Extracellular Production of Hydrogen Selenide Accounts for Thiol-assisted Toxicity of Selenite against Saccharomyces cerevisiae

Received for publication, October 27, 2006, and in revised form, January 24, 2007. Published, JBC Papers in Press, January 29, 2007, DOI 10.1074/jbc.M610078200

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Administration of selenium in humans has anticarcinogenic effects. However, the boundary between cancer-protecting and toxic levels of selenium is extremely narrow. The mechanisms of selenium toxicity need to be fully understood. In Saccharomyces cerevisiae, selenite in the millimolar range is well tolerated by cells. Here we show that the lethal dose of selenite is reduced to the micromolar range by the presence of thiols in the growth medium. Glutathione and selenite spontaneously react to produce several selenium-containing compounds (selenodiglutathione, glutathioselenol, hydrogen selenide, and elemental selenium) as well as reactive oxygen species. We studied which compounds in the reaction pathway between glutathione and sodium selenite are responsible for this toxicity. Involvement of selenodiglutathione, elemental selenium, or reactive oxygen species could be ruled out. In contrast, extracellular formation of hydrogen selenide can fully explain the exacerbation of selenite toxicity by thiols. Indeed, direct production of hydrogen selenide by d-cysteine desulphydrase induces high mortality. Selenium uptake by S. cerevisiae is considerably enhanced in the presence of external thiols, most likely through internalization of hydrogen selenide. Finally, we discuss the possibility that selenium exerts its toxicity through consumption of intracellular reduced glutathione, thus leading to severe oxidative stress.

Selenium deserved additional interest when an inverse correlation between cancer mortality rates and geographic distribution of selenium in forage crops was observed (2). Later, numerous epidemiological data, as well as animal studies and supplementation trials, have reinforced the idea of anticarcinogenic properties of selenium (3–5).

Although anticarcinogenic properties of selenium have become a field of intensive investigations, their causes remain elusive. Different non-exclusive explanations have been proposed, including (i) inhibition of DNA synthesis by selenocompounds (6, 7), (ii) induction of DNA repair (8), (iii) induction of apoptosis in cancer cells (9), and (iv) action of selenoproteins (10). However, because the cancer-protective effects keep on increasing at selenium intake levels exceeding that required to maximize the activity of selenoproteins (11), it is unlikely that selenoproteins alone mediate the protection against cancer.

The boundary between cancer-protecting and toxic levels of selenium is extremely narrow. Indeed, protection was observed with a daily allowance of 200 μg of total selenium (4), whereas the tolerable upper intake level is estimated to be 400 μg for adults (12). The use of selenium-enriched supplements may therefore be beneficial but becomes dangerous if doses are too high. The mechanisms of selenium toxicity remain largely unknown and need to be further investigated. To this end, we chose the yeast Saccharomyces cerevisiae to study the effect of selenite on viability. This model organism has the advantage to be devoid of selenoproteins (13). Therefore, its growth can be compared in the presence or absence of selenium without any interfering effect of selenoproteins. The impact of selenium on yeast has already been studied (14–20). Dual effects of selenium are observed. Low concentrations protect the cell against mutagenesis, whereas millimolar concentrations are toxic.

In mammals, selenium is translationally incorporated in a small number of proteins under the form of selenocysteine. Several of these selenoproteins display antioxidant activities. Others are involved in immune function, sperm motility, or production of thyroid hormones (1). As a clue of the miscellaneous role of selenoenzymes, selenium deficiency is implicated in various pathologies (1).
product of the reduction of selenite, must be at the origin of the thiol-assisted selenite toxicity.

EXPERIMENTAL PROCEDURES

Materials—Glutathione, sodium selenite, l-cysteine, dithiothreitol, DL-selenocystine, xanthine, 2-morpholinoethanesulfonic acid (MES),6 milk xanthine oxidase, Escherichia coli thioredoxin reductase were from Sigma. HNO3 (Suprapur) was from Merck. NADH, NADPH, lactate dehydrogenase from rabbit muscle, catalase from beef liver and superoxide dismutase (SOD) from bovine erythrocytes were purchased at Roche Applied Science. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) was from Promega (Madison, WI).

Strain and Media—The S. cerevisiae strain DTY7 (MATα ura3–52 leu2–3,112 his6 CUP1R-3) was kindly provided by Dr. D. J. Thiele (University of Michigan Medical School). Rich YT medium contained 1% yeast extract (Difco), 1% Bacto-Trypton (Difco), and 2% glucose. Synthetic dextrose (SD) minimal medium contained 1% yeast extract (Difco), 1% Bacto-Trypton (Difco), and 2% glucose. Synthetic dextrose (SD) minimal medium contains 0.67% yeast nitrogen base (Difco), 2% glucose and 50 μg/liter of histidine, leucine, and uracil. This medium was buffered at pH 6.0 by the addition of 50 mM MES-NaOH.

Preparations of Selenodiglutathione and of Red Elemental Selenium—Selenodiglutathione was obtained from selenite and glutathione as described previously (26), with the following modifications. The reaction mixture was made by successive additions of 30 μl of 0.2 mM HCl, 10 μl of 50 mM sodium selenite, and 20 μl of 100 mM glutathione. After 10-min incubation at room temperature, reaction products were applied on an Alltech high-performance liquid chromatography column (0.32 × 15 cm, 5 μm, from Alltech) equilibrated in 0.05% (v/v) acetic acid/water. Elution was performed at a flow rate of 0.4 ml/min using a linear gradient from 0.05% acetic acid to 100% methanol in 20 min. Concentration of selenodiglutathione was calculated using a light absorption coefficient of 1.87 A280 units mM−1 (27).

To obtain red elemental selenium, 1 ml of 20 mM potassium phosphate (pH 6.5) containing 1 mM sodium selenite and 10 mM glutathione was incubated for 5 min at room temperature and then centrifuged for 10 min at 18,000 × g. The pellet was washed twice with 20 mM potassium phosphate (pH 6.0) and resuspended in 0.5 ml of this buffer. Concentration of elemental selenium was determined after oxidation into selenite by concentrated nitric acid and fluorometric quantitation of resulting selenite with the help of diaminonaphtalene (28).

Purification of Proteins—E. coli thioredoxin was purified from E. coli JM101TR cells (29) harboring plasmid pFP1TRX, as described previously (30). Concentration of thioredoxin was calculated using a light-absorption coefficient of 12.65 A280 units mM−1 (30). E. coli d-cysteine desulfhydrase was purified from E. coli XL1-Blue cells (Stratagene) harboring plasmid pKKyedO (31). Specific activity of purified d-cysteine desulfhydrase was 34 s−1, when assayed in the presence of 250 mM d-cysteine under previously described conditions (31). Concentration of d-cysteine desulfhydrase was calculated using a light-absorption coefficient of 0.599 A260 units mg−1 ml and a Mw of 70,044 (31). According to SDS-PAGE analysis, thioredoxin, and d-cysteine desulfhydrase were at least 95% homogeneous.

Toxicity Assays—S. cerevisiae strain DTY7 was pregrown overnight at 30 °C in SD minimal medium. Cells were then inoculated in the same medium to obtain an OD650 of 0.02 and left to grow at 30 °C. When the OD650 reached 0.1, the compounds under study were added to the culture. After 1 h at 30 °C under agitation, samples were diluted 1000-fold in water. An aliquot of 200 μl of this dilution was plated onto rich YT agar plates to monitor cell viability. Plates were left to grow for 2 days at 30 °C prior to scoring. When toxicity assays included extra-cellular presence of enzymes, bovine serum albumin (25 μg/ml) was added to the medium to prevent adsorption on the culture tube walls.

Total Selenium Determination—Selenium incorporated in cells was determined by inductively coupled plasma mass spectrometry. To ensure dissolution of elemental selenium particles that may be produced extracellularly during the reduction of selenite by thiols, harvested cells (1.5 ± 0.7 mg of dry weight) were washed three times with 0.1 M potassium sulphite, a solvent of elemental selenium (32), then rinsed with water and lyophilized.

Samples were digested in a closed Ethos Touch Control microwave device from Milestone. Before use, vessel was cleaned by addition of 10 ml of 65% HNO3 and heating at 200 °C during 20 min. Blanks were performed to verify the absence of reagent pollution. Each sample was placed into a 50-ml Teflon digester. Then, 1 ml of 69% HNO3, 2 ml of 30% H2O2 (Trace Select, from Fluka), and 5 ml of water were added. The digester was closed, progressively heated until 180 °C in the microwave (30 min), and then maintained at 180 °C for 15 min. After cooling, mineralized samples were placed in volumetric flasks and analyzed by inductively coupled plasma mass spectrometry on a PQ Excell spectrometer from VG Elemental. The mass spectrometer was fixed on the ratio m/z = 82. Standard used for determinations was the inductively coupled plasma mass spectrometry certified multielement solution 2A, provided by Spex. Intracellular selenium concentrations were calculated by assuming that 1.0 OD650 (Shimadzu spectrophotometer UV-2101PC) corresponds to 0.4 μl of intracellular volume (33).

Enzymatic Assays—Activity of d-cysteine desulfhydrase was monitored through measurement of pyruvate produced from d-cysteine or DL-selenocysteine by using a spectrophotometric method involving lactate dehydrogenase and NADH (31). The overall reaction was performed at 30 °C in a reaction mixture containing 130 μM NADH, 10 units/ml lactate dehydrogenase, 250 μM d-cysteine or 40 μM DL-selenocysteine, and various concentrations of desulfhydrase. Consumption of NADH was deduced from the decrease in absorbance at 340 nm.

SOD activity was deduced from the inhibitory effect of this enzyme on Pyrogallol auto-oxidation (34). Reaction mixtures (490 μl) contained 50 mM Tris-HCl (pH 8.2), 1 mM EDTA, and catalytic amounts of SOD. The reaction was started by the addition of Pyrogallol (10 μl) at a final concentration of 200 μM. The

6 The abbreviations used are: MES, 2-morpholinoethanesulfonic acid; ROS, reactive oxygen species; SD, synthetic dextrose minimal medium; SOD, superoxide dismutase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.
initial rate of Pyrogallol oxidation was calculated by following light absorbance at 420 nm during 10 min.

Initial rates of catalase activity were obtained by measuring the consumption of hydrogen peroxide at 240 nm (35). Initial concentration of hydrogen peroxide in the reaction mixture was 10 mM.

Assay of Superoxide Radical Formation—Production of superoxide ions was monitored spectrophotometrically at 490 nm using the tetratolium dye MTS (36). Assays were performed in SD medium containing MTS at 190 μM, in a double beam spectrophotometer. A reference cuvette with 190 μM MTS alone in SD medium was systematically used.

RESULTS

Glutathione Exacerbates the Toxicity of Selenite toward S. cerevisiae Cells—To evidence an effect of glutathione on the sensitivity of S. cerevisiae to selenite, cell survival was estimated after 1-h incubation in SD minimal medium containing varying concentrations of both glutathione (0–400 μM) and sodium selenite (0–50 μM). Cells were plated on rich medium and left to grow for 2 days. Their ability to form colonies was used as an indicator of viability. In the absence of glutathione, selenite concentrations as high as 5 mM reduced survival by <60% (Fig. 1A). Glutathione addition strongly enhanced the toxicity of selenite (Fig. 1B). For instance, in the presence of 400 μM glutathione and 20 μM selenite, only 12% of the cells survived. Besides, under such conditions of high mortality, we noted that a large proportion of survivors (over 25%) displayed the phenotype of the petite mutant (37).

To know whether the effect of glutathione was mediated by its thiol function, cell survival was monitored after incubation in SD medium containing selenite and supplemented by either l-cysteine or dithiothreitol. Like glutathione, these thiol-containing compounds dramatically increased the toxicity of selenite (Table 1). On the other hand, oxidized glutathione or l-cystathionine, which do not carry reduced thiol groups, did not enhance the toxicity of selenite (Table 1).

Selenite (SeO₃²⁻) is known to react spontaneously with glutathione (GSH) (27, 38–40). The first product is selenodiglutathione (GS-Se-SG, Reaction 1 below). In the presence of excess glutathione, selenodiglutathione is further reduced by glutathione into glutathioselenol (GS-SeH/HS-Se⁻, Reaction 2). Glutathioselenol either spontaneously dismutates into elemental selenium (Se⁰) and glutathione (Reaction 3) or is further reduced by glutathione to yield volatile hydrogen selenide (H₂Se/HSe⁻/Se²⁻, Reaction 4). Finally, H₂Se is readily oxidized by oxygen into elemental selenium (Reaction 5). Excess glutathione can shield hydrogen selenide from oxidation (Reaction 6):

\[ \text{SeO}_3^{2-} + 4\text{GSH} + 2\text{H}^+ \rightarrow \text{GS-} \text{Se-SG} + \text{GSSG} + 3\text{H}_2\text{O} \]  
REACTION 1

\[ \text{GS-} \text{Se-SG} + \text{GSH} \rightarrow \text{GS-} \text{SeH} + \text{GSSG} \]  
REACTION 2

\[ \text{GS-} \text{SeH} \rightarrow \text{Se}^{0} + \text{GSH} \]  
REACTION 3

\[ \text{GS-} \text{SeH} + \text{GSH} \rightarrow \text{H}_2\text{Se} + \text{GSSG} \]  
REACTION 4

\[ \text{H}_2\text{Se} + \frac{1}{2}\text{O}_2 \rightarrow \text{Se}^{0} + \text{H}_2\text{O} \]  
REACTION 5

\[ \text{H}_2\text{Se} + \frac{1}{2}\text{O}_2 + 2\text{GSH} \rightarrow \text{H}_2\text{Se} + \text{GSSG} + \text{H}_2\text{O} \]  
REACTION 6

FIGURE 1. Toxicity of selenite against S. cerevisiae. Strain DTY7 was grown in SD minimal medium. When the optical density reached 0.1 at 650 nm, selenite (A) or selenite plus glutathione (B) were added to the culture. After 1-h incubation at 30 °C, samples were plated onto YT agar to monitor cell viability. In A, glutathione concentrations were as follows: ○, 0 μM; □, 50 μM; △, 100 μM; ◻, 150 μM; ●, 200 μM; ▲, 300 μM; and ▼, 400 μM. Results are expressed as percentage of survival compared with control samples incubated in SD medium alone. Bars represent mean and range for two independent experiments.

TABLE 1

<table>
<thead>
<tr>
<th>Assayed compound</th>
<th>Concentration</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>%</td>
</tr>
<tr>
<td>Oxidized glutathione</td>
<td>200</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>l-Cystathionine</td>
<td>400</td>
<td>115 ± 10</td>
</tr>
<tr>
<td>l-Cysteine</td>
<td>200</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>400</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>
TABLE 2

<table>
<thead>
<tr>
<th>Added compounds(s)</th>
<th>Absorbance at 490 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD</td>
</tr>
<tr>
<td>400 μM glutathione</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>20 μM selenite, 400 μM glutathione</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>15 μM xanthine, xanthine oxidase</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>80 μM xanthine, xanthine oxidase</td>
<td>0.05 ± 0.01</td>
</tr>
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</table>

Along this series of reactions, reactive oxygen species (ROS) such as superoxide ions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^\cdot$), are likely to be produced (24, 41–43). Further experiments described below were designed to evidence which product(s) may account for the high toxicity of selenite:glutathione mixtures.

Extracellular Formation of Reactive Oxygen Species Is Not at the Origin of the High Toxicity of Selenite in the Presence of Glutathione—First, we asked whether ROS were indeed produced upon mixing selenite and glutathione in the synthetic minimal medium. For this purpose, putative superoxide ion production was searched for by using MTS as spectrophotometric probe. This probe is highly sensitive to superoxide at pH 6.0, the pH of the growth medium (36). Incubation of MTS in SD medium containing 20 μM selenite and 400 μM glutathione resulted in a time-dependent increase in the absorbance at 490 nm (Table 2). This signal was quenched by the addition of SOD (Table 2), thus evidencing the generation of superoxide ions by the selenite:glutathione mixture. A part of the MTS signal resisted to SOD addition (Table 2). This remaining signal possibly reflects the reaction of the MTS probe with other reducing agents such as glutathione, glutathioseelenol, or hydrogen selenide. In agreement with this idea, we found that incubation of MTS with glutathione alone in the SD medium promoted a slight but continuous increase in the absorbance at 490 nm (Table 2).

Superoxide ions can be produced by mixing xanthine and xanthine oxidase. Using the MTS assay, we established that a mixture of 15 μM xanthine and 7.5 × 10$^{-3}$ units/ml xanthine oxidase sustained, within 1 h, a signal at 490 nm equivalent to the SOD-quenchable signal produced by the mixture of 400 μM glutathione with 20 μM selenite (Table 2). Cells in SD medium at 30 °C were exposed for 1 h to these concentrations of xanthine and xanthine oxidase, and their survival was surveyed. Yeast cells fully resisted this treatment (Table 3). An additional experiment was performed where xanthine concentration was increased 5-fold. Despite the subsequent increase in ROS production, cell survival remained equal to nearly 100%.

In another set of experiments, free radical scavengers were added to the growth medium at the same time as selenite, glutathione, and S. cerevisiae cells. Neither the O$_2^-$ scavenger SOD, the H$_2$O$_2$ scavenger catalase, nor the OH$^\cdot$ scavenger mannitol, alone or in combination, lowered the toxicity of the selenite:glutathione mixture (37). We verified that added catalase and SOD had remained fully active after the incubation in the presence of the cells. Altogether, these experiments enabled us to conclude that enhancement of selenite toxicity by glutathione cannot be ascribed to the presence of ROS in the growth medium.

Toxicity of Selenodiglutathione for S. cerevisiae Is Low in the Absence of Glutathione—The first step of the reduction of selenite by glutathione is the formation of selenodiglutathione. Because this compound can be easily isolated (27), we monitored S. cerevisiae survival after 1-h incubation in SD medium containing varying concentrations (0–50 μM) of selenodiglutathione. Whatever the added concentration of selenodiglutathione, survival remained larger than 90% (Fig. 2), thus establishing the low toxicity of selenodiglutathione. Nevertheless, in the presence of additional 10–400 μM glutathione, selenodiglutathione induced high mortality (Fig. 2). These results indicate that selenite toxicity in the presence of glutathione must arise from a step beyond the formation of selenodiglutathione.

Actually, such a low toxicity of selenodiglutathione could be expected from the results in Fig. 1. In the presence of 200 μM glutathione, paradoxically, 50 μM selenite is significantly less toxic than 10 μM selenite. Four molecules of glutathione are required to produce one molecule of selenodiglutathione from one molecule of selenite. Consequently, at a 200:50 selenite:glutathione stoichiometry, the main reaction product is selenodiglutathione (39). At a 200:10 stoichiometry, selenodiglutathione is also produced but is allowed to combine downstream with excess glutathione. Products of the further reactions can be expected to contain the agent(s) responsible for the mortality of the cells.
selenodiglutathione, the reaction between glutathione and selenite and before elemental selenium. Beyond the formation of responsible for toxicity are thus located after selenodiglutathione and glutathione were added to the culture. After 1 h at 30 °C, samples were plated onto YT agar to monitor cell viability. Results are expressed as percentage of survival compared with control samples incubated in SD medium alone. Glutathione concentrations were as follows: ○, 0 μM; ●, 10 μM; ■, 20 μM; and ▲, 400 μM. Bars represent mean ± S.D. of three independent experiments.

FIGURE 2. Toxicity of selenodiglutathione in the presence of varying concentrations of glutathione. Strain DTY7 was grown in SD minimal medium. When the optical density reached 0.1 at 650 nm, various combinations of selenodiglutathione and glutathione were added to the culture. After 1 h at 30 °C, samples were plated onto YT agar to monitor cell viability. Results are expressed as percentage of survival compared with control samples incubated in SD medium alone. Glutathione concentrations were as follows: ○, 0 μM; ●, 10 μM; ■, 20 μM; and ▲, 400 μM. Bars represent mean ± S.D. of three independent experiments.

Selenodiglutathione (μM)

Survival (%)

0 10 20 30 40 50

FIGURE 3. Effect of elemental selenium on the viability of S. cerevisiae. Strain DTY7 was grown in SD minimal medium. When the optical density reached 0.1 at 650 nm, various combinations of elemental selenium were added to the culture, either alone (○) or in the presence of 400 μM glutathione (●) or of 400 μM glutathione plus 20 μM selenite (■). After 1 h at 30 °C, samples were plated onto YT agar to monitor cell viability. Results are expressed as percentage of survival compared with control samples incubated in SD medium alone. Bars represent mean and range for two independent experiments.

Selenium protected the cells against the toxicity of a selenite:glutathione mixture. For instance, addition of 200 μM elemental selenium to SD medium containing 20 μM selenite and 400 μM glutathione significantly increased the survival of the cells (Fig. 3). This protective role of elemental selenium will be debated in the discussion part.

Hydrogen Selenide Is Toxic for S. cerevisiae—In the cascade of reactions between selenite and glutathione, the products responsible for toxicity are thus located after selenodiglutathione and before elemental selenium. Beyond the formation of selenodiglutathione, the reaction between glutathione and selenite produces intermediary products such as glutathioselenol or hydrogen selenide (Reactions 2 and 4). These compounds are thought to be short-lived and so should be the toxicity of a selenite:glutathione mixture if they were at its origin. To assess a possible involvement of short-lived products in toxicity, cells were added in tubes in which the reaction between 20 μM selenite and 400 μM glutathione had been triggered earlier. Fig. 4 shows that a selenite:glutathione mixture prepared less than 30 min before addition of the cells was fully toxic. Indeed, after incubation of cells for 1 h in such tubes, survival was <10%. When the mixtures were older than 30 min, toxicity progressively decreased with their age. For instance, upon exposure to a 45-min-old mixture, cell survival exceeded 70%. Hence, toxicity must be associated with transitory products of the reduction pathway.

Thioredoxin reductase catalyzes reduction of selenite into hydrogen selenide according to the following global reaction (Reaction 7 (26)).

\[
\text{SeO}_3^{2-} + 3\text{NADPH} + 5\text{H}^+ \rightarrow \text{H}_2\text{Se} + 3\text{NADP}^+ + 3\text{H}_2\text{O}
\]

REACTION 7

We observed that incubation of S. cerevisiae cells in SD medium containing 20 μM selenite, 500 μM NADPH, 1.4 μg/ml E. coli thioredoxin reductase, and thioredoxin concentrations higher than 1 μM resulted in a high mortality (Fig. 5). Control experiments without NADPH or without thioredoxin reductase did not indicate any significant lethality. The toxicity of the above mixture is thus likely to result from hydrogen selenide. Because reduction of selenite by an excess of thiols also produces H₂Se, we suspected this compound to be at the origin of the exacerbation of selenite toxicity by thiols.

To further assess the toxicity of H₂Se, we produced this compound directly in the growth medium through the catalytic action of E. coli d-cysteine desulphhydrase on d-selenocystine (44) in Reaction 8.
Thiol-assisted Toxicity of Selenite

FIGURE 5. Enhancement of selenite toxicity by the thioredoxin/thioredoxin reductase system. Strain DTY7 was grown in SD minimal medium. When the optical density reached 0.1 at 650 nm, 20 μM selenite, 500 μM NADPH, 1.4 μg/ml thioredoxin reductase, and various concentrations of thioredoxin were added. Results are expressed as percentage of survival compared with control samples incubated in SD medium alone. Bars represent mean and range for two independent experiments. Control samples incubated in SD medium alone.

FIGURE 6. Effect of DL-selenocystine plus D-cysteine desulfhydrase on the viability of S. cerevisiae. Strain DTY7 was grown in SD minimal medium. When the optical density reached 0.1 at 650 nm, DL-selenocystine (40 μM) and various concentrations of D-cysteine desulfhydrase were added to the culture. After 1 h at 30 °C, samples were plated onto YT agar to monitor cell viability. Results are expressed as percentage of survival compared with control samples incubated in SD medium alone. Bars represent mean ± S.D. of four independent experiments.

D-Selenocystine + 2H₂O → 2 pyruvate + 2H₂Se + 2NH₃

REACTION 8

At first, the specific activity of D-cysteine desulfhydrase in SD medium containing 40 μM DL-selenocystine was measured by following formation of pyruvate from DL-selenocystine. This activity was found equal to 0.03 s⁻¹ at 30 °C. Then, we evaluated survival of S. cerevisiae cells after 1-h incubation in SD medium containing 40 μM DL-selenocystine and concentrations of D-cysteine desulfhydrase ranging from 0 to 150 nM. A high mortality was observed at D-cysteine desulfhydrase concentrations larger than 50 nM (Fig. 6). We estimated that such an enzyme concentration catalyzes the overall production of nearly 5 μM H₂Se within 1 h. Finally, we verified that cells survived if DL-selenocystine or D-cysteine desulfhydrase were omitted. Altogether, the above results evidence that H₂Se is highly toxic toward S. cerevisiae. Noticeably, H₂Se doses causing toxicity, as evaluated here, lie in the same range of concentrations as the lethal doses of selenite in toxic selenite-glutathione mixtures.

Production of H₂Se Causes a Large Uptake of Selenium—To further investigate the mechanism of the toxicity, uptake of selenium by yeast cells was followed during a 4-h incubation in growth medium containing either selenite alone or selenite plus glutathione. In the presence of 20 μM or 10 mM selenite alone, selenium in the cells reached 75 μM and 5 mM, respectively (Fig. 7). If selenite (20 μM) was in the presence of glutathione (400 μM), then cellular selenium went up to 5 mM within 4 h (Fig. 7). Other assayed thiols stimulated selenium uptake in a similar manner (Table 4).

Thioredoxin, in the presence of thioredoxin reductase and NADPH, was shown above to promote cellular mortality in the presence of selenite. Thus, we investigated whether selenium uptake by the cells was associated with this mortality. Upon 1-h
incubation in the presence of selenite (20 μM), thioredoxin (1 μM), thioredoxin reductase and NADPH, internalized selenium amounted to more than 3 mM (Table 4).

Finally, to evidence whether hydrogen selenide was the precursor of the selenium uptakes observed above, cells were left to incubate for 1 h in the presence of both D,L-selenocystine (40 μM) and D-cysteine desulphhydrase (50 nM). A marked uptake of selenium (11 mM) was observed. Incubation in the absence of the desulphhydrase resulted in a much smaller increase (0.3 mM) (Table 4).

These results show that hydrogen selenide enters in the cell much more efficiently than selenite. A massive accumulation of selenium may explain the thiol-assisted toxicity of selenite.

**DISCUSSION**

**Role of H₂Se in the Thiol-assisted Toxicity of Selenite**—Spontaneous reaction between thiols and selenite generates ROS (24, 41–43). However, extracellular production of ROS is unlikely to be at the origin of the enhancement of selenite toxicity. First, under our experimental conditions, H₂O₂ concentrations are expected to remain in the micromolar range (28). Because millimolar concentrations of H₂O₂ are required to kill *S. cerevisiae* (45), an involvement of H₂O₂ in the toxicity of a selenite:glutathione mixture can be ruled out. Second, we show here that superoxide ions produced from xanthine and xanthine oxidase are not toxic. Finally, addition of ROS scavengers such as mannitol, catalase or SOD, alone or in combination, does not protect *S. cerevisiae* against the effect of the selenite:glutathione mixtures.

Reduction of selenite by glutathione produces compounds such as selenodiglutathione, glutathioselenol, elemental selenium, and hydrogen selenide (27, 38–40). The toxicity of either selenodiglutathione or elemental selenium is shown here to be negligible. On the other hand, hydrogen selenide is strongly toxic, as evidenced by the high mortality of yeast cells exposed to hydrogen selenide enzymatically generated from D,L-selenocystine. Furthermore, we observed that elemental selenium relieves the toxicity of the selenium:glutathione mixtures. Because it catalyzes the oxidation of hydrogen selenide by oxygen (46, 47), elemental selenium is likely to protect the cells by quenching the accumulation of hydrogen selenide originating from the reaction of selenite with glutathione.

A strong argument in favor of an involvement of hydrogen selenide in toxicity comes from our measurements of selenium intake. Incubation of yeasts in the presence of either (i) selenite and glutathione or (ii) selenite and thioredoxin plus thioredoxin reductase or (iii) D,L-selenocystine and D-cysteine desulphhydrase always resulted in a marked uptake of selenium by the cells. Because these various mixtures have in common the capacity to generate H₂Se, it may be proposed that enhancement of selenite toxicity by thiols results from the entry of selenium inside the cell under the form of hydrogen selenide.

Another short-lived product of the reaction of selenite with glutathione is glutathioselenol. This unstable compound reacts with glutathione to form H₂Se. Consequently, it is difficult to assess whether glutathioselenol contributes by itself to the massive entry of selenium in the presence of glutathione. In this study, we observed that addition to selenite of thioredoxin plus thioredoxin reductase caused high toxicity and promoted a marked increase in cellular selenium concentration. Because of the large size of thioredoxin, it is difficult to imagine that any selenol derivative of thioredoxin corresponding to glutathioselenol can be massively taken up by the cell. Being insoluble, elemental selenium, also, is not likely to cross the cell membrane. Therefore, at least in this case, the only compound likely to enter the cell is H₂Se.

Exposure of yeast cells to micromolar concentrations of selenite in the absence of added glutathione results in a weak selenium uptake (Table 4). A transport of selenite in *S. cerevisiae* has already been reported (19), but no transporter has been identified in this organism so far. The question thus arises to know whether internalization of selenium from selenite is still mediated by H₂Se, although no thiol is added in the growth medium. Possibly, the yeast cells display or excrete some thiol-containing compounds able to react with selenite extracellularly and to reduce it into H₂Se. Another possibility would be that membrane-bound sulphydryl groups participate to the transport. Interestingly, in agreement with this idea, an increase in the amount of sulphydryl groups of such proteins in brush-border membrane vesicles from chick duodenum enhances selenite uptake (48). Otherwise, an H₂Se-independent pathway for selenite internalization has to be discovered. Anyway, in the latter case, once selenium is internalized, it should be allowed to react with intracellular thiols, thus producing H₂Se inside the cell. Finally, H₂Se might be involved in toxicity also when selenite is added without glutathione.

**Fates of H₂Se inside the Cell**—As soon it comes through the membrane of the cell, hydrogen selenide is expected to undergo various chemical or enzymatic transformations. Below, we consider major stable products, which are formed upon such transformations, and discuss their potential to exert cellular toxicity. A first type of modification comes out from the resemblance between selenium and sulfur. Thus, H₂Se may enter the sulfur assimilation pathway and be at the origin of selenoamino acids. Actually, selenomethionine has been shown to accumulate in yeast cells grown in the presence of selenite (49). Once there, selenomethionine can take the place of methionine in polypeptides (50). However, yeast cells appear rather insensitive to the incorporation of this amino acid into their proteins (50). On the other hand, selenocysteine does not accumulate in selenized yeast cells, and its incorporation into proteins is negligible (50). Oxidation of H₂Se by oxygen yields elemental selenium. A redening of yeast cells upon incubation with selenite alone has been reported (19). This color most probably comes from the formation of red elemental selenium inside the cell. Because elemental selenium is not soluble, it is not likely to exert any toxicity unless it has been previously reduced.

**Cellular Uptake of Selenium in Yeast Might Cause Oxidative Stress**—Hydrogen selenide not only enters the cell with an efficiency far larger than that of selenite, but also is susceptible to be the species responsible for toxicity. Possibly H₂Se promotes a change in the balance between reduced and oxidized glutathione. Indeed, according to the Reaction 6 above, the presence of hydrogen selenide in the cell is likely to cause oxidation of glutathione. The cycle involving oxidation of hydrogen selenide by
oxygen (Reaction 5), and formation of glutathioselenol from elemental selenium and glutathione (reversal of Reaction 3) (27), followed by reduction of glutathioselenol by glutathione into hydrogen selenide (Reaction 4), may drive continuous consumption of glutathione. Moreover, ROS generated from the reaction of hydrogen selenide with molecular oxygen inside the cell (43) may also contribute to the oxidation of glutathione. Eventually, a decrease in the pool of intracellular reduced glutathione could yield an oxidative stress, thus possibly accounting for the genotoxic effects observed in yeast upon exposure to selenite or selenite-glutathione mixtures (14, 15).

Other mechanisms sustaining selenium toxicity may involve reactions of hydrogen selenide with metal-containing proteins. Indeed, the iron of lipoxygenase of human monoclonal B-lymphocytes has been shown in vitro to be sensitive to H₂Se (51). Apart from that, by analogy with the action of H₂S in rat hepatocyte mitochondria (52), H₂Se could promote inhibition of heme-containing enzymes belonging to the respiratory chain. Reaction of H₂Se would therefore lead to leakage of electrons and their capture by molecular oxygen and, eventually, to formation of superoxide ions. Superoxide ions, whether due to redox cycling or to inhibition of enzymes of the respiratory chain, might then be amplified by mitochondria, by analogy with what occurs in human hepatoma cells (53). In favor of an involvement of mitochondria in the toxicity of selenite-glutathione mixtures, we observed a high proportion of petite mutants in the survivors after exposure of yeast cells to selenite plus glutathione.

The Case of Mammals—Selenium as a diet supplement can be administered under inorganic (selenite) or organic (selenomethionine, selenized yeast cells) forms. At one step or another of their metabolism, all these forms can produce hydrogen selenide, as we will discuss below.

With selenite as the selenium source, reduction occurs early in the absorption process. When administered by perfusion inside the intestine, selenite is mainly recovered intact in the vasculature (54). However, small metabolites such as selenodiglutathione or selenodicysteine, as well as selenium associated to proteins, become also detectable in the intestine vasculature (54). All the above mentioned selenocompounds, including selenite, are likely to be further reduced in the vessels. Indeed, after intravenous injection, selenite is taken up within minutes by the red blood cells where it is reduced to selenide (55). Selenium is then released by the cells in the plasma. Eventually, through transport by blood, selenium can be widely distributed. When administered orally to pigs, selenomethionine is transported across the brush-border membranes of intestine (56) and addressed to nearly all organs in its intact form (57). Inside the cells, selenomethionine can be transformed in selenocysteine by the sulfur pathway (50) and then into alanine and elementary selenium by the selenocysteine β-lyase (58). Elementary selenium can be reduced in selenide by intracellular thiols.

Finally, selenized yeast cells contain various forms of selenium. Selenomethionine in proteins is by far the major form (59). Digestion of proteins and metabolization of selenomethionine will supply cells with hydrogen selenide.

Concluding Remarks—In liver cells, detoxification of selenium is ensured by the enzymatic conversion of hydrogen selenide into several methylated species (60–64). Such metabolic pathways do not occur in S. cerevisiae (65). However, according to the available literature, the toxicity of selenium toward animal cells and yeast cells shares many features. For instance, with animal cells: (i) thiols have been observed to enhance the accumulation of selenium (66–68) and the toxicity of selenite (21, 66, 69); (ii) hydrogen selenide toxicity has been recognized very early (70); and (iii) redox phenomena (24, 25, 71) as well as mitochondria (72) are involved in the toxicity mechanisms of selenocompounds. Interestingly, in bacteria also, redox phenomena are involved in the toxicity of selenium (73). In particular, in E. coli, the gene of SOD is essential to the defense against selenite (74).

We may therefore propose that the toxicity of selenite follows general rules involving perturbation by hydrogen selenide of the redox balance of the cell. In animal cells, the choice between apoptosis and proliferation is linked to the redox state. By decreasing the reducing power of the cell through hydrogen selenide action, selenite might exert its anticarcinogenic properties.
Thiol-assisted Toxicity of Selenite

1115–1124
Extracellular Production of Hydrogen Selenide Accounts for Thiol-assisted Toxicity of Selenite against Saccharomyces cerevisiae
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doi: 10.1074/jbc.M610078200 originally published online January 29, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M610078200

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