INTRACELLULAR LOCATION OF CAROTENOID PIGMENTS IN YEAST-PHASE CELLS OF *WANGIELLA DERMATITIDIS* AND CELL WALL MORPHOLOGY AFTER ENZYME TREATMENT

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Linda Ann Foster, B.S., M.S.
Denton, Texas

December, 1991

Foster, Linda Ann, Intracellular location of carotenoid pigments in yeast-phase cells of *Wangiella dermatitidis* and cell wall morphology after enzyme treatment. Doctor of Philosophy (Microbiology), December, 1991, 130 pp., 2 tables, 43 figures, bibliography, 29 titles.

Carotenoid pigments in W. dermatitidis, the first pathogenic, dematiaceous fungus in which carotenoid pigments have been reported, are located primarily (81%) in lipid organelles which floated on the surface of the supernatant fraction of lysed cells. Pigment in this fraction could be extracted with ethyl ether without prior treatment with acetone indicating the pigment is unbound in the lipid organelle. Eight percent remains after exhaustive ether extraction and is recovered after the sample is treated with acetone indicating this fraction is non-covalently bound to proteins in the membranes associated with the lipid organelle. The remaining pigment (about 12%) represents contamination of the supernatant with the lipid organelles.

Selectively degrading the cell wall of the yeast-phase cell of W. dermatitidis, individually or with combinations of pronase, beta-glucuronidase, and lysing enzyme, reveals a multilayered cell wall seen in transmission electron micrographs which stains unevenly with uranyl acetate and lead citrate or ruthenium red. The wall has at least three

layers with the inner layers consisting of microfibrils unevenly distributed in an amorphous matrix which is composed, at least in part, of protein since it is removed by pronase treatment.

The outer layer of the cell wall consists of a heterogeneous mixture of polymers which are unevenly distributed. Treatment with various enzyme mixtures leads to pitting of the wall. Treatment with all three enzymes results in complete digestion of the inner wall layers leaving distorted cells surrounded by a remnant of the outer wall layer which is breached in many places.

The results show that the inner two wall layers are responsible for cell shape and consist of microfibrils, presumably of chitin and/or cellulose, embedded in an amorphous matrix of protein, glucans and mannans. The outer layer consists of pockets of protein and beta-glucuronides susceptible to digestion by pronase and beta-glucuronidase respectively. These in turn are embedded in a matrix which is refractile to digestion by all the enzymes tested.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

Purpose.

The specific intracellular location and the nature of the pigment-substrate binding is an important step in determining the function of the carotenoid pigments in Wangiella dermatitidis. A previous study by Foster (1985) indicated that carotenoid pigments in carotenogenic, melanin-less mutants of W. dermatitidis were non-covalently bound to protein and located in the membranes associated with and surrounding the lipid organelles of yeast-phase cells. One purpose of this investigation was to determine the specific location of these pigments.

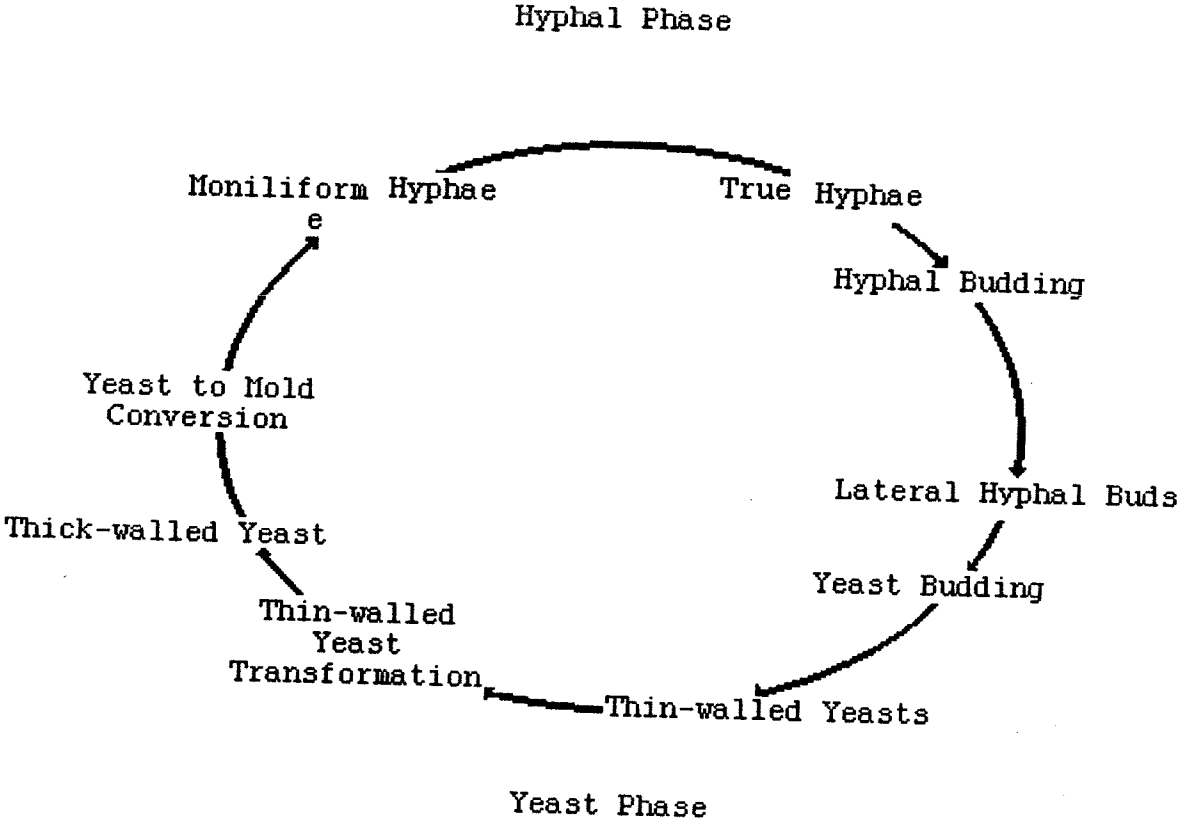
Electron microscopic examination of thin sections of yeast cells, before and after treatment with hydrolytic enzymes, and pigmented cell lysate revealed differences in the cell wall. A second purpose of this investigation was to study the ultrastructure of the cell wall of W. dermatitidis by selectively degrading portions of the wall with different wall degrading enzymes and examining treated cells by electron microscopy.

Literature Review of W. dermatitidis.

Wangiella dermatitidis [formerly Hormiscium dermatitidis (Kano, 1934), Fonsecaea dermatitidis (Carrion, 1950), Hormodendrum dermatitidis (Conant, et al., 1954) and Philaphora dermatitidis (Matthews & Sistrom, 1959)] is a polymorphic fungus that may produce hyphal elements or yeast growth (Oujezdsky, et al., 1973). The fungus is in the fungal-form family Dematiaceae and is one of the causative agents of phaeohyphomycosis (Dixon, et al., 1987). Matsumoto et al. (1984) considered W. dermatitidis to also be an important neurotrophic pathogen with a calculated mortality rate of nearly 50% in reported cases and 100% mortality when the central nervous system is involved. A study by Dixon et al. (1987) in murine phaeophypomycosis caused by W. dermatitidis showed 100% mortality for infections caused by the wild type strain, DMD 368, but no mortality for infections with the melanin-deficient mutant Mel 3.

The yeast-to-mold conversion of W. dermatitidis involves the development of five distinct cell types (Fig. 1) (Oujezdsky, et al., 1973): (1) Thin walled yeast cells predominate during exponentially growing cultures and are characterized by polarized, site specific cell wall synthesis that results in bud emergence. (2) Thick walled

Fig. 1. Transitions among the five cell type morphologies of W. dermatitidis (Oujezdsky, et al., 1973).


Hyphal Phase
Moniliform Hyphae
e
True Hyphae
Hyphal Budding
Lateral Hyphal Buds
Yeast Budding
Thin-walled Yeasts
Thin-walled
Yeast
Transformation
Thick-walled Yeast
Yeast to Mold
Conversion
Yeast Phase

yeasts found in stationary cultures have a thick cell wall that is composed of at least two layers, the outer layer being fragile. (3) Monoliform hyphae which arise from the thick walled yeast cell type, (4) true hyphae and (5) a multicellular morphology. The multicellular form may be induced *in vivo* during establishment of infection of the wild type strain in humans (McGinnis, 1983) or in experimental infections in mice (Nishimura & Miyaji, 1983). *In vitro*, the multicellular morphology is induced by incubation in an acidic medium or prolonged growth on a solid agar medium (Szansizlo, *et al.*, 1976).

The transition from thin walled yeast cell to thick walled cells results in intracellular changes as well as changes in the cell wall. The thin walled cell contains numerous mitochondria and ribosomes, but few vacuoles or storage material. As the wall thickness increases the ribosomes become less obvious in electron micrographs and the size and number of the vacuoles increases (Oujezdsky, *et al.*, 1973).

Geis and Szansizlo (1984) discovered the carotenoid pigments torulene and torularhodin in melanin-less mutants of *W. dermatitidis*. Carotenogenesis required light and an aerobic environment. The presence of the pigments was shown to protect cells against killing by UV radiation. This

protection appeared to involve a "shielding" of sensitive organelles rather than a neutralization of oxidants (Geis & Szansizlo, 1984).

Carotenoid Pigments in Fungi.

Carotenoid pigments are one of the most important classes of pigments found in living organisms. The pigments are found in bacteria, fungi, plants, insects and animals. The basic carotenoid molecule is symmetrical and is formed from eight isoprenoid units linked together so the two central units are in a 1,6 positional relationship and the remaining, nonterminal units are in a 1,5 positional relationship (IUPAC Commission, 1971). Different carotenoid pigments may be derived by hydrogenation, dehydrogenation, or oxidation of the isoprenoid units. The carotenoid molecules which have oxygenated modifications are xanthophylls and those which are hydrocarbons are carotenes (IUPAC Commission, 1971).

In plants, algae and photosynthetic bacteria, carotenoids are part of the photosynthetic system. They are accessory, light absorbing pigments associated with chlorophyll and protect the photosynthetic organism from photosensitized oxidation (Krinsky, 1971). The pigments are associated with chlorophyll in the lamella of chloroplasts in vascular plants (Conjeaud, et al., 1976). Mutants of the photosynthetic bacterium, Rhodospirillum capsulata which

accumulated phytoene, phytofuluene, e-carotene, and neurosporin showed a lethal aerobic photosensitivity when compared to wild-type cells which accumulated normal amounts of unsaturated carotenes (Biel & Marrs, 1985).

The pigments may exist in the cell in either the free or bound state. A true carotenoprotein is defined as a compound in which the carotene or xanthophyll is bound to protein in stoichiometric proportions (Zagalsky, 1978). Three carotenoproteins have been isolated: (1) crustacyanin, a blue protein found in lobster carapaces, (2) ovoverdin, the green storage protein found in lobster eggs and (3) ovorubin, a red protein isolated from the gastropod Promaceae canliculata (Thommen, 1971). Another class of carotenoid-substrate complexes exist in the yolk of avian eggs. The pigment is part of a large lipid prosthetic group and does not show specific interaction with the protein (Zagalsky, 1978).

Less work has been done on non-photosynthetic bacteria and fungi and the function of carotenoids in non-photosynthetic microorganisms is less understood, although they have been shown to protect against killing by white light. Krinsky (1971) proposed four mechanisms by which carotenoids might function as photoprotectors:

1. An absorbing system in the cell envelope to filter out potentially harmful light.
2. Systems to interact with and quench photosensitizer triplet states.
3. Systems to serve as preferred substrates for photosensitzed oxidations; and
4. Systems to stabilize membranes or repair damaged membranes.

The association of the carotenoids with a substrate can result in mutual stabilization of both the pigment and the substrate (Krinsky, 1971). For example, the carotene in Sarcina flava is covalently bound to a glycoprotein and is more resistant to elevated temperatures and acidic pH than the unbound pigment (Thirkell & Hunter, 1969).

The location of the pigment in the cell may give an indication of its function. In carotenogenic, non-photosynthetic bacteria, the carotenoid pigment has been found in the cell wall (Work & Griffiths, 1968) and in the cell membrane (Itschik & Reichenbach, 1978; Salton & Ehtisham-uh-Din, 1965; Steensland & Larson, 1969). In fungi, carotenoid pigments have been discovered in a number of subcellular components. Beta-carotene in Blakeslea trispora is found in a lipid pool (Hsu & Yokoyama, 1972) and is an important precursor to hormones important in mating (Davis, 1973). In Verticillium agaricinum the carotenoids are

associated with a lipid pool and a particulate fraction (Riley & Bramley, 1976). Neurospora crassa contains carotenoids in lipid globules and the endoplasmic reticulum (Mitzka-Schnabel & Rau, 1980).

In a study of the intracellular location of carotenoid pigments in W. dermatitidis Foster (1985) concluded: 1. The pigment accumulation in the cell was not concomitant with with growth of the cell, but appeared to be correlated with the increase in size of the lipid organelles in the yeast-phase cell. 2. The pigments were associated with membranes surrounding the lipid organelle. 3. Based on the extraction procedure outlined by Thirkell and Hunter (1969), it was concluded that the pigments were noncovalently bound to a substrate, presumably a protein.

Cell Wall Structure in Fungi.

Cell walls are complex structures surrounding the protoplasm of plant, fungi, and bacterial cells. In these organisms, the cell wall provides mechanical support, protection from osmotic lysis, and defines the characteristic shape of the cell (Burgess, 1985; Bold et al., 1987; Moore-Landecker, 1982;).

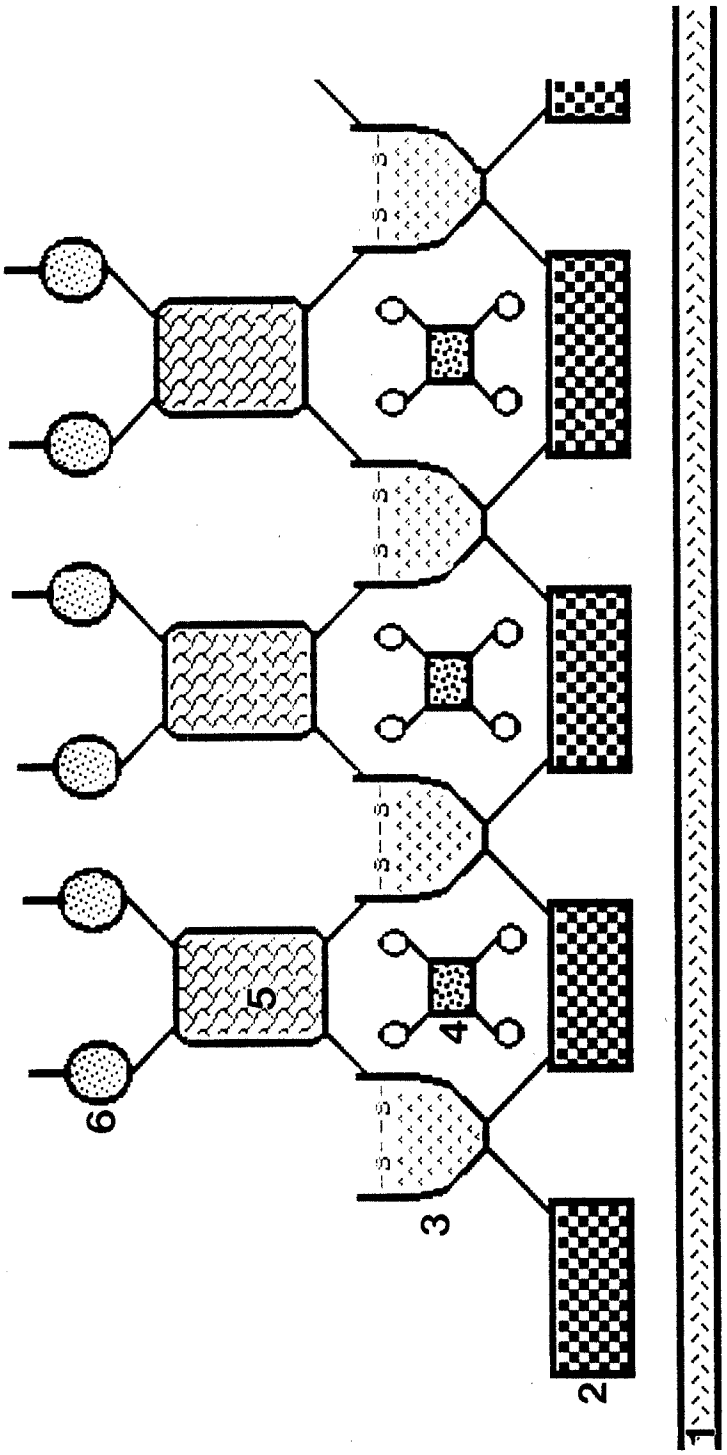
Although the wall has a similar function for the members of these kingdoms, the chemical composition and structure is different. Plant and fungal cells are similar in that the walls are composed of microfibrils embedded in a matrix of polysaccharide. The major polysaccharide in plant cell walls is cellulose (Burgess, 1985). The major chemical components of the fungal cell wall are chitin, cellulose or noncellulosic beta-glucans and alpha-glucans, mannose, mannoproteins, proteins and lipids (Moore-Landecker, 1982; Bartnicki-Garcia, 1968; Aronson, 1965). The fundamental difference between bacteria and fungi is the lack of peptidoglycan and glycerol or ribitol teichoic acids in fungal cell walls (Maresca & Kobayashi, 1989).

The chemical composition of fungal cell walls has a close correlation with the taxonomic position of fungi (Bartnicki-Garcia, 1968) and is used as part of the classification scheme for fungi. Cell wall composition is also important in the pathogenicity of fungal organisms. Attachment of the fungal parasite to the host is one of the first events required for parasitism (Manocha & Chen, 1990) and requires recognition of the specific surface topology and wall ultrastructure of the host. Avirulent strains of Histoplasma capsulatum lack alpha-(1->3)-glucans. It is thought that the glucans provide a proper substrate for the proteins involved in virulence (Klimpel & Goldman, 1988). Wall composition

also aids in the prevention of mycoparsitism by other fungi. Trichoderma harzianum easily overgrew fungal test colonies of Rhizotonia slanik, Schlerotium rolfsii, and Phythium aphanidermatun, but is unable to parasitize colonies of Fusarium oxysporum (Sivan & Chet, 1989). The walls of F. oxysoprum and Fusarium sp. contain a higher concentration of protein and are more resistant to the beta-(1->3)-glucanases synthesized by T. harzianum (Sivan & Chet, 1989; Barran, et al., 1975).

The interwoven matrix of microfibrils and polysaccharides which make up the fungal cell wall (Fig. 2) is arranged in different layers of the wall and may be seen as layers of different densities in specimens prepared for transmission electron microscopy. Fimbriae are fine, electron dense, hair-like structures found on the outermost portion of the cell wall and function in sexual conjugation and flocculation of Saccharomyces sp. (Day, et al., 1975). A polysaccharide capsule is present in some fungi on the outermost surface of the cell wall and in Cryptococcus neoformis it is composed of a matrix of fine microfilaments (Edwards, et al., 1967). The capsule is important in the virulence of C. neoformis and protects the cell from dehydration. Fibrillar or flocculent material that is

Fig. 2. Hypothetical model of the cell wall structure of the yeast S. cerevisiae. (1) Cell membrane, (2) Glucans, (3) Protein with disulfide linkage [S-S], (4) Enzymes, (5) Mannan, (6) Phosphate bridge (Kidby & Davis, 1970).


1
2
3
4
5
6

present on the surface of the mother cell of Candida albicans is not present on the surface of the bud cells. The layers of the cell wall are shed during growth of the cell and may have implications in pathogenesis and host-parasite interaction (Brawner & Cutler, 1986). The antigens found most reactive with monoclonal antibodies are associated with the flocculent layer and expression of this layer is dependent on the growth phase of the organism (Brawner & Cutler, 1986).

The cell envelope found between the microfimbriae and the cell membrane is an amorphous structure whose layers may only be distinguished in electron micrographs by the degree of retention of electron dense materials. Electron micrographs of the cell wall isolated from hyphae of Fusarium sulphureum and prepared for transmission electron microscopy by shadow casting showed the wall to consist of an electron dense outer layer and a broader, electron transparent inner layer (Barran, et al., 1975). The inner layer is the only layer that is contiguous with the cross walls of the hyphae of F. sulphureum. The conidia and chlamydospores of F. sulphureum have the same distinctive layering found in vegetative hyphae. The outer wall of the chlamydospore is 30% thicker than the corresponding layer in the conidia and the inner, electron transparent layer is 250% thicker in the chlamydospore (Schneider & Barran, 1977). A comparative

study of several fungi by Hunsley and Burnett (1970) found the cell wall of N. crassa to have three distinct layers in the mature hyphae, Schizophyllum commune four layers and Phytophthora parasitica two layers. Three noncontinuous bands were seen in the cell wall of Agaricus bisporum and a loosely structured, gelatinous polysaccharide was found on the outer most surface of the cell wall (Michalenko, et al., 1976). C. albicans has a cell wall that consists of five layers in the mother cell and mature blastospore, but only four layers in the cell wall of buds and young cells (Djaczenko & Cassone, 1971).

The polysaccharides most common in fungal cell walls are chitin, glucans, and mannose (Farkas, 1979). The cell wall is built around a rigid glucan layer with chitin microfibrils embedded in the glucan and concentrated at the bud scars (Shematek, et al., 1980). Mannan-protein complexes coat the surface and are interspersed with glucan (Balleu & Raschke, 1974).

Glucosamine is the major carbohydrate of F. sulpureum and is present as N-acetylglucosamine in chitin. In N. crassa, S. commune, and P. parasitica chitin or cellulose microfibrils must be formed at the apex of the hyphae and organized so a co-axial aggregation is formed. In the mature hyphae, the aggregation of the microfibrils increases and accounts for the increase in the diameter of the hyphae

(Hunsley & Burnett, 1970). The inner mycelial wall of A. bisporum is typical of other fungi and is composed of chitin microfibrils in a beta-glucan matrix (Michalenko, et al., 1976).

Chitin is not evenly distributed in the cell wall of fungi and will change during the life cycles of dimorphic fungi. The concentration of chitin changes during the yeast-to-hyphal transition of C. albicans with the mycelial form having three times the concentration of chitin as the yeast form. There is a net increase in chitin synthesis with the yeast to mycelial transition and another increase during germ tube formation (Sullivan, et al., 1983). Chitin is concentrated in the bud scars of S. cerevisiae (Roberts, et al., 1983) and the yeast-phase of W. dermatitidis (Harris & Szansizlo, 1986).

The second class of carbohydrates important in fungal cell walls are glucans. The polysaccharide may be composed of alkali soluble or insoluble alpha or beta glucans (Farkas, 1979). These carbohydrates interact closely with chitin and, like chitin, change in concentration in the cell wall during morphogenesis (Messner & Kubicek, 1990). Glucans are one of the contributing factors to cell shape (Benhamou, et al., 1990; Miyata, et al., 1985; Shematak, et al., 1980; Michalenko, et al., 1976;) and are thought to be the most important structural element in the yeast cell wall

(Yamamoto, et al., 1986). Beta-(1->3)-glucan is the principal structural component in yeast cell walls (Shematek, et al., 1980) and is part of a rigid layer of glucans that forms the foundation around which the rest of the cell wall is formed (Balleu, et al., 1974). The carbohydrate is synthesized by beta-(1->3)-glucan synthase which is attached to the cell membrane and is active at sites where the wall is expanding and inactive where the wall is quiescent (Shematak, et al., 1980). This enzyme in sensitive S. cerevisiae yeast cells is the target of the killer toxin of Hansenula mrakii (Yamamoto, et al., 1986), is activated by ATP and GTP (Shematek & Cabib, 1980), and is subject to cellular regulatory mechanisms. Ultrastructural localization of beta-(1->4)-glucan by Benhamou, et al. (1990) using gold-complexed exoglucanase showed widespread interaction in the innermost layer of the conidial wall of Trichoderma viride and in the outer wall layer of F. oxysporum and an absence of labeling in the vegetative hyphae of these organisms. Beta-glucan is present in mycelial walls of Trichoderma ressi, but is replaced by alpha-glucan in the regenerating protoplasts (Messner & Kubicek, 1990).

Binding studies done by exposing the plasma membrane of Phytophthora cinnamoni zoospores to glycoconjugates showed that conconavalin A bound to the membrane, indicating that glucosyl or mannosyl residues predominate in the accessible

saccharides present at the membrane (Hardmam & Suzaki, 1990) as well as being located in the cell wall. Lectins used for the visualization of D-mannose and D-glucose in the anaerobic rumen fungus, Sphaeromonas communis, demonstrated an absence of both these residues at the cell surface (Guillot, et al., 1990).

Antifungal agents which affect the synthesis of glucans result in a change in the shape of the fission yeast Schizosacchromyces pombe. S. pombe has a spherical rather than cylindrical shape when grown in the presence of aculeacin A (Miyata, et al., 1985), an antifungal agent produced by Aspergillus aculeatur (Yamaguchi, et al., 1982). This agent decreases the synthesis of alkali-insoluble glucans and mannans and increases the synthesis of alkali-soluble glucans. Aculeacin A resistant cells exhibited either a "dumb-bell" or "round-bottom flask" morphology. The spherical areas of the cell wall consisted of alpha-glucans and had a loosely arranged ultrastructure when compared to cylindrical regions of the cell wall (Miyata, et al., 1985). Tunicamycin inhibits the transport of glycoproteins from the endoplasmic reticulum (Tkacz & Lampen, 1975) which results in a porous cell wall and fungal cells that are more susceptible to osmotic lysis (Hasilik & Tanner, 1978; Lagunas, et al., 1986).

Manno-proteins are considered to be the major immunological determinant of fungi because of the high concentration found on the cell surface (Balleu & Raschke, 1974; Takashi, et al., 1981b). Mannan anchors invertase and acid phosphatase to the cell wall (Takashi, et al., 1981a) and plays a role in the structural integrity of the wall (Takashi, et al., 1981a; Hamada, et al., 1984). Nuclear magnetic resonance spectroscopy of baker's yeast mannan showed it to have an alpha-(1->6)-linked backbone with side chains of different lengths containing both alpha-(1->2) and (1->3)-linkages (Balleu & Raschke, 1974). The linkage region between mannan and protein in S. cerevesiae has an inner core that is near the point of attachment to the protein and is chemically different from the outer chain (Nakajima & Ballou, 1974). The mannose residues are linked to the protein via O-glycosidic linkages (Marriott, 1977). Pastor, et al. (1984) found that mannoproteins in S. cerevesiae are located briefly in the periplasm before being incorporated into the rigid wall structure. This transport of mannans and mannoproteins is blocked in yeast secretory mutants and results in a cell wall with a looser ultrastructure that that of the wild type (Novick & Schekman, 1983). M-7002 mutants of C. albicans contain no appreciable amount of mannan in the cell wall. The wall of the mutant, when visualized in scanning electron micrographs, is wrinkled when compared to the smoother wall

of the the wild type cell and the cell shape changed from the egg shape of the wild type cell to a round swollen cell (Takashi, et al., 1981a).

The arrangement and composition of glucan, mannoproteins and chitin are important in the morphogenesis of dimorphic fungi. The covalent linkage between glucans and the high molecular weight mannoproteins of C. albicans and H. capsulatum forms a scaffold into which other cell wall constituents could be incorporated (Elorza, et al., 1988). The alpha and beta glucans of peptidomannans of H. capsulatum are secured in the wall by chitin fibrils. The integrity of this structure and the arrangement of the chitin fibrils are important in the budding of the yeast phase of H. capsulatum (Maresca & Kobayashi, 1989).

Szansizlo, et al. (1983) noted that W. dermatitidis also had glucans and peptidomannans as major components of the cell wall. Chitin is present in the yeast phase cells around the bud sites and bud scars. In thin sections stained with colloidal gold-labeled chitinase, the complex is found in the inner areas of the cell wall, which would indicate that chitin is not transported far from the cell membrane (Harris & Szansizlo, 1986).

CHAPTER II

METHODS

Organism and growth conditions.

Cultures of melanin-less mutants of W. dermatitidis ATCC 58059, obtained from Dr. Paul J. Szansizlo, University of Texas at Austin, were maintained on slants containing (w/v): 2% glucose, 2% malt extract, 1% Bacto-peptone, and 1.5% agar and maintained at 4 °C. For long term storage, each strain was inoculated into a media containing 50% (v/v) glycerol, 2% (w/v) malt extract, 2% (w/v) glucose, and 1% (w/v) Bacto-peptone and stored at -22 °C. For experimental purposes, the cells were grown in a semi-defined medium as described by (Roberts & Szansizlo, 1978) which contained (w/v): 3% glucose, 0.3% $NaSO_4$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.0001% $FeSO_4$, and 1% yeast extract. The pH of the medium was adjusted to 6.5 with 1N HCl prior to the addition of $FeSO_4$ and yeast extract. Media used for experimental purposes was made within twenty-four hours of inoculation.

Inoculation.

Seed flasks containing 50 ml of semi-defined media were inoculated from slants and incubated for twenty-four hours under constant illumination (1300 $uJs^{-1}cm^{-1}$) provided by six

Phillips cool white fluorescent, 24T12 high output light bulbs on a reciprocal shaker (stroke length 16 cm;80 strokes/min) and 25 °C. After checking the purity of the culture by phase contrast microscopy, the absorbance of the seed culture was read on a Bausch and Lomb Spectronic 20 at 600 nm (1-cm light path). Absorbance was adjusted to 0.3 with sterile distilled water, and a standard inoculum of 1% (v/v) was used to inoculate experimental flasks. Routinely, two, 4-L flasks, each containing 1-L of semi-defined broth media were inoculated at the same time and grown under the same conditions as the seed cultures. After incubation for five days, each test flask was checked microscopically for purity and the two cultures pooled. The absorbance at 600 nm and total volume of the pooled culture was read and measured. The culture was divided into six 300 ml samples and harvested by centrifugation at 1085 **g** at 22- 24 °C in a Sorvall SS-3 centrifuge. The cell pellets were washed once with distilled water and stored at -22 °C. For experimental purposes, the pellets were used within 72 h of harvesting. Four pellets from each pooled culture were used for cell disruption and the remaining two pellets were used for pigment extraction of whole cells.

Cell disruption.

The pellets were thawed at room temperature, washed once with 1.2 M KCl at 1085 **g** for 15 minutes and resuspended in 1.2 M KCl to a concentration of 200 mg dry weight/ml. Beta-glucuronidase (type H-2 from Helix pomatia, Sigma Chemical Co., St. Louis) was added to a final concentration of 10,000 Fishman units/ml. The cell suspension was incubated for 120 min on a reciprocal shaker (8 rpm) at 25 °C. After incubation, the suspension was centrifuged and washed once with 1.2 M KCl followed by a second wash with $MgSO_4$-Na malate buffer (0.05 M malate and 0.5 M $MgSO_4$, pH 5.8 adjusted with 5 N NaOH). The buffer was made each week and stored at 4 °C. The pellet from the last wash was resuspended in 5 ml of $MgSO_4$-Na malate buffer to which was added 50 mg of lysing enzyme (lyophilized powder from Trichoderma harzianum, Sigma Chemical Co., St. Louis). The cell suspension was incubated under the same physical conditions as described for the incubation for beta-glucuronidase treatment. Fragile cell formation was followed by phase contrast light microscopy and osmotic sensitivity. The incubation period for cells in lysing enzyme solution averaged 60 min.

Osmotic lysis and sonication of fragile cells.

The enzyme treated cells were partially lysed osmotically by diluting 1:20 (v/v) in cold Tris buffer (50 mM Tris, 10 mM KCl, 2 mM EDTA, 0.1 mM PMSF, pH adjusted to 7.4

with 1 N HCl). The cold cell lysate was agitated for 30 minutes with a magnetic stir bar. The cells were kept cool during agitation by placing the flask in a crushed ice bath. During the 30 minutes of agitation, the probe of the sonicator (Braun-Sonic 2000) was cooled in crushed ice and ethanol. The cell suspension was poured into a plastic beaker embedded in crushed ice and sonicated with the large probe, diameter 20 mm, for five minutes at an output of 375 watts. The temperature of the cell lysate at the end of the sonication never exceeded 32 °C.

Division of Cell Lysate into Samples for Centrifugation and Solvent Extraction.

The first pellet from 300 ml of culture used for fragile cell formation was divided into two equal volumes after sonication. The first half was extracted immediately for total pigment concentration. The second half was stored for 24 hours in the dark at 4 °C before extraction to determine the total pigment concentration. The second pellet from 300 ml of culture used for fragile cell generation was centrifuged (Sorvall RBC-3, automatic refrigerated centrifuge) at 1040 **g** for 30 min at 0 °C after sonication . The pellet from this centrifugation was saved for total pigment extraction and the supernatant fluid was divided into two equal volumes. The first volume was stored in crushed ice for total pigment extraction. The second volume was

centrifuged at 17,300 **g** for 65 min at 0 °C. After this centrifugation, the pigment layer floating on top of the supernatant was separated from the supernatant with a pasteur pipette. Fig. 3 diagrammatically illustrates the procedure described above.

Extraction of pigments and pigment binding.

Extraction of the pigmented fractions of the lysate were carried out with ethyl ether after different treatments as described by Thirkell & Hunter (1969) to determine whether pigments were free or bound (Fig. 4). The supernatant was extracted with ethyl ether following treatment with an equal volume of acetone. The pigmented floating layer was first extracted with ether then with ether after treatment with acetone. Pigments from whole cells were extracted under subdued light by washing the pellet three times with methanol followed by repeated extractions with chloroform:methanol (2:1, v/v) until the cell pellet appeared colorless. Pigment extracts were dried over anhydrous Na_2SO_4, filtered (using a medium porosity Pyrex sintered glass filter) and evaporated to dryness in a Buchler flash evaporator. The dried pigment was redissolved in petroleum ether (b. p. range 35 °C to 60 °C) and chilled to -20 °C. Unpigmented lipid, which precipitated under these conditions, was removed from the extract by centrifuging the sample at -20 °C at 4340 **g** for 15 min and pipetting the soluble pigmented fraction from the

Fig. 3 Flow-chart of the division of cell lysate into samples for centrifugation and solvent extraction.

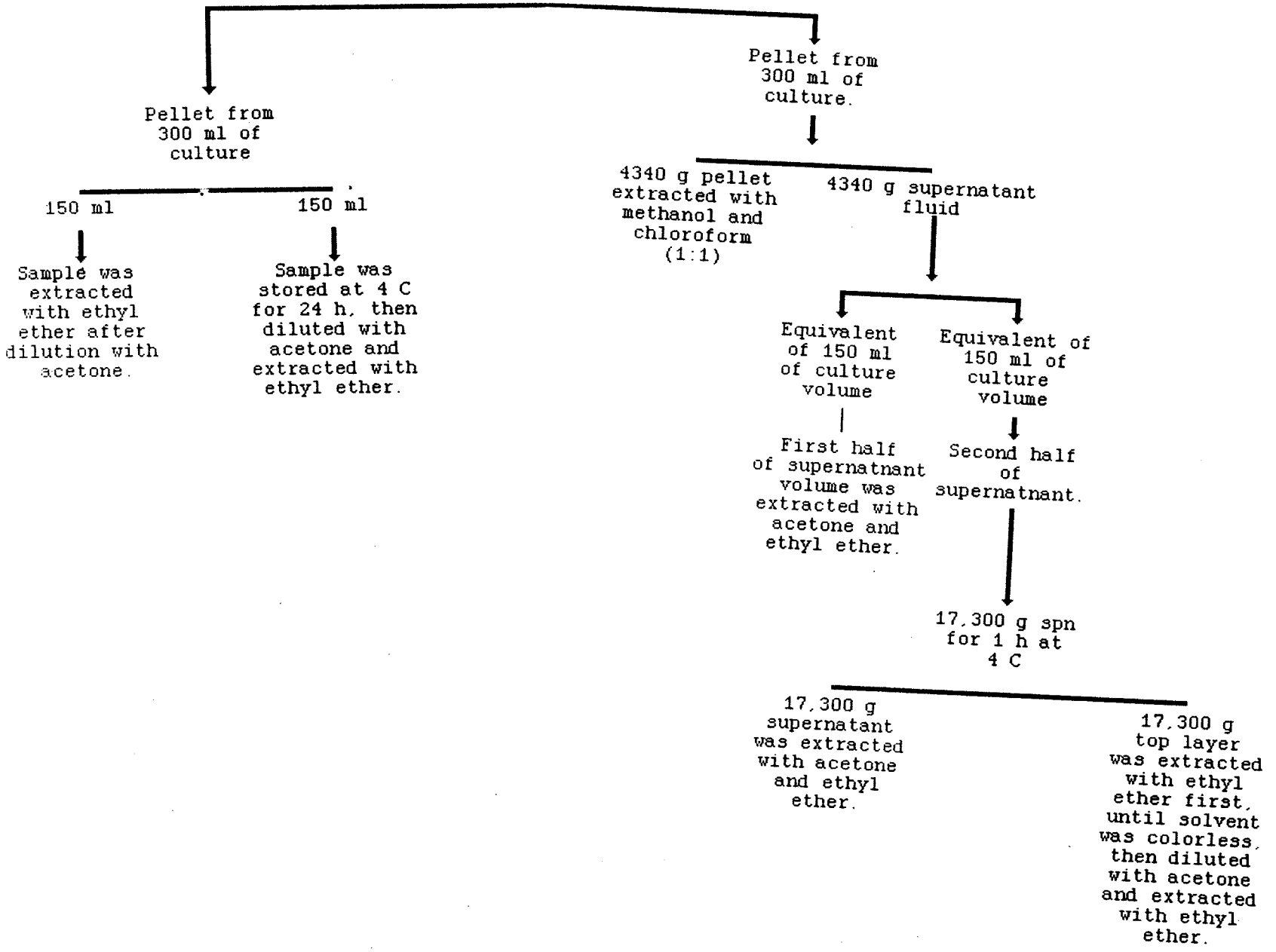
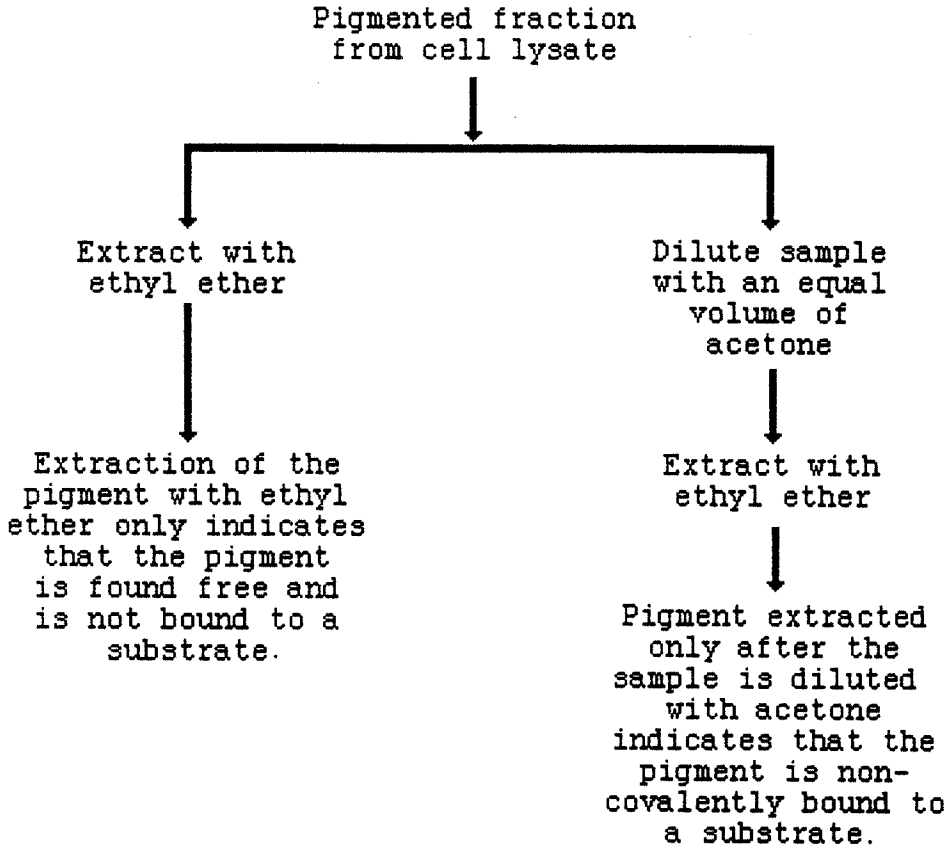

Pellet from 300 ml of culture
150 ml
150 ml
Sample was extracted with ethyl ether after dilution with acetone.
Sample was stored at 4 C for 24 h, then diluted with acetone and extracted with ethyl ether.
Pellet from 300 ml of culture.
4340 g pellet extracted with methanol and chloroform (1:1)
4340 g supernatant fluid
Equivalent of 150 ml of culture volume
Equivalent of 150 ml of culture volume
First half of supernatnant volume was extracted with acetone and ethyl ether.
Second half of supernatnant.
17,300 g spn for 1 h at 4 C
17,300 g supernatant was extracted with acetone and ethyl ether.
17,300 g top layer was extracted with ethyl ether first, until solvent was colorless, then diluted with acetone and extracted with ethyl ether.

Fig. 4 Flow-chart of the solvent extraction method outlined by Thirkell and Hunter (1969) showing the nature of pigment binding.


Pigmented fraction from cell lysate
Extract with ethyl ether
Extraction of the pigment with ethyl ether only indicates that the pigment is found free and is not bound to a substrate.
Dilute sample with an equal volume of acetone
Extract with ethyl ether
Pigment extracted only after the sample is diluted with acetone indicates that the pigment is non-covalently bound to a substrate.

pellet. Prior to determining pigment levels, the solvent was evaporated to dryness under a stream of dry nitrogen gas and the pigment redissolved in a known volume of petroleum ether. The absorbance of the solution was read in a Perkin-Elmer Lambda 3 spectrophotometer at 480 nm and the concentration determined by assuming $E_{cm}^{1\%} = 2200$.

Enzyme treatments for cell wall thin sections.

Method one-pronase. The organism was incubated as previously described, but the pellets were not frozen after harvest. The pellet was washed twice with distilled water and suspended at a concentration of 200 mg cells (wet weight)/ml in the following solution: 50 mM dithiothreitol, 5 mM EDTA, 0.1 M Tris, and 1 mg/ml Pronase (Streptomyces lyphilized powder, Sigma Chemical Co., St. Louis). The pH of the solution was adjusted to 8.9 with 1 N NaOH before the addition of the pronase. The cells were incubated in this solution for 90 min at 25 °C with gentle agitation. After incubation, the cells were centrifuged, washed with distilled water and prepared for transmission electron microscopy.

Method two-beta glucuronidase. Incubation and harvest of the organism was the same as with method one, except the pellet was washed with 1.2 M KCl and resuspended to a concentration of 200 mg (dry weight)/ml in 1.2 M KCl. Beta-glucuronidase (Type H-2 crude solution from Helix pomatia, Sigma Chemical Co., St Louis) was added to yield a

final concentration of 10,000 Fishman units/ml of cell suspension. The cells were incubated at 25 °C for 120 min. After incubation, the cell suspension was centrifuged at 4340 **g** for 10 min and prepared for transmission electron microscopy.

Method three-lysing enzyme. The cell incubation and harvest was the same for this method as for the previous two methods. The pellet was washed with $MgSO_4$-Na malate buffer (0.05 M malate and 0.5 M $MgSO_4$, pH 5.8 adjusted with 5 N NaOH). The pellet was resuspended in 5 ml of $MgSO_4$-Na malate buffer to which was added 50 mg of lysing enzyme. The cell suspension was incubated under the same physical conditions as the previous methods for 60 min. After incubation, the cell suspension was centrifuged at 4340 **g** for 10 min and the pellet prepared for transmission electron microscopy.

Method four-pronase and beta glucuronidase. Fragile cells were prepared by a modified method of Torres-Bauza & Riggsby (1980). The cells were harvested by centrifugation and washed with distilled water. The pellet was incubated in pronase as described in method one. After incubation, the cells were centrifuged, washed once with distilled water, once with 1.2 M KCl and resuspended to a concentration of 200 mg (dry weight)/ml and incubated with

beta-glucuronidase as described in method two. The fragile cells were harvested and prepared for transmission electron microscopy.

Method five-beta glucuronidase and lysing enzyme. The culture was harvested and washed once in 1.2 M KCl at 1085 **g** for 15 minutes and resuspended in 1.2 M KCl to a concentration of 200 mg dry weight/ml. Beta-glucuronidase was added to a final concentration of 10,000 Fishman units/ml. The cell suspension was incubated for 120 min on a reciprocal shaker (8 strokes/min) at 25 °C. After incubation, the suspension was centrifuged and washed once with 1.2 M KCl followed by a second wash with $MgSO_4$-Na malate buffer. The pellet from the last wash was resuspended in 5 ml of $MgSO_4$-Na malate buffer to which was added 50 mg of lysing enzyme. Fragile cell formation was followed by phase contrast light microscopy and osmotic sensitivity. The incubation period for cells in lysing enzyme solution averaged 60 min.

Method six-pronase, beta glucuronidase, and lysing enzyme. The organism was incubated as previously described, but the pellets were not frozen after harvest. The pellet was washed twice with distilled water and suspended at a concentration of 200 mg cells (wet weight)/ml in the following solution: 50 mM dithiothreitol, 5 mM EDTA, 0.1 M Tris, and 1 mg/ml Pronase (Streptomyces lyphilized

powder, Sigma Chemical Co., St. Louis). The pH of the solution was adjusted to 8.9 before the addition of the pronase. The cells were incubated in this solution for 90 min at 25 °C with gentle agitation. The cell suspension was washed once by centrifugation (1085 **g** for 10 min) with distilled water followed by a wash with 1.2 M KCl, after which the pellet was resuspended in 1.2 M KCl to a concentration of 200 mg dry weight/ml. Beta-glucuronidase was added to a final concentration of 10,000 Fishman units/ml. The cell suspension was incubated for 120 min on a reciprocal shaker (stroke length 15 cm; 80 strokes/min) at 25 °C. After incubation, the suspension was centrifuged and washed with $MgSO_4$-Na malate buffer. The pellet from the last wash was resuspended in 5 ml of $MgSO_4$-Na malate buffer to which was added 50 mg of lysing enzyme. Cells were incubated for an average of 60 min prior to harvesting and preparing for electron microscopy.

Differential centrifugation of cell lysate from enzymatic disruption methods four, five and six.

Each cell suspension was osmotically lysed as previously described. The cell lysate was centrifuged at 30 **g** at room temperature for 15 min. The pellet was saved for transmission electron microscopy (TEM) and the supernatant

fluid was centrifuged at 12,100 **g** for 30 min and the pellet was used for a second TEM sample Osmotically lysed and sonicated samples were taken for TEM analysis.

*Detergent extraction of 30 **g** pellet after treatment with pronase and beta glucuronidase.*

Fragile cells were prepared as described in the method for pronase and beta-glucuronidase. The fragile cells were harvested and the portion of the sample that pelleted at 30 **g** was used for extraction with detergent. The pellet was extracted five times with 10 ml of 1% Triton X-100. The extract was discarded and the pellet was washed twice with buffer and prepared for TEM analysis.

Transmission electron microscopy.

Samples for electron microscopy were enrobed in 2% (w/v) molten agar, cut into small blocks and fixed with 4% (v/v) glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.3) at 4 °C for 25 h, after which they were washed three times (15 min/wash) with cold sodium cacodylate buffer. The samples were post-fixed with 1% (w/v) OsO_4 in sodium cacodylate buffer for 2.5 h at 4 °C after which they were washed with cold buffer and dehydrated with increasing concentrations of acetone. Dehydrated samples were embedded in Luft's Epon (Dawes, 1979) and polymerized overnight at 65 °C. Seventy nanometer sections were cut with a diamond knife on a Sorvall 6000 ultramicrotome, stained with uranyl acetate and lead

citrate (Dawes, 1979). In some cases sections were stained with 1.0% ruthenium red (Dawes, 1979). Stained sections were examined in a JEOL 100 CX II transmission electron microscope at an accelerating voltage of 80 KeV.

Scanning electron microscopy.

Samples were centrifuged at 4340 **g** and a paste from the pellet was smeared on the surface of a 0.4 um membrane filter and placed immediately in 2% (v/v) glutaraldehyde in cacodylate buffer and fixed at 4 °C overnight. Samples were dehydrated in a graded series of ethanol ending with two washes of 100% ethanol and critical point dried with liquid CO_2. The dried samples were gold coated in a Polaron E5100 Series II 'Cool' Sputter Coater (Polaron Equipment Limited, Watford, Hertfordshire) and examined in a JEOL T300 scanning electron microscope at an accelerating voltage of 15 KeV.

CHAPTER III

RESULTS AND DISCUSSION

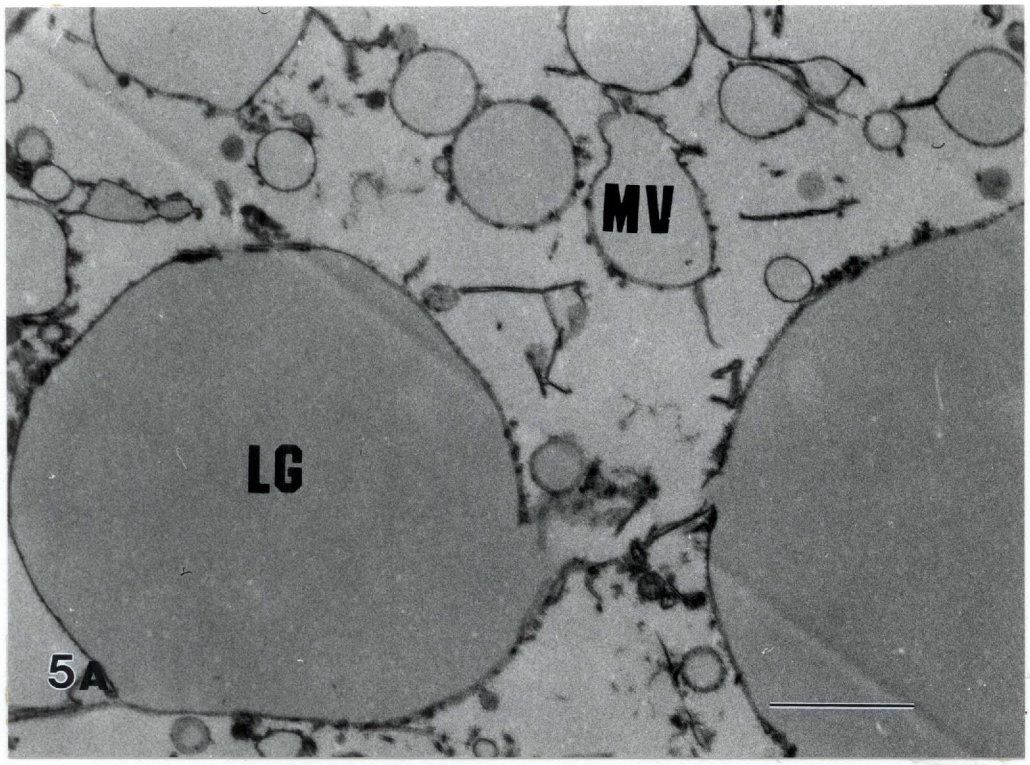
Light and electron microscopic examination of pigmented fractions of the cell lysate.

Carotenoid pigments were associated with two fractions of the sonicated cell lysate. The first fraction, found in the pellet after centrifugation at 4340 **g**, was composed of unlysed cells, as determined by phase contrast microscopy. The second fraction floated on the 4340 **g** supernatant after it had been centrifuged at 17,300 **g**. Light microscopic examination of this fraction showed it to consist of large lipid containing vesicles. Electron micrographs of the same fraction showed membranous vesicles interspersed with large coalesced lipid globules surrounded by a uni-membrane (Figs. 5A & B). The pellet from the 17,300 **g** centrifugation contained small membranous vesicles, with some entrained lipid, but no large lipid organelles (Fig. 6).

Pigment extraction and binding.

Pigment in the lysate, which could be quantitatively extracted with ethyl ether after treatment with acetone, was found to be unstable. Total pigment extraction of the lysate immediately after preparation showed a 38% loss of pigment

Fig. 5 Transmission electron micrograph of thin section of top pigmented layer from 17,300 **g** centrifugation of cell lysate obtained by incubation with beta-glucuronidase and lysing enzyme followed by osmotic lysis and sonication for 5 min. Stained with uranyl acetate and lead citrate. (A) Low magnification showing fused lipid globules (LG), membranous vesicles (MV) and debris. Bar = 1.0 µm. (B) Higher magnification showing unimembrane nature of lipid globules (arrows). Bar = 1.0 µm.


MV
LG
5A

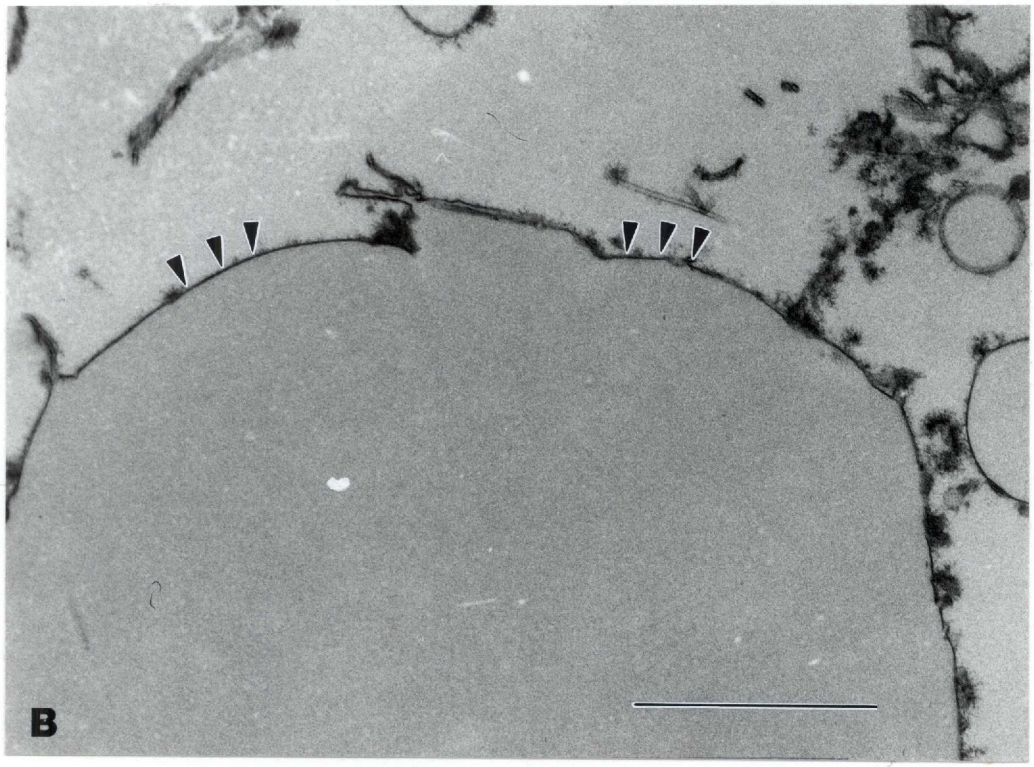
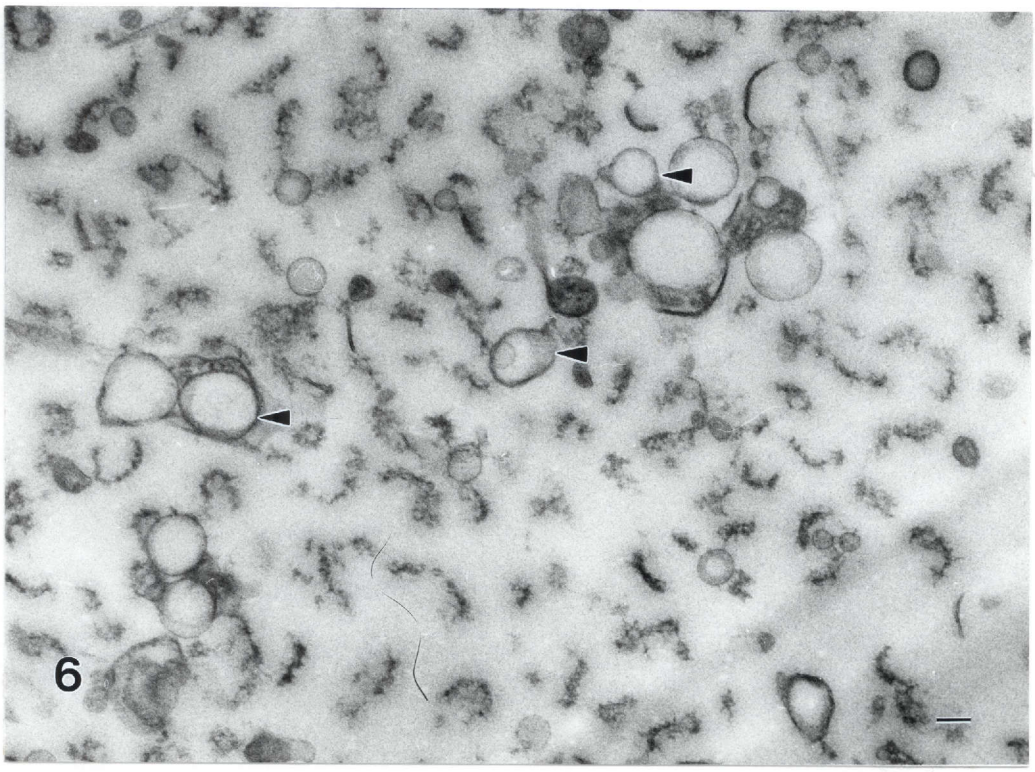

B

Fig. 6 Transmission electron micrograph of thin section of the pellet of cell lysate after centrifugation at 17,300 **g**. Sample was treated as described in Fig. 5. Stained with uranyl acetate and lead citrate. Arrows indicate membraneous vesicles. Bar = 1.0 μm


6

when compared to the concentration of pigment extracted from the same amount of whole cells (Table 1). Pigment loss increased to 56% in lysate stored at 4 °C.

The pigmented fraction floating on the supernatant was extracted first with ethyl ether. After the solvent phase appeared colorless, the sample was diluted with an equal volume of acetone and again extracted with ethyl ether. Sixty-four percent of the pigment was found in the ether extract of the top layer, 6% in the ether extract of the top layer following acetone treatment, and 9% in the supernatant under the top layer, giving an overall recovery of 79% based on total pigment (33.9 µg) in the whole supernatant from the 4340 **g** centrifugation (Table 2). Based only on the pigment recovered in the top layer and the supernatant under the top layer of the 17,300 **g** centrifugation (26.9 µg), recoveries were 81% in the ether extract of the top layer, 8% in the ether extract of the top layer following acetone treatment, and 9% in the supernatant under the top layer for a total recovery of 98%.

These data are different from those described earlier (Foster, 1985). Previously, the five day yeast cells were prepared for protoplast formation by a modified method of Torres-Bauza & Riggsby (1980) in which the osmotically ruptured sample was not sonicated. The pigment was found in one band that migrated to a 40-60% sorbitol interface after

Table 1. Pigment recovery after lysis of whole cells. A 5-day-old culture (450 ml) was divided into three-150 ml aliquots. Whole cells from one aliquot were extracted with cholorform:methanol as described in METHODS. The remaining 300 ml were treated with enzymes and sonicated as described in METHODS. One 150 ml aliquot of the sonicate was extracted immediately with ether after treatment with an equal volume of acetone and the second 150 ml aliquot was stored at 4 oC for 24 hrs before extracting with ether after acetone treatment.

Sample	µg Pigment	% Recovered (loss)
Whole cells	56.8	100
Lysate (immediate extraction)	35.4	62 (38)
Lysate (extraction after 24 hrs at 4 oC)	25.2	44 (56)

*Results represent the average of three experiments.

Table 2. Sub-cellular location of pigments in lysed cells. Three hundred ml of 5-day-old cultures were treated with cell wall degrading enzymes and sonicated as described in METHODS. The resulting lysate was divided into two 150 ml samples and centrifuged at 4340 **g**. In one sample, the pellet and supernatant fluid were extracted after treatment with acetone. For the other sample, the 4340 **g** supernatant fluid was centrifuged at 17,300 **g** and then extracted with ether after treatment with acetone. The supernatant fluid beneath the top layer was extracted with ether after treatment with acetone.

Sample	Extracting Solvent	µg Pigment	% Recovery*
4340 **g** Supernatant	Acetone/ether	33.9	86%
4340 **g** Pellet	Acetone/ether	5.6	14%
17,300 **g** Top layer	Ether	21.6	64%¶
17,300 **g** Top layer (after ether extraction)	Acetone/ether	2.1	6%¶
17,300 **g** Supernatant	Acetone/ether	3.1	9%¶

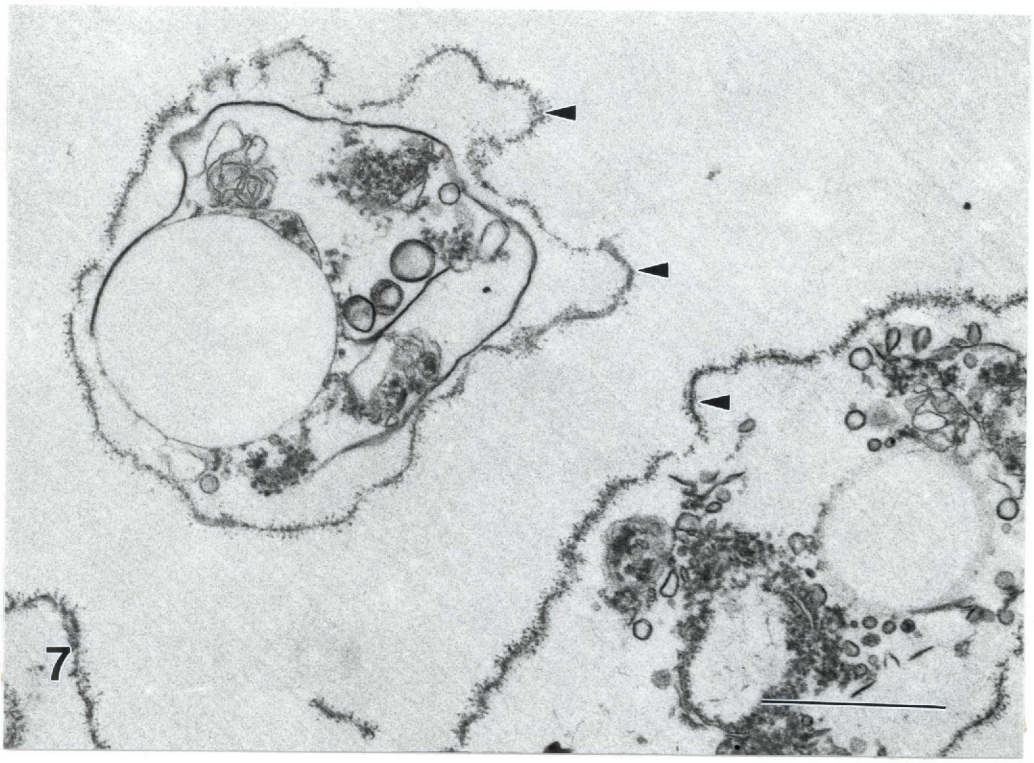
*Average of three experiments.

¶ Based on pigment in the supernatant fraction of 4340 **g** centrifugation (33.9 µg).

centrifugation at 17,300 **g** for one hour at 4 °C. Electron micrographs of this fraction showed it to contain lipid organelles surrounded by remnants of the cell wall (Fig. 7). These remnants also entrained endoplasmic reticulum and mitochondria which could be detected by assaying for enzymatic markers (data not shown). Futhermore, pigments in this fraction could only be extracted with ether after pre-treatment with acetone. This contamination of the pigmented fraction with other subcellular organelles resulted in an earlier assumption that the pigment was non-covalently bound to protein associated with membranes other that of the lipid organelles.

Other investigators have shown that carotenoid pigments in filamentous fungi are associated with lipid (Riley & Bramley, 1976; Osagie & Valadan, 1983; Cederberg & Neujahr, 1970; and Mitska-Schnabel & Rau, 1980). However, these same authors also implicate other sub-cellular organelles or fractions such as vacuoles, submicrosomal fractions, mitochondria, microsomes, cell walls and endoplasmic reticulum as possible sites. In, W. dermatitidis 81% of the total pigment of the lysed protoplasts was found to be in the lipid organelles and could be extracted by ethyl ether alone. The ease of pigment extraction from the lipid layer with ethyl ether without pretreatment with acetone, indicates the

Fig. 7 Transmission electron micrographs of pigmented fraction of cell lysate prepared by the protoplast method modified from Torres-Bauze & Briggs (1980). Arrows show remnant of cell wall. Bar = 1.0 μm.


7

pigments exist unbound in the lipid (Thirkell & Hunter, 1969). Another 8% appears to be non-covalently bound, presumably to membrane proteins in this fraction since they could only be extracted with ethyl ether after pretreatment with acetone (Thirkell & Hunter, 1969). The 12% found in the supernatant under the lipid layer resulted in part from the inability to quantitatively remove the lipid layer from the surface of the supernatant and probably represents contamination by this layer. The conclusions reached by Foster (1985) are erroneous and can be explained by the observation that only after sonication is the lipid released by the cell. Enzyme treatment alone leaves enough wall and membrane intact (Fig. 7) to prevent extraction by ether alone.

Carotenoids in the photosynthetic apparatus or in membranes of non-photosynthetic bacteria normally exist as carotenoproteins in which the pigments are non-covalently bound to protein(s) (Meckel & Kester, 1980; Drews & Oelze, 1981). In this bound state the pigments are involved in energy transfer and/or photoprotection. The role of the pigments in W. dermatitidis is less clear because it normally contains significant amounts of melanin, which masks the presence of carotenoids detectable only in melanin-less mutants. Geis & Szaniszlo (1984) found that colorless mutants of W. dermatitidis are more sensitive to UV-radiation

than wild type melanin-containing cells and that melanin-less mutants containing only carotenoids have intermediate protection against UV irradiation. Geis and Szansizlo (1984) felt that these pigments may protect by shielding sensitive sites. Since this study revealed most of the carotenoid pigments are not bound to any proteins or nucleic acids these results support the photoprotective mechanism proposed by Geis and Szansizlo (1984).

Studies on the nature of the cell wall of W. dermatitidis.

During the study just described on the location of pigments in W. dermatitidis, it was observed that only partial degradation of the cell wall was obtained by the enzyme treatments. It was felt that by selective treatment of yeast-phase cells with individual or combinations of various enzymes, information about the structure of the cell wall and distribution of wall polymers could be obtained. Using solubility, cytochemical, lectin binding and infrared spectroscopic data, Geis (1981) and Geis & Cooper (see Geis & Jacobs, 1985, pp 218-220) found that polymers in walls of yeast phase cells of W. dermatitidis are primarily protein, alpha-mannan, beta-glucan, and chitin. Some lipid (about 3%) was also found, but over 70% of the wall is sugar polymers. Chitosan and alpha-glucan were not detected. Alpha mannans were found in the outer layer and the beta-glucans throughout the cell wall. In a separate study, Harris & Szansizlo

(1986), using gold labeled chitinase, showed that chitin is primarily located in the bud scar.

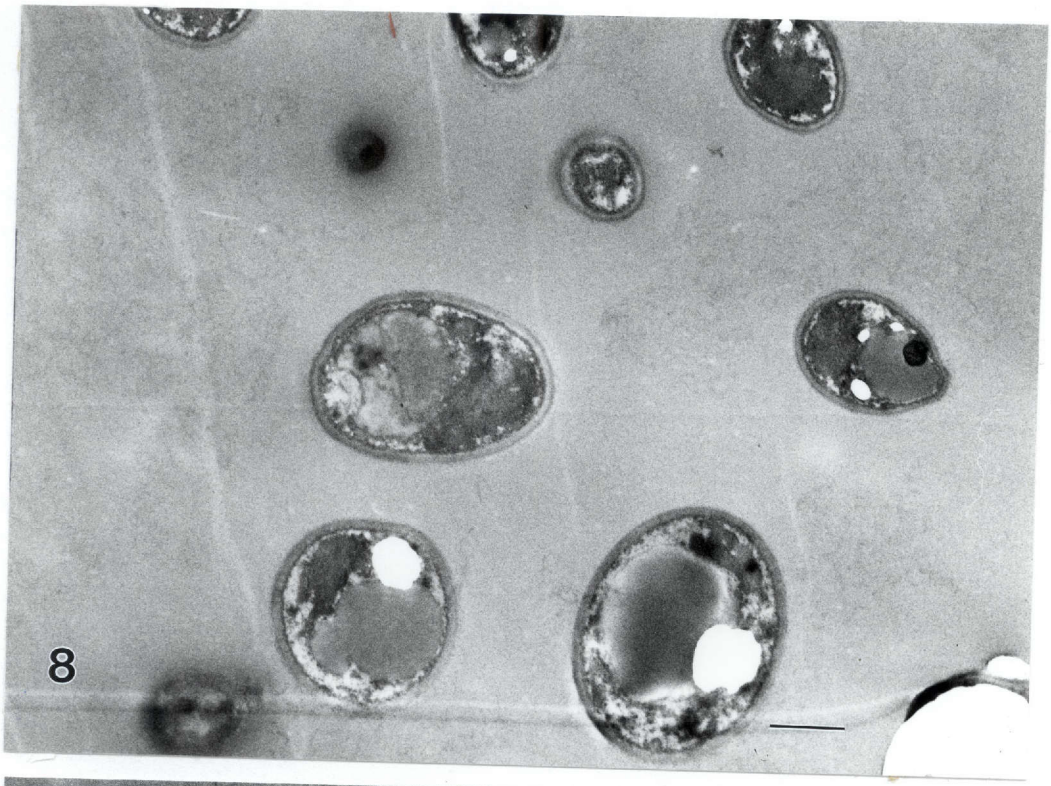
Initial attempts to digest the cell wall of melanin-less mutants of W. dermatitidis (Foster, 1985) used sequential enzyme treatments with the protease, pronase, followed by beta-glucuronidase as described by Torres-Bauza & Riggsby (1980) for C. albicans. Subsequent experiments used beta-glucuronidase followed by lysing enzyme from T. harzianum (DeVries & Wessels, 1973) which contains a mixture of alpha-(1,3)-glucanase, beta-(1,3) and (1,6)-glucanase, chitinase, cellulase and protease (Peberdy, 1979; DeVries & Wessels, 1973). This latter method was more effective in producing fragile cells. The following studies describe the structure of the cell wall of yeast phase cells of melanin-less mutants of W. dermatitidis as revealed by treatments with individual and combinations of the enzymes beta-glucuronidase, pronase, and lysing enzyme. In these studies, thin sections were stained with the conventional stains uranyl acetate followed by lead citrate and with ruthenium red. Uranyl acetate is an effective stain for nucleic acids, protein and phospholipid while lead citrate is a general stain in cells fixed with osmium tetroxide. Neither effectively stains carbohydrates. Ruthenium red enhances contrast in areas containing acid mucopoly-saccharide (Dawes, 1988).

Cell wall morphology of five-day yeast-phase cells before enzyme treatment.

Low magnification transmission electron micrographs of untreated cells show ellipsoidal cells and an occasional budding cell or bud site. Ruthenium red stained cell walls (Fig. 8) have a smooth wall with a darker outside margin, indicating an area containing acid mucopolysaccharides (Dawes, 1988 p. 187). Uranyl acetate and lead citrate stain combination also show a darker outer margin, though less distinct (Fig. 9). At higher magnifications, the ruthenium red stained sections (Fig. 10) show little definition in the cell wall, but bleb-like projections extending from the margin of the cell wall into the surrounding plastic can be seen. These projections are more prominent around bud sites (Fig. 11). The inner portion stains unevenly indicating non-uniform distribution of the wall polymers. The uranyl acetate and lead citrate stained cell wall do not clearly show distinct projections at the margins of either the cell wall (Fig. 12) or bud site (Fig. 13), but allow visualization of a faint second layer in the middle of the cell wall (Fig. 12). As with ruthenium red, the inner layer also stains unevenly (Fig. 13).

Fig. 8 Low magnification transmission micrograph of thin section from untreated whole cell sample. Stained with 1% ruthenium red. Bar = 1.0 μm.

Fig. 9 Low magnification transmission micrograph of thin section from untreated whole cell sample. Stained with uranyl acetate and lead citrate. Bar = 1.0 μm.


8

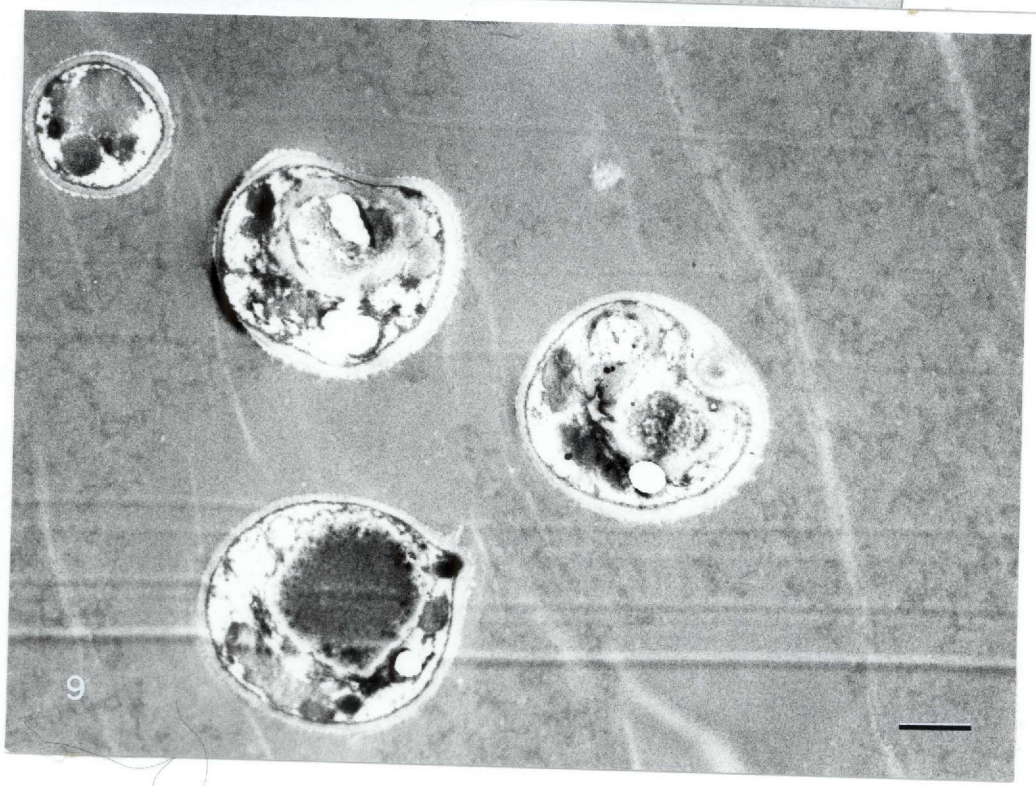
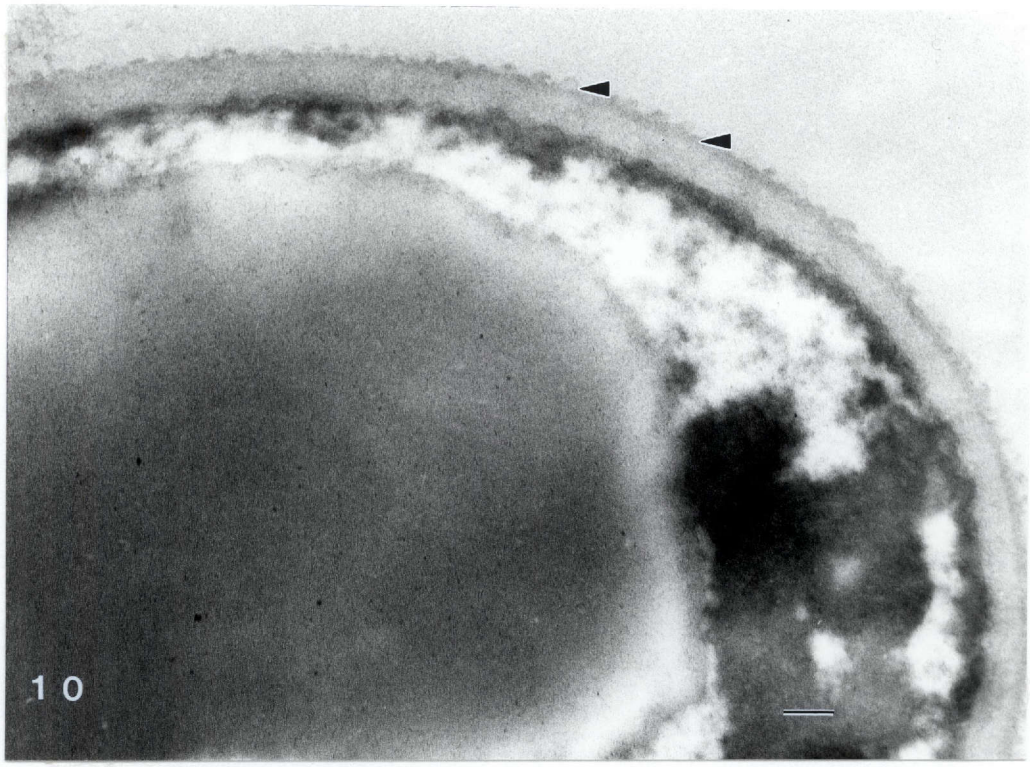

9

Fig. 10 Higher magnification transmission micrograph of whole cells not treated with enzyme. Stained with 1% ruthenium red. Arrows indicate bleb-like protrusion from the surface and uneven staining of the inner portion of the cell wall (CW). Bar = 0.1 μm.

Fig. 11. Higher magnification transmission electron micrograph of whole cell not treated with enzyme. Stained with 1% ruthenium red. Arrows indicated bud site (BS) and projections from the cell wall (CW). Bar = 0.1 μm.


10

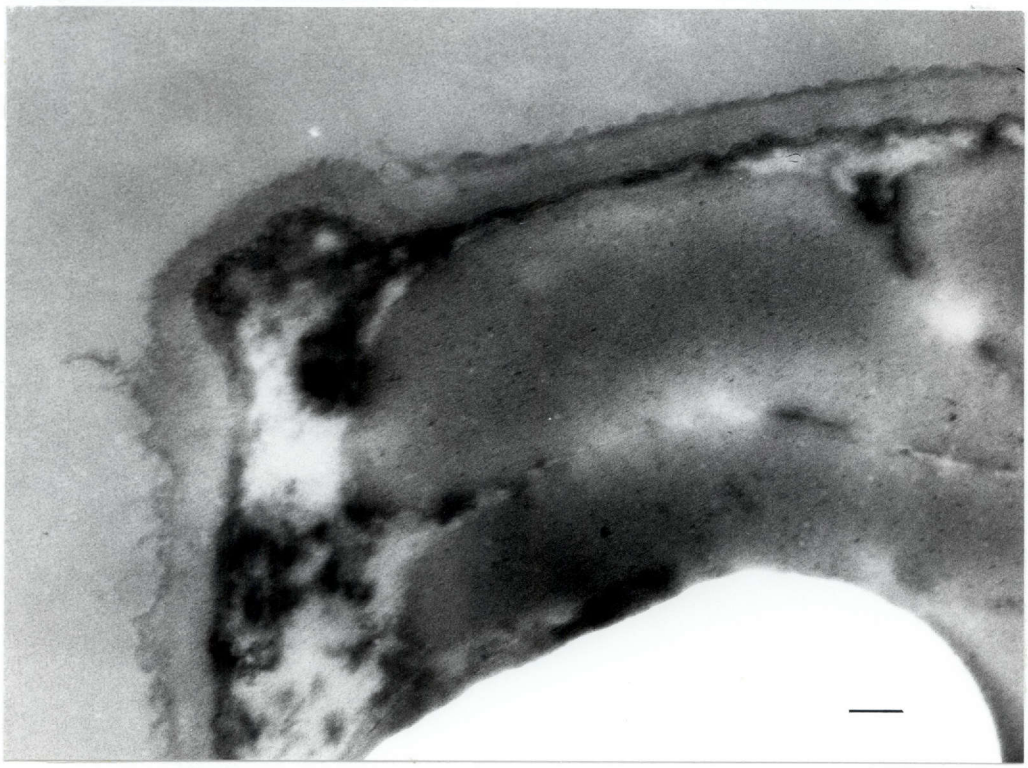
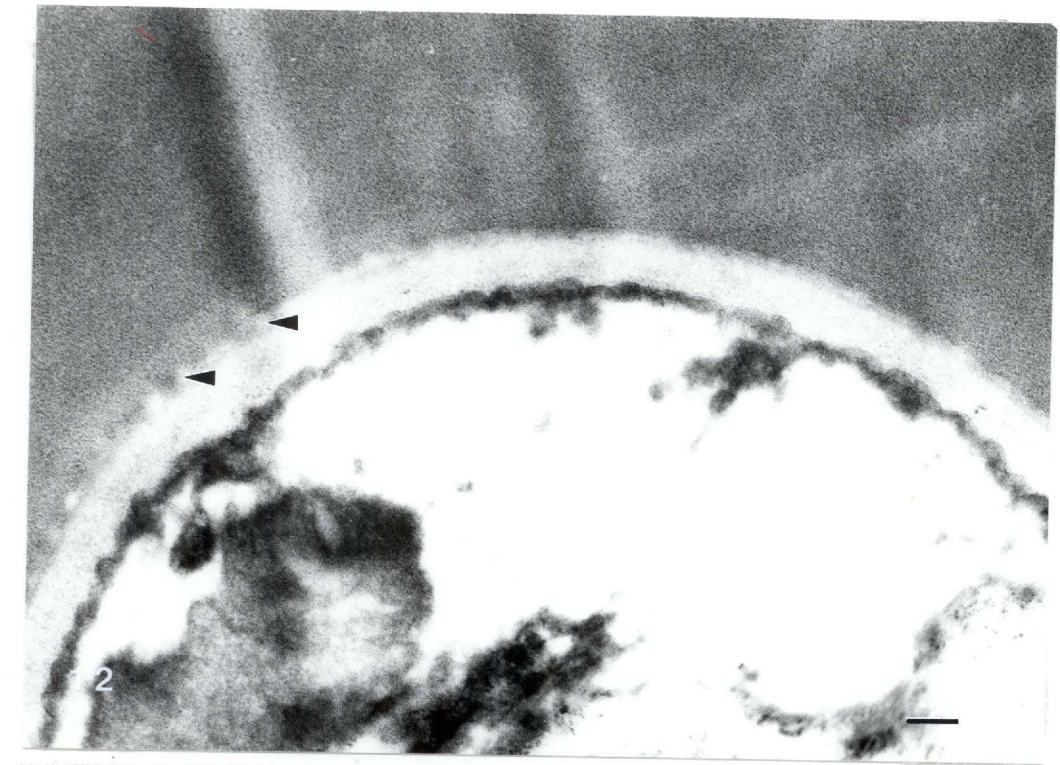
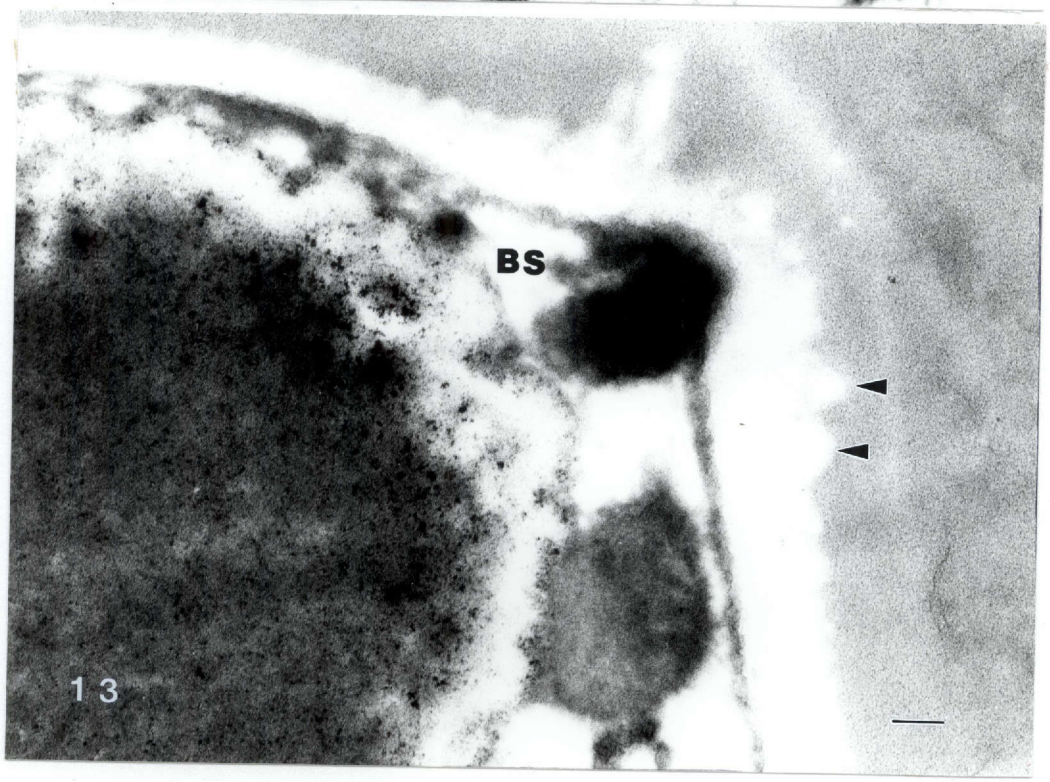


Fig. 12 Electron micrograph of untreated cell wall (CW) in a yeast phase cell. Stained with uranyl acetate and lead citrate. Arrows indicate faint bleb-like protrusions and a slightly darker staining band in the inner wall layer. Bar = 0.1 μm.

Fig. 13 Electron micrograph of bud site (BS) found in an untreated yeast-phase cell. Stained with uranyl acetate and lead citrate. Arrows indicate bleb-like protrusions at the cell wall (CW) surface and uneven staining of the inner layer. The darker staining band seen in Fig. 12 is not evident. Bar = 0.1 μm.



12



13

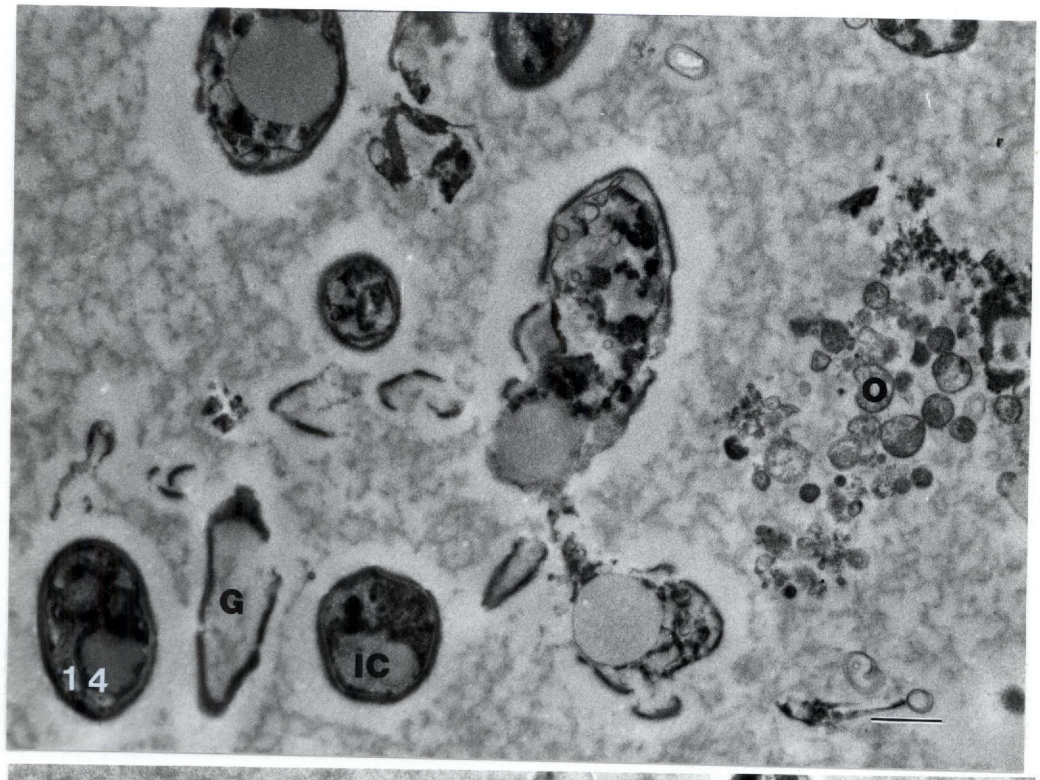
Ultrastructure of the cell wall after treatment with pronase.

Low magnification micrographs of sections stained with either ruthenium red (Fig. 14) or uranyl acetate and lead citrate (Fig. 15) show a mixture of whole cells with the cell wall mostly intact, some subcellular organelles, and cell wall ghosts from lysed cells. Fig. 15 shows breaches in the cell wall indicating non-homogeneity of the wall with some areas richer in protein than others.

Higher magnification of thin sections of yeast cells treated with pronase and stained with uranyl acetate (Fig. 16A) and lead citrate show a marked difference from untreated whole cells. The cell wall has a more open fibrous texture with the fiber of the inner wall roughly parallel to the cell contour. Three layers can to seen, the first layer is moderately electron dense and is found in the half of the wall next to the cell membrane. The second, middle layer, is about the same thickness as the first, but does not stain as intensely. The third layer is found at the outer margin of the cell wall and consists of a very electron dense fibrous layer with fibers running perpendicular to the contours of the cell. A higher magnification micrograph of this region shows better resolution of the fibrillar nature of this section (Fig. 16B). The intensity of staining by uranyl acetate and lead citrate indicates this outer layer is rich in ionic groups and/or proteins, proteoglycans or

Fig. 14 Low magnification electon micrographs of whole cells treated with pronase and stained with 1% ruthenium red showing intact cells (IC), subcellular organelles (O), and cell ghosts (G). Bar = 1.0 μm.

Fig. 15 Low magnification electron micrographs of whole cells treated with pronase and stained with uranyl acetate and lead citrate. Arrow indicate breaches in the cell wall. Bar = 1.0 μm.


G
IC
O
14

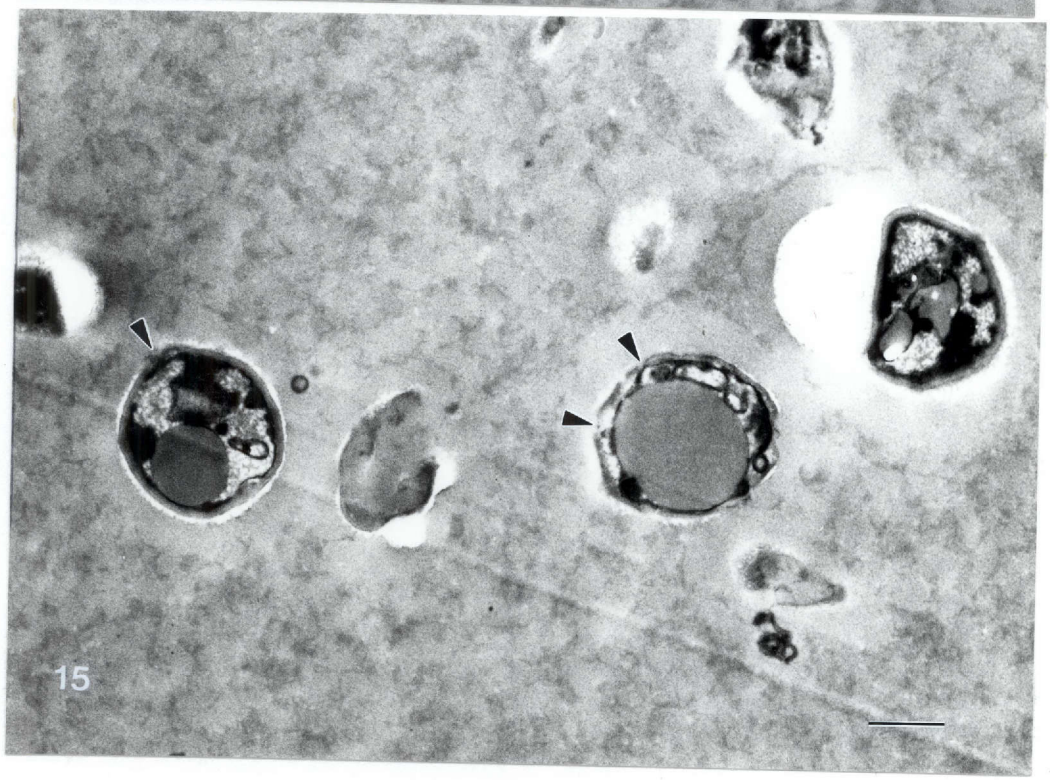
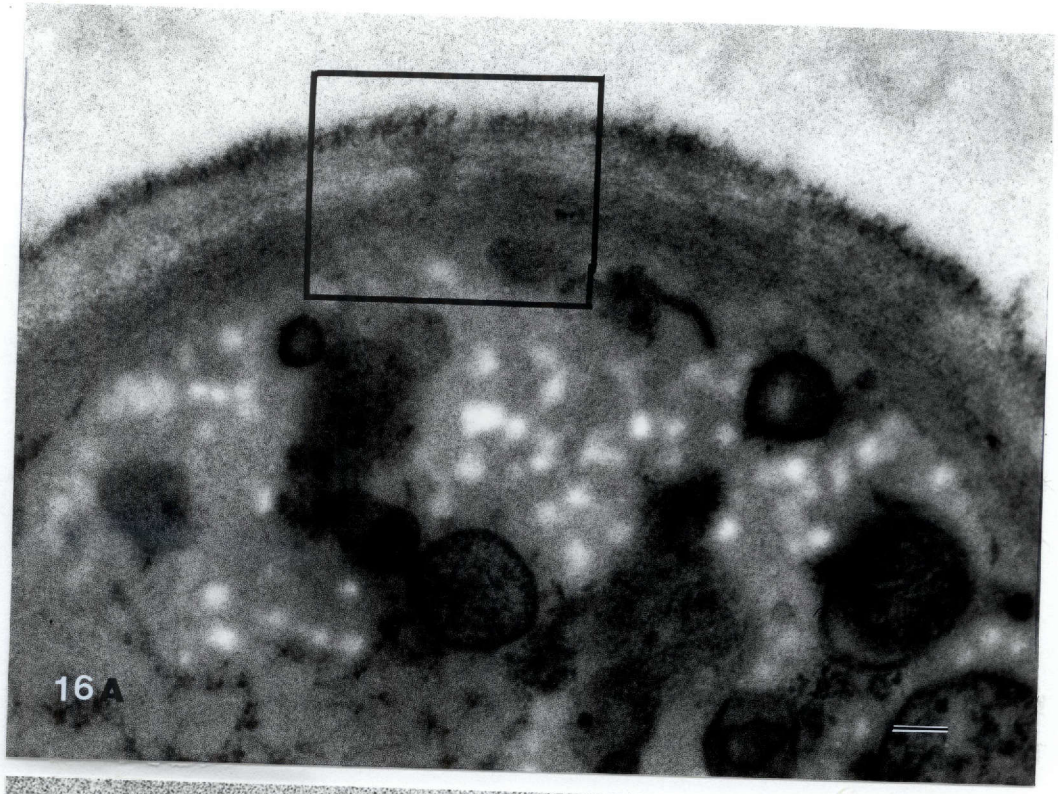
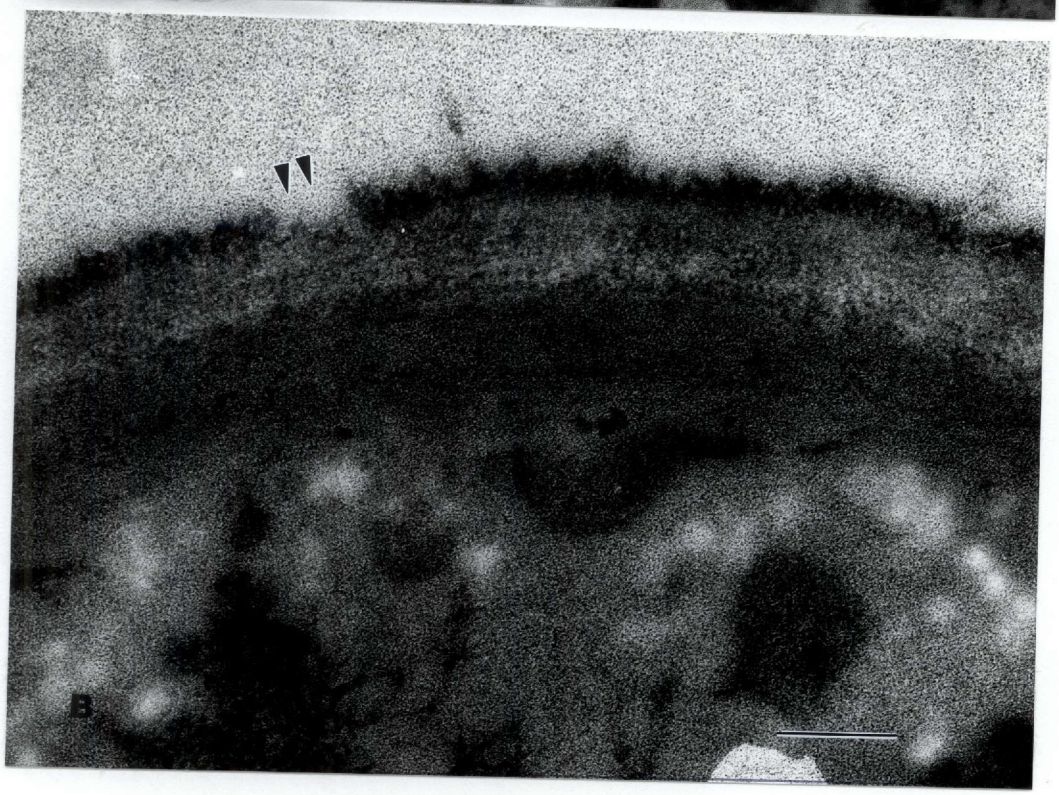

15

Fig. 16 Electron micrograph of whole cell wall after incubation with pronase showing fibrous layers (FL). Stained with uranyl acetate and lead citrate. (A) Wall section showing fibrous layer. Bar = 0.1 μm. (B) Higher magnification of boxed area in (A) showing breach in the outer layer of the cell wall. Bar = 0.1 μm.


16 A


B

glycoproteins refractile to pronase. Both Fig. 16A & B show uneven staining of the second wall layer indicating non-uniform distribution of the polymers.

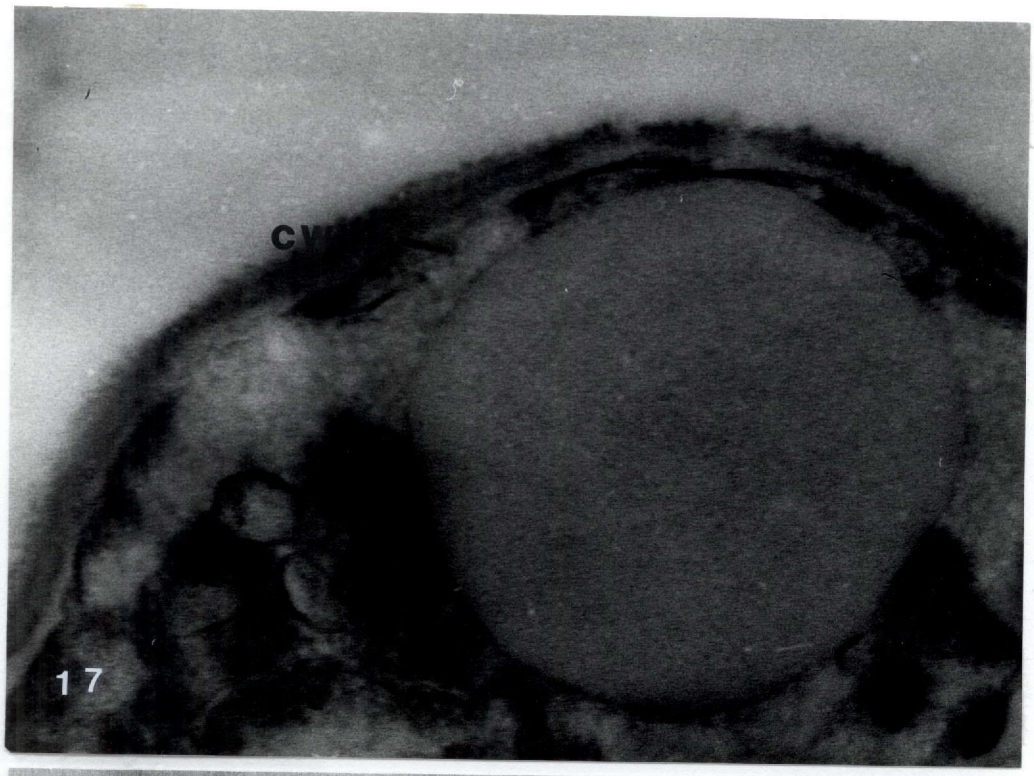
Thin sections of cells stained with ruthenium red show four distinct layers of the cell wall (Figs. 17 & 18), an electron dense inner layer, a less electron dense center layer, an electron dense layer underlying a bleb-like outer layer. However, the inner, less electron dense layer is the thickest of the four layers. Ruthenium red does not stain the outer layer as intensley as uranyl acetate-lead citrate indicating that carbohydrate and/or acid mucopolysaccharides are not predominant components of this layer. The fibrous nature of the inner wall layers are revealed by uranyl acetate-lead citrate is not evident. Breaches in the wall are evident with pits in the fourth layer that extend through the cell wall.

Beta-glucuronidase treated whole cells.

Low magnification micrographs of sections stained with either uranyl acetate and lead citrate or ruthenium red (Fig. 19A & B) show a mixture of whole cells with the cell wall mostly intact and some subcellular organelles. As with pronase, digestion of portions of the cell wall is also evident (Fig. 19B).

Fig. 17 Electron micrograph of whole cell wall (CW) after treatment with pronase. Stained with 1% ruthenium red. Bar = 0.1 μm.

Fig. 18 Electron micrograph of bud site (BS) after treatment with pronase. Stained with 1% ruthenium red. Bar = 0.1 μm.


17

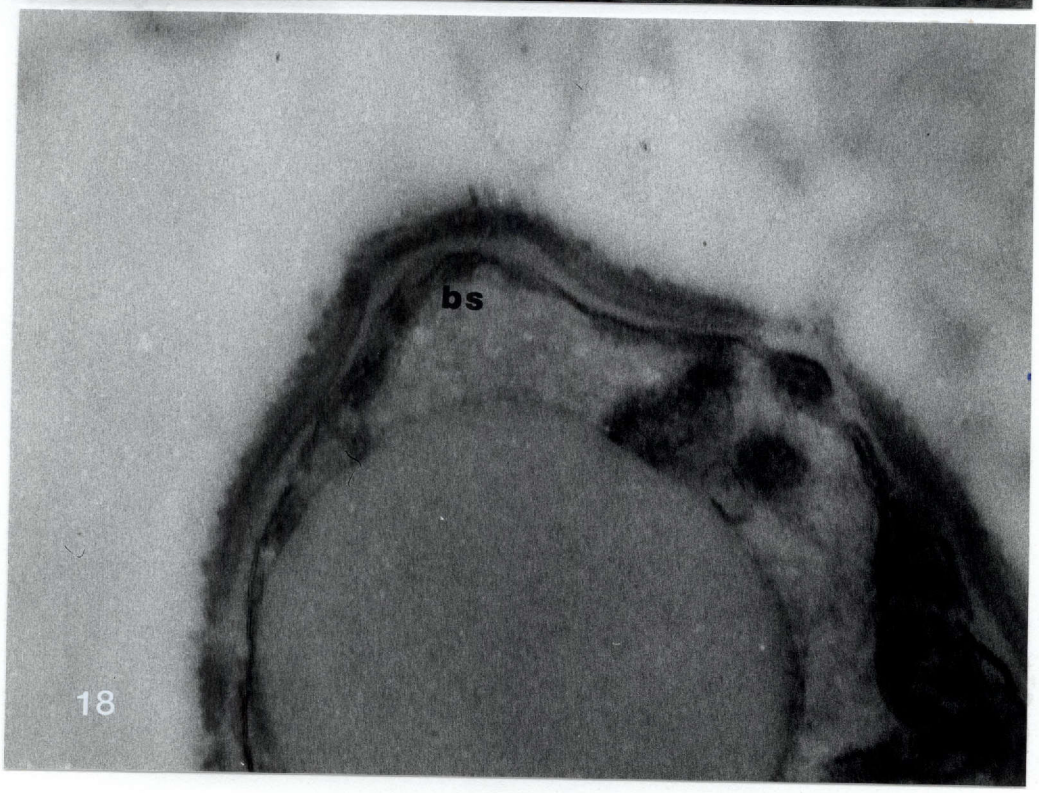
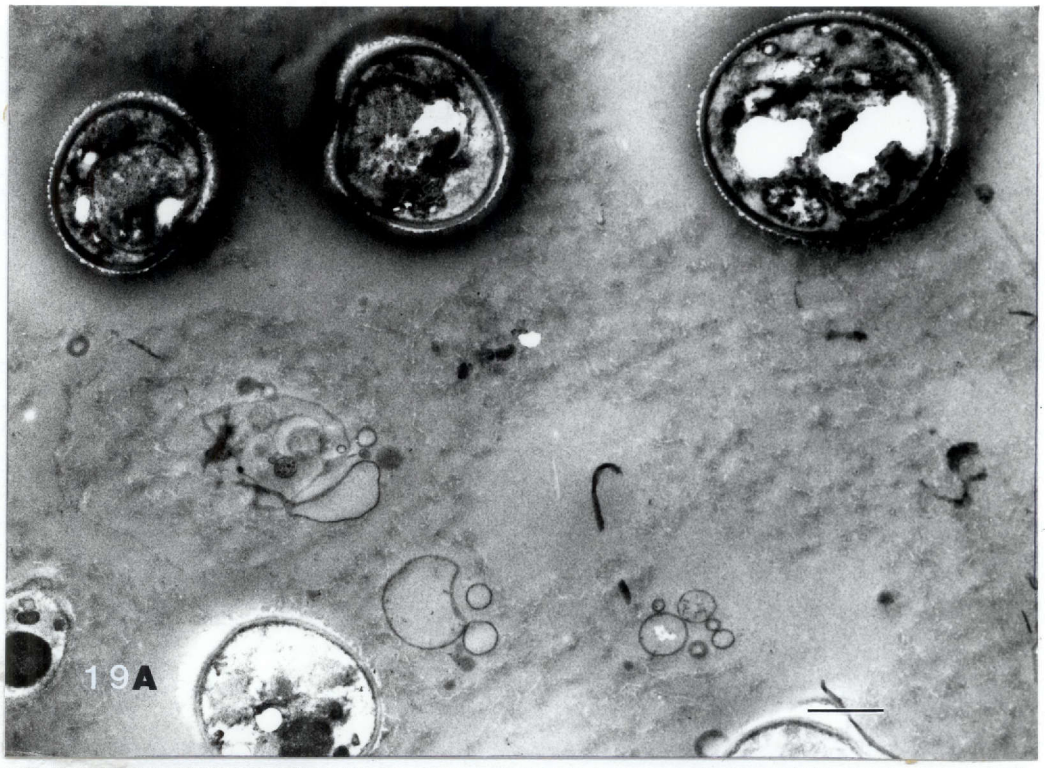
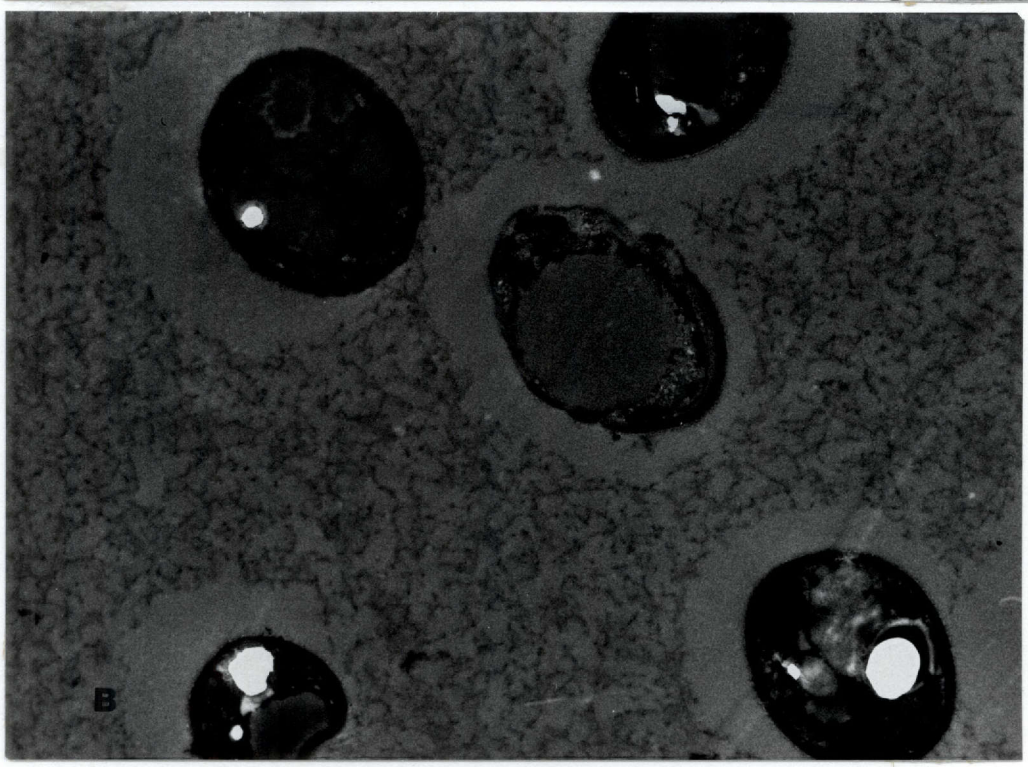

bs
18

Fig. 19 Low magnification micrographs of cells treated with beta-glucuronidase. (A) Stained with uranyl acetate and lead citrate. Bar = 1.0 μm. (B) Stained with 1% ruthenium red. Arrows indicate digestion of portions of the cell wall visable in this micrograph. Bar = 1.0 μm.


19A

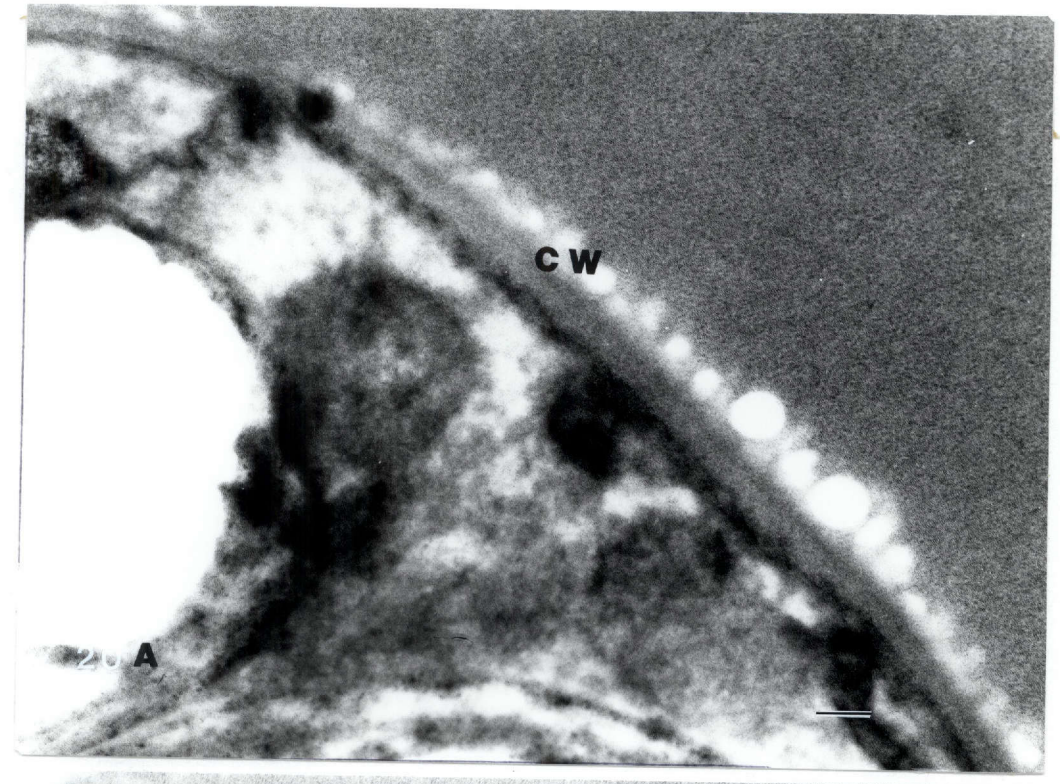

B

Higher magnification of these sections stained with uranyl acetate-lead citrate (Fig 20A & B) reveal the four layers observed when pronase treated cells were stained with ruthenium red. In ruthenium red stained sections (Fig. 21A & B) four layers are seen, but differ from previous sections consisting of a thick inner layer which stains lightly, a thinner more intensly staining layer, a narrow dark band and an outer bleb-like layer. Around the bud site only the outer bleb-like layer and thick, amorphous, unevenly stained inner layer are seen. The uneven staining reflects uneven distribution of stainable polymers in the wall. Around the bud site the outer bleb-like layer is removed in spots. The outer layer at the bud has a more fibrous appearance.

Lysing enzyme treated whole cells.

Thin sections of yeast cells treated with lysing enzyme and stained with uranyl acetate and lead citrate or ruthenium red revealed little new about the nature of the substructure of the wall (Figs. 22, 23, & 24). This enzyme mixture containing cellulase, protease, and chitinase does not appear to breach the outer layer as does beta-glucuronidase and protease. This may be due to masking of reactive sites by other polymers.

Fig. 20 Whole cell wall (CW) after incubation with beta-glucuronidase. (A) Stained with uranyl acetate and lead citrate. Bar = 0.1 μm. (B) Bud site (BS) stained with uranyl acetate and lead citrate. Bar = 0.1 μm.


CW
A


bs
B

Fig. 21 Whole cell wall after incubation with beta-glucuronidase and stained with 1% ruthenium red. (A) Micrograph of cell wall (CW) showing wall layers (1-3) and a breach in the outer layer of the wall (arrows). Bar = 0.1 µm. (B) Bud site (BS) showing lack of wall layers, uneven staining, and digestion of portions of the outer layer (arrows). Bar = 0.1 µm.

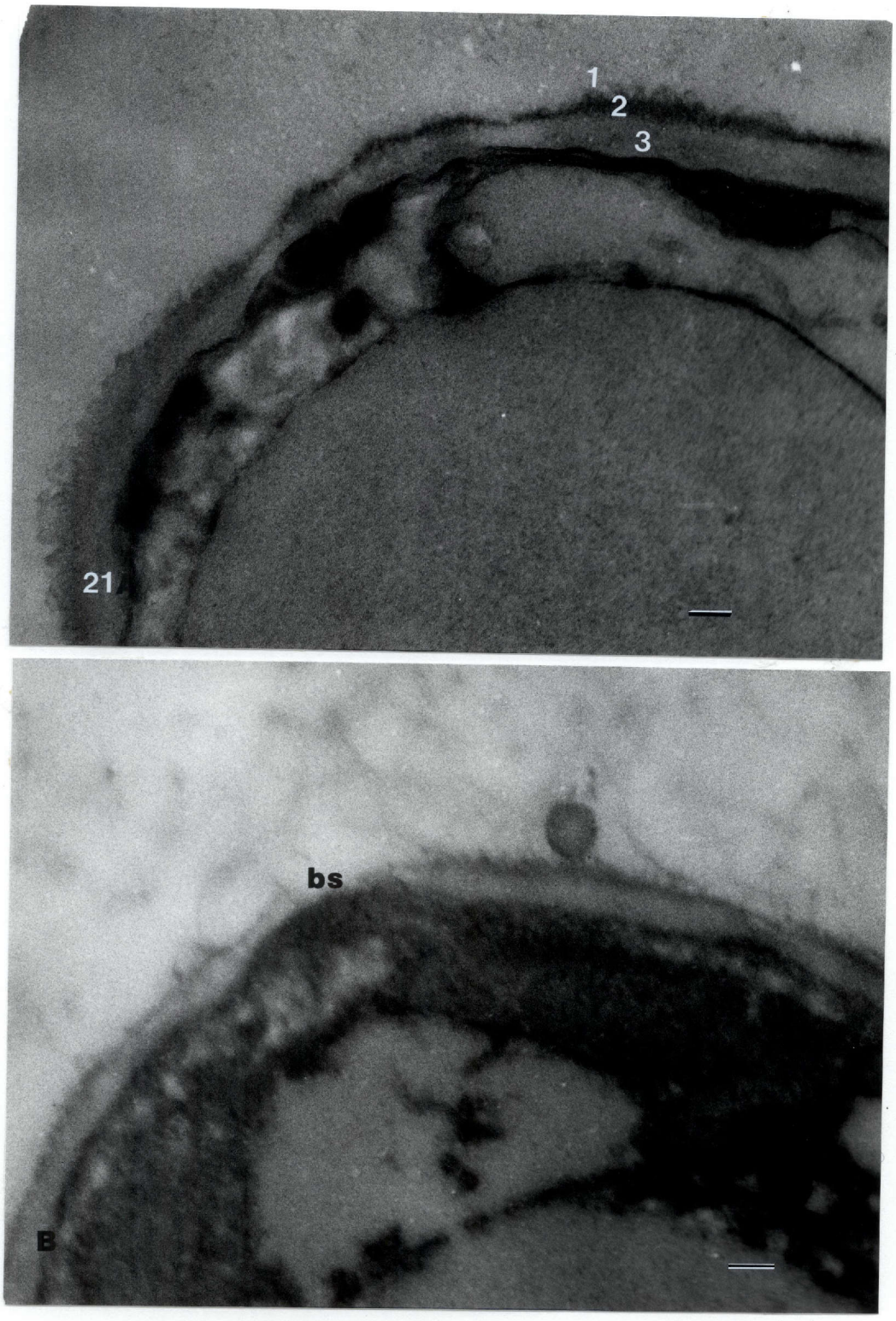
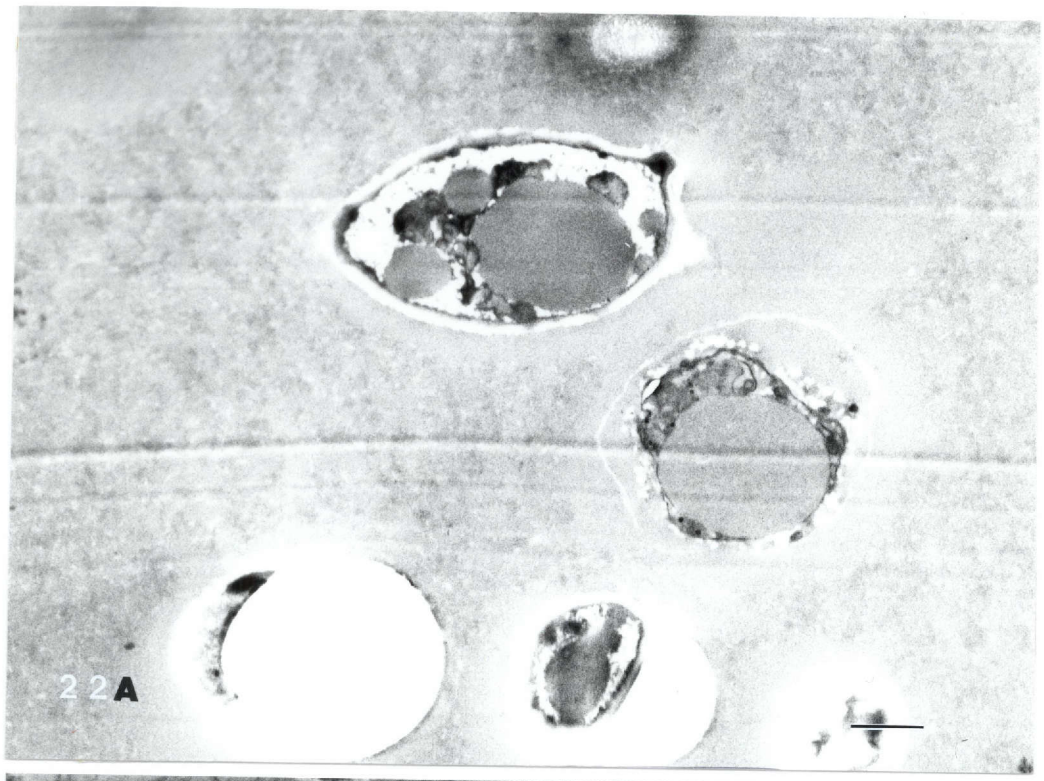

1
2
3
21
bs
B

Fig. 22 Low magnification electron micrograph of cells after incubation with lysing enzyme. (A) Stained with uranyl acetate and lead citrate. Bar = 1.0 μm. (B) Stained with 1% ruthenium red. Bar = 1.0 μm.


22A

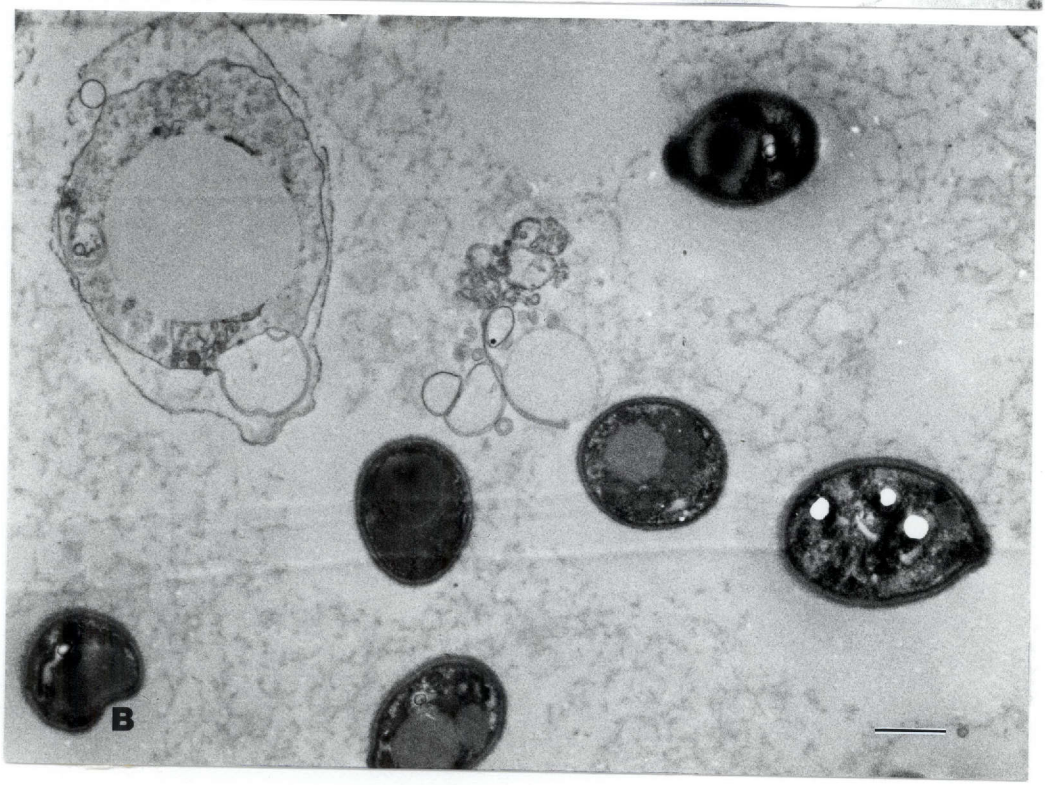

B

Fig. 23 Whole cell wall after incubation with lysing enzyme and stained with uranyl acetate and lead citrate. (A) Wall section of cell away from bud site. Bar = 0.1 µm. (B) Bud site (BS) showing two areas of differing electron densities (arrows). Bar = 0.1 µm.

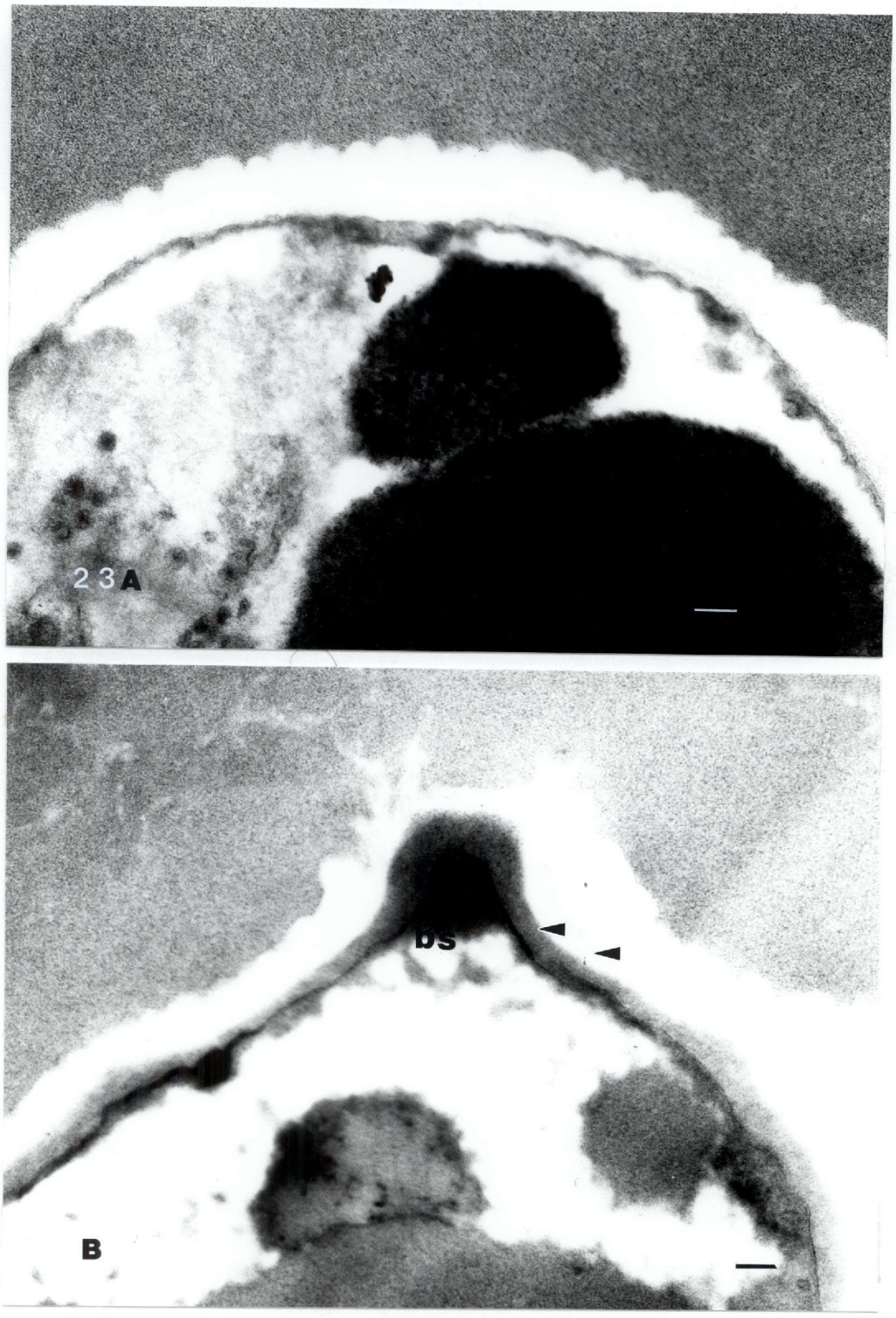
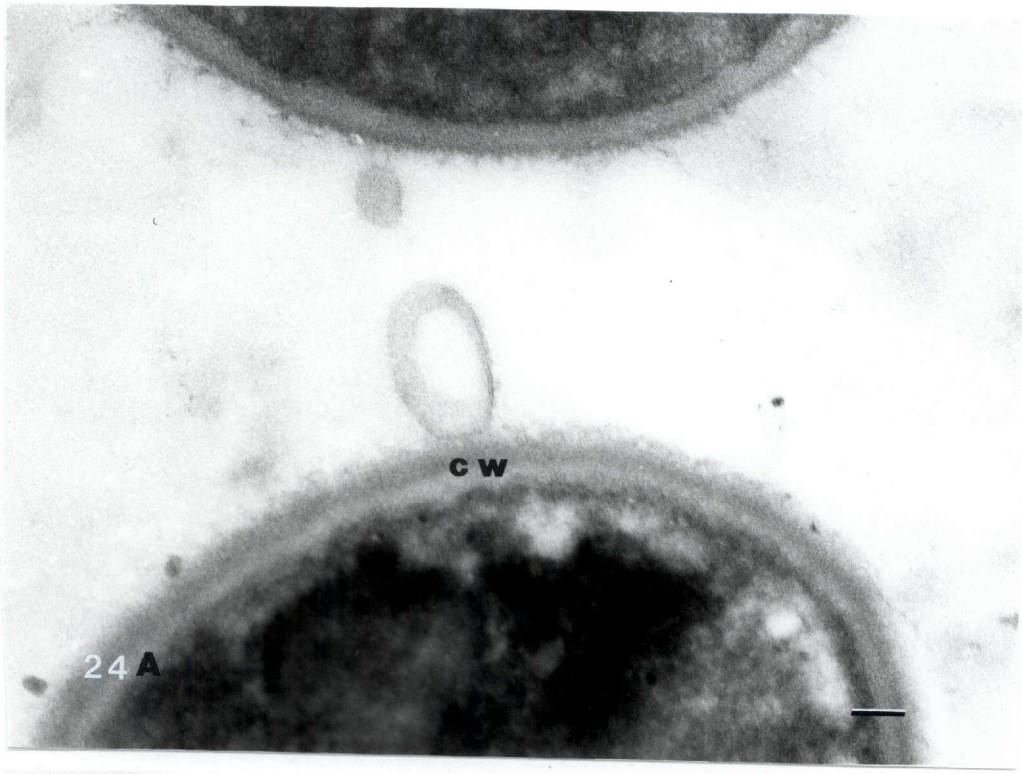
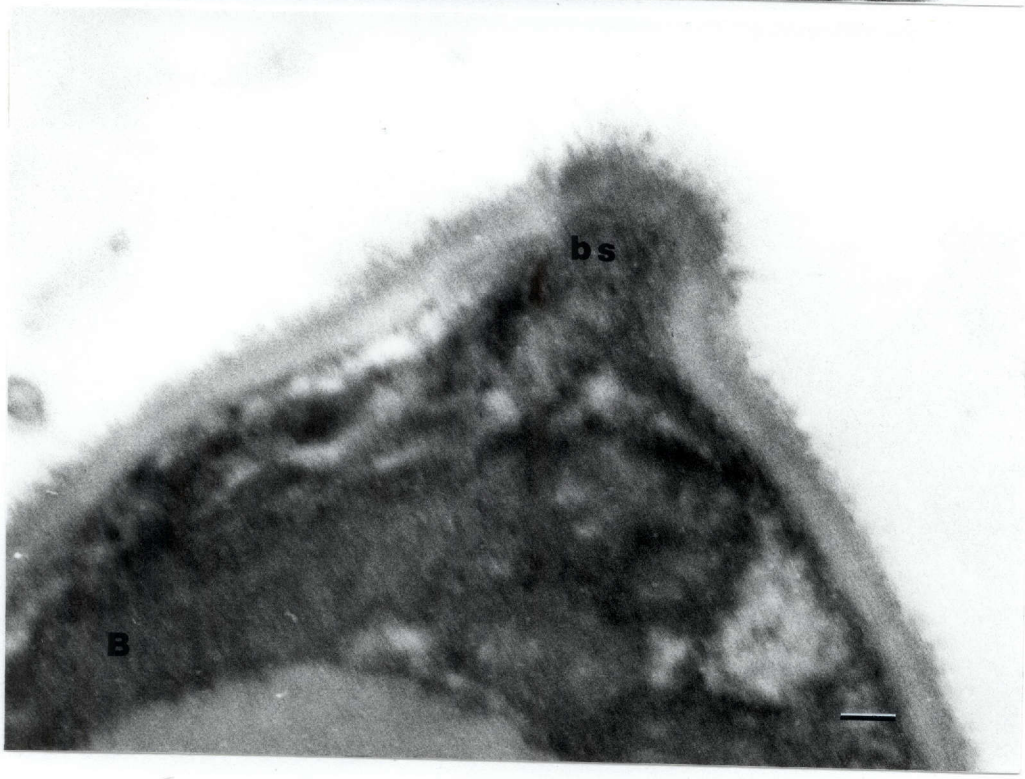

2 3 A
bs
B

Fig. 24 Yeast cell wall after treatment with lysing enzyme. Stained with 1% ruthenium red. (A) Cell wall (CW) area away from bud site. Bar = 0.1 μm. (B) Cell wall surrounding bud site (BS). Bar = 0.1 μm.


cw
24 A

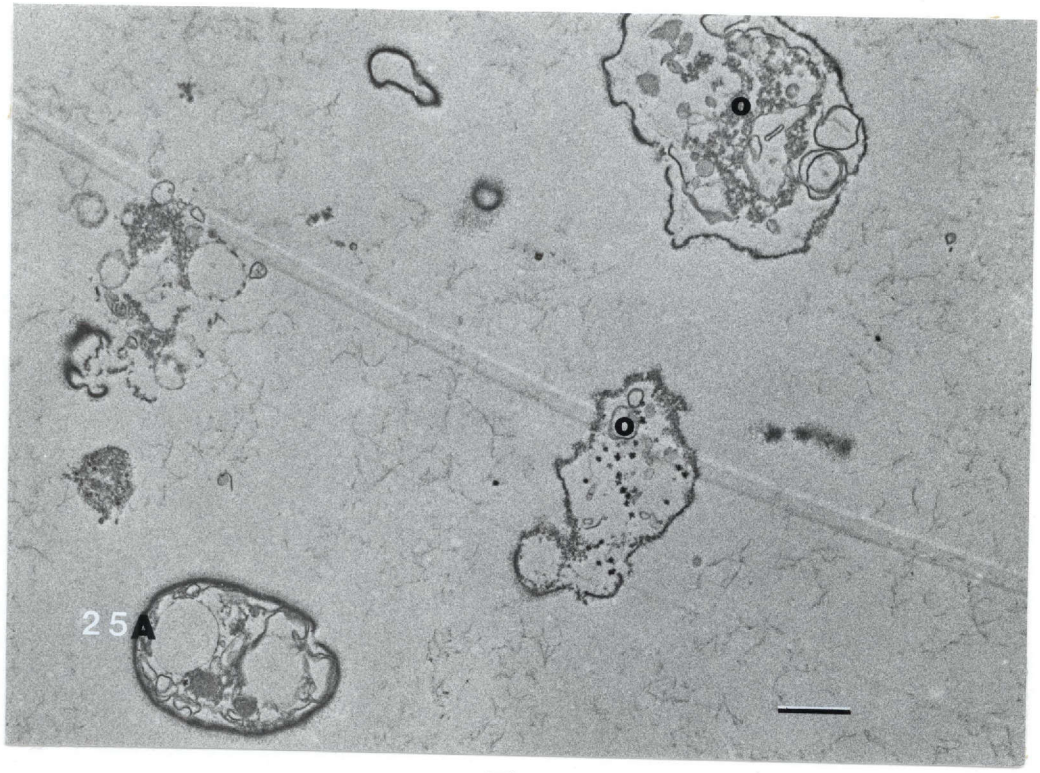

bs
B

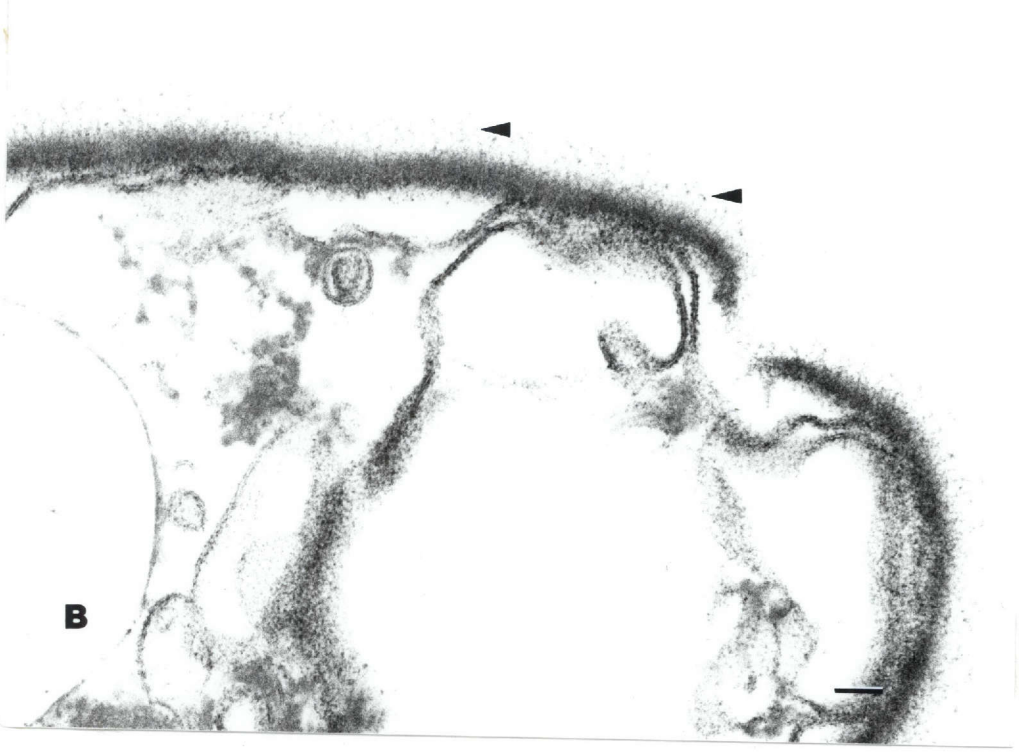
*Pronase and beta-glucuronidase treatment: 30 **g** pellet of treated sample.*

The studies just discussed dealt with yeast-phase cells treated with a single enzyme. The following studies are concerned with the effects of combinations of these enzymes on the cell wall when added in sequence. In each study two fractions were obtained after osmotic lysis. A fraction containing whole cells or cells with partially degraded walls which sedimented at 30 **g** and a second fraction, the pellet from the supernatant of the 30 **g** centrifugation, sedimenting at 12,100 **g** which contained wall, wall fragments or cell ghosts from which the cytoplasm has been lost.

Whole cells found in the 30 **g** pellet after treatment with protease and beta-glucuronidase look similar at low magnifications to the cells treated with either enzyme alone (Fig. 25A) when stained with uranyl acetate and lead citrate. Cytoplasm and cellular organelles are still contained within the wall, but significant breaching of the wall is evident. Higher magnifications of the cell wall stained with uranyl acetate and lead citrate show no differentiation of the inner cell wall, but the fibrillar nature of the outer wall is clearly seen (Fig. 25B).

Fig. 25 Electron micrographs of yeast-phase cells from 30 g pellet after treatment with pronase and beta-glucuronidase and osmotic lysis. Stained with uranyl acetate and lead citrate. (A) Low magnification showing breaching (arrows) of cell wall (CW), but retention of cytoplasmic organelles (O). Bar = 1.0 μm. (B) High magnification of showing fibrillar material (arrows) extending from cell wall. Bar = 0.1 μm.


25
A


B

Sonication of this sample destroyed the fragile cells which only had a portion of the wall remaining after enzymatic treatment. The only cells remaining are whole cells which were, for unknown reasons, refractile to the enzyme treatment (Fig. 26).

*Pronase and beta-glucuronidase treatment: 12,100 **g** pellet of treated sample.*

The 12,100 **g** pellet of the pronase and beta-glucuronidase treated sample contained distorted whole cells, cell ghosts, and wall fragments (Fig. 27). Apparently the 15 min used to remove whole cells at 30 **g** was not sufficient to remove all of them. Higher magnification of the wall reveals an electron dense layer with fibrous projections emanating from this layer (Fig. 28). A still higher magnification shows this fibrous material to be an extension of the electron dense center layer (Fig. 29). Fig. 28 also shows some portion of the inner wall remaining, but no substructure is evident.

Sonication of this sample for five minutes completely disintegrated the wall which had no recognizable wall fragments. Only membrane bound vesicles could be observed (Fig. 30A & B). However, when the sample was sonicated for only one minute, the fibrous wall could be seen in the sample (Fig. 31).

Fig. 26 Low magnification electron micrograph of cells after treatment with pronase, beta-glucuronidase, osmotic lysis and 5 min sonication showing only whole cells remaining. Stained with uranyl acetate and lead citrate. Bar = 1.0 μm.

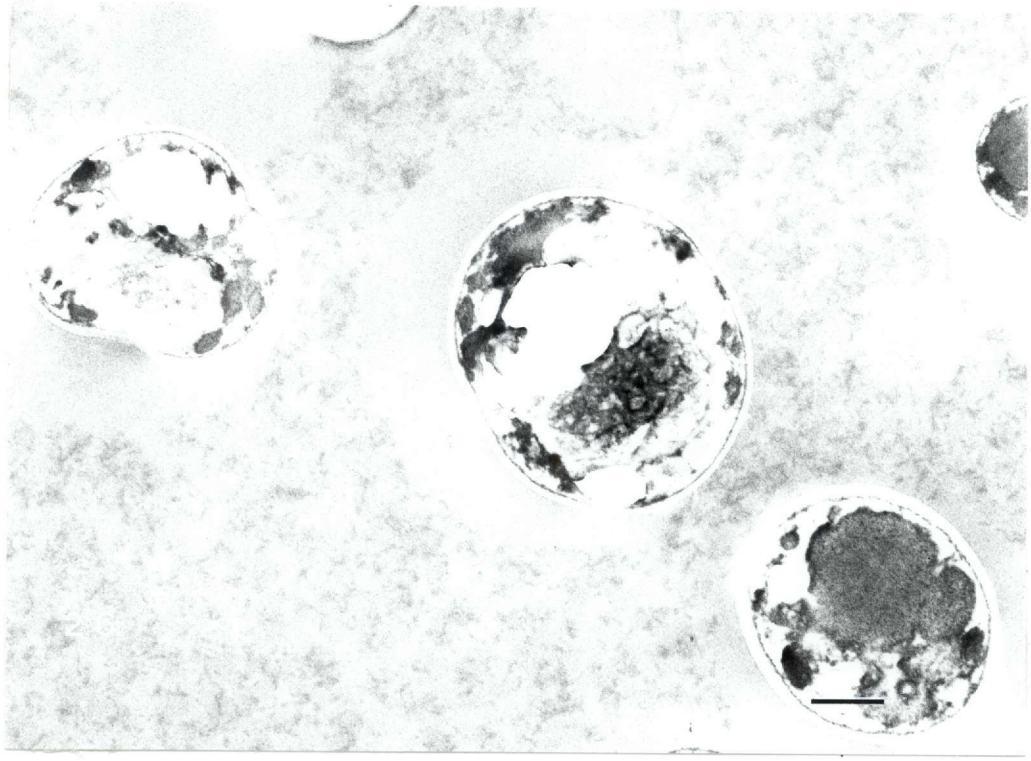
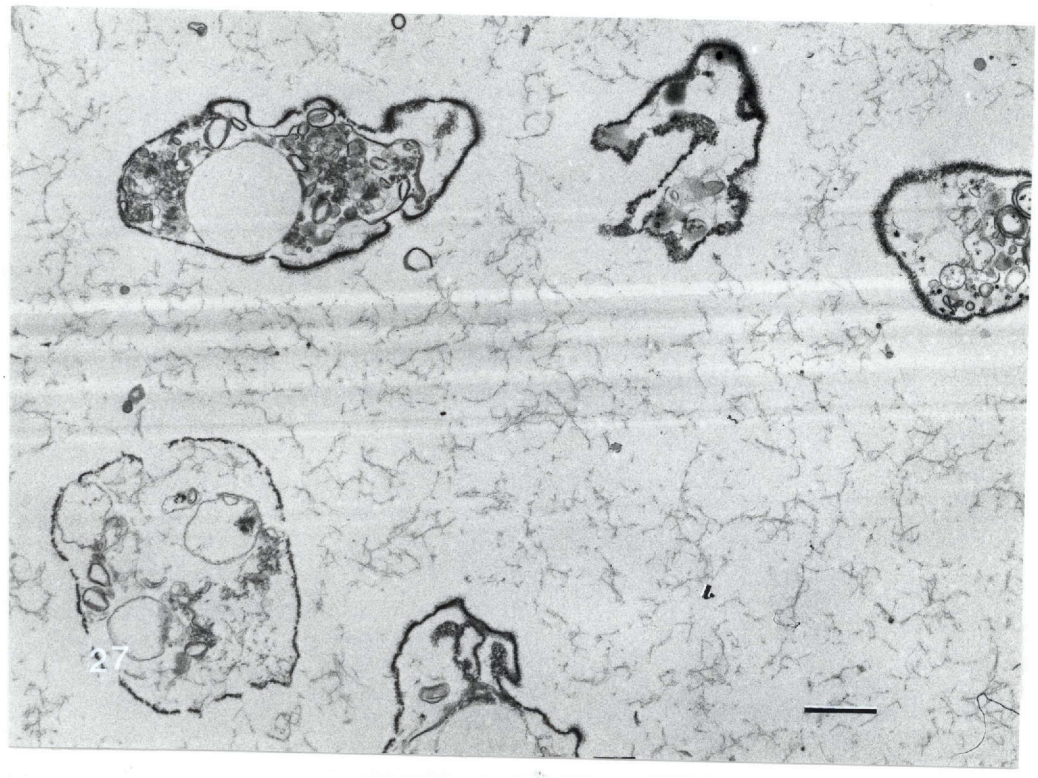
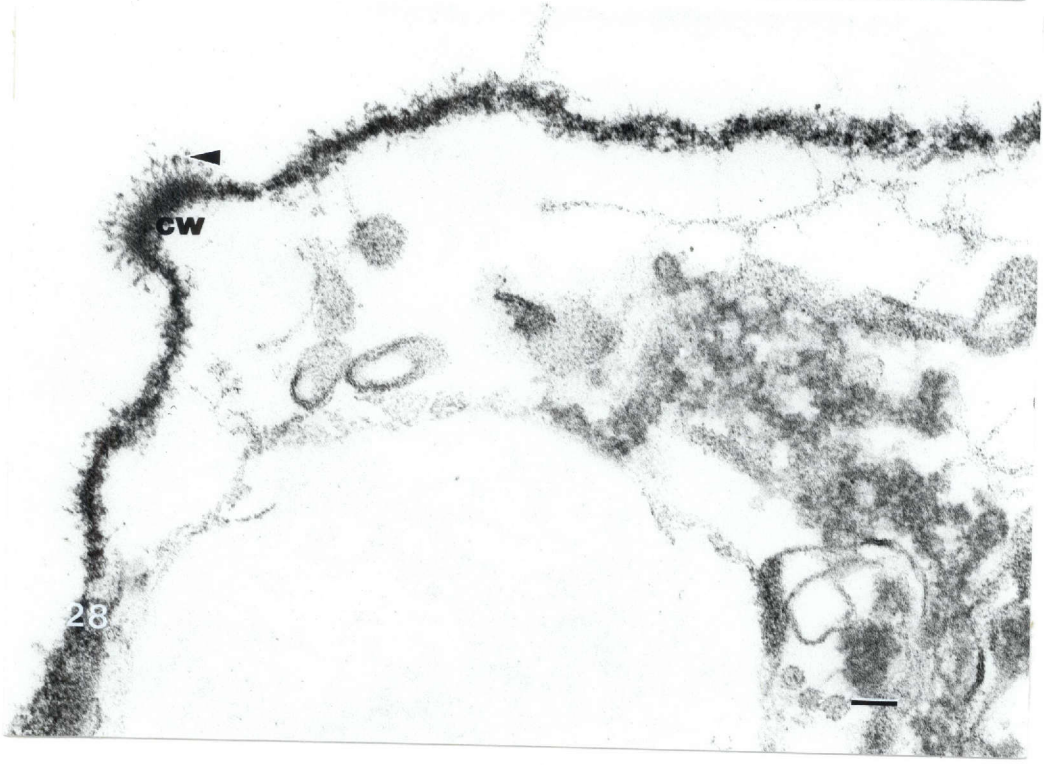


Fig. 27 Low magnification micrographs of 12,100 **g** cell lysate pellet after treatment with pronase and beta-glucuronidase. Sample was not sonicated. Stained with uranyl acetate and lead citrate. Bar = 1.0 µm.

Fig. 28 Cell lysate found in 12,100 **g** pellet after treatment with pronase and beta-glucuronidase. Arrow indicates the fibrous structures that extend from the cell wall (CW) into the area external to the cell. Bar = 0.1 µm.



27



28

Fig. 29 High magnification micrograph of fibrous structures (arrows) found on the remnants of the cell wall after treatment with pronase and beta-glucuronidase. Bar = 0.1 μm.

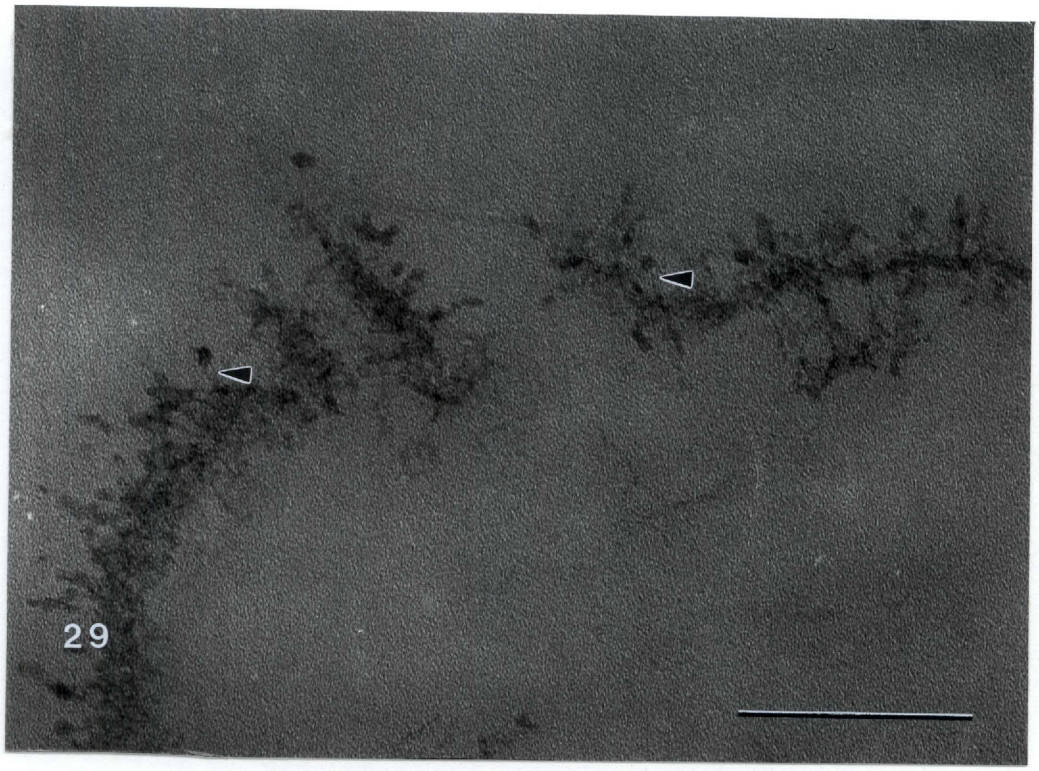

29

Fig 30 Cell lysate found in the 12,100 **g** pellet after treatment with pronase and beta-glucuronidase. Sample was sonicated for 5 min. Stained with uranyl acetate and lead citrate. (A) Low magnification showing absence of wall fragments. Bar = 1.0 µm. (B) High magnification showing vesicles released from cell. Bar = 0.1 µm.

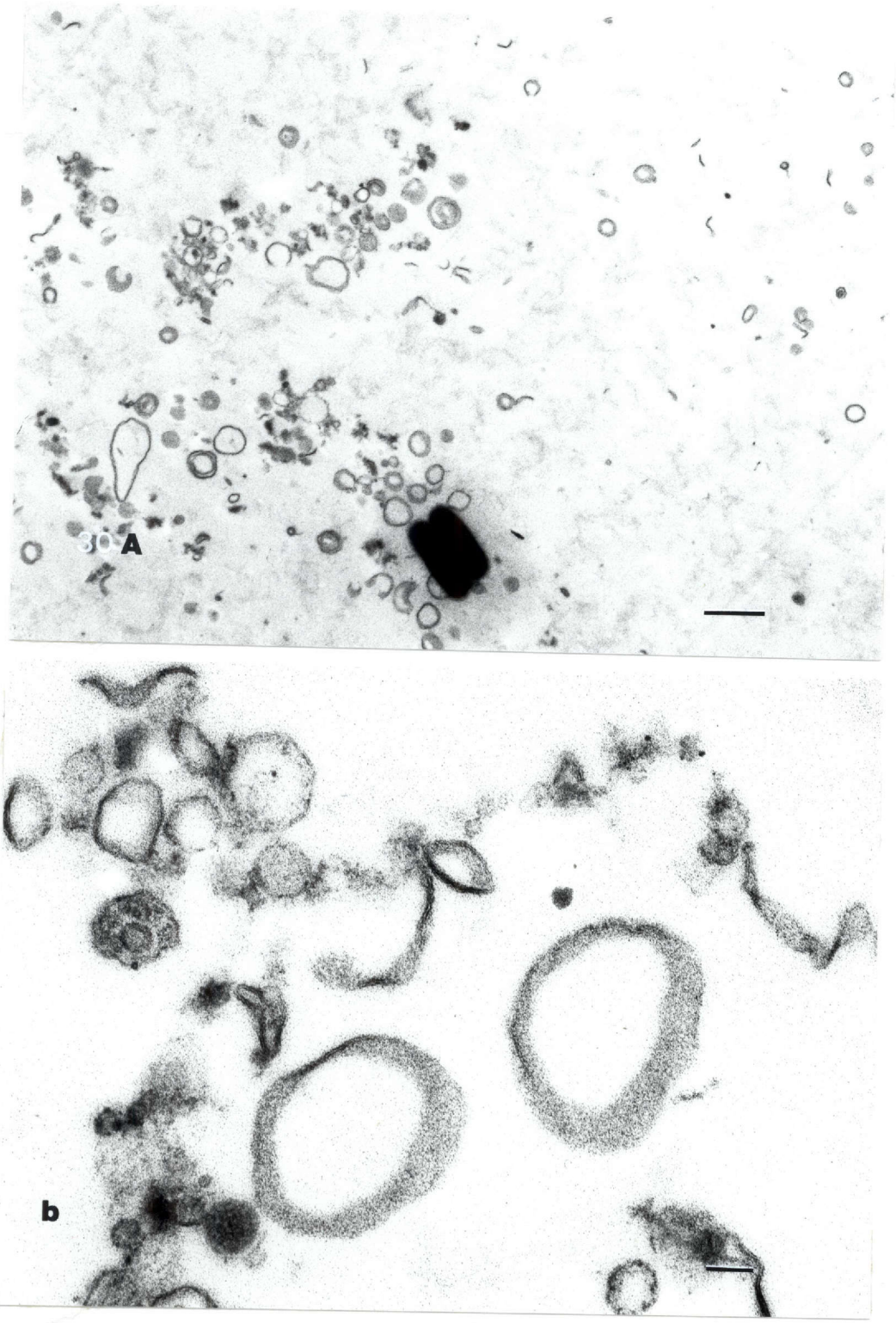
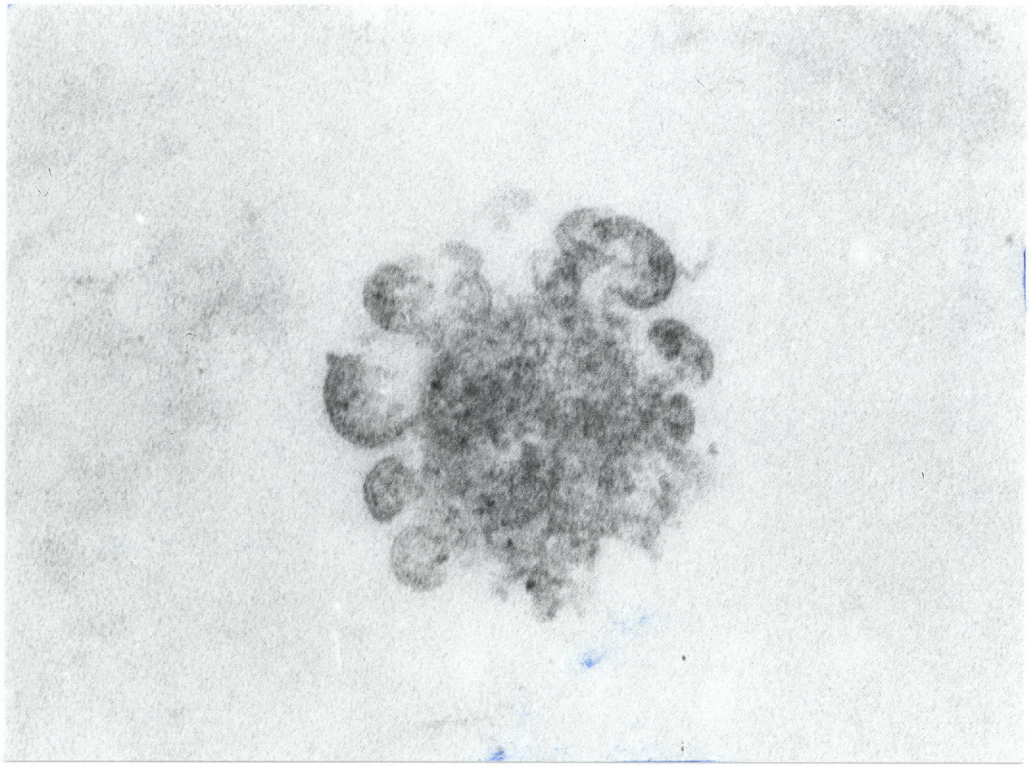

A
b

Fig. 31 Cell vesicle from cell lysate from 12,100 **g** centrifugation after treatment with pronase and beta-glucuronidase and sonicated for 1 min. Arrows indicate the fibrillar section of the wall fragment. Bar = 0.1 μm.



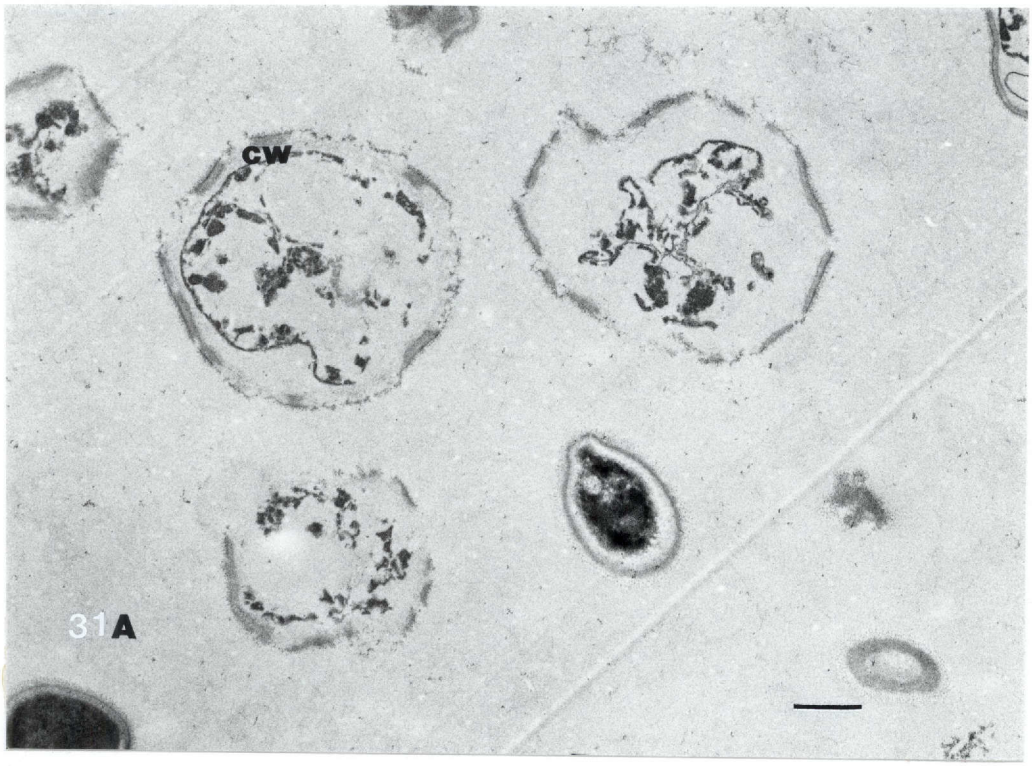
Extraction of the nonsonicated sample with Triton X-100 caused a partial extraction of the remaining portion of the wall leaving gaps in the wall (Fig. 32A). Higher magnification of these extracted areas (Fig. 32B) show fibrous nature of the inner wall and a thin electron dense outer layer with fibers projecting from it.

*Beta-glucuronidase and lysing enzyme treatment: 30 **g** pellet of treated sample.*

The whole cells found in the pellet of the sample treated with beta-glucuronidase and lysing enzyme show a faint striation at low magnifications (Fig. 33A) when stained with uranyl acetate. Higher magnification of this sample show a slightly more electron dense area next to the cell membrane and a lighter, or less electron dense portion of the cell wall on the exterior margin of the cell. This stratification is uniform whether at the cell bud site or at the wall away from the bud site (Fig. 33B). The electron dense outer layer seen after treatment with pronase and beta-glucuronidase is not seen and presumably has been digested.

When this sample was sonicated for five minutes the fragile cells which only have a remnant of the cell wall remaining are destroyed and only the cells with the majority of the wall intact are found in the pellet (Fig. 34).

Fig. 32 Pellet from cell lysate treated with pronase and beta-glucuronidase, extracted with 1% Triton X-100 and centrifuged at 12,100 **g** for 10 min. (A) Low magnification showing non-uniform digestion of cell wall (CW). Bar = 1.0 µm. (B) Higher magnification showing fibrous structures (arrows) of inner wall (1) and thin electron dense outer layer (2) with projecting fibrils. Bar = 0.1 µm.


CW
31A

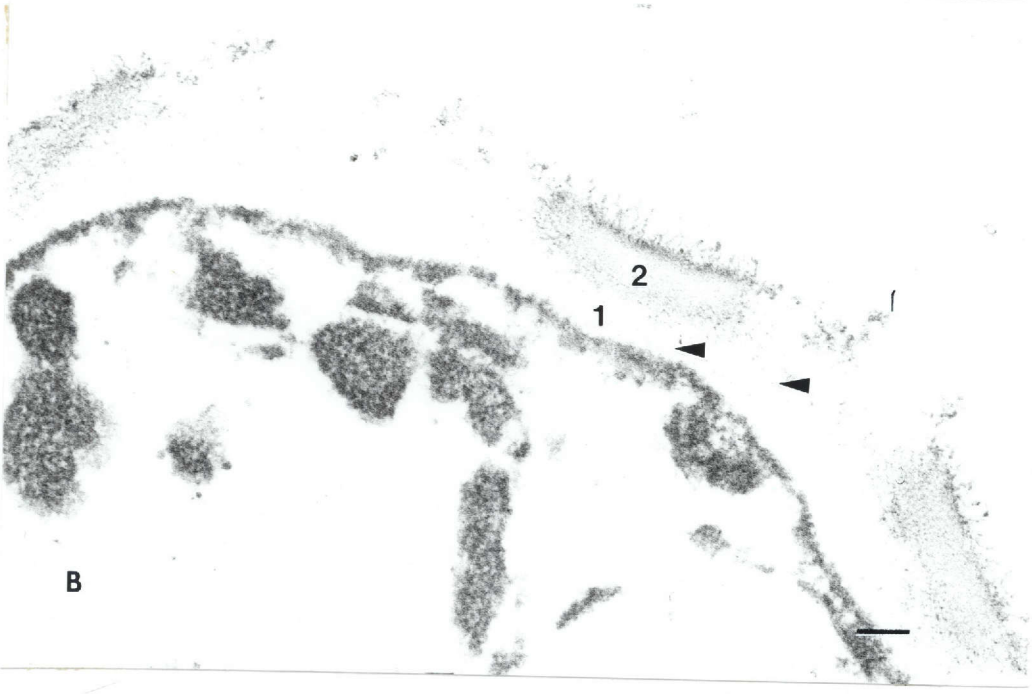
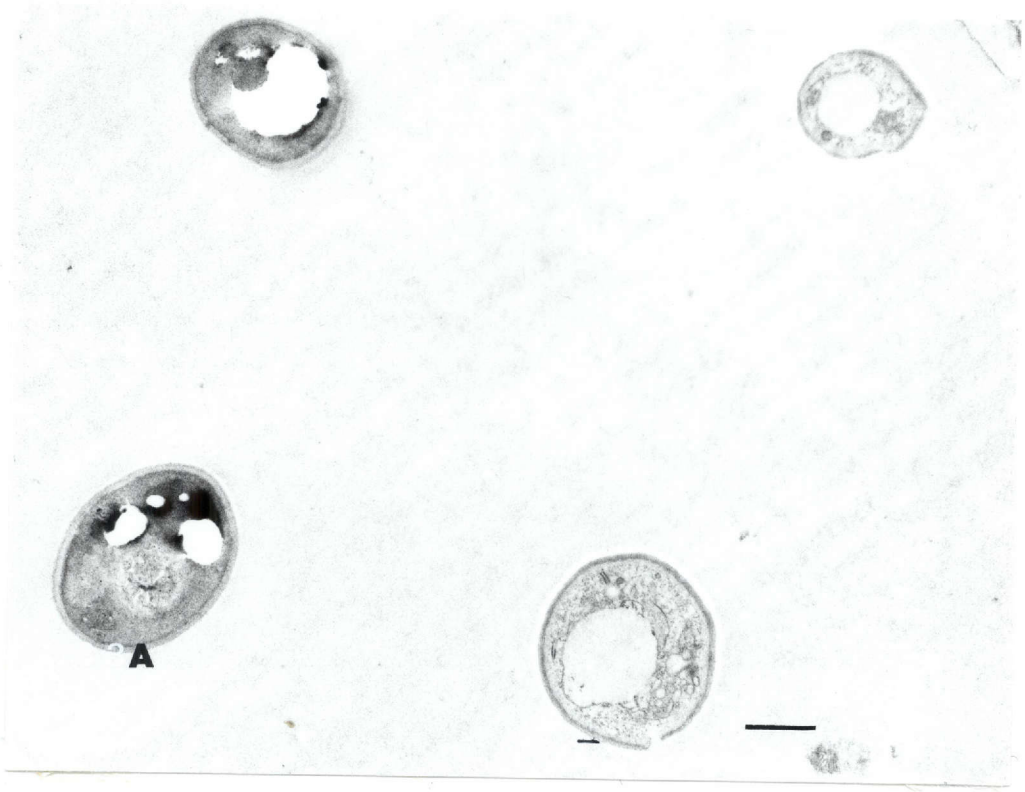

2
1
B

Fig. 33 Transmission electron micrographs of cells from 30 g pellet after treatment with beta-glucuronidase and lysing enzyme and osmotic lysis. Stained with uranyl acetate and lead citrate. (A) Low magnification showing whole cells. Bar = 1.0 µm. (B) High magnification showing areas of the cell wall with differing electron densities (arrows). Bar = 0.1 µm.


A

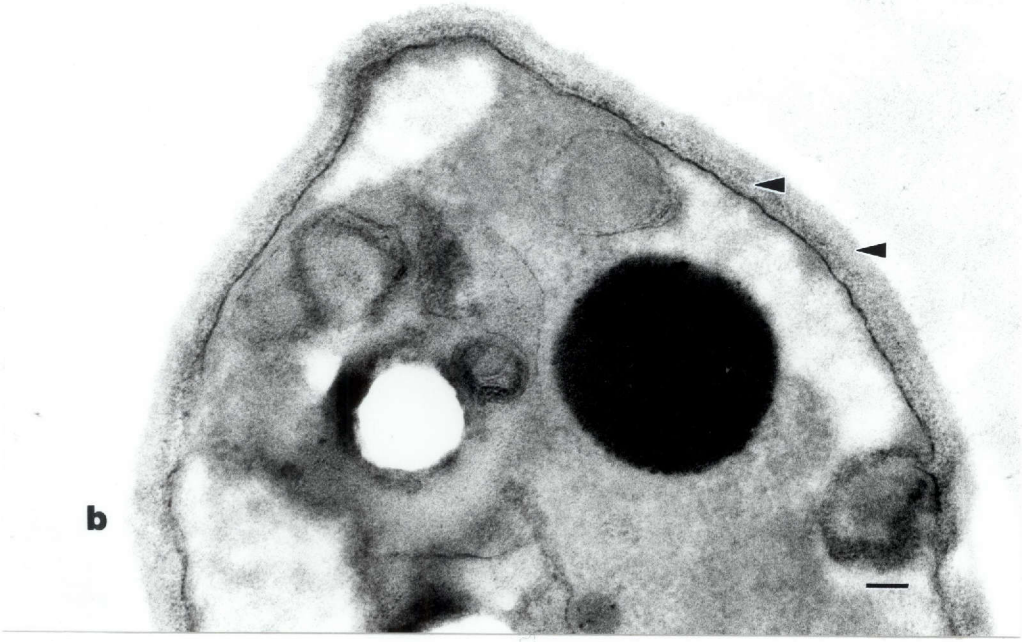
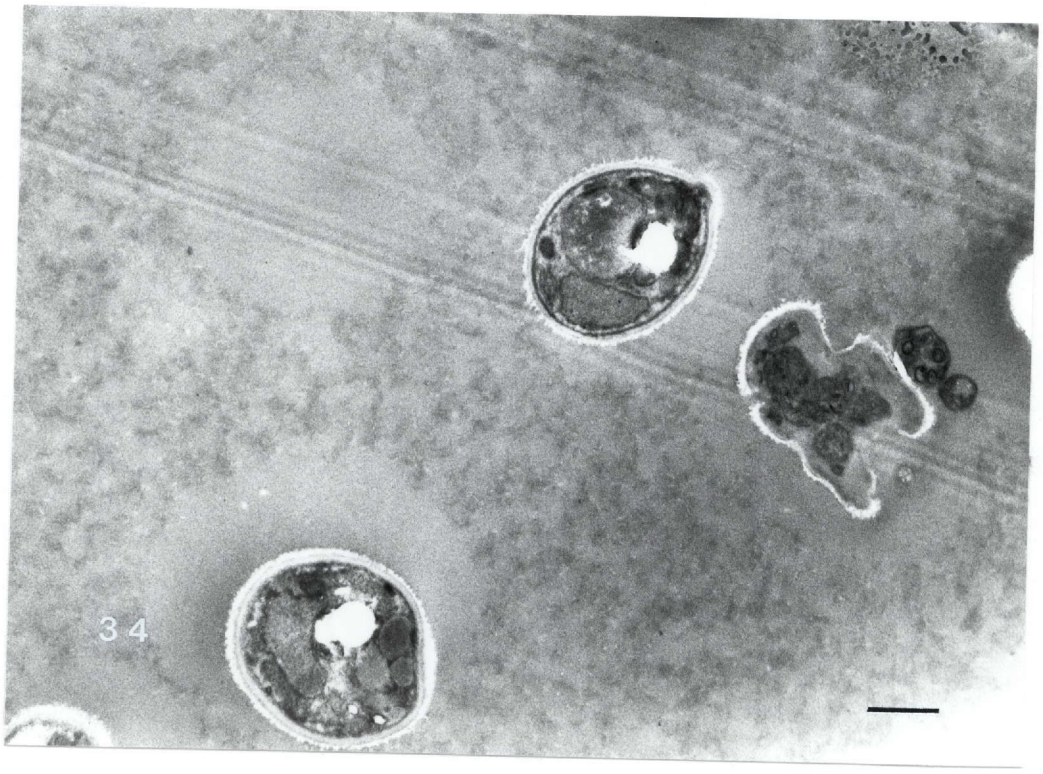

b

Fig 34 Low magnification micrographs of 30 **g** pellet after treatment with beta-glucuronidase and lysing enzyme, osmotic lysis and 5 min sonication. Stained with uranyl acetate and lead citrate. Bar = 1.0 μm.


34

*Beta-glucuronidase and lysing enzyme treatment: 12,100 **g** pellet of treated sample.*

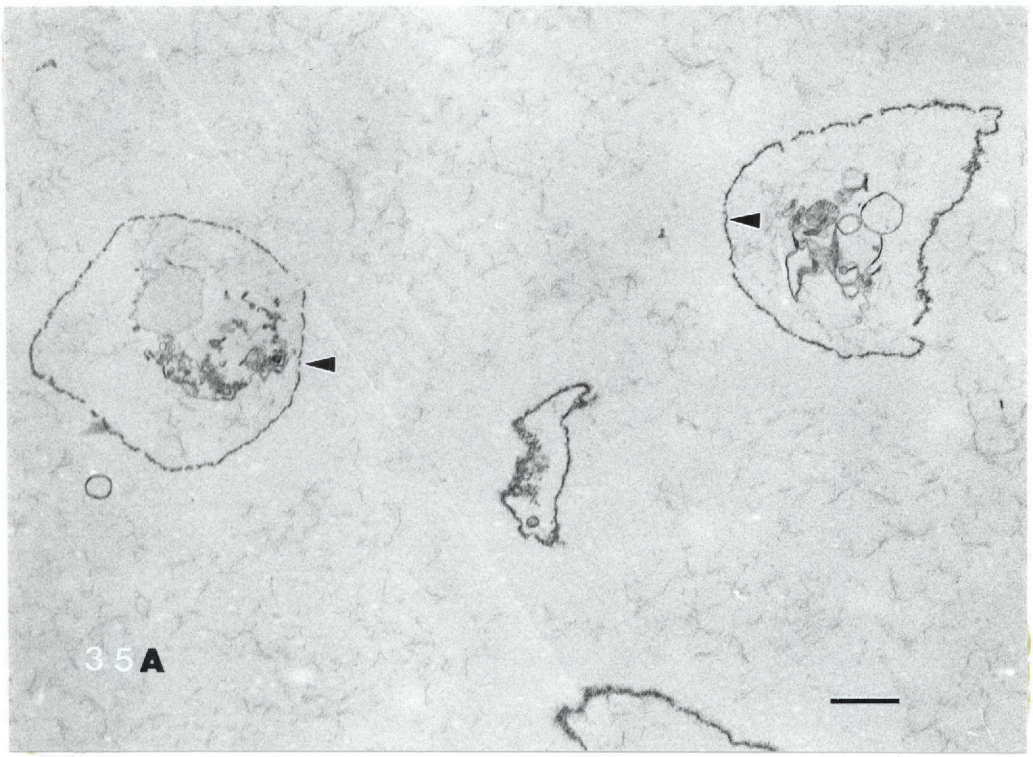
Samples removed from the 12,100 **g** pellet of culture which was treated with beta-glucuronidase and lysing enzyme and osmotically lysed showed remnants of the cell wall (Fig 35), but the fibrous material emanating from the electron dense area were not as evident as those seen after treatment with protease and beta-glucuronidase treatment (Fig. 28). Lysing enzyme is a mixture containing glucanases, cellulase, chitinase, and protease. Digestion of these fibers by this mixture indicates the fibers consist either of a protein which is refractile to pronase or cellulose and/or chitin. The fact that this layer stains intensely with uranyl acetate and some what intensely with ruthenium red (See Fig. 21) the fibers are presumably glycoprotein or mucopolysaccharide in nature and are resistant to digestion by the protease in this enzyme mixture.

Sonication of this sample for five minutes resulted in the formation of rounded vesicles (Fig. 36A & B).

*Pronase, beta-glucuronidase and lysing enzyme treatment: 30 **g** pellet of treated sample.*

Cells found in the sample treated with protease, beta-glucuronidase, and lysing enzyme (Fig. 37A) have only a very electron dense portion of the wall remaining. This sample was completely disrupted upon sonication. Higher

Fig. 35 Pellet from cell lysate treated with beta-glucuronidase and lysing enzyme and centrifuged at 12,100 **g**. (A) Low magnification showing only thin outer wall remaining (arrows). Bar = 1.0 μm. (B) High magnification of area shown in (A). Bar = 0.1 μm.


35A

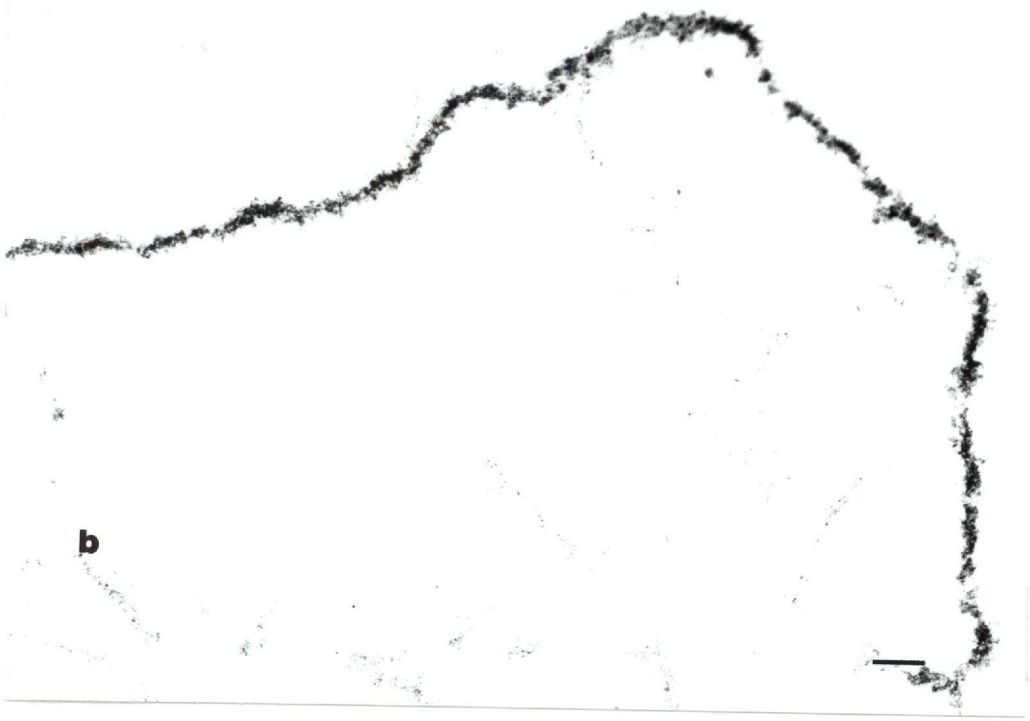
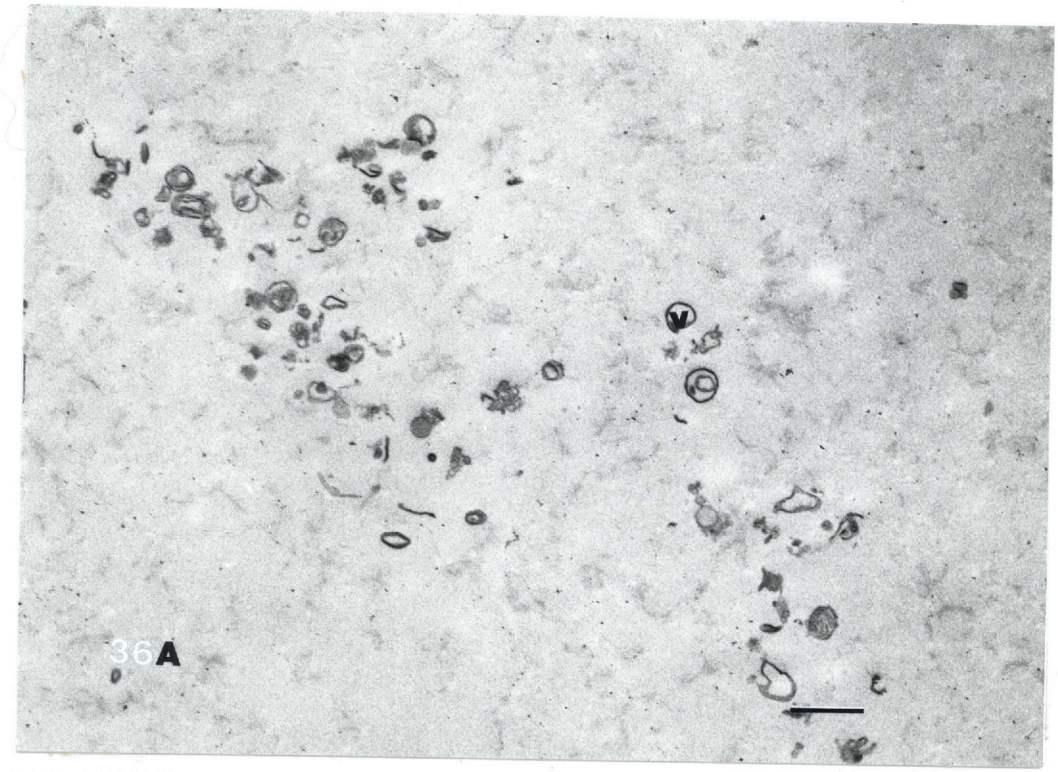
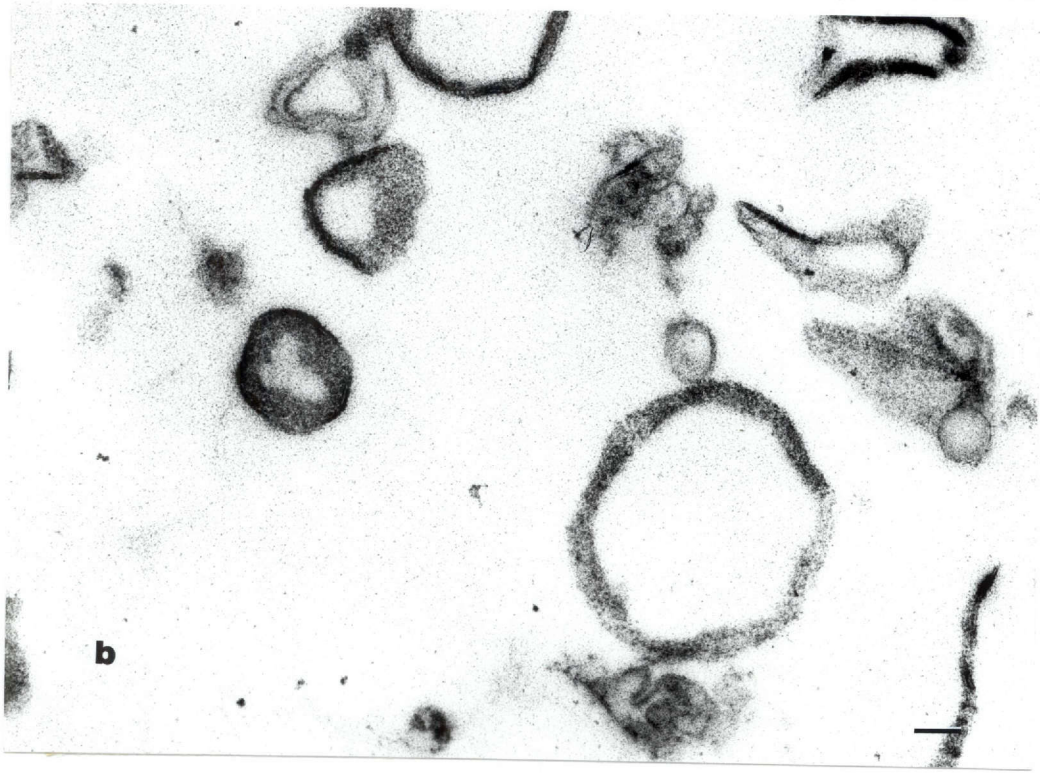

b

Fig. 36 Sample removed from 12,100 **g** pellet after treatment with beta-glucuronidase and lysing enzyme and sonicated for 5 min. (A) Low magnification micrograph showing vesicles (V) and cellular debris. Bar = 1.0 μm. (B) High magnification of vesicles and cellular debris. Bar = 0.1 μm.


36 A


b

magnification of the nonsonicated sample show a remnant of the wall that is very electron dense with fibrous protrusions of the wall extending into the area on both sides of the wall (Figs.36B & 37). The structure of this region of the wall resembles that seen in samples treated with protease and beta-glucuronidase (Fig. 27), but the inner wall is completely removed.

*Pronase,beta-glucuronidase and lysing enzyme treatment: 12,100 **g** pellet of treated sample.*

The 12,100 **g** pellet from the sample treated with protease, beta-glucuronidase, and lysing enzyme contained an electron dense remnant of the cell wall similar to the previous three treatments (Fig. 38A). This remnant did not have as many of the protruding fibers and was more diffuse with interupted portions (Fig. 38B). Sonication for five minutes resulted in the disruption of the cell wall remnant which did not have identifiable portions of the cell wall remaining.

Scanning electron micrographs of whole cells.

The effects of pronase, beta-glucuronidase and lysing enzyme treatments could also be seen with scanning electron microscopy. Untreated whole cells had a smooth, slightly rippled appearance, with no visible pores or breaches in the cell wall (Fig. 39).

After incubation with pronase, the cell wall appeared roughened and slight furrows could be seen in the cell wall surface (Fig. 40). The furrowing was more prominent in the cell wall surface after treatment with beta-glucuronidase (Fig 41), but less prominent in cell walls after treatment with lysing enzyme (Fig. 42). Pores were also visable in cell walls after incubation with beta-glucuronidase (Fig 41).

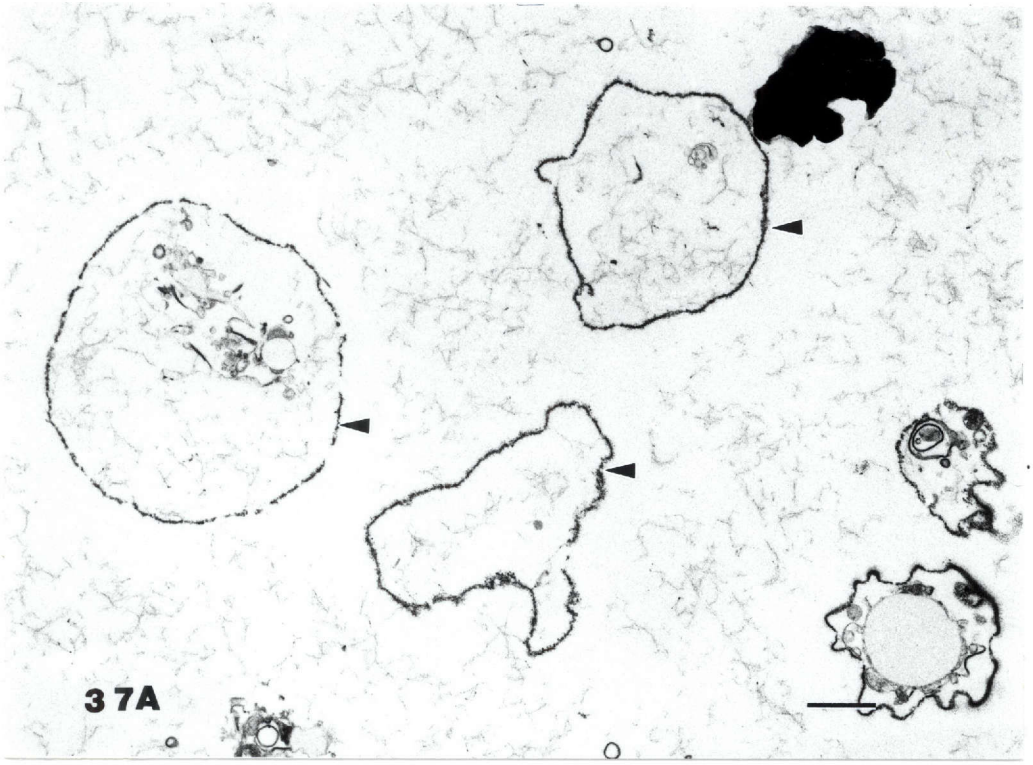
The most dramatic change in the cell wall was seen in cells treated with pronase followed by beta-glucuronidase (Fig. 43). The cells had large visible pits in the wall that did not appear to be confined to either the bud site or the wall of the mother cell and penetrated deep into the cell wall.

Thus it appears there is an external layer composed of polysaccharides (glucans), protein and/or mucopolysaccharide which is degraded by beta-glucuronidase and pronase. Pre-treatment with pronase hydrolyzes portions of the outer protein layer exposing the glucans in the inner layers to the action of beta-glucuronidase

Summary and conclusion of the cell wall ultrastructure.

The ultrastructure of the cell wall of yeast phase cells of W. dermatitidis has been revealed, in part, by selectively degrading the wall with enzymes. Visualization of the structure depends in part on the staining method used.

Fig. 37 Pellet from 30 **g** centrifugation of osmotic lysate after treatment with pronase, beta-glucuronidase, and lysing enzyme. (A) Low magnification micrograph showing cell ghosts with only thin outer layer (arrows). Bar = 1.0 μm. (B) High magnification showing thin outer wall layer with protruding fibers (arrows). Bar = 0.1 μm.


37A

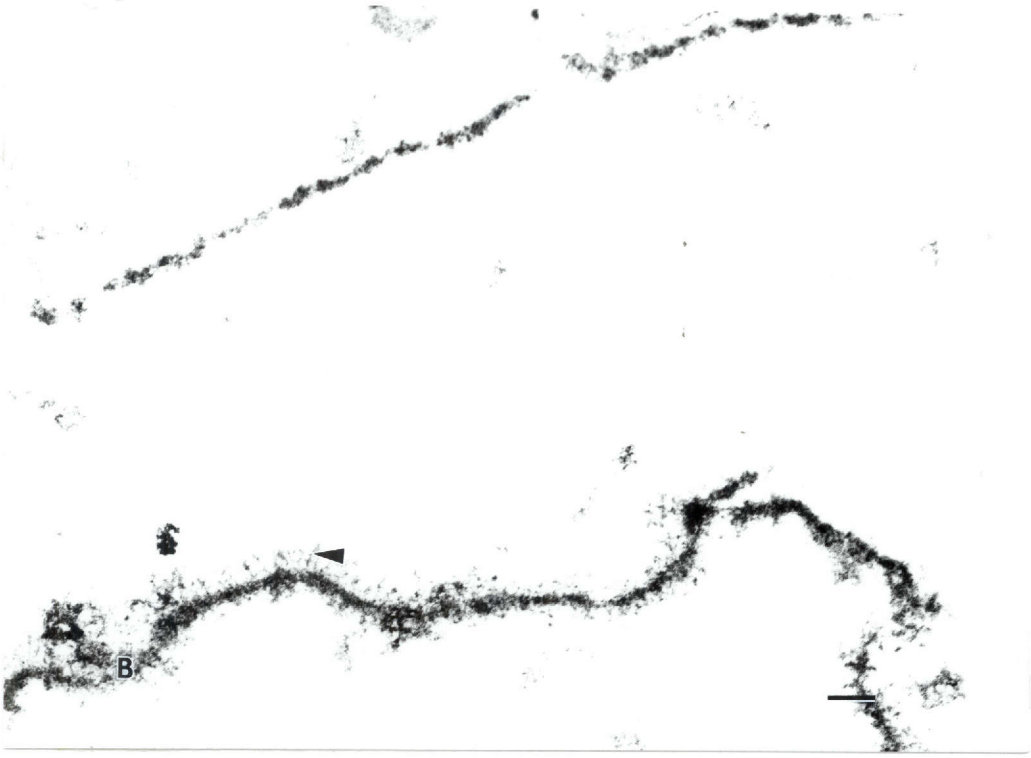
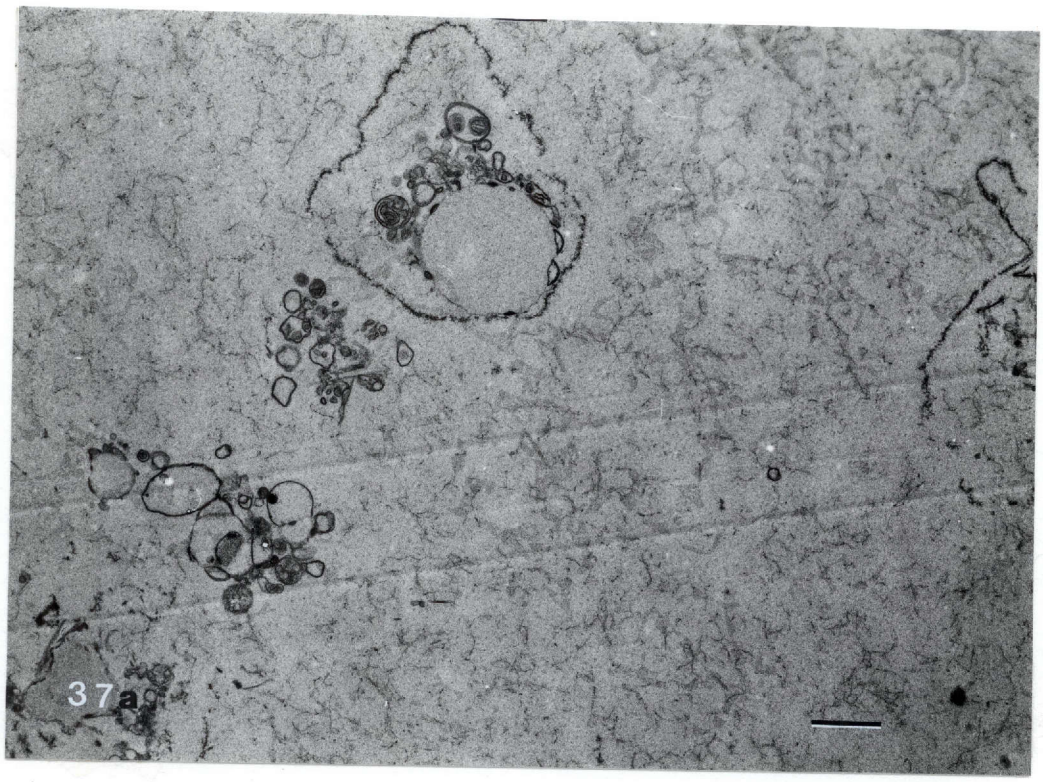

B

Fig. 38 Electron micrograph of cell lysate from 12,100 **g** pellet after treatment with pronase, beta-glucuronidase, and lysing enzyme. (A) Low magnification micrograph of sample Bar = 1.0 μm. (B) High magnification showing lack of inner wall. Cell wall remnant is noted by arrows. Bar = 0.1 μm.


37a

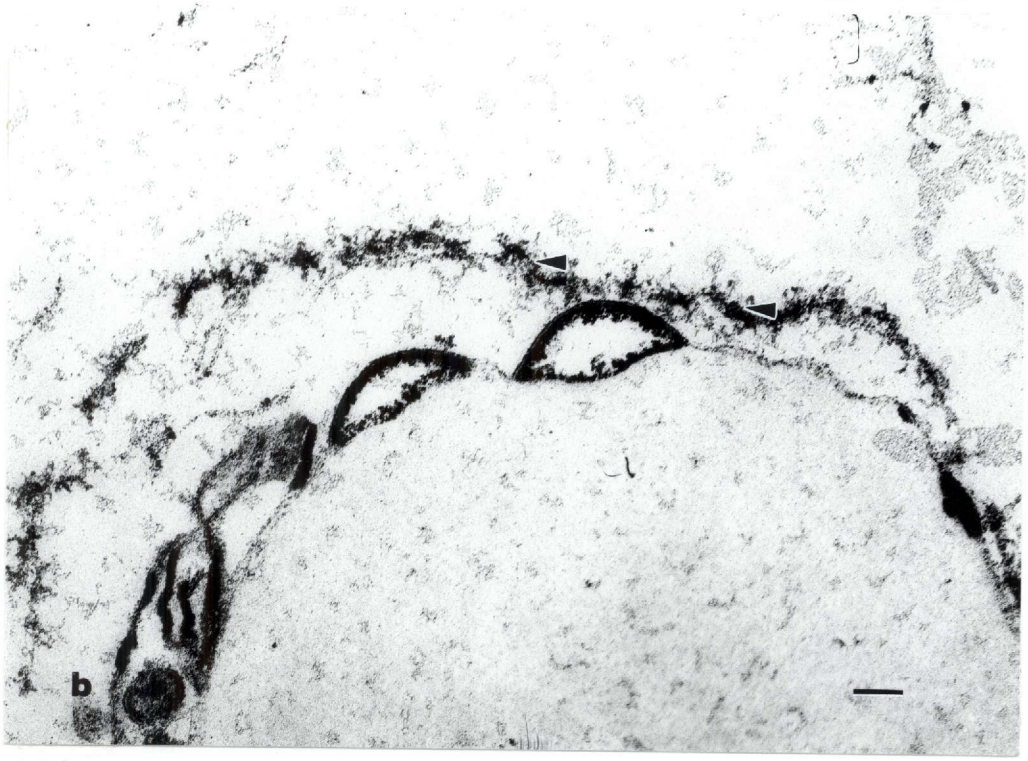
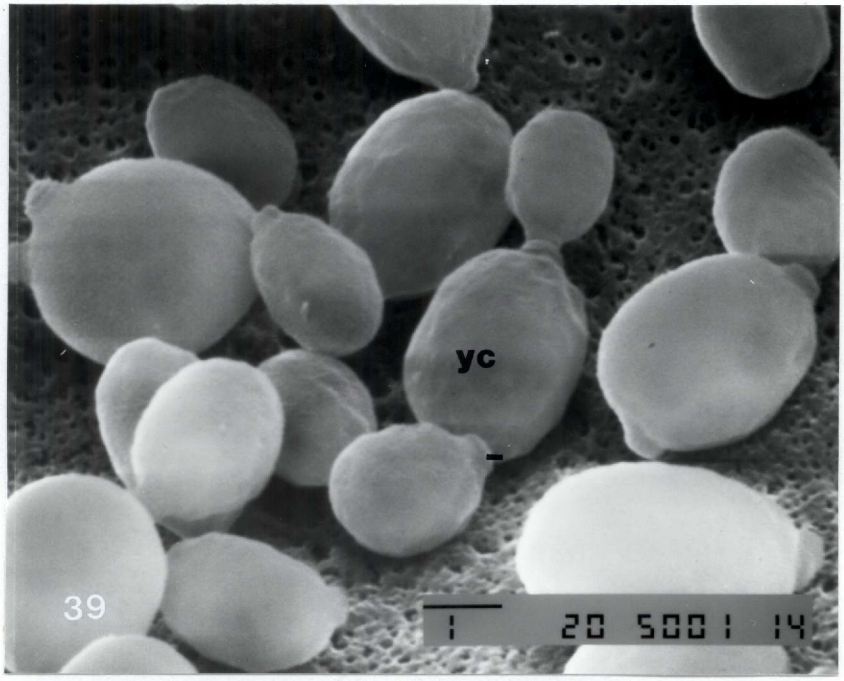

b

Fig. 39 Scanning electron micrograph of untreated yeast-phase cells (YC) showing smooth, slightly rippled cell wall surface. Bar = 1.0 μm.

Fig. 40 Scanning electron micrograph of yeast-phase cells after incubation with pronase. Cell wall appears slightly rugose. Bar = 1.0 μm.


yc
39
1 20 5001 14

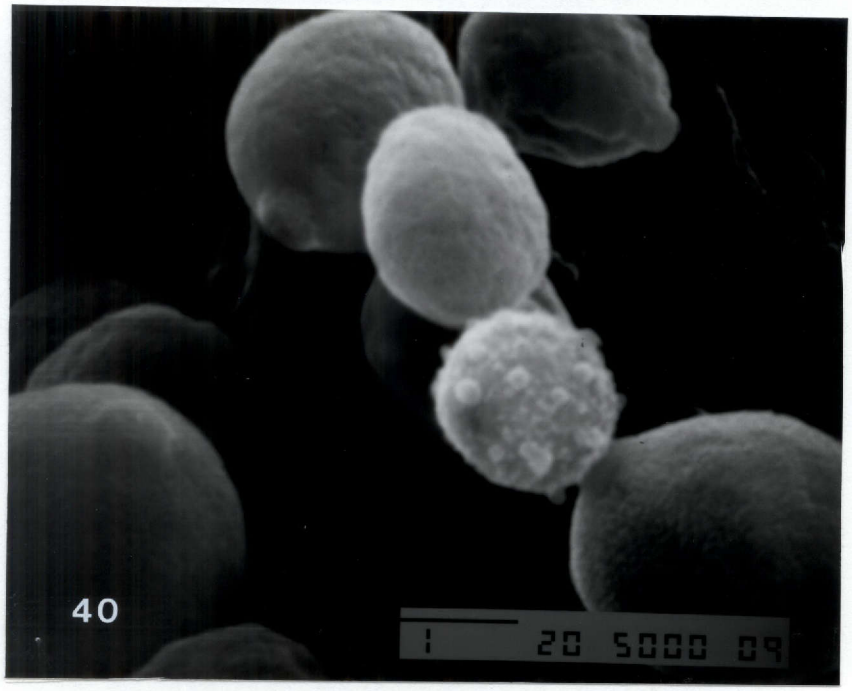
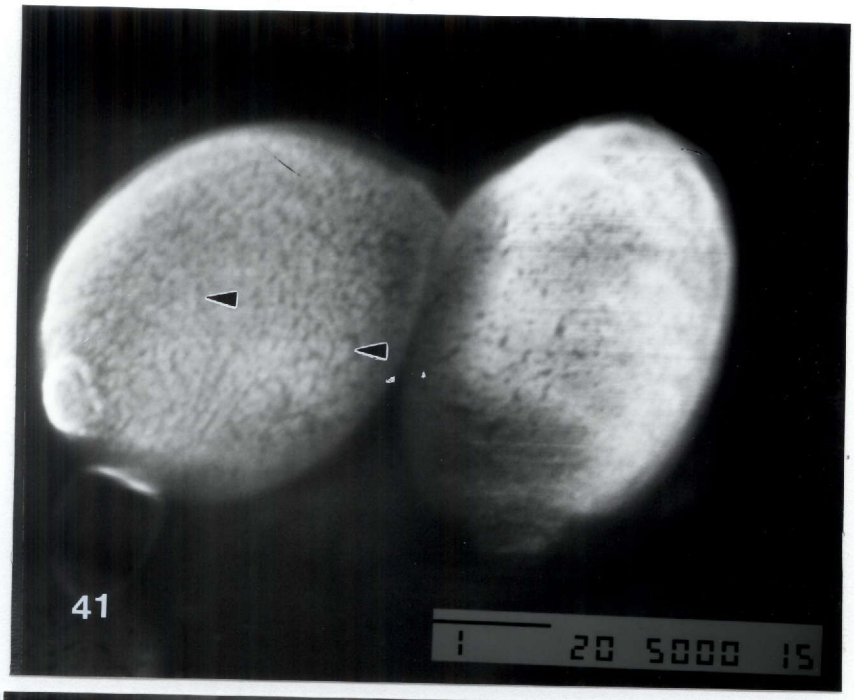

40
1 20 5000 09

Fig. 41 Scanning electron micrograph of yeast-phase cells after incubation with beta-glucuronidase. The wall surface is furrowed and small pores are evident (arrows) on some of the cells. Bar = 1.0 μm.

Fig 42 Scanning electron micrograph of yeast-phase cells after incubation with lysing enzyme. The cell wall surface appears similar to the sample treated with pronase (Fig. 42) with only a slight roughing of the surface. Bar = 1.0 μm.


41
1 20 5000 15

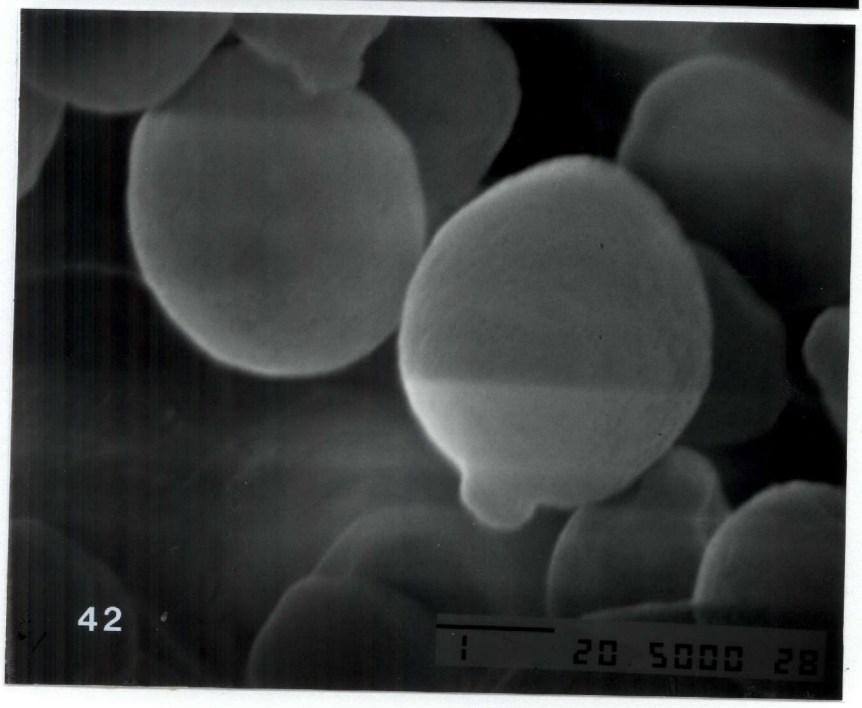
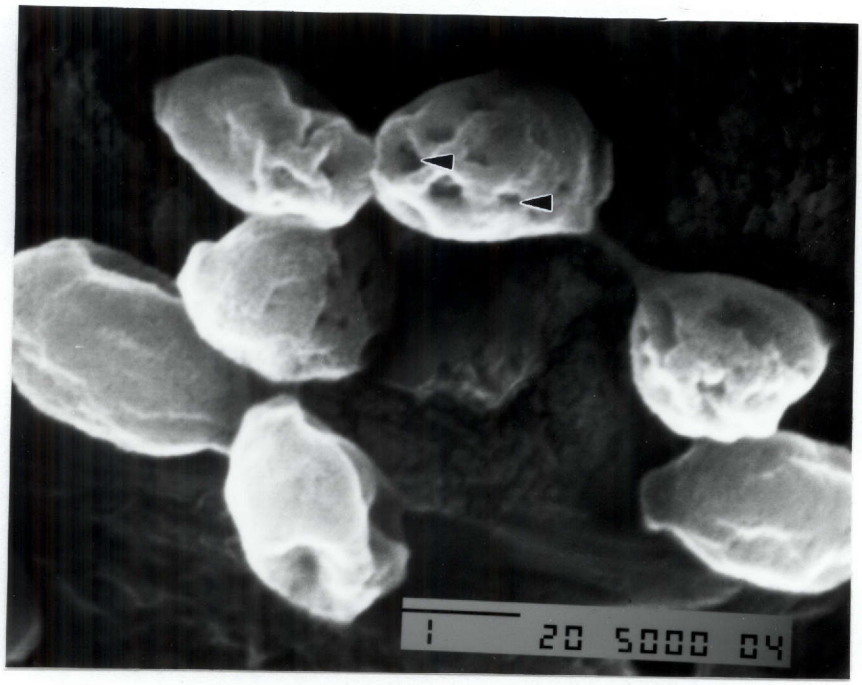

42
1 20 5000 28

Fig. 43 Scanning electron micrograph of yeast-phase cells after pretreatment with pronase followed by incubation with beta-glucuronidase. Large pores (arrows) are seen in most of the cells and deep furrows and folds are seen on the cell surface. Bar = 1.0 μm.


1 20 5000 04

Staining of untreated cells with ruthenium red reveals a two layered structure with a thick unevenly staining inner wall and a thin outer layer with fibrous projections with stain more intensely (Fig. 12, 13). With both stains the inner layer of untreated cells appears to be amorphous. Treatment with the protease, pronase, reveals a three layered fibrous structure with a more open texture when stained with uranyl acetate and lead citrate. When the same cells are stained with ruthenium red four layers are seen, but all layers, except the outer layer, appear amorphous, rather than fibrous. In general, it can be said that the wall consists of at least three layers with the inner layers consisting of microfibrils embedded in an amorphous matrix which is composed, at least in part, of protein since it is removed by pronase. Futhermore, the fibrils in the inner wall layers are not uniformaly distributed. This is particularly evident in cells treated with pronase and stained with uranyl and lead salts where the inner layers stain unevenly.

The outer layer of the cell wall also consists of a heterogeneous mixture of polymers which are unevenly distributed over the surface. Treatment with pronase, beta-glucuronidase, pronase followed by beta-glucuronidase, lysing enzyme, beta-glucuronidase followed by lysing enzyme or all three enzymes result in breaching of the cell wall at selected sites (Fig. 18, 19B, 21, 25, 32). Scanning electron

micrographs show these treatments to result in pitting of the cell wall. Despite this breaching of the outer wall it appears to be the most resistant to degradation by these enzymes. After treatment with all three enzymes the inner wall layers are completely degraded but the outer layer, which now stains intensely with uranyl acetate and lead citrate, remain relatively intact although distorted and breached in numerous places. Since removal of the inner layers only occurred after lysing enzyme was added following treatment with pronase and beta-glucuronidase it suggests that the inner layers are rich in glucans and chitin and/or cellulose. Distortion of the cell shape after treatment with lysing enzyme also indicated that the inner wall layers are responsible for the cell shape. The outer layer remaining after all three enzyme treatments presumably consists in large part of glycoprotein or mucopolysaccharide which is resistant to the enzymes in this mixture.

In summary, the results of this investigation have shown that the cell wall of W. dermatitidis consists of at least three layers. The inner two layers, responsible for cell shape consist of microfibrils, presumably of chitin, and/or cellulose embedded in an amorphous matrix of glucans and mannans. The outer layer consists of pockets of protein,

susceptible to digestion by pronase, and beta-glucuronides, susceptible to degradation by beta-glucuronidase. These in turn are embedded in a matrix of glycoprotein or mucopolysaccharide refractile to pronase and the protease in the lysing enzyme mixture.

LITERATURE CITED

1. Aronson, J. M. (1965). The cell wall. In The fungi: an advanced treatise, pp. 49-76. Edited by G. C. Ainsworth & A. S. Sussman. New York: Academic Press.

2. Balleu, C. E. & Raschke, W. C. (1974). Polymorphism of the somatic antigen of yeast. Science 184, 127-134.

3. Benhamou, N., Chamberland, H., Noel, S., & Ouellette, G. B. (1990). Ultrastructural localization of beta-1,4-glucan containing molecules in the cell walls of some fungi: a comparative study between spore and mycelium. Canadian Journal of Microbiology 36, 149-158.

4. Barran, L. R., Schneider, E. F., Wood, P. J., Madhosingh, C., & Miller, R. W. (1975). Cell wall of Fusarium sulphureum. I. Chemical composition of the fungal wall. Biochimica et Biophysica Acta 392, 148-158.

5. Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis and taxonomy in fungi. Annual Review of Microbiology 22, 87-108.

6. Bartnicki-Garcia, S. & Lippman, E. (1969). Fungal morphogenesis. Cell wall construction in Mucor rouxii. Science 165. 302-304.

7. Biel, A. J. & Marrs, B. L. (1985). Oxygen does not directly regulate carotenoid biosynthesis in Rhodopseudomonas capsulata. Journal of Bacteriology 162, 1320-1321.

8. Bold, H. C., Alexopoulos, C. J., & Delevoryas, T. (1987). Morphology of plants and fungi, pp. 12-13. New York, NY: Harper and Row.

9. Brawner, D. L. & Cutler, J. E. (1986). Ultrastructural and biochemical studies of two dynamically expressed cell surface determinants on Candida albicans. Infection and Immunity 51, 327-336.

10. Burgess, J. (1985). An introduction to plant cell development, pp. 3-7. Cambridge: Cambridge University Press.

10. Burgess, J. (1985). An introduction to plant cell development, pp. 3-7. Cambridge: Cambridge University Press.

11. Carrion, A. L. (1950). Yeastlike dermatiaceous fungi infecting the human skin. Archives of Dermatology Symposium (Chicago) 61, 996-1009.

12. Ceberberg, E. & Neujahr, H. Y. (1970). Distribution of beta-carotene in subcellular fractions of Blakeslea trispora. Experientia 26, 366-367.

13. Conant, N. F., Smith, D. T., Baker, R. D., Callway, J. L., & Martin, S. A. (1954). Manual of clinical mycology 2nd ed. Philadelphia: W. B. Saunders Co.

14. Conjeaud, H. M., Michel-Villaz, M. Vermeglio, A., & Mathis, P. (1976). Location of field-sensitive carotenoid molecules in the chloroplast membrane. Arguments from low-temperature studies. FEBS Letters 71, 138-141.

15. Day, A. W., Poon, N. H., & Stewart, G. C. (1975). Fungal fimbriae. III. The effect on flocculation in Saccharomyces. Canadian Journal of Microbiology 21, 558-566.

16. Davis, B. H. (1973). Carotene biosynthesis in fungi. Pure and Applied Chemistry 35, 1-28.

17. Dawes, C. J. (1979). Biological techniques for transmission and scanning electron microscopy, pp. 104-105. Burlington, VT: Ladd Research Industries, Inc.

18. DeVries, O. M. & Wessels, J. G. H. (1973). Release of protoplasts from Schizophyllum commune by combined action of purified alpha-1,3-glucanase and chitinase derived from Trichoderma viride. Journal of General Microbiology 76, 319-330.

19. Dixon, D. M., Polak, A., & Szansizlo, P. J. (1987). Pathogenicity and virulence of wild-type and melanin-deficient Wangiella dermatitidis. Journal of Medical and Veterinary Mycology 25, 97-106.

20. Doner, J. E. (1985). Blastomyces dermatitidis. In Fungal dimorphism with emphasis on fungi pathogenic for humans pp 51-67. Edited by P. J. Szansizlo. New York: Plenum.

21. Djaczenko, W. & Cassone, A. (1971). Visualization of new ultrastructural components in the cell wall of Candida albicans with fixatives containing TAPO. Journal of Cell Biology 52, 186-190.

22. Edwards, M. R., Gordon, M. A., Lapa, E. W., & Ghiorse, W. C. (1967). Micromorphology of Cryptococcus neoformis. Journal of Bacteriology 94, 766-777.

23. Elorza, M. V., Marcilla, A., & Sentandreu, R. (1988). Wall mannoproteins of the yeast and mycelial cells of Candida albicans: nature of the glycosidic bonds and polydiversity of their mannan moieties. Journal of General Microbiology 134, 2393-2403.

24. Farkas, V. (1979). Biosynthesis of cell walls of fungi. Microbiological Reviews 45, 117-144.

25. Foster, L. A. (1985). The intracellular location of carotenoid pigments in the yeast-phase of Wangiella dermatitidis. Masters thesis. Denton, TX: North Texas State University.

26. Geis, P. A. & Jacobs, C. W. (1985). Polymorphism of Wangiella dermatitidis. In Fungal dimorphism, pp 205-233. Edited by P. J. Szansizlo. New York, NY: Plenum.

27. Geis, P. A. & Szansizlo, P. J. (1984). Carotenoid pigments of the dermatiaceous fungus Wangiella dermatitidis. Mycologia 76, 268-273.

28. Gribanvshi-Sassu, O. & Foppen, F. H. (1969). The carotenoids of the fungus Epicoccum nigrum (Link). Phytochemistry 5, 907-999.

29. Guillot, J., Breton, A., Damez,M., Dusser, M., Gaillard-Martinie, B., & Millet, L. (1990). Use of lectins for a comparative study of the cell wall composition of different anaerobic rumen fungal strains. FEMS Microbiology Letters 67, 151-156.

30. Kidby, D. K. & Davis, R. (1970). Invertase and disulphide bridges in the yeast cell wall. Journal of General Microbiology 61, 327-333.

31. Hagsblom, P. & Unestam, T. (1977). Blue light inhibits mycotoxin production and increases total lipids and pigmentation in Alternaria alternate. Applied and Enviromental Microbiology 38, 1074-1077.

32. Hamada, T., Noda, F., & Hayashi, K. (1984). Structure of the cell wall and extracellular mannans from Saccharomyces rouxii and their relationship to a high concentration of NaCl in the growth medium. Applied and Enviromental Microbiology 48, 708-712.

33. Hardman, A. R. & Suzaki, E. (1990). Glycoconjugates on the surface of spores of the pathogenic fungus Phytophtora cinnamonis studied using fluorescence and electron microscopy and flow cytometry. Canadian Journal of Microbiolgy 36, 183-192.

34. Harris & Szansizlo (1986). Localization of chitin in walls of Wangiella dermatitidis using colloidal gold-labeled chitinase. Mycologia 78, 853-857.

35. Hasilik, A & Tanner, W. (1978). Carbohydrate moiety of carboxypeptidase Y and perturbations of its biosynthesis. European Journal of Biochemistry 91, 567-575.

36. Hunsley, D. & Burnett, J. H. (1970). The ultrastructural architechture of the walls of some fungi. Journal of General Microbiology 62, 203-218.

37. Hsu, W. J. & Yokoyama, H. (1972). Carotenoid biosynthesis in Blakeslea trispora. Phytochemistry 11, 2985-2990.

38. IUPAC commission on the nomenclature of organic chemistry and IUPAC-IUB commission on biochemical nomenclature: Tenative rules for the nomenclature of carotenoids. (1971). Biochemistry 10, 4827-4837.

39. Itschik, H. & Reichenbach, H. (1978). Intracellular location of flexirubins in Flexibacter elegans (Cytophagales). Biochimica et Biophysica Acta 510, 1-10.

40. Kano, K. (1934). A new pathogenic Hormiscium (Kunzo) causing chromoblastomycosis. Aichi Igakkai Zasshi 41, 1657-1673 (in Jap.).

41. Keegstra, K., Talmadge, K. W., Bauer, W. D., & Albershein, P. (1973). The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnection of the macromolecular components. Plant Physiology 51, 188-196.

42. Klimpel, K. R. & Goldman, E. (1988). Cell walls from avirulent variants of Histoplasma capsulatum lack alpha-(1->3)-glucan. Infection and Immunity 56, 2997-3000.

43. Krinsky, N. I. (1971). Function. In Carotenoids, pp. 703-705. Edited by Otto Isler. Stuttgart:.Birkhouser Verlag Basel.

44. Lagunas, R., DeJuan, C., & Benito, B. (1986). Inhibition of biosynthesis of Saccharomyces cerevisiae sugar transport by tunicamycin. Journal of Bacteriology 168, 1484-1486.

45. Manocha, M. S. & Chen, Y. (1990). Specificity of attachment of fungal parasites to their hosts. Canadian Journal of Microbiology 36, 69-76.

46. Maresca, B. & Kobayashi, G. (1989). Histoplasm capsulatum: a model for study of cell differentiation in pathogenic fungi. Microbiological Reviews 53, 186-209.

47. Marriott, M. S. (1977). Mannon-protein location and biosynthesis in plasma membrane from the yeast form of Candida albicans. Journal of General Microbiology 103, 51-59.

48. Matsumoto, T., Padhye, A. A., Apello, L., Standard, P. G., & McGinnis, M. R. (1984). Critical review of human isolates of Wangiella dermatitidis. Mycologia 76, 232-249.

49. Matthews, M. M. & Sistrom W. R. (1959). The function of carotenoid pigments of Sarcina lutea. Archives fur Mikrobiologie 35, 139-146.

50. McCam, M. C., Wells, B., & Roberts, K. (1990). Direct visulatization of cross-links in the primary plant cell wall. Journal of Cell Science 96, 323-334.

51. McGinnis, M. R. (1977). Wangiella, a new genus to accommodate Hormiscium dermatitidis. Mycotaxon 5, 353-563.

52. McGinnins, M. R. (1983). Chromomycosis and phaeohyomycosis: new concepts, diagnosis, and mycology. Journal of the American Academy of Dermatology 8, 1-16.

53. Messner, R. & Kubicek, C. P. (1990). Synthesis of cell wall glucan, chitin, and protein by regenerating protoplasts and mycelia of Trichoderma reesi. Canadian Journal of Microbiology 36, 211-217.

54. Michalenko, G. O., Hohl, H. R., & Rast, D. (1976). Chemistry and architecture of the mycelial wall of Agaricus bisporus. Journal of General Microbiology 92, 251-262.

55. Miyata, M., Kanbe, T., & Tanaka, K. (1985). Morphological alterations of the fission yeast Schizosaccharomyces pombe in the pressence of aculeacin A: spherical wall formation. Journal of General Microbiology 131,611-621.

56. Mitzka-Schnabel, U. & Rau, W. (1980). The subcellular distribution of carotenoids in Neurospora crassa. Phytochemistry 19, 1409-1404.

57. Moore-Landecker, E. (1982). Fundamentals of the fungi, pp 4-16. Englewood Cliffs, NJ: Prentice-Hall.

58. Nakajima, T. & Ballou, C. E. (1974). Structure of the linkage region between the polysaccharide and protein parts of Saccharomyces cerevisiae. Journal of Biological Chemistry 249,7685-7694.

59. Nishimura, K. & Miyaji, M. (1983). Defense mechanism of mice against Exophiala dermatitidis infection. Mycopathologia 81, 9-21.

60. Novick, P. & Schekman, R. (1983). Export of major cell surface protein is blocked in yeast secretory mutants. Journal of Cell Biology 96, 541-547.

61. Osagie, A. U. & Valadon, L. R. G. (1983). Carotenoids associated primarily with lipid globules in Verticillium agaricinum. Microbios Letters 24, 57-63.

62. Oujezdsky, K. B., Grove, S. N., & Szansizlo, P. S. (1973). Morphological and structural changes during the yeast-to-mold conversion of Philaphora dermatitidis. Journal of Bacteriology 113, 468-477.

63. Pastor, F. I., Valentin, E., Herrero, E., & Sentandreu, R. (1984). Structure of the Saccharomyces cerevisiae cell wall mannoproteins released by zymolase and their contribution to wall architecture. Biochimica et Biophysica 802, 292-300.

64. Peberdy, J. (1979). Fungal protoplasts: isolation, reversion and fusion. Annual Review of Microbiology 33, 21-39.

65. Riley, G. J. & Bramley, P. M. (1976). The subcellular distribution of carotenoids in Phycomyces blakesleeanus C115 car-42 mad-107 (-). Biochemistry Biophysica Acta 450, 429-440.

66. Roberts, R. L. & Szaniszlo, P. J. (1978). Temperature-sensitive multicellular mutants of Wangiella dermatitidis. Journal of Bacteriology 135, 622-632.

67. Salton, M. R. S. & Ehtisham-ud-Din, A. F. M. (1965). The localization of cytochromes and carotenoids in isolated bacterial membranes and envelopes. Ault. Journal of Experimental Biological Medical Science 43, 255-274.

68. Schneider, E. F. & Barran, L. R. (1977). Cell wall of Fusarium sulphureum. II. Chemical composition of the conidial and chlamydospore walls. Canadian Journal of Microbiology 23, 763-769.

69. Shematek, E., James, M., Braaty, A., & Cabib, E. (1980). Biosynthesis of the yeast cell wall. I. Preparation and properties of beta-(1->3)-glucan synthase. Journal of Biological Chemistry 255, 888-894.

70. Shematek, E. & Cabib, E. (1980). Biosynthesis of the yeast cell wall. II. Regulation of beta-(1->3)-glucan synthetase by ATP and GTP. Journal of Biological Chemistry 255, 895-902.

71. Sivan, A. & Chet, I. (1989). Degridation of fungal cell walls by lytic enzymes of Trichoderma harzianum. Journal of General Microbiology 135, 675-682.

72. Steensland, H. & Larson, H. (1969). A study of the cell envelope of the Halobacteria. Journal of General Microbiology 55, 235-336.

73. Sullivan, P. A., Yin, C. Y., Molloy, C.,.& Templeton, M. D. (1983). An analysis of the metabolism and cell wall composition of Candida albicans during germ-tube formation. Canadican Journal of Microbiology 29, 1514-1525.

74. Szaniszlo, P. J., Geis, P. A., Jacobs, C. W., Cooper, C. R., & Harris, J. L. (1983). Cell wall changes associated with yeast-to-multicellular form conversion in Wangiella dermatitidis. In Microbiology 1983, pp. 239-244. Edited by D. Schlessinger. Washington, DC: American Society for Microbiology.

75. Szansizlo, P. J., Hseih, P. H., & Marlowe, J. D. (1976). Induction of synchronous growth in the yeast phase of Wangiella dermatitidis. Journal of Bacteriology 141, 981-984.

76. Takashi, H., Nakajima, T., Izaki, K., & Matsuda, K. (1981a). Comparison of mannan structures from the cell-wall mutant Candida sp. M-7002 and its wild type. European Journal of Biochemistry 119, 365-371.

77. Takashi, H., Nakajima, T., & Matsuda, K. (1981b). Comparison of the mannan structure from the cell wall mutant Candida sp M-7002 and its wild type. II. Immunochemical properties of the mannans. European Journal of Biochemistry 119, 373-379.

78. Thirkell, D. & Hunter, M. I. T. (1969). Carotenoid-glycoprotein of Sarcina flava membrane. Journal of General Microbiology 58, 289-292.

79. Thommen, I. (1971). Metabolism. In Carotenoids, p. 663. Edited by Otto Isler. Stuttgart: Verlag Basel.

80. Tkacz, J. S. & Lampen, J. O. (1975). Tunicamycin inhibition of polyisoprenyl N-acetylglucosamine pyrophosphate formation in calf-liver microsomes. Biochemical and Biophysical Research Communications 65, 248-257.

81. Torres-Bauza, J. & Riggsby, W. (1980). Protoplast from yeast and mycelial forms of Candida albicans. Journal of General Microbiology 119, 341-349.

82. Van der Valk, P., Marchant, T., & Wessels, J. G. H. (1977). Localization of chitin synthase acetivity in subcellular fractions of Schizophyllum commune protoplasts. Journal of General Microbiology 114, 87-97.

83. Work, E. & Griffiths, H. (1968). Morphology and chemistry of the cell walls of Micrococcus radiodurans. Journal of Bacteriology 95, 641-657.

84. Yamaguchi, H., Hiratari, T., Iwatat, K., & Yamamoto, Y. (1982). Studies on the mechanism of antifungal action of aculeacin A. Journal of Antibiotics 35, 210-219.

85. Yamamoto, T., Hiratani. T., Hirata, H., Imai, M., & Yamaguchi, H. (1986). Killer toxin from Hansenula mrakii selectively inhibits the cell wall synthesis of a sensitive yeast. FEBS Letters 197, 50-54.

86. Yu, R. J., Bishop, C. T., Cooper, F. P., Hasenclever, H. F., & Blank, F. (1967). Structural studies of mannan from Candida albicans (Serotypes A and B), Candida parpsilosis, Candida stellatoidae, and Candida tropicalis. Canadian Journal of Chemistry 48, 2205-2211.

87. Zagalsky, P. F. (1978). Carotenoid-protein complexes. Pure and Applied Chemistry 47, 103-112.