INTRACELLULAR pH OF YEAST CELLS MEASURED WITH FLUORESCENT PROBES

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1. Introduction

Current techniques for the measurement of internal pH in cells or organelles are imperfect (review [1]). The application of pH-sensitive glass electrodes is limited to larger cells; distribution techniques are unsuitable for following rapid pH changes. The most promising methods appear to be spectroscopic, utilizing the pH-dependence of NMR, ESR, fluorescence and absorption spectra of selected elements and compounds inside cells or organelles. However, the measurement of both ESR spectra of special pH-sensitive extrinsic spin probes [2] and the determination of the pH-dependent chemical shift of ^{31}P in NMR spectroscopy [3] requires sophisticated expensive instruments and considerable experience. This fluorescence method is quick and simple requiring only measurement of the fluorescence intensity at two fixed wavelengths and computing the ratio of these two intensities. Not being a distribution method it allows in principle for monitoring rapid pH changes down to milliseconds.

2. Materials and methods

The following yeast species were used: *Saccharomyces cerevisiae* K (distillery strain; cf. [4]), *Rhodotorula glutinis* (= *Rhodosporidium toruloides;* ATCC [5]), *Endomyces magnusii* (kind gift of Dr E. Streiblová of this institute), *Torulopsis versatilis* and *Lodderomyces elongisporus* (collection from Dr E. Novák, Budapest).

The cells were grown in a synthetic medium with yeast extract and glucose as carbon source [4] at 30°C for 20 h, harvested, washed twice (in the centrifuge), aerated for 2 h and resuspended in buffer.

Prior to the fluorescence measurements the suspension was incubated at ~10 mg dry wt/ml at 30°C with the fluorescent dye for ⩾20 min. The fluorescent dyes used were 20–100 μM fluorescein diacetate, FDA (Koch-Light from 10 mM stock solution in acetone), 1 mM chromotropic acid (=4,5 dihydroxynaphthalene-2,7-bisulfonic acid) and 1 mM quinine sulphate (both from Lachema).

(*Caution:* fluorescein diacetate solution decomposes spontaneously to fluorescein in alkaline solutions; the stock solution of chromotropic acid should be kept in dark.)

After this incubation cells were thoroughly washed and resuspended in the original volume of buffer. Samples of 0.5 ml were placed in a cuvette of a Perkin-Elmer MPF 3 Spectrofluorometer and the fluorescence intensity was recorded (e.g., in the case of fluorescein at 520 nm after excitation at 490 and 435 nm); the measurement of one sample was completed in a few seconds. In most cases the fluorescence of cells without probe was already negligible after 5–10 min preincubation in 100 μM FDA; if not its intensity was subtracted from the total. The original right-angle illumination of the specimen was replaced with frontal illumination using a special adapter which changes the position of the cuvette relatively to the excitation beam. The application of this adapter minimizes the effects of light scattering and inner filter effects and thus allows the measurement of dense suspension of cells as well as of non-transparent specimens (fig.1). The values of intracellular pH were read from a calibration curve prepared as follows. Fluorescein was dissolved in a series of buffers from pH 2.5–7.5. At each pH the fluorescence intensity at 520 nm of the sample was recorded after excitation at 435 and 490 nm and the ratio of these two intensities ($I_{490/435}$) was plotted against pH (fig.2). The mea-

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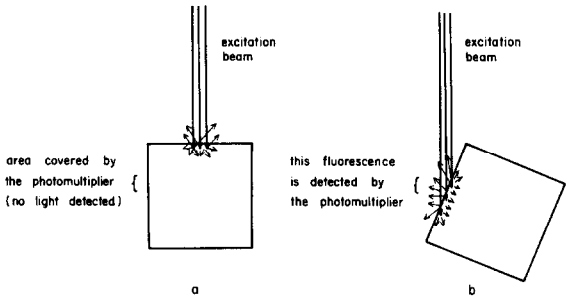

Fig.1. In the case of highly turbid (and/or highly absorbing) samples only a thin layer near the cuvette wall is hit by the excitation beam. The produced fluorescence cannot reach the photomultiplier in the usual right angle arrangement (a), but can do so in the frontal one (b). The frontal illumination also allows measurement of non-transparent samples (e.g., freshly-washed cells on membrane filter fixed on the external wall of the cuvette and covered with a microscopic cover slip). It is recommended to pose the cuvette so that the excitation light reflected from the cuvette wall is not registered by the photomultiplier, i.e., the angle of incidence should be >45°.

surement employs the monitoring of a monoanion–dianion transition of fluorescein [6]. The main reason why a diacyl derivative of fluorescein (FDA) was selected for the incubation of cells was that it does not fluoresce as such but only after decomposition to fluorescein through intracellular hydrolases [7]. This decomposition of FDA trapped inside cells is completed in several minutes. Another advantage was that fluorescein shows very small permeability across the cell plasma membrane and hence experiments extending for several tens of minutes can be performed without risk of losing the dye to the medium [7].

In fact there are two ways of application of FDA. Cells may be either preincubated with the dye and then washed prior to the measurement, or, utilizing the extracellular stability (with the mentioned exception of alkaline solutions) of non-fluorescent colourless FDA, the measurement is performed immediately after the addition of the dye without any washing. The steady increase of the fluorescence intensity in the latter case should not worry the experimenter because it is the ratio of intensities that is of interest.

Besides fluorescein two other dyes, chromotropic acid and quinine, were used (fig.3). Chromotropic acid has, depending on pH, two forms with different fluorescence spectra. The transition between the basic and acidic forms can be used for pH-measurement over pH 3–7. It is convenient to employ the ratio of fluorescence intensities measured at 360 and 430 nm (fluorescence maxima of acidic and basic forms, respectively) after excitation around 330–340 nm.

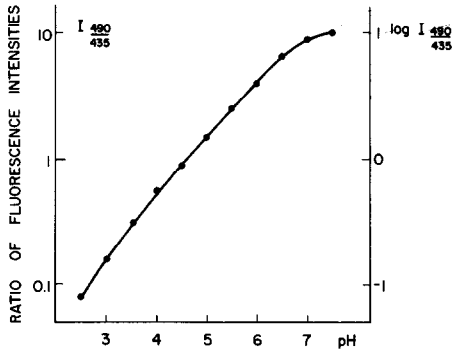

Fig.2. The fluorescence *vs* pH calibration curve. It shows the ratio of fluorescence intensities taken at 520 nm after excitation at 435 nm and 490 nm ($I_{490/435}$) of fluorescein in 0.1 M triethanolamine–phtalate buffer. Identical results were obtained with phosphate and citrate–phosphate buffers, independent of their ionic strength and of the concentration of fluorescein. The curve is plotted in a logarithmic scale and its shape may be considered universal for any apparatus as all differences between particular spectrofluorometers may be expressed merely by a shift of the whole curve in the direction of the ordinate scale (*y*-axis) (in fact they represent a multiplication of all values of the curve by a numerical constant).

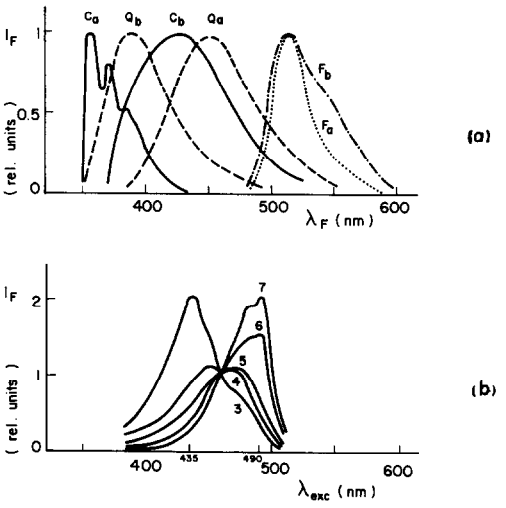

Fig.3. (a) Fluorescence emission spectra of chromotropic acid (C_a, C_b), quinine (Q_a, Q_b) and fluorescein (F_a, F_b). (Subscripts a and b denote acidic and basic forms, respectively; spectra are normalised and not spectrally corrected.) (b) The pH dependence of the excitation fluorescence spectrum of fluorescein. (Spectrally uncorrected spectra, taken at 520 nm and normalised at 460 nm.)

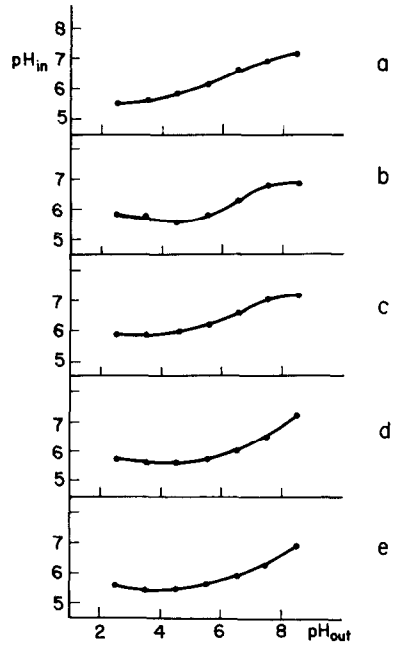

Fig.4. Dependence of internal pH on the extracellular pH in different yeast species: (a) *Rhodotorula glutinis;* (b) *Torulopsis versatilis;* (c) *Saccharomyces cerevisiae;* (d) *Endomyces magnusii;* (e) *Lodderomyces elongisporus.* (The error due to the efflux of fluorescein was <0.1 pH unit.)

Quinine has also a suitable transition between two forms with different fluorescence spectra. The measurement of the ratio of fluorescence intensities at 380 and 450 nm after excitation around 330–340 nm allows the determination of pH over 3–8.

3. Results

Intracellular pH was determined in 5 different genera of yeasts at different external pH where it was of interest for computing the protonmotive force at the external membrane [8]. The equilibrium pH values measured after 90 min incubation in buffer are shown in fig.4. The response of the internal pH to an abrupt change in the external pH was very similar in all species and is illustrated for *Endomyces magnusii* in fig.5. Apparently the internal pH is stabilized in ~10 min after the change in the external pH is completed.

For comparison, the measurement of the intracellular pH was also performed on the root cells of sugar beet (pH_{in} 6.9).

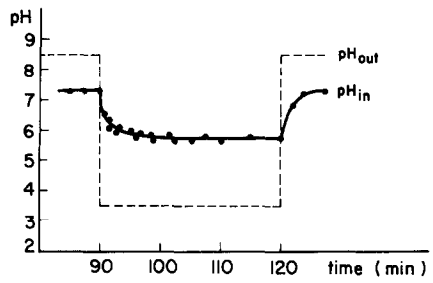

Fig.5. Internal pH of *Endomyces magnusii* after a 90 min incubation in a pH 8.5 buffer followed by a reversible 30 min drop in the external pH. (The error due to the efflux of fluorescein was <0.1 pH unit.)

4. Discussion

4.1. *Applicability of the technique*

The obvious advantages of using a probe that responds in situ to the ambient pH have been the Leitmotif of this work. Three fluorescent dyes were selected after screening about a dozen different compounds. The requirements were as follows: sufficient fluorescence change in the desired pH range responding solely to the actual $[H^+]$, small leak of the fluorescent dye out of yeast cells, negligible binding of the dye inside cells, no toxicity, a high quantum yield and excitation above the absorption band of proteins.

These conditions were best met by quinine, chromotropic acid and above all by fluorescein.

4.1.1. Chromotropic acid and quinine

The chromotropic acid seems not to be bound inside the cells (judged from absorption and fluorescence spectra) and its fluorescence responds solely to $[H^+]$, depending only slightly on the viscosity and on the ionic strength of the environment. Unfortunately, the high efflux of the dye out of yeast cells (in *Rhodotorula* ~50% in 1 min) made it unsuitable for measurements with yeast.

Similarly the sensitivity of the quinine fluorescence not only to the pH but also to the ionic strength of the solution and to the presence of Cl^- makes this dye hardly applicable to in vivo experiments.

Therefore, the absolute values of intracellular pH of yeast cells obtained using either chromotropic acid (because of rapid efflux) or quinine (because of ionic strength and chloride effects) were not considered reliable and therefore are not shown.

4.1.2. Fluorescein

The effects of the buffer composition and its ionic strength, as well as of the presence of protein (0.2 g albumin/ml) in the buffer solution and of its viscosity (probably thanks to the exceptionally small p*K* shift after the excitation [6]), are well below the expected accuracy of the method (0.1–0.2 pH unit). However, the method allows detection of pH changes as small as 0.01 pH unit.

Fluorescein retained inside the cells is supposed to remain mostly freely dissolved in the cytoplasm. The fraction of bound fluorescein is negligible on account of the relatively high concentration of the dye [9]. This was confirmed by fluorescence polarization measurements [9,10] (it reflects the mobility of chromophore molecules) and by the comparison of positions of excitation and emission peaks of intracellular fluorescein and fluorescein in solution (it reflects the interactions of chromophore molecules) when no differences were found ([9,11], these results).

Since retention of fluorescein is used as a criterion of cell viability and of the intactness of cell membranes [7,12,13], the efflux of intracellularly-generated fluorescein is expected to be very small. Indeed in cells such as *Euglena* [10] no leak was reported, but certainly there are exceptions (Ehrlich ascites tumour cells) with a relatively high efflux (>90% after 1 h at 20°C, but only 5% at 4°C [14]). With our yeast cells the highest efflux was found with *Saccharomyces* (some 20% after 1 h). However, if any significant efflux should occur and disturb the pH_{in} measurement it may be reduced by decreasing the temperature or by the application of fluorescein labelled compounds of high M_r. Derivatives of fluorescein bound to dextran [15,16], albumin [17], cytochrome [18] and chloroplasts [19] may be used for pH measurement in the same manner as fluorescein in this paper. Only the calibration curve might be slightly different [16].

4.2. *Actual values of intracellular pH*

Cells loaded with fluorescein resemble evenly luminous discs under the microscope as FDA penetrates into the entire cell water including organelles [14]. In this case it may be shown that the measured pH refers to the arithmetical mean value of intracellular pH, provided that the pH values of all these compartments lie on the linear middle part of the $I_{490/435}$ *vs* pH curve (like fig.2, but $I_{490/435}$ plotted on a linear scale) i.e., over pH of 5–7. Areas at pH <5 and >7 are underestimated because of the S-like shape of the curve.

It is evident in fig.4 that an effective buffering system maintains the internal pH within a limited pH range and thus allows the yeast cells to tolerate considerable changes in the external pH. The action of this internal buffering system is clearly visible on the acidic sides of all curves where, even at pH_{out} 2.5 the pH_{in} is still >5. The similarity of all these curves for different yeast genera suggests that, at least in acidic media, the buffering mechanism may be similar in all these species. The possible candidates are glutamate, phosphate, succinate, malate, lactate, acetate, arginine, polyphosphate and proteins. The system is likely to be related to the H^+ pool which plays a role in proton extrusion after addition of glucose and which includes a variety of organic acids (cf. [20]).

Bromophenol blue in *Saccharomyces cerevisiae* [21] and bromophenol blue and 5,5-dimethyloxazolidine-2,4-dione in *Rhodotorula glutinis* [5] generated the same S-shaped curve with plateaus on the alkaline and acidic sides as in fig.4. On the alkaline side the values of pH_{in} measured here are somewhat lower than those in [21,22] and seem to be closer to the actual pH_{in} values, as no mechanism is known to alkalify the cell interior in alkaline buffers up to pH_{in} 8.5 or 9 at pH_{out} 8. The discrepancy is probably caused by an error of the distribution technique due to low accumulation of the indicator compound at higher external pH.

Different permeabilization methods gave pH 5.8 and 6.35 for resting [22,23] and 6.1 and 7.1 [23] for fermenting yeast. Using ^{31}P NMR spectrometry a pH_{in} 6.5–6.8 in resting *Saccharomyces cerevisiae* cells (at a pH_{out} of 6.5) was reported [24] which agrees well with these results (fig.4).

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