules.

The curve in Fig. 2 C is the least-squares fit of a pH spectrum to the isotropic absorbance data. Two major points are apparent from the fit. First, the spectrum of the indicator in this fiber was approximately that expected if myoplasmic pH was ~7.17, a value similar to pH measured in frog skeletal muscle by pH-sensitive glass micro-electrodes (7.04, Bolton and Vaughan-Jones, 1977; 7.18, Abercrombie et al., 1983). Second, over much of the wavelength range the muscle data are red-shifted by ~10 nm in comparison with the curve. Such a spectral shift probably results from the binding of dye to intracellular constituents (Thomas et al., 1979; Chaillet and Boron, 1985; Baylor et al., 1986; Konishi et al., 1988).

To quantitate the red-shift in the phenol red spectrum, the muscle absorbance data ($\lambda \geq 480$ nm) were least-squares fitted with cuvette calibration curves red-shifted by variable amounts, and the "best shift" (in nanometers) was determined as that which minimized the sum-of-squares deviation between the data and the calibration curve. Fig. 3 A shows an example, for the same data given in Fig. 2 C, where the best fit was obtained after a red-shift of the calibration curves by 10 nm. It is clear that the overall shape of the muscle data is better fitted by the shifted calibration curves. The fitted value of pH estimated from these curves, referred to hereafter as apparent pH (pH$_{app}$), was 7.25. Under the assumption that 490 nm (rather than 480 nm) is actually the isosbestic wavelength of phenol red in myoplasm, and that $e(490) = 1.1 \times 10^4$ M$^{-1}$ cm$^{-1}$, the value of [D$_T$] in Fig. 3 A is estimated to be 1.68 mM. This may be compared with the value of 2.00 mM obtained from the fit to the data in Fig. 2 C (see legend of Fig. 2).

Table I, column 3 indicates that a red-shift close to 10 nm was found in all fibers. The pH estimated from the red-shifted calibration curves was on average 0.06 pH units higher than the estimate obtained from the unshifted curves (not shown). Although essentially all the conclusions in the remainder of the paper would hold with only minor modifications if the muscle data were fitted by unshifted calibration curves, the 10-nm shifted curves have been chosen for the routine analysis. The use of shifted curves presumably permits greater accuracy in characterization of the indicator spectra in the experiments. However, it is not known whether the use of shifted curves leads to more (or less) accurate estimates of intracellular dye concentrations and levels of myoplasmic pH.
**Fiber-to-Fiber Variation in the Phenol Red Isotropic Spectrum**

Fig. 3, B–D show phenol red isotropic spectra from three other experiments. The values of pH\(_{\text{app}}\) show significant variation, ranging from 6.84 to 7.53. As shown in column 7 of Table I, the mean value of pH\(_{\text{app}}\) for eight fibers was 7.17 (±0.08 SEM). The variation in pH\(_{\text{app}}\) presumably indicates some significant fiber-to-fiber difference in the myoplasmic environment, for which the most obvious possibility is a difference in pH\(_i\). However, two other explanations may be proposed. First, the possibility exists that a variation in the amount or properties of bound dye could explain the variation in pH\(_{\text{app}}\). This might be considered unlikely, however, since the wavelength shift (column 3 of Table I) that provided a best fit by the calibration curves, a number that is presumably related to dye-binding, was very similar for all fibers.

The second possibility is that phenol red might become compartmentalized in some internal space (e.g., within the sarcoplasmic reticulum) or metabolized or chemically altered in some way, and that such changes might proceed differently in different fibers. If the changes proceeded on a slow time scale, then pH\(_{\text{app}}\) in any given fiber might vary with time. The data in Fig. 4, which show pH\(_{\text{app}}\) as a function

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**Figure 3.** Examples of fiber-to-fiber variation in isotropic absorbance (circles) and corresponding fits of pH\(_{\text{app}}\) (curves). The muscle measurements were made with narrow-band filters in A and C, and with wide-band filters in B and D. The fits were obtained with 10-nm red-shifted calibration curves (see text); the number next to each curve is the best-fit value of pH\(_{\text{app}}\). Open circles indicate data points that were not included in the fits. A, Same data set as in Fig. 2 C, Fiber 111385.2; 16°C; run taken 8–11 min after injection. C, Fiber 122785.1; 16°C; run taken ~4–7 min after injection. D, Fiber 111385.1; 16°C; run taken 14–21 min after injection. See Table I for values of [D\(_i\)], etc.
Table I summarizes the analysis from each fiber of the most complete absorbance measurements made relatively early (5-22 min) after injection. Column 1, fiber identification; column 2, average fiber diameter; column 3, the red-shift (in nanometers) which, when applied to the cuvette calibration curves, provided a best-fit to the shape of the dye-related isotropic spectrum (λ = 480 nm). Columns 4 and 5, the average values of isotropic absorbance, (A₀ + 2A₁)/3, measured at 480 and 570 nm, respectively. Column 6, the corresponding estimate of total dye concentration. Column 7, the apparent pH of the isotropic spectrum (λ = 480 nm) obtained with 10-nm shifted calibration curves by the fitting procedure described in Methods. Columns 8 and 9, the average amplitude of the dichroic absorbances at 480 and 570 nm. Column 10 gives the lower limit for the concentration of oriented dye obtained, as described in the text, from the estimated dichroic absorbance at 490 nm. Column 11, the apparent pH of the dichroic spectrum obtained with 10-nm shifted calibration curves. Ringer pH was 7.10 in all experiments except for fiber 010386.3, for which it was 7.40 (see Methods). The pH of the injected phenol red solution was titrated to 7.0 in all experiments except for fiber 122785.1, for which it was untitrated (pH = 8.7). Fibers 111385.1, 122785.1, and 122785.4 were in 1.8 mM Ca²⁺ Ringer’s; all other fibers were in 11.8 mM Ca²⁺ Ringer’s. -, Data not measured; * data too noisy to resolve, either because of lamp instability or the small values of dye-related absorbance. For fibers 110785.2, 111385.1, 111385.2, and 051586.1 measurements were carried out with wide-band interference filters, and, for the remainder, with narrow-band filters.

Table 1

| Fiber no. | Fiber diameter | Best shift | A(480) | A(570) | [D₀] | pH_{isotropic} | A₀(A) - A₀(B) | Minimal oriented dye pH_{dichroic} | pH_{app} | pH_{app} |
|-----------|----------------|------------|--------|--------|------|----------------|----------------|-------------------------------|-----------|----------|
| 1         | 120            | 0.097      | 0.098  | 0.92   | 7.24 | 0.007          | 0.001          | 19                   | 10        | 11       |
| 111385.1  | 115            | 0.192      | 0.092  | 1.79   | 6.84 | 0.023          | 0.002          | 69                   | 5.89      |          |
| 111385.2  | 90             | 0.189      | 0.244  | 2.47   | 7.36 | 0.016          | 0.003          | 61                   | 6.43      |          |
| 122785.1  | 129            | 0.055      | --     | 0.52   | 7.51 | *              | *              | *                   | *         |          |
| 122785.4  | 99             | 0.204      | 0.203  | 2.30   | 7.15 | 0.030          | 0.005          | 103                  | 6.06      |          |
| 010386.1  | 101            | 0.110      | 0.119  | 1.22   | 7.21 | 0.011          | 0.002          | 39                   | 6.49      |          |
| 010386.3  | 84             | 0.124      | 0.146  | 1.68   | 7.25 | 0.017          | 0.004          | 71                   | 6.37      |          |
| 051586.1  | 74             | *          | 0.210  | 0.093  | 3.05 | 6.81           | 0.020          | 87                   | 6.24      |          |
| Mean      |                |            | 10.1   |        | 7.17 |                |                | 6.24                  | (±SEM)    | (±0.4)   |

For each fiber, measurements were obtained 0-150 μm from the site of dye injection.
of time after injection for five fibers, indicate that such changes do not take place in healthy fibers. Three of the fibers survived the injection extremely well and still responded in an all-or-none fashion more than 2 h later. In these fibers, pH$_{\text{app}}$ estimated shortly after injection was 7.26, 7.24, and 6.81, and remained near these levels for the duration of the experiments. It thus appears unlikely that phenol red became compartmentalized or metabolized during the time of the measurements, at least in a manner that produced an alteration in the spectral properties of the dye. In further support of this interpretation is the finding that the wavelength shift that provided a best fit of the muscle data with the cuvette spectra did not change significantly with time (fibers 110785.2 and 111385.2, data not shown). Although a chemical alteration that was complete within the first few minutes after injection, or a rapid and nonsaturable compartmentalization, cannot be ruled out, the most likely conclusion is that the variations in pH$_{\text{app}}$ in Table I do reflect fiber-to-fiber variations in pH$_{i}$.

One fiber in Fig. 4 did show a marked drop in pH$_{\text{app}}$ with time, falling from 7.15 to 7.04 in 10–15 min. However, this fiber ceased to respond in an all-or-none fashion shortly after the second measurement. Thus, the relatively rapid acidification observed was probably associated with injection damage. Interestingly, the mean value of pH$_{\text{app}}$ measured in seven fibers that had lost their all-or-none response as a result of the injection was 6.93 (±0.05 SEM; range, 6.80–7.17). This average value is significantly lower ($P < 0.05$, Student’s $t$ test) than the value of 7.17 (±0.08 SEM) measured from the fibers in Table I. Not surprisingly, therefore, injection damage appears to be associated with acidification, possibly directly, as a result of proton leaks, or as a result of increased metabolic activity (for instance of ATP-dependent membrane pumps working to maintain ionic concentration gradients). More acidic fibers are not necessarily damaged, however, at least as judged from functional parameters. For example, one of the fibers in Fig. 4 responded with an all-or-none tension response and a birefringence signal of normal amplitude for more than 2.5 h after injection, during which time pH$_{\text{app}}$ remained steady, between 6.8 and 6.9.

Changes in pH$_{\text{app}}$ with Acid or Alkaline Loads

The results described thus far indicate that variations in the isotropic absorbance of phenol red in myoplasm probably reflect variations in pH$_{i}$. To directly test this idea, fibers were exposed to acid or alkaline loads, achieved with CO$_2$ or NH$_3$, respectively, added to Ringer (Aickin and Thomas, 1977; Bolton and Vaughan-Jones, 1977; Abercrombie et al., 1983; Curtin, 1986; Putnam and Roos, 1986). Both CO$_2$ and NH$_3$ rapidly equilibrate across the plasma membrane, but the ionized forms do not. CO$_2$ in myoplasm will hydrate and release a proton, forming HCO$_3^-$ and H$^+$, while NH$_3$ will remove a proton by forming NH$_4^+$. 

Fig. 5 A shows examples of phenol red spectra obtained from a fiber before exposure to 5% CO$_2$ (solid circles), 2–3 min after exposure (open circles), and 33 min after return to normal Ringer (crosses). The spectra ($\lambda \approx 480$ nm) have been fitted in the usual way with the 10-nm shifted calibrations to estimate pH$_{\text{app}}$; the data are therefore displayed after normalization relative to an isosbestic point at 490 nm. The fitted values of pH$_{\text{app}}$ obtained from Fig. 5 A and from other measurements in the same fiber are plotted as a function of time in Fig. 6 A.
Figs. 5 A and 6 A clearly show that when a fiber is subjected to an acid load the isotropic absorbance of phenol red responds qualitatively in the manner expected of a myoplasmic pH indicator. A more quantitative assessment may be made by calculation of the apparent buffering power of myoplasm (denoted by B.P.), defined by

$$\text{B.P.} = \frac{\text{amount of acid produced}}{\text{change in pH}_i},$$

which may be calculated from the magnitude of the observed change in pH_{app} (denoted by ∆pH_{app}) after adoption of standard assumptions (cf. Aickin and Thomas, 1977; Bolton and Vaughan-Jones, 1977; Boron, 1977; Abercrombie et al., 1983; Curtin, 1986). The amount of acid produced is equal to the HCO₃⁻ concentration in myoplasm generated by the equilibration of CO₂ across the membrane. The HCO₃⁻ concentration can be calculated from the law of mass action (Henderson-Hasselbalch equation; assumed pK of 6.16 for the CO₂/HCO₃⁻ reaction at 16°C) if pH_i and the CO₂ concentration are known and, additionally, if it is assumed that HCO₃⁻ itself does not bind significantly to intracellular sites. For the data in Fig. 6 A, after identification of pH_{app} with pH_i, B.P. upon exposure to CO₂ is 14.67 meq of H⁺ per liter myoplasm/(7.30 - 6.95) = 42 meq per liter per pH unit; a similar value can be calculated from the ∆pH_{app} observed after removal of CO₂. A value of 42 meq per liter per pH unit for B.P. is close to the mean values of B.P. calculated for frog myoplasm based on pH-sensitive micro-electrode measurements (35, 26, and 38 meq per liter per pH unit reported, respectively, by Bolton and Vaughan-Jones, 1977, Abercrombie et al., 1983, and Curtin, 1986). Thus, the phenol red absorbance signal responds, at least on the slow time scale of these measurements, in the quantitative manner expected for a properly functioning indicator of pH_i.
The analogous experiment carried out with NH₃ to achieve an alkaline load is shown in Figs. 5B and 6B. In this case the dye spectrum also responded in the manner expected. Shortly after exposure to NH₃-Ringer's, pH_{app} had increased by ~0.25 pH units, whereas shortly after return to normal Ringer's, pH_{app} decreased by ~0.4 pH units and then slowly increased to the initial level. An undershoot and slow recovery in pHₐ upon removal of NH₃ was also observed with pH-sensitive micro-electrodes, in mammalian skeletal muscle by Aickin and Thomas (1977) and in frog skeletal muscle by Putnam and Roos (1986).

Again, B.P. may be calculated from the NH₃ experiment. If the pK of the NH₃/\text{NH}_4^+ reaction in myoplasm is 9.02 (Aickin and Thomas, 1977), the values calculated for the NH₃ exposure and removal are, respectively, 93 and 82 meq per liter per pH unit. These values for B.P. are higher than calculated from the CO₂ experiment, a difference that might be explained, at least in part, by a failure to detect a larger ΔpH_{app}, because of the temporal limitations of the procedure used to change the bathing solutions (see Methods). Even so, the value of 82 meq per liter per pH unit calculated upon removal of NH₃ is similar to that reported by Aickin and Thomas (1977) for mouse soleus muscle (66 meq per liter per pH unit; average of three entries in their Table I, which were measured at 28–37°C). Aickin and Thomas chose not to calculate B.P. from their data upon exposure to NH₃ because of the possibility of a rapid entry of NH₄⁺ from the extracellular space. It may also be noted that, as in Fig. 6B, their data show a peak increase in pHₐ upon exposure to NH₃ that was only 60–65% of the peak decrease in pHₐ observed upon removal of NH₃. Thus, the NH₃
The experiment of Figs. 5 and 6 is quantitatively close to that expected from previous work with pH-sensitive micro-electrodes.

Overall, both experiments in Figs. 5 and 6 support the conclusion that, in spite of the evidence for the presence of bound phenol red molecules (Figs. 2 and 3; also see below), the isotropic absorbance signal appears to change quantitatively in the manner expected for a properly functioning indicator.

**Relation of pH_{app} to Dye Concentration**

A basic question concerning the phenol red isotropic signal is whether pH_{app} is independent of [DT], as would be expected from the 1:1 stoichiometry observed in vitro for the indicator's reaction with protons. Such independence is suggested by the data in Fig. 4 for the three fibers with measurements up to 160 min after injection. In these experiments pH_{app} varied little over a time when [DT] decreased, by factors between 2.2 and 2.8, because of dye diffusion away from the site of injection. Information over a wider range of dye concentrations was obtained from these fibers and in one other fiber, from measurements of pH_{app} at different distances along the fiber axis from the site of dye injection. Since these fibers were stable, data collected at different times could also be included in the analysis, and pH_{app} could be estimated from measurements in which dye concentration varied over a nearly 10-fold range. The results of the analysis for the four fibers are shown in Fig. 7.

Fig. 7 A plots pH_{app} as a function of distance from the injection site. Overall, relatively little spatial gradient in pH_{app} is seen, with an average variation about the mean of <0.1 pH units for distances up to ~1,000 µm from the injection site. Beyond these distances, dye concentrations were too small, with dye-related absorbances <0.01 absorbance units, to accurately resolve the ratio of A(570)/A(480), the principal determinant of pH_{app} in these measurements. Data for three of the fibers in Fig. 7 A suggest that pH_{app} may have increased slightly with distance from the injection site. Such an increase is consistent with the possibility that slight damage due to the injection process can cause a small acidification at the injection site.

In Fig. 7 B the pH_{app} data from part A have been plotted directly as a function of [DT]; also included are other data points from the same fibers measured both before and after the spatial runs in part A. In general, most of the additional data points in part B were obtained near the injection site (±150 µm). The pooled data in Fig. 7 B indicate that there is little or no correlation between pH_{app} and [DT] for dye concentrations at least as high as 2–3 mM, as expected for 1:1 stoichiometry.

**The Apparent Diffusion Constant of Phenol Red in Myoplasm**

The red-shifted absorbance spectrum of phenol red in myoplasm (Fig. 2 C and column 3 of Table I) was attributed to the effects of bound dye. A quantitative estimate of the fraction of the dye that is bound can be obtained from D_{app}, the dye’s apparent diffusion constant in myoplasm (Kovacs et al., 1983; Irving et al., 1985; Baylor et al., 1986, 1989; Maylie et al., 1987a, b, c).
If a rapid equilibration is established between bound and free dye, then

\[ D_{\text{app}} = \frac{D}{1 + R}, \]  

where \( D \) denotes the actual diffusion constant of the unbound (i.e., freely mobile) dye and \( R \) denotes the ratio of bound to free dye, assumed to be independent of dye concentration (cf. Crank, 1956). Since estimates of \( D \) in myoplasm are available for several compounds spanning a range of molecular weights, both from skinned fibers (Kushmerick and Podolsky, 1969) and from cut fibers (Maylie et al., 1987a, b, c), measurement of \( D_{\text{app}} \) in intact fibers permits an estimate of \( R \). For example, \( D \) in myoplasm for phenol red (MW = 346) should be close to the value of \( D \) measured for: (a) sucrose (MW = 342), namely \( 2.1 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) at 20°C (Kushmerick and Podolsky, 1969), or \( 1.8-1.9 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) at 15-17°C (if \( Q_{10} \) for \( D_{\text{app}} \) is 1.3); and...
tetramethylmurexide (MW = 340), namely, $1.75 \times 10^{-6}$ cm$^2$ s$^{-1}$ (average of seven measurements by Maylie et al. (1987a) at 18°C, for which the range was $1.3-2.3 \times 10^{-6}$ cm$^2$ s$^{-1}$).

Estimates of $D_{app}$ for phenol red in intact fibers were made as previously described (Baylor et al., 1986). Fig. 8 plots values of $[D_T]$ (open circles; proportional to indicator absorbance) measured from a single fiber at different distances $x$ from the injection site. The points in panel A were measured relatively early in the experiment (36–47 min after injection), whereas those in panel B were measured considerably later (98–111 min after injection). As expected for a diffusing substance, the concentration profile measured at the later time has a smaller peak amplitude and a broader half-width. Either data set, or both sets combined, may be used to estimate $D_{app}$ (cm$^2$ s$^{-1}$), under the assumption that dye concentration as a function of distance and time, denoted by $[D_T](x, t)$, satisfies the solution to the one-dimensional diffusion equation (Crank, 1956):

$$[D_T](x, t) = \frac{M}{2 \sqrt{\pi D_{app}}} \exp\left(\frac{-x^2}{4D_{app}}\right). \quad (11)$$

In Eq. 11, $M$ (µmol cm$^{-2}$) is the total amount of dye per fiber cross-sectional area injected at location $x = 0$ and time $t = 0$. For the fitting of Eq. 11 to the data, both $D_{app}$ and $M$ were adjusted to minimize the sum-of-squares deviation between observed and predicted values of $[D_T]$. The solid circles in Fig. 8 show the best
least-squares fit to the combined data sets in Fig. 8. The data are well described by Eq. 11, with the fitted values of \( D_{\text{app}} \) and \( M \) being \( 0.34 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) and 0.118 \( \mu \text{mol cm}^{-2} \), respectively. Closely similar values were obtained for each data set analyzed separately (Fig. 8A: \( D_{\text{app}} = 0.35 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) and \( M = 0.121 \mu \text{mol cm}^{-2} \); Fig. 8B: \( D_{\text{app}} = 0.33 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) and \( M = 0.115 \mu \text{mol cm}^{-2} \)).

Table II summarizes results from five fibers. The average value of \( D_{\text{app}} \) was 0.37 \( \pm 0.03 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1} \). This value is about fivefold smaller than expected for \( D \) itself, and according to Eq. 10, is explained if ~80% of the phenol red molecules in myoplasm are bound to effectively immobile sites, with only 20% of the dye being freely diffusible.

### Table II

| Fiber no. | Temporal variation | Spatial variation | \([D_4]\) | \(\text{pH}_{\text{iso}}\) | \(M\) | \(D_{\text{app}}\) |
|-----------|--------------------|------------------|--------|----------------|------|---------------|
| 1         | 82-95              | 0, +1,250        | 0.5    | 7.22-7.25      | 0.07 | 0.32         |
| 110785.2  | 44-59              | 0, +1,300        | 1.2    | 6.83-6.95      | 0.16 | 0.50         |
| 111385.1  | 36-111             | -1,100, +1,350   | 0.7-1.2| 7.21-7.37      | 0.12 | 0.54         |
| 010386.1  | 57-68              | -800, +1,400     | 0.4    | 7.13-7.36      | 0.05 | 0.34         |
| 051586.1  | 133-144            | -800, +1,200     | 1.2    | 6.78-6.99      | 0.22 | 0.34         |
| Mean      |                    |                  |        |                |      | 0.37         |
| (±SEM)    |                    |                  |        |                |      | (±0.03)      |

Table II summarizes the results of fitting Eq. 11 in the text to measurements of isotropic absorbance made at 480 nm as a function of distance from the injection site and time after injection. The variation in time and distance is given, respectively, in columns 2 and 3. Column 4 gives the average dye concentration at the injection site during the measurement and column 5 the range in \(\text{pH}_{\text{iso}}\) (see text). Columns 6 and 7 give the best fit of the two parameters in Eq. 11 of the text to each fiber’s data set after least-squares adjustment. The temperature in all experiments was 16.0-16.5°C.

### The Phenol Red Dichroic Signal

Fig. 2D demonstrated the existence of a phenol red dichroic signal, \( A_0(\lambda) - A_{90}(\lambda) \). The most likely explanation for this signal is that some of the phenol red molecules are bound to oriented structures, such as the sarcoplasmic reticulum or the myofilaments. An analysis of the properties of the dichroic signal may give information about possible alterations in dye properties attributable to the binding (cf. Baylor et al., 1986) and/or possible pH differences in the local environment of the binding site(s).

Columns 8–11 of Table I summarize information about the dichroic signal. Columns 8–9 show that this signal was detected in all fibers in which the isotropic absorbance at 480 nm was at least 0.06 (or, equivalently, a total dye concentration, \([D_4]\), ≥ 0.6 mM). A dichroic signal was also detected in many of the later runs, not shown in Table I, when \(A(480)\) was <0.06. For all runs indicated in Table I, the dichroic absorbance spectrum for \(\lambda \geq 480 \text{ nm} \) was well fitted by a calibration curve.
for pH_{app} (denoted by pH_{app}^{dichroic}). The mean value (±SEM) for pH_{app}^{dichroic} was 6.24 (±0.08). A comparison of columns 7 and 11 indicates that of pH_{app}^{dichroic} was consistently more acidic than pH_{app}^{isotropic}, on average by ~0.9 pH units.

One interpretation of the difference between pH_{app}^{dichroic} and pH_{app}^{isotropic} is that the phenol red molecules that are bound to oriented structures can still exchange protons with the myoplasmic pool, but do so with a pK 0.9 units higher than that of the nonoriented dye molecules. If correct, this would clearly demonstrate that dye binding to intracellular constituents can markedly change the pK of the indicator. Alternatively, the oriented dye may be in an environment that is 0.9 units more acidic than is the myoplasm as a whole. For example, the oriented phenol red molecules may be bound to the myofilaments, which, because of their fixed negative charge (e.g., Naylor et al., 1985), may attract protons and therefore reduce pH in the local environment.

In either case, it is of interest to know if pH_{app}^{dichroic} responds to changes in pH. An analysis (not shown) of the dichroic signals from the fibers of Figs. 5 and 6 indicated that this was probably the case. In response to CO_2 exposure, pH_{app}^{dichroic} changed from an average value of 6.42 before, to a value ≤5.8 during, to a value of 6.64 just after removal of the acid load. Conversely, in the NH_3 experiment, pH_{app}^{dichroic} went from 6.44 before, to 6.51 during, to 6.44 after the alkaline load. These changes in pH_{app}^{dichroic}, while not distinguishing between the two hypotheses mentioned in the preceding paragraph, suggest that the oriented phenol red molecules are sensitive to pH.

A Lower Limit for the Concentration of Bound and Oriented Phenol Red Molecules

Column 10 of Table I gives the minimum concentration (in μM) of oriented phenol red molecules required to explain the observed value of A_0(480) − A_90(480) (column 8 of Table I). For the conversion of column 8 to column 10, the dichroic amplitude at 490 nm was estimated from the measurement in column 8 and the least-squares fitted curve corresponding to the pH_{app}^{dichroic} given in column 11. The ratio [A_0(490) − A_90(490)]/(3\times1.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1}), where \ell denotes optical path length and the factor of 3 reflects the three possible spatial dimensions for oriented dye (cf. Baylor et al., 1982b), then estimates the minimum concentration of oriented dye molecules (column 10 of Table I). The value of column 10 divided by column 6 for each experiment (not shown in the table) corresponds to the fraction of oriented dye and was 0.033 on average (n = 7). This lower limit for the fraction of oriented phenol red molecules is similar to the corresponding estimates (Baylor et al., 1986) made for two metallochromic dyes in intact frog fibers (antipyrilyazo III, 0.028–0.030; arsenazo III, 0.015–0.018). In the case of phenol red, the finding that the shape of the dichroic spectrum was quite different from that of the isotropic spectrum (yet both shapes could be described by calibration curves for pH_{app}) supports the interpretation that the dichroic signal is real and not, for example, the result of a path length artifact. The fact that the spectrum of the phenol red dichroic signal is quite different from that of the intrinsic dichroic signal (Baylor et al., 1986) also makes it unlikely that the phenol red dichroic signal is an artifact of the procedure used for subtraction of the fiber's intrinsic dichroism.
DISCUSSION

This paper reports on the first detailed examination of the properties of a pH indicator dye introduced into the myoplasm of single muscle cells. The properties measured include the spectral and polarization features of the dye's absorbance, the indicator's response to imposed changes in myoplasmic pH, and the dye's apparent diffusion constant in myoplasm. The results demonstrate both the strengths and weaknesses inherent in the intracellular use of indicator dyes and support a number of conclusions reached previously in the case of absorbance (Baylor et al., 1982a, b, c; 1985; 1986; Hollingworth and Baylor, 1986; Maylie et al., 1987a, b, c) and fluorescence (Baylor and Hollingworth, 1988; Klein et al., 1988; Konishi et al., 1988) indicators for Mg$^{2+}$ and Ca$^{2+}$. In general, indicator properties appear to be altered by the intracellular environment. The likely source of the alteration is the binding of dye to intracellular constituents, for example, soluble proteins (Beeler et al., 1980; Konishi et al., 1988) and oriented structures (Baylor et al., 1982a, 1986, and this study). In the case of phenol red, evaluation of the dye's behavior was facilitated by the fact that the dye in vitro responds to pH alone (and not to Ca$^{2+}$ and Mg$^{2+}$), and that detailed studies of pH$_i$ had previously been carried out in frog skeletal muscle fibers with pH-sensitive micro-electrodes (Bolton and Vaughan-Jones, 1977; Abercrombie et al., 1983; Curtin, 1986; Putnam and Roos, 1986).

Clear evidence was obtained for the existence of at least three subpopulations of phenol red in myoplasm. The measurements of $D_{app}$ (Fig. 8 and Table II) suggest that only 20% of the indicator is freely diffusible and that the majority of the indicator, 80%, is bound to relatively immobile intracellular constituents. Moreover, at least two populations of bound dye can be distinguished. A minimum of 3% of the dye is bound and oriented, as evidenced by the dichroic absorbance signal. It is very unlikely, however, that all of the bound dye is oriented. If this were so, the dichroic and isotropic spectra should be similar, differing only by the contribution of free dye. In fact, the dichroic spectrum is very different from the isotropic spectrum (cf. Fig. 2, C and D), indicating that a second population of bound dye is quantitatively important. This dye is presumably bound to soluble myoplasmic constituents and may constitute the largest population of dye within the fiber. A further indication of bound dye (either oriented or unoriented) is the red-shift of 9–12 nm detected in the isotropic spectrum (Figs. 2 and 3 and Table I). If only 3% of the phenol red is oriented, then the red-shift must be a property of the bound but unoriented dye. If the percentage of oriented dye is substantially larger, then either or both of the bound populations might contribute to the shift.

Estimates of pH$_i$ from the Isotropic Signal

The largest and most easily measured signal from phenol red in myoplasm is its isotropic spectrum, $[A_0(\lambda) + 2A_{iso}(\lambda)]/3$, which represents the average absorbance of all phenol red molecules independent of their orientation. The experiments show that this spectrum does not report pH$_i$ in a simple fashion and that each of the three identified populations of dye probably have different affinities for protons. Values of pH$_i$ in frog fibers have been reported previously from measurements with pH-sensitive micro-electrodes (7.05 at 25°C and equilibrated with 100% O$_2$, when the
pH of Ringer was 7.23; Bolton and Vaughan-Jones, 1977; 7.18 at 22°C and also equilibrated with 100% O₂, when Ringer's pH was 7.35; Abercrombie et al., 1983) and a value of 6.9–7.1 might be expected under the conditions of our experiments (Malan et al., 1976). The freely diffusible dye, which presumably senses myoplasmic pH with a pK close to that measured in the in vitro calibrations (7.73), thus probably indicates a pH close to 7.0. Since the oriented dye indicates a pH of 6.24, the pK of this dye might be 8.5, ~0.76 (7.0–6.24) units higher than that of free dye. Since pH_{isotropic} is ~0.2 units more alkaline than expected, and since pH_{app} is more acidic than pH_{isotropic}, the conclusion follows that the pK of dye bound to soluble constituents is lower than that of free dye. An estimate of the value of this third pK would depend on the size of the oriented dye fraction, which is uncertain. Given this complexity, the absolute calibration of resting pH from the phenol red isotropic absorbance is clearly uncertain.

Our findings in muscle may be compared with related results reported from other preparations. An increase in the pK of phenol red of 0.4 units has been reported from Limulus photoreceptor cells (Bolsover et al., 1986) and an increase of 0.3 units was reported for the pK of the indicator dimethyl-carboxyfluorescein in salamander proximal tubule cells (Chaillet and Boron, 1985). In both cases the changes were attributed to dye binding. A red-shift in the cytoplasmic absorbance of dimethyl-carboxyfluorescein was also reported (Chaillet and Boron, 1985; cf. Thomas et al., 1979). There was no evidence, however, for a red-shift of the phenol red absorbance spectrum in Limulus photoreceptors.

In spite of the altered properties of phenol red in muscle, the results of Figs. 5 and 6 show that when fibers were subjected to acid and alkaline loads, the isotropic absorbance changed approximately in the quantitative manner expected if the effective pK and the changes in extinction coefficient of the indicator were similar in myoplasm to those measured in the in vitro calibrations. This suggests that the indicator should be useful for the estimation of changes in myoplasmic pH under experimental conditions where pH-sensitive micro-electrodes are of limited use, for example, when fiber movements threaten the stability of micro-electrode recordings, or in tracking the rapid changes in pH that possibly accompany excitation-contraction coupling (Baylor et al., 1982b, 1987; Irving et al., 1989; Hollingworth and Baylor, 1990; Pape et al., 1990).

Fiber-to-Fiber Variations in pH_{app}

The results in column 7 of Table I indicate that there is a significant variation in pH among fibers judged by other criteria to be normal. Fiber-to-fiber variations in pH probably represent an important source of variability in the signals observed with some metallochromic dyes, for example, the myoplasmic-free [Mg²⁺] signals from arsenazo III and antipyrylazo III (Baylor et al., 1982b, c, 1986; Irving et al., 1985), which are quite pH sensitive. Although the phenol red isotropic signal has given no reliable new information about the absolute mean level of myoplasmic pH under the conditions of our experiments, the results in Table I suggest that the standard deviation about the mean is ~0.2–0.3 pH units. Variations in pH of this magnitude are sufficient to explain the two- to threefold fiber-to-fiber variations in the estimates of free [Mg²⁺] found previously with either arsenazo III or antipyrylazo III.
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