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Cytosolic and vacuolar Ca^{2+} concentrations in yeast cells measured with the Ca^{2+}-sensitive fluorescence dye indo-1

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Cells of *Saccharomyces cerevisiae* were loaded with indo-1, by incubation in a medium of pH 4.5, which contained penta-potassium indo-1. Cells were then washed and resuspended in a buffer of pH 4.0. The emission fluorescence spectra were recorded between 390 and 500 nm (excitation at 355 nm) and the autofluorescent spectra of the matched controls were subtracted. A 19-fold cellular accumulation of indo-1 was achieved. By permeabilization of plasma membranes, leaving the vacuolar membrane intact, it was proved that indo-1 was accumulated in the cytosol. It was also shown that intracellular indo-1 did not leak out of the cells and was not modified by cellular metabolism. Using the emission fluorescence ratio at 410/480 nm, the concentration of a free cytosolic Ca^{2+} was found to be 346 nM. Vacuolar Ca^{2+} concentration, calculated from indo-1 fluorescence after lysis of vacuolar and cellular membranes, was found to be 1.3 mM.

Ca^{2+}, cytosolic; Yeast; *Saccharomyces cerevisiae*; Indo-1

1. INTRODUCTION

It is well established that the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in eukaryotic cells is a major signal transducing element, by which extracellular factors induce a variety of physiological responses [1]. Understanding the mechanisms of signal transduction by $[Ca^{2+}]_i$ depends on the availability of non-disruptive methods for measuring $[Ca^{2+}]_i$ in intact cells. The family of Ca^{2+}-sensitive fluorescent dyes, developed by Tsien and his co-workers, such as quin 2, fura 2, and indo-1 [2,3], provided the method suitable for measuring $[Ca^{2+}]_i$ in animal cells.

In the yeast, *Saccharomyces cerevisiae*, the function of the key regulatory proteins such as GTP-regulatory proteins, adenylate cyclase, and different protein kinases, is similar to mammalian cells [4–6]. Calmodulin, the Ca^{2+}-binding regulatory protein, was found to be an essential protein in *S. cerevisiae* [7], but the regulatory role of $[Ca^{2+}]_i$ in yeast is not yet known. A major element in understanding the signalling role of $[Ca^{2+}]_i$ would be the measurement of $[Ca^{2+}]_i$. The failure to load the Ca^{2+}-sensitive fluorescent indicators into yeast cells hindered the research along these lines. In mammalian cells, these indicators are applied to the cells as membrane-permeable, acetoxymethylester derivates, which upon penetration into the cytosol, are de-esterified by the cellular esterases, yielding the Ca^{2+}-sensitive dye [2]. In yeast, the acetoxymethylester derivates are not de-esterified and, therefore, the dyes cannot accumulate intracellularly in their Ca^{2+}-sensitive non-esterified form. In the present work, we describe a new non-disruptive technique for loading indo-1 into intact yeast cells and show that indo-1 accumulates in the cytosol. Using this method, we determine the concentration of free Ca^{2+} in the cytosol and calculate the concentration in vacuole.

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Abbreviations: $[Ca^{2+}]_i$, concentration of free cytosolic Ca^{2+}; ΔpH, pH difference across plasma membranes; DMG, dimethylglutaric acid; Mes, 4-morpholineethanesulfonic acid

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2. MATERIALS AND METHODS

2.1. *Loading the cells with indo-1*

S. cerevisiae, strain N123 (MAT a/α, his 1) was maintained, grown overnight and washed as described previously [8]. To load the cells with indo-1, cells were suspended (10^9 cells/ml) in loading solution containing dimethyl glutaric acid (10 mM, pH 4.5), KCl (50 mM), glucose (100 mM) and pentapotassium indo-1 (20 μM). Cells were incubated for 1.5 h at 30°C in the dark, with shaking. The cells were then spun down, washed 3 times with distilled water and resuspended in DMG buffer (10 mM, pH 4.0). Control samples were treated similarly but without indo. The loaded and unloaded cells were then brought to the same cell density (2×10^8 cells/ml). Emission spectra of the loaded and unloaded suspensions were recorded between 390 and 500 nm (slit 5.0 nm) (excitation at 355 nm, slit 2.5 nm) using a Perkin Elmer fluorescence spectrometer.

2.2. *Permeabilization of cell membranes*

Permeabilization of both vacuolar and plasma membrane was achieved by addition of DEAE-dextran (2 mg/ml) to cells suspended in DMG buffer. Differential permeabilization of plasma membrane, leaving the vacuolar membranes intact was achieved by suspending the cells in a solution containing mannitol (0.7 M) and DMG (10 mM, pH 4.0) at 0°C, DEAE-dextran (0.6 mg/ml) was then added for 0.5 min, and the reaction was terminated by the addition of dextran sulfate (0.6 mg/ml) as described previously [8].

2.3. *Ionic composition of the cells*

Washed cells were preincubated in DMG buffer pH 4 for 2 h, then washed, resuspended in distilled water and lysed by incubation at 80°C for 10 min. Cell debris were spun down and the concentrations of K^+, Na^+ and Mg^{2+} were determined using a Perkin Elmer atomic absorption spectrometer, essentially as described previously for K^+ [9].

2.4. *Calculations of $[Ca^{2+}]_i$*

Concentrations of Ca^{2+} were determined from fluorescence ratio at 410/480 nm, according to Grynkiewicz et al. [3]. R_{min} (free dye fluorescence ratio) and R_{max} (Ca^{2+}-saturated fluorescence ratio) were determined always in the solution in which R was measured by addition of indo-1 (0.1 μM) to the solution. R_{min} was measured after the addition of NaOH to adjust the pH to 8 and EGTA (1 mM). R_{max} was determined after the addition of $CaCl_2$ (3 mM). To determine R_{max} and R_{min} for calculating $[Ca^{2+}]_i$ in indo-1 loaded cells, solution of KCl (170 mM), $NaCl_2$ (35 mM), $MgCl_2$ (25 mM) and Mes buffer (10 mM, pH 6.2) was used. Autofluorescence was always measured and subtracted from the fluorescence data.

3. RESULTS

3.1. *Loading indo-1 into yeast cells*

Indo-1 was loaded into yeast cells by incubating the cells with the pentapotassium salt of indo-1, in a medium of an acidic pH. It is suggested that at pH 4–4.5, the dye, in its acidic form, is permeable through cell membranes, whereas while exposed to the higher intracellular pH, the dye accumulates as impermeable ions. Loading the cells with indo-1 is analogous, therefore, to the accumulation of weak acids such as propionic or isobutyric acid, into cells exposed to acidic medium; a method which is widely used to measure intracellular pH in yeast [10].

Since the autofluorescence of yeast suspensions at the indo-1 excitation and emission wavelengths is fairly high, every measurement done on indo-1 loaded cells was followed by a similar measurement on a carefully matched control, which consisted of a suspension of exactly the same cell density, which underwent all preincubations and washing procedures as the suspension of indo-1 loaded cells, but without indo-1. All experimental procedures were carried out on both loaded and unloaded cells. The difference between the fluorescence spectra of indo-1 loaded cells and the matched control represented indo-1 fluorescence.

Fig.1a shows the emission spectra of indo-1 loaded cells and the matched control (excitation at 355 nm). The difference between the two spectra showed a peak at 440 nm (fig.1b). Photobleaching of indo-1 fluorescence is shown in fig.1c. Cell suspensions used to obtain fig.1a were subjected to illumination at 650 nm (inside the spectrophotometer), for 2 min. Indo-1 fluorescence was completely abolished whereas the autofluorescence was not affected. Therefore, during all experimental procedures, the loaded cells were protected from light and the intensity of excitation light was minimized as much as possible.

3.2. *Indo-1 accumulates intracellularly, and is not modified by the cells*

The next group of experiments was designed to determine whether indo-1 was accumulated intracellularly, and whether it retained its Ca^{2+}-sensitive form. The presence of indo-1 in the medium was examined by spinning down the loaded and unloaded cells and measuring the emission spectra of the supernatants. To examine whether indo-1 leaks from the cells the same procedure was repeated after maintaining the suspensions at 4°C for various lengths of time up to 2.5 h. Fig.2 shows the emission spectra of the medium in which the loaded and unloaded cells were incubated for 2.5 h. The similarity between the intensities in-

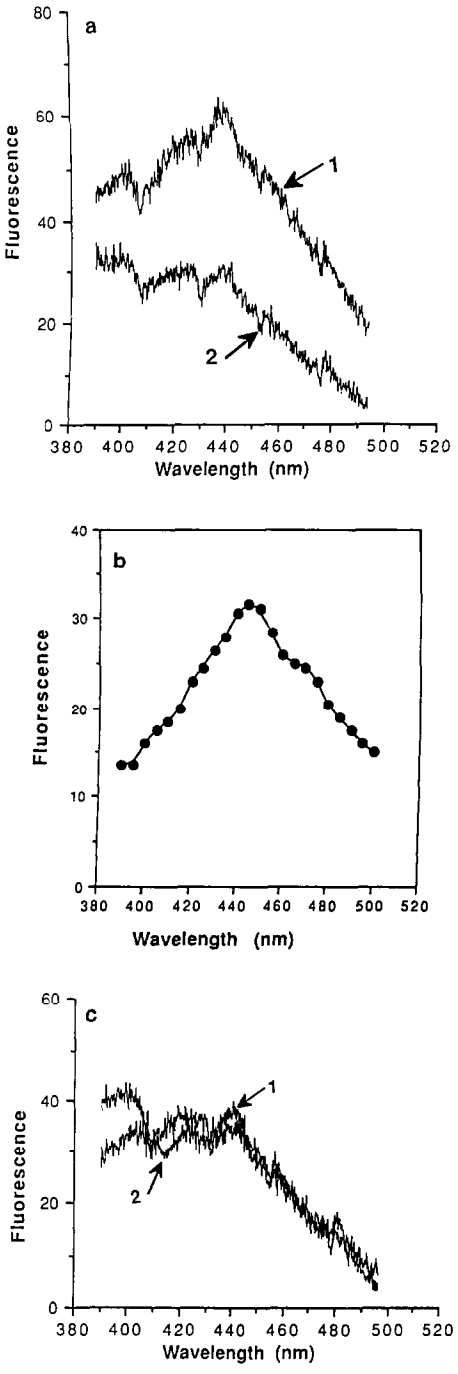

Fig.1. Typical emission spectra of indo-1 loaded cells. Fluorescence intensities (in relative units) of (a) indo-1 loaded (1) and unloaded (2) cells. The difference between curves 1 and 2 is shown in b. Photobleaching of the suspensions (measured in fig.1a) by exposure to 650 nm light in a spectrophotometer for 2 min is shown in c. Curves 1 and 2 are indo-1 loaded and unloaded cells respectively.

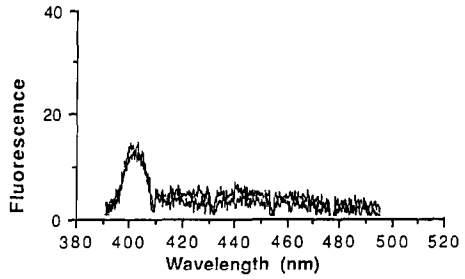

Fig.2. Fluorescence spectrum of DMG buffer in which indo-1 loaded cells (upper curve) and unloaded cells (lower curve) were incubated for 2.5 h at 4°C. Measurements were done on the supernatant after spinning down the cells.

dicates that indo-1 is associated with the cells and that the leakage is not appreciable.

To examine whether indo-1 is located intracellularly or alternatively bound to the cells externally, the cells were exposed to DEAE-dextran, at a concentration sufficient to permeabilize cellular and vacuolar membranes [8]. Following DEAE-dextran application, the fluorescence intensity increased several fold and the peak was shifted from 440 nm to between 400 and 420 nm. The spectra of the control suspensions were not affected by DEAE dextran. We interpreted the increase in the intensity of fluorescent light as caused by the liberation of indo-1 from the cells, since when inside the cells, fluorescence intensity was presumably reduced by cell walls. This assumption was examined by spinning down the lysed cells, measuring the fluorescence of the supernatants and the resuspended pellets. All the fluorescence was found now in medium, and the spectrum was changed to form a peak at 400–420 nm (fig.3a).

To examine whether indo-1, which was liberated from the cells, was not modified by cellular metabolism, we added EGTA (after adjusting the pH to 8 by NaOH) to obtain the spectrum of the free dye, then $CaCl_2$, to obtain the spectrum of the Ca^{2+}-saturated dye, followed by the addition of $MnCl_2$, to quench indo-1 fluorescence (fig.3b). As a control, we measured the spectra of a solution of the same composition as above, to which indo-1 was added (fig.3c). The similarity between the spectra shown in fig.3b and c indicates that indo-1, which was trapped intracellularly and liberated by cell lysis was not modified by cellular metabolism.

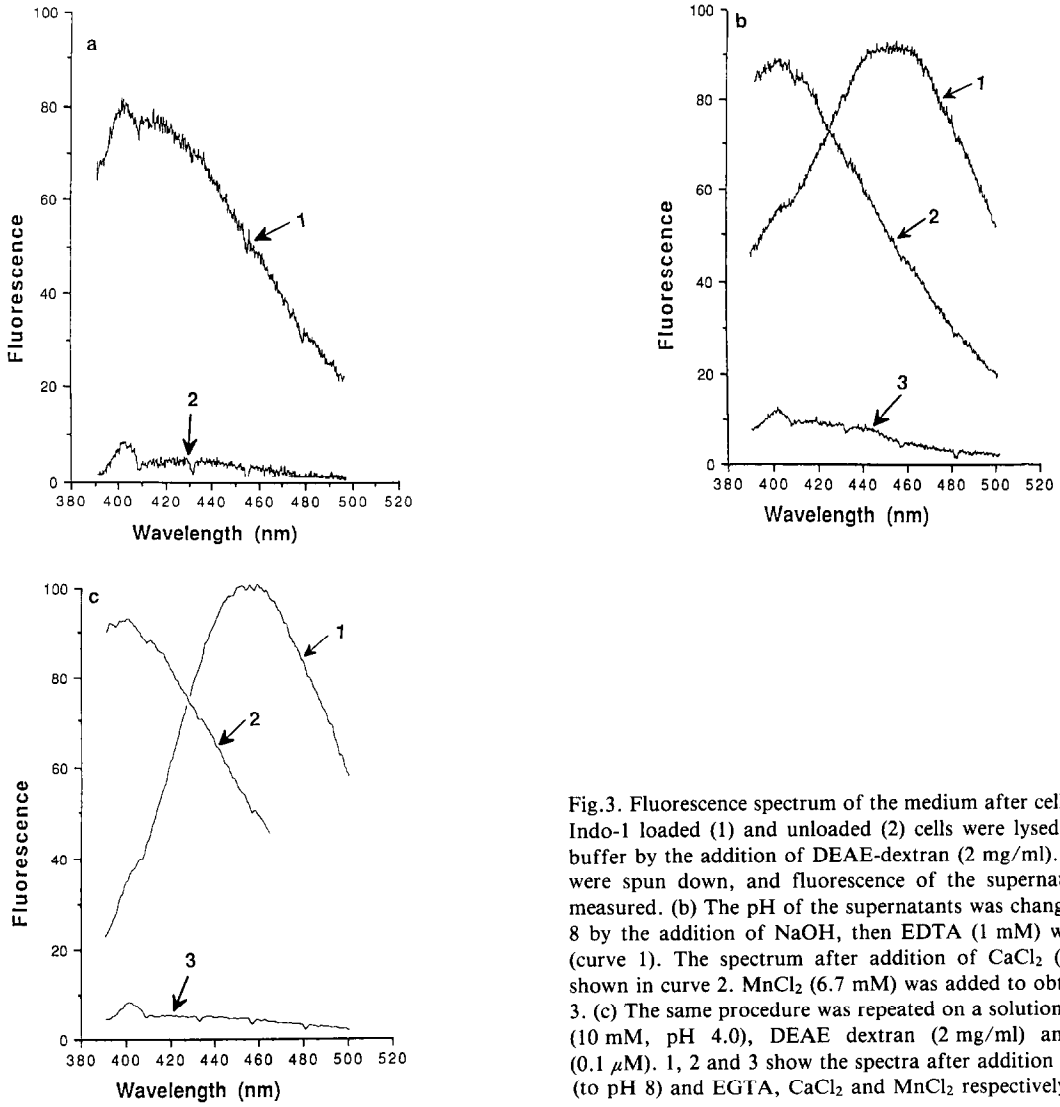

Fig.3. Fluorescence spectrum of the medium after cell lysis. (a) Indo-1 loaded (1) and unloaded (2) cells were lysed in DMG buffer by the addition of DEAE-dextran (2 mg/ml). The cells were spun down, and fluorescence of the supernatants was measured. (b) The pH of the supernatants was changed to pH 8 by the addition of NaOH, then EDTA (1 mM) was added (curve 1). The spectrum after addition of $CaCl_2$ (3 mM) is shown in curve 2. $MnCl_2$ (6.7 mM) was added to obtain curve 3. (c) The same procedure was repeated on a solution of DMG (10 mM, pH 4.0), DEAE dextran (2 mg/ml) and indo-1 (0.1 μM). 1, 2 and 3 show the spectra after addition of NaOH (to pH 8) and EGTA, $CaCl_2$ and $MnCl_2$ respectively as in b.

3.3. *Indo-1 is accumulated in the cytosol*

Using methods based on differential extraction of the cytosol and vacuoles of *S. cerevisiae* (N123 strain), it was previously shown that the concentration of Ca^{2+} in the vacuoles is about 10^3-fold higher than that in the cytosol [8]. Therefore, the presence of indo-1 in the vacuoles may lead to a serious overestimation of cytosolic Ca^{2+}. We examined the presence of indo-1 in the cytosol and the vacuoles of loaded cells by differential permeabilization of plasma membranes under conditions which render the vacuolar membrane intact [8]. The cells were then spun down and the presence of indo-1 in the supernatant and in the pellet was examined. Very little fluorescence was associated with the pellet, which contained cells with intact vacuoles (fig.4b), while the supernatant contained most of indo-1 fluorescence (fig.4a). The difference between the spectra of loaded and unloaded cells before and after permeabilization of cytoplasmic membrane indicated that 90% of cellular indo-1 was accumulated in the cytosol (fig.4c). Since the remaining 10% of indo-1 fluorescence, which was associated with the

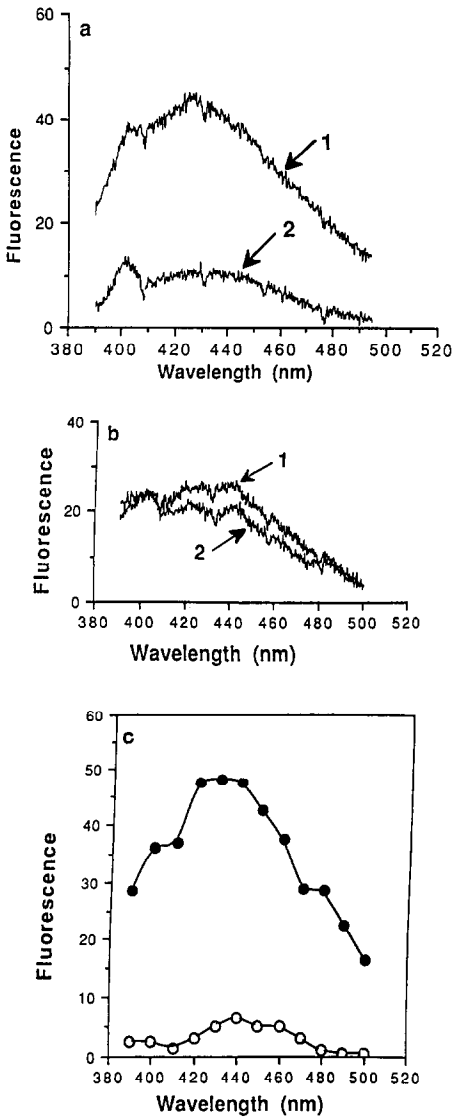

Fig.4. Fluorescence of cells and medium after differentially permeabilizing the plasma membranes. Indo-1 loaded (1) and unloaded (2) cells, suspended in mannitol (0.7 M) and DMG (10 mM, pH 4.0) were differentially permeabilized by exposure to DEAE-dextran (0.6 mg/ml) for 0.5 min at 0°C, followed by addition of dextran sulfate (0.6 mg/ml). The cells were spun down and the fluorescence of the supernatants (a) and the pellets, resuspended in mannitol and DMG solution (b), were measured. In c, the differences between the spectra of loaded and unloaded cells before (●), and after (○) permeabilization of plasma membranes and resuspending the pellets, are shown.

vacuolar pellet, showed a peak at 440 nm, it seems that even this small amount of indo-1 was not located inside the vacuole, where it would be Ca^{2+}-saturated (with a peak at 400–420 nm), but bound to cytosolic or membranal sites outside the vacuoles.

As a control, cells from the same suspension were lysed completely by applying high concentration of DEAE-dextran and incubating at room temperature for 10 min. The lysed cells were spun down and the fluorescence spectrum determined. A shift in the peak from 440 to 400–420 nm was observed, presumably due to the liberation of Ca^{2+} from the vacuoles (fig.5).

3.4. *The concentration of Ca^{2+} in the cytosol and the vacuoles of the yeast cells*

Concentration of Ca^{2+} in the cytosol was determined from the difference between spectra of loaded and unloaded cells in 9 different experiments, by calculating the ratio of fluorescence intensities at 410/480 nm. To determine the values of R_{min} and R_{max} in a solution of ionic composition as close as possible to the intracellular one, we measured the content of K^+, Na^+ and Mg^{2+} in cells preincubated in DMG buffer, pH 4.0, for 2 h, by lysing the cells and measuring the content of these ions in the lysate. Using the value of cell volume previously determined, 2.8 μl per 10^8 cells [8], cellular concentration of 170 mM K^+, 35 mM Na^+ and 25 mM Mg^{2+} were determined. To obtain R_{min} and R_{max}, indo-1 was added to a solution of the above composition. The fluorescence intensity ratios at 410/480 nm, of the free dye (R_{min}) and Ca^{2+}-saturated dye (R_{max}) were determined after the addition of EGTA (at pH 8) and $CaCl_2$, respectively. Using these values, a mean value of the concentration of free cytosolic Ca^{2+} of 364 ± 42 nM (SE, n = 9) was determined.

The concentration of Ca^{2+} in the solutions in which the cells were incubated was determined by addition of indo-1 and measuring the fluorescence ratio 410/480 nm. R_{min} and R_{max} were determined in the same solutions. Values between 350 and 700 nM were obtained in different experiments.

The concentration of Ca^{2+} in the solution after differential permeabilization of cytoplasmic membranes, leaving the vacuoles intact (fig.3a) was not significantly different from the concentration of Ca^{2+} in the same solution before permeabilizing the cells. Since the concentration of cytosolic Ca^{2+} was found to be low, 10^3-fold dilution by lysing plasma membranes would yield too low a Ca^{2+}

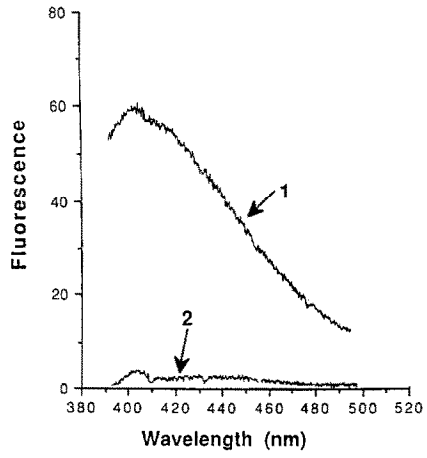

Fig.5. Fluorescence of cell medium after complete cell lysis. Indo-1 loaded (1) and unloaded (2) cells, suspended in mannitol and DMG solution as in fig.4 were fully lysed by the addition of DEAE-dextran (2 mg/ml) for 10 min at room temperature. The fluorescence of the supernatants, after cell centrifugation, is shown.

concentration to be distinguished above solution background.

To calculate the concentration of Ca^{2+} in the vacuoles, Ca^{2+} concentration was measured in the solutions before and after complete lysis of the cells, which led to the liberation of Ca^{2+} from the vacuoles (fig.2). R_{min} and R_{max} were determined in the same solution. Mean concentration of Ca^{2+} in solution after cell lysis, minus Ca^{2+} concentration which was present in the solution before cell lysis, was 1.15 μM. Using vacuolar volume of 1.3 μl per 10^8 cells, which was determined by us previously in the same strain of yeast [8] a concentration of 1.3 mM Ca^{2+} in the vacuoles was calculated.

3.5. *Concentration of indo-1 in the cytosol*

To determine the concentration of indo-1 in the cytosol, a calibration curve was constructed. Increasing amounts of indo-1 were added to the solution of DMG and DEAE-dextran, to which $CaCl_2$ (3 mM) was added. The graph of fluorescence intensities at 400 nm vs indo-1 concentration was used to determine the amount of indo-1 in the solution in which the cells were lysed, by its fluorescence intensity at 400 nm after addition of $CaCl_2$ (fig.3b). Using the value of water-volume of the cytosolic compartment, previously determined, 1.5 μl/10^8 cells [8], a concentration of 0.38 mM indo-1 in the cytosol was calculated. Thus, at least a 19-fold cellular accumulation was obtained during loading in a solution of 0.02 mM indo-1.

4. DISCUSSION

We have shown in the present work that indo-1 can accumulate in yeast cells when applied as pentapotassium salt of indo-1 in a medium of pH 4.5. The conditions necessary for measuring $[Ca^{2+}]_i$ in indo-1 loaded cells were found to be fulfilled: (i) indo-1 does not leak out of the cells when the cells are immersed in a buffer of pH 4.0; (ii) indo-1 accumulates in cytoplasm and is not present appreciably in the vacuoles; and (iii) intracellular indo-1 is not modified by cellular metabolism.

Fluorescence spectra of indo-1 loaded cells, after subtracting the autofluorescence, were, therefore, used to determine the concentration of free cytosolic Ca^{2+} in yeast. A value of 364 nM was obtained. Thus, the levels of $[Ca^{2+}]_i$ in yeast are of the same order of magnitude as in mammalian cells [11]. The concentration of Ca^{2+} in the vacuoles was calculated to be 1.3 mM; the concentration gradient of Ca^{2+} across vacuolar membranes is therefore 2×10^3.

The concentration of Ca^{2+} in the cytosol of yeast cells was not directly determined previously. Using methods based on differential extraction of yeast cells labelled with $^{45}Ca^{2+}$, we have previously shown cytoplasmic Ca^{2+} homeostasis at low Ca^{2+} levels, and accumulation of Ca^{2+} in the vacuoles [8]. Whereas the values of vacuolar Ca^{2+} concentration found before were similar to the values found in the present work, the values of cytoplasmic Ca^{2+} found by differential extraction were higher, probably since the differential extraction method is much less sensitive at low Ca^{2+} concentrations.

Indo-1 accumulates in the cells probably due to the large ΔpH across plasma membranes of yeasts immersed at pH 4.0. Vacuolar pH was found to be more acidic than cytosolic pH, since the ΔpH is maintained by vacuolar H^+ ATPase which pumps protons into the vacuoles [12]. It appears that the pH gradient across vacuolar membranes prevents indo-1 from entering the vacuoles.

A method of loading indo-1 into plant protoplasts, based on similar principles was previously published [13]. In contrast to plant cells, in yeast

undisrupted cells with cell wall intact were loaded with indo-1. This method therefore opens new possibilities for investigations on signal transduction by changes in $[Ca^{2+}]_i$ in yeast cells. Experiments in this direction are in progress in our laboratory.

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REFERENCES

[1] Rasmussen, H. (1986) N. Engl. J. Med. 17, 1094–1169.
[2] Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell Biol. 94, 325–334.
[3] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[4] Watson, J.D., Hopkins, N.H., Roberts, J.W., Steiz, J.A. and Weiner, A.M. (1987) in: The Molecular Biology of the Gene, vol.1, pp.550–618, Benjamin/Cummings Publishing Company.
[5] Engelberg, D., Perlman, R. and Levitzki, A. (1989) Cell. Signalling 1, 1–7.
[6] Matsumoto, K., Uno, I. and Ishikawa, T. (1985) Yeast 1, 15–24.
[7] Davis, T.N., Urdea, M.S., Masiarz, F.R. and Thorner, J. (1986) Cell 47, 423–431.
[8] Eilam, Y., Lavi, H. and Grossowicz, N. (1985) J. Gen. Microbiol. 131, 623–629.
[9] Eilam, Y., Lavi, H. and Grossowicz, W. (1985) J. Gen. Microbiol. 131, 2555–2564.
[10] De La Peña, P., Barros, F., Gascon, S., Ramos, S. and Lazo, P. (1982) J. Biochem. 121, 447–453.
[11] Tsien, R.Y., Rink, T.J. and Poenie, M. (1985) Cell Calcium 6, 145–157.
[12] Kakinuma, Y., Ohsumi, Y. and Anraku, Y. (1981) J. Biol. Chem. 256, 10859–10863.
[13] Bush, D.S. and Jones, R.L. (1987) Cell Calcium 8, 455–472.