

## Cytoplasmic $\text{Ca}^{2+}$ Homeostasis Maintained by a Vacuolar $\text{Ca}^{2+}$ Transport System in the Yeast *Saccharomyces cerevisiae*

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Differential extraction of  $\text{Ca}^{2+}$  from the cytoplasmic and vacuolar pools of the yeast *Saccharomyces cerevisiae*, using DEAE-dextran, revealed that most of the cellular  $\text{Ca}^{2+}$  was bound, precipitated or sequestered within the vacuole. When the concentration of  $\text{Ca}^{2+}$  in the medium was raised from  $10^{-6}$  M to  $10^{-3}$  M, cytoplasmic  $\text{Ca}^{2+}$  homeostasis was maintained at  $5.8 \times 10^{-6}$  to  $2.3 \times 10^{-5}$  M, whereas the vacuoles accumulated higher concentrations of  $\text{Ca}^{2+}$ . The results indicate that the vacuoles function as a cytoplasmic  $\text{Ca}^{2+}$  buffering system and as the major sequestering organelle for  $\text{Ca}^{2+}$ . A respiratory-deficient mutant ( $\rho^0$ ) displayed a similar intracellular distribution of  $\text{Ca}^{2+}$  to the wild-type. When cells were permeabilized by DEAE-dextran the vacuoles were still capable of  $\text{Ca}^{2+}$  uptake. This uptake proceeded without the addition of ATP or glucose in fresh preparations but required the addition of ATP after incubation of the permeabilized cells in buffered sorbitol for 2 h. The results are consistent with the proposed  $\text{Ca}^{2+}/\text{H}^{+}$  antiport in the vacuolar membrane, which is driven by  $\Delta\mu_{\text{H}^{+}}$  formed by the  $\text{H}^{+}$ -ATPase pumping  $\text{H}^{+}$  into the vacuole.

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### INTRODUCTION

Intracellular calcium compartmentalization in eukaryotic cells plays a major role in maintaining the cytoplasmic  $\text{Ca}^{2+}$  homeostasis which is essential for the function of calcium as a regulator of many cellular processes (Carafoli & Crompton, 1978). In higher eukaryotic cells cellular  $\text{Ca}^{2+}$  concentration is kept constant and low by calcium transport systems in the plasma membrane (Schatzmann & Vincenzi, 1969), the mitochondrial inner membrane (Mela, 1977; Nicholls & Akerman, 1982) and the endoplasmic reticulum (Ebashi & Endo, 1968). In non-excitable cells the mitochondria are the major  $\text{Ca}^{2+}$ -sequestering organelles and act as a buffering system for cytoplasmic calcium (Carafoli & Crompton, 1978).

Yeasts (lower eukaryotic cells) can accumulate high amounts of calcium without any apparent damage to the cells (Conway & Gaffney, 1966). However, *in vitro* experiments revealed that the mitochondria of *Saccharomyces cerevisiae* are incapable of  $\text{Ca}^{2+}$  uptake (Carafoli *et al.*, 1970) and it remained unclear whether cytoplasmic  $\text{Ca}^{2+}$  homeostasis is maintained. In view of the recent discovery of calmodulin in yeasts (Hubbard *et al.*, 1982) the problem of free  $\text{Ca}^{2+}$  concentration in the cytoplasm is of interest as a starting point for the investigation of the role of calcium in the regulation of cellular processes in yeasts.

In the present work we show that in intact yeast cells the vacuoles function as a calcium-buffering system maintaining the cytoplasmic calcium concentration at a constant and low level, equivalent to the function of the mitochondria in higher eukaryotic cells.

### METHODS

**Organism and culture conditions.** *Saccharomyces cerevisiae* strain N123 (genotype *MATa/α his1*) was maintained at 4 °C on YPD-agar slopes and grown with shaking at 30 °C in YPD broth (Bacto yeast extract, 10 g l<sup>-1</sup>; Bacto peptone, 20 g l<sup>-1</sup>; and glucose, 20 g l<sup>-1</sup>). Cells were collected from the overnight culture by centrifugation, washed three times by resuspension in distilled water, and finally resuspended in the indicated medium at a cell density of  $5 \times 10^7$  cells ml<sup>-1</sup>.

The respiratory deficiency mutation 'petite' was induced as described by Goldring *et al.* (1970). Ethidium bromide ( $10 \mu\text{g ml}^{-1}$ ) was added to exponentially growing yeast cells in medium containing Difco yeast nitrogen base ( $6.7 \text{ g l}^{-1}$ ), glucose ( $20 \text{ g l}^{-1}$ ) and histidine ( $40 \text{ mg l}^{-1}$ ), and the cells were incubated for 2 h. The cells were then spun down and washed by centrifugation, diluted, and plated on YPD agar plates. Criteria used to identify the petite colonies were: (1) the ability to grow on glucose-containing plates (YPD) but not on the same plates containing 1% (v/v) glycerol instead of glucose; and (2) the inability to reduce tetrazolium chloride when colonies grown on glucose-containing plates were overlaid with the dye.

*Differential extraction of soluble pools from the cytosol and vacuole.* This was done essentially as described by Huber-Wälchli & Wiemken (1979) for *Candida utilis*, with minor variations. Cells were collected by filtration on membrane filters ( $0.45 \mu\text{m}$  pore size, 25 mm diameter,  $10^8$  cells per filter) and washed three times with  $\text{MgCl}_2$  (20 mM) to remove the cell-wall bound  $\text{Ca}^{2+}$  (Eilam, 1982a, b), followed by three washes with buffered sorbitol solution containing MES/Tris pH 6 (10 mM) and sorbitol (0.7 M). The filters with the cells were each immersed in a flask containing 3 ml buffered sorbitol and the cells were resuspended by a vortex shaker. The cell suspensions of five flasks were refiltered, each on a membrane filter, to determine the amounts of  $\text{Ca}^{2+}$  and  $\text{K}^+$  in the intact cells. DEAE-dextran (0.5 ml of a  $2 \text{ mg ml}^{-1}$  solution in buffered sorbitol) was added to each of the next five flasks at  $0^\circ\text{C}$ , and the suspensions were incubated at  $0^\circ\text{C}$  for 30 s, filtered on membrane filters and washed four times with buffered sorbitol. The filtrates were collected and the amounts of ions in the cells on the filters and in the filtrates were determined. Methanol (4 ml) was added to the next five flasks, containing cell suspensions, which were then incubated for 5 min at room temperature to release the unbound ions from the vacuoles. The cells were collected by filtration and washed once with 60% (v/v) methanol and three times with water. In order to release the bound ions, in an additional five flasks of cells the suspensions were incubated with 0.5 M- $\text{HClO}_4$  at  $60^\circ\text{C}$  for 10 min, filtered on membrane filters and washed with water.

The amounts of  $\text{Ca}^{2+}$  in the cells after the differential extraction were determined from the radioactivity of the filters measured in toluene-based scintillation fluid, and the radioactivity of the filtrates measured in toluene/Triton-based scintillation fluid. The amount of  $^{40}\text{Ca}^{2+}$  in the medium was measured by a Perkin-Elmer atomic absorption spectrometer. The differences between the amounts of  $\text{Ca}^{2+}$  in the intact cells and in the DEAE-dextran-treated cells were equal to the amounts of  $^{45}\text{Ca}^{2+}$  recovered in the filtrates after DEAE-dextran treatments. In experiments to determine the amounts of  $\text{Ca}^{2+}$  in the intracellular pools, the cells were preincubated in medium containing MES/Tris pH 6.0 (10 mM), glucose (80 mM) and  $\text{CaCl}_2$  at the desired concentration labelled with  $^{45}\text{CaCl}_2$  until equilibrium in the distribution of the radioactivity between the medium and the cells was obtained. Thus the amounts of  $\text{Ca}^{2+}$  in the cells could be calculated from the cell-associated radioactivity. In a control experiment differential extraction was carried out on cells similarly incubated but without  $^{45}\text{Ca}^{2+}$  and the content of  $\text{Ca}^{2+}$  was determined using the atomic absorption spectrometer. Since larger quantities of cells had to be used for a single measurement ( $5 \times 10^8$  cells) the cells were collected by centrifugation instead of by filtration. The results obtained were similar to those reported in Tables 1 and 2.

In order to determine the distribution of  $\text{K}^+$  between the pools, the filters with the cells obtained after the differential extraction (as described above) were each immersed in 3 ml distilled water, boiled to release the ions from the cells and centrifuged to remove the debris.  $\text{K}^+$  was determined in the supernatant after appropriate dilution, using a Perkin-Elmer atomic absorption spectrometer.

*Staining the cells with berberine sulphate.* The washed cells were incubated for 30 s in buffered sorbitol containing DEAE-dextran ( $0.6 \text{ mg ml}^{-1}$ ) at  $0^\circ\text{C}$ . The reaction was stopped by the addition of dextran sulphate ( $0.6 \text{ mg ml}^{-1}$ ) (Dürr *et al.*, 1975). The fluorescent dye berberine sulphate was added to the suspensions ( $10 \mu\text{g}$  per ml cell suspension) and the cells were immediately observed in a Zeiss fluorescence microscope.

*Uptake of  $\text{Ca}^{2+}$  into the vacuoles of broken cells.* Cells were washed by centrifugation and incubated in medium containing MES/Tris pH 6.0 (10 mM) and sorbitol (0.7 M). DEAE-dextran was added at a final concentration of  $0.6 \text{ mg ml}^{-1}$ ; after incubation for 30 s at  $0^\circ\text{C}$  the reaction was stopped by the addition of dextran sulphate ( $0.6 \text{ mg ml}^{-1}$ ). The cells were collected by centrifugation and resuspended in the indicated medium, which always contained 0.7 M-sorbitol (Fig. 2).  $\text{CaCl}_2$  ( $10^{-6} \text{ M}$ , labelled with  $^{45}\text{Ca}^{2+}$ ,  $0.5 \mu\text{Ci ml}^{-1}$ ,  $18.5 \text{ kBq ml}^{-1}$ ) was added to the cells and 1 ml samples were removed at the indicated times, and the cells were collected by filtration and washed on the filters four times with buffered sorbitol. The radioactivity was determined in toluene-based scintillation fluid.

*Determination of the water volume of the cells and the vacuoles.* The water volumes were determined according to Rottenberg (1979). Cells were suspended ( $2 \times 10^9$  cells  $\text{ml}^{-1}$ ) in medium containing MES/Tris pH 6.0 (10 mM), glucose (80 mM),  $^3\text{H}_2\text{O}$  ( $5 \mu\text{Ci ml}^{-1}$ ;  $185 \text{ kBq ml}^{-1}$ ) and [ $^{14}\text{C}$ ]sorbitol ( $0.5 \mu\text{Ci ml}^{-1}$ ;  $18.5 \text{ kBq ml}^{-1}$ ), and incubated for 10 min at room temperature. Samples of 0.6 ml were removed and centrifuged using a microfuge and 0.1 ml of each supernatant was transferred to 1 ml 1 M- $\text{HClO}_4$ . The rest of the supernatant was removed and 1 ml 1 M- $\text{HClO}_4$  was added to the cell pellets. The cells were resuspended and incubated for 30 min. All samples in  $\text{HClO}_4$  were centrifuged again and the radioactivity in the supernatant was determined. To determine the volume of the vacuoles, the cells were first incubated in buffered sorbitol containing DEAE-dextran ( $0.6 \text{ mg ml}^{-1}$ ) for 30 s

at 0 °C, and the reaction was stopped by the addition of dextran sulphate (0.6 mg ml<sup>-1</sup>). Double labelling was carried out as above, and the volumes of the cells and the vacuoles from each of the experiments were calculated according to the equation

$$[(^3\text{H}_p/^3\text{H}_s) - (^{14}\text{C}_p/^14\text{C}_s)] \times V = V_i$$

where  $^3\text{H}_p$  and  $^{14}\text{C}_p$  are the radioactivities in the pellet,  $^3\text{H}_s$  and  $^{14}\text{C}_s$  are the radioactivities in the supernatant,  $V$  is the volume of the sample used, and  $V_i$  is the cellular or vacuolar volume impermeable to sorbitol.

**Preparation of spheroplasts.** Cells were incubated at 30 °C in medium containing sorbitol (1 M), EDTA (1.5 mM), MES/Tris pH 7.0 (7.5 mM), mercaptoethanol (7 mM) and liticase (5 mg ml<sup>-1</sup>). The formation of spheroplasts was monitored by measuring the osmotic fragility of the cells by diluting, with water, small samples taken from the cell suspension at different times during incubation with liticase. Spheroplast formation was completed after 60 min incubation and the spheroplasts were centrifuged and subjected to differential extraction as described previously, but the media always contained sorbitol (1 M).

## RESULTS

It was previously reported that the soluble pools of amino acids and  $\text{K}^+$  can be differentially extracted from the cytosol and the vacuoles of *Candida utilis* by a method based on the use of DEAE-dextran (Huber-Wälchli & Wiemken, 1979). Fig. 1 shows that treatment of *S. cerevisiae* cells with isotonic DEAE-dextran rendered the plasma membrane permeable to the fluorescent dye berberine sulphate whereas the vacuoles remained impermeable to the dye. Additional evidence for the integrity of the vacuoles after DEAE-dextran treatment was the presence of 93% of the cellular arginine in the DEAE-dextran-stable fraction. Arginine is considered to be a marker for the vacuole (Ohsumi & Anraku, 1981).

Cells of strain N123 and the respiratory-deficient mutant  $\rho^0$  prepared from this strain were grown overnight in YPD medium (containing 10<sup>-4</sup> M- $\text{Ca}^{2+}$  labelled with  $^{45}\text{Ca}^{2+}$ ). The prolonged incubation with  $^{45}\text{Ca}^{2+}$  was intended to achieve an equilibrium distribution of the labelled  $\text{Ca}^{2+}$  so that the amounts of cellular  $\text{Ca}^{2+}$  could be determined from the cell-associated radioactivity. Cells were washed repeatedly with 20 mM-MgCl<sub>2</sub> to remove calcium ions bound to the cell walls, and were differentially extracted by isotonic DEAE-dextran. Over 97% of the cellular  $\text{Ca}^{2+}$  was found in the DEAE-dextran-stable fraction. Similar results were obtained in the wild-type and in the respiratory-deficient mutant. The DEAE-dextran-stable fraction was further extracted with 60% methanol to determine the amounts of free and bound, or precipitated,  $\text{Ca}^{2+}$ ; 87% of the vacuolar  $\text{Ca}^{2+}$  was liberated by the methanol, the remaining  $\text{Ca}^{2+}$  was liberated by incubation with 0.5 M-HClO<sub>4</sub> at 60 °C for 30 min (Table 1).

In order to determine whether the  $\text{Ca}^{2+}$  which remained in the cellular fraction after methanol treatment represented ions bound to cell walls or to intracellular and intravacuolar sites, we prepared spheroplasts and subjected them to similar differential extractions. The results obtained were similar to those in Table 1. We conclude, therefore, that the 'bound  $\text{Ca}^{2+}$ ' represented either binding to intracellular sites, or intracellular precipitation, probably as calcium polyphosphate, which is present in large quantities within *S. cerevisiae* vacuoles (Solimene *et al.*, 1980).

In parallel measurements, the distribution of  $\text{K}^+$  between the vacuoles and the cytosol was determined. DEAE-dextran treatment liberated 59% of the cellular  $\text{K}^+$  in the wild-type and 68% in the respiratory-deficient mutant  $\rho^0$ . Only about 8% of the cellular  $\text{K}^+$  remained in the cellular fraction after extraction with methanol (Table 1).

Cells of strain N123 and the respiratory-deficient mutant  $\rho^0$  derived from this strain were incubated in media containing buffer, glucose and different concentrations of  $\text{Ca}^{2+}$  labelled with  $^{45}\text{Ca}^{2+}$  until a steady state in the distribution of  $^{45}\text{Ca}^{2+}$  was achieved (4 h). The cells were then subjected to the differential extraction described in Methods. The water volumes of the cells and the vacuoles were determined after similar incubations in media containing different concentrations of  $\text{Ca}^{2+}$  but without  $^{45}\text{Ca}^{2+}$ , by double labelling the cells with  $^3\text{H}_2\text{O}$  and [ $^{14}\text{C}$ ]sorbitol. The water volumes of the cells and vacuoles were not affected by the concentration of  $\text{CaCl}_2$  in the incubation media; the values obtained for strain N123 were  $2.8 \pm 0.1 \mu\text{l}$  per  $10^8$  cells for cellular water volume, and  $1.28 \pm 0.05 \mu\text{l}$  per  $10^8$  cells for vacuolar volume. The

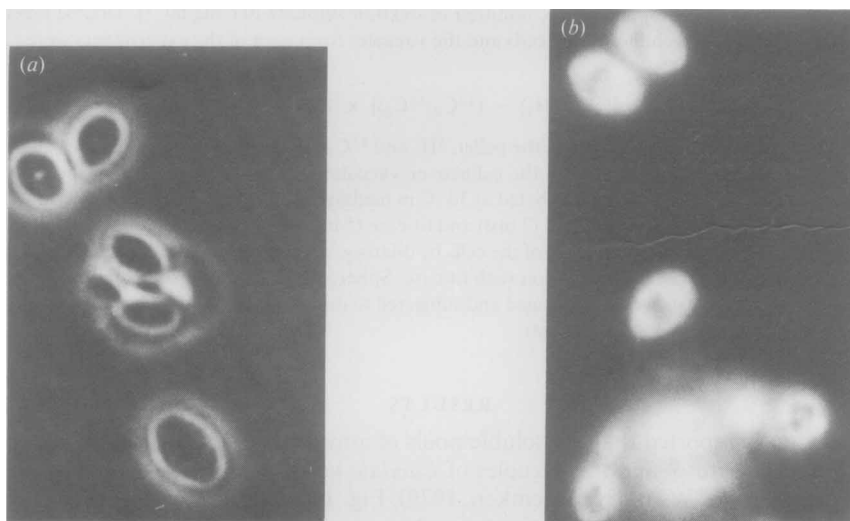


Fig. 1. Fluorescence micrograph (a) and phase-contrast micrograph (b) of cells incubated with DEAE-dextran for 30 s at 0°C and stained by the fluorescent dye berberine sulphate (not the dark vacuoles in b). Cells which had not been incubated with DEAE-dextran appeared black after staining. Magnification  $\times 2000$ .

Table 1. Intracellular distribution of  $\text{Ca}^{2+}$  and  $\text{K}^+$  as determined by DEAE-dextran and methanol fractionation.

	Values represent means $\pm$ SEM ( $n = 15$ ).		
	DEAE-dextran-labile fraction (mol per $10^8$ cells)	DEAE-dextran-stable fraction (mol per $10^8$ cells)	Methanol-stable fraction (mol per $10^8$ cells)
Strain N123			
$\text{Ca}^{2+}$	$(3.18 \pm 0.09) \times 10^{-11}$	$(2.32 \pm 0.04) \times 10^{-9}$	$(3.04 \pm 0.06) \times 10^{-10}$
$\text{K}^+$	$(158.4 \pm 4.7) \times 10^{-9}$	$(110.8 \pm 2.1) \times 10^{-9}$	$(22.1 \pm 0.42) \times 10^{-9}$
$\rho^0$ mutant			
$\text{Ca}^{2+}$	$(7.51 \pm 0.22) \times 10^{-11}$	$(1.65 \pm 0.03) \times 10^{-9}$	$(2.51 \pm 0.52) \times 10^{-10}$
$\text{K}^+$	$(175.1 \pm 5.3) \times 10^{-9}$	$(82.2 \pm 1.51) \times 10^{-9}$	$(21.4 \pm 0.38) \times 10^{-9}$

Table 2. Concentrations of  $\text{Ca}^{2+}$  in the cytoplasmic and vacuolar pools in strain N123 and the respiratory-deficient mutant  $\rho^0$ .

	Values represent means $\pm$ SEM ( $n = 15$ ).			
	Medium $\text{Ca}^{2+}$ (M)	Cytoplasmic $\text{Ca}^{2+}$ (M)	Vacuolar free $\text{Ca}^{2+}$ (M)	Bound or precipitated $\text{Ca}^{2+}$ (mol per $10^8$ cells)
Strain N123	$10^{-3}$	$(2.30 \pm 0.09) \times 10^{-5}$	$(3.36 \pm 0.08) \times 10^{-3}$	$(4.20 \pm 0.13) \times 10^{-10}$
	$10^{-4}$	$(2.04 \pm 0.07) \times 10^{-5}$	$(1.48 \pm 0.03) \times 10^{-3}$	$(3.10 \pm 0.06) \times 10^{-6}$
	$10^{-5}$	$(1.56 \pm 0.08) \times 10^{-5}$	$(7.14 \pm 0.14) \times 10^{-4}$	$(3.63 \pm 0.07) \times 10^{-11}$
	$10^{-6}$	$(5.81 \pm 0.29) \times 10^{-6}$	$(2.25 \pm 0.04) \times 10^{-4}$	$(2.32 \pm 0.07) \times 10^{-11}$
$\rho^0$ mutant	$10^{-4}$	$(6.02 \pm 0.24) \times 10^{-5}$	$(1.06 \pm 0.02) \times 10^{-3}$	$(2.24 \pm 0.04) \times 10^{-10}$
	$10^{-5}$	$(6.13 \pm 0.12) \times 10^{-5}$	$(4.41 \pm 0.09) \times 10^{-4}$	$(1.34 \pm 0.03) \times 10^{-10}$
	$10^{-6}$	$(2.12 \pm 0.04) \times 10^{-5}$	$(1.32 \pm 0.03) \times 10^{-4}$	$(2.38 \pm 0.05) \times 10^{-11}$

corresponding values for the respiratory-deficient mutant were  $2.7 \pm 0.09 \mu\text{l}$  per  $10^8$  cells and  $1.50 \pm 0.06 \mu\text{l}$  per  $10^8$  cells. (The values represent means  $\pm$  SEM,  $n = 15$ .)

The concentrations of  $\text{CaCl}_2$  in each cellular compartment were determined from the amounts of  $\text{Ca}^{2+}$  in each fraction (calculated from the radioactivity) and the measured cellular and vacuolar volumes (Table 2). In strain N123, cytoplasmic  $\text{Ca}^{2+}$  homeostasis was maintained

Table 3. Effect of medium  $\text{K}^+$  on the concentrations of  $\text{Ca}^{2+}$  and  $\text{K}^+$  in the cytoplasmic and the vacuolar pools

Values represent means $\pm$ SEM ( $n = 15$ ).				
Incubation conditions	Ion measured	Concentration in cytoplasmic pool (M)	Concentration in vacuolar pool (M)	Amount of bound or precipitated ions (mol per $10^8$ cells)
A. YPD medium	$\text{Ca}^{2+}$	$(2.08 \pm 0.08) \times 10^{-5}$	$(1.77 \pm 0.05) \times 10^{-3}$	$(2.98 \pm 0.07) \times 10^{-10}$
	$\text{K}^+$	$(105.3 \pm 5.2) \times 10^{-3}$	$(84.6 \pm 2.5) \times 10^{-3}$	$(22.4 \pm 0.5) \times 10^{-9}$
B. 3 h incubation in: glucose (80 mM) + MES/Tris pH 6.0 (10 mM)	$\text{Ca}^{2+}$	$(1.93 \pm 0.09) \times 10^{-5}$	$(1.38 \pm 0.06) \times 10^{-3}$	$(2.46 \pm 0.06) \times 10^{-10}$
	$\text{K}^+$	$(74.0 \pm 3.0) \times 10^{-3}$	$(121.1 \pm 6.0) \times 10^{-3}$	$(21.2 \pm 0.48) \times 10^{-9}$
C. As in B, + KCl (0.2 M)	$\text{Ca}^{2+}$	$(1.86 \pm 0.11) \times 10^{-5}$	$(0.52 \pm 0.02) \times 10^{-3}$	$(1.03 \pm 0.12) \times 10^{-10}$
	$\text{K}^+$	$(102.3 \pm 6.1) \times 10^{-3}$	$(133.4 \pm 7.9) \times 10^{-3}$	$(20.3 \pm 0.61) \times 10^{-9}$

at between  $5.8 \times 10^{-6}$  M and  $2.3 \times 10^{-5}$  M  $\text{Ca}^{2+}$ , while the concentrations of  $\text{Ca}^{2+}$  in the medium were altered 1000-fold, between  $10^{-3}$  M and  $10^{-6}$  M. In the respiratory-deficient mutant  $\rho^0$  the concentration of cytoplasmic  $\text{Ca}^{2+}$  was somewhat higher, between  $6.0 \times 10^{-5}$  M and  $2.1 \times 10^{-5}$  M when the medium  $\text{Ca}^{2+}$  was altered between  $10^{-4}$  M and  $10^{-6}$  M. The vacuoles, on the other hand, contained large quantities of  $\text{Ca}^{2+}$ , and their concentrations of  $\text{Ca}^{2+}$  were always higher than the concentration of  $\text{Ca}^{2+}$  in the medium. The results were similar for the wild-type and the respiratory-deficient mutant, but the concentrations of vacuolar and bound  $\text{Ca}^{2+}$  were somewhat lower in the mutant than in the wild-type.

Table 3 shows the changes in the concentrations of  $\text{Ca}^{2+}$  and  $\text{K}^+$  in each cellular compartment following a 3 h incubation in media containing MES/Tris pH 6.0 (10 mM) and glucose (80 mM), with or without KCl. In the absence of  $\text{K}^+$  in the medium, there was a small efflux of  $\text{Ca}^{2+}$  (not shown) with 7% decrease in the concentration of  $\text{Ca}^{2+}$  in the cytoplasm, a 22% decrease in the concentration of  $\text{Ca}^{2+}$  in the vacuoles and a 17% decrease in the concentration of bound  $\text{Ca}^{2+}$ . Under the same conditions  $\text{K}^+$  transferred from the cytoplasm to the vacuoles, but  $\text{K}^+$  efflux was not observed. In the presence of KCl (0.2 M) in the medium, a substantial  $\text{Ca}^{2+}$  efflux was observed as was reported in our previous studies (Eilam, 1982*b, c*). Vacuolar  $\text{Ca}^{2+}$  concentration decreased by 71%, the bound and precipitated  $\text{Ca}^{2+}$  decreased by 65% while the cytoplasmic  $\text{Ca}^{2+}$  concentration decreased comparatively little (11%). The decrease in the vacuolar  $\text{Ca}^{2+}$  was accompanied by a 58% increase in the concentration of  $\text{K}^+$  in the vacuole with no change in the concentration of  $\text{K}^+$  in the cytoplasm. These results indicate that the cytoplasmic  $\text{Ca}^{2+}$  homeostasis is maintained even under conditions when substantial  $\text{Ca}^{2+}$  efflux is induced.

The ability of the vacuolar calcium transport system to transport  $\text{Ca}^{2+}$  in cells in which the plasma membrane was permeabilized by DEAE-dextran is shown in Fig. 2. In the presence of isotonic medium containing 100 mM-KCl, 1 mM- $\text{MgCl}_2$  and 80 mM-glucose the uptake of calcium into the vacuoles proceeded linearly until the end of the experiment at 30 min. The rate of uptake was higher in the absence of  $\text{MgCl}_2$ , but decreased considerably when  $\text{K}^+$  was omitted from the medium. Glucose was not necessary during the time of the experiment when fresh preparations were used. Maintaining the broken cells for 2 h in buffered sorbitol caused a reduction in the ability to take up  $\text{Ca}^{2+}$  into the vacuoles but this ability was partially restored by the addition of ATP to the medium. Diethylstilboestrol, an inhibitor of plasma membrane  $\text{H}^+$ -ATPase, completely inhibited calcium uptake into the vacuole in both the presence and the absence of ATP.

#### DISCUSSION

The present work demonstrates that most of cellular calcium in *S. cerevisiae* is bound, precipitated or sequestered within an intracellular compartment that is stable to isotonic DEAE-dextran treatment. This compartment cannot be the mitochondria, as the intracellular distribution of  $\text{Ca}^{2+}$  of the respiratory-deficient mutant  $\rho^0$  is similar to that of the wild-type. Microscopic observations using the fluorescent dye berberine sulphate, and the finding that

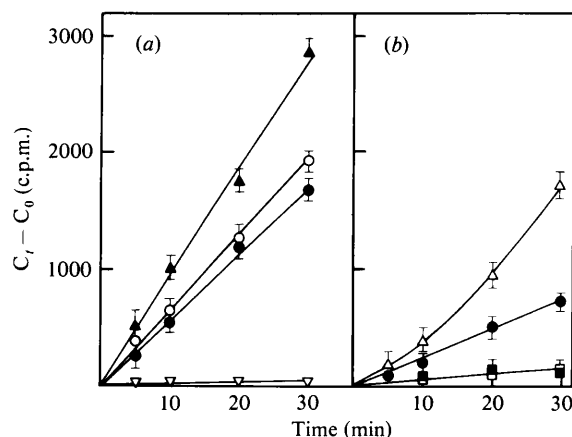


Fig. 2.  $\text{Ca}^{2+}$  uptake into the vacuoles of permeabilized cells. Cells were permeabilized by incubation with DEAE-dextran for 30 s at  $0^\circ\text{C}$  followed by the addition of dextran sulphate (see Methods). The permeabilized cells were separated by centrifugation and suspended in media containing sorbitol (0.7 M) and MES/Tris (10 mM, pH 6.0). Experiments were done on permeabilized cells either (a) immediately after preparation or (b) after incubation in sorbitol/MES/Tris solution for 2 h at  $30^\circ\text{C}$ .

The uptake medium consisted of sorbitol (0.7 M), MES/Tris pH 6.0 (10 mM) and  $\text{CaCl}_2$  ( $1\ \mu\text{M}$ ,  $0.5\ \mu\text{Ci ml}^{-1}$ ) plus the following: ●, KCl (100 mM),  $\text{MgCl}_2$  (1 mM) and glucose (80 mM); ○, KCl (100 mM) and  $\text{MgCl}_2$  (1 mM); ▲, glucose (80 mM) and KCl (100 mM); ▽, no additions; △, KCl (100 mM),  $\text{MgCl}_2$  (1 mM) and ATP (1 mM); ■, KCl (100 mM),  $\text{MgCl}_2$  (1 mM) and diethylstilboestrol ( $100\ \mu\text{M}$ ); or □, KCl (100 mM),  $\text{MgCl}_2$  (1 mM), diethylstilboestrol ( $100\ \mu\text{M}$ ) and ATP (1 mM).

At the indicated times cells were separated by filtration and washed four times with buffered sorbitol, and the radioactivity on the filters was determined.  $C_0$  is the radioactivity (c.p.m.) associated with the cells after 1 min of incubation,  $C_t$  is the radioactivity (c.p.m.) associated with the cells after  $t + 1$  min of incubation. The values of  $C_0$  were subtracted from the values of  $C_t$  in order to correct for binding of  $^{45}\text{Ca}^{2+}$  to the cell walls, which was different in cells incubated in different media ( $\text{Mg}^{2+}$ , and to a lesser extent  $\text{K}^+$ , reduced the binding). The bars represent SEM ( $n = 10$ ).

93% of the cellular arginine is concentrated in the DEAE-dextran stable fraction, indicate that the vacuoles constitute this compartment.

The distributions of amino acids,  $\text{K}^+$ ,  $\text{Na}^+$  and ATP between the cytosol and the vacuoles have been studied in *Candida utilis* (Huber-Wälchli & Wiemken, 1979) and the distributions of  $\text{Mn}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{K}^+$  have been studied in *S. cerevisiae* using the DEAE-dextran method (Nieuwenhuis *et al.*, 1981). Okorokov *et al.* (1977, 1980), using isotonic cytochrome *c* to differentiate between the cytosolic and vacuolar pools in *Saccharomyces carlsbergensis*, found that over 75% of the accumulated  $\text{Mn}^{2+}$ , and high concentrations of  $\text{Mg}^{2+}$ ,  $\text{K}^+$  and orthophosphate, were sited in the vacuoles. The intracellular distribution of  $\text{Ca}^{2+}$  in yeast had not been previously determined. Suggestions that calcium might be sequestered within the vacuoles were based on the following results. (1) Analysis of the kinetics of  $\text{Ca}^{2+}$  efflux revealed two intracellular exchangeable  $\text{Ca}^{2+}$  pools which turned over at different rates (Eilam, 1982a, b). The respiratory-deficient mutant  $\rho^0$  displayed similar kinetics to the wild-type (Y. Eilam, unpublished data), indicating that the mitochondria were not involved in  $\text{Ca}^{2+}$  exchange. (2) Ohsumi & Anraku (1983) demonstrated the presence of a  $\text{Ca}^{2+}$  uptake system in the vacuolar membrane vesicles of *S. cerevisiae*. This  $\text{Ca}^{2+}$  uptake is driven by  $\Delta\mu_{\text{H}^+}$  (acid inside) which is formed by a specific  $\text{H}^+$ -ATPase situated in the vacuolar membrane.

In the present work it is shown that cells permeabilized by DEAE-dextran still maintain the ability to take up  $\text{Ca}^{2+}$  when suspended in isotonic medium which also contains  $\text{K}^+$ . When  $\text{Ca}^{2+}$  uptake was measured in permeabilized cells immediately after preparation,  $\text{Ca}^{2+}$  uptake proceeded without the addition of  $\text{Mg}^{2+}$  or ATP, probably because  $\Delta\mu_{\text{H}^+}$  across the vacuolar membrane was still maintained. Incubation of the permeabilized cells for 2 h in buffered sorbitol led to a marked decrease in  $\text{Ca}^{2+}$  uptake, but addition of ATP and  $\text{Mg}^{2+}$  restored the uptake ability. This uptake could be inhibited by diethylstilboestrol, an inhibitor of plasma membrane ATPase (Serrano, 1980) and probably also of vacuolar membrane  $\text{H}^+$ -ATPase

(Kakinuma *et al.*, 1981). Our results are therefore in full agreement with the results of Ohsumi & Anraku (1983) on vacuolar membrane vesicles.

The main findings of the present work are the maintenance of cytoplasmic  $\text{Ca}^{2+}$  homeostasis at low concentrations of  $\text{Ca}^{2+}$  and the accumulation of  $\text{Ca}^{2+}$  within the vacuoles. It is clear from the results that the vacuoles function as a buffering system for cytoplasmic  $\text{Ca}^{2+}$  and as the major sequestering organelle for  $\text{Ca}^{2+}$ .

In higher eukaryotic non-excitable cells the mitochondria function as the main  $\text{Ca}^{2+}$  sink and as a regulator of cytoplasmic  $\text{Ca}^{2+}$  concentration (Nicholls & Akerman, 1982). It is interesting that in yeast a similar function is performed by the vacuoles at the expense of metabolic energy in the form of  $\Delta\bar{\mu}_{\text{H}^+}$ . Whether cytosolic free  $\text{Ca}^{2+}$  concentration functions as a messenger for cellular processes in yeasts is not yet known; although calmodulin has recently been detected in yeasts (Hubbard *et al.*, 1982) the functions that it regulates have not yet been defined. The present results, demonstrating cytosolic  $\text{Ca}^{2+}$  homeostasis in yeast, indicate that regulatory mechanisms based on  $\text{Ca}^{2+}$  concentration are possible in yeasts.

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