Cytochemical Localization of Mercury in *Cryptococcus albidus* Grown in the Presence of Mercuric Chloride

By T. A. BROWN AND D. G. SMITH

Department of Botany and Microbiology, University College London,
London WC1E 6BT

(Received 29 October 1976)

INTRODUCTION

Many metals are known to be toxic and, in particular, the harmful effects of mercury compounds on all groups of micro-organisms (Vallee & Ulmer, 1972), as well as on mammalian systems (Brun *et al.*, 1976), are well documented. However, only two previous investigations on the location of mercury in treated yeast cells have been reported (Brunker & Bott, 1974; Murray & Kidby, 1975). Several cytochemical and histochemical techniques have been used to demonstrate mercury by light microscopy, as summarized by Sakai *et al.* (1975), but there has been only one previous electron microscope study, on rat nervous tissue (Chang & Hartmann, 1972). This paper describes a simple cytochemical method for the localization of mercury, which is suitable for electron microscopy, and demonstrates the binding sites for mercury within the cells of the yeast *Cryptococcus albidus*.

METHODS

Preparation of cultures. *Cryptococcus albidus* NCYC448 was grown in a liquid medium containing 2.0% (w/v) glucose, 0.5% (w/v) yeast extract (Oxoid) and 0.21% (w/v) KH_2PO_4 , final pH 5.8. This medium was sterilized by autoclaving and freshly prepared filter-sterilized HgCl_2 was added to give a final concentration of 20 mg Hg^{2+} l^{-1} .

One ml of an overnight culture of *C. albidus* was inoculated into 25 ml growth medium in a 250 ml side-arm flask, to give a concentration of 10^5 to 2.5×10^5 cells ml^{-1} , and shaken at 20°C; growth was followed turbidimetrically. Samples for electron microscopy were removed at the start of the exponential phase of growth.

Preparation for electron microscopy. Cells were washed twice in citrate/phosphate buffer, pH 5.8, and fixed in 1.5% (w/v) KMnO_4 at room temperature for 3 h. After two further washes in buffer, a firm pellet was obtained by centrifugation, dehydrated in acetone and embedded in Araldite. Gold sections were examined with a Hitachi HS-9 electron microscope.

Cytochemical technique. Grids with sections were floated section-side down on drops of ammonium hydroxide solution (sp. gr. 0.880) for 5 min, washed carefully in distilled water and dried on filter paper.

RESULTS

The normal ultrastructure of *C. albidus* is shown by a cell grown in the absence of HgCl_2 (Fig. 1a). A well-defined nucleus and three mitochondrial profiles can be seen with several pieces of endoplasmic reticulum, part of a vacuole and numerous small vesicles. The cytoplasm is enclosed by the plasmalemma and a rigid wall about 0.1 μm thick.

Fig. 1. Sections of *C. albidus* grown: (a) in the absence of HgCl_2 ; (b) in the presence of HgCl_2 , section not treated with NH_4OH ; (c, d) in the presence of HgCl_2 , sections treated with NH_4OH . The location of the mercury is shown by electron-opaque granules mainly on membranes. Bar markers represent $0.5\ \mu\text{m}$.

Few significant ultrastructural changes occur during early growth in the presence of HgCl_2 (compare Fig. 1 a, b). However, sections treated with NH_4OH show electron-opaque granules indicating that mercury is present in various parts of the cell (Fig. 1 c, d). These granules are associated mainly with the wall and membranes. Mercury is bound to both the inner and outer mitochondrial and nuclear membranes, to the endoplasmic reticulum

and to the various vesicles. The presence of granules specifically on the plasmalemma is not so definite, but in Fig. 1(d) there is an invagination of the cell membrane and several spots of the cytochemical marker are associated with it. Freeze-etching studies have shown such invaginations to be infoldings of the plasmalemma (Bauer, 1970), so the presence of mercury here may be indicative of its presence elsewhere on the plasmalemma. Few granules are seen in the general cytoplasmic matrix, within the nucleus, or within the vacuole.

Ammonium hydroxide treatment had no effect on cells grown in medium without HgCl_2 : such cells had a similar appearance to those in Fig. 1(a).

DISCUSSION

The main problem with cytochemistry is to ensure the specificity of the chemical reaction, in this case for mercury. The reaction between mercuric compounds and NH_4OH produces an amido derivative with the basic formula $(\text{HgNH}_2\text{X})_n$, where X is a halide group such as chloride. This compound is made up of long zig-zag molecules of alternating Hg and NH_2 units, with the X^- anions between the separate chains (Aylett, 1973):

With the concentration of HgCl_2 used in our experiments, there is insufficient density of mercury atoms to give enough electron scattering to produce an image in the electron microscope. The formation of the long-chain amido compound draws together separate mercury atoms and this concentration effect gives rise to the electron-opaque granules (Fig. 1c, d). Theoretically reactions resulting in precipitates of sufficient density to be seen under the electron microscope are also possible between NH_4OH and some other elements but, unlike the mercury-ammonia complex, these are all highly soluble in water and so likely to be removed when the grids are washed following NH_4OH treatment. The consistent absence of deposits in NH_4OH treated control cells suggests that this test is specific for mercury under the conditions used.

A similar technique has been described by Chang & Hartmann (1972), the basis of which is the reaction between mercuric salts and $(\text{NH}_4)_2\text{S}$ leading to the formation of a mercury-ammonium-sulphur complex. This reaction is routinely used in inorganic analysis and in the controlled conditions of the test tube is specific for mercuric ions. However, with the less-defined conditions of a yeast cell, it is still necessary to ensure that no reaction occurs in sections of cells grown in the absence of HgCl_2 before drawing conclusions on the presence of mercury in treated cells. Another difference between the procedure described here and that of Chang & Hartmann (1972) involves the stage at which the material is exposed to the reactant. Chang & Hartmann worked with nervous tissue and immersed the sample in $(\text{NH}_4)_2\text{S}$ immediately after fixation and before dehydration and embedding. The problems involved in the preparation of yeast cells for electron microscopy are well known; the wall forms a barrier to penetration of the reagents and this must also be taken into account with this technique. Because of such difficulties it is probably advisable to use the cyto-

chemical reagent as a post-stain rather than introducing it during the preparative stages. This precludes the use of copper grids with $(\text{NH}_4)_2\text{S}$ due to the formation of mixed copper sulphides and disintegration of the grid in the electron beam. For these reasons NH_4OH is recommended for work with yeast cells.

The results obtained using the NH_4OH technique are consistent with those of Murray & Kidby (1975) who showed by autoradiography that the major fraction of bound mercury was present in the walls of *Saccharomyces cerevisiae* treated with lower concentrations of HgCl_2 ($5.0 \text{ mg Hg}^{2+} \text{ l}^{-1}$) than used in our study. They also found that a small fraction of the mercury was associated with the cytoplasm and showed this to be bound to molecules with a molecular weight greater than 1000 — which is what one would expect if mercury were bound to membranes as suggested here. The affinity of Hg^{2+} for yeast cell membranes was first suggested by Passow & Rothstein (1960) who demonstrated that irreversible membrane damage was caused by concentrations of Hg^{2+} as low as 0.2 mg l^{-1} and resulted in a loss of K^+ and other anions to the medium. Mercury has low affinity for membrane phospholipids (Vallee & Ulmer, 1972) but binds to protein sulphhydryl groups and sugar phosphoryl sites, the latter being involved in ion transport (Passow, Rothstein & Clarkson, 1961).

The presence of mercury on the mitochondrial membranes is interesting as Hg^{2+} ions have been implicated in inhibiting ATP-driven NAD reduction by succinate in phosphorylating beef heart submitochondrial particles (Schuurmans-Stekhoven, Sani & Sanadi, 1970), and in inducing proton release in complete beef heart mitochondria (Southard, Blondin & Green, 1974).

The wall binding sites were suggested by Murray & Kidby (1975) to be glucan-linked proteins and this study throws no further light on this. The present state of knowledge on the organization of the cell wall of yeasts is still somewhat rudimentary but what is known, especially concerning the role of glucan in cross-wall formation during budding (Matile, Moor & Robinow, 1969), may be useful in future studies using the cytochemical technique.

There are numerous other possible binding sites for mercury in the cell, notably nucleic acids (Vallee & Ulmer, 1972), and the affinity of mercury for sulphhydryl groups provides a means of binding to any protein with free SH groups. This may explain why the electron micrographs of yeasts grown in higher concentrations ($100 \text{ mg Hg}^{2+} \text{ l}^{-1}$) of HgCl_2 than used in this study (Brunker & Bott, 1974) appear to show mercury present in most parts of the cell.

The cytochemical technique described here provides a simple and rapid way of locating mercury in HgCl_2 -treated cells, and further studies should provide more detailed information on the precise binding sites. Work is in progress with electron microscopic X-ray microanalysis which we hope will confirm the results presented and provide quantitative data on mercury binding in the cell.

One of us (T.A.B.) acknowledges the support of a research studentship from the Science Research Council.

REFERENCES

- AYLETT, B. J. (1973). Chemistry of Group II B. In *Comprehensive Inorganic Chemistry*, vol. III, pp. 187–328. Edited by J. C. Bailar, H. J. Emeléus, R. Nyholm and A. F. Trotman-Dickenson. Oxford: Pergamon Press.
- BAUER, H. (1970). A freeze-etch study of membranes in the yeast *Wickerhamia fluorescens*. *Canadian Journal of Microbiology* **16**, 219–222.

- BRUN, A., ABDULLA, M., ISLE, I. & SAMUELSSON, B. (1976). Uptake and localisation of mercury in the brain of rats after prolonged oral feeding with mercuric chloride. *Histochemistry* **47**, 23-29.
- BRUNKER, R. L. & BOTT, T. L. (1974). Reduction of mercury to the elemental state by a yeast. *Applied Microbiology* **27**, 870-873.
- CHANG, L. W. & HARTMAN, H. A. (1972). Electron microscopic histochemical study on the localisation and distribution of mercury in the nervous system after mercury intoxication. *Experimental Neurology* **35**, 122-137.
- MATILE, PH., MOOR, H. & ROBINOW, C. F. (1969). *Yeast cytology*. In *The Yeasts*, vol. I, pp. 220-302. Edited by A. H. Rose and J. S. Harrison. London and New York: Academic Press.
- MURRAY, A. D. & KIDBY, D. K. (1975). Sub-cellular location of mercury in yeast grown in mercuric chloride. *Journal of General Microbiology* **86**, 66-74.
- PASSOW, H. & ROTHSTEIN, A. (1960). The binding of mercury by the yeast cell in relation to changes in permeability. *Journal of General Physiology* **43**, 621-623.
- PASSOW, H., ROTHSTEIN, A. & CLARKSON, T. W. (1961). The general pharmacology of the heavy metals. *Pharmacological Reviews* **13**, 185-224.
- SAKAI, K., OKABE, M., ETO, K. & TAKEUCHI, T. (1975). Histochemical demonstration of mercury in human tissue cells of Minamata disease by use of autoradiographic procedure. *Acta histochemica et cytochemica* **8**, 257-264.
- SCHUURMANS-STEKHOFEN, F. M. A. H., SANI, B. P. & SANADI, D. R. (1970). Energy-linked NAD reduction in phosphorylating submitochondrial particles from heavy-layer beef heart mitochondria. *Biochemical and Biophysical Research Communications* **39**, 1026-1036.
- SOUTHARD, J. H., BLONDIN, G. A. & GREEN, D. E. (1974). Induction of trans-membrane proton transfer by mercurials in mitochondria. *Journal of Biological Chemistry* **249**, 678-682.
- VALLEE, B. L. & ULMER, D. D. (1972). Biochemical effects of mercury, cadmium and lead. *Annual Review of Biochemistry* **41**, 91-128.