Reduction of Mercury to the Elemental State by a Yeast

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A yeast of the genus *Cryptococcus* has been isolated from a stream and was shown to be capable of reducing mercury to the elemental state. The organism grows in Wickerham broth supplemented with high concentrations of mercury (II) chloride (180 mg of mercury per liter) and will metabolize [^{14}C]glucose in this medium as do cells in the absence of mercury. Mercury was associated with the cell wall and membrane, and in vacuoles within the cytoplasm.

It is generally recognized that mercury present in the environment will eventually accumulate in the cells of microorganisms and higher forms (3). Studies of the effect of mercury on photosynthesis and growth of the algae *Chlamydomonas* and *Chlorella* have been made (1, 5), and the metabolism of mercurials has been studied using various resistant bacteria (4, 6, 10). A yeast that is resistant to the toxic effects of mercury and appears to bring about the reduction of the divalent mercury to the elemental state has been isolated from a freshwater stream (White Clay Creek, Chester County, Pa.). The organism has been identified as a *Cryptococcus* sp. using the key of Phaff and Fell (8). In this paper we describe the results of experiments demonstrating the tolerance to mercury exhibited by the organism and initial studies of the metabolism of the organisms under mercury stress.

MATERIALS AND METHODS

Culture studies. The organism was maintained on Wickerham medium (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 1.0% glucose 2.0% agar) containing 30 mg of mercury (as $HgCl_2$) per liter. Experiments were conducted in liquid medium of the same composition, but with varying mercury concentration. To circumvent abiotic modification and precipitation of the mercurial during experiments, the medium was prepared in the following manner. Mercury (as $HgCl_2$) was added to the medium lacking glucose at a concentration of 500 mg per liter (2.5×10^{-3} M Hg) after which the medium was autoclaved for 2.5 h. A grey-black precipitate formed as a result of reactions of constituents of the medium with the mercurial reducing the mercury concentration of the liquid medium to approximately 20 mg per liter, a level below that desired in the experimental cultures. Extreme care was taken not to comtaminate the

laboratory atmosphere with vapors from the medium during this procedure. Further abiotic depletion of mercury concentration in solution did not occur. Glucose was added after having been autoclaved separately for 30 min. Preliminary studies showed that, despite the loss and alteration of some of its constituents during prolonged autoclaving with mercury, the medium supported the growth of the yeast nearly as well as the medium prepared without added mercury and autoclaved for 20 min.

Growth studies were made in this medium. After determining the concentration of residual mercury present after autoclaving, additional $HgCl_2$ was added to provide mercury concentrations up to 250 mg per liter. An inoculum of 10^5 to 5×10^5 cells into 100 ml of medium was used routinely. Experiments were carried out, in duplicate, in 250-ml Erlenmeyer flasks in a shaker bath (180 oscillations per min) at 25 C.

In some experiments the glucose concentration was halved, and uniformly labeled [^{14}C]glucose was added (New England Nuclear, Boston, Mass., specific activity 15.5 mCi/mmol, final concentration 0.01 μCi/ml). The flasks were closed with a neoprene stopper into which a cotton swab saturated with phenethylamine was inserted. This served to trap carbon dioxide evolved by the organisms. By replacing the swabs at selected intervals, CO_2 was monitored during an experiment. Radioactivity determinations were made with a liquid scintillation counter (model LS-133, Beckman Instruments, Fullerton, Calif.). Carbon dioxide traps were counted in an Omnifluor (New England Nuclear, Boston, Mass.)-toluene-Triton X-100 solution (Beckman Instruments, Fullterton, Calif.).

At the same time carbon dioxide traps were changed, duplicate 4-ml samples of culture were collected. One sample was used for mercury determinations as described below. The other was used for determination of cell numbers and radioactivity associated with the cells. Direct counts by phase microscopy were made on two portions using a Levy hemocytometer. Independent studies showed a close correla-

tion between cell numbers and cell yield determined as dry weight. The sample was then centrifuged for 10,000 × g for 10 min, and the cells were washed with distilled water. Radioactivity associated with cells was determined on portions of the cells resuspended into an Omnifluor-Triton X-toluene cocktail for liquid scintillation counting.

The viability of cells containing mercury was tested by staining with primulin and examining by fluorescence microscopy (Zeiss Universal Microscope, Zeiss Instruments, Oberkochen, W. Germany; 2).

At the end of the experiment the cells were harvested, washed in phosphate buffer, and dried, and the weight and mercury content were obtained. From this information a concentration factor (parts per million mercury in cells per parts per million mercury in medium) was calculated.

Mercury determination. Mercury determinations were made using atomic absorption spectrophotometry (Perkin Elmer model no. 303) with flame detection. A hollow cathode lamp (Perkin Elmer no. 6044) was employed and absorption was measured at 253.7 nm. Corollary studies showed no interferences from other constituents in the samples. Samples were centrifuged at 10,000 × g for 10 min to separate cells from culture medium after which the cells were washed in 0.1 M mercaptoethanol to remove mercury adsorbed to cell surfaces. Independent studies showed that mercaptoethanol did not affect the integrity of the cell. Mercury was assayed after resuspension of the cells in 4 ml of 0.02 M phosphate buffer. Determinations on the supernatant fluid from centrifugation were made directly. All mercury concentrations are given as total mercury; no attempt was made to determine the nature of the mercurial.

Electron microscopy. Cells in early exponential growth were collected by centrifugation from cultures containing 100 μg of Hg per ml and washed with distilled water. After fixing for 16 h in 4% formaldehyde by the method of Morgan et al. (7), they were dehydrated through an ethanol series (50, 70, 80, 95%, and absolute), followed by hexylene glycol, and infiltrated in a slurry of 50% hexylene glycol and 50% 60-40 Epon in a 15-ml Corex centrifuge tube in a desiccator overnight. Cells were then compacted by centrifugation (10,000 × g, 10 min), and the pellet was removed using a small spatula and pressed between the fibers of a stip of paper toweling (ultra-high absorbancy, Scott Paper Co.) that had previously been saturated with freshly prepared Epon. This cell-impregnated toweling strip was immediately shredded with a pair of scissors into strips approximately 0.5-mm wide, which were then individually embedded in Epon and allowed to harden at 40 C for 48 h. (The paper fibers had no effect on sectioning.) Silver sections were mounted on uncoated grids (400 mesh) to enhance contrast. Specimens were not treated with an electron-dense stain of any kind with the expectation that mercury in the cells would serve this purpose. Photography was accomplished using a Phillips model 200 electron microscope.

RESULTS AND DISCUSSION

The results shown in Fig. 1 are representative of those obtained in several experiments. With mercury concentrations of 100 and 200 mg per liter, the lag phase of growth is lengthened when compared to control cultures grown in the presence of 20 mg of mercury per liter (the
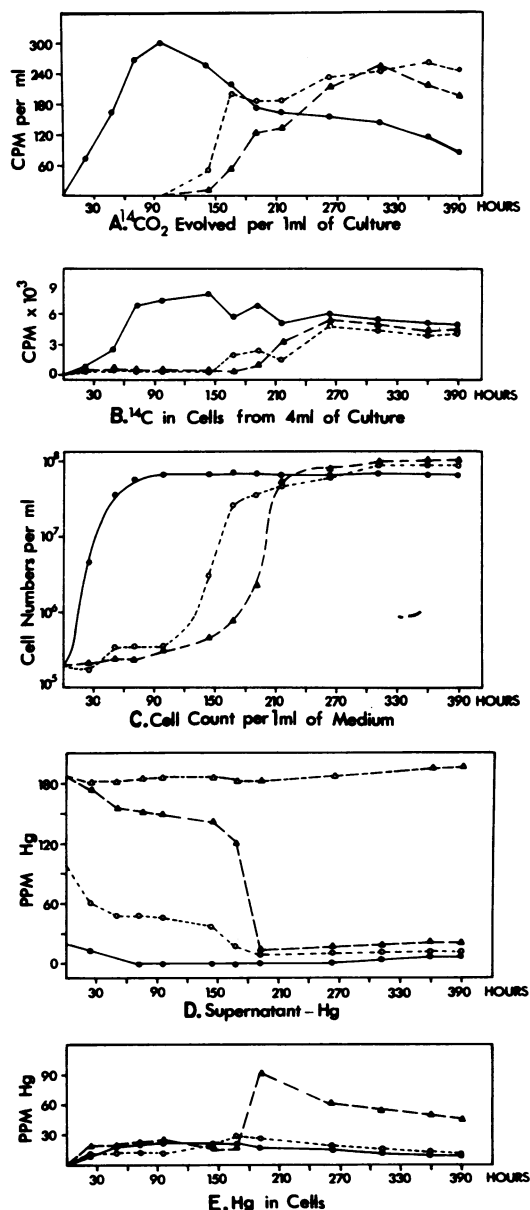

FIG. 1. *Change in cell numbers, respiratory activity, glucose utilization, and mercury content of cells and medium in cultures containing varying concentrations of mercury (as HgCl₂). Symbols: closed circles, solid line, 20 mg/liter; open circles, dashed line, 120 mg/liter; closed triangles, dashed line, 180 mg/liter; open triangles, dashed line, 180 mg/liter uninoculated control.*

residual remaining after autoclaving, Fig. 1C). However, the cell numbers ultimately obtained in cultures containing as much as 200 mg of mercury per liter were of the same order of magnitude as the number obtained when only a residual amount was present. A burst of CO_2 evolution took place just prior to, or concomitant with, the increase in cell numbers (Fig. 1A) for all concentrations of mercury tested. Carbon 14 uptake (Fig. 1B) paralleled the rise in cell numbers and demonstrated that cells in the presence of mercury are capable of utilizing hexose substrate as do cells in the absence of mercury.

The concentration of mercury in the medium declined at a relatively slow rate until growth commenced, at which time there was a dramatic reduction in its concentration in the medium and an association with the cells (Fig. 1D and 1E). The mercury concentration in the uninoculated control remained unchanged for the duration of the experiment demonstrating that the organisms mediate any reduction of level of mercurial in solution.

At the end of the experiment the mercury associated with cells was determined and compared with that present in the medium. This quotient (or concentration factor) ranged from 10 (in 180 ppm medium) to 30 (in 20 ppm medium), with a mean of 14.5 for the duplicate cultures at all concentrations studied. The presence of high levels of mercury in viable cells indicates that they have the ability to adjust to the presence of this heavy metal by converting it to a nontoxic form, probably elemental mercury.

The direct counting procedure also revealed that some cells from experimental cultures had associated with either their cell walls or cytoplasm small dark granules. Cells cultured in Wickerham medium without mercury had no similar associations. The viability of these cells was confirmed by staining with the fluorochrome primulin. In broth cultures these cells accumulated as a grey sediment. On solid Wickerham medium containing mercury, a silvery metallic sheen developed on the surfaces of colonies after 4 days of incubation at room temperature. The presence of mercury associated with colony was confirmed by atomic absorption spectrophotometry, and the silver sheen suggested reduction to the elemental state.

Mercury was the only electron-dense material in preparations made for electron microscopy. Electron micrographs revealed that mercury was associated both with the cell surface and within the cells in vacuoles (Fig. 2). Note that,
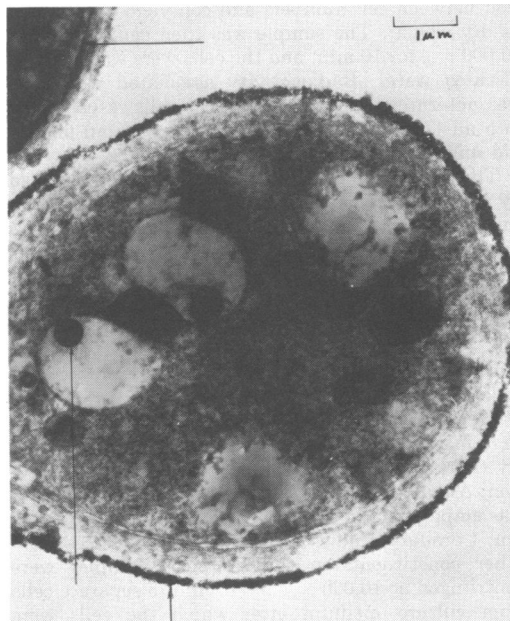

FIG. 2. *Electron micrograph of unstained cells showing mercury associated with cell wall, membrane, and intracellular vacuoles.* $\times 25,600$.

in one cell, mercury is associated with the cell wall and in vacuoles in the cytoplasm. In the other cell, the mercury appears to be associated with the membrane. Confirmatory evidence that the electron-dense material was mercury was obtained by examining unstained preparations of cells grown in the absence of mercury. No electron-dense constituent was discerned.

The association of mercury with the yeast cell wall has been reported and the kinetics have been studied (9), but the residence of significant levels of mercurials in yeast cytoplasm has not been reported. The isolation of this mercury-resistant eukaryotic microbe provides a unique opportunity to explore the mechanism of resistance to and metabolism of mercury-containing compounds, and further experiments are in progress.

ACKNOWLEDGMENTS

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