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A micro-spectrophotometric study on the spectral properties of phenol red injected into cytoplasm for pH_i determination

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Abstract

The spectral properties of phenol red, used to determine intracellular pH (pH_i), have been studied in the myoplasm of cultured rat skeletal muscle cells, by means of a micro-spectrophotometric technique. Two sets of intracellular calibration spectra at different dye concentrations were established in the presence of nigericin. Intracellular spectra of phenol red differ from the absorption spectra obtained in buffered solutions in the following ways: (1) Considerably greater absorbance in the violet region, owing to scattering of light by intracellular particles, which can be corrected by applying an energy transfer model derived from the work of Kubelka and Munk (*Z. Tech. Phys.* 12 (1931) 593). (2) Slight spectral red-shift and an increase in the dye pK_d, assumed to be due to interaction of the dye with intracellular constituents, especially soluble proteins, since the same spectral modifications are observed in buffered solutions containing bovine serum albumin. The potential advantages of using phenol red to determine pH_i are the simple dye-H^+ interaction, both in buffered solutions and in myoplasm, and the absence of dye selfassociations at the high dye concentrations required to perform intracellular measurements. Finally, our results show that absolute pH_i can be measured to an accuracy of ± 0.02 pH units using phenol red, if complete absorption spectra and intracellular spectral calibrations are available.

Keywords: Micro-spectrophotometry; Phenol red; Intracellular pH (pH_i); Myoball; Nigericin; Light scattering

1. Introduction

Intracellular pH (pH_i) is an essential determinant of the finely balanced metabolic and synthetic activities of the cell. Knowledge of pH_i values is therefore required in order to investigate the various intracellular and membrane mechanisms, and for precise calibration of many optical methods for studying the intracellular environment.

Phenol red, a classic acid-base indicator commonly used in analytical chemistry, has been employed for over a decade to measure pH_i and to monitor its variations in excitable cells [1–4]. Little information is available, however, about its behavior in the intracellular environment. Intracellular signals are often interpreted on the basis

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of *in vitro* calibrations, assuming that the dye indicates pH in cytoplasm in the same way as it does in buffered solutions.

Recently, Baylor and Hollingworth [5] studied intracellular absorbance of phenol red in frog skeletal muscle fibers, using a multi-wavelength measurement technique. They concluded that the properties of the dye may be altered to an unknown extent inside the cell, making measurements of pH_i uncertain.

In the study reported here, the absorption spectra of phenol red, injected under pressure into the myoplasm of cultured rat skeletal muscle cells, were recorded using the microspectrophotometric technique recently developed in our laboratory [6]. The spectra of intracellular phenol red were found to be of quite different shape from those obtained in buffered solutions in thin cuvettes. The mechanisms of the spectral deformations observed in cells were examined by establishing intracellular calibration spectra for two concentrations of dye ([D]).

2. Materials and methods

2.1. Microspectrophotometer

Basically, we used the system described previously [6], in which a cell preparation, mounted on an Olympus IMT-2 microscope bench, is illuminated by a 75-W, forced air-cooled, quartz tungsten–halogen lamp. A heat filter and a wide-band blue filter are used to equalize the light emission intensities over a 385–665 nm wavelength range, calibrated with a series of interference filters, and linearized by means of a three-term polynomial adjustment.

A portion of the observation field, representing an area of about 20 μm^2, is focused on the input slit of a prism monochromator, and the transmitted spectrum is projected onto an array of 512 photodiodes (PCD Hamamatsu type S2301). The charges due to photon energy transfer are collected through a specially designed interface, and converted into 12-bit digital values, at a rate of one spectrum per second in the present study.

With long acquisition times, the thermal charges (darkness level) can alter the spectral shape, especially in the violet range, when the sensitivity of the photodiodes is low. The darkness level is therefore recorded prior to spectrum acquisition, and taken into account in calculations.

2.2. Cell preparation and treatment

Primary culture of skeletal muscle cells were prepared from one-day-old rats as already described [6]. In order to obtain higher absorption signals at the same dye concentration, the long, thin myotubes were transformed into rounded myoballs by the application of colchicine (20 n*M*) to the culture medium from day 5. The myoballs used in this study were about 40 μm in diameter, and had several nuclei in the peripheral region.

The colchicine-treated myoballs show normal contraction, normal response to electrical and chemical stimuli, and normal structural properties, apart from the absence of myotubules and disorganization of the contractile material [7,8]. For measurements of pH_i, the culture medium was replaced by a Tyrode solution containing 137 m*M* NaCl, 5.4 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.33 m*M* NaH_2PO_4, 10 m*M* glucose, 4 m*M* mannitol, 100 mg thiamine, buffered with 10 m*M*-HEPES to pH 7.4. The bath temperature was regulated to 25°C or 37°C.

2.3. Dye injection and measurement of intracellular transmission spectra

Phenol red, dissolved to 10 m*M* in a solution of 30 m*M* KCl and 5 m*M* K_2PIPES (pH 7.2), was injected under pressure into the myoplasm through a microelectrode containing a silver wire to ensure electrical contact for monitoring membrane potential.

The intracellular transmission spectra of the dye were obtained by recording the spectrum in the central region of an uncolored myoball, considered to be the reference spectrum, and then recording the spectrum after the dye was injected and distributed homogeneously inside the myoplasm. The second recording was divided di-

rectly "on line" by the reference spectrum, so that the spectra recorded in colored myoballs were already corrected for the system response and for the intrinsic absorbance of cells.

Intracellular dye concentrations, [D], were estimated according to the Beer–Lambert law:

$$A = \varepsilon l[\mathrm{D}]$$

where A is the absorbance measured at 485 nm, the isosbestic point of intracellular dye; ε, the molar extinction coefficient of the dye at the isosbestic point; and l, the cell thickness evaluated with the microscope micrometer under phase contrast conditions. A value of 1.16×10^4 M^{-1} cm^{-1} for ε, determined in a buffered solution on the microspectrophotometer, was considered to be convenient for estimating the intracellular dye concentrations.

2.4. *In vitro spectral calibration*

In vitro spectral calibrations were carried out on a Shimadzu spectrophotometer as well as on our microspectrophotometer, in buffered solutions with and without bovine serum albumin (BSA, from Sigma). The buffered solutions contained 120 mM KCl, 10 mM HEPES and 10 mM K_2PIPES. To prepare the buffered solution containing BSA, 1.5% of BSA (about 0.25 mM) were added and stirred gently for half a day, in order to dissolve completely the protein. The pH of the solutions was set to the desired values, and read directly on a digital pH meter (S93313, Bioblock Scientific), which was calibrated precisely before the preparation of solutions, to an accuracy of ± 0.01 pH unit.

2.5. *Intracellular spectral calibration*

In order to calibrate the dye intracellularly, i.e., to record the phenol red spectra in myoplasm at known pH_i, a method capable of imposing a pH_i at the desired values is required. Nigericin, an electroneutral ionophore, has been shown to carry one H^+ across cell membranes in exchange for one K^+, with a high selectivity [9–11]. It was therefore used to equilibrate the pH_i with the well-buffered extracellular pH (pH_0) in the present study.

As nigericin acts as a K^+–H^+ exchanger, it establishes a transmembrane H^+ gradient that depends on the K^+ concentration gradient across the membrane ($[K^+]_i/[K^+]_0$) [12]. In order to achieve transmembrane H^+ equilibrium, the $[K^+]_i/[K^+]_0$ gradient must be abolished. For this purpose, the Tyrode solution was modified in the following ways: (1) 140 mM K^+ (approximately equal to the $[K^+]_i$) in the form of K-aspartate (120 mM) and of K_2PIPES (10 mM), was used to replace almost all of the external Na^+. K_2PIPES, combined with 10 mM of HEPES, was used to buffer the pH_0 effectively. (2) 1 μM of ouabain was added to block the Na^+/K^+-ATPase, in order to facilitate the transmembrane K^+ equilibrium by both passive diffusion and the action of nigericin. (3) 10 mM of NaCl (close to the intracellular concentration), was maintained in the solution. (4) No Ca^{2+} was added but 0.1 mM EGTA was present, in order to prevent cell contracture due to the entry of Ca^{2+} in the low Na^+ tyrode solution.

The intracellular phenol red spectra recorded at the known pH_i imposed by nigericin are referred to in this paper, as intracellular calibration spectra. All were acquired after addition of nigericin (50–100 μM) to extracellular solutions for at least 30 min, and after the recorded spectra (corresponding to given pH_is) had become stable. All intracellular spectral calibrations were performed at 25°C.

2.6. *Calculation of pK_d*

For the one step, 1:1 reaction of phenol red with H^+,

$$\mathrm{pH} = \mathrm{p}K_\mathrm{d} - \log[(1-\alpha)/\alpha] \qquad (1)$$

where α is the fraction of ionized dye to total dye (dissociation coefficient). A linear titration curve can be obtained by plotting $\log[(1-\alpha)/\alpha]$ versus pH, and the pK_d values are thus equivalent to the pHs at which half of the dye is ionized.

The absorption spectra of phenol red recorded on the spectrophotometer under different

physico-chemical conditions were analyzed as described above. The variations in dye absorption at 560 nm as a function of pH were used to derive titration curves. For the spectral series obtained from the microspectrophotometer, pK_ds were computed and plotted from titration curves over a wide range of wavelengths, using a personal computer.

3. Results and discussion

3.1. Spectral characteristics of intracellular phenol red

The bold lines in Fig. 1A show a set of absorption spectra recorded in one myoball containing about 4.5 m*M* phenol red. pH_i was modified by adding a few microliters of KOH (2 *M*) to the extracellular medium, in the presence of nigericin. The pH_i changed so rapidly (usually in less than one minute) that any variation in intracellular dye concentration, due to possible diffusion from the microinjection electrode (0.5 μm tip diameter) into the cell, can be considered negligible. The isosbestic point, at which absorption depends only on the dye concentration, and not on pH_i, can thus be determined in myoplasm. It was found to be located at 485 nm for the spectral set shown in Fig. 1A. The pH_i values indicated on the spectra were determined later on the basis of a set of intracellular calibration spectra (see below). Isosbestic points around 485 nm (±3.5 nm) were found in similar experiments with 7 myoballs.

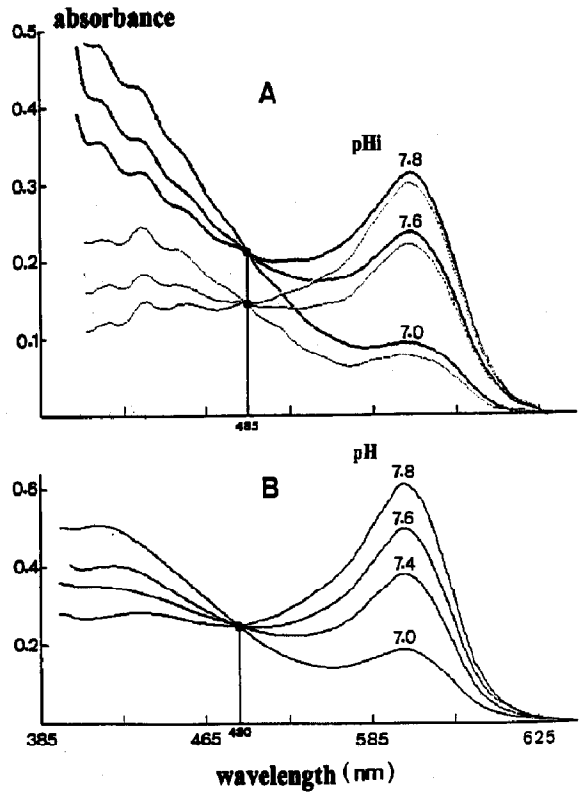

Fig. 1. Phenol red absorption spectra recorded with a microspectrophotometer: (A) in a myoball before correction of light scattering (bold lines), and after correction (thin lines); (B) in a thin cuvette containing a buffered solution. Temperature: 25°C. Dye concentrations: 4.5 m*M* in (A) and 0.6 m*M* in (B).

The absorption spectra of the intracellular dye showed a slight red shift (Fig. 1A), in comparison with those obtained in a thin cuvette containing a buffered solution (Fig. 1B), and striking differences in spectral shape appeared over the 385–560 nm wavelength range. The absorption spectra recorded in the cuvette showed a steeper "valley" between the absorption peak near 560 nm and the isosbestic point at any given pH. In the spectral region below the isosbestic point, intracellular dye spectra showed a markedly ascending slope (Fig. 1A, bold lines). Considerably greater absorbance in the violet region was also observed by Ahmed and Connor [2], who used phenol red to monitor changes in pH_i in molluscan neurons, but provided no explaination for the finding.

3.2. Effect of light scattering in cells on spectral shape

The deformation of intracellular dye absorption spectra seen in Fig. 1, particularly evident in the shorter wavelength region, was thought to be due to the scattering of light by intracellular particles, since the amount of scattered light is more important at shorter wavelengths. In order to confirm this supposition, a correction of the light scattering effect has been tried for the spectral set shown in bold lines in figure 1A, by applying an energy transfer model derived from the work of Kubelka and Munk [13], as described in the Appendix.

The corrected spectra (Fig. 1A, thin lines) look more like, but are not identical to, that recorded in buffered solution (Fig. 1B). The red shift is still evident. The ratios of absorption around 560 nm and at the isosbestic point (A_{560}/A_{iso}) were smaller for intracellular dye than in buffered solution at the same pH. These findings suggest that light scattering is not the only factor responsible for deformation of the spectra of intracellular dye, and that some physico-chemical properties of the dye must be altered in the intracellular environment.

3.3. Possible dependence of spectral properties on dye concentration

In view of the high dye concentration required for intracellular measurements, the effect of [D] on spectral properties was examined in both the spectrophotometer and the microspectrophotometer. No change in spectral shape was seen in the spectrophotometer, at [D] from 10 μM to 3 mM. The titration data fit very well, with dye pK_d values around 7.6, to a straight line, plotted assuming the stoichiometry of the dye: H^+ reaction to be 1:1. An example obtained with 50 μM of phenol red is shown on fig. 2.

Phenol red concentrations up to 6 mM were tested in the microspectrophotometer, using a thin cuvette containing buffered solution. Fig. 3 gives the spectra obtained at 6 mM phenol red in the pH range 6.8 to 8.4. The isosbestic point is well defined at 480 nm. The computed pK_d re-

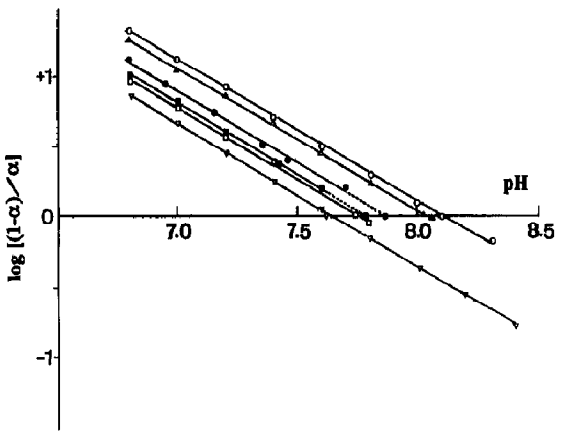

Fig. 2. Titration curves (according to eq. 1) obtained under different physico-chemical conditions: (▽) buffered solution without BSA, with 50 μM phenol red and molar concentration ratio of BSA:dye (R) = 0; (□) buffered solution containing 0.25 mM BSA, 2.5 mM phenol red, R = 0.1; (△) buffered solution containing 0.25 mM BSA, 50 μM phenol red, R = 5; (○) buffered solution containing 0.25 mM BSA, 10 μM phenol red, R = 25; (●) myoplasm containing about 2.5 mM phenol red; and (■) myoplasm containing about 5 mM phenol red.

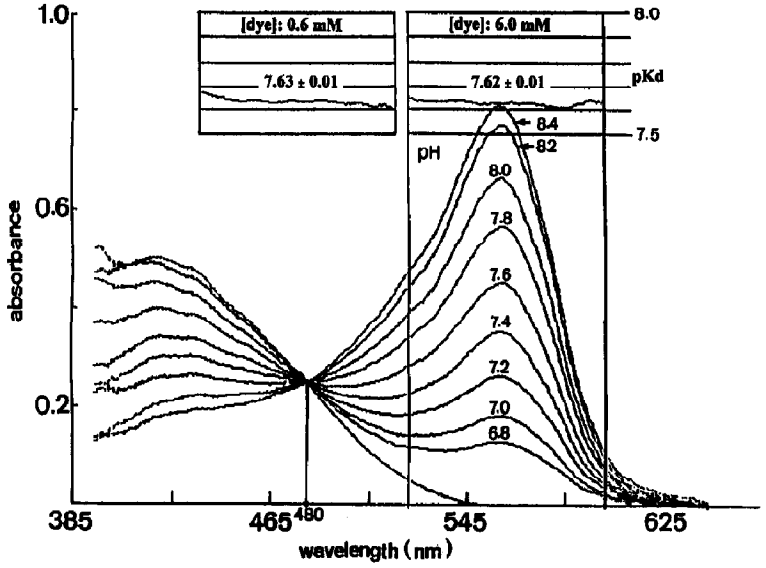

Fig. 3. Absorption spectra of phenol red at a concentration of 6 mM, recorded on the microspectrophotometer. The computed pK_d values are plotted versus wavelength above the spectra for 6 mM and 0.6 mM dye concentrations. Temperature: 25°C.

mained constant over a wide spectral region, as illustrated on the top of the spectra, indicating that only one type of dye–H^+ association takes place, and no dye self-associations occur, even at high dye concentration. The result obtained at 0.6 m*M* phenol red is shown on the same figure for comparison: Clearly, the pK_d of phenol red was not significantly different at the two dye concentrations, whatever wavelength was chosen for its calculation, demonstrating the absence of an effect of [D] on the spectral properties of phenol red, up to 6 m*M*. In contrast, a deviation of pK_d observed at high dye concentrations has been reported for some pH indicators of the carboxyfluorescein family [14].

3.4. Effects of interactions with soluble proteins on the spectral properties of phenol red

Dyes and fluorescent indicators bind substantially to cytoplasmic constituents, especially soluble proteins, under intracellular conditions [15–18]. We therefore investigated wether spectral properties of phenol red are modified in the presence of soluble proteins, such as occur in the myoplasm (Fig. 1A), using readily available and well known bovine serum albumin (BSA), chosen as a model of soluble proteins in the present study.

In the presence of BSA, the spectra of phenol red exhibits a slight red shift (isosbestic point at 485 nm), and lower A_{560}/A_{iso} ratios at any given pH than in the absence of proteins. The spectral modifications were very similar to those observed in myoballs (Fig. 1A, thin lines).

The titration data obtained at different molar concentration ratios (R) of BSA:dye all fit well to straight lines (Fig. 2), but the pK_d shifted towards the basic pH values in the presence of BSA. These results suggest a non-specific dye–protein binding, with no modification of dye protonation stoichiometry, but with a greater affinity of the protein-bound dye than of free dye for H^+.

3.5. Intracellular calibration of phenol red absorption spectrum

The finding that modification of the intracellular spectrum of phenol red is very similar to that observed in buffered solution containing BSA,

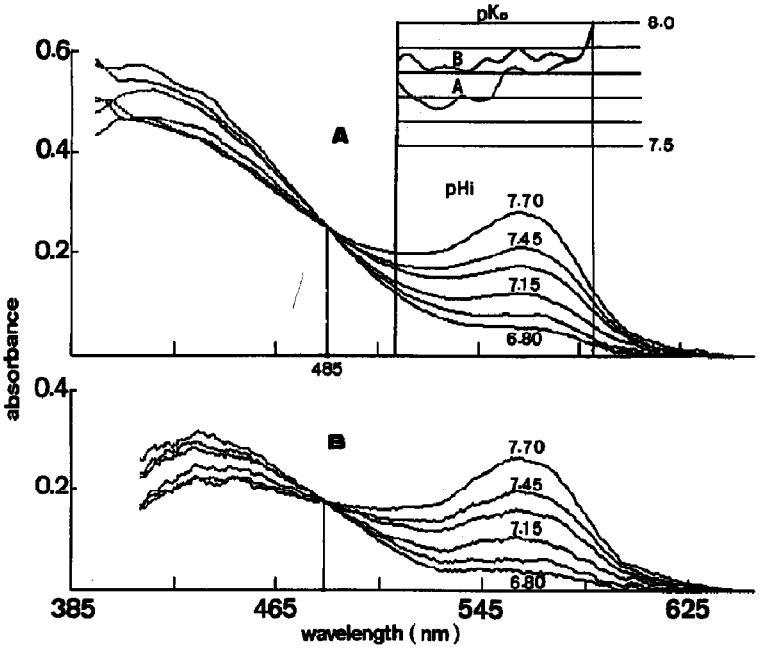

Fig. 4. Intracellular calibration spectra obtained at about 2.5 m*M* of intracellular dye: (A) before correction of light scattering, (B) after correction. Temperature: 25°C.

indicates that a large fraction of the dye must become bound to soluble proteins and eventually to other intracellular constituents, such as myofibrillar proteins and the membranes of organelles, after it has been introduced into the myoplasm. We considered therefore that the dye may behave differently in the intracellular environment. The pK_d of phenol red, for instance, may be changed to an extent that is impossible to evaluate, since the number of intracellular binding sites and their dissociation constants for the dye cannot be known exactly. We undertook

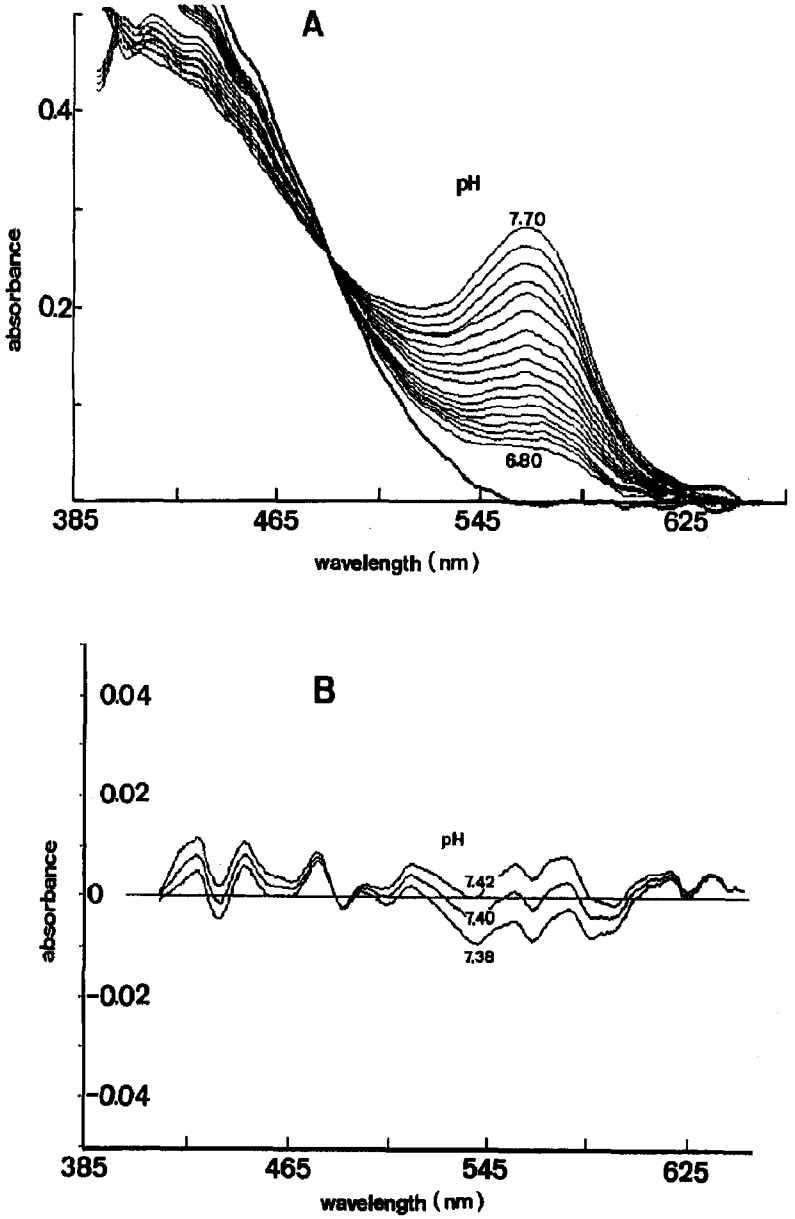

Fig. 5. Determination of pH_i. (A) interpolation at 0.05 pH units intervals, computed from an initial intracellular calibration spectral set with 2.5 mM phenol red. The spectrum in bold line represents the interpolation of limiting acid pH. (B) Difference spectra between a spectrum recorded in a myoball under physiological conditions, and three intracellular calibration spectra at the pHs indicated. Temperature: 25°C.

therefore calibrations of intracellular phenol red in order to interpret accurately the dye absorption spectra obtained in myoballs in terms of absolute pH_i. Two sets of spectra were established over the pH ranges 6.8–7.7 and 6.8–7.8, at intracellular dye concentrations of about 2.5 and 5 mM, respectively, in the presence of nigericin.

In order to verify that the pH_i is well equilibrated with the pH_0 by nigericin, the transmembrane potential was monitored during the experiments. If equilibrium of K^+ ions across the cell membrane, which determines the pH_i–pH_0 equilibrium, is achieved, the transmembrane potential should be zero; if not, the pH_i will differ from the known pH_0, according to the Nernst equation. In the present study, myoballs always showed a drop in membrane potential to zero, after immersion in the calibration tyrode solution, (the average resting potential of this type of cells is about −50 mV, according to our measurements and another report [8]), and remained to zero after the addition of nigericin. The pH_i can thus be considered equal to the well-buffered pH_0, and no correction is needed.

The spectra obtained at 2.5 mM of intracellular dye are shown in Fig. 4A. They have been adjusted to the same base line in the wavelength range above 620 nm (since phenol red does not absorb in that spectral region), and then normalized to the same optical density by fixing the isosbestic point at 485 nm, which is the average value measured from seven myoballs (see above). Figure 4B shows the same spectral set, corrected for light scattering. Without these corrections, the pK_d computed over a wide spectral region (insert curve A) appeared to vary with wavelength, but no significant deviation of the pK_d from the average value was observed after correction, at the accuracy of our measurements, over the entire wavelength range (insert curve B). These findings indicate that only one type of dye–H^+ association occurs in myoplasm, and underline the potential importance of correcting for light scattering when evaluating the behaviour of dyes inside living cells.

The dye absorption changes at 560 nm as a function of pH were also plotted for two sets of intracellular calibration spectra (Fig. 2), and found to fit well to straight lines. Although the protonation mechanism of phenol red is not altered in the myoplasm, its pK_d value is modified. As was to be expected from the results obtained with a buffered solution containing BSA, the pK_d shifts towards higher pHs in a [D]-dependent manner, being 0.24 and 0.12 pH units at estimated intracellular [D] of 2.5 mM and 5 mM, respectively (Fig. 2, see also Fig. 4).

3.6. Determination of pH_i

The procedure used to determine pH_i involves an interpolation of the intracellular calibration spectra and a calculation of difference spectra between the intracellular calibrations and spectra measured in myoballs under physiological conditions. The interpolation is done at intervals of 0.02 pH units according to simple first order dye protonation, as discussed above. Figure 5A gives an example of such an interpolation, using larger (0.05 pH units) steps for clarity. The calibration spectrum that has the same shape as that measured in myoballs corresponds to the same pH, and their difference spectrum should exhibit the smallest statistical deviation from zero line (as evaluated by a least square criterion). Figure 5B gives an example, in which pH_i is determined to be 7.40. As it can be seen in the figure, the accuracy of the method can reach ±0.02 pH unit.

Since the pK_d of intracellular dye has been found to vary with the dye concentrations in myoplasm, it is important to use the same dye concentration both for intracellular dye calibration and for pH_i measurement. In the present study, pH_i was measured at an intracellular dye concentration of around 2.5 mM, and the intracellular calibration spectra shown in Fig. 4 were chosen for interpreting the experimental results. The average pH_i was found to be 7.35 ± 0.05 in 36 myoballs at 25°C, and 7.19 ± 0.09 in 30 myoballs at 37°C. Figure 6 illustrates the distribution of pH_i measured at these two temperatures.

These variations of pH_i with temperature can be explained by the displacement of the dye pK_d. The importance of this effect was verified in a 5 mM phenol red solution: When the initial pH was ajusted to the pK_d value of 7.60 at 25°C, it

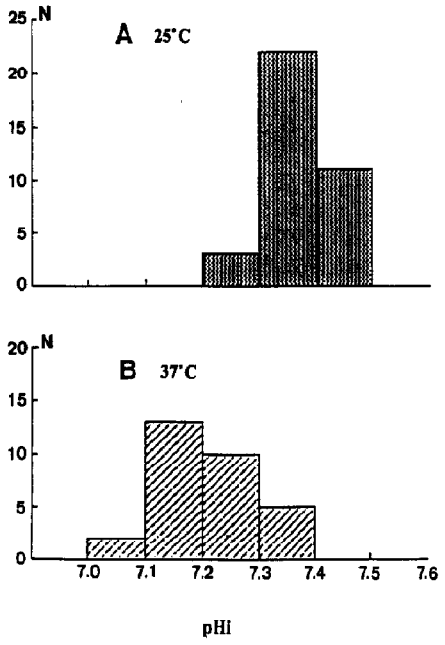

Fig. 6. Distribution histograms of pH_i values in 36 myoballs at 25°C (A), and in 30 myoballs at 37°C (B). *N*: number of cells per class of 0.1 pH unit.

fell by 0.06 when the temperature rose to 37°C. If a similar shift occurs inside the myoplasm, the average pH_i at 37°C should be around 7.25 instead of 7.19. But this displacement of the pK_d cannot account for all of the observed variations in pH_i, and Rink et al. [19] proposed that a difference in the Q_{10} for H^+ transport and acid production was involved. They obtained a pH_i of 7.05 at 37°C, and of 7.35 at 20°C in pig lymphocytes, using a digitonin null-point technique.

Appendix

Light scattering correction

The light beam impinging on a cell interacts with scatterers of widely dispersed sizes compared to the incident wavelength, ranging from small molecules (Rayleigh scattering) to proteins (Rayleigh–Gans) and even particles (Mie scattering). In order to take into account this complex situation, an energy transfer model such as proposed by Kubelka and Munk [13] can be used, assuming an homogeneous medium, parallel incident lights and an optical thickness much greater than the wavelength.

If R and T are the reflectance and the transmittance of the sample, and K and S the absorption and scattering coefficients, they are related by the following equation:

$$K/S + 1 = A = (1 + R^2 - T^2)/2R$$

The transmittance is obtained after integration of a set of differential equations involving inward and outward fluxes through elementary slabs, for which a first order attenuation law is applied:

$$T = (1-B)[(R+1)/4 + (R-1)/(4B)] \times \exp(\Gamma d) + (1+B)[(R+1)/4 - (R-1)/(4B)] \exp(-\Gamma d)$$

In this relation,

$$B = [K/(K+2S)]^{1/2}$$

$$\Gamma = [K(K+2S)]^{1/2}$$

and d is the thickness of the sample.

Let us consider now the case of a dye injected into a non-absorbing scattering medium: Its pure absorption spectrum is a measure of $K(\lambda)$, while the absorption spectrum of the medium gives the transmittance $T(\lambda)$. The two unknowns R and S can be determined from the set of equations shown above, by means of a numeric iterative procedure for any wavelength (λ) inside the range.

This procedure was used in the present work to determine S and the ratio of $S:K$ at several pHs, starting from the absorption spectra of phenol red in a cuvette and inside the cell. The ratio of $S:K$ was shown clearly not to depend on pH, but to an intrinsic optical property of the cell (in fact, it did not change significantly from one myoball to another).

When $S(\lambda)$ is calculated as described above, the reverse computation can be done in order to obtain a corrected set of spectra corresponding to intracellular absorption of the dye, in the absence of scattering effects added by the cell.

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