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SOME CYTOLOGICAL AND PATHOGENIC PROPERTIES OF SPHEROPLASTS OF *CANDIDA ALBICANS*

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ABSTRACT

KOBAYASHI, GEORGE S. (Tulane University, New Orleans, La.), LORRAINE FRIEDMAN, AND JUDITH F. KOFROTH. Some cytological and pathogenic properties of spheroplasts of *Candida albicans*. J. Bacteriol. **88**:795-801. 1964.—Spheroplasts of *Candida albicans* were prepared by use of an enzymatic mixture from the digestive tract of the snail *Helix pomatia*. Untreated cells exhibited well-defined cell walls, whereas such structures were absent from spheroplasts. The intravenous inoculation of either spheroplasts or intact cells into rabbits produced a fever which was apparent within 30 min, the "immediate" fever response characteristic of microbial endotoxin. Cell-wall fragments of enzyme-treated cells did not induce a convincing pyrogenic response. When the inoculum was viable, body temperatures did not return to normal but remained elevated until death of the animal 1 or more days later, exhibiting the "delayed" fever of infection. The gross pathological picture in animals succumbing to infection by viable spheroplasts was similar to that obtained with untreated yeast cells.

Giaja (1914, 1919, 1922) first described the denudation of a *Saccharomyces* sp. by removal of its rigid cell wall with an enzyme mixture obtained from the gut of the snail *Helix pomatia*. Since then, protoplasts and spheroplasts of *S. cerevisiae*, *S. carlsbergensis*, *Schizosaccharomyces pombe*, and *Candida utilis* have been obtained in this manner (Eddy and Williamson, 1957; Eddy, 1958; Holter and Ottolenghi, 1960; Svihla, Schlenk, and Dainko, 1961). Garcia-Mendoza and Villaneuva (1962) reported the isolation of another enzymatic preparation, from a *Streptomyces* sp., which could remove the cell wall from various nonpathogenic yeasts but with varying degrees of effectiveness. None of the previous reports on the susceptibility of yeasts to these enzymatic preparations has included *C. albicans*.

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In this study, spheroplasts of *C. albicans* were prepared with snail enzymes and were isolated by differential centrifugation. The denuding process was followed by phase-contrast and by ordinary-light microscopy; the fine structure of untreated and enzyme-treated cells was studied by examination of uranium-shadowed preparations and ultrathin sections with an electron microscope; and the disease-producing capabilities of these denuded cells were investigated by intravenous inoculation into rabbits.

MATERIALS AND METHODS

Cultural methods. C. albicans strain 374, isolated from a human infection, was maintained by transfer every 2 weeks on Sabouraud glucose agar slants at room temperature, between 24 and 27 C. For growth of cells used in the preparation of spheroplasts, the synthetic medium A of Ramachandran and Walker (1957) was modified as follows: 100 ml of buffer, containing 37.5 mg of $(NH_4)_2HPO_4$ and 30 mg of KH_2PO_4, were dispensed into 500-ml Erlenmeyer flasks and autoclaved; after the buffer had cooled, 10 ml of $MgSO_4 \cdot 7H_2O$ (15 mg) and 30 ml of glucose (15 g), both sterilized by autoclaving, were added aseptically to each flask; finally, 10 ml of a solution containing $FeSO_4 \cdot 7H_2O$ (1.7 mg) and biotin (0.0015 mg), sterilized by Seitz filtration, were added to each of the flasks to yield a final volume of 150 ml of medium per flask (pH 6.5). Preparation in this manner prevented the formation of insoluble precipitates and the breakdown of glucose. To check for possible contamination, all of the flasks were incubated at 37 C for 24 hr and then at room temperature for 72 hr prior to use. All of a 7-day-old culture of *C. albicans*, grown on a Sabouraud glucose agar slant, was inoculated into 150 ml of medium A and incubated for 72 hr at 37 C on a rotary shaker. The cells were harvested by centrifugation, and resuspended in fresh medium A. After three successive 72-hr passages and a final 16- to 18-hr passage in this manner, the growth was harvested by centrifuga-

tion, washed three times with cold saline, and suspended in isotonic buffer (0.75 M potassium chloride buffered with 0.05 M phosphate at pH 5.5) to a final concentration of 5% (wet w/v).

Preparation and isolation of spheroplasts. The lytic material, gastric juice of the snail *H. pomatia*, was obtained from L'Industrie Biologique Francais (Seine, France), and the ampoules were kept at −20 C. Prior to use, the thawed enzyme was centrifuged at 1,300 × *g* for 30 min to remove particulate matter, and then was diluted 1:3 with distilled water. The methods employed for the conversion of yeast cells to spheroplasts were essentially those of Holter and Ottolenghi (1960) and Svihla et al. (1961). The procedure was performed at room temperature, and the quantity of enzyme employed ranged from 6.7 to 8.4 mg (dry weight) per ml of a 5% yeast suspension. The denuding process, complete in 6 to 10 hr, was followed by periodic examination of samples under phase contrast with a Reichert Zetopan microscope equipped with bright-field positive and anoptral phase-contrast optics, and under an ordinary light microscope. When the perimeters of about 90% of the cells lost refractiveness and a large number of refractive granules became strikingly apparent within the cytoplasms, the treated cells were recovered by centrifugation at 1,300 × *g* for 15 min. They were washed three times with buffered isotonic salt solution and stored in this milieu at 4 C until used. The washed, treated cells were diluted to read 220 to 250 Klett units at 660 mμ in the salt solution, and were carefully layered over an equal volume of 40% sucrose (w/v). (Suspensions of greater concentration did not separate well in the sucrose.) After centrifugation at 1,300 × *g* for 30 min at 20 C, the spheroplasts were concentrated at the sucrose-salt interphase which was carefully removed by suction, and washed four times with buffered salt solution. Formaldehyde, to yield a final concentration of 0.5%, was added to a portion of the fresh spheroplast suspension, and after 12 hr the then dead spheroplasts were washed three times in cold saline. The material which sedimented in the 40% sucrose solution, termed "sucrose sediment," was recovered and washed in the same manner as that described for spheroplasts. Both the spheroplasts and the "sucrose sediment" were stored at 4 C until used.

The reaction of viable spheroplasts to osmotic shock was observed by placing them in distilled water and watching them under a light microscope for 15 min, after which time a small drop of alcohol was placed under the cover slip and the cytological changes were recorded. Also, 1 ml of 95% ethanol was added to 9.5 ml of water containing 1.5 × 10^9 spheroplasts, the mixture was centrifuged at 1,300 × *g* for 10 min, and the sediment was examined microscopically.

Studies with an electron microscope. Untreated cells, enzyme-treated cells before separation into fractions by centrifugation on sucrose solution, and spheroplasts suspended in distilled water, were placed on Formvar grids and shadowed with uranium. Samples of these suspensions were also processed according to the method of Fukushi (1959), i.e., washed three times with distilled water, fixed in 2% OsO_4 for 18 to 24 hr at 4 C, washed once with cold distilled water, suspended in melted 2% agar at 45 to 50 C, dispensed in small drops (diameter, 0.5 to 1.0 cm) onto a clean glass slide, allowed to solidify, cut into squares measuring about 0.125 cm^3, dehydrated in alcohol, and finally impregnated, according to the method of Freeman and Spurlock (1962), with Maraglass. Ultrathin sections were cut with a Porter Blum Ultra Microtome (Ivan Sorvall, Inc., Norwich, Conn.) equipped with a 45-degree glass knife (cutting angle, 3 to 5 degrees), and were stained with lead hydroxide according to the method of Karnovsky (1961). The stained sections were examined in an RCA model EMU–2D electron microscope.

Animal studies. Male New Zealand white rabbits (Albino Farms, Red Bank, N.J.), weighing 2 to 3 kg, were housed individually at a thermostatically controlled temperature of approximately 21 C. The animals were permitted to acclimatize to their new surroundings for at least 1 week before experimental use, and they were conditioned prior to pyrogenic assay by placing them in wooden stocks for increasing periods on 3 successive days. During an 8-hr period, rectal temperatures were taken at approximately hourly intervals, by inserting the thermometer to a depth of 6.5 to 7.5 cm and allowing it to remain for a period of at least 3 min. On the fourth day, the animals were placed in the stocks, allowed to adjust for 2 hr, and then were inoculated. Rectal temperatures were taken at 30-min intervals for 2 hr prior to and 3 hr after the inoculation, and then hourly until the tenth hour, at which time the animals were returned to their cages. Wher-

ever applicable, the animals were returned to the stocks on succeeding days to determine the outcome of the pyrogenic response.

The pyrogenicity of 10^9 particles of viable whole cells, viable and nonviable spheroplasts, and "sucrose sediment" was determined by injecting 1 ml into the right marginal ear vein of the conditioned animal.

Since the "sucrose sediment," which was rich in cell-wall material but also contained partially hydrolyzed cells, intact cells, and spheroplasts, i.e., a mixture of all cellular components, induced fever which seemed greater than that observed with spheroplasts alone, although less than from untreated cells, an additional experiment was performed. To determine whether the cell-wall fragments were pyrogenic *per se*, a cell-wall preparation devoid of soluble protoplasmic material was obtained by exposure of the "sucrose sediment" to sonic treatment at maximal efficiency in a 10-kc Raytheon sonic oscillator for 15 min. The cell-wall fragments and any other particulate debris (e.g., membranes) were recovered by centrifugation, washed several times with saline, and suspended in this milieu to the original volume; 1 ml was injected intravenously into each of three rabbits. As a control, "sucrose sediment" not sonically treated, at the same concentration, was injected into another group of three rabbits and the febrile responses were noted.

Concurrent with each animal experiment, three normal rabbits were injected with the saline used in the preparation of the test materials. The temperatures of these normal rabbits never fluctuated more than 0.5 F; therefore, only changes greater than this were accepted as significant.

Miscellaneous procedures. All glassware, syringes, and needles were heated at 160 to 170 C in a hot-air oven overnight to destroy any contaminating pyrogens. Doubly glass-distilled water was used in the preparation of all media, reagents, and solutions for injection purposes. Physiological saline so prepared was checked for pyrogenic activity by injection into rabbits.

RESULTS AND DISCUSSION

Treatment of a young, actively growing culture of *C. albicans* with an enzymatic mixture obtained from the digestive tract of *H. pomatia* resulted in the removal of the cell walls. The progress of digestion as followed by light microscopy is shown in Fig. 1. Cell membranes were seen to have pulled away from the cell wall during this process and to have become gradually filled

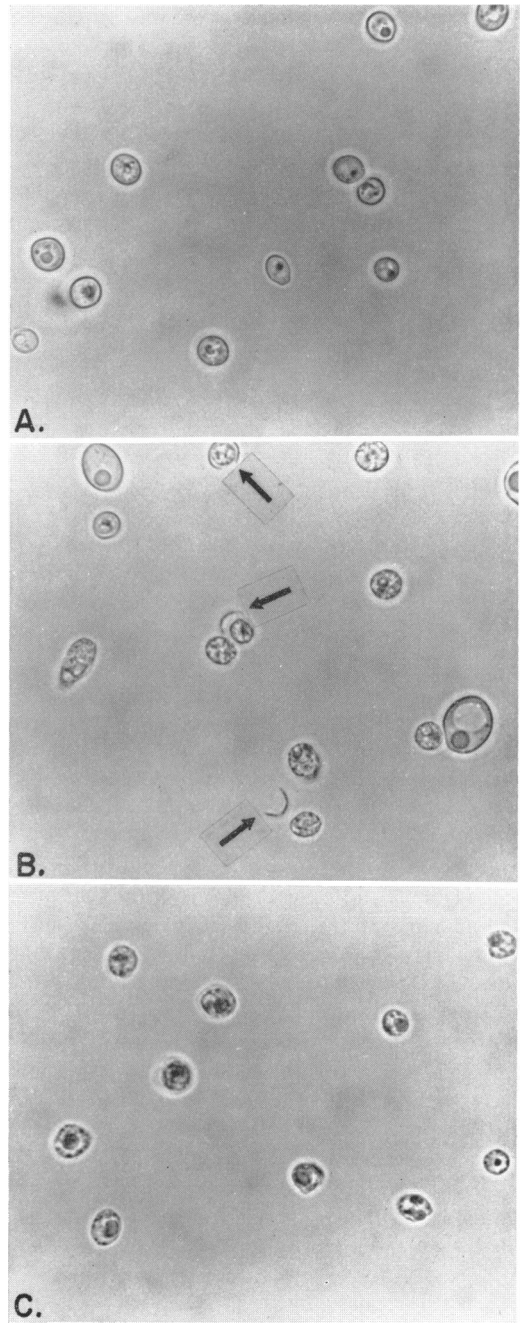

FIG. 1. *Light photomicrographs of Candida albicans. (A) Untreated cells, (B) cells undergoing various phases of hydrolysis of cell wall (see arrows), (C) spheroplasts. 430×.*

with highly refractive, granular material. Hydrolysis of the wall appeared to have begun at one edge of the cell, but apparently only a portion eroded, and when sufficient wall was hydrolyzed the spheroplast emerged, leaving a hollow eggshell structure. The separation of the spheroplasts from partially hydrolyzed cells and cell-wall debris by centrifugation on sucrose, removal of the interphase, and repeated washing with isotonic buffer did not alter their morphology (Fig. 1C), and transfer into a hypotonic environment of distilled water resulted only in swelling, not bursting. This observation of nonsusceptibility to osmotic shock seems to be in contradiction to the observations of other investigators (Eddy and Williamson, 1957). When, however, the surface of the spheroplast membranes was altered by addition of alcohol, the swollen cells seemed to burst, although the action was so fast the actual process could not be discerned. Also, after suspension of the spheroplasts in ethanol, intact cells could not be found in the sediment. Presumably, this was because the cell membrane contains lipoprotein and the organic solvent altered at least a portion of it, thus lowering the surface cohesive forces of the membrane. Cells before hydrolysis by enzyme were refractory to such treatment. The observation that spheroplasts will lyse in the presence of alcohol provides a rapid but mild method for disruption of these structures as compared with sonic vibration previously used (Svihla et al., 1961).

Electron microscopic studies of ultrathin sections (Fig. 2), in general, also confirm that after the enzymatic treatment and the various purification steps the spheroplasts still possessed structural integrity. They were enveloped by cytoplasmic membranes, as shown in Fig. 2D, and nothing resembling cell walls or fragments thereof was evident. While these denuded cells might seem to fulfill the criterion of "protoplasts," in that enzymatic digestion seemed to have removed all of the cell walls, the term "spheroplast" is used because more definitive work (such as immunological studies) to confirm the total absence of cell wall was not performed. Also, they were not osmotically sensitive to water.

Electron microscopic studies of uranium-shadowed whole yeast cells and spheroplasts confirmed the observations made by light microscopy, i.e., untreated cells possessed a well-defined perimeter whereas the spheroplasts did not, confirming the absence of cell wall in the latter. The electron density of the spheroplasts was irregularly reduced at the periphery of the cell with an accumulation of electron-dense material at the center. Cells not treated with enzymes gave no evidence of such collapse during dehydration by vacuum, presumably because of the structural strength afforded by their intact walls; in a study by Nickerson (1963), dehydrated yeast cells of *C. albicans* similarly withstood such treatment. It is suggested, therefore, that collapse of the spheroplasts can be taken, in combination with other results of this study, as evidence of the loss of cell wall through enzymatic treatment. It should be noted, however, that Hurst (1952) presented data showing that structural changes could be brought about by removal of certain lipids of the cell wall without removal of the wall *per se*. Nevertheless, there seems little doubt that walls of untreated cells remain rigid throughout vacuum dehydration.

The data presented in Fig. 3 confirm the pyrogenicity of *C. albicans* (Braude, McConnell, and Douglas, 1960); these data also show that this is not a manifestation solely of intact cells, for both viable and formaldehyde-killed spheroplasts had the ability to produce an immediate fever. These immediate febrile responses were indistinguishable from those observed with bacterial endotoxins (Keene, Silberman, and Landy, 1961) or zymosan (Freedman and Sultzer, 1961), in that they began as early as 30 min after inoculation, reached a peak at about 3 to 4 hr, and then declined somewhat at about 8 hr. They were elicited by intravenous injection of intact cells, viable spheroplasts, killed spheroplasts, or the enzymatically treated mixture of forms (Fig. 3). However, cell-wall fragments and cellular debris alone (particulate material removed from sonically treated "sucrose sediment") did not elicit a convincing rise in temperature (only 0.1 F above normal fluctuation). Since this material which contained only cell walls and particulate cellular debris was nonpyrogenic, and since the spheroplasts were pyrogenic, it would seem that the cell wall does not possess any of the substances causing the pyrogenic activity of the cell and that the component responsible for the fever resides solely in the intracellular material. One could postulate, however, that the enzymatic treatment followed by sonic vibration may have removed or destroyed the pyrogenically active materials from the cell-wall fragments. If this were the case, it is still not possible that regeneration of cell wall

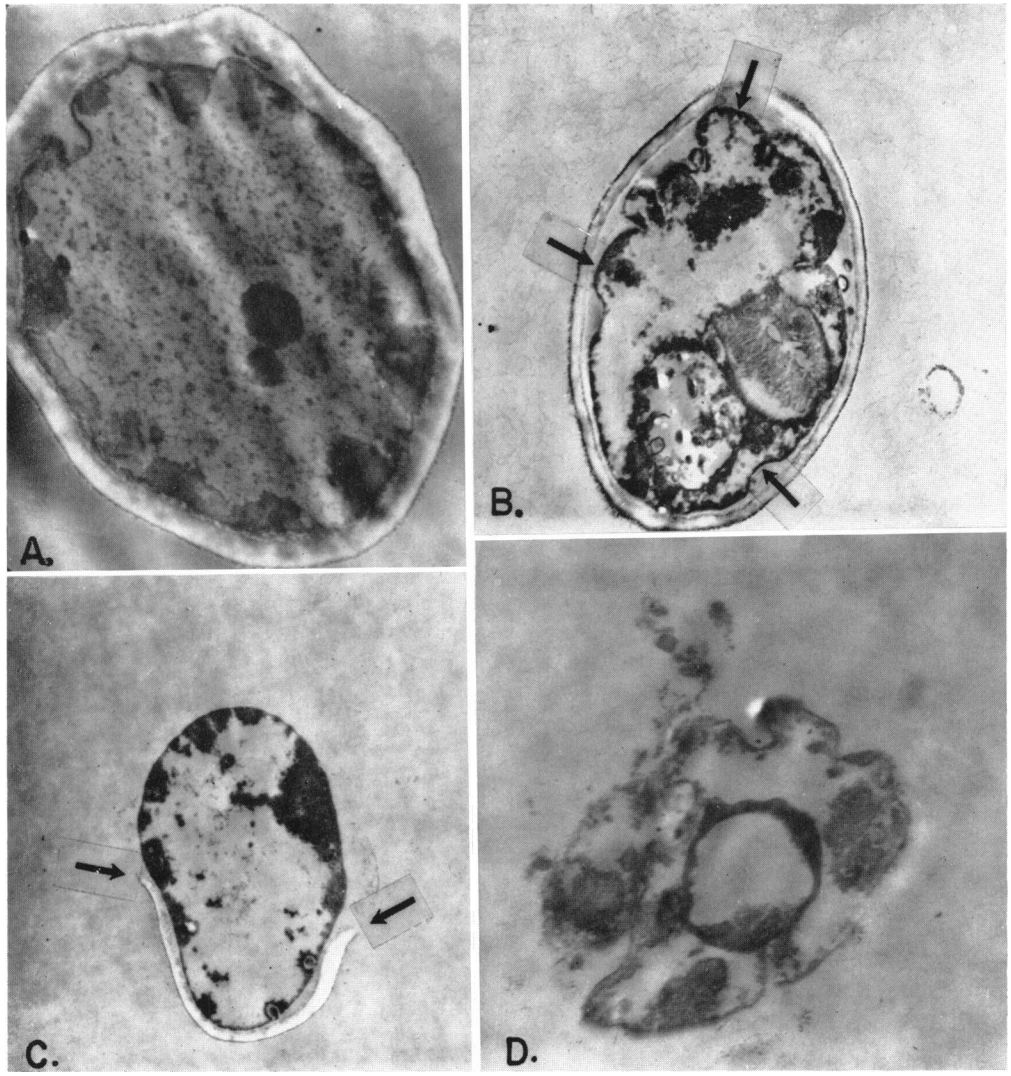

FIG. 2. *Electron photomicrographs of ultrathin sections of Candida albicans stained with lead hydroxide. (A) Untreated control cell, 6,000 ×; (B) enzyme-treated cell showing cytoplasmic membrane detaching from cell wall, 5,000 ×; (C) digestion of a portion of the cell wall, 4,000 ×; (D) spheroplast, 5,000 ×.*

could have accounted for the immediate febrile response when viable spheroplasts were inoculated, because an immediate fever still occurred when killed spheroplasts were injected into rabbits (Fig. 3). These experiments do not resolve whether or not the pyrogenic material of the spheroplasts was of a particulate or soluble nature because, unfortunately, the supernatant fluid of the sonically treated mixture was not tested in animals, nor were the spheroplasts disrupted and suitable studies made.

Only those animals given viable cells (either spheroplasts, intact yeast cells, or a mixture of these forms) experienced a delayed fever in addition to the immediate febrile response (Fig. 3). This response persisted for 1 or more days after injection, and those animals experiencing it succumbed to infection. At necropsy, there were bilateral lesions of the kidneys, microscopic examination of which revealed massive numbers of filamentous organisms; cultures of these lesions were positive for *C. albicans*.

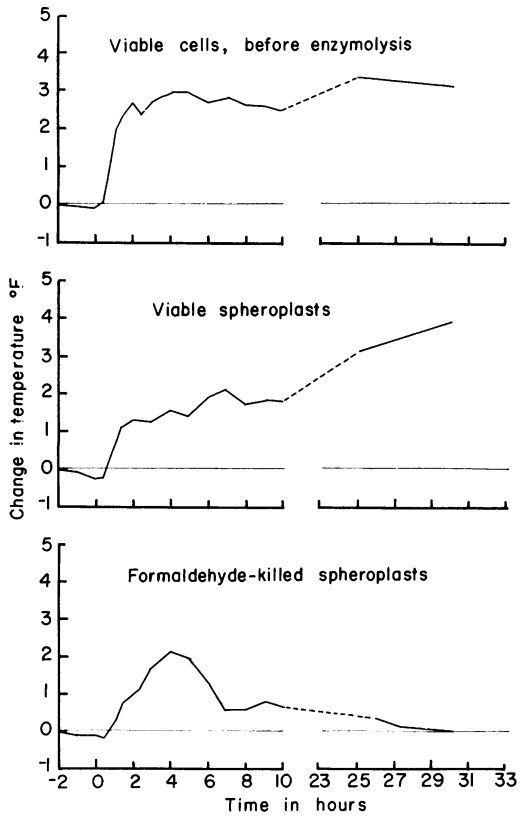

FIG. 3. *Similarity of the immediate febrile responses in rabbits inoculated intravenously with 10^9 enzymatically induced morphological forms of Candida albicans. Each curve represents the average febrile response of three rabbits. The broken lines represent intervals during which temperatures were not observed.*

It could be reasoned that the spheroplast suspension was pathogenic because of the presence of undetected intact cells, for it was technically not feasible to eliminate the possibility of such cells being present. If intact cells were present, however, they were there in such small number that repeated microscopic examinations failed to reveal them. It is inconceivable that such small numbers of intact cells, even if present, could have possessed the virulence displayed by the spheroplast suspension, for this suspension produced an acute infection, which low dosages of intact cells are incapable of causing. Therefore, it seems reasonable to conclude that pathogenicity was not lost by removal of the cell wall.

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LITERATURE CITED

BRAUDE, A. K., J. MCCONNELL, AND H. DOUGLAS. 1960. Fever from pathogenic fungi. J. Clin. Invest. **39**:1266–1276.

EDDY, A. A. 1958. The structure of the yeast cell wall. II. Degradative studies with enzymes. Proc. Roy. Soc. London Ser. B **149**:425–440.

EDDY, A. A., AND D. H. WILLIAMSON. 1957. A method of isolating protoplasts from yeast. Nature **179**:1252–1253.

FREEDMAN, H. H., AND B. M. SULTZER. 1961. Modification of lethality of endotoxin in mice by zymosan. Proc. Soc. Exptl. Biol. Med. **106**:495–498.

FREEMAN, J. A., AND B. O. SPURLOCK. 1962. A new epoxy embedment for electron microscopy. J. Cell Biol. **13**:437–443.

FUKUSHI, K. 1959. Electron microscopic studies of tubercle bacilli. V. Studies on fixation in ultrathin sectioning. Sci. Rept. Res. Inst. Tohoku Univ. Ser. C **9**:1–17.

GARCIA-MENDOZA, C., AND J. R. VILLANEUVA. 1962. Accion litica de un *Streptomyces* sobre levaduras: obtencion de protoplastos. Microbiol. Espan. **15**:139–148.

GIAJA, J. 1914. Sur l'action de quelques ferments sur les hydrates de carbone de la levure. Compt. Rend. Soc. Biol. **77**:2–4.

GIAJA, J. 1919. Emploi des ferments dans les études de physiologie cellulaire: le globule de levure dépouillée de sa membrane. Compt. Rend. Soc. Biol. **82**:719–720.

GIAJA, J. 1922. Sur la levure dépouillée de membrane. Compt. Rend. Soc. Biol. **86**:708–709.

HOLTER, H., AND P. OTTOLENGHI. 1960. Observations on yeast protoplasts. Compt. Rend. Trav. Lab. Carlsberg **31**:409–422.

HURST, H. 1952. An electron diffraction study of the structure and orientation of the lipids in yeast and bacterial cell walls. J. Exptl. Biol. **29**:30–53.

KARNOVSKY, M. J. 1961. Simple method for "staining with lead" at high pH in electron microscopy. J. Biophys. Biochem. Cytol. **11**:729–732.

KEENE, W. R., H. R. SILBERMAN, AND M. LANDY. 1961. Observations on the pyrogenic response

and its application to the bioassay of endotoxin. J. Clin. Invest. **40**:295–301.

NICKERSON, W. J. 1963. Symposium on biochemical bases of morphogenesis in fungi. IV. Molecular bases of form in yeasts. Bacteriol. Rev. **27**:305–324.

RAMACHANDRAN, K., AND T. K. WALKER. 1957. Glucose metabolism in *Candida* species. Biochem. J. **65**:20–24.

SVIHLA, G., F. SCHLENK, AND J. L. DAINKO, 1961. Spheroplasts of the yeast *Candida utilis*. J. Bacteriol. **82**:808–814.