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Proton Pumping and the Internal pH of Yeast Cells, Measured with Pyranine Introduced by Electroporation

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The internal pH of yeast cells was determined by measuring the fluorescence changes of pyranine (8-hydroxy-1,3,6-pyrene-trisulfonic acid), which was introduced into the cells by electroporation. This may be a suitable procedure for the following reasons. (i) Only minor changes in the physiological status of the cells seemed to be produced. (ii) The dye did not seem to leak at a significant rate from the cells. (iii) Different incubation conditions produced large fluorescence changes in the dye, which in general agree with present knowledge of the proton movements of the yeast cell under different conditions. (iv) Pyranine introduced by electroporation seemed to be located in the cytoplasm and to avoid the vacuole, and therefore it probably measured actual cytoplasmic pH. (v) Correction factors to obtain a more precise estimation of the internal pH are not difficult to apply, and the procedure may be useful for other yeasts and microorganisms, as well as for the introduction of other substances into cells. Values for the cytoplasmic pHs of yeast cells that were higher than those reported previously were obtained, probably because this fluorescent indicator did not seem to penetrate into the cell vacuole.

The measurement of internal pH in microorganisms, in yeast cells and in cells in general, has been studied for many years. Several mechanisms are involved in the regulation of the internal pH of the cell, many cellular processes are regulated by the internal pH, and many transport processes depend on the H^+ cycle. In yeast cells, very crude procedures were initially used, with disruption of the cells by boiling or freezing and thawing, after which the pH of the resulting sap was measured. In 1950, the group of Conway used the distribution of weak acids, such as carbonic or propionic acid, to measure the internal pH of yeast cells (5, 6). By these methods, the pH was probably obtained as an average of that of the entire cell interior, including all internal compartments. More recently, other methods have been used; among them, the shift of the P_i peak in nuclear magnetic resonance spectra has been useful but complicated and expensive (1, 3).

In yeast cells, the use of ionizable fluorescent probes capable of crossing the membrane and distributing between the cells, organelles, or vesicles depending on the internal and external pH (16) has proven useless. Slavík (17) first introduced indicators into yeast cells whose fluorescence depends on the surrounding pH; some of these, which are available commercially, can be introduced into the cells as acetoxymethyl esters (the permeant form), which are cleaved inside by esterases, transforming them into an impermeant form and preventing their efflux. A recent report on the use of one of these dyes to measure the internal pH of yeast cells has appeared (9). However, Slayman et al. (18) have pointed out some of the drawbacks of these dyes; because of the concentration of esterases in some intracellular compartments, they are preferentially hydrolyzed and accumulated in vacuoles or other internal compartments which accumulate hydrolytic enzymes.

Kano and Fendler (10) introduced the use of pyranine (8-hydroxy-1,3,6-pyrene-trisulfonic acid), a fluorescent dye with a ionizable -OH group, which shows remarkable pH dependence in fluorescence. The dye could be trapped in liposomes and used to estimate their internal pH (see also reference 4), the most important advantage being the relatively low interaction with the bilayer or proteins, because of its hydrophilic nature.

Unfortunately, this property makes its use with whole cells difficult, because of problems with entry. However, a controlled electric shock of high intensity and short duration appears to produce transient openings of the cell membrane that are closed in a short time after the treatment (11). This phenomenon has been used to introduce even substances with high molecular weights into cells by a procedure known as electroporation. This method has been successfully used in yeast cells, and great improvements have been made recently (2, 8).

The present communication deals with the use of pyranine to measure the internal pH of yeast cells.

MATERIALS AND METHODS

Cells from an isolated colony of commercial yeast cells (La Azteca, S. A.) were grown in 500 ml of the medium described by De Kloet et al. (7) for 24 h, collected by centrifugation, washed with distilled water by centrifugation, washed once with distilled water, and resuspended in 250 ml, and the suspension was shaken for 12 to 14 h. The cells were collected and washed again and resuspended at 0.5 g of yeast per ml of water, and the suspension was kept in ice for use the same day. Some results were found to be unaffected by 1 day's storage of the suspension.

Electroporation was according to the recommendation of the manufacturer (Bio-Rad [Gene Pulser, with the pulse controller attachment]). Preliminary experiments showed that variations in the pyranine concentration from 0.6 to 4.1 mM were not clearly important. A range of voltages from 300 to 1,800 showed penetration at more than 1,200 V, the highest penetration being at 1,800 V; a value of 1,500 V was chosen to minimize cell damage. The standard conditions, therefore, were as follows. A 0.7-ml volume of the cell suspension plus 20 μl of 100 mM pyranine were mixed in a cell with a 4-mm gap, and one 1,500-V pulse with a capacitance of 25 μF, a resistance of 200 Ω, and a duration of around 3.1 to 3.5 ms was applied. Then, the cells were centrifuged in a Beckman Microfuge for 10 s, washed twice with water, resuspended in the original ratio of 0.5 g/ml of water, and employed as described for the individual experiments.

Fluorescence changes were monitored at 460 to 520 nm, with a slit width of 4 nm, in a DMX-100 spectrofluorometer with a thermostated chamber at 30°C and a magnetic stirrer, which was connected to an acquisition and processing system (SLM Instruments, Urbana, Ill.).

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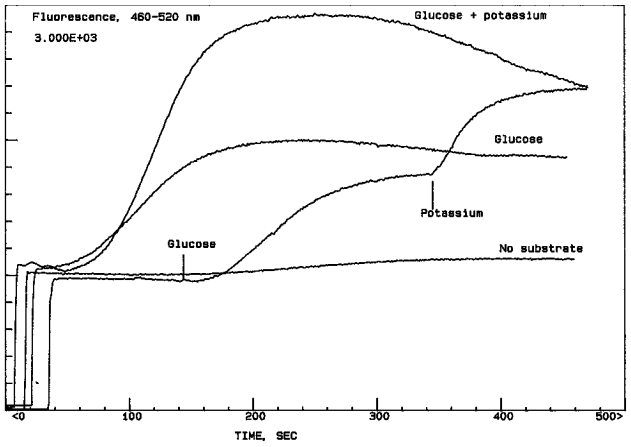

FIG. 1. Fluorescence changes observed under different conditions of yeast cells loaded with pyranine introduced by electroporation. Starved cells were electroporated by using 20 μl of 100 mM pyranine, with 0.7 ml of the yeast cell suspension, containing 350 mg (wet weight) of cells. One voltage pulse of 1,500 V was applied, with a capacitance of 25 μF and a resistance of 200 Ω, which lasted for 3.4 ms. After centrifuging and washing of the cells, they were incubated in 10 mM MES-TEA buffer, pH 6.0, in a final volume of 2.0 ml. When indicated, 10 mM glucose or 10 mM KCl was added. The measurements were made in the spectrofluorometer with a cell compartment at 30°C. Tracings were started by the addition of the cells.

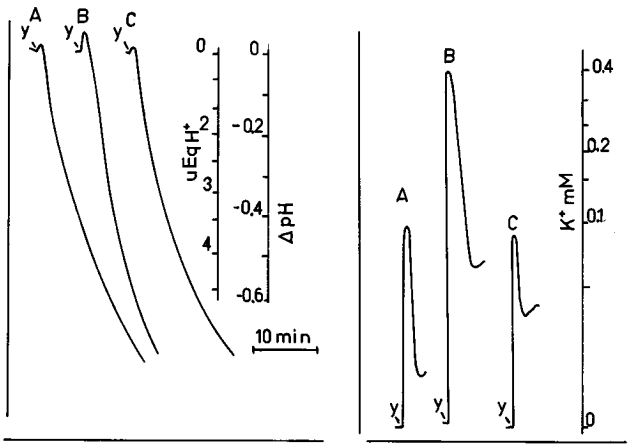

FIG. 2. Proton pumping (left panel) and potassium transport (right panel) by cells electroporated with pyranine as described in the legend to Fig. 1. A 250-μl sample of the cells was added (at point y) to 3 mM MES-TEA buffer, pH 6.0, and 10 mM glucose. The temperature was 30°C, and the final volume was 10.0 ml. The time course of potassium concentration and the pH of the medium were monitored by suitable electrodes (see Materials and Methods). A, control cells; B, cells electroporated and not washed; C, cells electroporated, washed, and resuspended in water. uEgH$^+$, microequivalents of H$^+$.

Respiration was monitored by means of a Clark oxygen electrode with a polarization device and a recorder.

The pH or monovalent cation changes of the medium were monitored by a pH or a cationic (Corning 476220) electrode, which was connected to a pH meter and a recorder. The total potassium content of the cells was determined by measurement with the cationic electrode after disruption of 50 μl of the cell suspension containing 25 mg (wet weight) of yeast cells by the addition of 200 μM cetyltrimethylammonium bromide (CTAB). Viability was tested by colony counting 24 h after plating close to 200 cells per plate in solid YPD medium with adenine (1% yeast extract, 1% Bacto Peptone, 2% glucose, 2% agar, 8 mg of adenine per 100 ml).

The leakage of the dye from the cells was determined by measuring the fluorescence at 460 to 520 nm of the supernatant after centrifuging the electroporated cells in the Microfuge at time zero or after 90 min and after adding 50 μl of the supernatant to 2.0 ml of 10 mM Tris base. To determine the percentage of the efflux during this time, the extra amount of dye appearing with time in the supernatant was compared with that existing in the supernatant after treatment of the cells with CTAB in the presence of 50 mM Tris base and centrifugation of the cells.

Micrographs of the electroporated cells were kindly obtained by A. Hernández-Cruz of the Neurosciences Department of our Institute from a suitable dilution of the suspension of the cells in water by use of a Nikon Diaphot inverted microscope with an epifluorescence system and a camera attached. An excitation filter with a wavelength band of 450 to 490 nm was used. The emission filter had a maximum at 520 nm. The ×100 glycerol immersion objective had a numeric aperture of 1.3. The dilution of the cells was such that a single layer of cells was produced after sedimentation to keep them from moving.

Protein contents were measured by the method described by Lowry as modified by Markwell et al. (12).

The buffers used for the different pH values were prepared as 100 mM solutions of either tartaric acid, adjusted to pH 4.0 with triethanolamine (TEA); 2-(*N*-morpholino)ethanesulfonic acid (MES); adjusted to pH 5.0, 5.5, or 6.0 with TEA; TEA adjusted to pH 7.0 or 8.0 with MES; or Tris adjusted to pH 8.5 with MES.

RESULTS

Characterization of the system. Figure 1 shows that in a simple medium at pH 6.0 containing only buffer (i) there was a significant and stable signal in the absence of added substrate, (ii) glucose caused a substantial increase in signal after a delay of ca. 1 min (a small decrease in fluorescence was sometimes observed in that initial period), and (iii) the addition of potassium ion after glucose produced a further increase in fluorescence, and simultaneous addition of glucose and potassium ion gave additional fluorescence.

The physiological effects of electroporation were assessed in several ways. First (not shown), results for respiration rates of the cells were practically the same after and before electroporation. Second (Fig. 2, left panel), acidification of the medium after addition of cells to buffer with glucose was normal (trace C versus trace A [control]); acidification was abnormally rapid if the washing step after electroporation was omitted (trace B). Third (Fig. 3A), there was relatively normal K^+ uptake after the treatment (trace C versus trace A, i.e., efflux followed by reuptake), but the lowest levels of the cation reached in the medium were somewhat higher in the cells that had been electroporated; omission of the washing step produced higher levels of K^+ in the medium and an apparent impairment of K^+ transport (trace B). Cell viability determined by colony formation in plates showed no great difference between the electroporated and the control cells; in two experiments, viability results were 82 and 80% for the control cells and 89% according to both measurements for the electroporated cells.

Control experiments showed that washed (1,500 V) electroporated cells had lost between 10 and 35% of the internal potassium to the medium. The cation remaining in the cells was not lost to the medium when the cells were incubated in buffer for 5 to 10 min; the concentration values of the cation obtained when 25 mg (wet weight) of cells was added to 10 ml of 10 mM MES-TEA buffer remained less than 25 μM. Treatment with 200 μM CTAB released all of the internal K^+. In addition, by monitoring the efflux of the dye as described in Materials and Methods, it was found that no more than 2% of the internal pyranine was lost to the medium in 1 h (not shown). Measurement of the concentration of pyranine inside the cells gave somewhat variable results, in the order of 40 nmol of pyranine per g (wet weight) of cells. Because the distribution seemed to exclude mainly the vacuole (see below), estimation of the actual concentration in the intracellular water is difficult.

With the knowledge of the system described above, two characteristic situations known to affect internal pH were tested. First, changes caused by substrate addition to starved

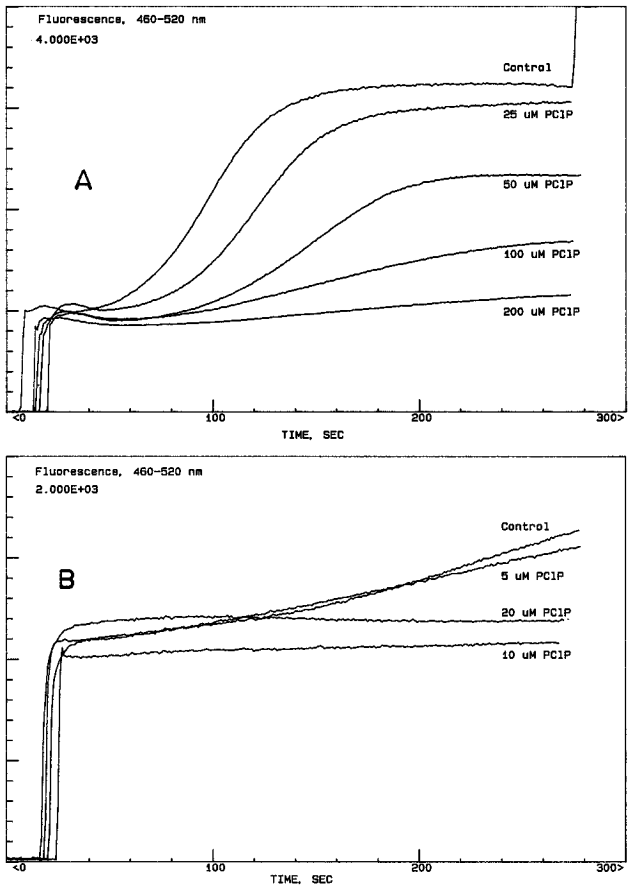

FIG. 3. Effects of various concentrations of PClP on the fluorescence changes of yeast cells electroporated with pyranine, with glucose (A) or ethanol (B) as substrate, at pH 6.0. The experiment was carried out as described in the legend to Fig. 1. Substrates were either 10 mM glucose or 100 mM ethanol.

cells are blocked by uncouplers (13). For cells in pH 6 buffer with glucose, titration with pentachlorophenol (PClP) showed ca. 50% inhibition of the fluorescence change at 50 μM and complete inhibition at 200 μM (Fig. 3A); a very much smaller effect of ethanol on fluorescence (ethanol is known to be a poor substrate for energizing transport [13, 14]) was completely blocked by PClP (Fig. 3B). The difference in sensitivity likely reflects plasma membrane versus mitochondrial uncoupling (15).

Second, it is known that in the presence of substrates, internal pH depends on external pH (14). As Fig. 4 shows, the fluorescence for cells without substrate was greatest at pH 8 or 7 compared with pH 4; the same result, although much more pronounced, applied to the increase caused by glucose at pH 6, 7, or 8. An analogous effect was obtained for cells incubated with potassium ion and glucose together (data not shown), which produced an increased fluorescence even at pH 4.0.

Calibration of fluorescence versus pH. It was first necessary to observe the fluorescence of pyranine in cells for situations of complete ionization (i.e., high pH) and of complete protonation (i.e., low pH). These were obtained, respectively, by treatment of the cells with 50 to 100 mM Tris base or with 265 mM propionic acid (Fig. 5). The two spectra are shown in Fig. 6. (The acidic fluorescence spectra were similar, while the alkaline spectrum of pyranine within the cells showed a more emphatic peak around 410 nm than the free material.)

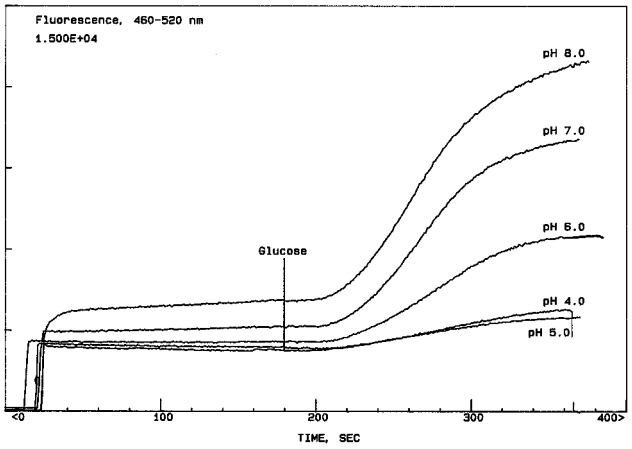

FIG. 4. Fluorescence changes of yeast cells electroporated with pyranine at different pH values, without substrate and with glucose added. After electroporation, the cells were incubated as described in the legend to Fig. 1, without substrate, but 10 mM glucose was added at 180 s after the cells. For the different pHs, 15 mM buffers were used with the following compositions: 10 mM tartaric acid adjusted with TEA to pH 4.0; 10 μM MES adjusted with TEA to pH 5.0 or 6.0; and 10 mM TEA adjusted with MES to pH 7.0 or 8.0.

Since the nonionized form of pyranine does not fluoresce at 460 to 520 nm and since pH is related to the dissociation constant by the Henderson-Hasselbalch equation [pH = pK − log (dissociated/protonated)], pH can be evaluated from fluorescence as follows. For any one situation, the amount of internal dissociated pyranine is the total fluorescence signal minus the fluorescence signal for external pyranine observed immediately after the addition of cells (i.e., before any contribution of metabolism) and also with subtraction of the small fluorescence observed after acidification with propionic acid. The amount of total internal pyranine, on the other hand, is obtained from measurement of total pyranine using Tris for complete dissociation minus external pyranine in its full dissociated status. The latter value is derived from the same signal (above) for external dissociated pyranine but corrected according to the Henderson-Hasselbalch equation to include the protonated, nonfluorescing fraction (for a pK of 7.2 [10], the correction would be a factor of 1.16 for the fluorescence ob-

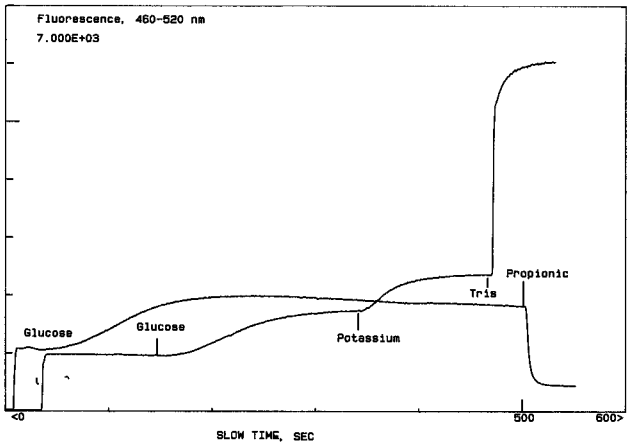

FIG. 5. Effects of Tris or propionic acid on the fluorescence of pyranine introduced into yeast cells by electroporation. After incubation of the cells as shown in Fig. 1 with 10 mM glucose plus 10 mM potassium, 100 mM Tris base or 265 mM propionic acid was added when indicated.

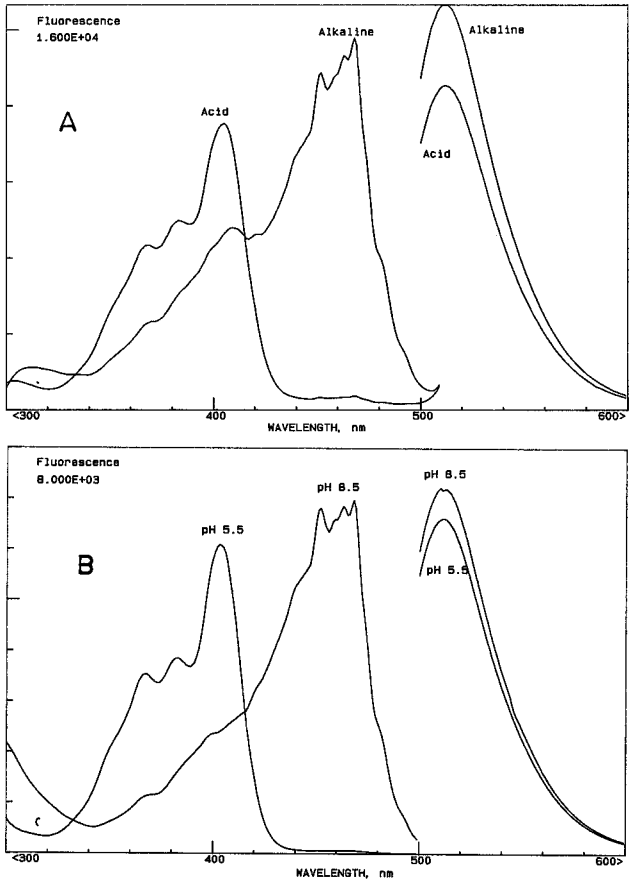

FIG. 6. (A) Excitation (left) and emission (right) spectra of pyranine captured from yeast cells after electroporation under alkaline or acidic conditions. Electroporated cells were incubated in the presence of glucose for 5 min, and then either 100 mM Tris base or 265 mM propionic acid was added. Then, excitation and emission spectra were obtained. (B) Excitation (left) and emission (right) spectra of 1 μM pyranine placed in either 10 mM Tris (adjusted to pH 8.5 with MES) or 10 mM MES (adjusted to pH 5.5 with Tris). The excitation spectra were obtained at an emission wavelength of 520 nm. The emission spectra in Tris base and in acid were obtained with excitation wavelengths of 460 and 407 nm, respectively. Excitation spectra were obtained at an emission wavelength of 520 nm. Emission spectra were obtained at excitation wavelengths of 460 and 407 nm under alkaline and acidic conditions, respectively.

served immediately after the cell addition to the medium at pH 8.0 because at this external pH, according to the Henderson-Hasselbalch equation, 86.2% of external pyranine is ionized). This value also has to be corrected by the small fluorescence observed after the addition of propionic acid, which is not zero (Fig. 5), and may reflect either bound pyranine or inaccessibility to the acid or light scattering. Hence, total internal pyranine = $F_{Tris} - F_{propionic\ acid} -$ corrected $F_{external\ pyranine}$.

Figure 7 shows the results of these calculations applied to three situations within a range of external pHs: cells without substrate or with glucose (as in Fig. 4) and cells with potassium and glucose together. When no substrate was added, the internal pH (fluorescence) was only slightly modified by the incubation of the cells at various external pHs. These values showed the highest deviation, because in spite of starvation, some batches of cells seemed to keep some level of endogenous substrates, which, particularly at the high pHs, allowed them to pump some protons out. In the presence of glucose or with glucose plus potassium, the cells showed rather large changes in internal pH. Glucose alone requires a high pH for

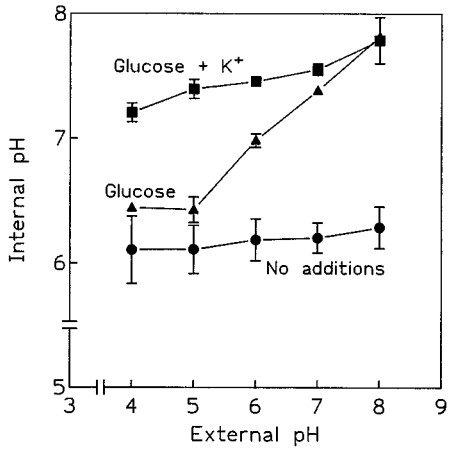

FIG. 7. Calculated values for the internal pH of yeast cells electroporated with pyranine as described in the legend to Fig. 8, from data from three similar experiments. Each point is the mean of the three values ± standard deviations.

increase of the internal pH, and potassium can overcome this difficulty even at low external pHs. It should be stressed that the values of the internal pH shown in Fig. 7 were intentionally obtained with three batches of yeast cells which incorporated different amounts of pyranine and that the scattering of the data was very small, indicating that this method may not require a constant amount of the dye incorporated within the cell.

Photographs of the electroporated cells were taken with a fluorescence microscope, and a typical example is shown in Fig. 8. The cells have large vacuoles, as the light micrographs show. It can be observed that, first, not all of the cells were stained by the dye, second, some of the cells were stained more intensely than others, and, finally, but most important practically in all of them, including those more intensely stained, the dye was excluded from the vacuole. This is most interesting, since what would appear to be measured by the procedure is the actual cytoplasmic pH. The images shown were consistently observed in three different batches of electroporated yeast cells.

DISCUSSION

The results of these experiments indicate that electroporation is a suitable procedure to introduce hydrophilic fluorescent indicators of pH or other molecules into yeast cells without producing significant harm. Hydrophilic dyes are much better indicators of pH, because interactions (aside from those with positive sites in macromolecules) are much less intense for the yeast hydrophobic molecules than used in other kinds of studies (15, 16) that are employed for their ability to cross the membrane.

Pyranine is also advantageous because at the wavelengths of approximately 460 to 520 nm selected for the measurements, both by us and by other authors (4, 10), the ionized form is fluorescent, while the un-ionized form is not fluorescent. The reported pK value of 7.2 is particularly useful for the pH values observed under different conditions for yeast cells and many other cells.

The tests of some physiological functions indicate that under the conditions used, electroporation is a rather mild treatment; it produced an efflux of potassium, as well as some decrease in the ability of the cells to concentrate the cation, which is logical in view of the nature of electroporation. However, a high

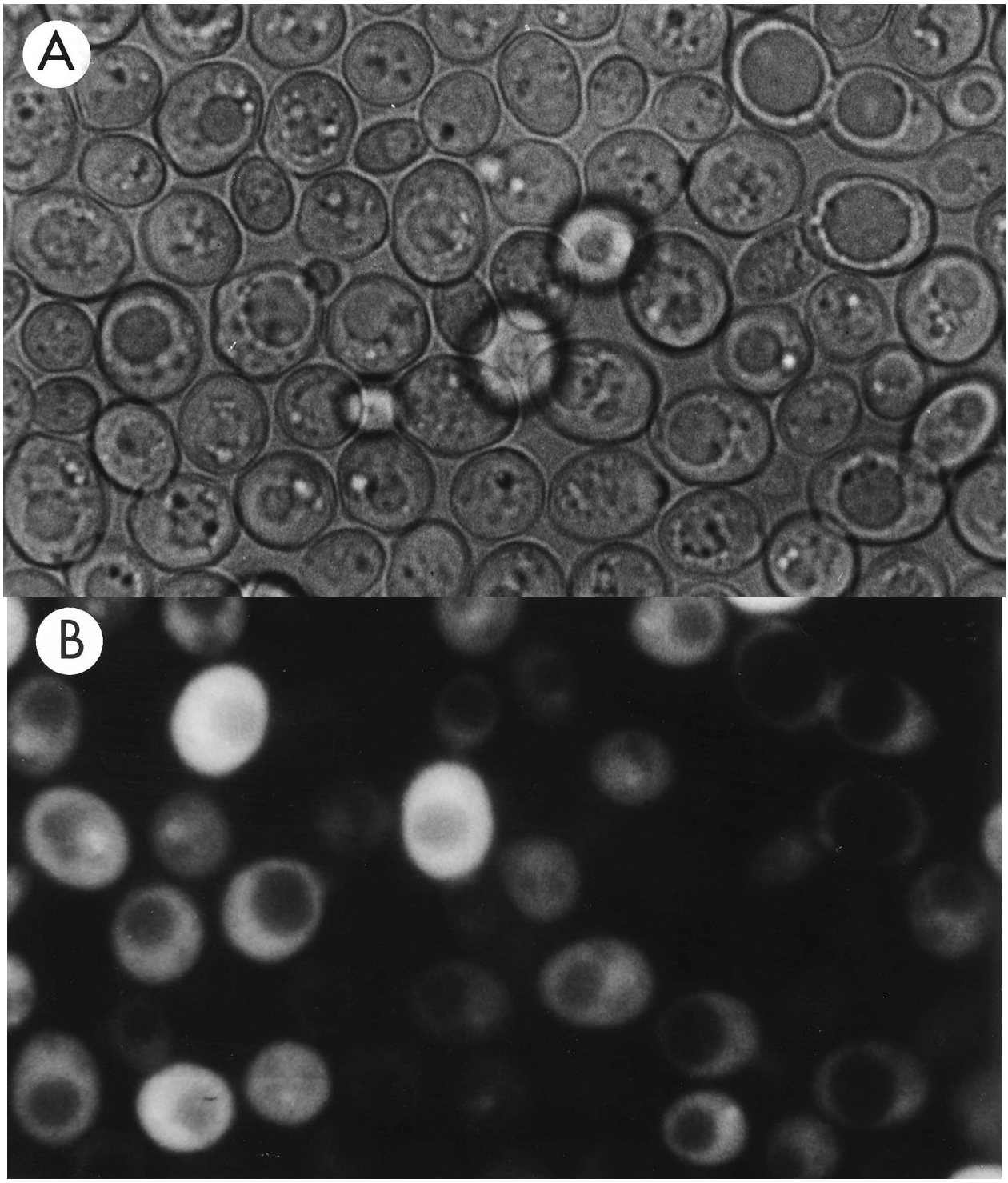

FIG. 8. Micrographs of yeast cells electroporated with pyranine. Micrographs of a suitable dilution of the cells in water were obtained with transmitted light (A) or fluorescence (B) as indicated in Materials and Methods.

capacity for potassium transport was maintained; the cells were able to take potassium up so as to leave only micromolar external concentrations. In addition, the ability of the cells to pump protons and to respire was maintained at an approximately normal level. Viability, which was measured as the capacity to grow, was actually slightly higher for the electroporated than for the control cells.

By centrifuging the electroporated cells and measuring the fluorescence of the supernatant (not shown), it was also found that pyranine in these cells leaked out only slowly during pe-

riods of several hours (approximately 2% of the internal dye per hour), which also indicates a reasonable recovery of the membrane impermeability. In addition, all fluorescence changes observed are consistent with the permanence of the dye inside; the micrographs obtained from the cells are good proof that the dye remains for long periods of time inside the cells.

The values obtained for the internal pH of the cells agree to some extent with those from other reports (5, 6, 9, 14, 17) but appear to be somewhat higher. This may be because pyranine was located essentially in the cytoplasm, while other procedures, such as the distribution of weak acids, provide information about the entire internal compartment of the cell; it is well known that the vacuole is more acidic than the rest of the cell. The report of Slavík (17) mentioned an even distribution of a dye (fluorescein acetate) in all compartments of the cell. It is also interesting that the values for the internal pH that were calculated from the fluorescence values of pyranine did not show large variations, as can be seen from the results shown in Fig. 7, in spite of the fact that the total fluorescence values observed in the three different experiments varied from approximately 5,000 to more than 50,000 arbitrary units, with the same spectrofluorometer amplification factors. The experiment shown in Fig. 4 gave unusually large fluorescence values; however, in most experiments, after alkalinization with Tris, values of 8,000 or less were observed, so that the procedure appears to be more reliable than would be expected from these values.

These results are encouraging, and this procedure may offer other possibilities for the study of many aspects of the H^+ cycle, which has such an important role in microorganisms. One important application may be monitoring the internal pH changes during the operation of symport or antiport systems, with other microorganisms also being studied. At this moment, we have already succeeded (not shown) in introducing pyranine by electroporation into cells of other yeasts, including *Saccharomyces cerevisiae* 757, kindly donated by R. Gaber, and XT300.3A, kindly provided by A. Rodríguez Navarro, as well as strain WM37 of *Kluyveromyces lactis* from our local collection, which was originally provided by J. R. Matoon. In some of these experiments, the cells that were used, which were different from those used in the present work, were collected in the late log phase of growth.

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