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Lead toxicity in *Saccharomyces cerevisiae*

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Abstract The effect of Pb on *Saccharomyces cerevisiae* cell structure and function was examined. Membrane integrity was assessed by the release of UV-absorbing compounds and by the intracellular K^+ efflux. No leakage of UV_{260}-absorbing compounds or loss of K^+ were observed in Pb (until 1,000 μmol/l) treated cells up to 30 min; these results suggest that plasma membrane seems not to be the immediate and primary target of Pb toxicity. The effect of Pb on yeast metabolism was examined using the fluorescent probe FUN-1 and compared with the ability to reproduce, evaluated by colony-forming units counting. The exposition of yeast cells, during 60 min to 1,000 μmol/l Pb, induces a decrease in the ability to process FUN-1 although the cells retain its proliferation capacity. A more prolonged contact time (120 min) of yeast cells with Pb induces a marked (> 50%) loss of yeast cells metabolic activity and replication competence through a mechanism which most likely requires protein synthesis.

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Introduction

Heavy metals are widely used in industrial processes and can be released in the environment, which causes harmful effects on ecosystems and public health. In the case of Pb, this metal occurs in the environment as a consequence of the natural weathering process of rocks and mainly due to human activities. Several environmental regulations have been proposed in USA and European Union in order to eliminate or reduce the use of Pb namely in gasoline addictives, paints, solders and water systems. At the present, examples of Pb applications are as follows: production of accumulators and lead–acid batteries used in the cars, colouring element in ceramic glazes, polyvinyl chloride plastic, bullets and in radiation shield. Thus, it is not surprising that the production and consumption of Pb is increasing worldwide; in 2009, the total annual production was about 8.8 million tonnes (International Lead and Zinc Study Group, http://www.ilzsg.org/static/statistics.aspx).

In opposition to Cu and Zn, which are essential metals for optimal growth of cells, although may also be toxic in certain conditions, Pb, Cd and Hg have no known biological roles (Gadd 1993). Lead poisoning was documented in ancient Greece and China and has been linked to the fall of the Roman Empire (Gilfillan 1965). In humans, Pb can cause blood and brain disorders. It is accumulated in "soft tissues" and bone over time; it remains in a semi-permanent reservoir and can be released long term after the initial absorption (ATSDR 2007). Pb is considered as a priority pollutant by the US Environmental Protection Agency (US-EPA 2006).

Yeast cells, particularly *Saccharomyces cerevisiae*, are a suitable model for studying Pb toxic effects. The yeast *S. cerevisiae* is a eukaryote cell that can be easily manipulated and has the genome completely sequenced (Goffeau et al. 1996). It is known that Pb reduce viability (Suh et al. 1999; Soares et al. 2002; Soares et al. 2003), cell growth, DNA/RNA ratio and impair ammonium assimilation in yeast cells (Chen and Wang 2007).

In this work, the effect of Pb on yeast membrane integrity (given by the efflux of K^+ and the release of UV-absorbing compounds) and metabolic activity (evaluated by the ability to process the fluorescent probe FUN-1) was studied. Using a kinetic approach, the loss of metabolic activity induced by Pb was compared with the loss of proliferation capacity, assessed by the classical plate count technique. The role of protein synthesis in Pb-induced toxicity was also studied.

Materials and methods

Strain, media and culture conditions

In this work, the brewing strain of *S. cerevisiae* National Collection of Yeast Culture (NCYC) 1214 was used. The strain was routinely maintained at 4°C on YEPD agar slants (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose and 20 g/l agar).

Pre-cultures were prepared in 10 ml of YEPD broth in 100-ml Erlenmeyer flasks. Cells were incubated at 25°C on an orbital shaker Sanyo Gallenkamp IOC 400 (West Sussex, UK), at 150 rpm, during 24 h.

Cultures were obtained by inoculating 40 ml of YEPD broth, in 100 ml Erlenmeyer flasks, with 1 ml of pre-culture. The flasks containing the culture were incubated under the same conditions as the pre-culture. The time of incubation was 16 h, which corresponds to the end of the exponential respiro-fermentative growth phase/beginning of the diauxic lag phase (data not shown).

Preparation of cell suspensions

After growth, cells were harvested by centrifugation (2,000×*g*, 5 min) and washed twice with 30 mmol/l ethylenediaminetetraacetic acid (EDTA) solution (Merck, Darmstadt, Germany). Subsequently, cells were washed twice with deionised water and resuspended in 2-(*N*-morpholino) ethanesulfonic acid (MES) pH buffer (Sigma-Aldrich, St. Louis, MO, USA) 10 mmol/l, at pH 6.0, with 2% (*w*/*v*) of glucose at approximately 1×10^7 cells/ml. MES is a suitable pH buffer for heavy metal toxicity studies because it does not complex with several metal ions, such as Cd, Cu, Pb and Zn (Soares et al. 1999a; Soares et al. 1999b); in addition, yeast cells maintain viability, and no detectable amount of protein or inorganic phosphate is observed in the filtrates when cells are incubated in this buffer for 48 h (Soares et al. 2000).

Cells concentration was determined spectrophotometrically (Unicam, Helios γ) at 600 nm after appropriate dilution of the samples in EDTA solution (30 mmol/l) to prevent cell aggregation. Calibration curves (absorbance versus number of cells) were previously made.

Contact of yeast cells with Pb

Cell suspensions (40 ml) containing 1×10^7 cells/ml, in 10 mmol/l MES buffer (pH 6.0) with 2% (*w*/*v*) of glucose and the appropriate volume of Pb solution ($Pb(NO_3)_2$) from a stock standard solution of 1 g/l (Merck, Darmstadt, Germany) were shaken in 100-ml Erlenmeyer flasks at 150 rpm, in an orbital shaker at 25°C.

To study the effect of protein inhibition, 25 μg/ml of cycloheximide (Cyh) (Sigma) was added to the cell suspensions (1×10^7 cells/ml), at the same time of Pb solution and under the same conditions described above.

Leakage of UV_{260}-absorbing materials

At defined intervals of time, 4 ml of cell suspension treated with each metal was filtered through a 0.45-μm pore-size filter (Advantec, Japan). Optical density at 260 nm was determined in the filtrates of cell suspensions; buffer solution with the same metal concentration was used as a blank.

Measurement of K^+ efflux

Cell suspensions (20 ml) containing 1×10^7 cells/ml, in 10 mmol/l MES buffer (pH 6.0) with 2% (*w*/*v*) of glucose and 50 μmol/l KCl, were incubated at 25°C, with a magnetic stirring, 5 min prior to the addition of the metals or water (control). Measurement of K^+ efflux was carried out using a potassium combined electrode (Phoenix Electrode Co.) connected to Orion 420A pH/mV metre (Orion, Boston, MA, USA). K^+-selective electrode was calibrated using known additions of KCl.

Assessment of metabolic activity (staining with FUN-1)

Prior or after contact with Pb, cells were washed with deionised water and with 10 mmol/l 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Sigma-Aldrich, St. Louis, MO, USA), pH 7.2, containing 2% (*w*/*v*) glucose; finally, cells were resuspended in HEPES buffer, pH 7.2, with 2% (*w*/*v*) glucose at 1×10^7 cells/ml. FUN-1 (Molecular Probes, Invitrogen, CA, USA) was added to a final concentration of 16 μmol/l from a working solution of 200 μmol/l; FUN-1 working solution was prepared, before use, by diluting a stock solution of

10 mmol/l in HEPES buffer, pH 7.2, with 2% (*w*/*v*) glucose. The cell suspension was incubated in the dark, at 25°C, during 30 min. Then, cells were examined using a Leica DLMB epifluorescence microscope (Leica Microsytems, Wetzlar GmbH, Germany) equipped with a HBO-100 mercury lamp and a filter set I3 (excitation filter BP 450-490, dichromatic mirror 510 and suppression filter LP 515) from Leica. For each sample, at least 400 cells were scored in randomly selected microscope fields.

Images were acquired with a Leica DC 300F camera (Leica Microsystems, Heerbrugg, Switzerland) using a N plan ×100 objective; the images were processed using Leica IM 50-Image manager software.

Evaluation of cell proliferation capacity

Before and after the addition of the metal, samples (100 µl; two replicates) were taken at defined intervals of time, serially diluted with sterile deionised water and plated on YEPD agar (two replicates of 100–200 µl from convenient dilutions). After 3–4 days of incubation at 25°C, the colonies were counted. Original cell suspensions had viability higher than 99%.

Results

Action on membrane integrity

Yeast cell wall is a porous matrix, which allows the passage of most of ions and molecules of molecular mass higher than 760 Da (Klis et al. 2002). In practice, it means that cell membrane is the first structure which is in contact with most of the chemical species. Thus, first of all, the impact of Pb on yeast cell membrane integrity was evaluated. For this purpose, two independent techniques were used: measurement of UV_{260}-absorbing compounds and intracellular K^+ efflux. As it can be seen in Fig. 1, no appreciable amount of UV_{260}-absorbing compounds was released by yeast cells during the 30 min of Pb exposition

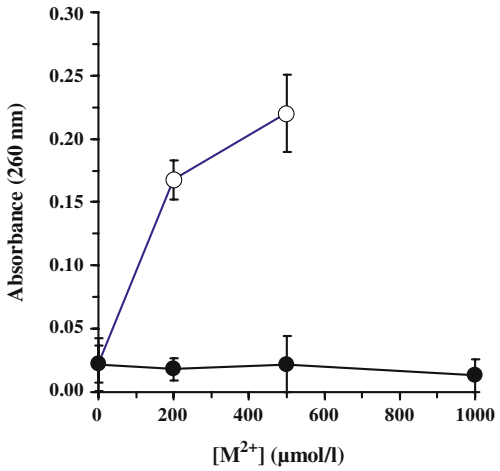

Fig. 1 Leakage of UV_{260}-absorbing cellular components from *S. cerevisiae* NCYC 1214; 1×10^7 cells/ml was suspended in 10 mmol/l MES pH buffer (pH 6.0) with 2% (*w*/*v*) of glucose. Cells were treated with different concentrations of $Pb(NO_3)_2$ (*filled circles*) or $CuCl_2$ (*empty circles*) (control ion), during 30 min. UV-absorbing compounds were determined in the filtrates. Each point represents the mean of three independent experiments performed in duplicate; standard deviations are presented ($n=6$)

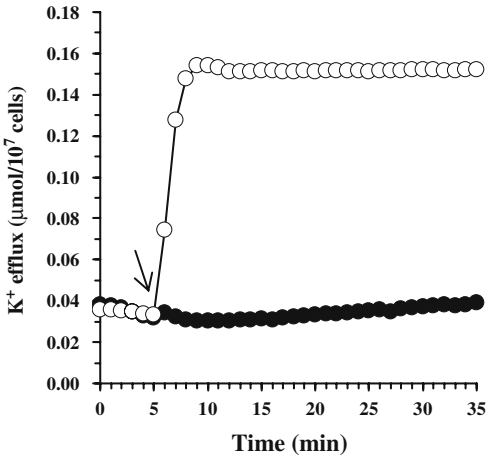

Fig. 2 K^+ efflux from *S. cerevisiae* NCYC 1214, in response to metals; 1×10^7 cells/ml was suspended in 10 mmol/l MES pH buffer (pH 6.0) with 2% (*w*/*v*) of glucose. At the time point indicated by the *arrow*, 1,000 µmol/l $Pb(NO_3)_2$ (*filled circles*) or 200 µmol/l $CuCl_2$ (*empty circles*) (control ion) were added. The efflux of potassium from cells was measured using a K^+-selective electrode. This is a typical example of an experiment performed three times

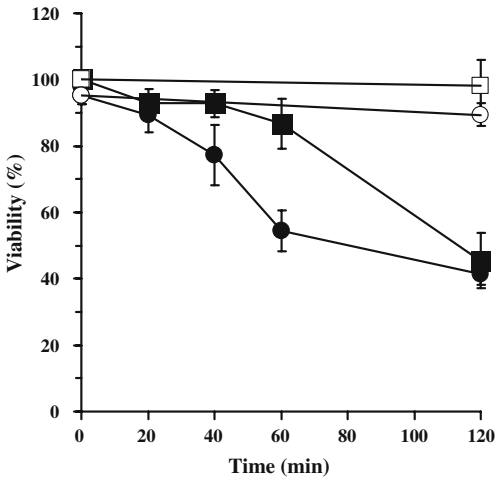

Fig. 3 Viability of *S. cerevisiae* NCYC 1214 during incubation with 1,000 µmol/l $Pb(NO_3)_2$; 1×10^7 cells/ml was suspended in 10 mmol/l MES pH buffer (pH 6.0) with 2% (*w*/*v*) of glucose. Viability was estimated by colony-forming units (c.f.u.) counting (*filled squares*) or by fluorescence microscopy after staining with FUN-1 (*filled circles*). Control: cells incubated in the buffer in the absence of Pb (*open symbols*). Each point represents the mean of three independent experiments performed in duplicate; standard deviations are presented ($n=6$)

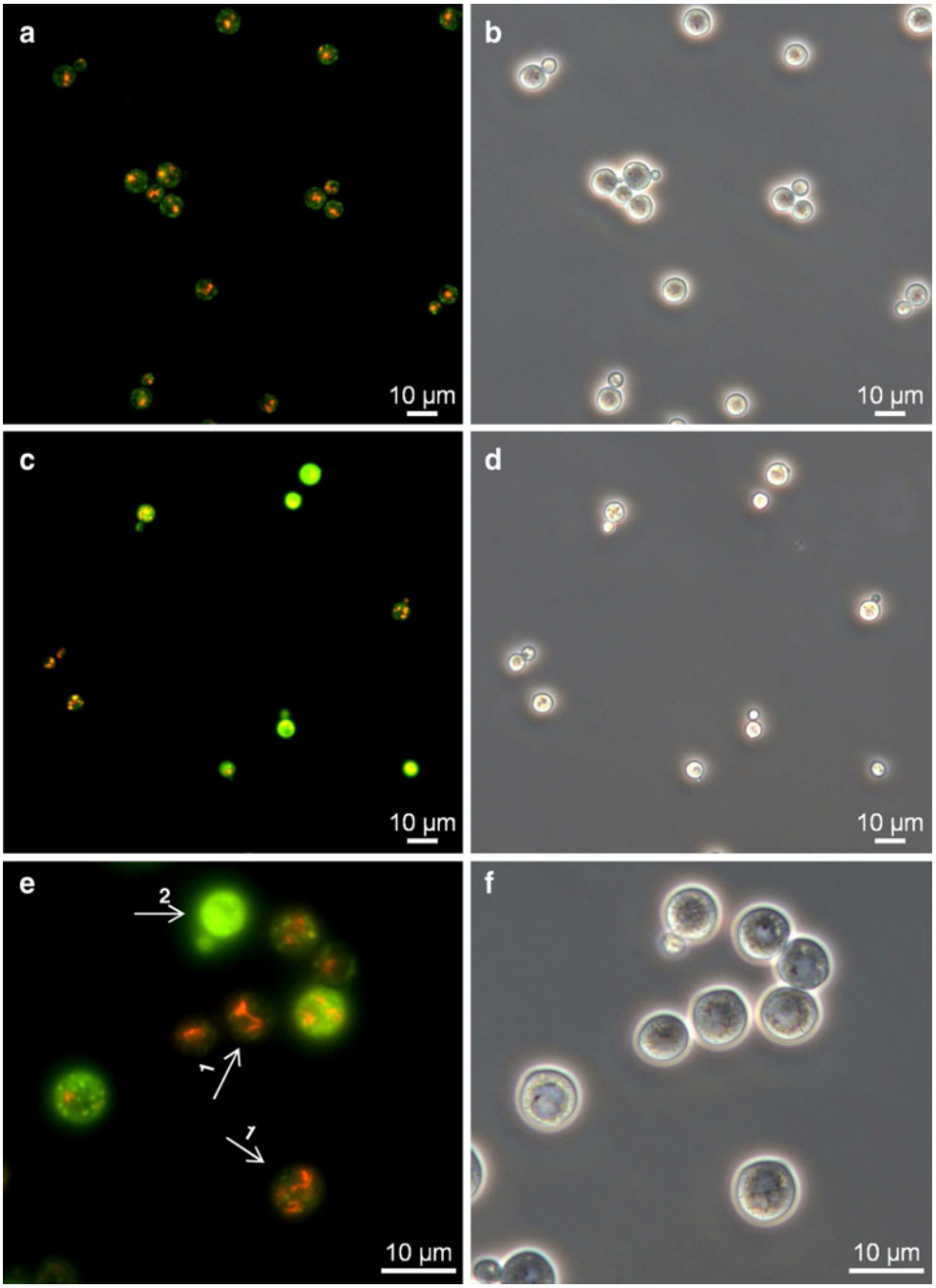

Fig. 4 Cells of *S. cerevisiae* NCYC 1214 stained with FUN-1. Fluorescence micrographs (**a**, **c**, and **e**); phase contrast micrographs of the same cells (**b**, **d**, and **f**). Micrographs of cells before being exposed (**a** and **b**) or after being exposed, during 2 h to 1,000 µmol/l $Pb(NO_3)_2$ (**c**–**f**). *Arrow 1*: metabolic active cells with CIVS; *arrow 2*: metabolic inactive cells (cells without CIVS)

concentrations up to 1,000 µmol/l. Similarly, no induction of the efflux of K+was observed (Fig. 2); for K^+ efflux, results for 0 (water addition) and 500 µmol/l Pb were not shown for pictorial clarity. In both experiments, Cu was used as positive control; in this case, a strong disruption of cell membrane integrity which provoked a fast (within 1–2 min) K^+ efflux (Fig. 2) and the release of UV_{260}-absorbing compounds was observed (Fig. 1). These results strongly suggest that yeast cell membrane is not the first target of Pb toxic effects.

Action on yeast cell metabolism and proliferation capacity

Subsequently, the effect of Pb on yeast metabolic activity was assessed using FUN-1 and compared with the cell proliferation capacity evaluated by classical spread technique (colony-forming units (c.f.u.) counting). The loss of cells viability provoked by the presence of 1,000 µmol/l Pb^{2+}, evaluated by the staining of yeast cells with FUN-1 probe, follows the same pattern of the loss of cell proliferation capacity (Fig. 3).

Metabolic active yeast cells are able to process and concentrate FUN-1 within the vacuoles, originating orange-red cylindrical intravacuolar structures (CIVS) (Fig. 4a, c, and e); on the other hand, non-active cells, with intact membrane, show a diffuse green cytoplasmatic fluorescence (Fig. 4c, e) (Millard et al. 1997). Before Pb treatment, more than 95% of cells display CIVS (Fig. 3 and 4a, b). Pb interferes with FUN-1 intracellular processing (Fig. 3 and 4c, e). This interference seems to be an early response to Pb toxic effects. After 1 h of yeast cells exposition to Pb, 40% of cells lost the ability to process the fluorochrome FUN-1, but retained their proliferation capacity. Nevertheless, a similar loss of 55% of viability was observed by the two techniques after 2 h of contact with Pb (Fig. 3).

The requirement of protein synthesis on the loss of cell proliferation capacity Pb-induced was evaluated by exposing the yeast cells simultaneously to the action of several Pb concentrations and 25 μg/ml of Cyh. Control experiments have shown that 25 μg/ml Cyh was not toxic to yeast cells (data not shown). The presence of Cyh reduces the loss of cell viability (accessed by c.f.u. counting), induced by 750 and 1,000 μmol/l Pb (Fig. 5). These results indicate that the loss of viability induced by Pb is dependent of protein synthesis.

Discussion

In the last two decades, a considerable research effort has been done related to the interaction of *S. cerevisiae* yeast cells with heavy metals, particularly those aspects associated with its use in metals removal (Wang and Chen 2006).

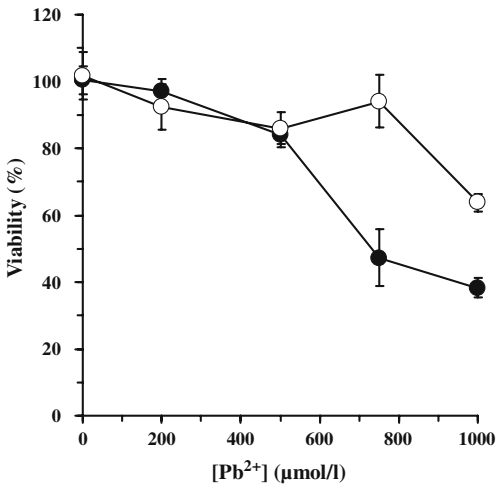

Fig. 5 Influence of cycloheximide in lead-induced toxicity on *S. cerevisiae* NCYC 1214. Cells were incubated with different Pb^{2+} concentrations, under the conditions described in Fig. 1, in the absence (*filled circles*) or in the presence (*empty circles*) of 25 μg/ml of cycloheximide. Viability was estimated by the spread plate technique, as described in "Materials and methods". Each point represents the mean of three independent experiments performed in duplicate; standard deviations are presented (n=6)

It is well known that yeast cell membrane is the initial site of the toxic action of several metals like Cd, Co, Cu, Hg and Zn (Norris and Kelly 1977; Kuypers and Roomans 1979; Gadd and Mowll 1983; Mowll and Gadd 1983; Soares et al. 2003). In the present work, the action of Pb on yeast cell membrane was evaluated by the release of UV-absorbing compounds and K^+ efflux. UV-absorbing compounds are mainly composed by nitrogenous compounds derived from nucleotides and related molecules (Delisle and Phaff 1961). It was shown that the injury of cell membrane by heavy metals (Cd, Co, Cu and Zn) induce the leakage of UV_{260}-absorbing compounds (Joho et al. 1984; Ohsumi et al. 1988; Brady and Duncan 1994; Soares et al. 2002). As a positive control, Cu was used to induce damage on yeast membrane. As a consequence of plasma membrane permeabilization, there is a rapid leakage of UV_{260}-absorbing compounds. This is a very sensitive index of metal toxicity (Soares et al. 2003). In the present work, the exposition of yeast cells up to 1,000 μmol/l Pb, during 30 min, did not induce the leakage of UV_{260}-absorbing compounds.

Metal accumulation in yeast cells is often accompanied by K^+ efflux in an approximate ratio $1Metal^{2+}(in)/2K^+(out)$, in order to maintain the ionic balance (Norris and Kelly 1977; Borst-Pauwels 1981; De Rome and Gadd 1987). Nevertheless, K^+ efflux can occur in a no-stoichiometric relationship, as a consequence of the disruption of yeast cell membrane integrity of *S. cerevisiae*, due to the action of heavy metals, such as Cu Cd, Co and Zn (Norris and Kelly 1977; Gadd and Mowll 1983; Mowll and Gadd 1983; Ohsumi et al. 1988; Brady and Duncan 1994). In the present study, the exposition of yeast cells up to 1,000 μmol/l Pb, during 30 min, did not provoke a K^+ efflux. Cu was also used as a positive control. Together, the data obtained by two independent techniques evidenced that yeast plasma membrane is not the immediate and primary target of Pb toxicity in *S. cerevisiae*.

When metabolic active cells are stained with FUN-1, red-orange CIVS could be observed (Fig. 4e); the mechanism of FUN-1 processing seems to involve the reaction with reduced glutathione (GSH), in cytosol, in an enzyme-mediated process, originating the formation of glutathione conjugates. After the biochemical modification, the dye is transported and sequestered in the vacuole as CIVS (Millard et al. 1997). The exposition of yeast cells to 1,000 μmol/l Pb, during 1 h, hindered the CIVS formation (Fig. 3); however, these cells retained their ability to reproduce after being plated on YEPD agar medium without Pb, which ultimately define the cell viability (Fig. 3). Since the decrease in the ability to process FUN-1 preceded the loss of replication competence, these results suggest that intracellular FUN-1 processing gives an early indication of toxicity induced by Pb.

It is described the involvement of GSH and vacuole in the detoxification of several metals. The sequestration of metals in the vacuole seems to be a form of cytosolic regulation of essential ions or a mechanism of the detoxification; examples of metals that are sequestered in the vacuole are Co, Mn, Ni and Zn (Gadd 1993; Ramsay and Gadd 1997). In the case of toxic metals, the compartmentalisation in the vacuole prevents its presence in the cytosol and the consequent toxic effects. GSH is also involved in the detoxification of several metals, such as Cd and Se (Gharieb and Gadd 2004), most likely by complexation; as a consequence, there is a decreasing of metals availability for toxic interactions, such as the inhibition of thiol-containing enzymes. Taking into account the mechanism underlying the cell staining with FUN-1 probe, the inhibition of CIVS formation in Pb exposed cells may suggest that one of them or both mechanisms of defence reported above could be implicated in Pb detoxification.

After 2 h of Pb exposition, the percentage of cell population unable to process FUN-1 fluorochrome was in agreement with the percentage of cells that loss proliferation capacity; these results evidence a more deep toxic effect of Pb on yeast cells.

The mechanism of Pb-induced toxicity seems to involve protein synthesis. This is supported by the fact that the incubation of cells in the presence of Cyh (in eukaryotes, Cyh inhibits cytoplasmatic protein synthesis at the ribosomal level) (Kuo et al. 1973) attenuated the toxic effect (assessed by c.f.u. counting) induced by high Pb concentrations. The reduction of Pb bioavailability due to its complexation by Cyh cannot be excluded; however, taken into account the molar ratios ([Cyh]/[Pb]) used (0.12 and 0.089, for 750 and 1,000 μmol/l Pb concentrations, respectively), the alleviation of Pb toxic effects cannot be attributed to Pb complexation. In the limit (if all the Pb is complexed with Cyh), a small decrease of Pb availability (12% and 9% for 750 and 1,000 μmol/l, respectively) could be provoked; this means that the presence of Cyh would reduce Pb concentrations from 750 and 1,000 to 660 and 911 μmol/l, respectively. For these concentrations, in the absence of Cyh, a viability of 62% and 44%, respectively, is predicted (Fig. 5). These results are markedly different from those obtained in the presence of Cyh (94% and 64%, respectively).

In conclusion, the results presented in this study evidence that plasma membrane is not the immediate and primary target of Pb toxicity in *S. cerevisiae*. The loss of cell metabolic activity (evaluated by the ability to process FUN-1) seems to be an early indicator of Pb toxicity. A more prolonged contact (2 h) of yeast cells with Pb provokes a marked (>50%) loss of yeast cell metabolic activity and proliferation capacity through a mechanism which requires protein synthesis.

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