Sub-cellular Location of Mercury in Yeast Grown in the Presence of Mercuric Chloride

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SUMMARY

The distribution of $^{203}\text{Hg}$ in Saccharomyces cerevisiae grown in the presence of mercuric chloride has been examined by physical and chemical fractionation procedures and autoradiography. The major fraction of the bound mercury is tightly bound to the wall. A significant quantity of mercury penetrates to the cytoplasm but only a minor fraction is present as low molecular weight components. The wall-associated mercury is not readily released by extraction with sodium hydroxide or ethylenediamine but a major fraction is solubilized by Pronase and Helicase treatment. Isolated walls are capable of binding their own weight of mercury to high-affinity adsorption sites. The major role of the cell envelope in the in vivo binding of mercury and the penetration to the cytoplasm of mercury was confirmed by autoradiography.

INTRODUCTION

Growth of yeasts is inhibited by most heavy metals which have been examined (White & Munns, 1951). Of the group comprising cadmium, lead and mercury, the last has been extensively characterized as an inhibitor of enzymes due to its binding to a number of functional groups (Vallee & Ulmer, 1972). The extracellular $\beta$-glucosidase and $\beta$-fructosidase of yeast have been shown to be extremely sensitive to mercury (Mealor & Townshend, 1968; Kaplan & Tacreiter, 1966). However, despite the well-documented phenomenon of mercury inhibition of yeast, at both the molecular and cell level, there has been no investigation of the distribution of mercury within the cell. We attempted to define the distribution of mercury in growing yeasts and to indicate the general nature of the mercury-binding sites.

METHODS

Organism. Saccharomyces cerevisiae strain Y95 (Department of Microbiology Yeast Culture Collection), originally isolated from a baker's yeast preparation obtained from Sigma, was maintained on agar slants containing 2% (w/v) glucose, 0.18% Difco yeast extract and inorganic salts. All experiments were performed upon cultures grown through two transfers of the defined liquid medium described below.

Growth medium. The medium contained the following components (g/l): $\text{KH}_2\text{PO}_4$, 1.76; $(\text{NH}_4)_2\text{HPO}_4$, 0.88; $(\text{NH}_4)_2\text{PO}_4$, 0.88; $\text{MgSO}_4\cdot7\text{H}_2\text{O}$, 0.16; $\text{NaCl}$, 0.008; $\text{FeSO}_4\cdot7\text{H}_2\text{O}$, 0.004; $\text{ZnSO}_4\cdot7\text{H}_2\text{O}$, 0.004; $\text{MnSO}_4\cdot3\text{H}_2\text{O}$, 0.004; $\text{CuSO}_4\cdot5\text{H}_2\text{O}$, 0.0004; inositol, 0.00299; pantothentic acid, 1.72 x 10⁻⁴; biotin, 1.97 x 10⁻⁷; glucose, 8.0. The medium was filter-sterilized with a 0.4 μm Millipore filter. Mercury, as HgCl₂, was freshly prepared and filter-

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sterilized similarly. Radioactive mercury was obtained as $^{203}$HgCl$_2$ from International Chemical & Nuclear Corp., Irvine, California, U.S.A. Samples from all fractionation steps were counted in duplicate. Five replicated samples were counted for adsorption isotherms.

Inoculation. Growth medium (50 ml) was loop-inoculated from agar slant culture and incubated at 30 °C with shaking until turbidity reached an absorbance reading of 0.4 for a 1:10 dilution in a 1.0 cm light path at 600 nm. The mercury-containing medium (46 ml) was then inoculated with 4.0 ml of this culture. Mercury concentrations were in all cases $2.5 \times 10^{-8}$ M-HgCl$_2$ at a specific activity of approximately 0.6 Ci/g-atom mercury. Mercury toxicity, as evidenced by a reduction in growth rate, is not acute at this concentration but does result in an approximate doubling of the mean generation time.

Cell harvesting and fractionation. Cultures were usually harvested after about 15 h, at which time the turbidity had reached approximately 2.0 absorbance units. Cells were harvested by centrifugation at 1000 g for 10 min at 4 °C, washed with 25 ml 0.1 M-KCl and quantitatively transferred to 40 ml glass homogenizing bottles containing 50 g glass beads (Glasperlen 0.45 to 0.50 mm, Canlab, Toronto, Canada). Cells were homogenized for 90 s in a Braun MSK 2876 homogenizer cooled with solid carbon dioxide. The extent of breakage was checked by phase microscopy. The broken-cell suspension was quantitatively removed from the homogenizing bottles by repeated rinsings with 0.1 M-KCl. Walls were sedimented by centrifugation as before and washed with four changes of 25 ml 0.1 M-KCl. Centrifugal fractionation was performed as indicated. Ultracentrifugation was performed with a Beckman Spinco L2-65B and SW-40 rotor. Ultrafiltrations were performed with Diaflo UM-10 membrane filters at 4 °C. Dialysis was performed with dialysis tubing which had been washed with hot ethylenediamine-tetra-acetic acid and rinsed thoroughly with glass-distilled water.

Radioactivity measurements. All samples were counted on a Nuclear Chicago 4233 automatic counting system. Coincidence corrections were performed where necessary and all results computed as percentages of total label at zero time. Uninoculated controls were also counted in each experiment.

Chemical and enzymic extraction of walls. Walls, isolated as described previously, were freeze-dried and a 0.276 g sample extracted by shaking with 69 ml ethylenediamine for 8 h at 37 °C, and then for a further 72 h with periodic shaking three times each day (Korn & Northcote, 1960). The suspension was fractionated by centrifuging at 10000 g for 15 min and the supernate decanted. The pellet was then washed successively with similar volumes of ethylenediamine, methanol and diethyl ether. The washed ethylenediamine-insoluble fraction, 0.068 g, was then extracted by shaking with 1 N-NaOH for 72 h at 40 °C. The suspension was sedimented by centrifuging at 10000 g for 5 min. The supernate appeared slightly turbid and so was re-centrifuged at 10000 g for 15 min at 4 °C. The remaining supernate was neutralized with HCl to pH 7.08, adjusted to 50 ml volume and dialysed against 1500 ml distilled water in a rocking dialyser at 4 °C to reduce the salt concentration. The dialysis sac contents were then concentrated to 3 ml by addition of polyethylene glycol to the dialysate. The concentrated residue was then adjusted to pH 6.2 with HCl, and 20 mg of Helicase (Industrie Biologique Franqaise S. A.) dissolved in 10 ml of 0.025 M-tris-citrate buffer at pH 6.5 were added. After shaking at 30 °C for 3 h, a further 13.8 mg Helicase in 1.5 ml buffer were added and the sample re-incubated for 7 h to establish whether or not the initial 3 h digestion had been adequate.

Enzymic extraction of cell walls. Freeze-dried walls (0.095 g) were suspended in 10 ml of Helicase solution (1 mg Helicase/ml 0.025 M/tris-citrate buffer pH 6.5). The suspension was shaken for 3 h and completeness of digestion checked by the addition of a further 5 mg of
Fractionation of disrupted cells harvested at the late logarithmic phase of growth revealed that nearly all the mercury present was associated with the cell, little remaining in the spent growth medium (Fig. 1). The major fraction of the bound mercury was associated with the wall and only a small proportion of this appeared to be in weakly bound ion-exchange positions, as evidenced by the small quantity removable by washing with potassium chloride. Very little of the total cell-associated mercury was soluble, as defined by failure to sediment in 3 h at 180,000 g. The cytoplasmic fraction was further characterized by an experiment in which ultrafiltration was used to establish the amount of mercury associated with molecules of less than 10^3 Daltons in size (Fig. 2). It is evident that mercury is not extensively bound to
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Hg-grown S. cerevisiae, 100.0 %

$1_{10}$ (medium), 1.2 %  $1_{10}$ (cells)

$1_{10}$ (wash), 0.7 %  $1_{10}$ (cells), 88.9 %

Homogenizer treatment (disrupted cells), 86.4 %

$1_{10}$ (cytoplasm), 17.8 %  $1_{10}$ (cell walls)

UM-10 filtration

Filtrate, 1.2 %  Filter, 14.3 %  $1_{10}$, 7.0 %  $1_{10}$, 55.1 %

Filter rinsing, 14.1 %  Adsorbed to filter, 0.2 %

Fig. 2. Percentage distribution of total $^{203}$Hg: fractionation of Hg-grown S. cerevisiae by centrifugation and ultrafiltration after cell disruption. $1_{10}$ and $1_{10}$, 1000 g for 10 min, supernate and pellet respectively. See Methods for details.

Fig. 3. Distribution of cell-associated $^{203}$Hg: electron microscope autoradiography of Hg-grown S. cerevisiae. Distances are measured from the cell centre. The regions differentiated are: W-1, grains lying outside of the wall within one grain diameter of the wall; W-0, grains overlying the wall; W+1, grains lying inside of the wall within one grain diameter of the wall; C, grains overlying the cytoplasm at distances greater than one grain diameter from the wall. (a) 5 h culture; (b) 3 h culture; (c) 0 h culture.
Table 1. Loss of $^{203}$Hg during fixation and embedding procedures

<table>
<thead>
<tr>
<th>Incubation period (h)</th>
<th>Percentage of total incorporated $^{203}$Hg appearing in fixation supernatates</th>
<th>Percentage of total incorporated $^{203}$Hg appearing in embedding supernates*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.1</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>6.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* This value does not include the amount released into the final embedding medium but from the autoradiography this was crudely estimated always to have been $\leq 15\%$ of the incorporated $^{203}$Hg. Generally, this proportion was probably $5\%$.

Walls from Hg-grown $S.\ \text{cerevisiae}$, 100%

- Ethylenediamine digestion (37 °C)
  - P, $69.9\%$
  - S, $4.0\%$
- Ethylenediamine wash
  - P, $6\%$
  - S, $0.0\%$
- Methanol wash
  - P, $0.0\%$
  - S, $0.0\%$
- Diethyl ether wash
  - P, $0.9\%$
  - S, $0.9\%$
- Sodium hydroxide digestion (40 °C)
  - P, $7.9\%$
  - S, $24.1\%$
- Helicase digestion (30 °C)
  - P, $62.8\%$
  - S, $14.0\%$
  - Non-dialysable, $18.1\%$
  - Dialysate, $0.0\%$

Recovery from dialysis sac, $14.3\%$

Adsorbed to dialysis sac, $3.8\%$

Fig. 4. Distribution of wall-associated $^{203}$Hg: fractionation of walls from Hg-grown $S.\ \text{cerevisiae}$ by chemical and enzymic extraction and dialysis. See Methods for details. P, pellet; S, supernate.

small molecules since little of the cytoplasmic mercury passed through a UM-10 ultra-filter. It was established that the mercury on the filter was in fact retained owing to limiting porosity, rather than to binding to the filter, by subsequently demonstrating that this could be readily removed from the filter surface (Fig. 2).

An attempt was made to verify the predominant cell-surface distribution of mercury with electron microscope autoradiography. The results (Fig. 3) support the view that the major
Table 2. Enzymic solubilization of mercury from yeast walls

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pronase</th>
<th>Helicase</th>
<th>Final*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helicase</td>
<td>—</td>
<td>37.8</td>
<td>37.8</td>
</tr>
<tr>
<td>Denatured Helicase</td>
<td>—</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Pronase + Helicase</td>
<td>6.1</td>
<td>44.5</td>
<td>49.5</td>
</tr>
</tbody>
</table>

* After incubation, a further quantity of Helicase was added to establish that Helicase activity was not limiting.

mercury fraction was associated with the cell envelope. It was also apparent that as the incubation progressed there was a net movement of mercury from the wall to the cytoplasm. Slight differences between widths of comparable regions in the different histograms reflect actual differences in cell diameter and wall thickness. A qualification which should be applied to these results is that due to the loss, and possible redistribution, of $^{203}\text{Hg}$ during fixation and embedding. While it is not possible to determine the extent of redistribution, it was shown that losses were relatively small (Table 1). The results support the distribution determined by cell fractionation. The choice of regions, on either side of the wall, corresponding to a single grain diameter (Fig. 3) permits the histogram to reflect the approximate degree of resolution obtainable in the present study, i.e. the degree of resolution obtained is approximately equal to one grain diameter.

After the establishment of the wall as a major site of mercury binding, a characterization of this fraction in terms of its chemical extractability was attempted (Fig. 4). Ethylenediamine digestion released very little of the wall-associated mercury. Methanol and diethyl ether yielded almost negligible solubilization of mercury. Sodium hydroxide solubilized a significant fraction which was essentially a high molecular weight component, as revealed by dialysis. Helicase solubilized a significant fraction of the sodium hydroxide-extracted residue, suggesting that there may have been a considerable protein fraction not released by sodium hydroxide because of association with wall glucan. However, since the activity of Helicase upon yeast cell walls has been deduced from studies on unextracted walls (Anderson & Millbank, 1966), there is a great deal of uncertainty as to the correct interpretation of this result. For this reason a further experiment using both Pronase and Helicase digestion was performed upon unextracted walls (Table 2). The results reinforce the opinion that protein associated with wall glucan was responsible for a major fraction of the bound mercury. The failure of Pronase alone to solubilize appreciable quantities of mercury probably reflects substrate inaccessibility. In each of these experiments (Table 2), a final addition of Helicase was made and the incubation prolonged to ensure that mercury release was not limited by enzyme activity. The control, using denatured Helicase, was employed to establish that Helicase-induced release was not simply due to exchange of mercury from the wall to the soluble enzyme preparation.

In order to define further the binding of mercury to walls ($v$), adsorption of mercury to isolated walls was examined. Under the conditions used in these experiments it was established that equilibrium binding of mercury from initial solution concentrations of $1 \times 10^{-2}$ to $1 \times 10^{-10} \text{M-}\text{HgCl}_2$ could be established within about 25 min. An adsorption isotherm covering this range is shown in Fig. 5. A Scatchard plot for adsorption over the concentration ($C$) range employed in the previous whole cell experiments suggests that there was a strong primary binding up to $v \approx 4 \times 10^3 \mu\text{g-atom Hg bound/g wall}$, followed by a
Fig. 5. Binding of mercury to isolated walls, \( v \) (\( \mu \)g-atom Hg bound/g wall), as a function of mercury concentration, \( C \) (\( \mu \)g-atom Hg free/l).

Fig. 6. Scatchard plot for binding of mercury to isolated walls. \( v \), \( \mu \)g-atom Hg bound/g wall; \( C \), \( \mu \)g-atom Hg free/l. Horizontal bar lines indicate two standard deviations.
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weaker secondary binding (Fig. 6). Although primary binding appeared to involve sites of essentially the same order of affinity it is certainly not claimed that these sites are homogeneous. Unfortunately, at these very low mercury levels the results do not permit a definitive statement about putatively linear regions in the Scatchard plot. A good deal of experimentation, including attempts to perform equilibrium dialysis, was addressed to this problem but, because of the propensity of mercury for binding to a variety of materials including ultrafilters and even exhaustively washed dialysis membranes, none of the methods attempted was satisfactory.

The saturation value for the primary adsorption (Fig. 6) indicates that yeast walls are capable of tightly binding approximately their own weight of mercury. This result seems somewhat surprising but has since been verified in a number of independent experiments using different preparations of walls.

DISCUSSION

Rothstein (1959) has reported the binding of mercury to the plasma membrane of yeast but intracellular penetration of mercury was not observed. Kaplan & Tacreiter (1966) also reported the failure of mercury to penetrate the yeast cell envelope. However, in neither case was there direct proof that mercury did not enter the cytoplasm. In the present study it is apparent that intracellular mercury is not due to the release of envelope-bound mercury during the disruption process, since electron microscope autoradiography supports the results of the physical separation procedures. Penetration of the cell by mercury may occur by a number of possible mechanisms. If mercury is associated with a high-affinity anion such as chloride, two quite probable mechanisms are that the mercury may permeate the envelope as an essentially uncharged species (Cotton & Wilkinson, 1962) or may bind to the membrane itself and, as a consequence, render the membrane permeable to mercury. The present study sheds no light upon these alternatives but does indicate the fact of mercury penetration.

Since mercury is not only taken up preferentially into the wall but may be bound at high-affinity sites in quantities similar to the weight of the wall itself, it becomes of considerable interest to discover the nature of the binding sites. Knowledge of the chemical composition and structure of the yeast cell walls is still somewhat rudimentary and the models advanced are crude' (Kidby & Davies, 1970). However, our information is sufficient to preclude the possibility that this large quantity of mercury could be mon-atomically bound at individual binding sites. Wall protein and phosphate groups (Kidby & Davies, 1970) would not supply enough high-affinity binding sites. The fact that ethylenediamine, which has a \( \log K_a \) of 14.3 for 1:1 mercury complexes (Vallee & Ulmer, 1972), failed to remove more than a small fraction of the wall-bound mercury arouses great interest in the explicit chemical identity of these binding sites.

The failure of sodium hydroxide to release more than a small fraction of the bound mercury suggests the involvement of the structural protein of the wall. This opinion is reinforced by the significant release by Helicase, which would be expected to release glucan-linked protein in particular (Anderson & Millbank, 1966). The Pronase-facilitated Helicase release suggests that at least one limitation on the action of Helicase is substrate inaccessibility. The small but significant release by Pronase alone further suggests that protein is directly involved in the binding of mercury. It is difficult to imagine that binding sites other than those associated with the wall protein would provide sufficiently tight binding of mercury to account for these results. While there are too few data on the chemistry of mercury complexes to support this view unequivocally, the ranking of known association
constants does suggest that ligands associated with non-protein wall components are less probable candidates for such tight binding (Madsen, 1963).

We are indebted to Dr R. J. Douglas for helpful advice during the course of this work.

REFERENCES


