

Kinetic and Morphological Observations on the Yeast Phase of *Histoplasma capsulatum* During Protoplast Formation

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Protoplast formation by *Histoplasma capsulatum* yeasts using high concentrations of $MgSO_4$ occurs either by lysis of the bud or lysis of the entire cell wall. Both mechanisms may also occur simultaneously. Neither the protoplast emerging through a hole in the cell wall nor the freshly released protoplast has a recognizable cell wall or the remnant of such. The protoplast contains all the organelles of the normal cell except for mesosomes. During protoplast formation the nucleus increases in size and produces several nuclear masses by the invaginations of the internal layer of the nuclear membrane. All these nuclear masses are surrounded by the external layer of the nuclear membrane. Several nuclei with a normal nuclear membrane are formed later.

In recent years protoplasts have been obtained from bacteria (16), yeasts (8), and plant cells (6). Only recently protoplasts from human pathogenic fungi (*Histoplasma capsulatum* and *Blastomyces dermatitidis*) have been obtained by using a snail enzyme (2) and a nonenzymatic method for the yeast phase of *H. capsulatum* (3). We were interested in studying the relationship between intracellular structures and biodegradation and biosynthesis of the cell wall of human pathogenic fungi. Protoplast formation and the regeneration of the cell wall provide a good model for this study. It is the aim of the present paper to study the ultrastructural mechanism of protoplast formation and the fine structure of the protoplast, and to compare the submicroscopic structure of the protoplast with the structure of the normal yeast cell of *H. capsulatum*.

MATERIALS AND METHODS

The procedure of Berliner and Reza (3) was followed with some modifications to obtain protoplasts from the yeast phase of *H. capsulatum*. The yeast phase of strain G184B of *H. capsulatum* incubated at 37 C for not less than 48 hr and not more than 120 hr on brain heart infusion (BHI) slants containing 0.1% cystine in tubes stoppered with cotton plugs saturated with paraffin, was streaked heavily on fresh BHI plus cystine and incubated at 37 C for 48 hr. Surface growth was removed from two slants by

washing each with 1.0 ml of modified Sabouraud broth (MSAB) and added to a 300-ml nephelometric flask (Bellco Glass, Inc.) with a metal cap containing 50 ml of MSAB (Baltimore Biological Laboratories), pH 5.7. The flask was incubated at 37 C on a reciprocal shaker for 72 hr. Ten milliliters of the 72-hr culture was transferred to a fresh flask of MSAB and incubated at 37 C on a reciprocal shaker for 18 hr. Five milliliters of the 18-hr culture was transferred to a 300-ml nephelometric flask containing 50 ml of MSAB, 1.5 M $MgSO_4$, 2.5 mg of 2-deoxyglucose, pH 5.2, and incubated on the shaker at 37 C for 48 hr. Protoplasts began to be produced after 12 to 24 hr. At 48 hr abundant protoplasts were observed and were collected at this time. No appreciable increase in the amount of protoplasts was detected after 48 hr. A mixture of the medium (1:5, v/v) containing the protoplasts and 0.8 M mannitol was made and was centrifuged at 3,000 rev/min for 30 min. The resulting pellet was collected for electron microscopy studies. For comparative purposes, yeasts were grown in MSAB at 37 C for 18 and 66 hr, centrifuged at 3,000 rev/min for 30 min, and the pellet also was embedded for electron microscopy.

The pellets either from the protoplasts or from the normal yeast were embedded in 2% agar with mannitol as a stabilizer and cut into small blocks. Agar blocks were fixed for 3 hr in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, plus 0.8 M mannitol. The latter was used as an osmotic stabilizer for the protoplasts. Postfixation was in 2% osmium tetroxide for 2 hr, followed by dehydration in an alcohol series and embedding in Maraglas (10) and Epon 812 accelerated with 1.5 or 2% tris(dimethyl-

aminomethyl)phenol (DMP-30, Ernest F. Fullam, Inc.). Sections showing silver to gray interference colors were obtained by using a Porter-Blum MT-2 ultramicrotome equipped with a diamond knife (IVIC). The sections were picked up on parlodion-coated or uncoated 300-mesh copper grids, stained with uranyl acetate (15) and lead nitrate (13), and examined with either a Philips 300 or a Hitachi HU11-B electron microscope. Semithin sections were cut and stained with toluidine blue for light microscopy.

RESULTS

In general, our observations on the ultrastructure of the untreated cells of *H. capsulatum* confirmed those of a previous report (9). Yeasts grown for both 18 and 66 hr have cytoplasm densely packed with free ribosomes surrounded by an undulated cytoplasmic membrane. In several places, however, the plasmalemma is not clearly delimited. The number of nuclei varies from one to (seldom) two and exhibits an irregular outline with a homogeneous matrix. Mitochondria with a diffuse outline, few cristae, and many membranous profiles are scattered in the cytoplasm. Several layers of the membranes parallel to the cytoplasmic membrane are frequently seen. Typical mesosomes are observed. The cell wall seems to be rather even in thickness, smooth in outline, and appears layered (Fig. 1).

Fresh protoplasts vary in size from 4 to 15 μm in diameter (Fig. 2, 3). They are round or slightly oval (this last feature due to compression artifact). Their cell membrane is clearly outlined and more distinct than in intact cells (Fig. 2, 3, 4). Small vesicles between 80 and 150 nm are often extruded from the protoplast (Fig. 2, 3) as well as larger ones of 200 to 300 nm attached to the cell membrane and with smaller vesicles inside (Fig. 4). These are most common when the protoplast is emerging (Fig. 4) and sometimes greatly deform the shape of the protoplast. There is always a big noncentral vacuole and its contents have varying electron densities and different degrees of granulations. The vacuole occupies less than 50% of the cytoplasm volume and is clearly delimited by a single membrane (Fig. 2, 3). The mitochondria are small with few cristae and of high electron density (Fig. 3, 4, 7). Small bodies of 0.7 μm surrounded by a single membrane and with a homogeneous matrix are found clustered near the sparse endoplasmic reticulum (Fig. 2, 3). They are very similar to the microbodies described in *Saccharomyces cerevisiae* (1). Abundant free ribosomes are scattered throughout the cytoplasm (Fig. 3). Intracytoplasmic membrane systems such as mesosomes are not found in freshly formed proto-

plasts. The nuclei are prominent and delimited by two membrane units which form the nuclear envelope (Fig. 2, 9). The number of nuclei varies between one and five, not only in the protoplasts (Fig. 2, 3) but also in the stage prior to their release (Fig. 6).

These nuclei are formed in the following way. During protoplast formation there is a pronounced increase in the size of the nucleus of the yeast and the nucleoplasm is then divided by the invaginations of the internal layer of the nuclear membrane. The resulting nuclear masses are surrounded by the external layer of the nuclear membrane (Fig. 7). When they separate, each has a complete double nuclear membrane (Fig. 2). The nucleolus is not clear during the formation of the new nuclei. Instead, masses of high electron density are seen scattered in the divided nucleoplasm (Fig. 6, 7). Chromosomes are not observed during this abnormal nuclear division.

The release of the protoplast seems to occur by two different mechanisms which may occur simultaneously or separately. In the first instance, a mass of cytoplasm without a recognizable cell wall emerges through an undifferentiated small hole in an otherwise intact cell wall (Fig. 1), whereas Fig. 5 shows a yeast cell in which the cell wall becomes discontinuous and peels in layers. Figure 4 shows the two mechanisms working together. Most of the cytoplasm has emerged through the hole in the cell wall and the cell wall itself is very thin. When emerging through the hole in the yeast cell wall, most of the protoplasts are devoid of any cell wall (Fig. 1, 4, 6). During protoplast formation the cytoplasm loses the characteristic high density observed in the intact yeast, and the nuclear envelope becomes clearly outlined (Fig. 9). The final escape of the protoplast from the cell wall remnants is probably caused by a change in the osmotic conditions and by surface tension (14).

Not infrequently a cell wall is seen surrounding a protoplast and a yeast cell (Fig. 8), and the space around these structures is filled with membranes and amorphous material. This is similar to the intrahyphal hyphae and intrayeast hyphae described in *Paracoccidioides brasiliensis* (4).

DISCUSSION

The early log-phase yeasts that are used to produce protoplasts are 18 hr old and are mainly uninucleate. The intact yeasts in medium containing 1.5 M MgSO_4 plus 2-deoxyglucose also have one or two nuclei. However,

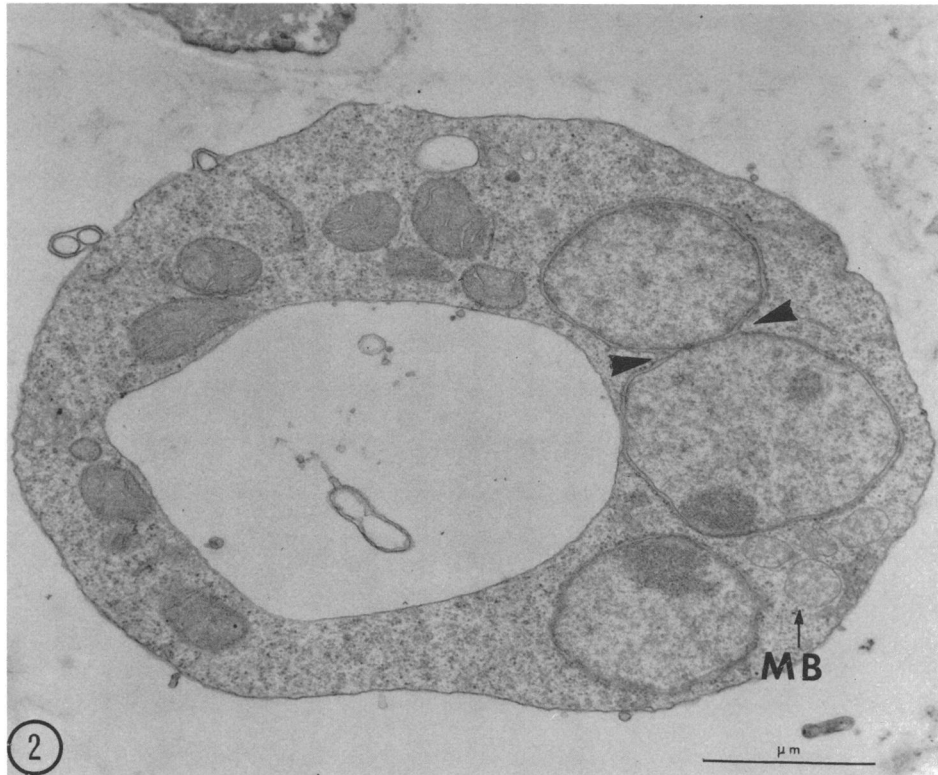
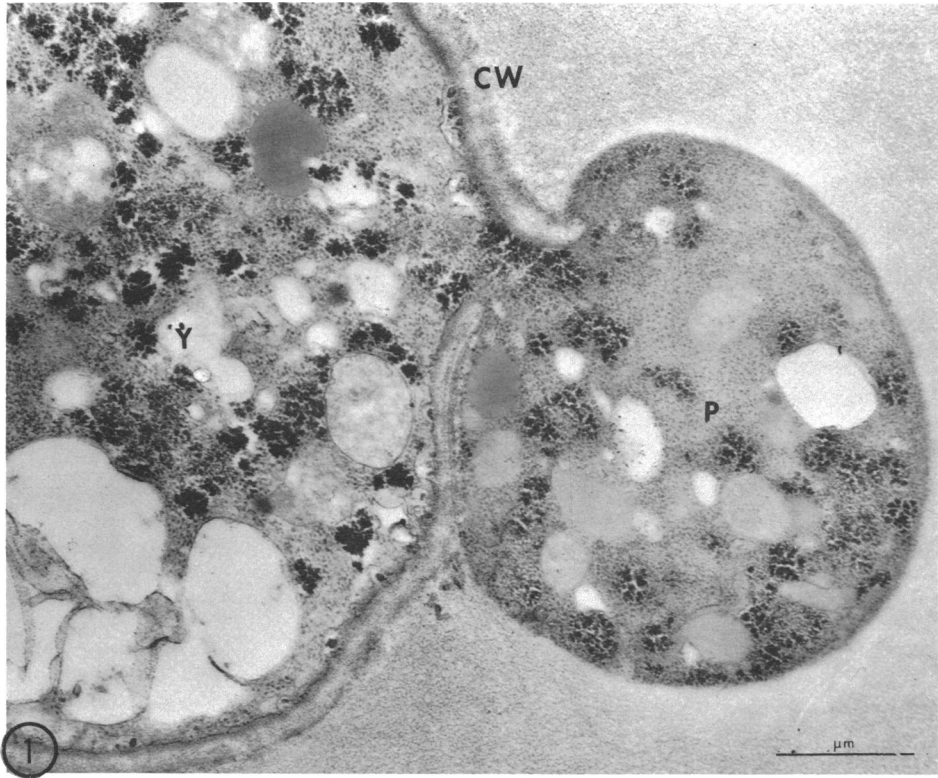
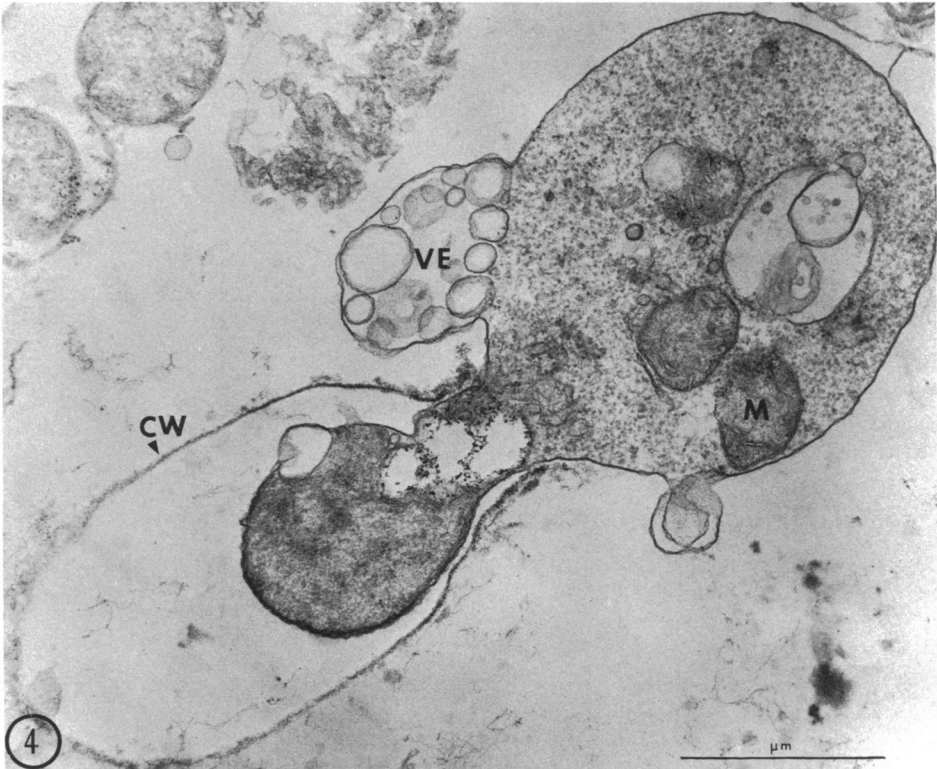
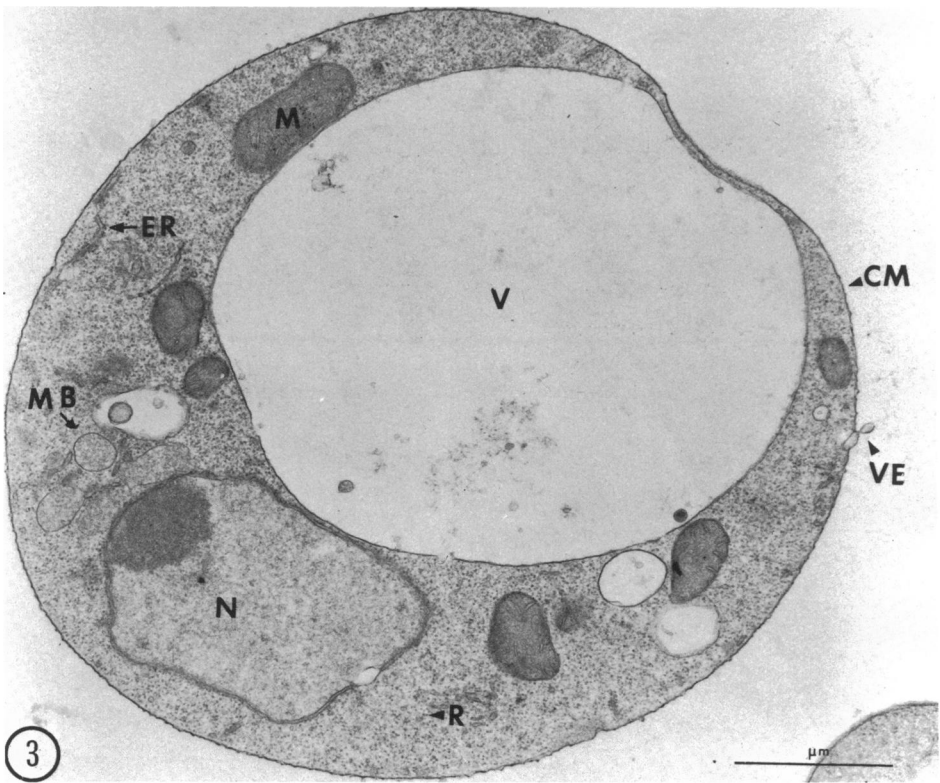


FIG. 1. Early stage of protoplast (P) release through a hole at the apex of the yeast cell (Y). CW: cell wall. Scale marker = 1 μ m.

FIG. 2. Fresh protoplast with three nuclei. Two of them (arrows) are separating within a common external nuclear lamella. MB: microbodies. Scale marker = 1 μ m.



FIGS. 3 AND 4
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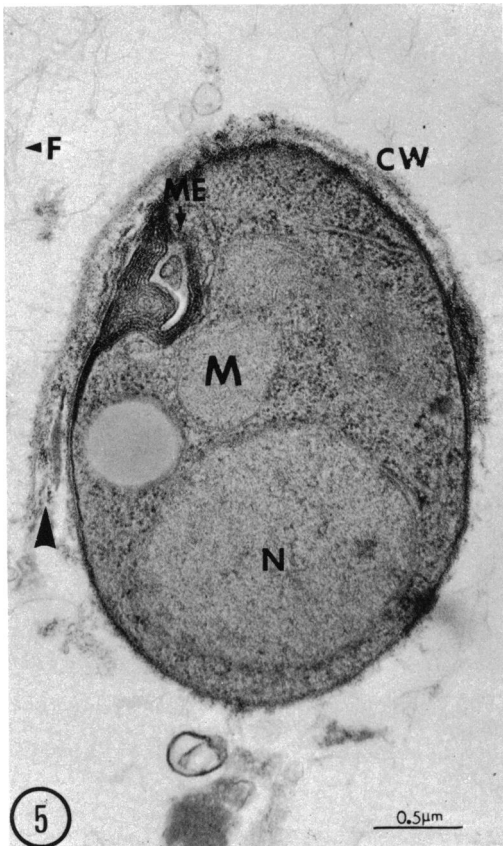


FIG. 5. Early stage in the production of the protoplast. The cell wall (CW) becomes discontinuous and peels in layers (arrow). F: fibers, derived from cell wall. ME: mesosome; M: mitochondria; N: nucleus. Scale marker = 500 nm.

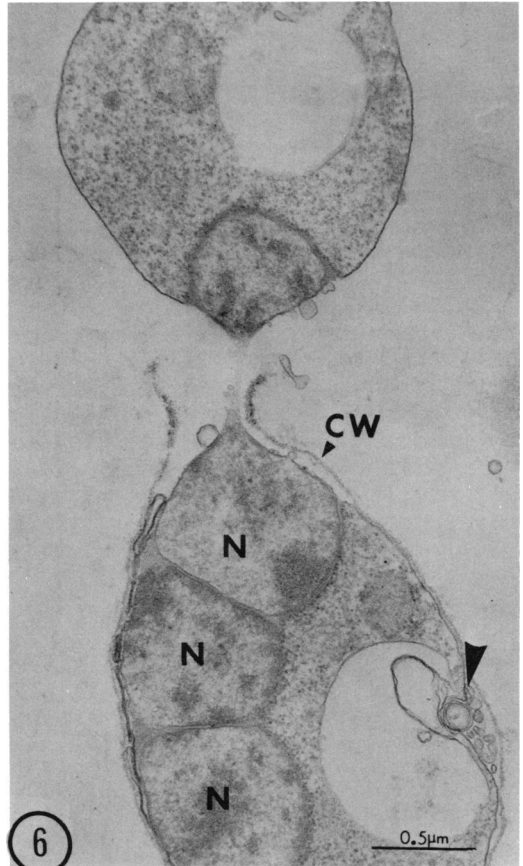


FIG. 6. Release of the protoplast. In this picture the tangential section does not allow a clear relation to be seen between the upper part of the protoplast which has emerged and the trinucleated (N) lower part that still is inside the very thin cell wall (CW). Arrow indicates the clear continuity of the cell membrane with the mesosomes. Scale marker = 500 nm.

FIG. 3. Fresh protoplast without wall remnants attached to the cell membrane (CM); however, small vesicles (VE), are present. The vacuole (V) is off center. Mitochondria (M) with clearly delimited cristae have a high electron density. Ribosomes (R) are abundant. MB: microbodies; ER: endoplasmic reticulum; N: nucleus. Scale marker = 1 μm.

FIG. 4. Release of the protoplast. The protoplast is almost out of the hole in the cell wall, and the cell wall (CW) itself is very thin. There is a big vesicle (VE) enclosing smaller vesicles. M: mitochondria. Scale marker = 1 μm.



FIG. 7. Fresh protoplast with three nuclear masses (N) within a common envelope (arrow) originating from the external layer of the nuclear membrane. Each mass is bounded by the internal layer of the same nuclear membrane. F: fibers; M: mitochondria. Scale marker = 500 nm.

abnormal nuclear divisions which result in more than two nuclei are seen both in stages leading to protoplast formation, prior to the complete disappearance of the cell wall, and in newly formed protoplasts. Since this abnormal division is not seen in protoplasts that regenerate a cell wall (5) in a medium lacking Mg^{2+} , the role played by the high concentration of $MgSO_4$ in promoting abnormal division cannot be overlooked. The fate of these nuclei will be studied in regeneration experiments. Fresh protoplasts of *S. cerevisiae* have one nucleus, but when they regenerate a cell wall they become multinucleate (12). In contrast, *H. capsulatum* has several nuclei in the medium used for protoplast formation and prior to evidence of cell wall regeneration.

The bud in *H. capsulatum* starts as a thickening of the cell wall around a small nipple-



FIG. 8. Protoplast (P) and a yeast (Y) inside the cell wall of a dead yeast. This structure could be called an intra-yeast-protoplast-yeast. Scale marker = 1 μ m.

like evagination of the cytoplasm. This thickening is always located with a bulging of the cytoplasm at one of the apices of the oval yeast (9). Because protoplasts of *H. capsulatum* emerge through one of the apices of the yeast (3) and because at 18 hr most of the cells are at different stages of budding, it is reasonable to suppose that the cell wall is more easily degraded at the bud.

Stages such as the prospheproplasts and spheroplasts of *S. cerevisiae* (7) cannot be clearly detected in *H. capsulatum* because the protoplast becomes spherical and osmotically sensitive immediately after emerging from the cell wall.

The presence of mesosomes and lomasomes in the yeast, and the lack of these organelles in the protoplast and their reappearance in the regenerated protoplast (5), probably indicate

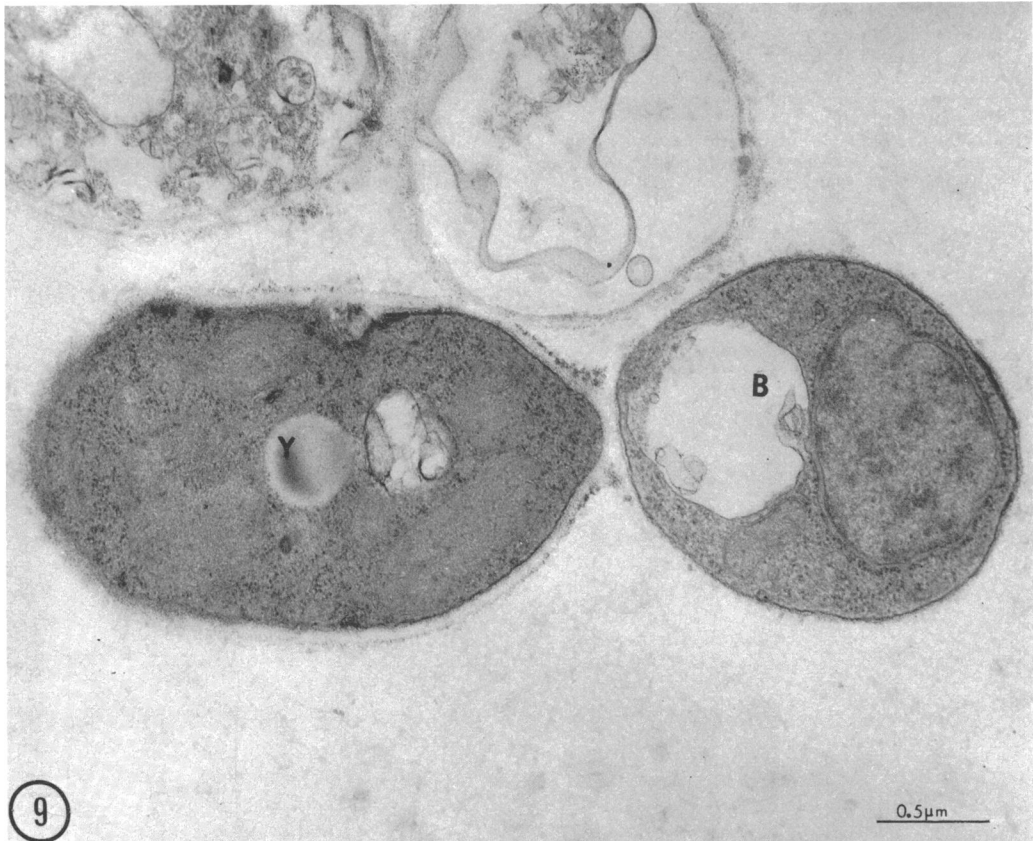


FIG. 9. Bud (B) with a very thin cell wall and cytoplasm showing a lower density than that of the cytoplasm of the mother cell (Y). This bud will later form a protoplast through the degradation of the cell wall. Note that the cell wall of the yeast (Y) is almost intact. Scale marker = 500 nm.

that they play a role in building the cell wall as it has been demonstrated for the lomasomes in *Penicillium vermiculatum* (17) and for mesosomes in *P. brasiliensis* (11).

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