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Influence of Nitrilotriacetic Acid on Cd^{2+} Uptake by Yeast

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During the past decade increased concern has developed over the ecological effects of toxic heavy metal pollutants and particularly over the release of cadmium into the environment. Although several studies on heavy metal transport into various organisms suggest a close connection between metal uptake and toxicity (Gutknecht 1983), there exists little information on the mechanism of Cd^{2+} uptake and the effects of cadmium on the molecular level (for review see Degraeve 1981). In addition to the possibility that the toxic effect may be due to an inhibition of enzymes within the cell (Vallee and Ulmer 1972) or to an interaction with DNA (Berthe-Corti et al. 1984), there are also indications that the presence of cadmium ions leads to irreversible membrane damage (Gadd and Mowll 1983). Since the mechanism and rate of transport through the plasma membrane is of great importance with regard to bioavailability and possible toxic effects of an environmental chemical we have studied the uptake of Cd^{2+} by the eucaryotic microorganism Saccharomyces cerevisiae in a project testing the influence of environmental chemicals on biomembranes.

We recently found that nitrilotriacetic acid (NTA), as well as its calcium complex, are taken up readily at the same rate by yeast (Rösick et al. 1985). As NTA is supposed to substitute pentasodiumtriphosphates in detergents, considerable amounts of this chemical are found in the environment. Although we observed no indications of toxicity of NTA at concentrations that can be expected in the environment (Rösick et al. 1985), the possibility exists that NTA might form complexes with heavy metal ions also present in waste water, thus preventing their sedimentation. Moreover, the large amounts of heavy metal ions which are deposited in form of insoluble salts in the sediments of almost every river in industrial nations (usually 300 -400 mg/kg, but sometimes more than 800 mg/kg (Stoeppler 1984)) might be dissolved by NTA and thus become available to living organisms. We therefore were interested in investigating the influence of NTA on cadmium uptake.

MATERIALS AND METHODS

The yeast Saccharomyces cerevisiae (strain R XII, a kind gift of Dr. Kotyk, Prague) was grown under aerobic conditions at 30 °C in a medium containing 2% glucose, 1% Difco yeast extract and 5% peptone and harvested during the stationary phase. In some cases as indicated in the text the cells were energized by 1 hour treatment with 2 % (w/v) glucose in 50 mM citrate, pH 5, under aeration. To test the influence of energy poisons or inhibitors on Cd^{2+}-uptake, we added 1 mM dinitrophenol, 5 mM azide or 6 µg/ml cycloheximide. These substances were added either 15 - 60 minutes before the uptake reaction or at the time indicated on the figures. For uptake experiments 2 x 10^6 - 4 x 10^7 cells/ml were incubated at 25 °C with $CdCl_2$, CdNTA or NTA in 10 mM 2-(N-morpholino)ethane-sulfonic acid (MES), pH 6, in the presence or absence of 2 % (w/v) glucose. Aliquots of 1 ml were collected at the indicated times on glass fiber filters (GF 92, Schleicher + Schüll, Dassel, Germany) previously soaked with stopping solution (10 mM $CdCl_2$ or NTA or CdNTA in 20 mM acetate, pH 3.5) and washed with 30 ml of this solution. The filters were assayed for radioactivity in a Beckman scintillation counter in 8 ml dioxan cocktail.

The internal concentrations of Cd^{2+} and NTA were calculated assuming a volume of 1.2 x 10^{-13} l/cell (Winter 1972). The dry weight of a cell was estimated to 3 x 10^{-8} mg.

To test the influence of Cd^{2+} on growth, cells from the stationary phase were added to the growth medium, which was supplemented with various concentrations of Cd^{2+} or Cd^{2+} + NTA.

Chemicals: Cadmium-115m (0.12 - 0.02 mCi/mg, Amershan-Buchler), Nitrilotri(1-^{14}C)acetic acid (58Ci/mole, Amersham-Buchler), Cadmium chloride-1-hydrate (Riedel-de-Haen), NTA, free acid (Merck), sodium azide (Serva), dinitrophenol (Merck) and cycloheximide (Serva) were of the highest purity available.

RESULTS AND DISCUSSION

Cd^{2+}-uptake by the yeast Saccharomyces cerevisiae was rapid and largely dependent on incubation conditions. Figure 1 shows the time-course of Cd^{2+} incorporation obtained with 15 µM and with 65 µM cadmiumchloride in the presence and absence of glucose. An influence of divalent cation adsorption at the outer face of the cell described in the literature (Fuhrmann and Rothstein 1968, Ponta and Broda 1970) was minimized by extensive washing of the cells with cold Cd^{2+}. From experiments with dead cells (60 min. heated on 70 °C, resulting in a cell viability of less than 0.005%) it could be shown that unspecific adsorption and/or incorporation of Cd^{2+} was rather low. A possible effect of complexing agent capacity of the pH buffer was counteracted by using MES, which displays only a weak affinity to metal ions. We performed the measurements in phosphate-free medium as inorganic phosphate effects uptake of metal ions (Fuhrmann and Rothstein 1968, Roo-

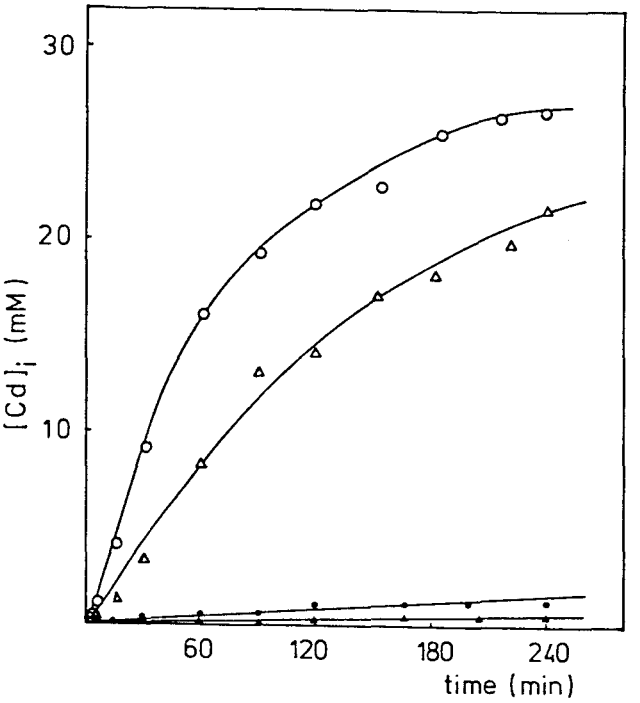

Figure 1. Time curves of Cd^{2+} - uptake.
o = 0.065 mM Cd^{2+}; Δ = 0.015 mM Cd^{2+}
open symbols: presence of glucose
closed symbols: absence of glucose

mans et al. 1979). It can be seen, that in the presence of glucose the initial uptake rate is enhanced 40 fold (Table 1). Figure 1 also shows that the cadmium uptake is more or less completed after 4 hours when glucose was present. This is not due to a significant decrease in the external medium, but solely to the increase of

Table 1. Initial rates of Cd^{2+}-uptake and accumulation factors f[a]

presence of glucose during preincubation	uptake	[Cd] (mM)	v (n mol/ min x mg dry wt.)	f
-	-	0.015	0.01	32
+	+	0.015	0.44	1500
-	-	0.065	0.03	20
+	+	0.065	1.20	530
-	+	0.065	1.20[b]	530

[a] ratio of $[Cd]_i/[Cd]_e$ determined after 4 hours of incubation, substrate consumption was taken into account
[b] with a lag time of 5 - 7 minutes.

cadmium within the cell. For full stimulation of uptake it is essential that glucose is present in the uptake medium. An additional preincubation is not necessary. However, without preincubation a lag time of 5 - 7 minutes was observed before full activity is achieved, similar to results reported by Roomans et al. (1979). This lag phase indicates that a direct coupling of glucose and cadmium transport is not the reason for the observed stimulation. Moreover, as the lag time is rather short, it is not likely that the glucose effect on initial rate is due to a stimulation of the synthesis of transport systems. Instead this result supports the view that the uptake of Cd^{2+} is energy dependent. The small but significant uptake observed in the absence of glucose may be due to passive diffusion, possibly of undissociated $CdCl_2$ as discussed by Gutknecht (1983) or to other energy sources of the cell. The relatively high accumulation ratio in the presence of glucose may be explained either by an energy dependent transport system or by the binding of Cd^{2+} to special heavy metal binding proteins. The enhancement of accumulation by glucose may be due to a stimulation of biosynthesis of these proteins.

To obtain more information about the energy dependence of Cd^{2+} transport and accumulation we repeated the experiments described above in the presence of glucose and energy poisons, such as dinitrophenol or azide and also in the presence of cycloheximide, an inhibitor of protein synthesis. Cycloheximide was already present during preincubation and had no influence on the results, indicating that the glucose effect cannot be related to synthsis of metal-ion binding proteins or transport systems. Dinitrophenol or azide lead to a strong inhibition of cadmium uptake regardless of whether the inhibitors were added 15 minutes before the radioactive cadmium isotope or during the course of accumulation (Fig. 2). With azide the uptake reaction stopped within less than 4 minutes after application. In contrast dinitrophenol lead to a large decrease in the cadmium uptake rate, but did not abolish it totally. The fact that cadmium uptake is inhibited by azide, which blocks the mitochondrial ATPase thus preventing the synthesis of ATP, and by dinitrophenol, a proton translocator favours the view, that this transport is not directly coupled to the ATP concentration. Instead, it is probably driven by the electrochemical potential of the membrane, possibly by the proton gradient across the membrane, which is created by the plasma membrane ATPase through ATP consumption (Goffeau and Slayman 1981). Similar results were described by Seipel (1978) for the uptake of xanthine by yeast. She observed a dissipation of the proton gradient across the plasma membrane and an inhibition of xanthine transport as the immediate consequence of the addition of proton tanslocators. Interestingly, the cadmium ions do not leak from the cells after inhibition of uptake, although they were already accumulated to a considerable amount within the yeast cell (Fig.2). This observation is in accordance with the assumption that cadmium ions are immobilized within the cell by binding to SH- groups of proteins (Vallee and Ulmer 1972).

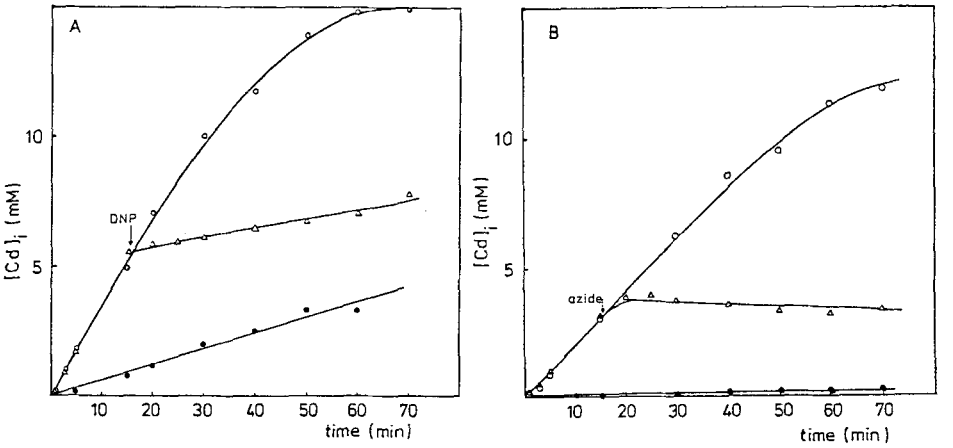

Figure 2. Influence of dinitrophenol (A) or azide (B) on Cd^{2+} - uptake in the presence of glucose. Cd^{2+} : 0.065 mM
o = control, Δ = 1 mM DNP (A) or 5 mM azide (B) added at the indicated time, • = presence of DNP (A) or azide (B) 15 minutes prior to the addition of Cd^{2+}.

Reports on the influence of complexing agents on cadmium uptake by various organisms given in the literature are contradictary. Some authors found a decrease of cadmium uptake by oysters and clams in the presence of NTA or EDTA (Mc Leese and Ray 1984, Hung 1982), others report that EDTA doubles the rate of cadmium uptake in mussels (George and Coombs 1977). Scharpf et al. (1972) found that the toxic effect of Cd^{2+} on rats is suppressed by NTA. There are still no reports on the molecular mechanism of these effects. Therefore we investigated the influence of NTA on the kinetics of Cd^{2+} transport.

Table 2. Initial rates of Cd/NTA uptake and accumulation factors

Nr	glu-cose[a]	[Cd] (mM)	[NTA] (mM)	measurement via	v (n mol/ min x mg dry wt)	f[b]
1	-	0.065	0.130	Cd-115m	0.002	1.3
2	+	0.065	0.130	Cd-115m	0.130	130.0
3	-	0.065	0.130	^{14}C-NTA	0.032	3.7
4	+	0.065	0.130	^{14}C-NTA	0.080	3.3
5	-		0.130	^{14}C-NTA	0.032	3.8
6	+		0.130	^{14}C-NTA	0.080	4.9

[a] absence or presence of glucose during preincubation and uptake
[b] ratio $[Cd]_i/[Cd]_e$ (exp. 1 - 2) or $[NTA]_i/[NTA]_e$ (exp. 3 - 6) after 4 hours of incubation.

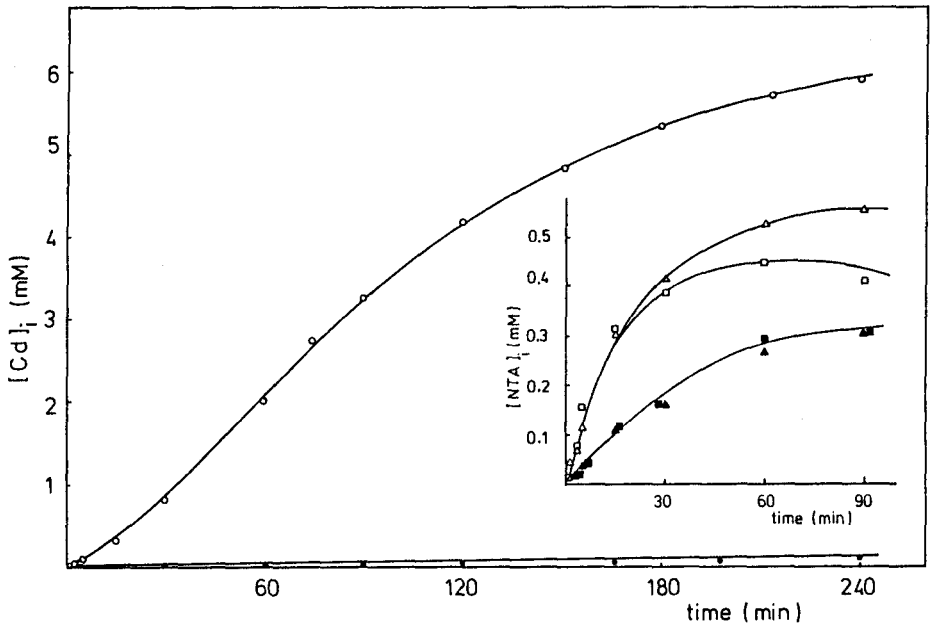

Figure 3. Time curves of Cd/NTA uptake
open symbols: presence of glucose,
closed symbols: absence of glucose
○ = 0.065 mM Cd^{2+} + 0.130 mM NTA measured via Cd-115m
Inset: △ = 0.13 mM NTA; □ = 0.065 mM Cd^{2+} + 0.13 mM NTA measured via ^{14}C-NTA.

In the presence of NTA we observed a significant decrease in cadmium uptake. By comparison of the rates given in Figs. 1 and 3 or Tables 1 and 2, it can be seen that the initial rates are reduced 10-fold when the ratio of Cd:NTA was 1:2 . Under these conditions, practically all Cd^{2+} is bound by the complexing agent NTA. However, we observed a strong energy dependency, similar to the cadmium uptake results shown in Fig.1 and Table 1. When the uptake was measured with ^{14}C labelled NTA, we observed only small differences in the absence or presence of Cd^{2+} (inset in Fig.3). However, surprisingly, this rate is smaller than in the same experiment, in which the uptake was measured through radioactive Cd^{2+} in the presence of glucose (Figure 3, Table 2). We assume that despite of the high affinity of NTA to Cd^{2+} part of the cadmium ions are transported into the cell by a carrier system, because of its high affinity to Cd^{2+} and/or because of its high activity. In accordance with this interpretation, the presence of glucose has similar effects as discussed for the results presented in Fig.1, when the rate was measured via radioactive Cd^{2+} (Table 2). When it was measured via ^{14}C-NTA we observed a positive effect of glucose on the initial rate but only a small influence on the accumulation factor (Table 2), similar to the results described for NTA uptake (Rösick et al., 1985). Our results are in good agreement with the observation of Mc Leese and Ray (1984) that a chelation of Cd^{2+}

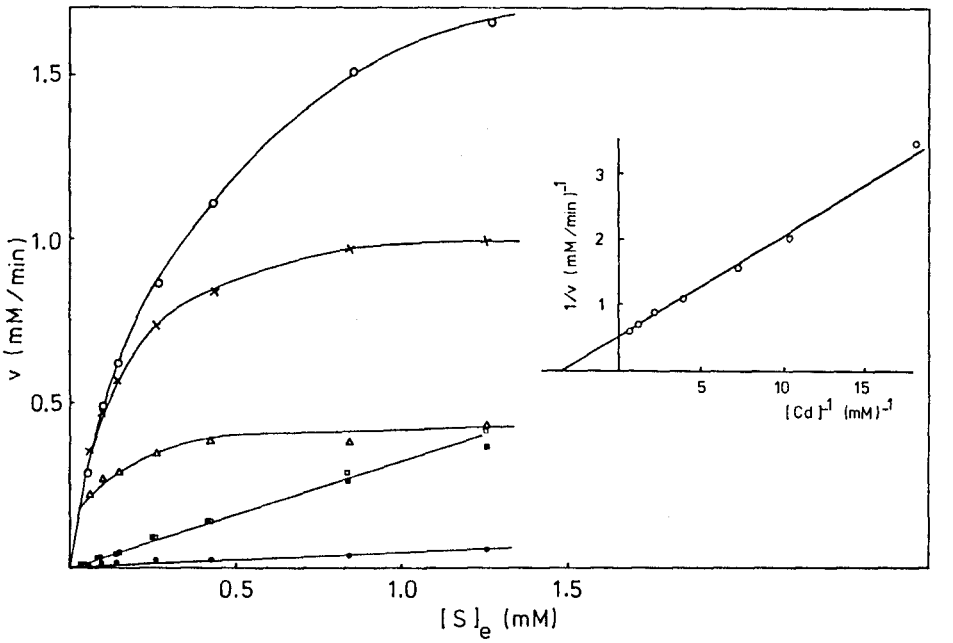

Figure 4. Influence of the external substrate concentration on the initial uptake rate in the presence of glucose.
○ : Cd^*, × : Cd^*/NTA = 2/1,
Δ : Cd^*/NTA = 1/1, ● : Cd^*/NTA = 1/2,
□ : NTA*, : Cd/NTA* = 1/1
* indicates the radioactively labeled substrate.
Inset: double reciprocal plot of Cd^{2+} - uptake.

with EDTA reduces the uptake of cadmium ions by clams and that of Hung (1982), who related the decrease in Cd^{2+} accumulation by the American oyster in the presence of NTA or EDTA to the stability constants of the cadmium-complexing agent complexes.

To gain a deeper insight into the mechanism of Cd^{2+} and CdNTA uptake we performed substrate variations. Figure 4 displays the dependence of the initial uptake rate on the concentration of the substrates Cd^{2+}, CdNTA or NTA in the presence of glucose. In the absence of NTA we observed saturation kinetics and straight lines in the reciprocal plots (inset). From these reciprocal plots a Km of 0.3 ± 0.1 mM was obtained and a $V_{max.}$ of 5.2 ± 2.4 n mol/min x mg dry weight. The presence of increasing concentrations of NTA reduced the rate of Cd^{2+} transport. When measuring the uptake rate via ^{14}C-NTA, straight lines instead of saturation kinetics were observed (Fig.4), in agreement with recent observations that the uptake of NTA occurs by free diffusion (Rösick et al., 1985). Again there were no differences in the uptake rates of NTA or Cd + NTA, when measurement was via ^{14}C-NTA.

From our results it is clear, that Cd^{2+} is taken up by a transport system the rate of which is largely dependent on the presence of glucose. Addition of NTA reduces the uptake of Cd^{2+} due to formation of CdNTA complexes. Despite the high affinity of NTA for Cd^{2+}, still most of the cadmium is incorporated into the cell via the transport system, probably due to its very high transport rate. Only in the presence of an excess of NTA does the concentration of unbound Cd^{2+} seem to be too low to permit considerable uptake via the transport system.

In addition to the rates and mechanisms of uptake, we were interested in finding out whether these data correlate with physiological effects of Cd^{2+} and NTA on yeast cells. Therefore we examined the kinetics of cell growth in the presence of 0 to 50 µM $CdCl_2$ and various amounts of NTA. We observed inhibition of cell growth with increasing concentrations of cadmium (Table 3). The lowest effective concentration was 2 µM (= 230 µg/ml), whereas at 50 µM (= 6 mg/l) the cells ceased growth completely. These results are in accordance with those of Gadd and Mowll (1983), who observed no decrease in the viability of yeast cells incubated with 5 µM $CdSO_4$ in the absence or presence of glucose and of those incubated with 50 µM $CdSO_4$ in the absence of glucose, whereas at higher concentrations or at 50 µM Cd^{2+} in the presence of glucose a rapid loss of cell viability was detected.

Table 3. Growth rates of yeast cells

conditions	growth rate without preincubation (%)	growth rate after 15 h preincubation with Cd^{2+} (%)
control	100[a]	100
2.5 µM Cd^{2+}	80	85
5 "	71	77
10 "	57	70
20 "	36	68
20 " + 20 µM NTA	45	
20 " + 40 "	52	

[a] doubling time was 1.2 hours

Addition of NTA at a ratio NTA/Cd of 1 had a small but significant protective effect. When NTA was present in excess this effect was enlarged, but a total reversal of growth inhibition could not be achieved, although under these conditions the concentration of free Cd^{2+} ions is rather low. NTA by itself has no effect on growth under these conditions (Rösick et al. 1985). These results agree with our uptake studies, which showed a considerable transport of Cd^{2+} under similar conditions. When the growth rate was measured with yeast cells, which had been preincubated for 15 hours with the same cadmium concentrations, the cadmium effect was considerably smaller than without preincubation. These results can be explained by the synthesis of cadmium binding proteins, which reduce the effective concentration of unbound Cd^{2+} within the

cell, in agreement with the observation of Berthe-Corti et al. (1984) ie., that the permanent presence of cadmium is necessary to achieve inhibition of growth.

Our physiological studies support the results from Cd^{2+} uptake measurements in the absence and presence of NTA, indicating that complexing agents do not effectively protect cells from incorporation of metal ions. Therefore, in general, the presence of NTA will enhance the environmental hazard of cadmium by preventing sedimentation of Cd^{2+} in waste water or by dissolving low soluble cadmium salts from the sediments and thus making this and other metal ions available to living cells. A considerable protective effect occurs only under special conditions, when NTA is present in large excess. Although no significant amounts of the CdNTA complex permeate the membrane, binding and transport proteins will effectively compete with NTA for metal ions and consequently will lead to an incorporation of these ions.

Moreover it is evident that the cadmium concentrations, permitted in waste water by environmental acts (in Germany: 1 mg/l) would have a drastic effect on the growth of yeast and possibly also of other organisms.

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