Cyclic Accumulation of Zinc by Candida utilis during Growth in Batch Culture

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SUMMARY

Intracellular accumulation of zinc by Candida utilis NRRL-Y-7634 was mediated by an energy- and temperature-dependent, highly specific process exhibiting saturation kinetics. In zinc-supplemented medium, uptake occurred only during the lag and late-exponential phases; this type of transport did not occur with zinc in bacteria nor with iron in either yeast or bacteria. Cells of C. utilis did not possess a zinc-efflux system; they could reduce their level of intracellular zinc only by dilution of the metal into daughter cells. Zinc-deficient organisms accumulated 12 times more zinc than did cells of the same culture age grown in zinc-supplemented medium. The varied, but experimentally reproducible levels of intracellular zinc that occurred in response to the physiological and environmental parameters had no detectable effects on respiration, rate of growth, total cell yield, or cell viability. Neither the mechanism underlying the cyclic accumulation of zinc nor the function of such behaviour are understood.

INTRODUCTION

Numerous functions of zinc at the molecular and cellular levels are now well established (Failla, 1977) and homeostatic mechanisms have evolved to regulate the distribution and metabolism of the metal in multicellular organisms (Kowarski, Blair-Stanek & Schachter, 1974; Richards & Cousins, 1975). This study is concerned with possible homeostatic regulation of zinc accumulation by a unicellular organism. We have previously described the uptake of zinc by Candida utilis as an energy-, temperature- and pH-dependent, highly specific process that requires reactive sulphydryl groups and an intact membrane (Failla, Benedict & Weinberg, 1976). Zinc transport also exhibits saturation kinetics and the cells accumulate the metal against a concentration gradient. Thus, zinc uptake has the characteristics of an active transport system.

In the previous work, cells were isolated from mid-exponential phase cultures and incubated in the absence of glucose to reduce energy pools. However, we also observed that in batch cultures C. utilis does not accumulate zinc simply in proportion to the increase in cell mass, but rather at certain stages of the growth cycle, namely the lag and late-exponential phases. In this paper we report that the ability of the cells to take up the metal is determined not only by the phase of the culture but also by the content of intracellular zinc.

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METHODS

Organisms and growth. The conditions for maintenance and growth of Candida utilis NRRL-Y-7634 have been described (Failla et al., 1976). The organisms were transferred from a stock slant to Sabouraud's dextrose broth (SDB; Difco) and incubated at 30°C on a gyratory shaker at 200 rev. min⁻¹. Cells were subsequently inoculated into a synthetic medium consisting of (mM): glucose, 16·5; NH₄Cl, 18·7; K₂HPO₄, 10·0; MgSO₄·7H₂O, 1·0; sodium citrate, 5·0; biotin, 0·004; and piperazine-N,N'bis-(2-ethanesulphonate) buffer, 5·6. The medium was adjusted to pH 6·6 with 1·2 M-NaOH. The reagent grade salts contributed 0·1 μM-zinc to the medium as determined by atomic absorption spectrophotometry.

Samples from 24 h cultures were diluted into fresh medium to give 1 × 10⁶ colony forming units (c.f.u.) ml⁻¹. Assays were done with cells in the third subculture. Cell concentrations were determined by measuring extinctions with a Klett-Summerson photoelectric colorimeter (filter no. 54; Klett Manufacturing Co., New York, U.S.A.); Klett units were converted to cell numbers and/or cell mass using calibration curves. The number of viable cells was determined at the beginning and end of each experiment using pour plates. The pH, measured with a Corning pH meter, did not alter during growth or on addition of test substances.

Assay of zinc accumulation. Various quantities of ZnCl₂ enriched with carrier-free ⁶⁵Zn (New England Nuclear, Boston, U.S.A.) were added to the growth medium. At intervals, the yeast was collected by centrifuging at 3000 g for 10 min at 5°C, washed twice in distilled, deionized water and resuspended in fresh growth medium to give approx. 10⁶ c.f.u. ml⁻¹ (50 μg dry wt ml⁻¹). Potential metabolic inhibitors were added 4 min later and, after a further 1 min, ZnCl₂ containing ⁶⁵Zn (usually 1·0 μCi) was added. Samples (0·5 ml) were taken at intervals; the cells were collected on Millipore filters (0·45 μm) and washed three times with 3·0 ml ice-cold 0·2 mM-ZnCl₂ at pH 3·5. The filters were air-dried and their radioactivity determined with a Beckman Biogamma counter. In general, the yeast suspensions were shaken at 150 strokes min⁻¹ in a water bath at 30°C.

Assay of iron accumulation. Carrier-free ⁵⁹Fe (New England Nuclear, Boston, U.S.A.) was added to the medium which already contained 0·15 μM-iron as determined by atomic absorption spectrophotometry. The cellular content of ⁵⁹Fe was assayed as for ⁶⁵Zn except that the wash solution contained 0·2 mM-FeCl₃ at pH 3·5.

Phosphate assay. Orthophosphate and polyphosphate were extracted by a modification (Witney, Failla & Weinberg, unpublished) of the method of Weimberg (1975). Inorganic phosphate was determined by the method of Fiske & Subbarow (1925).

Cytochrome spectra. The yeast (500 mg wet wt) was mixed with aqueous 50% (v/v) glycerol to give 3 ml final volume. The suspensions were examined at room temperature in a Cary 14 scanning spectrophotometer equipped with an accessory to compensate for light scattering.

Chemicals. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was purchased from Calbiochem; cycloheximide from Sigma; and nystatin (Mycostatin) from Squibb, New York, U.S.A.

RESULTS

Zinc uptake in batch culture

The intermittent nature of zinc uptake by Candida utilis in batch culture is illustrated in Fig. 1 (a). Cells accumulated the metal during the lag phase (stage I) and again during the late-exponential phase (stage III) but did not take up zinc during either the early-exponential
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Fig. 1. Accumulation of (a) zinc (•) and (b) iron (□) by *C. utilis* in batch cultures. Stationary-phase cells were collected and prepared as described in Methods before transfer to growth medium (25 ml in 125 ml Erlenmeyer flasks) containing 1·0 μM-ZnCl₂. Either ⁶⁵Zn or ⁵⁶Fe (5·0 μCi) was added and the accumulation of metal and cell concentrations (○, □) were measured at intervals.

(stage II) or stationary (stage IV) phases. The following experiments showed that in stages II and IV there is no uptake rather than no net accumulation. The organisms were grown in the presence of various levels of ZnCl₂ with ⁶⁵Zn; they were collected during stages II and IV, washed, and resuspended in growth medium enriched with 100 μM non-radioactive ZnCl₂. The amount of radioactivity associated with the yeast did not decrease.

Similarly, addition of 10 mM-KCN alone or with 100 μM-ZnCl₂ had no effect on the level of retention of previously accumulated zinc. When the uptake of ⁶⁵Zn was inhibited at various times during stages I and III by the addition of either KCN or excess non-radioactive ZnCl₂, exit-exchange reactions did not occur. These results are in agreement with earlier observations (Failla et al., 1976) that this organism lacks an efflux system for zinc.

When stationary phase yeasts from 24 h cultures were diluted 200-fold into low-zinc medium that contained only 0·1 μM-zinc (the background level contributed by zinc contamination of the reagent grade chemicals) plus carrier-free ⁶⁵Zn (less than 10⁻⁴ μM-Zn²⁺), all the radioactivity was taken up during the lag phase. Although the zinc content per cell decreased throughout the growth period, neither the growth rate nor the cell yield differed from that in zinc-supplemented medium. The zinc level of the medium was increased 10- to 500-fold, the accumulation of zinc during the lag phase, compared with that in low-zinc medium, increased only twofold [40 to 45 nmol (mg dry wt)⁻¹]. Regardless of the amount of zinc in the medium, the maximum amount accumulated was only 6 nmol (mg dry wt)⁻¹ after 24 h.

In contrast to the pattern of zinc accumulation, *C. utilis* did not take up iron during the lag period but accumulated it linearly throughout the exponential phase of growth (Fig. 1b).

**Effect of the time of zinc supplementation on zinc uptake**

At various times during the growth cycle the medium was made 10 μM with respect to ZnCl₂ and the quantity of zinc that accumulated was determined as a function of the increase in the number of cells. When Zn²⁺ was added after cell multiplication had begun.
Fig. 2. Effect of time of addition of ZnCl₂ on zinc uptake by C. utilis in batch culture. (a) Growth curve. (b) At 0 h (○), 3 h (■), 6 h (□) and 9 h (■) ZnCl₂ was added to batch cultures to a final concentration of 10 µM. When the metal was withheld for 9 h, it was entirely accumulated within the next 1 h.

(at 3 or 6 h), the organisms immediately began to accumulate the metal (Fig. 2b). This first period of uptake stopped when the quantity of zinc per c.f.u. was approximately equal to that of yeasts growing in medium supplemented with ZnCl₂ at zero time. In the latter cultures, cells were in stage II at 3 and 6 h, i.e. the metal was not accumulated and the intracellular level was decreased with increasing cell number. These results suggest that the actual capacity of each cell for zinc may have decreased after the lag period.

At 8 to 9 h, the second phase of zinc uptake (stage III) was initiated in cultures supplemented with ZnCl₂ at 0, 3 or 6 h. In each of the three cultures (Fig. 2b), the final amount of zinc accumulated per cell was the same, i.e. about 6 nmol (mg dry wt)⁻¹. In contrast, when zinc supplementation was delayed until 9 h, all of the zinc provided was accumulated by the cells in less than 1 h. Thus, at 10 h, these organisms had four times more zinc than did those from medium supplemented at earlier times (0, 3 and 6 h). Stationary-phase cells grown in low-zinc medium retained the ability to accumulate elevated quantities of the metal.

It was important to determine if the observed differences in the uptake of zinc by C. utilis during growth in batch culture resulted from changes in the ability of the cells to transport the metal or, simply, to alterations in the medium. The organisms were therefore grown in low-zinc medium, and the cultures were collected at various cell concentrations and resuspended in fresh growth medium at a uniform density of 50 µg dry wt ml⁻¹. Zinc chloride was added and the uptake of the metal was measured for 40 min. The results (Fig. 3) show that yeast cells from the late-exponential phase (when the cell concentration was 80 x 10⁶ c.f.u. ml⁻¹) accumulated 10 to 20 times more zinc than did cells from the
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Table I. Effect of culture medium and number of transfers on zinc uptake by late-exponential phase cells of C. utilis

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>No. of transfers</th>
<th>Zinc accumulated in 1 h [nmol (mg dry wt)-1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDB</td>
<td>0</td>
<td>11.8</td>
</tr>
<tr>
<td>Defined</td>
<td>0</td>
<td>89.9</td>
</tr>
<tr>
<td>Defined</td>
<td>1</td>
<td>98.6</td>
</tr>
<tr>
<td>Defined</td>
<td>2</td>
<td>100.8</td>
</tr>
<tr>
<td>Defined</td>
<td>3</td>
<td>98.7</td>
</tr>
</tbody>
</table>

Stationary phase cells grown in SDB were washed and diluted 200-fold into defined low-zinc growth medium. When the concentration of cells was 400 to 450 μg dry wt (80 to 90 x 10⁴ c.f.u.) ml⁻¹, they were collected and resuspended in fresh growth medium. Zinc chloride was added to a final concentration of 20 μM and the quantity of metal that accumulated was determined after 1 h.

Early- or mid-exponential periods. Also, the total amount of zinc accumulated was 14 times greater than that taken up by cells grown in zinc-supplemented medium [84 compared with 6 nmol (mg dry wt)⁻¹]. In contrast, there was no change in ⁵⁹Fe uptake by yeasts collected at different points in the growth cycle.

Requirements for enhanced zinc transport

The conditions required for enhanced uptake of zinc by cells from late-exponential phase cultures in low-zinc medium were determined. Candida utilis grown in low-zinc medium and harvested during the late-exponential phase accumulated about eight times more zinc in 1 h than did organisms at the same cell concentrations which had been grown in SDB containing 3.4 μM-Zn²⁺ (Table I). Continued subculturing of yeast cells in low-zinc medium did not affect the amount of metal accumulated from the resuspension system. These results suggested that the enhanced ability to accumulate zinc might be the result of a zinc-deficient status.

To test this hypothesis, defined medium was supplemented with various amounts of ZnCl₂ at zero time and the quantity of zinc taken up from the resuspension system by late-exponential phase cells was determined (Fig. 4). Zinc accumulation was identical in cells from medium containing 0.1 to 1.1 μM-Zn²⁺. As the quantity of zinc in the growth medium increased, the ability of cells to remove it from the resuspension system decreased. Cells from medium containing 5.1 μM-Zn²⁺ or higher were unable to accumulate any zinc from the test system. At the time of harvest, such cells contained about 12 nmol zinc (mg dry wt)⁻¹ whereas organisms from low-zinc medium were able to accumulate about 96 nmol (mg dry wt)⁻¹ in the resuspension flasks. The latter type of cells are termed zinc-deficient and the process whereby they accumulate elevated levels of the metal is termed the activated zinc transport system.

The following experiments were designed to detect possible zinc-phosphate interactions. In media containing 0.5 to 20.1 μM-Zn²⁺, both the biphasic pattern of zinc uptake and the total quantity of metal accumulated during the growth cycle were unchanged in the presence of either 1.0, 10 or 100 mM-KH₂PO₄. The levels of intracellular orthophosphate and polyphosphate were also similar in cells from early-, mid- and late-exponential phase cultures grown in medium containing the three different concentrations of phosphate (Table 2). However, the amount of orthophosphate decreased and the amount of polyphosphate increased during the stationary phase in cells from medium containing either 10 or 100 mM-phosphate. These results suggest that zinc uptake by either zinc-sufficient or zinc-deficient yeasts is independent of phosphate metabolism.
Fig. 3. Ability of *C. utilis* to take up zinc at various times in the growth cycle. Low-zinc batch cultures were started with $1.8 \times 10^6$ c.f.u. ml$^{-1}$. At various cell concentrations, the organisms were collected, washed and resuspended in growth medium to a uniform concentration (50 µg dry wt ml$^{-1}$). Zinc chloride was added to a final concentration of 15 µM and the accumulated metal was measured at intervals. The cell concentrations at harvest were ($10^{-6}$ x c.f.u. ml$^{-1}$): 6 (○), 10 (●), 23 (□), 48 (■) and 80 (△).

Fig. 4. Effect of Zn$^{2+}$ content of the medium on zinc accumulation by cells in the late-exponential phase. At the time of inoculation the growth medium contained ZnCl$_2$ at ($\mu$M): 1.1 (○), 1.6 (●), 3.1 (□), 5.1 (■). When the cells had grown to $85 \times 10^6$ c.f.u. ml$^{-1}$, they were collected, washed and resuspended in fresh medium. Zinc chloride was added to a final concentration of 20 µM and zinc uptake was measured.

Fig. 5. Characteristics of activated transport by zinc-deficient cells of *C. utilis*. Yeasts were grown in low-zinc medium to $80 \times 10^6$ c.f.u. ml$^{-1}$. Cells were collected, washed and resuspended in fresh growth medium, and test substances were added. One min later, the resuspended culture was made 20 µM with respect to ZnCl$_2$ and the accumulation of zinc was measured. All experiments were done at 30°C except for one series (□) at 10°C. The agents tested were: 10 mM-CN$^-$ (○), 0.2 mM-Cd$^{2+}$ (■) and 0.2 mM-Ca$^{2+}$ (△). Control (○).

Table 2. Orthophosphate and polyphosphate levels in *C. utilis* grown in medium containing various levels of phosphate

Yeasts were harvested, washed three times with growth medium without phosphate, and assayed for both orthophosphate and polyphosphate during the exponential and stationary phases. Values listed are averages from three separate cultures. The exponential phase values are averages of samples of early-, mid- and late-exponential phase cells.

<table>
<thead>
<tr>
<th>Phosphate concn in medium (µM)</th>
<th>Total orthophosphate [nmol (mg dry wt)$^{-1}$]</th>
<th>Total polyphosphate [nmol (mg dry wt)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exponential</td>
<td>Stationary</td>
</tr>
<tr>
<td>1.0</td>
<td>290</td>
<td>290</td>
</tr>
<tr>
<td>10</td>
<td>350</td>
<td>230</td>
</tr>
<tr>
<td>100</td>
<td>360</td>
<td>170</td>
</tr>
</tbody>
</table>
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Fig. 6. Kinetics of zinc uptake by cells of mid- and late-exponential phase cultures of C. utilis. Yeasts were collected from low-zinc medium at 5, 10, 20 and 80 \times 10^6 c.f.u. ml$^{-1}$ and incubated in fresh growth medium at 500 \(\mu\)g dry wt ml$^{-1}$. After 10 min, the yeasts were diluted 10-fold into the reaction medium containing ZnCl$_2$ from 0.2 to 10 \(\mu\)M. Samples were taken at intervals for 10 min. The results obtained with cells collected at 5, 10 and 20 \times 10^6 c.f.u. ml$^{-1}$ were similar. Data from only one concentration (10 \times 10^6) are presented (○) for comparison with cells from the late-exponential phase collected at 80 \times 10^6 c.f.u. ml$^{-1}$ (●). The kinetic parameters $K_m$ and $V_{max}$ were determined by a modification of the method of Cleland (1967). These values were used to determine the intercepts in the Lineweaver-Burk (1934) plot.

Characteristics of activated zinc transport

Uptake of zinc was linear during 50 min in the resuspension system containing glucose (Fig. 5). Removal of glucose decreased the total amount of metal accumulated (data not shown). Activated zinc transport was completely inhibited by addition of either 10 mM-KCN (Fig. 5) or 0.1 mM-CCCP. The amount of accumulated zinc decreased sevenfold when the temperature was reduced from 30 to 10°C (Fig. 5). The presence of 0.2 mM-Ca$^{2+}$ (Fig. 5), Mn$^{2+}$, Fe$^{2+}$ or Co$^{2+}$ had no effect on zinc uptake, but the amount of zinc accumulated in the presence of 0.2 mM-Cd$^{2+}$ was only 17% of the control (Fig. 5). The zinc status of the cells had no effect on their ability to accumulate iron.

Zinc uptake in C. utilis exhibited Michaelis-Menten saturation kinetics (Fig. 6). However, the rate of uptake by cells harvested at different times during exponential growth varied (Figs 3 and 6). The kinetic parameters of zinc uptake by yeasts harvested from low-zinc medium at cell concentrations of 5 \times 10^6 (early-exponential), 10 \times 10^6 and 20 \times 10^6 (mid-exponential) and 80 \times 10^6 (late-exponential) c.f.u. ml$^{-1}$ were determined. The results for early- and mid-exponential phase cells were similar: the transport system of these cells had an apparent $K_m$ of 2.0 \(\mu\)M-Zn$^{2+}$ and a $V_{max}$ of 0.22 nmol Zn$^{2+}$ min$^{-1}$ (mg dry wt)$^{-1}$ at 30°C. The affinity constant of the late-exponential phase cells was about the same (1.8 \(\mu\)M-Zn$^{2+}$), but $V_{max}$ was increased 17-fold [3.65 nmol min$^{-1}$ (mg dry wt)$^{-1}$].
Table 3. Zinc uptake by stationary phase cells of C. utilis grown in normal and low-glucose medium

Yeast were grown to the stationary phase in low-zinc medium with either 16.5 or 5.5 mM-glucose as the limiting nutrient. Samples were then removed at intervals and the cells were collected and resuspended to give 50 μg dry wt ml⁻¹ in homologous medium from which cells had been removed. The medium was made 20 μM with respect to ZnCl₂ and zinc accumulation was measured for 1 h. Cells from the medium containing 16.5 mM-glucose accumulated the metal at a similar rate to that of zinc-deficient yeasts from late-exponential phase cultures. However, the duration of uptake progressively decreased with increased time spent in the stationary phase. Zinc uptake was inhibited by 10 mM-KCN.

<table>
<thead>
<tr>
<th>Time in stationary phase (h)</th>
<th>16.5 mM-glucose</th>
<th>5.5 mM-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>77.0</td>
<td>5.8</td>
</tr>
<tr>
<td>12</td>
<td>51.4</td>
<td>3.3</td>
</tr>
<tr>
<td>24</td>
<td>19.0</td>
<td>2.7</td>
</tr>
<tr>
<td>48</td>
<td>18.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.

The maximum amount of zinc accumulated by zinc-deficient organisms via the activated transport system was determined by suspending late-exponential phase cells in medium containing 5.0 to 50 μM-ZnCl₂. The experiment was also done with 25 μM-cycloheximide in the resuspension flasks; this concentration is 10 times greater than that required to inhibit growth. The organisms removed all the zinc from medium containing 5.0 μM-Zn²⁺ in 40 min and from medium containing 10 μM-Zn²⁺ in 80 min. In media with 15 to 50 μM-Zn²⁺, the cells became saturated with zinc within 2 h at a level of 121.6 ± 7.6 nmol (mg dry wt)⁻¹ without cycloheximide and 122 ± 4.7 in the presence of cycloheximide. These quantities are equivalent to 0.78% of the total dry wt of the cells. The accumulation required neither cell division nor protein synthesis.

Earlier studies showed that the wash solution (0.2 mM non-radioactive ZnCl₂ at pH 3.5) removed all non-specifically bound Zn²⁺ from the surface of filtered yeast cells. However, since the quantity of zinc accumulated by zinc-deficient organisms was so great, the following experiment was done to see if the location of the metal was, indeed, intracellular. Zinc-deficient cells were incubated in the presence of 20 μM-ZnCl₂ for 1 h. After centrifuging, the pellet was washed with water, EDTA and low-zinc medium. The washed cells were resuspended in a solution of 0.2 mM non-radioactive ZnCl₂ for 30 min, or 0.1 mM-EDTA for 30 min, or 1 mM-HCl for 15 min. Samples were filtered and washed with 18 vols of 0.2 mM-ZnCl₂ at pH 3.5 and their radioactivity was compared with that of cells washed three times with acidic ZnCl₂. Resuspension in excess Zn²⁺, EDTA or acid did not reduce the quantity of cell-associated zinc. When the cells were mechanically disrupted (greater than 99.9% breakage) and the homogenate centrifuged at 3000 g for 10 min, less than 5% of the zinc was present in the pellet. Heat treatment (100°C for 10 min) of cells before addition of ZnCl₂ inhibited uptake; heat treatment after uptake caused complete release of accumulated metal.

Status of intracellular zinc

The high intracellular level of zinc following activated transport did not adversely affect the cells. Spectra of non-reduced versus H₂O₂-oxidized whole cells at room temperature before and after zinc uptake indicated that cytochromes and electron transfer processes
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Fig. 7. Zinc accumulation by cells in the lag phase in cultures of C. utilis. Stationary phase (24 h) cultures of three types were obtained: ○, low-zinc medium; ●, 20 μM-ZnCl₂ added at time of inoculation; and □, 20 μM-ZnCl₂ added when the cell concentration was 8.0 x 10⁶ c.f.u. ml⁻¹. Cells from these three cultures were collected, washed and resuspended in fresh growth medium. Ten min after resuspension, the medium was made 5 μM with respect to ZnCl₂ and the accumulated metal was measured at intervals.

remained functional. The rate of cell growth, total cell yield and culture viability were identical, regardless of the zinc concentration in the growth medium or the time of zinc supplementation. In yeasts that had accumulated 95 nmol (mg dry wt)⁻¹, 50% of the intracellular zinc was rapidly released on addition of nystatin (20 μg ml⁻¹). These results suggest that the intracellular zinc is localized and that much of it exists as the free ion.

Zinc uptake during the stationary phase

Stationary phase yeasts from glucose-limited medium containing 5.1 μM-ZnCl₂ or higher did not take up zinc, although stationary phase cells from low-zinc medium were capable of accumulating the metal. The quantity of zinc which accumulated decreased the longer the cells were in the stationary phase (Table 3). Nevertheless, even after 48 h in the stationary phase, zinc-deficient yeasts accumulated three times as much zinc as that taken up during the entire growth cycle by cells grown in zinc-supplemented medium [18 and 6 nmol (mg dry wt)⁻¹, respectively].

When the glucose content of the medium was reduced to 5.5 mM, the culture reached the stationary phase at a cell concentration (300 μg dry wt ml⁻¹) slightly less than that at which activated transport was observed (about 350 μg dry wt ml⁻¹). Non-growing cells from the low-glucose cultures were far less able to accumulate zinc (Table 3). The addition of glucose (to 16.5 mM) to 24 h cultures of stationary phase cells grown on medium containing either 16.5 or 5.5 mM-glucose, caused increases in zinc accumulation of 180% and 11%, respectively. These observations indicate that although glucose limitation causes a decrease in the quantity of zinc accumulated, the actual capacity of the organisms to accumulate the metal is not determined directly by their energy status. The data support the hypothesis that the amount of zinc already contained in the cells determines the quantity that they can subsequently accumulate.
Zinc uptake during the lag phase

The maximum quantity of zinc which accumulated when cells from a 24 h low-zinc culture were transferred to fresh medium was 40 nmol (mg dry wt)\(^{-1}\). However, yeasts grown in zinc-supplemented medium failed to accumulate the metal either during the stationary phase or after transfer (Fig. 7). In low-zinc medium, when 20 \(\mu\text{M-ZnCl}_2\) was added during the late-exponential phase, the cells accumulated this quantity without being saturated. When resuspended in fresh medium, they were able to take up 5 nmol (mg dry wt)\(^{-1}\) (Fig. 7). Thus, the uptake of zinc during the lag period is determined by the history of the cells in the previous growth cycle.

DISCUSSION

The intermittent nature of zinc accumulation in \(C.\) \textit{utilis} is in direct contrast to the linear uptake of iron by this organism throughout the growth cycle. To our knowledge, this sequence of accumulation \(\rightarrow\) dilution \(\rightarrow\) accumulation has not been reported previously for micro-organisms. We have observed linear uptake of zinc in batch growth cultures of various bacterial species (unpublished). However, in newly replated confluent 3T3 mouse fibroblasts, zinc uptake occurred in a cyclical manner (Schwarz & Matrone, 1975). In \(C.\) \textit{utilis}, the rate of growth and final cell yield were identical in low-zinc and zinc-supplemented medium. Similarly, excess zinc was not essential for growth of a strain of Euglena (Kempner & Miller, 1972); as with \(C.\) \textit{utilis}, all of the metal in low-zinc medium was accumulated by the algal cells during the lag phase.

Zinc-deficient, stage III cells can accumulate as much as 122 nmol \(\text{Zn}^{2+}\) (mg dry wt)\(^{-1}\) which is \(0.78\%\) of their dry wt. This high level of zinc does not affect their viability or growth rate. The zinc loading also does not inhibit cytochrome redox reactions. In contrast, as little as \(1.1 \mu\text{M-Zn}\) increased the respiratory quotient of \(\text{Saccharomyces pastorianus}\) (Lomander, 1965) and \(2.5 \mu\text{M-Zn}\) inhibited adaptation of \(S.\) \textit{cerevisiae} from an anaerobic to aerobic environment (Ohanian & Chaix, 1966), while \(15 \mu\text{M-Zn}\) or less inhibited animal mitochondrial activity (Skulachev \textit{et al.}, 1967; Kleiner, 1974), electron transfer in \(\text{Salmonella typhimurium}\) membrane fractions (Singh & Bragg, 1974), and proline and leucine transport in \(\text{Escherichia coli}\) (Kasahara & Anraku, 1974; Anraku, Goto & Kin, 1975). Thus, our data suggest that the high levels of zinc in \(C.\) \textit{utilis} are either localized or in some other way effectively detoxified. Electron dense regions, presumably containing zinc, were observed just inside the plasma membrane of unstained hyphae of \(\text{Neocosmospora vasinj2cta}\) (Paton & Budd, 1972). Cysts of \(\text{Entamoeba invadens}\) contained as much as \(1.0\%\) zinc by dry wt; much of the metal was localized in large aggregates of ribosomes called chromatoid bodies (Morgan & Satillaro, 1972). In response to increased levels of intracellular zinc, animal cells synthesize a storage protein called metallothionein (Richards & Cousins, 1975).

Correlations between uptake, localization, and metabolism of phosphate and divalent cations such as calcium (Jones, 1967; Rosenberg & Munk, 1969), magnesium (Rothstein \textit{et al.}, 1958), iron (Okorokov & Kulaev, 1968) and zinc (Paton & Budd, 1972) have been reported with various yeasts, filamentous fungi and protozoa. However, with \(C.\) \textit{utilis} (this study), \(\text{Serratia marcescens}\) (unpublished), or \(\text{Pseudomonas aeruginosa}\) (unpublished), neither the characteristics of uptake of zinc or iron nor the total quantities accumulated were influenced by the amount of phosphate in the growth medium. There were no rapid changes in the level of polyphosphate before or after accumulation of high quantities of zinc by zinc-deficient \(C.\) \textit{utilis}. Okorokov & Kaelev (1968) and Okorokov \textit{et al.} (1975) suggested that formation and degradation of polymeric orthophosphate complexes of magnesium and iron
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have an important role in maintaining steady-state levels of these metals in the cytoplasm. It is possible that insoluble zinc–phosphate complexes in C. utilis are formed.

Unidirectional influx of any substance must be regulated since unrestricted accumulation would eventually impair cell functions and viability. Steady-state intracellular levels might be maintained by coordinated control of influx–efflux processes. The manganese-transport system of Bacillus subtilis is subject to such control (Fisher et al., 1973; Scribner et al., 1975). Manganese-deficient cells accumulate high, potentially toxic, intracellular levels of this metal via a hyperactive uptake system. Reduction of the manganese content of the cell is mediated by synthesis of a modulator protein that decreases the activity of the influx system without altering the rate of manganese efflux. Once the steady-state intracellular level of manganese is attained, growth continues. In contrast to this type of control, our results show that intracellular zinc in C. utilis, as in Candida albicans (unpublished observations), Dunaliella tertiolecta (Parry & Haywood, 1973), Neocosmospora vasinfecta (Paton & Budd, 1972) and S. cerevisiae (Ponta & Broda, 1970), neither exits nor exchanges with extracellular zinc. Also, inhibition of energy metabolism in C. utilis by the addition of either KCN or CCCP fails to reduce the zinc content of pre-loaded cells. Therefore, the zinc transport system, at least in fungi, must be regulated through control of the influx system itself.

Micro-organisms can regulate accumulation of essential metabolites by the process of transinhibition. In this type of feedback, uptake of a substance is inhibited by binding of the material to its carrier on the inner surface of the membrane. Thus, the size of the intracellular pool determines the activity of the permease; for example, at higher pool levels, more permease molecules are inactivated. The transport of various amino acids (Hunter & Segel, 1973; Rytha, 1975; Morrison & Lichstein, 1976), adenine (Cummins & Mitchison, 1967), uracil (Grenson, 1969), choline-O-sulphate (Bellenger et al., 1968), sulphate (Yamamoto & Segel, 1966) and biotin (Becker & Lichstein, 1972) in fungi, methionine in E. coli (Kadner, 1975), and neutral amino acids in Streptomyces hydrogenans (Ring, Gross & Heinz, 1970) are controlled by transinhibition.

However, the marked differences in zinc content of the yeast cells at different times in the growth cycle are difficult to explain solely on the basis of either transinhibition or repression of permease synthesis. Possibly, a cytoplasmic zinc-binding or zinc-storage protein possessing metallothionein-like properties might be important in controlling the quantity of zinc accumulated by cells (Failla et al., 1976). Although the rapid release of recently accumulated zinc induced by nystatin (Failla et al., 1976) argues against a direct role for storage proteins in zinc uptake, further studies may reveal a regulatory role for such a protein in the zinc metabolism of C. utilis. Since the characteristics of growth are identical in zinc-deficient and zinc-sufficient cells, the purpose of activated zinc transport is not immediately obvious. The phenomenon is perhaps associated in some manner with the stringent quantitative requirements of zinc for such post-exponential phase events as secondary metabolism and differentiation (Weinberg, 1970).

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REFERENCES


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SKULACHEV, V. P., CHISTYAKOV, V. V., JASAITIS, A. A. & SMIRNOVA, E. G. (1967). Inhibition of the respiratory chain by zinc ions. *Biochemical and Biophysical Research Communications* 26, 1–6.

WEINBERG, R. (1975). Polyphosphate levels in nongrowing cells of *Saccharomyces mellis* as determined by magnesium ion and the phenomenon of 'überkompensation'. *Journal of Bacteriology* 121, 1122–1130.
