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Changes Induced in the Permeability Barrier of the Yeast Plasma Membrane by Cupric Ion

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A specific effect of Cu^{2+} eliciting selective changes in the permeability of intact *Saccharomyces cerevisiae* cells is described. When 100 μM $CuCl_2$ was added to a cell suspension in a buffer of low ionic strength, the permeability barrier of the plasma membranes of the cells was lost within 2 min at 25°C. The release of amino acids was partial, and the composition of the amino acids released was different from that of those retained in the cells. Mostly glutamate was released, but arginine was mainly retained in the cells. Cellular K^+ was released rapidly after $CuCl_2$ addition, but 30% of the total K^+ was retained in the cells. These and other observations suggested that Cu^{2+} caused selective lesions of the permeability barrier of the plasma membrane but did not affect the permeability of the vacuolar membrane. These selective changes were not induced by the other divalent cations tested. A novel and simple method for differential extraction of vacuolar and cytosolic amino acid pools by Cu^{2+} treatment was established. When Ca^{2+} was added to Cu^{2+}-treated cells, a large amount of Ca^{2+} was sequestered into vacuoles, with formation of an inclusion of a Ca^{2+}-polyphosphate complex in the vacuoles. Cu^{2+}-treated cells also showed enhanced uptake of basic amino acids and *S*-adenosylmethionine. The transport of these substrates showed saturable kinetics with low affinities, reflecting the vacuolar transport process in situ. With Cu^{2+} treatment, selective leakage of K^+ from the cytosolic compartment appears to create a large concentration gradient of K^+ across the vacuolar membrane and generates an inside-negative membrane potential, which may provide a driving force of uptake of positively charged substances into vacuoles. Cu^{2+} treatment provides a useful in situ method for investigating the mechanisms of differential solute pool formation and specific transport phenomena across the vacuolar membrane.

Some heavy metal cations, especially Hg^{2+} and Cu^{2+}, are known to be toxic to the cell. Passow and Rothstein (13) reported that in yeast cells, Hg^{2+} causes loss of cellular K^+ with an all-or-none response and that Cu^{2+} induces partial leakage of K^+ from the cells. Of interest is the effect of Cu^{2+}, which is toxic at high concentrations but is essential at lower concentrations for various oxidase reactions and is accumulated by a specific, energy-dependent transport system (15). The primary sites for the toxic effects of Hg^{2+} and Cu^{2+} seem to be on the membranes, but the specific targets of these two most toxic ions have not yet been elucidated biochemically.

In this paper, we report evidence that Cu^{2+} causes a selective change in the permeability barriers of membranes of yeast cells. At low concentrations, Cu^{2+} causes a loss of the barrier function for ions and metabolites of the plasma membrane but not of the vacuolar membrane.

Vacuoles are important in maintaining the ionic and osmotic environments of the cytoplasm in fungi and plant cells. In vitro studies using purified vacuolar membrane vesicles revealed that the vacuolar membrane has an H^+-translocating ATPase (7, 19) and facilitates active transport of Ca^{2+} (11) and amino acids by a mechanism of substrate/nH^+ antiport (10, 16, 17). By virtue of the selective toxic effect of Cu^{2+} on the plasma membrane, in situ vacuolar functions can be examined in Cu^{2+}-treated cells.

This paper describes the nature of changes induced by Cu^{2+} in the permeability barrier of the yeast plasma membrane and the properties of the in situ vacuolar functions that could be elicited in selectively permeabilized cells by appropriate treatment with Cu^{2+}.

MATERIALS AND METHODS

Strains and culture conditions. The haploid strain of *Saccharomyces cerevisiae*, X2180-1A, from the Yeast Genetic Stock Center, Berkeley, Calif., mainly was used. Various regulatory mutants of phosphatases [YAT647 *pho81*(Con), YAT655 *pho80*, W764-C *pho85*] were generous gifts from A. Toh-e, Hiroshima University, Japan. Cells were grown in YEPD medium containing 1% yeast extract (Difco Laboratories, Detroit, Mich.), 2% polypeptone (Daigo-eiyo, Saitama, Japan), and 2% glucose or YNBD medium containing 0.67% yeast nitrogen base (Difco) and 2% glucose (with amino acids when indicated) at 30°C with shaking.

Chemicals. ^{14}C-labeled compounds and $^{45}CaCl_2$ were purchased from Amersham Corp., Arlington Heights, Ill. Their specific activities (mCi/mmol) were as follows: [U-^{14}C]arginine, 336; [U-^{14}C]lysine, 330; [U-^{14}C]glutamate, 285; *S*-adenosyl-L-carboxy-[^{14}C]methionine, 59.5; tetra[U-^{14}C]phenylphosphonium bromide, 31.4; and $^{45}CaCl_2$, 608. Gramicidin S, cycloheximide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), poly-L-lysine, and cytochrome *c* were obtained from Sigma Chemical Co., St. Louis, Mo. DEAE-dextran was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. The other chemicals used were commercial products of analytical grade.

Standard procedure for differential extractions of cytosolic and vacuolar amino acid pools. Cells (3×10^8) were harvested and washed twice with distilled water. They were then suspended in 1.5 ml of 2.5 mM potassium phosphate buffer

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(pH 6.0) containing 0.6 M sorbitol, 10 mM glucose, and 0.2 mM $CuCl_2$. After incubation for 10 min at 30°C, 1.0 ml of the cell suspension was filtered on a membrane filter (pore size, 0.45 μm; Sartorius) and washed four times with 0.5 ml of the solution described above but without glucose or $CuCl_2$. The filtrates were combined (3.0 ml) and used as the cytosolic extract. The cells retained on the filter were suspended in 3.0 ml of distilled water and boiled for 15 min. The suspension was centrifuged for 3 min at 5,000 rpm, and the supernatant was collected as the vacuolar extract.

Assays of Ca^{2+} and amino acid uptakes by intact cells. Exponentially growing cells were harvested, washed twice with distilled water, and suspended in buffer A (10 mM MES [morpholinoethanesulfonic acid]-Tris [pH 6.4], 2 mM $MgCl_2$, 25 mM KCl) or in distilled water at a cell density of 1.5×10^8/ml. At zero time, 1.0 μCi of $^{45}CaCl_2$ or ^{14}C-labeled amino acid per ml was added and incubation at 30°C was started. Samples (100 μl) were withdrawn and diluted with 3 ml of ice-cold buffer A. The mixture was promptly filtered with a membrane filter. The cells on the filter were washed three times with 5 ml of buffer A, and the radioactivity was measured in a toluene base scintillator in an LS-9000 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Assays of Ca^{2+}, amino acid, and tetraphenylphosphonium ion (TPP^+) uptakes by Cu^{2+}-treated cells. Exponentially growing cells were harvested, washed twice with distilled water, and suspended in buffer B (20 mM MES-Tris [pH 6.0], 0.6 M sorbitol) at a cell density of 1.5×10^8/ml. Cells were treated with 100 μM $CuCl_2$ for 15 min, washed with the same buffer, and resuspended at a cell density of 1.5×10^9/ml in this buffer on ice. Immediately before the assay, the cell suspension was diluted 10-fold with the same buffer and labeled substrate was added. For measurement of Ca^{2+} or amino acid uptake, samples (100 μl) were withdrawn, diluted with 3 ml of ice-cold buffer B, promptly collected on a membrane filter, and washed three times with 5 ml of buffer B. TPP^+ was assayed by the method of de la Peña et al. (2) by using a glass fiber filter (GF/C; Whatman, Inc., Clifton, N.J.).

Other analytical procedures. The potassium content of cells was determined by using a 370 atomic absorption spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.). Amounts of amino acids were measured by a ninhydrin method (23). The composition of amino acids in cell extracts was analyzed with a Hitachi 835 amino acid analyzer. The K^+ concentrations of sample solutions were recorded with a K^+ ion electrode F2312K (Radiometer, Copenhagen, Denmark). Protein content was determined by the method of Lowry et al. (8), with bovine serum albumin as a standard.

RESULTS

Morphological changes induced by Cu^{2+} treatment. *S. cerevisiae* X2180-1A cells suspended in buffer A were incubated at 25°C with 30 μM $CuCl_2$. Within 10 min, distinct morphological changes of almost all the cells were detectable by light microscopy. The cytosol of each cell became refractive, and the central vacuole in each cell became swollen and more clearly visible. The cells then began to shrink. The packed cell volume, determined in a hematocrit tube, decreased during incubation for 30 min to 77% of that of untreated cells.

Consistent with these observations, the turbidity of the cell suspension increased with Cu^{2+} treatment. When the cell suspension in distilled water was incubated with 30 μM $CuCl_2$, the optical density increased sigmoidally after a lag of 1 min and reached a plateau in 3 min. The rate and extent of increase in the optical density depended on the $CuCl_2$ concentration, and the plateau level of increase observed with more than 30 μM $CuCl_2$ was 25% more than the initial value. When a cell suspension in buffer A was treated similarly, the same extent of turbidity increase was observed, but the lag time was longer and about three times more $CuCl_2$ (100 μM) was required (data not shown). In general, cells suspended in distilled water were more sensitive to Cu^{2+} than cells suspended in buffer A and showed about 20% of the turbidity change even with 1 μM $CuCl_2$.

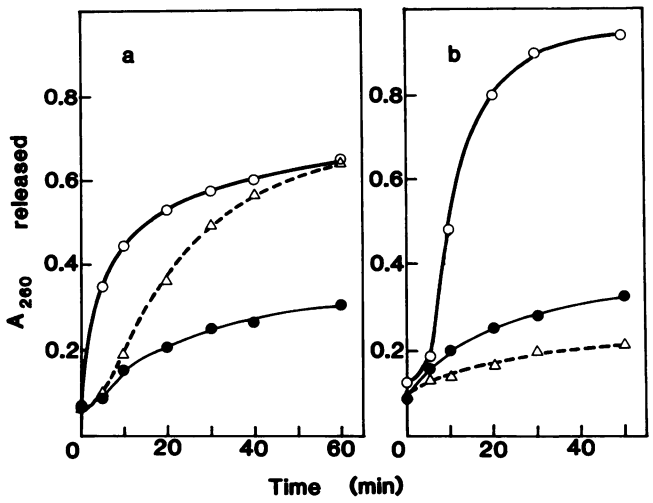

FIG. 1. Release of nucleotides induced by Cu^{2+} treatment. (a) Washed cells were suspended in distilled water at 1.5×10^8 cells per ml. To samples of cell suspension, 100 μM $CuCl_2$ (○) or 0.1 mg of DEAE-dextran per ml (△) was added. At the indicated times, 2-ml samples were withdrawn and filtered on a membrane filter and the A_{260} of the filtrates was measured. (b) Cells were suspended in buffer A instead of distilled water and treated with 100 μM $CuCl_2$ or 0.1 mg of DEAE-dextran per ml. Symbol: ●, control.

Among the other divalent cations tested, only Hg^{2+} elicited slow and gradual turbidity changes. Zn^{2+} had no effect. $LaCl_3$ was a potent inhibitor of the effect of Cu^{2+} on cell shape and turbidity (data not shown).

Release of ions and metabolites from cells. Cu^{2+} treatment appeared to cause loss of the barrier function of the plasma membrane, because it resulted in significant leakage of low-molecular-weight substances from the cells. DEAE-dextran is known to permeabilize the yeast plasma membrane (6), but with wild-type cells, treatment with 0.1 mg of DEAE-dextran per ml caused only slow leakage of nucleotides and required longer incubation than with 100 μM $CuCl_2$. Moreover, DEAE-dextran had no effect on cells in buffer A (Fig. 1b). For unknown reasons, total A_{260} release from the cells on treatment with 100 μM $CuCl_2$ was 1.8 times higher in buffer A than in distilled water (Fig. 1a).

Upon incubation with 100 μM $CuCl_2$, the time course of leakage of amino acids from the cells was similar to that of nucleotides (Fig. 1). However, leakage of amino acids was never complete, and 50 to 70% of the amino acids always remained in Cu^{2+}-treated cells. Furthermore, the compositions of amino acids in the supernatant and that in the cells were entirely different: arginine mainly remained in the cells, while glutamate was almost completely released from the cells. Since arginine is a vacuolar constituent while gluta-

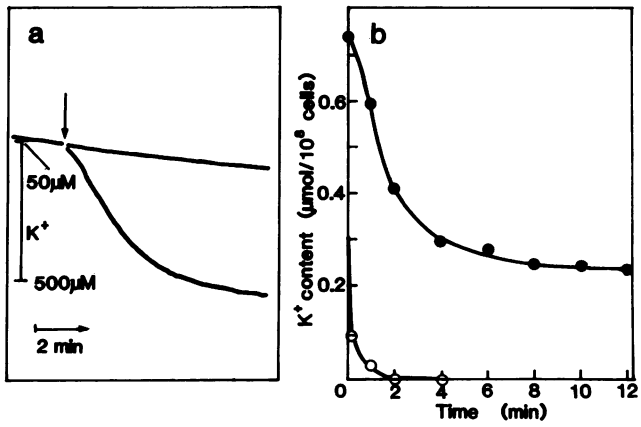

FIG. 2. Release of K^+ induced by Cu^{2+} treatment. (a) K^+ release from cells was monitored with a K^+ selective electrode at 25°C. Cells suspended in distilled water at 1.5×10^8/ml were treated with 100 μM $ZnCl_2$ or 100 μM $CuCl_2$. Arrow, Time of $CuCl_2$ addition. (b) Extent of K^+ release by Cu^{2+} or gramicidin S treatment. Cells suspended in 10 mM MES-Tris (pH 6.0)–0.6 M sorbitol (buffer B) at a density of 1.5×10^8 cells per ml were treated with a final concentration of 100 μM $CuCl_2$ (●) or 10 μg of gramicidin S (○) per ml at 30°C. At the indicated times, 1-ml samples were withdrawn and immediately filtered on a membrane filter. The filter was washed twice with 3 ml of buffer B, and then the cells on the filter were suspended in 3 ml of distilled water. The cell suspensions were diluted appropriately with distilled water, and their K^+ contents were measured in an atomic absorption spectrophotometer.

mate is present only in the cytoplasm (21, 22), these findings strongly suggested that Cu^{2+} treatment did not affect the barrier function of the vacuoles.

The leakage of K^+ from Cu^{2+}-treated cells was measured directly with a K^+-specific electrode (Fig. 2a). K^+ was released immediately after the addition of 100 μM $CuCl_2$, and it was the earliest detectable event, but again, K^+ release was not complete. $ZnCl_2$ (100 μM) had no effect on K^+ release. Gramicidin S (10 μg/ml) caused prompt and complete loss of cellular K^+, but about 30% of the total K^+ remained in the cells after treatment with 100 μM $CuCl_2$ (Fig. 2b). No protein or nucleic acids were detected in the supernatant after incubation with 100 μM $CuCl_2$ even after 30 min.

All these results indicated that Cu^{2+} causes loss of the barrier function of the plasma membrane but not of the intracellular organelle membranes or, at least, the vacuolar membrane.

Mode of action of Cu^{2+} on yeast cells. The morphological and turbidity changes were associated with leakages of ions and metabolites and were induced specifically by Cu^{2+}. Other divalent cations tested, such as Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, and Sn^{2+} had no effects, although Hg^{2+} caused a slow increase of turbidity with no leakage of amino acids or nucleotides. The presence of an isotonic concentration of sorbitol or mannitol did not influence either reaction much, but the presence of monovalent cations, especially K^+, slowed down the whole process. The turbidity increase and nucleotide release showed similar pH dependence: the effects of Cu^{2+} were most rapid and had the shortest lag period at pH 6.0, and neither reaction proceeded at low temperatures (0 to 4°C). In the presence of 250 mM KCl, no turbidity change was observed even with 1 mM $CuCl_2$. The change in turbidity was also not observed with the presence of the protonophore uncouplers carbonyl cyanide *m*-chlorophenylhydrazone (0.1 mM) and 2,4-dinitrophenol (0.2 mM). These results suggested that the specific lesion induced by Cu^{2+} requires an energized state of target cells or a membrane potential imposed across the plasma membrane.

$LaCl_3$ (10 μM) protected the cells from the effect of Cu^{2+} (data not shown), and when $LaCl_3$ was added during the turbidity change, it promptly blocked further increase in turbidity. These facts suggested that for induction of turbidity change, Cu^{2+} must be taken up by cells via an energy-dependent transport system which is probably sensitive to $LaCl_3$ and that loss of the barrier function of the plasma membrane occurs when the concentration of Cu^{2+} within the cells reaches some critical value. The prompt cessation of turbidity increase on addition of $LaCl_3$ suggested that this change took place in each single cell instantaneously and in an all-or-none manner.

Differential determination of the vacuolar and cytosolic amino acid pools. All the results described above strongly suggest that Cu^{2+} treatment provides a novel method for determination of cytosolic and vacuolar constituents. For selective extractions of the two pools, it was essential to determine the conditions in which the plasma membrane was permeabilized completely while the vacuolar membrane remained intact. By changing various parameters, standard conditions were established. For this purpose, we used mainly cells grown in YNBD medium supplemented with 10 mM arginine; arginine and glutamate were used as markers of vacuolar and cytosolic amino acids, respectively. The parameters were as follows. (i) Temperature. The turbidity change and release of amino acids did not proceed at low temperatures (0 to 4°C), so the temperature was set at 30°C. (ii) pH and buffer system. The action of Cu^{2+} on yeast cells was pH dependent; the stability of the vacuolar membrane also depended on the pH value. The effects of Cu^{2+} treatment did not depend on the buffer system. Besides, several buffers which chelate Cu^{2+} were of no use. Since MES-Tris buffer disturbs the ninhydrin reaction of amino acid analysis, potassium phosphate was chosen. The rate of amino acid liberation from the cytosolic pool was fastest at pH 6.0 and reached a plateau after incubation for 10 min at 30°C. Under these conditions, as much as 90% of the total glutamate was extracted with less than 6% of the total arginine. At lower or higher pH values, the amounts of glutamate and total amino acids extracted in the cytosolic fraction were reduced. With 10 or 2.5 mM potassium phosphate (pH 6.0), cytosolic pools of almost the same composition were obtained, but a longer incubation period was required with the former. In distilled water alone, the cytosolic extract contained a significant amount of arginine, probably due to some lesion of the vacuolar membranes. The presence of 0.6 M sorbitol stabilized the steady level of the extraction. Therefore, we chose 2.5 mM potassium phosphate buffer (pH 6.0)–0.6 M sorbitol as the standard extraction medium. (iii) Time course of extraction. Figure 3 shows the time course of extractions of the cytosolic and vacuolar pools with the standard extraction medium containing 0.2 mM $CuCl_2$. When cells were grown in YNBD medium supplemented with 10 mM arginine, the vacuolar amino acid pool, including arginine, was greatly increased. About 30% of the total amino acids were recovered as a cytosolic pool fraction after 10 min of incubation (Fig. 3). Differential extraction of both amino acid pools was achieved, as judged from the time courses of extraction of glutamate, aspartate, and arginine. (iv) Effect of Cu^{2+} concentration. Under the standard conditions with extraction for 10 min at 30°C, 0.2 mM $CuCl_2$ was sufficient for separation of the two pools of cells grown in YNBD medium, while

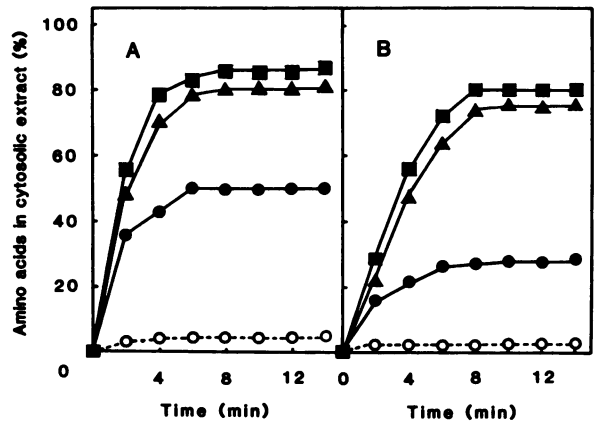

FIG. 3. Time courses of release of amino acids with Cu^{2+} treatment. X2180-1A cells grown in YEPD medium (A) or YNBD medium supplemented with 10 mM arginine (B) were treated with 0.2 mM $CuCl_2$ for the indicated times, and released amino acids were analyzed with an amino acid analyzer. Releases of total amino acids (●), aspartate (■), glutamate (▲), and arginine (○) are shown as percentages of those in the total cellular pool.

0.1 mM $CuCl_2$ was sufficient when cells were grown in YEPD medium (Fig. 4). (v) Effect of glucose. Cells in the stationary phase of growth seemed to acquire resistance to Cu^{2+} treatment, and the addition of 10 mM glucose was effective for quantitative separation of the pools. No change in extraction efficiency or amino acid distribution in the two pools of cells in the logarithmic phase was observed with the addition of 10 mM glucose. Therefore, 10 mM glucose was routinely included in the standard extraction medium to assure wide applicability of the methods.

Under the standard conditions described in Materials and Methods, Cu^{2+} treatment caused selective loss of the permeability barrier of the plasma membrane of almost all the cells (>98%), as judged by neutral red staining of the cells.

Ca^{2+} accumulation induced by Cu^{2+} treatment. The vacuolar membrane has a specific Ca^{2+}/H^+ antiport system, and

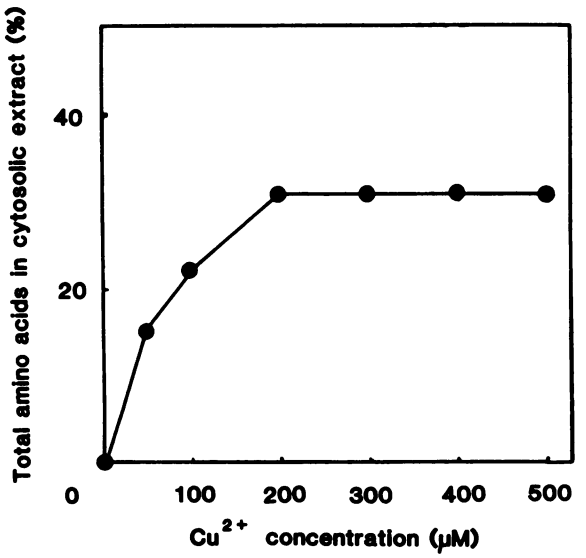

FIG. 4. Effect of Cu^{2+} concentration on differential extraction. X2180-1A cells grown in YNBD medium supplemented with 10 mM arginine were treated with $CuCl_2$ at the indicated concentrations for 10 min.

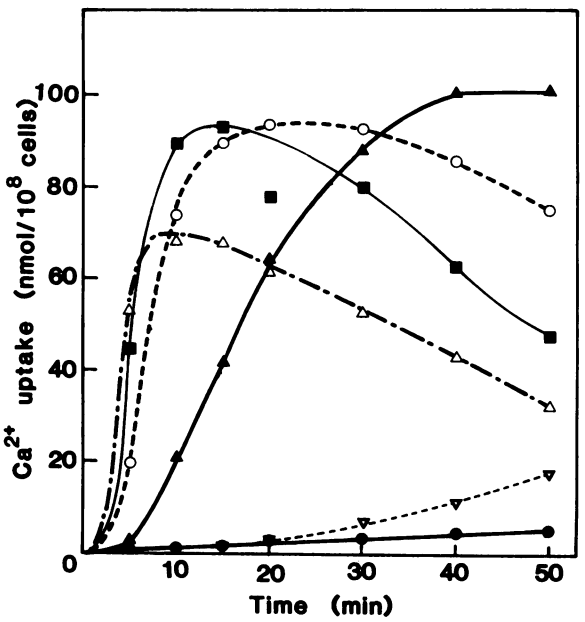

FIG. 5. Ca^{2+} uptake induced by Cu^{2+} treatment. Cells (1.0 ml, 1.5×10^8/ml in 10 mM MES-Tris [pH 6.4]) were treated with various concentrations of $CuCl_2$ or 100 μM $HgCl_2$ in the presence of 1 μCi of $^{45}CaCl_2$ (20 mCi/mmol). Uptake of Ca^{2+} was measured as described in Materials and Methods. The final concentration of $CuCl_2$ added was 3 (●), 10 (▲), 30 (○), 100 (■), or 300 (△) μM. The final concentration of $HgCl_2$ added was 100 μM (▽).

vacuoles may function as a regulatory pool for Ca^{2+} in yeast cells (11). We examined the effect of Cu^{2+} treatment on induction of Ca^{2+} uptake. Large amounts of Ca^{2+} were sequestered in the cells, with the amount depending on the amount of Cu^{2+} added. With 100 μM $CuCl_2$, Ca^{2+} sequestration started after a short lag period but was followed by slow leakage of sequestered Ca^{2+} from the cells during further incubation (Fig. 5). Light microscopic examination showed that cells treated with 30 μM $CuCl_2$ had one swollen central vacuole per cell, while cells treated with 30 μM $CuCl_2$ in the presence of 0.5 mM $CaCl_2$ had an inclusion within the central vacuole (data not shown). This inclusion may have been a Ca^{2+} polyphosphate complex for the following reasons. (i) Polyphosphates are known to be localized exclusively in the vacuoles of yeast cells (20). (ii) Structures exactly the same as Ca^{2+} inclusions were formed in vitro by mixing polyphosphates (average chain length, >15) and $CaCl_2$ solution. (iii) Much larger Ca^{2+} inclusions were seen in the vacuoles of mutant strains (*pho80*, *pho81*, and *pho85* strains) that contained larger amounts of polyphosphates. (iv) After phosphate starvation, the formation of Ca^{2+} inclusions in these cells became negligible (data not shown).

Effect of Cu^{2+} on amino acid uptake. We next examined the effect of Cu^{2+} on vacuolar amino acid uptake activities in Cu^{2+}-treated cells. The uptake of arginine increased greatly 2 min after the addition of $CuCl_2$ (Fig. 6). In contrast, uptake of glutamate stopped and accumulated glutamate leaked out completely in 10 min. These results were interpreted to mean that the arginine taken up by intact cells becomes located mainly in the vacuoles while the glutamate taken up stays in the cytosol and that, therefore, the addition of Cu^{2+} selectively impairs the barrier function of the plasma membrane but not that of the vacuolar membrane.

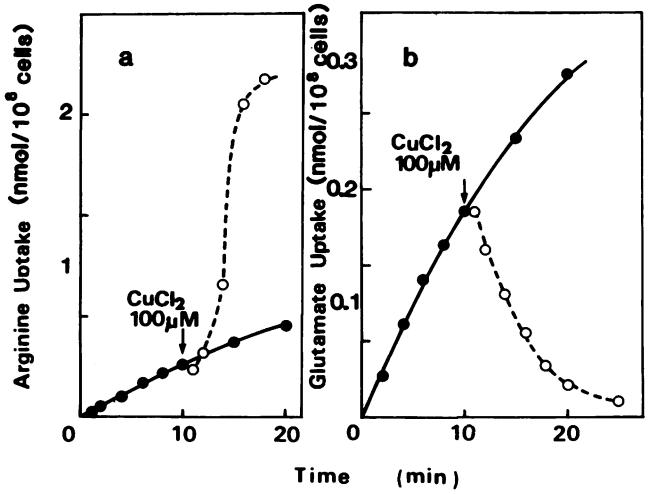

FIG. 6. Effect of Cu^{2+} on arginine and glutamate uptake by intact cells. Cells suspended in buffer A supplemented with 10 μg of cycloheximide per ml were mixed with 1.0 μCi of [^{14}C]arginine (10 mCi/mmol) (a) or [^{14}C]glutamate (10 mCi/mmol) (b) per ml. Samples were withdrawn and filtered. At 10 min, 0.7 ml of each reaction mixture was transferred to a tube and 100 μM $CuCl_2$ was added. Symbols: ●, control; ○, $CuCl_2$, added.

The Ca^{2+} transport activity of cells pretreated with 100 μM $CuCl_2$ was enhanced even after the cells were washed, although their steady level of uptake was about half that by cells treated with Cu^{2+} in the presence of Ca^{2+} (data not shown). The uptake activities of arginine, lysine, histidine, and *S*-adenosylmethionine (SAM) were also enhanced after Cu^{2+} treatment, especially when assayed with high concentrations of substrates (data not shown). These uptake reactions were temperature dependent, and no accumulation was observed at 0°C. Furthermore, these reactions by Cu^{2+}-treated cells showed saturable kinetics: the K_t values for Ca^{2+}, arginine, and SAM were determined to be 0.1, 0.7, and 0.5 mM, respectively. These values were quite different from the K_t values of transport by intact cells, which were 10 μM for arginine (5) and 3.3 μM for SAM (9) but similar to those of the amino acid/H^+ antiport systems of the vacuolar membrane, which were 0.1 mM for Ca^{2+} and 0.6 mM for arginine (16). The uptake of Ca^{2+} and arginine by Cu^{2+}-treated cells was insensitive to the protonophore uncouplers SF6847, CCCP, and 2,4-dinitrophenol. Valinomycin slightly stimulated Ca^{2+} uptake.

Mechanisms of uptake of Ca^{2+} and basic amino acids induced by Cu^{2+} treatment. After Cu^{2+} treatment, cytosolic K^+ was rapidly released but vacuolar K^+ remained rather stable, possibly resulting in the formation of a large concentration gradient of K^+ across the vacuolar membrane and causing the formation of a diffusion potential of K^+ (inside negative). To investigate this possibility, we examined the uptake of the membrane permeable cation TPP^+ by Cu^{2+}-treated cells. TPP^+ uptake was greatly enhanced by Cu^{2+} treatment, and the enhanced uptake activity was reduced in the presence of KCl (data not shown). This large membrane potential indicated by the accumulation ratio (170 mV; inside negative) may be part of the driving force of in situ vacuolar transport activities for Ca^{2+}, arginine, lysine, and SAM in Cu^{2+}-treated cells. The pH dependence of the vacuolar activities of histidine and arginine uptake in situ was studied by using 25 mM MES-Tris buffer. Histidine uptake activity was maximal at pH values below 6.0, while arginine uptake activity was high at pH values above 6.6 (data not shown). Efflux of the [^{14}C]arginine accumulated in Cu^{2+}-treated cells was observed when KCl was added to the reaction mixture (Fig. 7a). Moreover, the K^+ content in Cu^{2+}-treated cells decreased when Ca^{2+} or arginine was added to the reaction mixture, but the addition of leucine had no effect on the content of K^+ (Fig. 7b). These results suggested that a K^+

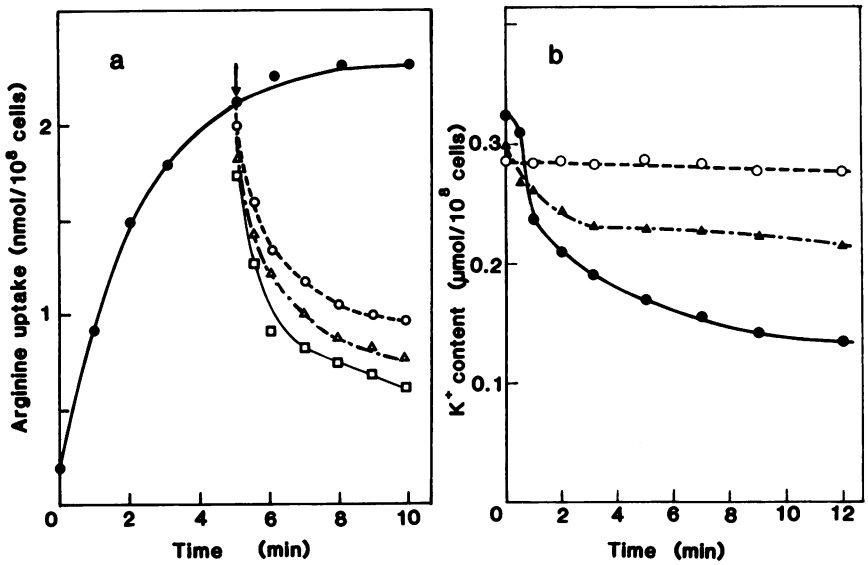

FIG. 7. Efflux of [^{14}C]arginine from Cu^{2+}-treated cells induced by KCl (a) and efflux of K^+ from Cu^{2+}-treated cells induced by $CaCl_2$ or arginine (b). (a) Cu^{2+}-treated cells (1.5×10^8/ml) in buffer B were prepared. [^{14}C]arginine was added, and the uptake reaction was measured. At 6 min, 0.3 M KCl (□), 0.1 M KCl (△), or 0.1 M KCl (○) was introduced and [^{14}C]arginine efflux from the cells was measured. (b) To Cu^{2+}-treated cells resuspended in the original volume of buffer B, 1 mM $CaCl_2$ (●), arginine (▲), or leucine (○) was added. At the indicated times, 1-ml samples were withdrawn and promptly filtered and the filters were washed twice with 3 ml of buffer B. Cells on the filter were resuspended in 3 ml of distilled water, and the K^+ contents of the suspensions were determined by atomic absorption spectroscopy.

channel or a cation-exchange transport system in the vacuolar membrane, whose functions are dependent on and regulated by a membrane potential, may have an essential role in vacuolar pool formation.

DISCUSSION

This paper reports evidence that Cu^{2+} has a specific action on yeast cells. At low concentrations, Cu^{2+} makes the plasma membrane permeable and causes the selective release of ions and low-molecular-weight substances from the cytosolic compartment. This selective event, or Cu^{2+} method, is a simple and reliable method for separating the cytosolic and vacuolar pools of ions and metabolites.

Passow and Rothstein (13) demonstrated that Hg^{2+} caused leakages of K^+ and anions from yeast cells. We examined this effect and found that Hg^{2+} did not induce either rapid turbidity increase or leakage of metabolites. Moreover, Hg^{2+} inhibited the rapid release of nucleotides from Cu^{2+}-treated cells. Gramicidin S induced complete loss of cellular K^+ but with no change in turbidity. Thus, the integrity of the vacuolar membrane during selective modification of the plasma membrane barrier by Cu^{2+} may be important in inducing turbidity change.

We also examined the effects of various cell surface-acting reagents (24) in the specific release of low-molecular-weight substances from the cytosolic compartment and in the modification of Ca^{2+} uptake activity. Cytochrome *c*, DEAE-dextran, and polylysine required restricted conditions to release amino acids from the cytosol and did not work on stationary-phase cells. DEAE-dextran enhanced Ca^{2+} uptake activity but in more restricted conditions. For instance, DEAE-dextran had no effect on the activity in the solution containing salts such as Mg^{2+} or K^+ and had no effect on stationary-phase cells. The effect of nystatin was partial. In contrast with these chemicals, Cu^{2+} appeared to exert a selective effect on the plasma membrane barrier in a buffer containing salts. To determine the cytosolic pool, K^+ in the extraction buffer must minimize a redistribution of amino acids into vacuoles. Therefore, Cu^{2+} treatment provides a reliable and widely applicable method of differential extraction of ion and metabolite pools of yeast cells.

The mechanism of resistance to copper has been studied extensively, and copper metallothionein has been shown to be essential in the detoxification of Cu^{2+} (1, 4). The present work was done mainly with a haploid strain of *S. cerevisiae*, X2180-1A. This strain has a copper resistance marker, *CUP1*. We have done experiments similar to those described in the text, using various unrelated yeast strains, and have found that all these strains showed essentially similar responses to Cu^{2+}. It should be mentioned that X2180-1A cells grown in YEPD medium supplemented with 0.5 mM $CuCl_2$ acquired resistance to the standard Cu^{2+} treatment used in this study. Consequently, we concluded that the *CUP1* gene itself does not play an important role in exhibiting the effect but that overproduction of metallothionein may raise the threshold Cu^{2+} concentration required for lesion of the plasma membrane barrier.

From the following results we concluded that Cu^{2+} caused the selective permeability loss of the yeast plasma membrane. (i) In Cu^{2+}-treated cells, the transport systems for Ca^{2+} and arginine in the vacuolar membrane functioned as normally as standard vacuolar membrane vesicles in vitro (10, 11), whereas the active glutamate transport system in the plasma membrane was inhibited (Fig. 6). (ii) The extent of in situ sequestration of Ca^{2+} and arginine by vacuoles in Cu^{2+}-treated cells depended on and were affected by the concentration of KCl (Fig. 7), suggesting that the K^+ channel in the vacuolar membrane (20a) remained normal and regulated vacuolar transport functions. K^+ is the most abundant cation in most cells and is believed to play an essential role in maintenance of an ionic or osmotic environment in the cytoplasm. By microprobe analysis, Roomans and Seveus (14) demonstrated an even distribution of K^+ across the vacuolar membrane. Okorokov et al. (12) reported a stable pool of 470 mM K^+ in vacuoles, which establishes an eightfold concentration gradient across the vacuolar membrane. In the present paper, we showed that perturbation of the cytosolic K^+ content induced by Cu^{2+} treatment, although this was unphysiological, created a large inside-negative membrane potential across the vacuolar membrane. Schwencke and De Robichon-Szulmajster (18) found that SAM uptake by isolated intact vacuoles did not require any energy supply and was insensitive to azide or 2,4-dinitrophenol. These properties and kinetic parameters obtained for SAM transport in vitro resemble those of SAM transport in Cu^{2+}-treated cells. Their observations can be interpreted by supposing that when the intact vacuoles were suspended in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer containing 0.8 M sorbitol, an inside-negative membrane potential across the vacuolar membrane could easily be formed, as in Cu^{2+}-treated cells, and that the membrane potential imposed via a K^+ channel (20a) may drive active SAM uptake against a concentration gradient. Similar phenomena of Ca^{2+} uptake by DEAE-dextran treated cells were reported by Eilam et al. (3). Cu^{2+}-treated cells provide a simple and useful system for in situ studies on intracellular compartmentation of solutes and the active transport phenomena associated with intact vacuoles.

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LITERATURE CITED

1. **Butt, T. R., E. J. Sternberg, J. A. Gorman, P. Clark, D. Hamer, M. Rosenberg, and S. T. Crooke.** 1984. Copper metallothionein of yeast, structure of the gene, and regulation of expression. Proc. Natl. Acad. Sci. USA **81**:3332–3336.
2. **de la Peña, P., F. Barros, S. Gascon, and S. Lazo.** 1982. The electrochemical proton gradient of *Saccharomyces cerevisiae*: role of potassium. Eur. J. Biochem. **123**:447–453.
3. **Eilam, Y., H. Lavi, and N. Grossowicz.** 1985. Cytoplasmic Ca^{2+} homeostasis maintained by a vacuolar Ca^{2+} transport system in the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. **131**: 623–629.
4. **Fogel, S., and J. M. Welch.** 1982. Tandem gene amplification mediates copper resistance in yeast. Proc. Natl. Acad. Sci. USA **79**:5342–5346.
5. **Grenson, M., M. Mousset, J. M. Wiame, and J. Bechet.** 1966. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **127**:325–338.
6. **Huber-Wälchi, V., and A. Wiemken.** 1979. Differential extraction of soluble pools from the cytosol and the vacuoles of yeast (*Candida utilis*) using DEAE-dextran. Arch. Microbiol. **120**: 141–149.
7. **Kakinuma, Y., Y. Ohsumi, and Y. Anraku.** 1981. Properties of H^+-translocating adenosine triphosphatase in vacuolar membranes of *Saccharomyces cerevisiae*. J. Biol. Chem. **256**:

10859–10863.
8. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
9. **Murphy, J. T., and K. D. Spence.** 1972. Transport of *S*-adenosylmethionine in *Saccharomyces cerevisiae*. J. Bacteriol. **109**:499–504.
10. **Ohsumi, Y., and Y. Anraku.** 1981. Active transport of basic amino acids driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. J. Biol. Chem. **256**:2079–2082.
11. **Ohsumi, Y., and Y. Anraku.** 1983. Calcium transport driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. J. Biol. Chem. **258**:5614–5617.
12. **Okorokov, L. A., L. P. Lichko, and I. S. Kulaev.** 1980. Vacuoles: main compartments of potassium, magnesium, and phosphate ions in *Saccharomyces carlsbergensis*. J. Bacteriol. **144**: 661–665.
13. **Passow, H., and A. Rothstein.** 1960. The binding of mercury by the yeast cell in relation to changes in permeability. J. Gen. Physiol. **43**:621–631.
14. **Roomans, G. M., and L. A. Seveus.** 1976. Subcellular localization of diffusible ions in the yeast *Saccharomyces cerevisiae*: quantitative microprobe analysis of thin freeze-dried sections. J. Cell Sci. **21**:119–127.
15. **Ross, I. S.** 1977. Effect of glucose on copper uptake and toxicity in *Saccharomyces cerevisiae*. Trans. Br. Mycol. Soc. **69**:77–81.
16. **Sato, T., Y. Ohsumi, and Y. Anraku.** 1984. Substrate specificities of active transport systems for amino acids in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. J. Biol. Chem. **259**:11505–11508.
17. **Sato, T., Y. Ohsumi, and Y. Anraku.** 1984. An arginine/histidine exchange transport system in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. J. Biol. Chem. **259**:11509–11511.
18. **Schwencke, J., and H. De Robichon-Szulmajster.** 1976. The transport of *S*-adenosyl-L-methionine in isolated yeast vacuoles and spheroplasts. Eur. J. Biochem. **65**:49–60.
19. **Uchida, E., Y. Ohsumi, and Y. Anraku.** 1985. Purification and properties of H^+-translocating adenosine triphosphatase in vacuolar membranes of *Saccharomyces cerevisiae*. J. Biol. Chem. **260**:1090–1095.
20. **Urech, K., M. Durr, T. Boller, A. Wiemken, and J. Schwencke.** 1978. Localization of polyphosphate in vacuoles of *Saccharomyces cerevisiae*. Arch. Microbiol. **116**:275–278.
20a. **Wada, Y., Y. Ohsumi, M. Tanifuji, M. Kasai, and Y. Anraku.** 1987. Vacuolar ion channel of the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **262**:17260–17263.
21. **Wiemken, A., and M. Dürr.** 1974. Characterization of amino acid pools in the vacuolar compartment of *Saccharomyces cerevisiae*. Arch. Microbiol. **101**:45–57.
22. **Wiemken, A., and P. Nurse.** 1973. Isolation and characterization of the amino-acid pools located within the cytoplasm and vacuoles of *Candida utilis*. Planta **109**:293–306.
23. **Yemm, D. A., and E. C. Cooking.** 1955. The determination of amino-acids with ninhydrin. Analyst **80**:209–213.
24. **Yphantis, D. A., J. L. Dainko, and F. Schlenk.** 1967. Effect of some proteins on the yeast cell membrane. J. Bacteriol. **94**:1509–1515.