THE PRESERVATION OF SUBCELLULAR ORGANELLES OF *CANDIDA ALBICANS* WITH CONVENTIONAL FIXATIVES

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INTRODUCTION

In an approach to a study of the action of fungicidal drugs on the morphology of *Candida albicans*, we were faced with the problem of the preservation of substructures in untreated control cells. In contrast to results obtained with other yeast cells such as *Cryptococcus neoformans* (2, 9), chemical fixation with OsO_4 or with glutaraldehyde proved to be unsatisfactory for the preservation of the ultrastructural morphology of *C. albicans* (10, 11). The use of conventional fixatives offers good information about the cell wall and the plasmalemma but, with the exception of permanganate, chemical fixatives permeate very slowly so that the internal structures are barely made visible (8). Removal of cell wall constituents by mechanical or enzymatic treatment before fixation partly solves this problem, but all information about the cell wall and plasmalemma is lost.

In this study an attempt has been made to adapt conventional fixation procedures in such a way that the cell components of both the periphery and the interior are well visible. This was achieved by using a sectioning procedure in the presence of the fixative. Further interest in this preparation procedure lies in its application to enzyme cytochemistry.

MATERIALS AND METHODS

C. albicans cells (strain R. V. 4688), were inoculated at 37°C in a medium containing 0.5% casein hydrolysate, 0.5% yeast extract, and 0.5% glucose (CYG) (15). The cells in growth and stationary phases were harvested 7 and 24 h, respectively, after inoculation. In order to permit freeze-sectioning of the yeast cell pellet, embedding and solidification were done analogous to Anderson's technique for buffy coats (4). This was done as follows: the pellets of *C. albicans* were resuspended in a few drops of rat serum. After centrifugation, the supernate was discarded and 2% glutaraldehyde (buffered to pH 7.4 with 0.1 M Na-cacodylate) was gently added without disturbing the pellet. Solidification of the pellets proceeded for 15 min. To obtain an immediate fixation, the pellets were frozen on a drop of 2% Na-cacodylate-buffered glutaraldehyde placed on the microtome table. 7.5-μm thick sections were prepared and immediately dropped into 2% Na-cacodylate-buffered glutaraldehyde for 15 min. After a 15-min wash in 0.1 M Na-cacodylate + 0.22 M sucrose, the sections were postfixed for 30 min in 1% phosphate-buffered OsO_4. Other sections were immersed directly in 1% phosphate-buffered OsO_4 or in Chang's fixative (4% glutaraldehyde + 1% OsO_4) (7) for 30 min. After a brief wash in the appropriate buffer, the sections were impregnated in 0.5% uranium acetate in Michaelis buffer at pH 5.2 for 10 min, dehydrated in graded series of ethanol, and routinely embedded in Epon.

Ultrathin sections were stained with uranium acetate

and lead citrate before examination in an EM 300 electron microscope.

RESULTS

The percentage of cells that had been cut through the cytoplasm in a 7.5-μm thick frozen section was estimated to be about 70–80%. This was calculated on a theoretical basis, assuming a medium cell diameter of 5-μm and a random distribution of cells in the pellet. A survey of cross-sectioned cells embedded in the serum matrix is seen in Fig. 1.

The general appearance of ultrastructural organelles differed very little with the three different modes of fixation. The cytoplasm of a small number of cells appeared very electron-dense and no detailed morphology could be observed (Fig. 2). These represent probably the unbroken cells during 7.5-μm sectioning. In the presumptive intersected cells, on the other hand, the organelles were well visualized and most of the substructures were quite similar to those described in other yeast species (1, 3, 9).

Cell Wall and Plasmalemma

The definition into three distinct layers of the cell wall was observed in most cells (Fig. 3). No artifactual separation of the wall from the plasmalemma due to freeze-sectioning was noted. The plasmalemma presented short invaginations into the cytoplasm during the growth phase (Fig. 5) to rather extended ones during the stationary phase (Figs. 3, 6).

Intimate contacts between the plasmalemmal invaginations and the large cytoplasmic vacuoles were frequent (Fig. 6).

Nucleus

The ultrastructure of the nuclei of cells in the stationary phase is shown in Figs. 1, 4, and 6, revealing irregularly shaped nuclei limited by discontinuous double membranes. The excentric nucleolus was composed of intermingled granular and agranular electron-dense material (Fig. 4).

Cytoplasm

The density of the cytoplasm depended largely upon the amount of ribosomes, distributed in polysomal arrays or as single units. Owing to the less densely packed ribosomes, the visualization of the other cytoplasmic components was generally better in cells during the stationary phase than in growing cells.

Mitochondria, containing many cristae, were randomly distributed throughout the cytoplasm (Figs. 2 and 8). Central constriction of their double limiting membrane, suggestive of mitochondrial division, was occasionally observed (Fig. 4). Well-defined ribosomes, single or in small clusters, were seen in the matrix of almost all mitochondria (Figs. 2 and 4).

A vacuolar apparatus, mostly very electron-lucent and containing amorphous and vesicular material, occupied the central part of the cytoplasm (Figs. 6, 7). Part of the limiting membrane appeared flattened and could easily be interpreted as part of the membrane material generally designated as smooth endoplasmic reticulum. Short fragments of smooth membranes and vesicles were more frequent in cells in the growth phase and in budding cells than in resting cells in the stationary phase.

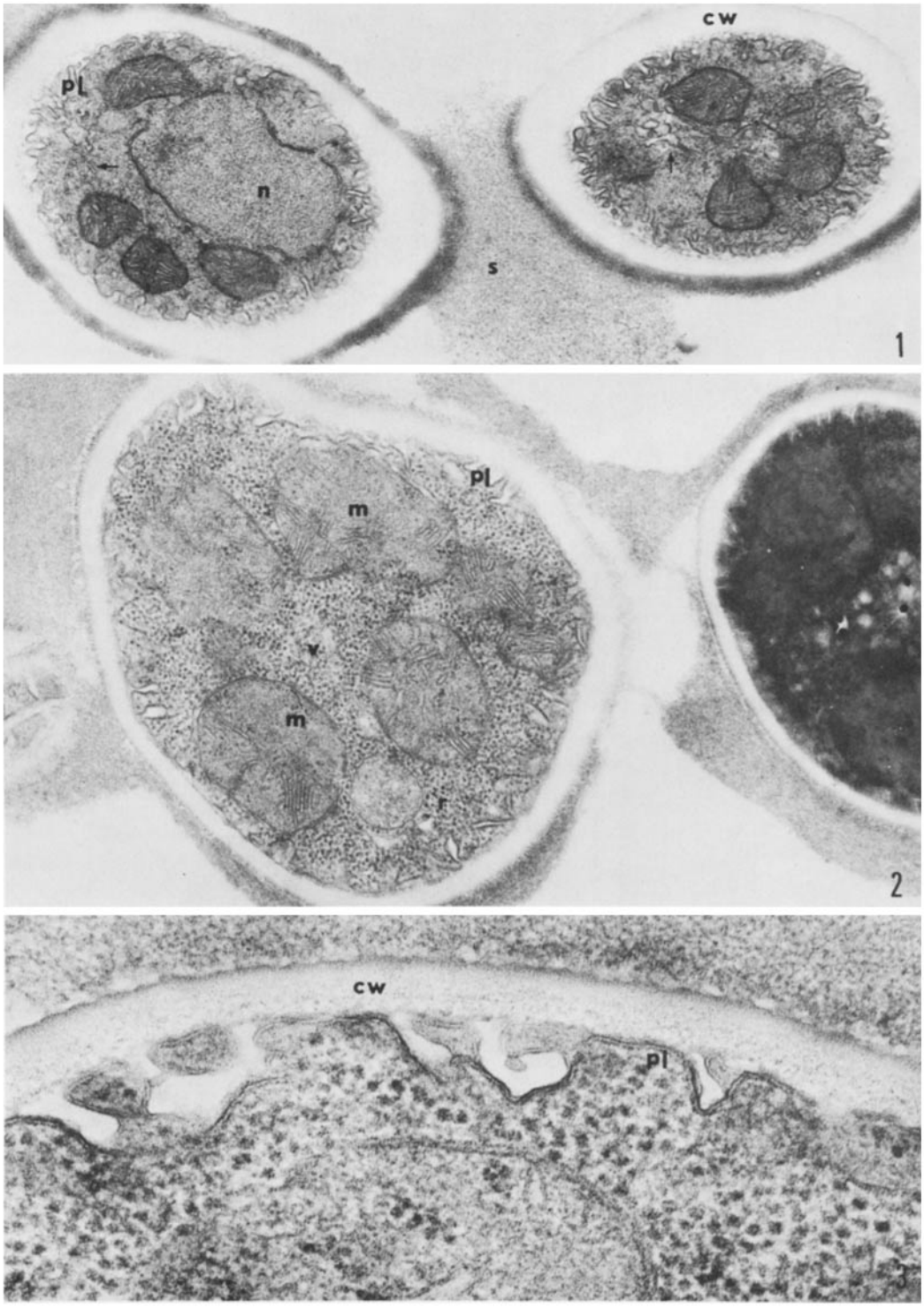
Round- to ovoid-shaped bodies, characterized by a single limiting membrane, a moderately dense matrix, and very often a central electron-dense core were present in most of the cells (Fig. 8). Lipid-like droplets were seen in the cytoplasm as well as in the central vacuole (Fig. 8). There was no evidence for the presence of a Golgi apparatus, multivesicular bodies, microtubules, or microfilaments in cells during either phase of growth.

DISCUSSION

The slow permeation of chemical fixatives into the cytoplasm poses a serious problem in the morphologic identification of the organelles of *C. albicans*. The fact that no adequately preserved untreated control cells could be obtained makes the interpretation of drug-induced morphologic alterations very difficult.

Permanganate, the commonly employed fixative for *C. albicans*, revealed fairly well the membranous components but failed to display ribosomes and the various nuclear and nucleolar substructures (6, 10, 13, 14). Moreover, this fixative cannot be used for the preservation of cells for enzyme cytochemistry.

Previous observations on 7.5-μm sections of mammalian tissues prepared for enzyme cytochemistry, frozen on a drop of fixative and immediately afterwards immersed in the fixative, revealed a normal ultrastructure in the cells cut

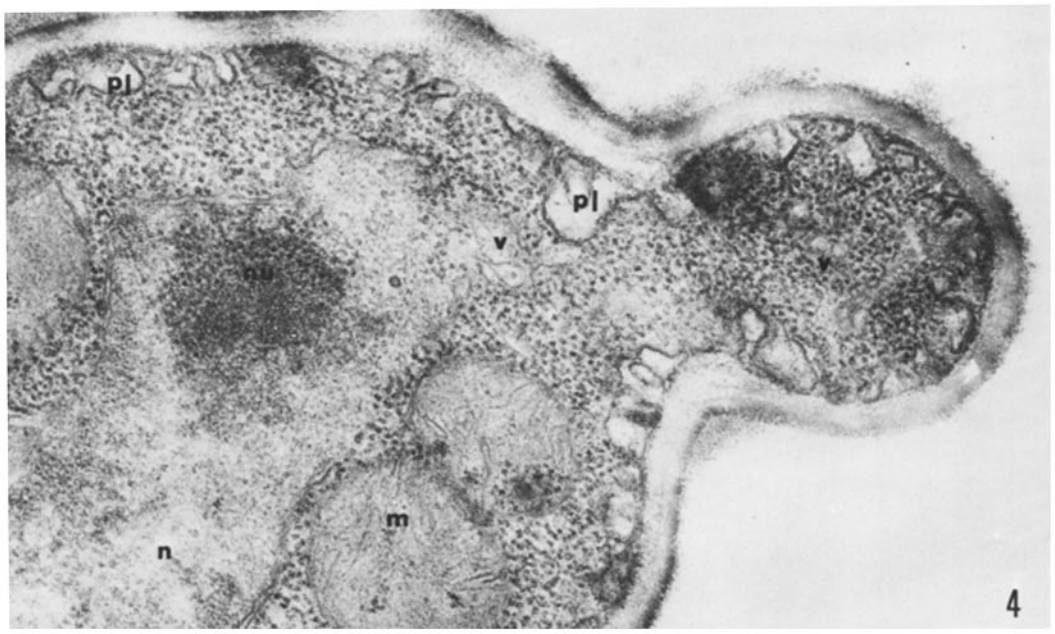


All figures are from cells in stationary phase unless indicated.

FIGURE 1 Survey picture of two cells, cut through the cytoplasm during freeze-sectioning. *cw*, Cell wall; *pl*, plasmalemma; *n*, nucleus, *s*, serum matrix. The arrows point to cytoplasmic membrane fragments. 2% glutaraldehyde, 15 min, followed by 1% OsO_4, 30 min. × 31,155.

FIGURE 2 This micrograph shows the difference in organelle visualization in a cross-sectioned cell (left) and a nonintersected cell (right). Plasmalemma (*pl*), mitochondria (*m*), vesicles (*v*), and abundant ribosomes (*r*) are noted in the cell on the left side whereas substructures are barely seen in the right cell. 2% glutaraldehyde, 15 min, followed by 1% OsO_4, 30 min. × 29,128.

FIGURE 3 Portion of the cell periphery. The different layers of the cell wall (*cw*) and irregular protrusions of the plasmalemma (*pl*) are seen. Chang's fixative, 30 min. × 98,208.



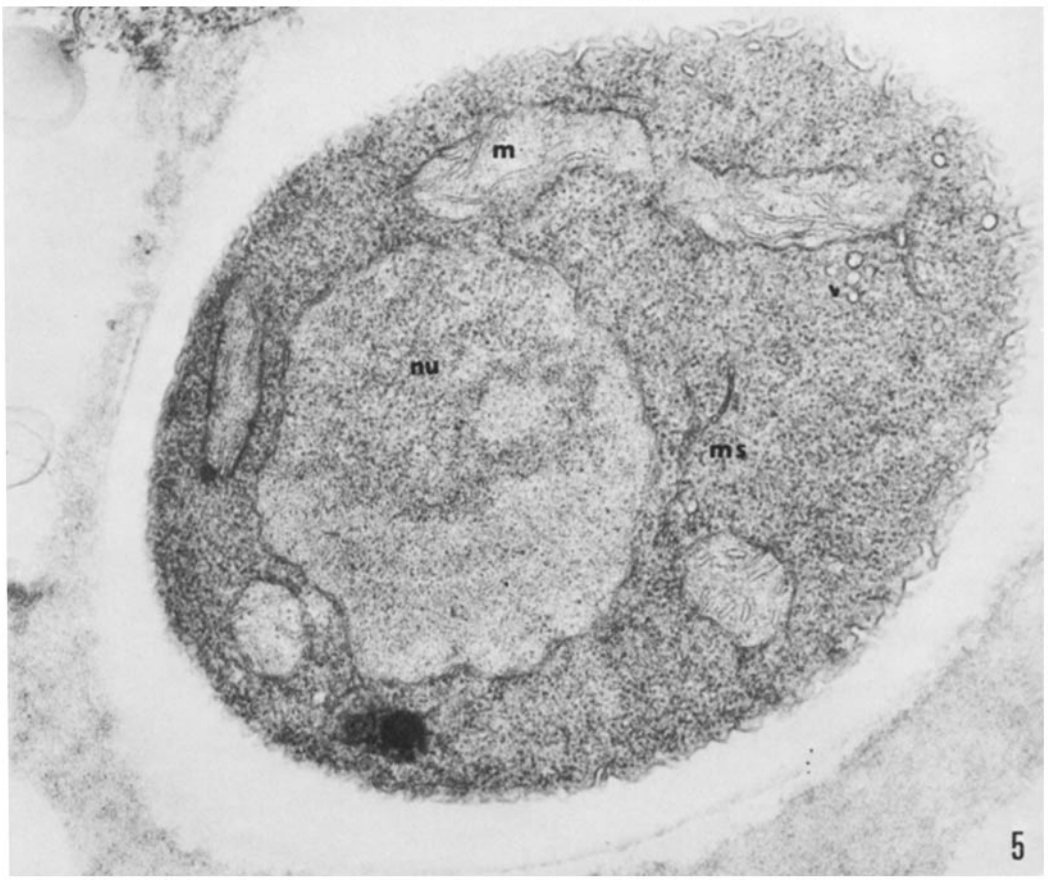


FIGURE 4 A budding cell showing abundant ribosomes, a mitochondrion (*m*) with central constriction containing small clusters of ribosomes, some vesicles (*v*), and a nucleus (*n*) with a prominent nucleolus (*nu*) composed of granular and agranular electron-dense structures. 1% OsO_4, 30 min. × 44,640.

FIGURE 5 A cell in the growth phase. The electron opacity of the cell cytoplasm is due to the densely packed ribosomes. The irregularly shaped mitochondria (*m*) possess only few cristae. Vesicular (*v*) and membranous (*ms*) structures are present in a moderate amount. Note the extended nucleolar mass (*nu*) in the nucleus. 2% glutaraldehyde, 15 min, followed by 1% OsO_4, 30 min. × 29,574.

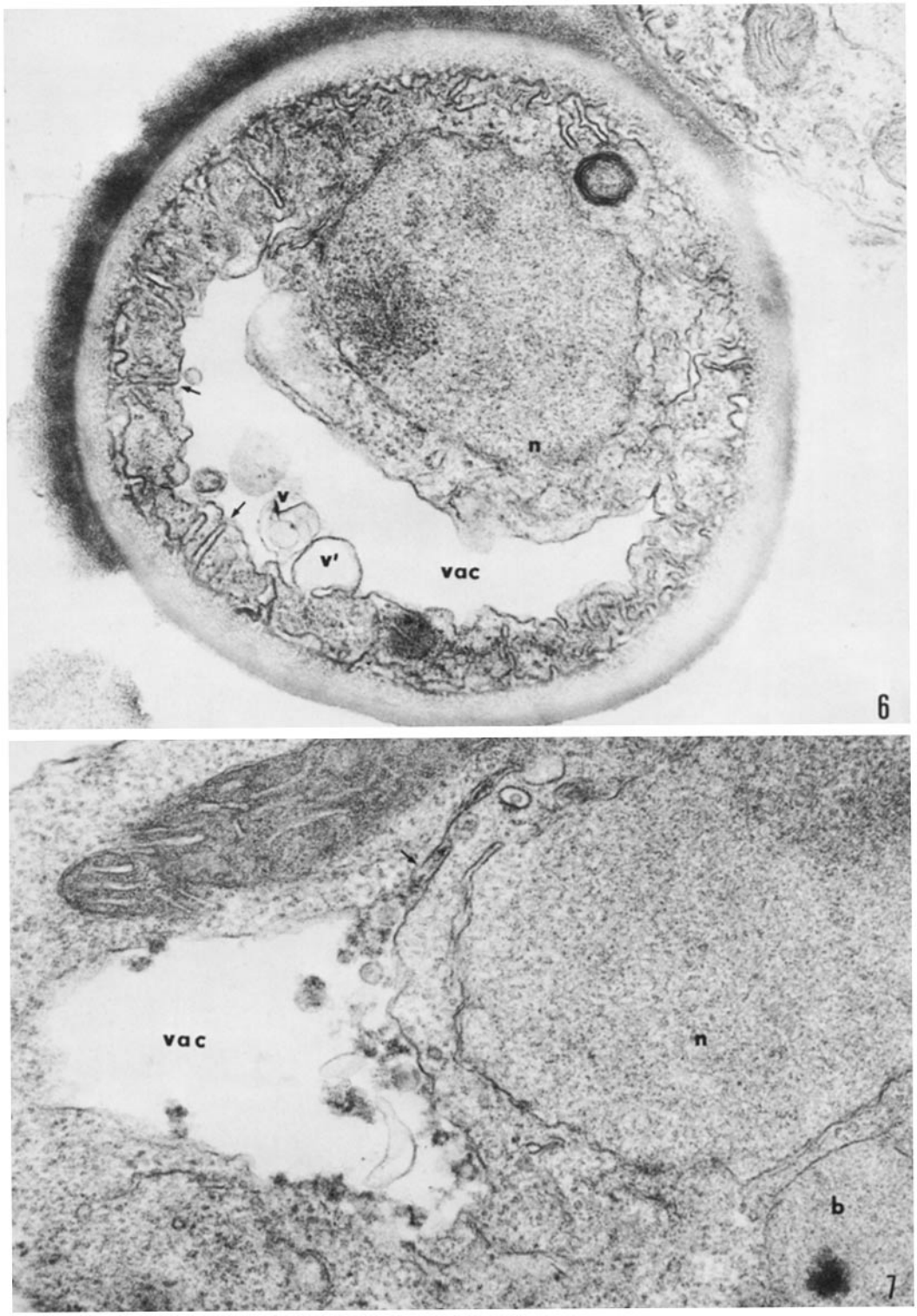


FIGURE 6 The nucleus (*n*) is partly surrounded by a large vacuole (*vac*) containing only a few vesicular structures (*v*). Protrusion of a cytoplasmic vesicle into the vacuole is indicated as *v'*. The arrows point to areas of close contact between the vacuolar membrane and the plasmalemmal invaginations. 1% OsO_4, 30 min. × 38,688.

FIGURE 7 Detailed appearance of the vacuolar apparatus presenting a dilated part with a vesicular and agranular content and a collapsed part (arrow). A portion of the nucleus (*n*) limited by a discontinuous double membrane and a single membrane-limited body (*b*) are seen. Chang's fixative, 30 min. × 52,499.

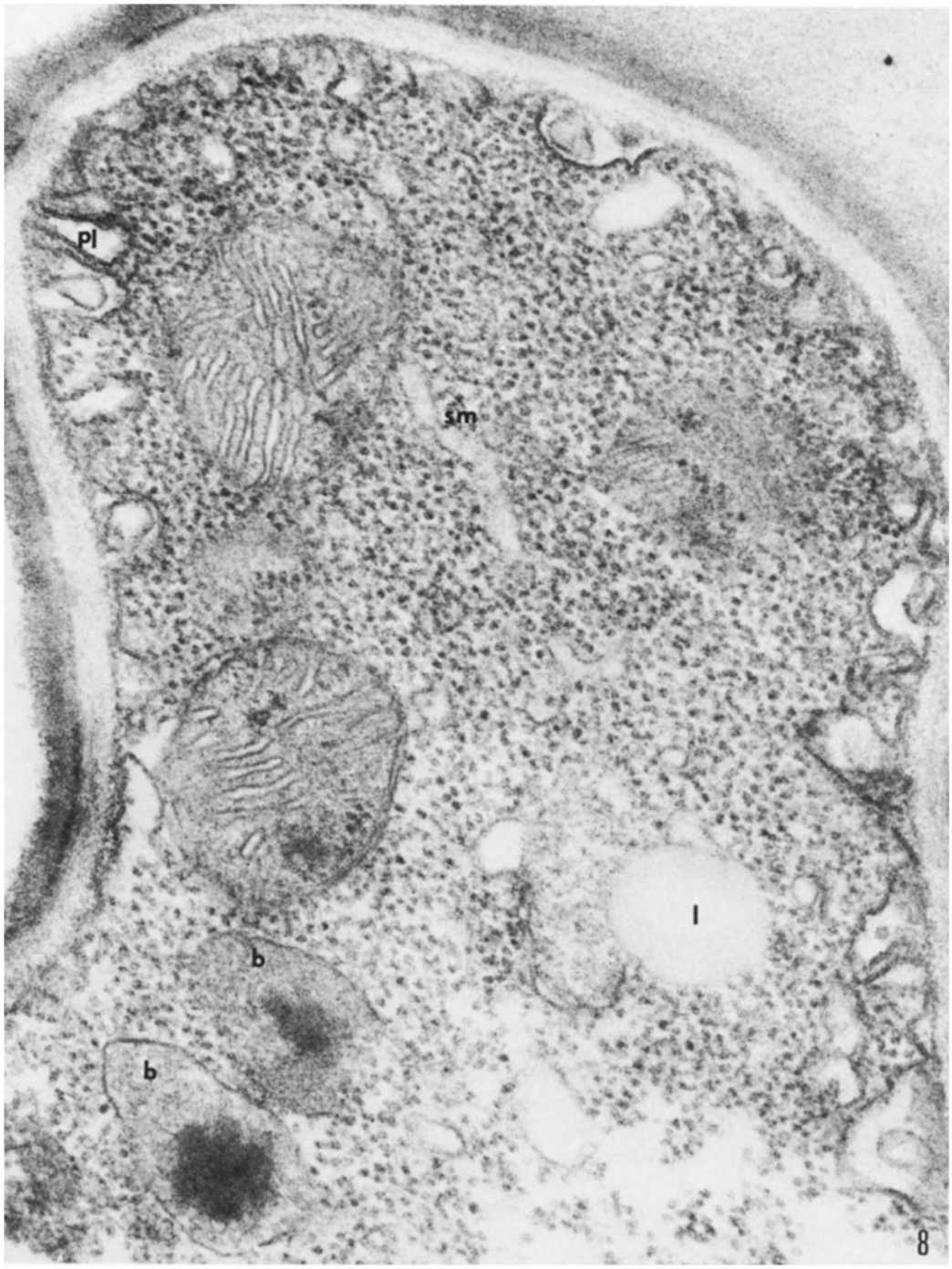


FIGURE 8 Detailed appearance of a few intracytoplasmic inclusions such as mitochondria with multiple cristae and a few ribosomal clusters, short strands of smooth membranes (*sm*), two ovoid-shaped bodies limited by a single membrane and presenting a dense core (*b*), and a lipid-like droplet (*l*). Note the large, somewhat dilated protrusions of the plasma membrane (*pl*) into the cytoplasm. 1% OsO_4, 30 min. × 70,122.

through the cytoplasm without an apparent change in organelle preservation.

In this study we tried to adapt this procedure to a solidified pellet of *C. albicans*, allowing by this procedure the free entrance of fixatives into the cell cytoplasm.

The application of the fixatives for short periods preserved adequately the 7.5-μm thick sections. Prolongation of fixation up to 24 h in either procedure did not alter the final preservation, although the subcellular organelles appeared slightly denser.

As far as the degree of preservation of subcellular organelles obtained with this procedure is concerned, the results are quite comparable to those for *C. neoformans* (2, 9). With this yeast there is apparently no major problem of permeation of chemical fixatives, since all cytoplasmic organelles were nicely demonstrated without the aid of freeze-sectioning.

Some of the cytoplasmic subtructures of *C. albicans*, although clearly visible, remained difficult to interpret in terms of their nature and origin. The ovoid or round bodies limited by a single membrane could represent lysosomal structures, as reported by Günther et al. (12) in *Saccharomyces cerevisiae*, and by Montes et al. (13) in *C. albicans*, or microbody-like particles as found by Avers and Federman (5) in *S. cerevisiae*. Whether the short membrane fragments found in variable amounts from one cell to another are strands of smooth endoplasmic reticulum, or represent, in fact, flattened sacs of the vacuolar apparatus remains an unanswered question already posed by Agar and Douglas for *S. cerevisae* (1).

The possible intermembranous connections between the plasmalemmal protrusions and the vacuolar membranes are difficult to elucidate by morphologic observation.

It is our aim to explore further the usefulness of this preparation procedure in the field of cytochemistry, in order to localize specific marker enzymes for the different cell organelles of *C. albicans*, and thereby to characterize more definitely the nature of these structures and the possible membranous interrelationships.

SUMMARY

The application of conventional fixatives for the visualization of the ultrastructure of *Candida albicans* has been described. The problem of inadequate permeation of chemical fixatives was solved by sectioning solidified pellets of the yeast in the presence of the fixative, a procedure that yields fairly well-preserved subcellular structures.

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REFERENCES

1. AGAR, H. D., and H. D. DOUGLAS. 1957. Studies on the cytological structure of yeast: electron microscopy of thin sections. *J. Bacteriol.* **73**:365.
2. AL-DOORY, Y. 1971. The ultrastructure of *Cryptococcus neoformans*. *Sabouraudia.* **9**:113.
3. AL-DOORY, Y., and C. A. BAKER. 1971. Comparative observation of ultrastructure of five species of *Candida*. *Mycopathol. Mycol. Appl.* **44**:355.
4. ANDERSON, D. R. 1965. A method for preparing peripheral leucocytes for electron microscopy. *J. Ultrastruct. Res.* **13**:263.
5. AVERS, C. J., and M. FEDERMAN. 1968. The occurrence in yeast of cytoplasmic granules which resemble microbidies. *J. Cell Biol.* **37**:555.
6. BAKERSPIGEL, A. 1964. Some observations on the cytology of *Candida albicans*. *J. Bacteriol.* **87**:228.
7. CHANG, J. H. T. 1972. Fixation and embedment, in situ, of tissue culture cells for electron microscopy. *Tissue Cell.* **4**:561.
8. DJACZENKO, W., and A. CASSONE. 1971. Visualization of new ultrastructural components in the cell wall of *Candida albicans* with fixatives containing Tapo. *J. Cell Biol.* **52**:186.
9. EDWARDS, M. R., M. A. GORDON, E. W. LAPA, and W. C. GHIORSE. 1967. Micromorphology of *Cryptococcus neoformans*. *J. Bacteriol.* **94**:766.
10. GALE, G. R. 1963. Cytology of *Candida albicans* as influenced by drugs acting on the cytoplasmic membrane. *J. Bacteriol.* **86**:151.
11. GHOSH, B. K. 1971. Grooves in the plasmalemma of *Saccharomyces cerevisiae* seen in glancing section of double aldehyde-fixed cells. *J. Cell Biol.* **48**:192.
12. GÜNTHER, TH., W. KATTNER, and H. J. MESHER. 1966. Über das Verhalten und die Lokalisation der sauren Phosphatase von Hefezellen bei Repression und Derepression. *Exp. Cell Res.* **45**:133.
13. MONTES, L. F., T. A. PATRICK, S. A. MARTIN, and M. SMITH. 1965. Ultrastructure of blastospores of

Candida albicans after permanganate fixation. *J. Invest. Dermatol.* **45**:227.

14. SUGAWARA, S. 1967. Cellular damage in *Candida albicans* caused by Azalomycin F. Electron microscopic observations. *Annals of the Sankyo Research Laboratory.* **19**:96.
15. VAN DEN BOSSCHE, H. Biochemical effects of miconazole on fungi. *Biochem. Pharmacol.* In press.