Accumulation and Storage of Zn^{2+} by *Candida utilis*

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SUMMARY

Starved cells of *Candida utilis* accumulated Zn^{2+} by two different processes. The first was a rapid, energy- and temperature-independent system that probably represented binding to the cell surface. The cells also possessed an energy-, pH-, and temperature-dependent system that was capable of accumulating much greater quantities of the cation than the binding process. The energy-dependent system was inhibited by KCN, Na_2HAsO_4 , *m*-chlorophenyl carbonylcyanide hydrazone, *N*-ethylmaleimide, EDTA and diethylenetriaminepenta-acetic acid. The system was specific inasmuch as Ca^{2+} , Cr^{3+} , Mn^{2+} , Co^{2+} or Cu^{2+} did not compete with, inhibit, or enhance the process. Zn^{2+} uptake was inhibited by Cd^{2+} . The system exhibited saturation kinetics with a half-saturation value of $1.3 \mu\text{M}$ and a maximum rate of $0.21 (\text{nmol } \text{Zn}^{2+}) \text{ min}^{-1} (\text{mg dry wt}^{-1})$ at 30°C . Zn^{2+} uptake required intact membranes since only the binding process was observed in the presence of nystatin, toluene, or sodium dodecyl sulphate. Cells did not exchange recently accumulated ^{65}Zn following the addition of a large excess of non-radioactive Zn^{2+} . Similarly, cells pre-loaded with ^{65}Zn did not lose the cation during starvation, and efflux did not occur when glucose and exogenous Zn^{2+} were supplied after the starvation period. Efflux was only observed after the addition of toluene or nystatin, or when cells were heated to 100°C . Cells fed a large quantity of Zn^{2+} contained a protein fraction resembling animal cell metallothionein. In batch culture, cells of *C. utilis* accumulated Zn^{2+} only during the lag phase and the latter half of the exponential-growth phase.

INTRODUCTION

Candida utilis is the yeast most commonly used as a source of single-cell protein since it can ferment sulphite waste liquor, whey, and hydrocarbons (Bhattacharjee, 1970). Yeasts accumulate various metal ions, one of which, Zn^{2+} , stimulates nitrogen incorporation during the production of single-cell protein (Pecilius *et al.*, 1969). Specifically, the metal: (i) activates at least 20 enzymes (Parisi & Vallee, 1969), including DNA and RNA polymerase (Valenzuela *et al.*, 1973; Coleman, 1974) and reverse transcriptase (Auld *et al.*, 1974); (ii) stabilizes nucleic acids, ribosomes, lysosomes, microtubules, and cell membranes (Tal, 1969; Chvapil, 1973); and (iii) is essential for fungal secondary metabolism and differentiation (Weinberg, 1970).

However, Zn^{2+} often exists as an insoluble complex in nature (Jurinak & Inouye, 1962; Zirino & Healy, 1970) and thereby presents unicellular organisms with the challenging problem of obtaining this highly essential nutrient. We are examining the means whereby *C. utilis* solubilizes, transports, and stores Zn^{2+} . In this paper, we present evidence for the existence of an energy-coupled transport system and a storage protein.

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METHODS

Organism. The strain used in the study was obtained from the departmental culture collection. It has been designated as inulin-negative *Candida utilis* NRRL-Y-7634 by Dr C. P. Kurtzman, USDA, Peoria, Illinois, U.S.A.

Media and culture conditions. The yeast was cultured at 30 °C on slopes of nutrient agar (Difco) enriched with 0.5 % glucose, stored at 4 °C and transferred fortnightly. For experiments, the organism was grown in Sabouraud's glucose broth (Difco) at 30 °C on a gyratory shaker at 200 rev./min. At 24 h, the organisms were transferred to a defined medium to obtain an initial concentration of 10^5 colony forming units (c.f.u.)/ml. The medium contained (mM): glucose, 16.5; NH_4Cl , 18.7; K_2HPO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; sodium citrate, 5.0; biotin, 0.004; piperazine-*N-N'*-bis-(2-ethane sulphonate) buffer, 5.6. The medium was adjusted to pH 6.6 with 1.2 M-NaOH. The pH was not altered during growth of the organism nor by the addition of substances whose effect on Zn^{2+} accumulation was being studied. All chemicals employed were reagent grade; aqueous solutions were prepared in distilled water that had been demineralized by passage through a Barnstead ion exchange column (Scientific Products Co., Chicago, U.S.A.).

During exponential growth in the defined medium, when the culture had attained a density of 1×10^7 to 2×10^7 c.f.u./ml (as determined by direct count using a haemocytometer), the cells were collected by centrifugation at 3000 *g* in a Sorvall RC-2B unit at 5 °C. The cells were washed once with sterile water and resuspended at a 10-fold concentration in the defined medium minus glucose. The culture was replaced on the shaker at 30 °C for 7 h to reduce endogenous energy pools.

Zn^{2+} accumulation. Generally, 1.0 ml starved cells at a density of 1×10^8 to 2×10^8 c.f.u./ml was added to 7.8 ml defined medium minus glucose in 50 ml flasks on a shaker at 175 rev./min in a water bath at 30 °C. Cells were allowed to equilibrate with the environment for 5 min before the addition of either 1.0 ml water or glucose. The cultures were then incubated for an additional 15 min at which time 0.1 ml of a solution of the substance to be tested for its effect on Zn^{2+} accumulation was added. One minute later, designated T_0 , 0.1 ml of a solution of 100 μM - ZnCl_2 , enriched with 25 μCi carrier-free ^{65}Zn (New England Nuclear, Boston, U.S.A.), was added to each flask. The total volume of fluids in each experimental or control flask was 10 ml. The background quantity of Zn^{2+} due to contamination of components of the medium was 0.1 μM as determined by atomic absorption spectrophotometry. Thus the final concentration of Zn^{2+} in the cultures was 1.1 μM with an activity of 0.025 $\mu\text{Ci ml}^{-1}$.

At intervals, 0.5 ml samples were removed, filtered through 0.45 μM HA filters (Millipore), and washed twice with either chilled defined medium that contained 10 μM non-radioactive ZnCl_2 and no glucose or with a solution of 10 μM non-radioactive ZnCl_2 at pH 3.0. The excess quantity of non-radioactive Zn^{2+} was used to exchange with any ^{65}Zn that was non-specifically attached to the surface of the cells. Additional washings with non-radioactive Zn^{2+} did not further alter the amount of radioactivity retained on the filters. Preliminary studies had shown that the use of wash medium at 4 °C would not induce loss of previously-accumulated Zn^{2+} . The radioactivity was counted in a Beckman Biogamma Counter (Fullerton, California, U.S.A.).

The number of c.f.u. in the flasks at the beginning and end of the Zn^{2+} -accumulation experiments was monitored by conventional pour-plate procedure. Dry weights were determined by passing volumes of known cell concentration through pre-weighed membrane filters. The filters were then washed with water, dried overnight at 80 °C, and cooled in a desiccator before weighing.

Zn²⁺-binding substance. Stationary-phase cells grown in Sabouraud's glucose broth enriched with 100 μ M-ZnCl₂ were harvested and resuspended in cold 0.1 M-K₂HPO₄, pH 7.4, and passed through a motor-driven pressure cell (Power Lab Press, American Instrument Co., Silver Spring, Maryland, U.S.A.) five times. To remove cellular debris, the suspension was then centrifuged twice at 33 000 g for 15 min. Metallothionein was extracted by a modification of the methods of Cherian (1974) and G. W. Evans (personal communication). The supernatant was heated to 60 °C, maintained at that temperature for one min and chilled immediately. After being centrifuged at 6800 g for 15 min, the supernatant was extracted with 1.2 parts (v/v) chilled 95 % (v/v) ethanol and 0.1 part chloroform. The mixture was then centrifuged at 4300 g for 15 min and the supernatant re-extracted with two parts 95 % (v/v) ethanol. After a precipitate was formed, the mixture was centrifuged at 4300 g for 15 min, the supernatant discarded, and the pellet extracted with water for 10 min. Insoluble material was then removed by centrifugation at 20 000 g for 10 min. The supernatant was enriched with 1.0 μ Ci ⁶⁵Zn, gently stirred for 30 min, lyophilized, resuspended in a small volume of 0.01 M-ammonium formate, and applied to a 25 $\times$ 450 mm Sephadex G-75 column equilibrated with 0.01 M-ammonium formate at pH 7.4. The material was eluted with the buffer at a rate of 35 ml h⁻¹ and 50 drop fractions were collected. The effluent was monitored at 254 nm by means of an LBK-Uvicord II and the radioactivity in each fraction was determined. Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

Zn²⁺ accumulation

As shown in Fig. 1, Zn²⁺ accumulation by starved cells of *C. utilis* was biphasic. First, Zn²⁺ was rapidly bound during the initial 5 min after addition of the cation, even in the absence of an energy source. To observe the second phase, which is energy-dependent, glucose had to be added. Both the rate and quantity of Zn²⁺ accumulated in the second phase were controlled by the glucose concentration. For example, to obtain linear uptake at the maximum rate, at least 5.5 mM-glucose was required. Moreover, before energy-dependent Zn²⁺ uptake could begin, the cells had to be provided with glucose for at least 16 min. Therefore, in all subsequent Zn²⁺ uptake experiments to be described, 16.5 mM-glucose was added 16 min before Zn²⁺ was added, i.e. at time $T_0 - 16$.

To test the hypothesis that glucose transport and subsequent catabolism does, in fact, yield energy for Zn²⁺ uptake, we added metabolic inhibitors at various times during the experimental period. When the poisons were added after energy-dependent Zn²⁺ uptake had been initiated, KCN and Na₂HAsO₄ inhibited the rate of Zn²⁺ transport (Fig. 2). Similarly, energy-dependent transport was inhibited when these poisons were added at the same time as either glucose ($T_0 - 16$) or Zn²⁺ (T_0). The addition of 100 μ M-*m*-chlorophenyl carbonylcyanide hydrazone (CCCP), an uncoupler of oxidative phosphorylation (Heytler & Prichard, 1962; Harold, Baarda & Pavlasova, 1970) not only suppressed uptake but actually induced a loss of the recently accumulated Zn²⁺.

Effect of temperature on Zn²⁺ accumulation

Cells grown and starved at 30 °C were resuspended at 30 °C in defined medium that contained glucose. The cells were energized in the presence of glucose for 10 min and were then transferred to water baths set at either 6, 16, 23, 30, 37, or 44.5 °C. After 7 min, Zn²⁺ was added. Cells similarly treated, but incubated without glucose, served as controls. The results in Table 1 show that energy-independent Zn²⁺ binding was not altered as a function

Fig. 1

Fig. 2

Fig. 1. Effect of glucose on Zn^{2+} uptake. Zn^{2+} was added at time T_0 . $\circ$, Glucose added at T_0-16 ; $\bullet$, no glucose; $\square$, glucose added at T_0-5 ; $\blacksquare$, glucose added at T_0 ; $\triangle$, glucose added at T_0+6 ; $\blacktriangle$, glucose added at T_0-16 to cells that had been heated at $100^\circ C$ from T_0-20 to T_0-16 .

Fig. 2. Effect of metabolic inhibitors on Zn^{2+} uptake. Glucose added at T_0-16 , Zn^{2+} added at T_0 , inhibitors added at T_0+16 . $\circ$, Glucose; $\bullet$, no glucose; $\square$, glucose plus 10 mM-KCN; $\blacksquare$, glucose plus 10 mM- Na_2HAsO_4 ; $\triangle$, glucose plus 100 μM -*m*-chlorophenyl carbonyl cyanide hydrazone.

of temperature. In contrast, energy-dependent Zn^{2+} uptake was eliminated at both 5 and $44.5^\circ C$. Neither of these temperatures affected cell viability during the experimental period. Inasmuch as the cells had been pre-incubated with glucose at $30^\circ C$, failure to accumulate Zn^{2+} at 5 and at $44.5^\circ C$ was not due to an energy-depleted state. At the intermediate temperatures listed in Table 1, the quantity of Zn^{2+} uptake was increased in a linear manner with increasing temperature, with a maximum at $37^\circ C$.

Effect of pH on Zn^{2+} accumulation

Zn^{2+} forms complexes with poly-phosphates, -carbonates and -hydroxides to yield insoluble precipitates at pH 6.8 in soil (Jurinak & Inouye, 1962) and colloids at pH 7.0 in sea water (Zirino & Healy, 1970). Therefore, we tested Zn^{2+} uptake at pH values above and below 6.6 (Fig. 3). It may be noted that as the hydrogen ion concentration was increased, thus increasing the solubility and effective concentration of Zn^{2+} , the rate and quantity of cation accumulation was increased. Moreover, even at the lower pH values, Zn^{2+} uptake required the presence of glucose.

Fig. 3. Effect of pH on Zn²⁺ uptake. The hydrogen ion concentration was adjusted with NaOH or HCl before addition of glucose at T_0-16 . Zn²⁺ was added at T_0 . ○, pH 4.8; ●, pH 5.6; □, pH 6.6; ■, pH 7.3; △, pH 8.2.

Table 1. *Effect of temperature on energy-independent and energy-dependent accumulation of Zn²⁺*

Temperature (°C)	Zn ²⁺ accumulated in 60 min [nmol (mg dry wt) ⁻¹]	
	Without glucose	With glucose
5	0.18	0.16
16	0.17	1.37
23	0.18	2.17
30	0.24	2.48
37	0.20	3.07
44.5	0.18	0.22

Specificity of Zn²⁺ uptake system

The specificity of energy-dependent Zn²⁺ uptake was studied first by the addition of single chloride salts of Na⁺, Ca²⁺, Cr³⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Cd²⁺ and Hg²⁺, and of the sulphate of Fe²⁺ and the nitrate of Ag⁺, at a concentration of 10 μ M. ⁶⁵Zn uptake was increased twofold by Ni²⁺ and Fe²⁺; these ions possibly displaced Zn²⁺ at non-specific wall sites, thereby increasing the effective concentration of the cation available for uptake. In contrast, Ag⁺, Hg²⁺, Cd²⁺ and non-radioactive Zn²⁺ inhibited ⁶⁵Zn uptake. However, 10 μ M-Ag⁺ and -Hg²⁺ killed the cells, whereas Zn²⁺ and Cd²⁺ were not toxic even at 100 μ M. Each of the six other cations tested had no effect on Zn²⁺ uptake or viability at either 10 or 100 μ M. Thus we concluded that the energy-dependent uptake system for Zn²⁺ is highly specific.

The inhibitory action of Cd²⁺ on Zn²⁺ accumulation was further studied by altering the

Table 2. *Effect of Cd²⁺ on energy-independent and energy-dependent accumulation of Zn²⁺*

Cd ²⁺ (μ M)	Zn ²⁺ accumulated in 60 min [nmol (mg dry wt) ⁻¹]			
	Without glucose		With glucose	
	Zn ²⁺	% inhibition*	Zn ²⁺	% inhibition*
0.0	0.18	—	2.44	—
0.1	0.24	—	2.20	9.0
1.0	0.15	14.9	1.63	33.0
10.0	0.125	30.6	0.68	72.3

* As compared with control flasks that contained no added Cd²⁺. All flasks contained 1.1 μ M-Zn²⁺.

Table 3. *Kinetics of Zn²⁺ uptake in C. utilis*

Zn ²⁺ (μ M)	Rate of uptake [nmol min ⁻¹ (mg dry wt) ⁻¹]
0.6	0.051
0.8	0.068
1.0	0.082
1.3	0.111
1.6	0.138
2.1	0.177
3.1	0.203
5.1	0.210
10.1	0.216

concentration of the former while keeping that of Zn²⁺ constant at 1.1 μ M. As the ratio of Cd:Zn was increased from approximately 0.1 to 1.0 to 10, the amount of inhibition of both binding and uptake of Zn²⁺ was increased (Table 2). This effect of Cd²⁺ could be overcome by the addition of exogenous Zn²⁺, thereby lowering the Cd:Zn ratio.

Kinetics of Zn²⁺ accumulation

The addition of increased quantities of non-radioactive Zn²⁺ before, at the same time as, or after radioactive-enriched Zn²⁺ resulted in a reduction of ⁶⁵Zn accumulation/mg dry wt cell material in a given period of time. This indicates that a limited number of transport sites are present. To determine if the energy-dependent transport system exhibited saturation kinetics, different concentrations of ⁶⁵Zn-enriched ZnCl₂ were added to the cell suspensions and portions were filtered at intervals of 30 s for 5 min (Table 3). Standard plots of these results reveal that simple Michaelis-Menten kinetics were not followed. For example, a plot of rate versus Zn²⁺ concentration yielded a half-saturation concentration of 1.3 μ M and a maximum rate of 0.21 nmol min⁻¹ (mg dry wt)⁻¹. Analysis by the Cleland method (Cleland, 1967) gives an apparent K_m of 1.83 ± 0.46 μ M and a V_{max} of 0.28 ± 0.03 (nmol Zn²⁺) min⁻¹ (mg dry wt)⁻¹. Both theoretical and technical considerations can be invoked to explain the discrepancy in kinetic values obtained by the two methods. Possibly the transport system is multicomponent and subjected to controls at various levels. Also, since the affinity of the system is very high, as indicated by the low half-saturation value, relatively low concentrations of Zn²⁺ must be employed in the kinetic studies. At such concentrations, errors that result from non-specific binding of Zn²⁺ to the reaction vessel and to

the cell walls can significantly contribute to a reduction in the actual amount of Zn²⁺ that is available to the uptake system.

Effect of chelators on Zn²⁺ accumulation

Of seven synthetic chelating agents tested, only the polyaminopolycarboxylic acids, EDTA and diethylenetriaminepenta-acetic acid (DTPA), prevented Zn²⁺ accumulation. At pH 7.0, the Zn²⁺ stability constants of these two agents are 11.9 and 10.7, respectively (Ringbom, 1963). These constants are at least four log units greater than those of the chelators that were inactive in the test system. The latter consisted of 4,5-dihydroxybenzene-1,3-disulphonic acid, 2-hydroxyethyliminodiacetic acid, 8-hydroxyquinoline-5-sulphonic acid, iminodiacetic acid and diethyldithiocarbamic acid.

The growth and experimental media contained two substances capable of complexing such cations as Zn²⁺, namely citrate and phosphate. Each of these anions was required for growth; however, in the absence of phosphate, Zn²⁺ uptake into starved cells was twofold greater than in the presence of 10 mM-phosphate. Likewise, 'spent' supernatant obtained from cultures at the end of the growth phase supported greater accumulation of Zn²⁺ than did fresh experimental medium; the former contained less phosphate than did the latter. In contrast to phosphate, removal of citrate from the test medium had no effect on Zn²⁺ uptake during short-term experiments.

Requirement for a functional membrane for Zn²⁺ uptake

Since carrier-mediated transport systems require intact membranes as well as a functional 'permease' for substrate translocation, we examined the effect on Zn²⁺ uptake of various substances that alter membrane integrity. A detergent (sodium dodecyl sulphate), an organic solvent (toluene), and a polyene (nystatin) were added after energy-dependent Zn²⁺ uptake had been initiated. These substances prevented further uptake and caused release of some of the recently translocated Zn²⁺ (Fig. 4). Nystatin was solubilized in dimethylformamide; the quantities of antibiotic and solvent used did not affect the viability of the yeast cells during the experimental period. Toluene and nystatin have been shown to interact with membrane components to produce 'leaky' cells from which cations and low molecular weight substances are released from the intracellular milieu (Jackson & DeMoss, 1965; Hamilton-Miller, 1973). The release of Zn²⁺ by each of the three substances indicates that some of the recently translocated Zn²⁺ exists either as a free cation or is complexed with low molecular weight compounds.

Formaldehyde, which 'seals' the cell membrane surface and thereby inactivates transport systems (Koch, 1963), prevented Zn²⁺ uptake but not binding. Likewise, 1.0 mM concentrations of the sulphhydryl-binding agents *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate, when added after energy-dependent Zn²⁺ accumulation had begun, suppressed further uptake of the cation by 89 and 88 %, respectively, compared with cells in the presence of glucose alone.

Efflux of Zn²⁺

Initially, starved cells were resuspended in medium with glucose plus radioactive ZnCl₂ and the organisms were permitted to accumulate the cation for 40 min. At that time, non-radioactive ZnCl₂ was added to a final concentration of 200 μM and the amount of radioactivity retained by the cells was monitored for the subsequent 60 min. Such cells did not lose the previously acquired ⁶⁵Zn. In subsequent experiments, cells were pre-loaded with ⁶⁵Zn during growth, harvested and starved as described in Methods. As may be noted in Fig. 5, radioactivity was not lost from the cells during a 7 h period of glucose deprivation.

Fig. 4

Fig. 5

Fig. 4. Effect of substances that alter cell membrane integrity on Zn^{2+} uptake. Glucose added at $T_0 - 16$, Zn^{2+} added at T_0 , substances added at $T_0 + 18$. $\circ$, Glucose; $\bullet$, no glucose; $\square$, glucose plus $5.34 \mu\text{M}$ -nystatin; $\blacksquare$, glucose plus $330 \mu\text{M}$ -sodium dodecyl sulphate; $\triangle$, glucose plus 57 mM -toluene.

Fig. 5. Effect of various test substances on Zn^{2+} efflux. Cells were incubated for 7 h without glucose (left-hand side of Figure). The broken vertical line designates the time of addition of test substances or of heat. $\circ$, Glucose; $\bullet$, no glucose; $\square$, glucose plus $5.34 \mu\text{M}$ -nystatin; $\blacksquare$, glucose plus $100 \mu\text{M}$ - Zn^{2+} ; $\triangle$, glucose plus 57 mM -toluene; $\blacktriangle$, glucose preceded by heating of cells at 100°C for 5 min.

After this starvation period, efflux did not occur following the addition of either glucose or glucose plus a large amount of exogenous ZnCl_2 .

In contrast, if the cells were treated with nystatin or toluene after the 7 h starvation period, approximately 55 % of their ^{65}Zn was released (Fig. 5). Treatment of the cells by heat resulted in a release of 87 % of the radioactive Zn^{2+} . With nystatin, a lag period was required before loss of the cation; however, no lag was needed for nystatin to cause efflux of recently-translocated Zn^{2+} (Fig. 4).

Requirement for protein synthesis for Zn^{2+} uptake

We consistently noted a delay between the time of addition of glucose and the initiation of energy-dependent Zn^{2+} translocation (Fig. 1). This lag might be due to the requirement for synthesis of one or more components of the Zn^{2+} transport system that might have been degraded during the starvation procedure. To test this hypothesis, we observed the effect on Zn^{2+} uptake of cycloheximide (CH), an inhibitor of protein synthesis in eukaryotic organisms. Cells starved for 0, 2, 4, 5.5 and 7 h were resuspended in medium with or without glucose and CH was added to a final concentration of $100 \mu\text{M}$, a quantity that inhibited

Fig. 6

Fig. 7

Fig. 6. Effect of cycloheximide on Zn²⁺ accumulation by non-starved cells and by cells starved for 2 h. Glucose and/or 100 μM-cycloheximide were added to cells at T_0-16 , Zn²⁺ was added at T_0 . ○, Glucose to non-starved cells; ●, glucose and cycloheximide to non-starved cells; □, glucose to starved cells; ■, glucose and cycloheximide to starved cells; △, no additions to starved cells.

Fig. 7. Fractionation through Sephadex G-75 of material extracted from cells of *C. utilis* that had been grown in presence of 100 μM-Zn²⁺. ○, Extinction; ●, radioactivity. Fraction volume: 2 ml; void volume: fraction no. 1; total volume: fraction no. 70.

growth completely but which had no effect on cell viability for at least 10 h. CH did not alter the ability of unstarved cells to accumulate Zn²⁺ (Fig. 6). In contrast, with starved cells, the inhibitor prevented uptake of Zn²⁺ above the level of that bound by starved cells that were deprived of glucose. Identical results were obtained with cells starved for each of the four periods of starvation. These data indicate that *de novo* protein synthesis was required for membrane translocation of Zn²⁺ by cells of *C. utilis*.

Extracellular Zn²⁺-sequestering molecules (analogous to the siderophores secreted by many micro-organisms to facilitate Fe³⁺ uptake), membrane permeases, and intracellular macromolecules that bind Zn²⁺ are substances for which protein synthesis would be required. To examine the first possibility, we monitored the culture medium for the presence of extracellular natural chelators. We observed that the rate and extent of Zn²⁺ uptake was the same in starved cells resuspended in (i) fresh growth medium, (ii) 'spent' starvation medium enriched with glucose and phosphate, and (iii) growth medium collected at various times during the growth cycle and enriched with glucose and phosphate. These observations indicated that, in our system, the cells did not form extracellular solubilizing or sequestering agents that would enhance accumulation of Zn²⁺. The presence of citrate, a natural complexing agent, might have offset the necessity for the production of such molecules.

In regard to the possible production of an intracellular Zn²⁺-binding macromolecule,

Fig. 8. Accumulation of Zn^{2+} during growth of cells of *C. utilis* in batch culture. $\circ$, Cell growth; $\bullet$, Zn^{2+} accumulated by cell population; $\square$, Zn^{2+} accumulated/ 10^6 cells.

animal cells synthesize metallothioneins, a family of proteins of about 6600 daltons that contain 30 to 35 % cysteine residues and which bind six to nine atoms of Cd^{2+} , Zn^{2+} or Cu^{2+} per molecule (Margoshes & Vallee, 1957; Webb, 1972; Evans, 1973; Buhler & Kagi, 1974). To determine if *C. utilis* contained a protein similar to metallothionein, cells were grown and treated by means of a procedure that is relatively specific for the extraction of metallothionein as described in Methods. A single peak that absorbed maximally at both 215 and 254 nm, contained protein, and had 70 % of the radioactive Zn^{2+} was eluted from the Sephadex G-75 column (Fig. 7).

Zn^{2+} accumulation during batch growth

The rate of accumulation of Zn^{2+} by cells of *C. utilis* in batch culture is shown in Fig. 8. The cation was taken up rapidly during the first 2 h incubation, a period during which total cell number did not increase. In the first half of the exponential phase, the total Zn^{2+} content of the cells remained constant (and was thereby diluted in individual cells), whereas a significant increase in Zn^{2+} accumulation occurred during the second half of the growth phase. Note, however, that the actual quantity of Zn^{2+} per cell continued to be diluted. No further accumulation occurred during the stationary phase.

DISCUSSION

Zn²⁺ transport in microbial cells has been studied in *Chlorella pyrenoidosa* (Matzku & Broda, 1970; Ponta & Broda, 1970; Findenegg, Paschinger & Broda, 1971), *Dunaliella tertiolecta* (Parry & Haywood, 1973), baker's yeast (Fuhrmann & Rothstein, 1968), *Neocosmospora vasinfectum* (Paton & Budd, 1972), and *Escherichia coli* (Bucheder & Broda, 1974). As with the present study on *Candida utilis*, the previous investigations generally demonstrated the two distinct processes in accumulation of the cation. First, there is a rapid, but limited, energy- and temperature-independent binding to the cell surface. The amount of such binding in our system was low in comparison with values reported in similar studies (Fuhrmann & Rothstein, 1968; Paton & Budd, 1972). These authors have shown that cations compete for non-specific anionic sites on the cell surface. Our low results were probably due to the presence of a 1000-fold excess of Mg²⁺ over Zn²⁺ in the medium plus our use of acidic washes following cell filtration.

The second process is that of the slower, but sustained, energy- and temperature-dependent translocation across the cell membrane. Energy-dependent Zn²⁺ uptake is a highly specific process that exhibits saturation kinetics. The half-saturation value of 1.3 µM that we observed for *Candida* is similar to the apparent K_m values of 5.7 to 8.7 µM, 20 µM and < 10 µM reported for *Chlorella* (Matzku & Broda, 1970), *Escherichia* (Bucheder & Broda, 1974), and baker's yeast (Fuhrmann & Rothstein, 1968), respectively. As with *Candida*, Cd²⁺ was the only competitive inhibitor of Zn²⁺ uptake in *Escherichia*. With *Neocosmospora*, Mn²⁺ was reported to inhibit Zn²⁺ uptake competitively (Paton & Budd, 1972); because a non-physiological quantity (500 µM) of Mn²⁺ was employed, the observation is difficult to evaluate.

Our results indicate that *Candida* does not possess an efflux system for Zn²⁺. The cation was released only after treatment with agents that alter cell membrane integrity; this observation was made also by other authors with *Dunaliella* and baker's yeast. However, with *Chlorella*, Matzku & Broda (1970) reported that EDTA caused partial release of the cation; inasmuch as EDTA does not enter intact cells (Rahman & Wright, 1975), the Zn²⁺ released must have been derived from metal bound to the cell surface. With *Escherichia*, 25 % of intracellular Zn²⁺ exchanged with exogenous ⁶⁵Zn in 2 h (Bucheder & Broda, 1974); unfortunately, in that system a 50 % loss in viability occurred during the experimental period.

In the system described in the present paper, cells of *C. utilis* at a density of 10⁷ c.f.u./ml occupied 0.1 % (v/v) of the medium. If we assume that 80 % of each cell consists of water and that all of the intracellular Zn²⁺ exists as the free cation, the uptake of 2.5 (nmol Zn²⁺) h⁻¹ (mg dry wt)⁻¹ represents a concentration factor of approximately 125. The release of intracellular Zn²⁺ following exposure of the cells to organic solvents or to nystatin indicates that some of the metal exists as either the free cation or in low molecular weight complexes with, for example, amino acids (Perkins, 1953) or nucleotides (Weser & Brauer, 1970). Such complexes would be capable of passing through the cell wall barrier. However, when the cells were heated at 100 °C for 50 min, approximately 30 % more Zn²⁺ was released than with either toluene or nystatin. Presumably, at least some of the intracellular Zn²⁺ is bound to macromolecules that are denatured by heat. Our studies indicate, moreover, that the cells do not contain a natural efflux system for Zn²⁺.

Our results indicate that Zn²⁺ is translocated in *C. utilis* via an energy-dependent, highly specific, saturatable process that requires an intact membrane. Following influx, however, it is possible that Zn²⁺ may be complexed at a rate sufficient to result in 'downhill' transport; ultimately, the cation might be deposited in a storage protein. Animals employ metallothio-

neins for the storage and/or detoxification of Zn^{2+} , Cd^{2+} , Cu^{2+} and possibly Hg^{2+} . Such proteins would be advantageous to micro-organisms that have quantitatively different trace-metal requirements during primary and secondary metabolism (Weinberg, 1970). Moreover, microbial cells may be exposed to rapidly changing micro-environments during their growth cycle in nature.

The data indicate that there was a 16 min lag between the time of glucose addition and the initiation of energy-dependent Zn^{2+} uptake. Likewise, Fuhrmann & Rothstein (1968) observed that energy-dependent cation transport by starved yeast cells required pre-treatment with glucose. However, in that study, transport was enhanced by the addition of phosphate to the pre-treatment solution. In our study, both the starvation and experimental media contained phosphate. The systems differ in that the former authors removed phosphate before cation addition to prevent the formation of metal-phosphate complexes, whereas we provided a weak, metal-complexing agent (5 mM-citrate) to solubilize Zn^{2+} . When we deleted phosphate from the medium or when we employed 'spent' medium from which phosphate had been removed as a result of cell growth, the quantity of energy-dependent Zn^{2+} accumulated was increased twofold over that in the presence of phosphate.

In addition to the requirement for a source of energy before the initiation of Zn^{2+} uptake, protein synthesis was needed. Proteins may be required for accumulation at one or more steps of the overall translocation process. Initially, auto-sequestering agents might be synthesized and released from the cells for the purpose of either solubilizing Zn^{2+} from colloids or precipitates, or transferring it from non-specific ligands and the subsequent 'presentation' of the cation to membrane permeases. Micro-organisms that live in alkaline or neutral environments are faced with the problem of solubilizing essential micronutrients. Much work has been performed on the fungal and bacterial siderophores that solubilize and translocate Fe^{3+} (Lankford, 1973). Moreover, Lange (1974) has observed that blue-green algae can grow at alkaline pH by producing natural chelators. While there was no evidence for such substances being produced to facilitate Zn^{2+} uptake in our system, the production of zinc-chelating agents in natural environments is quite plausible.

A second process that would require protein synthesis before Zn^{2+} uptake would be replacing Zn^{2+} permease(s) or intracellular Zn^{2+} -binding proteins that have been degraded during starvation. However, toluene was more effective in releasing recently accumulated Zn^{2+} than that which had been acquired several hours earlier (cf. Figs. 4, 5). This observation suggests that, once accumulated, Zn^{2+} itself may induce synthesis of a Zn^{2+} -storage macromolecule. In rat cells, intracellular Fe^{3+} and Zn^{2+} have been shown to induce the formation, respectively, of ferritin and metallothionein (Millar *et al.*, 1970; Richards & Cousins, 1975*a*). Our data indicate that a metallothionein-like Zn^{2+} -binding protein may be produced by *C. utilis*. Similar proteins have been reported in *Anacystis nidulans* (MacLean *et al.*, 1972) and in baker's yeast (G. W. Evans, personal communication).

Since Zn^{2+} efflux does not occur in *C. utilis*, the cell must regulate the quantity of cation accumulated through some type of homeostatic control of the uptake process. The results shown in Fig. 8 support the presence of such a regulatory mechanism. Cells actively accumulated Zn^{2+} before the initiation of growth, but did not translocate the cation again until later in the growth cycle. Conceivably, an inducible, intracellular Zn^{2+} -binding protein similar to metallothionein has a specific role in regulation. In rats, metallothionein synthesis is induced by the presence of sub-acute toxic concentrations of Cd^{2+} (Webb, 1972) as well as by dietary Zn^{2+} or Cu^{2+} (Mills, 1974; Chen, Eakin & Whanger, 1974; Richards & Cousins, 1975*b*; Bremner & Davies, 1975). We are presently attempting to isolate and purify such a protein from *C. utilis* following the addition of excess Zn^{2+} or Cd^{2+} at various

times during the batch growth cycle and to study the overall regulation of Zn²⁺ metabolism in this organism.

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