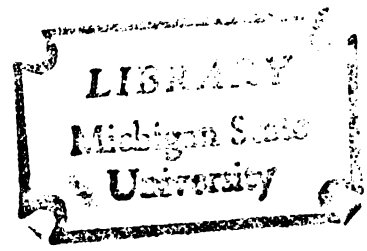
THE ULTRASTRUCTURE OF TORULOPSIS GLABRATA AND MORPHOLOGICAL CHANGES INDUCED BY AMPHOTERICIN B

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
DAVID A. DUPREY
1976



ABSTRACT

THE ULTRASTRUCTURE OF *TORULOPSIS GLABRATA*
AND MORPHOLOGICAL CHANGES INDUCED
BY AMPHOTERICIN B

By

David A. Duprey

The ultrastructure of *Torulopsis glabrata* and the cytological effects of amphotericin B, as seen in ultrathin sections, are described and illustrated with electron micrographs. Several fixatives and fixation techniques were employed. However, only a combination of glutaraldehyde and osmium tetroxide provided sufficient cytological detail for *Torulopsis glabrata*.

The ultrastructure was similar to that reported by authors who have studied other yeasts. A relatively thick cell wall [180 nanometers (nm)] and a double-layered cytoplasmic membrane were consistent features. Mesosomal-like appendages were frequently extended into the cytoplasm. Ribosomes, vacuoles, storage granules, mitochondria, and endoplasmic reticulum were also present within the cytoplasm.

The cytological effects of amphotericin B on *Torulopsis glabrata* were quite striking. The most dramatic effect of the drug was an

alteration of the cytoplasmic membrane. Following 48 hours of exposure to amphotericin B, the cytoplasmic membrane had separated from the cell wall but appeared intact. Cytoplasmic density was reduced and was attributed to a leakage of intracytoplasmic material. Since decreased electron scattering can only be explained by a reduction in cell density, it follows then that such a decrease in density can occur only through leakage of material across a permeable membrane.

THE ULTRASTRUCTURE OF *TORULOPSIS GLABRATA* AND MORPHOLOGICAL CHANGES INDUCED BY AMPHOTERICIN B

By

David A. Duprey

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1976

Dedicated to

my beloved wife,

Christine

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to the following for their role in making the completion of this research and thesis possible.

To Dr. John W. Dyke is extended deepest gratitude for his continuous support, guidance, and enthusiasm throughout this study.

Sincere appreciation is given to Mrs. Martha Thomas for her assistance in the preparation of this manuscript and guidance in the pursuit of the graduate degree.

Indebtedness is recognized to Dr. Stuart Sleight for his efforts and suggestions in the preparation of this thesis.

I am especially grateful to Dr. G. R. Hooper for providing the facilities of his electron optics laboratory and for giving some of his valuable time to the efforts of this research.

Sincere thanks are given to Mrs. June P. Mack for her assistance with electron microscopic techniques and to Mr. Richard Slocum and Mrs. Margaret Haynes for their assistance with special photographic procedures.

I am also deeply indebted to E. W. Sparrow Hospital and Dr. W. E. Maldonado for allowing me the opportunity to pursue this graduate program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

This research was undertaken for two reasons: (1) to study the ultrastructure of *Torulopsis glabrata*, and (2) to demonstrate the ultrastructural effects of amphotericin B on *Torulopsis glabrata* after a period of exposure to the drug. A similar study was done by Gale (1963) in which he illustrated the cytological effects of amphotericin B on *Candida albicans*.

Torulopsis glabrata was chosen as the organism for this study for several reasons. Recently, the medical profession has recognized an increase in the frequency of infections involving *Torulopsis glabrata*, particularly in urinary tract infections and in septicemias. To this author's knowledge, there have been no published reports on the ultrastructure of *Torulopsis glabrata*. Therefore, this was an opportunity to study the ultrastructure in an effort to provide new information about the organism that might be useful in understanding its pathogenicity.

Amphotericin B was selected as the antifungal agent primarily because of its widespread usage in systemic fungal infections. Since the mechanism of action of amphotericin B is known, certain cytological effects could be anticipated. Standard electron microscopic techniques would demonstrate these effects on *Torulopsis glabrata* after sufficient exposure to the drug.

In summary, this research was designed to supply basic information on the ultrastructure of *Torulopsis glabrata* that might be significant in understanding its pathogenic role in infections. In addition, previous reports on the cytological effects of amphotericin B would be confirmed utilizing standard electron microscopic procedures.

OBJECTIVES

The objectives of this research were:

1. To demonstrate, as accurately as possible, the ultrastructure of *Torulopsis glabrata*.

2. To determine the minimal inhibitory concentration (MIC) value for the strain of *Torulopsis glabrata* to be used in this research.

3. To demonstrate the ultrastructural effects of amphotericin B on *Torulopsis glabrata* after a period of exposure to the drug.

LITERATURE REVIEW

This literature review will be divided into two parts: (1) *Torulopsis glabrata* - history and classification, characteristics, epidemiology, and antifungal susceptibility; (2) amphotericin B - origin, chemical and physical properties, pharmacodynamics, toxicity and side effects, and clinical efficacy and precautions for drug use.

Torulopsis glabrata

History and Classification

Torulopsis glabrata was first isolated from human feces. It was originally named *Cryptococcus glabrata* (Anderson, 1917) based on its morphological appearance on solid culture media. The organism was later classified under the genus *Torulopsis* (Lodder and DeVries, 1938).

Torulopsis glabrata is widely distributed in nature. It has been isolated from cattle, horses, swine, and goats (Van Uden *et al.*, 1958; Van Uden, 1960). Cooke (1961) discovered the organism in the soil, while Hasenclever and Mitchell (1962) recovered it from creeks, ponds, and various other water sources. More recently, Kocan and Hasenclever (1972) found *Torulopsis glabrata* in pigeons and doves. White *et al.* (1972) isolated the organism from rats and lambs.

Torulopsis glabrata is currently classified under the family *Cryptococcaceae*, which includes both mycelial and non-mycelial forms of asporogenous yeasts. It reproduces by budding but does not produce septate hyphae or ascospores. This lack of ascospore formation separates *Torulopsis glabrata* from the true yeasts, such as *Saccharomyces cerevisiae* (Grimley, 1965).

Characteristics

On Sabouraud's dextrose agar (Figure 1), the colonies of *Torulopsis glabrata* are raised, glabrous, glistening, cream-colored and homogeneous in appearance (Grimley, 1965). Marks and O'Toole (1970) described the colonial morphology of the organism on blood agar as tiny, white, raised and non-hemolytic, remaining small despite prolonged incubation. Growth in liquid media lacks a pellicle or ring (Lodder, 1952) that is frequently formed with cultures of certain *Candida* species.

Microscopically (Figure 2), *Torulopsis glabrata* is regularly ovoid or elliptical generally measuring 3 x 4-5 microns (Lodder, 1952). It reproduces by budding. These buds are frequent and usually single, but occasionally are found dipolar (Grimley, 1965). In contrast to *Cryptococcus neoformans*, *Torulopsis glabrata* lacks a thick capsule (Grimley, 1965; Marks and O'Toole, 1970).

Torulopsis glabrata is almost biochemically inert (see Appendix C). It ferments only dextrose and trehalose with the production of acid and gas. In addition, it can assimilate only dextrose and trehalose (Marks and O'Toole, 1970). These biochemical reactions alone are sufficient to differentiate *Torulopsis glabrata*

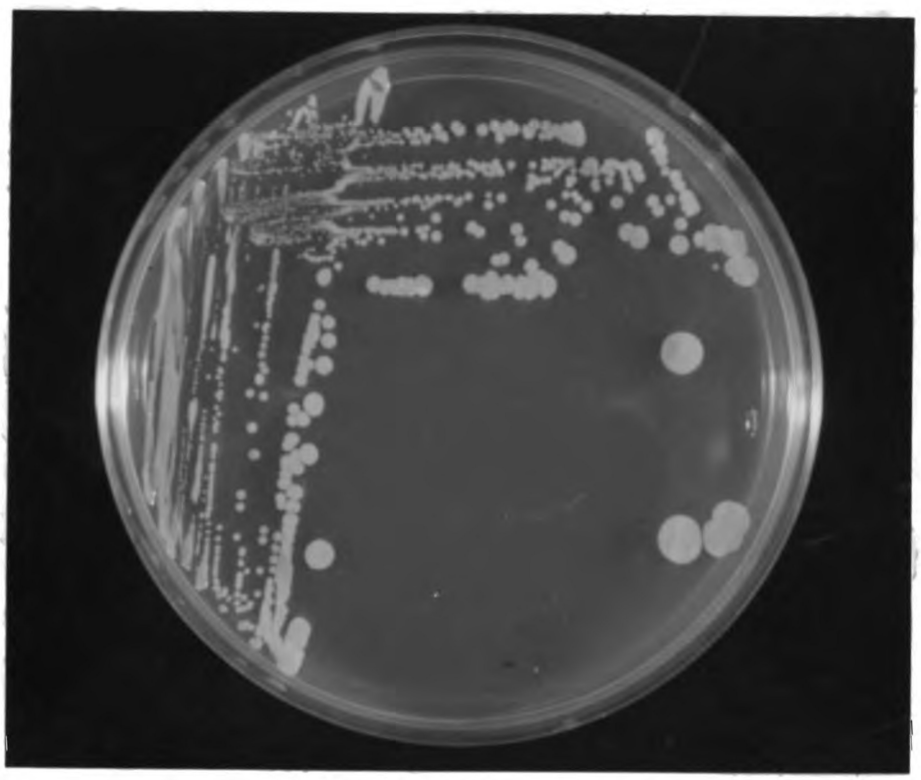


Figure 1. *Torulopsis glabrata* colonies appear as small to medium, cream-colored colonies on Sabouraud's dextrose agar.

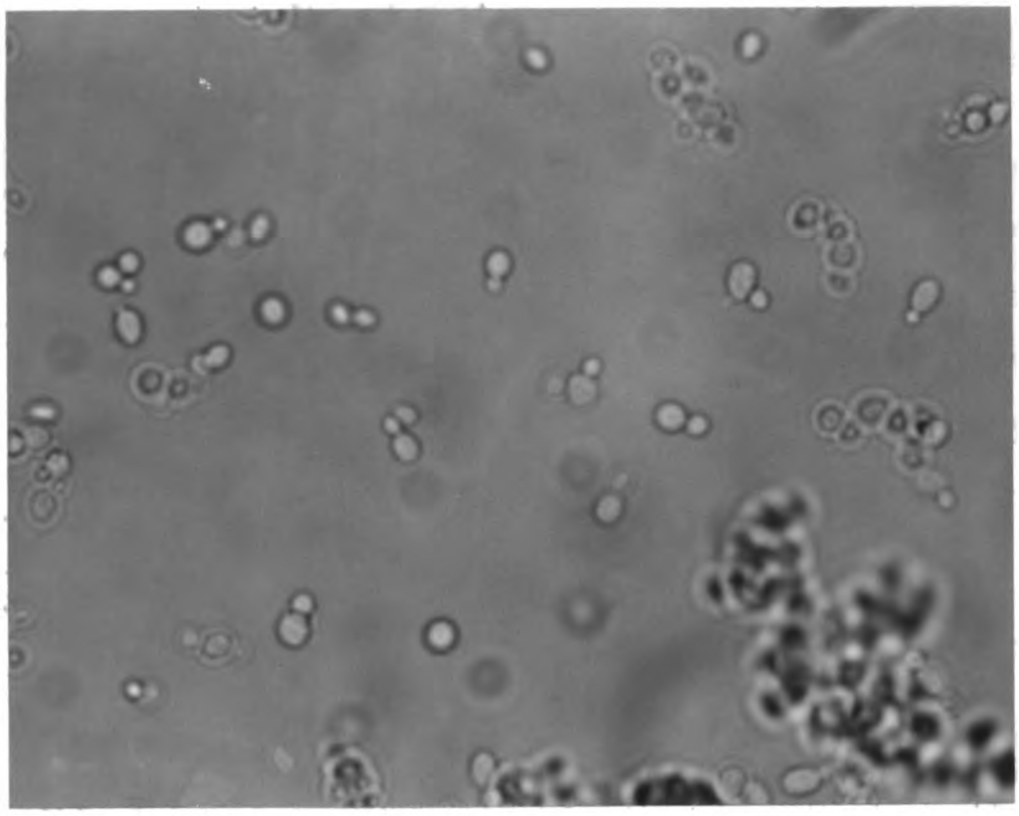


Figure 2. Microscopically, *Torulopsis glabrata* appears ovoid or elliptical measuring 3 x 4-5 microns with occasional buds attached to the parent cell. X500

from other yeasts. The organism also lacks the enzyme urease and the ability to synthesize starch (Marks and O'Toole, 1970).

Torulopsis glabrata can be identified serologically as well as biochemically. Benham (1935) demonstrated that *Candida albicans* and *Torulopsis glabrata* possessed common antigens. In addition, Hasenclever and Mitchell (1960) showed that the organism had its own specific antigen. Tsuchiya *et al.* (1961) classified *Torulopsis glabrata* serologically and observed that it possessed 5 thermostable antigens and 1 thermolabile antigen. The organism shared 4 of the 5 thermostable antigens with other yeasts in its class. It is the only member in its group, however, to possess a thermolabile antigen which Tsuchiya designated as the k antigen.

Epidemiology

Torulopsis glabrata, frequently isolated from human material, is ordinarily considered a non-pathogen (Pankey and Deloviso, 1973). Stenderup (1962) reported on its frequency of isolation from human sources and found it to be the second most commonly isolated yeast. These observations were in agreement with those of Barthe and Barthe (1973), who found *Torulopsis glabrata* ranked second in isolation frequency to that of *Candida albicans*. The order of frequency of isolation for the organism was as follows: urine, sputum, vagina, stool, gastric lavage, throat, mouth and skin. Dolan (1971) reported *Torulopsis glabrata* the most frequent isolate from the urine and vagina, respectively, when compared with *Candida albicans* and other yeasts from these sources (see Appendix B).

Several authors have studied the pathogenic role of *Torulopsis glabrata*. Hasenclever and Mitchell (1960) demonstrated that the yeast would not produce a progressive infection in normal mice, but multiplication of the yeast was observed in steroid-treated and diabetic mice.

Marks *et al.* (1970) indicated that *Torulopsis glabrata* is an opportunistic pathogen in the debilitated host. They isolated the yeast 130 times from 37 patients over a 16 month period. The yeast was considered a pathogen in 11 of the 37 patients. Ten of the 11 patients suffered major underlying illnesses and had received antibiotics, steroids, and other immunosuppressive drugs before or during recovery of the yeast.

The organism was considered pathogenic if one or more of the following criteria was present: (1) tissue invasion that could be demonstrated histologically; (2) *Torulopsis glabrata* fungemia present on two or more consecutive days; (3) repeated isolation of the organism from multiple sources; or (4) persistent cultivation of the yeast from catheterized urine specimens.

Torulopsis glabrata has been reported as the causative agent of a number of clinical disease states. It is commonly associated with urinary tract infections (Edebo and Spetz, 1965; Guze and Haley, 1958). It was considered the causative agent in two cases of bronchopneumonia (Black and Fisher, 1937; Oldfield, 1968). Plaut (1950) reported on uterine and fallopian tube invasion by *Torulopsis glabrata*.

There are approximately 23 reported cases of *Torulopsis glabrata* fungemia. In almost every case the patient suffered underlying

disease or had received antibiotics, steroids and other immunosuppressive drugs, or radiation therapy prior to onset of the disease. The most common clinical manifestations of *Torulopsis glabrata* fungemia are fever and hypotension resembling bacterial endotoxic shock (Pankey and Daloviso, 1973). This suggests that an endotoxin-like substance is produced by the yeast; however, there is nothing in the literature to support this hypothesis.

Torulopsis glabrata has been implicated in other disease conditions. Lees *et al.* (1971) reported a case of *Torulopsis glabrata* endocarditis. In 1963, Minkowitz *et al.* reported a case of meningoencephalitis caused by this organism. There are several reports of *Torulopsis glabrata* being associated with contaminated intravenous catheters (Rose and Heckman, 1970; Marks *et al.*, 1970; Pankey and Deloviso, 1973). These are but a few of the many reports of torulopsosis in humans.

Antifungal Susceptibility

Within the last 20 years, there has been a significant increase in the frequency of infections with *Torulopsis glabrata* (Wickerham, 1957). This can probably be attributed to the extensive use of antibiotics to treat bacterial infections. Consequently, investigators and clinicians have begun to realize the necessity for testing the susceptibility of *Torulopsis glabrata* to various antifungal agents.

In 1968, Hahn *et al.* reported on the successful treatment of a *Torulopsis glabrata* septicemia. The organism was isolated and tested for its susceptibility to amphotericin B. A quantitative method, the

minimal inhibitory concentration (MIC) test, was performed and a MIC value of 0.15 micrograms (mcg) of amphotericin B per milliliter (ml) was reported. The test was done to monitor the drug dosage so that effective serum levels could be attained without causing toxic reactions for the patient.

A similar report was described by Rose and Heckman (1970). Their patient was treated with amphotericin B as in the previous case. A MIC test was performed and a value of 0.10 mcg of amphotericin B per ml was reported. In both cases, *Torulopsis glabrata* was highly sensitive to amphotericin B.

Marks *et al.* (1971) investigated the susceptibility of 35 strains of *Torulopsis glabrata* to amphotericin B, clotrimazole (Bay 5097), and 5-fluorocytosine using a tube dilution technique (MIC test). Clotrimazole was shown to be virtually ineffective at achievable serum levels. Over 90% of the isolates had a MIC value of $\leq$1 mcg of amphotericin B per ml and a minimal fungicidal concentration (MFC) of 2 mcg/ml. 5-Fluorocytosine (5-FC) inhibited over 90% of the strains at a concentration of 0.2 mcg/ml. Three of the test strains required $\geq$7.8 mcg/ml for a "killing" effect. Vandevelde *et al.* (1972) reported similar sensitivity values for 5-FC. Their study showed that the yeast was sensitive to 0.2 mcg to 0.4 mcg of 5-FC per ml. 5-Fluorocytosine (Ancobon, Hoffmann-La Roche)* possesses two important advantages over the use of amphotericin B: (1) less toxicity, and (2) oral administration. A major disadvantage,

---

*Hoffmann-La Roche, Inc., Nutley, N.J.

however, is that development of resistance to 5-FC is common among certain yeasts and other fungi (Shadomy, 1970).

Amphotericin B

Origin and Introduction

Amphotericin B, an antifungal agent, is produced by the actinomycete, *Streptomyces nodosus*. The isolation and initial properties of amphotericin B have been previously described (Vandeputte *et al.*, 1956; Gold *et al.*, 1956). The antibiotic is the drug of choice in the treatment of cryptococcosis, histoplasmosis, coccidioidomycosis, blastomycosis, and candidiasis (Drutz *et al.*, 1968). On the other hand, it has virtually no effect against nocardiosis, actinomycosis, and bacterial infections (Kagan, 1970). Amphotericin B is highly nephrotoxic and therefore must be used with caution (Utz *et al.*, 1964; Butler *et al.*, 1964; Weismann *et al.*, 1966; Butler, 1966).

Chemical and Physical Properties

The chemical properties of amphotericin B are quite complex as they are for most antibiotics. Amphotericin B is an amphoteric, conjugated, polyene antibiotic with the probable empirical formula, $C_{46}H_{73}NO_{20}$ (Dutcher *et al.*, 1957). The presence of both amino and carboxyl groups make the molecule amphoteric, hence the name (Bennett, 1964). It is classified under the polyene group of antibiotics based upon the presence of a macrocyclic, lactone ring of carbon atoms and a series of conjugated double bonds (Hamilton-Miller, 1973). By ultraviolet (UV) absorption a polyene antibiotic, such as amphotericin B, can be subclassified as triene, tetraene,

pentaene, hexaene, or heptaene (Walker and Hawkins, 1952; Crashnik and Mebane, 1963).

Amphotericin B belongs to the subclass, heptaenes. There are three subgroups of heptaenes: (1) the amphotericin B-candidin group; (2) the candimycin group; and (3) the antifungin group (Hamilton-Miller, 1973). One factor which separates the amphotericin B-candidin group from the other groups is the presence of only one atom of nitrogen per molecule (Hamilton-Miller, 1973).

Mechlinski *et al.* (1970) and Ganis *et al.* (1971) described the chemical structure of amphotericin B. The total structure (Figure 3) was determined by X-ray single crystal analysis.

Amphotericin B has some unique physical properties. Kinsky (1967) found that polyene antibiotics, such as amphotericin B, possessed detergent-like properties. This was attributed to the presence of both hydrophilic (polyol) and hydrophobic (polyene) structures within the molecule.

Bennett (1964) observed that amphotericin B was relatively insoluble in water, but that it would dissolve quite readily in a few polar solvents, such as dimethylsulfoxide and dimethylformamide. In 1957, Bartner *et al.* reported that amphotericin B in combination with sodium desoxycholate formed a complex which could be dissolved and dispersed as a colloid in water.

This colloidal form of the drug is marketed today under the trade name, Fungizone Intravenous--Squibb.* The drug is supplied as a lyophilized powder in ampoules that contain 50 milligrams (mg) of

---

*E. R. Squibb, Inc., Princeton, N.J.

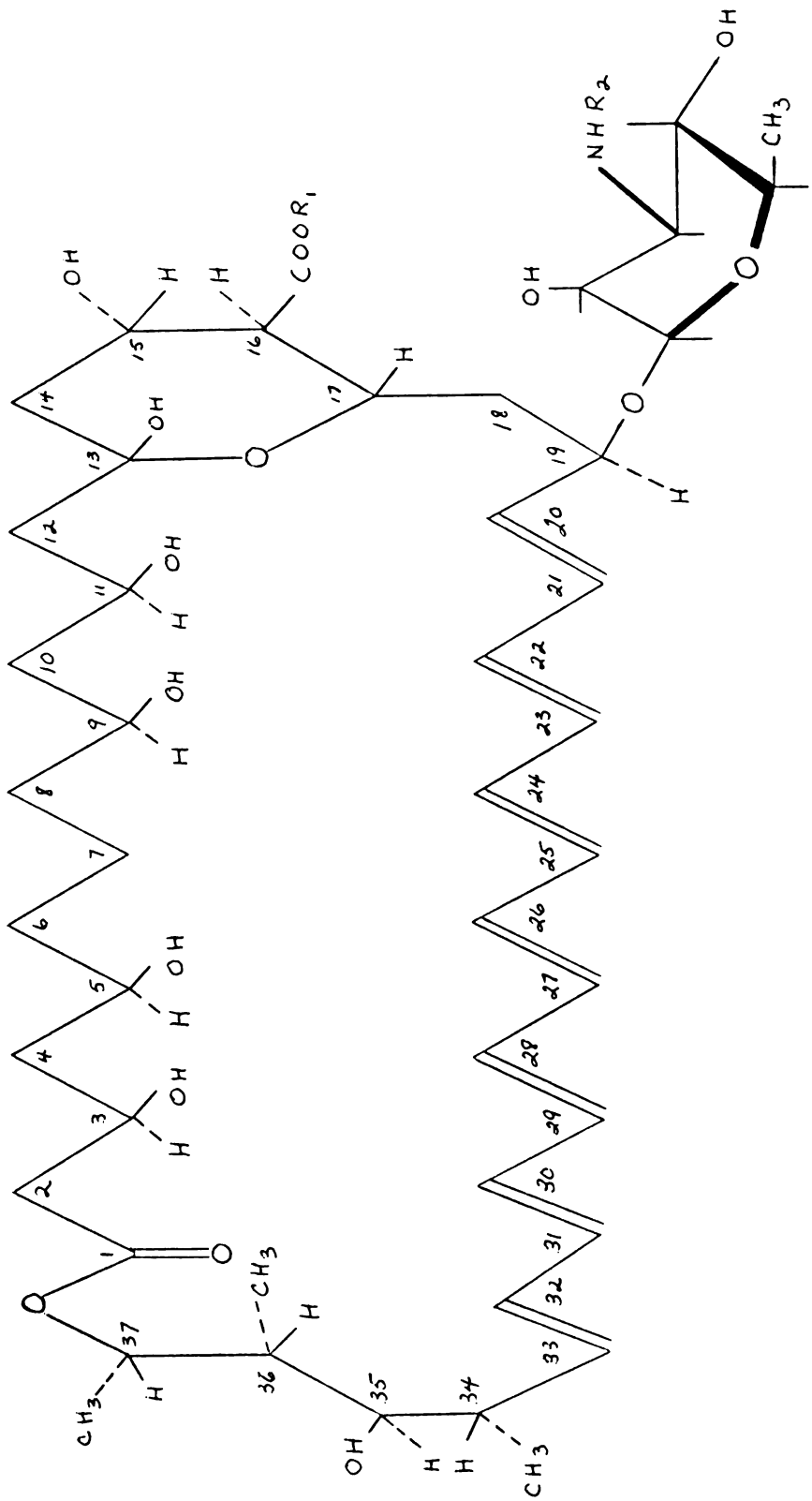


Figure 3. Structure of amphotericin B as determined by X-ray single crystal analysis.

amphotericin B, approximately 41 mg of sodium desoxycholate, and 25.2 mg of sodium phosphate as a buffer.

Another significant property of amphotericin B is its photoreactivity when exposed to light. In the presence of light with wavelengths of 380 and 410 nanometers (nm), it has been demonstrated that the drug becomes partially inactivated and loses some of its potency (Hamilton-Miller, 1973). Therefore, it is recommended that it be stored in the dark at refrigerator temperatures prior to use.

Pharmacodynamics

It has been demonstrated that organisms sensitive to polyene antibiotics, such as amphotericin B, bind to these substances probably by way of sterols in the cell membrane (Lampen *et al.*, 1959; Kinsky, 1962). This binding of the antibiotic with the membrane causes distortion and malfunction of the membrane resulting in leakage of essential metabolites (Sutton *et al.*, 1961; Zygmunt, 1966). Other effects, such as inhibition of glycolysis, growth, respiration, and eventual cell death, are considered sequelae to the initial injury (Hamilton-Miller, 1973).

There appears to be good correlation between the mode of action of the polyenes and the presence of sterols in the cell membrane. Yeasts, algae, protozoa, flatworms, and mammalian cells contain sterols in their cell membranes and all are susceptible to the polyene antibiotics (Weber *et al.*, 1965; Feingold, 1965). Bacteria, which lack membrane-bound sterols, are insensitive to the polyene antibiotics (Kinsky, 1962).

Studies with artificial membrane systems, monitored by changes in conductance and permeability, indicate that the sterol-polyene reaction involves hydrogen bonding with 3 β-OH groups of sterols and is dependent upon the structure of the antibiotic molecule (Cass *et al.*, 1970; Dennis *et al.*, 1970).

Because amphotericin B is not absorbed from the gastrointestinal tract, the drug is administered intravenously by slow infusion (e.g., over a period of 6 hours). The recommended concentration is 0.1 mg/ml of 5% dextrose utilizing an initial dose of 0.25 mg/kg of body weight. Depending upon the patient's tolerance to the drug, the dosage can be adjusted to an average daily dose of 1.0 mg/kg never exceeding 1.5 mg/kg. Once the optimum dosage level is reached, the drug may be administered on alternate days (Kagan, 1970).

Like many other drugs, amphotericin B is unable to penetrate the blood-brain barrier. Therefore, the drug should be administered intrathecally to patients suffering from fungal meningitis. In adults, an initial dose of 0.1 mg is used, which is then increased to 0.5 mg given every 72 hours (Kagan, 1970), depending upon the patient's condition.

Little is known about the distribution of amphotericin B once it leaves the bloodstream. There is some indirect evidence that the drug is taken up by the reticuloendothelial cells (Bennett, 1964).

Only a small portion of amphotericin B is excreted in an active form in the urine. About 40% of a single dose is slowly excreted via the kidneys in a 7 day period. Two months after terminating therapy, its presence can still be detected in the urine (Kagan, 1970).

Toxicity and Side Effects

Intravenous administration of amphotericin B is frequently associated with side effects and can be accompanied by serious toxicosis. Therefore, the drug must be given under the supervision of a hospital nursing staff.

Some side effects commonly encountered with amphotericin B administration are fever, anorexia, nausea, vomiting, headache, abdominal pain, weight loss, thrombophlebitis, and stiffness (Kagan, 1970). The most important toxic effects are nephrotoxicity, some hepatotoxicity, and anemia.

In recent years, there have been attempts to minimize the toxicity of amphotericin B. Nephrotoxicity from the drug has been reduced in laboratory animals by concomitant administration of intravenous mannitol or sodium bicarbonate (Keim *et al.*, 1973; Bonner *et al.*, 1972).

Mechlinski and Schaffner (1972) prepared amphotericin B methyl ester (AME) hydrochloride, a water-soluble derivative of amphotericin B. This derivative has been shown, in experimental animals, to be significantly less toxic than the parent compound (Keim *et al.*, 1973).

Howarth *et al.* (1975) reported that AME activity *in vitro* was slightly lower than the parent compound when tested against 61 isolates of pathogenic and potentially pathogenic fungi.

Bonner *et al.* (1975) reported similar activity *in vivo* for AME as compared with amphotericin B. They observed that the chemotherapeutic activity of AME was slightly lower than that of the parent compound. When they compared ED_{50} values, they discovered that AME was one-fifth as active against *Candida* infection and one-tenth as

effective as amphotericin B against other systemic mycoses. At the same time, they observed that AME was one-tenth as nephrotoxic as the parent compound when used to treat histoplasmosis, blastomycosis, cryptococcosis, and candidiasis in experimental mice.

It is theorized that decreased efficacy of AME is associated with its greater water solubility and diffusibility. In addition, it is apparently unstable in aqueous solutions, particularly at an acid pH, as compared with amphotericin B (Bonner *et al.*, 1975).

Combination therapy, utilizing amphotericin B and a less toxic agent, has been useful in minimizing toxicity due to amphotericin B. Kobayashi *et al.* (1972) showed that rifampin and 5-fluorocytosine could be used in combination with amphotericin B with good results and with minimal toxicity due to amphotericin B. When rifampin is used in combination with low concentrations of amphotericin B, RNA synthesis is inhibited. The amphotericin B attacks the cell membrane allowing the passage of rifampin into the cell to inhibit the RNA synthesis (Medoff and Kobayashi, 1975). Toxicity is reduced with lower concentrations of amphotericin B. Other examples of combination therapy are reported in the literature (Block and Bennett, 1973; Titsworth and Gruneburg, 1973; Huppert *et al.*, 1974; Garriques *et al.*, 1973).

Clinical Efficacy and Precautions for Drug Use*

As previously mentioned, amphotericin B is the drug of choice for most systemic mycoses including histoplasmosis, blastomycosis,

---

*Recommendations of E. R. Squibb & Sons, Inc., Princeton, N.J.

coccidioidomycosis, cryptococcosis, and candidiasis (Drutz *et al.*, 1968). Amphotericin B has little or no effect against bacteria (Kagan, 1970; Kinsky, 1962).

Certain precautions must be exercised when using amphotericin B. The drug is highly effective when prepared and administered under proper supervision. Corticosteroids, anti-neoplastic agents, and other nephrotoxic antibiotics should not be administered simultaneously except with extreme caution.

Renal function tests, blood urea nitrogen (BUN), and creatinine clearance should be done periodically while the patient is receiving amphotericin B therapy. Whenever possible, MIC studies and serum assays should be evaluated to determine the effectiveness of the drug as well as the potential for toxicity to the patient.

MATERIALS AND METHODS

General Plan and Considerations

Initially, *Torulopsis glabrata* was tested for its susceptibility to amphotericin B by using a method known as the minimal inhibitory concentration (MIC) test. Once the susceptibility had been determined, an appropriate drug concentration (e.g., 100X the MIC value) was added to samples of an overnight broth culture in preparation for electron microscopy studies.

Fungal Strain and Culture Conditions

A stock laboratory strain of *Torulopsis glabrata* maintained on Sabouraud's dextrose agar at 37 C was used as the test organism. It was checked periodically for purity by using a battery of biochemicals (see Appendix C).

Preparation of Drug Solutions

Commercial amphotericin B (Fungizone) was reconstituted with 100% dimethylsulfoxide (DMSO) to yield a concentration of 10,000 mcg of amphotericin B per ml. This stock solution was further diluted with Sabouraud's dextrose broth (Difco)* to 2,000 mcg/ml for use in the MIC procedure. A drug concentration of 10 mcg was utilized in the electron microscopy portion of the study.

---

*Difco Laboratories, Detroit, MI.

Minimal Inhibitory Concentration Test

Principle

A quantitative method known as the MIC test is often used to determine *in vitro* susceptibility of an organism to a particular antibiotic. The MIC is defined as the lowest concentration of the drug that inhibits growth of the organism. The drug is serially diluted, a specified concentration of organism is added, and the tubes are incubated at 37 C for 24 to 48 hours. They are examined macroscopically for growth and the MIC value is recorded as the lowest concentration (greatest dilution) showing no growth.

To determine the minimal fungicidal concentration (MFC), or the concentration that "kills" the organism, the tubes are subcultured to a recovery medium and examined for growth. The MFC is recorded as the lowest concentration (greatest dilution) of the drug that caused inhibition of growth upon subculture of the samples.

Procedure

The broth-dilution method of Marks and Eichkoff (1970) was used in this study. Twofold dilutions of the amphotericin B in Sabouraud's dextrose broth were made in sterile test tubes. Concentrations of the drug ranged from 1000 to 0.03 mcg/ml. The final volume in each tube was 1.0 ml. An inoculum of 0.05 ml of a 1:150 dilution of an overnight broth culture of *Torulopsis glabrata* was added to each tube and to a control tube containing only 1.0 ml of Sabouraud's dextrose broth. The test was done in duplicate. All tubes were incubated at 37 C for 24 to 48 hours.

The MIC value was defined as the lowest concentration of amphotericin B causing complete inhibition of growth. The MFC was determined by subculturing 0.05 ml of the solution in all tubes without visible growth onto Sabouraud's dextrose agar. These subculture plates were incubated at 37 C for 24 to 48 hours. The MFC value was defined as the lowest concentration of amphotericin B that caused complete inhibition of growth upon subculture to an appropriate recovery medium.

Transmission Electron Microscopy (TEM)

Principle

The transmission electron microscope (see Appendix D) utilizes electrons instead of light, and contains magnetic fields, not glass lenses to focus electrons through the specimen to form an image on a fluorescent screen. Since it works in a vacuum, its use is limited to dry specimens. After fixation, dehydration, and resin infiltration, the specimen is sectioned, stained and examined under the electron microscope. A beam of electrons directed onto the specimen will be only partly transmitted; the remainder will be reflected. Thus, particles which have taken up the stain appear dark whereas unstained components are transparent.

Procedure

Several colonies of the organism were inoculated into 100 ml of Sabouraud's dextrose broth and incubated for approximately 16 hours at 37 C. One milliliter of a 100 mcg concentration of amphotericin B per ml was added to 9.0 ml samples of the broth culture.

An equivalent concentration of 100% DMSO was added to the control tube. All tubes were wrapped in aluminum foil and incubated at 37 C. At specified time intervals (0, 4, 8, 12, 24 and 48 hours), the specimens were centrifuged, the supernatant discarded, and the pellet fixed in an equal mixture of 6% glutaraldehyde and 2% osmium tetroxide (modification of Trump and Bulger, 1966) in 0.2 M cacodylate buffer (pH 6.8) for 2.5 hours at room temperature. The material was rinsed three times with 0.1 M cacodylate buffer (pH 6.8) and then filtered onto a Seitz filter to obtain a pellet of the organism. The pellet was enrobed in 3% molten agar. The agar was allowed to harden and then cut into approximately 1.0 mm squares.

The cubes were dehydrated through a graded ethanol series (30, 50, 70, 95 and 100%) using 15 minute steps. They remained in the 100% alcohol overnight. An equal mixture of 100% alcohol and propylene oxide was used for 15 minutes. The samples were then transferred to full strength propylene oxide for 30 minutes. This was followed with equal proportions of propylene oxide and Epon-Araldite resin for 1 hour. The specimens were then placed in 100% Epon-Araldite resin for overnight infiltration. The following day, the material was transferred to BEEM capsules containing fresh resin, placed in an oven at 65 C, and allowed to polymerize for 48 to 72 hours.

Thin sections of the material were cut with a diamond knife on a Porter-Blum ultramicrotome.* The sections were placed on naked, 300-mesh grids, stained for 30 minutes with saturated uranyl acetate

---

*Ivan Sorvall, Inc., Norwalk, Conn.

and 5 minutes with saturated lead citrate (modification of Reynolds, 1963). The specimens were examined in a Philips 300 electron microscope* operating with a double condensor at 60 kV.

Other fixation methods (Edwards *et al.*, 1967; Karnovsky, 1965; Kreger Van-Rij and Veenhuis, 1971; Hofsten and Hofsten, 1974) proved to be inadequate for use in the electron microscopy study. Also, a number of fixatives used alone and in combination failed to provide satisfactory results. These included: (1) 3% glutaraldehyde - 3% acrolein, with and without sucrose; (2) 1.5% acrolein - 1% potassium dichromate, with and without sucrose; (3) 2.5% glutaraldehyde - 2% potassium permanganate; (4) 2% potassium permanganate; and (5) 2% osmium tetroxide.

In addition, the organism was exposed to these fixatives for various time periods (e.g., 30 min, 1 hr, 2 hrs, and 3 hrs). All attempts failed to yield adequate structural detail. In nearly every instance, extreme granularity was observed throughout the cell and no internal structures were present when the above methods were utilized. It is hypothesized that the cell wall may be wholly or partly responsible for these inadequacies.

Viability Study

Principle

When an organism is exposed to an antibiotic, the effectiveness of that antibiotic is related to the concentration and time of onset of action of the drug. This effectiveness can be qualitatively

---

*Philips Corp., Holland.

determined by subculturing a portion of the solution to a recovery medium at designated time intervals. After an appropriate incubation time, the plates are examined for colonial growth. The antibiotic is considered ineffective if any colonies are present upon examination of the plates. Absence of colonial growth would indicate sufficient time exposure and/or appropriate drug concentration to effectively "kill" the organism.

Procedure

Samples were withdrawn at specified time intervals, as previously described in the TEM section. A portion of the sample was washed with excess saline, centrifuged, and the supernatant discarded. The pellet was resuspended in saline and inoculated onto Sabouraud's dextrose agar plates. After 48 hours of incubation at 37 C, the plates were examined for colonial growth.

RESULTS AND DISCUSSION

General

In general, the objectives of this research were accomplished. The ultrastructure of *Torulopsis glabrata* possessed similar features to that observed in other yeasts. Examination of the effects of amphotericin B on *Torulopsis glabrata* showed that the drug induced an alteration of the cytoplasmic membrane. These effects were anticipated since it has been demonstrated that organisms sensitive to polyene antibiotics, such as amphotericin B, bind these substances specifically by way of the sterols in the cytoplasmic membrane (Lampen *et al.*, 1959; Kinsky, 1962). This binding results in distortion and malfunction of the membrane with subsequent leakage of cytoplasmic material (Sutton *et al.*, 1961; Zygmunt, 1966).

Minimal Inhibitory Concentration (MIC) for *Torulopsis glabrata*

The MIC for *Torulopsis glabrata* was established for two reasons. Initially, the MIC was performed to compare the results with previously published data. Marks *et al.* (1971) tested 35 strains of *Torulopsis glabrata* by a tube dilution method for susceptibility to amphotericin B, 5-fluorocytosine, and clotrimazole (Bay 5097). Over 90% of the isolates were sensitive to ≤1 mcg of amphotericin B per ml. The strain of *Torulopsis glabrata* used in this research was

sensitive to 0.12 mcg of amphotericin B per ml. This compared favorably with the results reported by Marks *et al*.

However, an unusual phenomenon occurred when the MIC test was performed which deserves mention. A yellow precipitate appeared in the series of tubes which contained from 31 mcg to 1000 mcg of amphotericin B. The size of the precipitate increased as the drug concentration increased. Fortunately, the test organism had a low MIC value which presented no difficulty in determining the end point. Nevertheless, the presence of the precipitate suggested potential problems. For reasons not at all clear, it appeared that the drug was precipitating out of solution.

Bennett (1964) noted that when amphotericin B was dissolved in an organic solvent and the resulting solution was mixed with water at neutral pH, the amphotericin B would generally precipitate unless it was quite dilute. Depending upon the concentration and the manner of mixing, the precipitated particles could be large or small.

Marks and his colleagues (personal communication), however, did not encounter a yellow precipitate like the one previously described. They concurred with Dr. W. B. McDowell of E. R. Squibb, Inc. (personal communication) that partial precipitation of amphotericin B resulted when the drug was diluted.

Therefore, a small experiment was conducted to determine if the precipitate was amphotericin B. A number of the tubes, which contained the yellow precipitate, was centrifuged and the supernatant discarded. A 100% solution of DMSO was added to half of the tubes. The precipitate redissolved immediately. The same results were

obtained when 100% dimethylformamide (DMFM) was added to the remaining tubes. This would seem to indicate that the precipitate was amphotericin B or at least a portion of it. Nevertheless, sufficient active drug was present in the tubes to inhibit growth as, when the MIC tubes were subcultured to determine the MFC value, no growth was observed in the tubes which contained the precipitate. Therefore, the results were considered valid.

The second reason for establishing the MIC value was in order to select a suitable drug concentration for the electron microscopy study. An appropriate drug concentration cannot be selected without some previous knowledge of the degree of sensitivity of the organism to the drug.

Owing to a difference in cell density in the tube dilution assay as compared with the electron microscopy study, the drug concentration was increased from 0.12 mcg to 10 mcg of amphotericin B per ml. This was considered an appropriate concentration to cause demonstrable cytological effects. The adequacy of this concentration was tested according to the viability studies mentioned in the Materials and Methods section. The results indicated that 10 mcg of amphotericin B was sufficient to "kill" the organism with 48 hours of exposure. Exposure for 4, 8, 12, and 24 hours failed to induce fungicidal effects on the organism.

Ultrastructure of *Torulopsis glabrata*

Control (Untreated) Cell

As far as this author knows, there have been no published reports on the ultrastructure of *Torulopsis glabrata*. Consequently,

every effort was made to establish an accurate description of this organism. Several fixation techniques were employed, and it is regretted that most of these did not sufficiently preserve the ultrastructure. Possibly, penetration of the fixatives was inadequate to cause complete fixation. This may be wholly or partly due to the thickness of the cell wall of the yeast.

Nevertheless, a mixture of glutaraldehyde and osmium tetroxide (modification of Trump and Bulger, 1966) proved to be an adequate, if less than ideal, fixative for *Torulopsis glabrata*. The cells of *Torulopsis glabrata* were generally round or oval (Figure 4). Some of the detail of the internal structure resembled that reported in yeasts of other species (Agar and Douglas, 1957; Edwards *et al.*, 1959; Robinow and Marak, 1966; Edwards and Edwards, 1960). For clarity, observations and descriptions of the cell components will be presented separately.

Cell Wall. It measures approximately 180 nanometers (nm) in thickness (Figure 5). As previously mentioned, *Torulopsis glabrata* reproduces by budding. Initially, a bulging and thickening of the cell wall occurs, which eventually results in the formation of a bud or daughter cell (Figure 6). Once the daughter cell has separated from the parent, a "bud scar" remains on the surface of the parent's cell wall (Figure 6).

Frequently, mitochondria and other organelles were observed at the budding site of the parent cell (Figure 7). Kreger-Van Rij and Veenhuis (1971) made similar observations with *Rhodotorula glutinis*, other ascomycetous yeasts, and certain basidiomycetous yeasts.

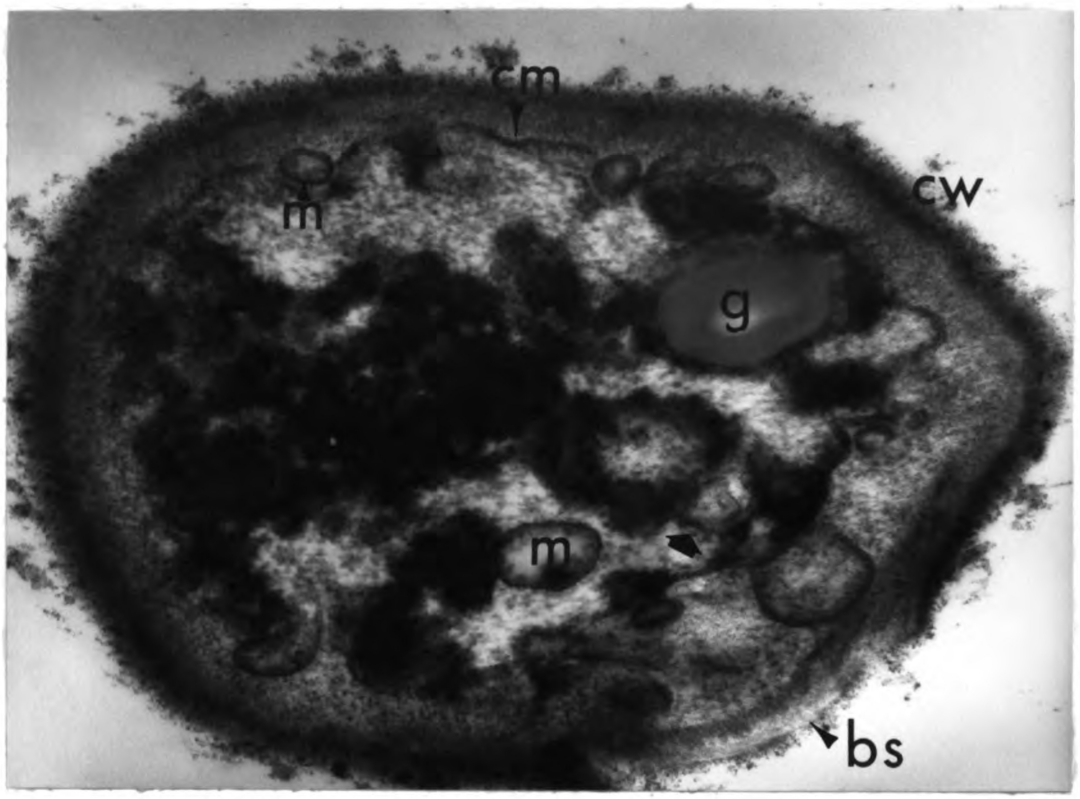


Figure 4. An untreated (control) cell of *Torulopsis glabrata* showing thick cell wall (cw), cytoplasmic membrane (cm), mitochondria (m), storage granule (g), bud scar (bs), and occasional tubules of endoplasmic reticulum (arrow). The nucleus is obscured by the large clump of dark, granular material. These clumps may represent nuclear material; however, no nuclear membrane can be seen. X60,000.

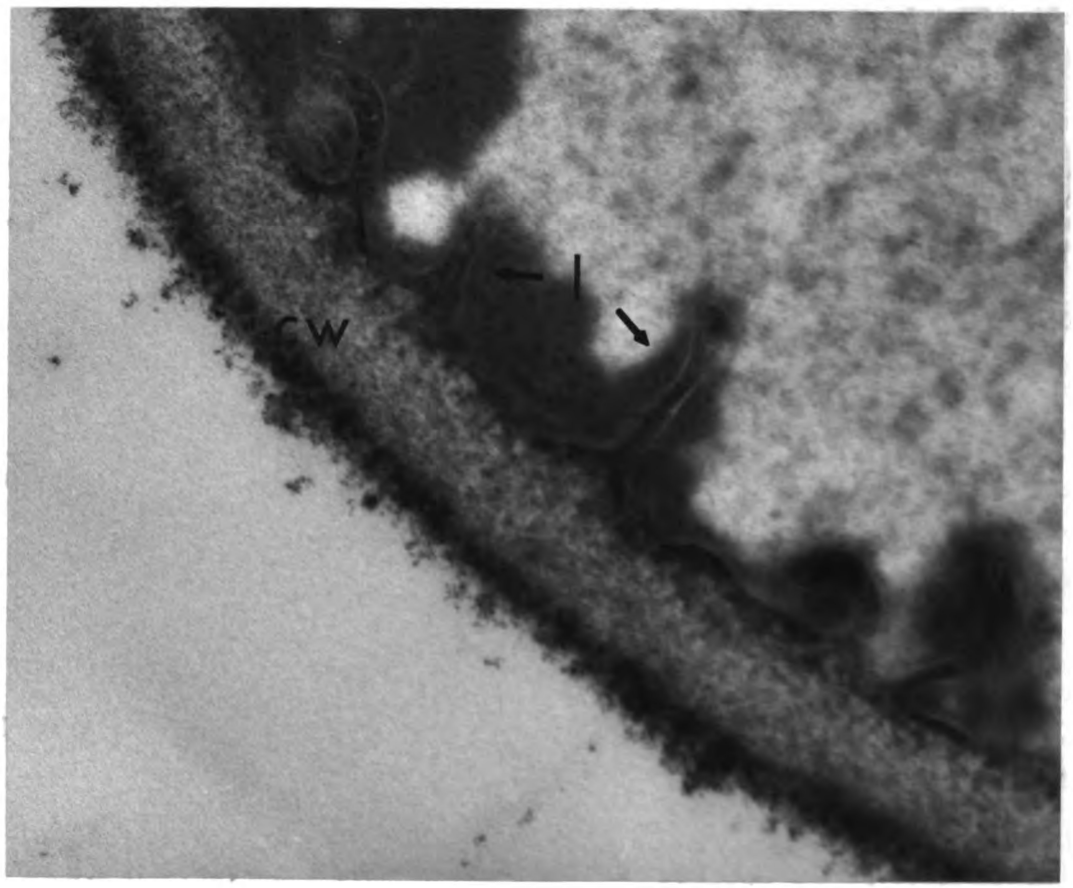


Figure 5. The cell wall is thick and measures approximately 180 nanometers in diameter. Invaginations, called lomasomes (l), are often extended into the cytoplasm from the inner surface of the cell wall. X112,500.

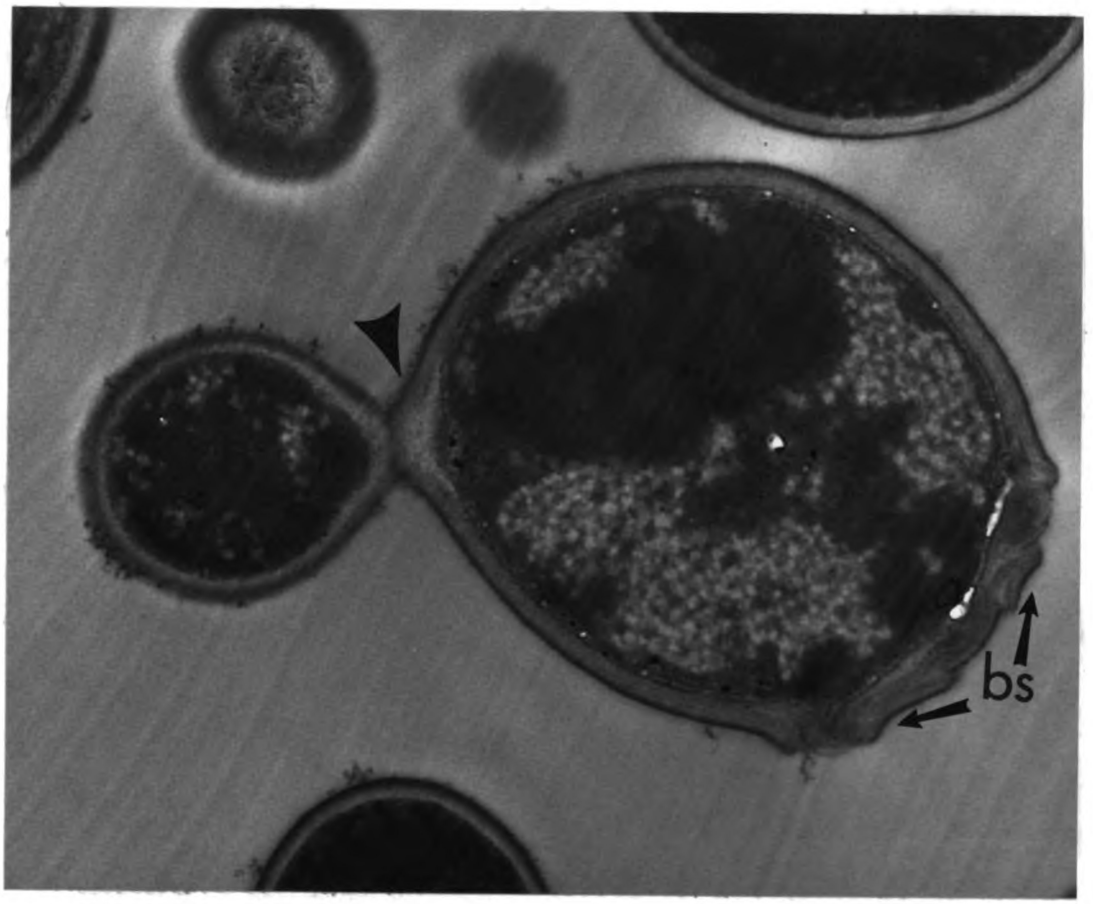


Figure 6. A parent cell of *Torulopsis glabrata* showing an attached bud or daughter cell (b). Note the thickened area at the place of attachment (large arrow). Also present are two bud scars (bs) which have formed following separation of other daughter cells. Internal detail is indiscernible. Holes (small arrow) represent artifacts of dehydration. X32,000.

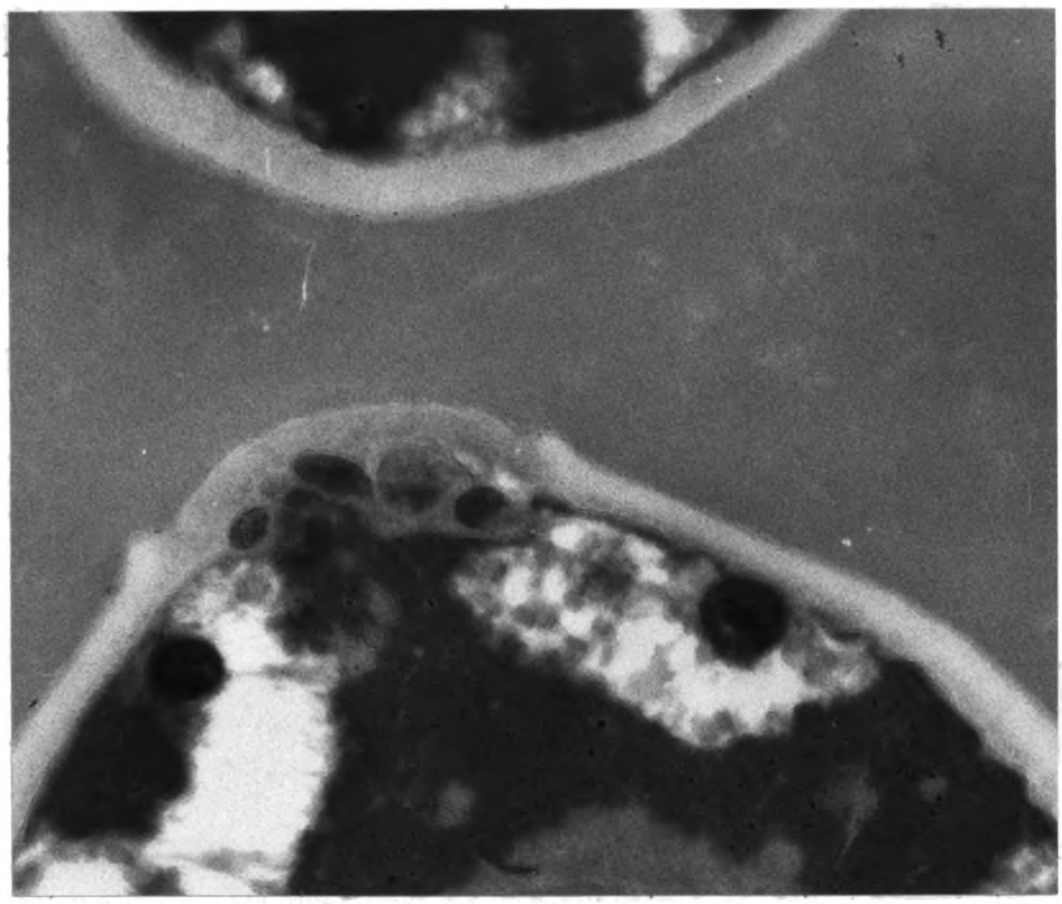


Figure 7. In early budding, mitochondria and other organelles can be seen near the bud area. These structures generally follow the nucleus to this area, but occasionally precede it prior to formation of the daughter cell. X56,250.

Capsule. Based upon examination of a number of electron micrographs, *Torulopsis glabrata* did not appear to have a capsule surrounding the cell wall. This observation was consistent and in agreement with earlier light microscopic findings (Marks and O'Toole, 1969; Grimley *et al.*, 1965).

Cytoplasmic Membrane. The cytoplasmic membrane appears to consist of two dark outer layers and a central lighter one (Figure 8). For years the membrane was believed to consist of a central layer of phospholipid molecules, and outer and inner layers of protein. Robertson (1962) showed that the reactions of the inner and outer layers to different fixatives are not identical. It is suggested that the inner layer may consist of protein and the outer layer of carbohydrate. Manton (1961) described the membrane as being really tripartite and suggested the use of the term "plasmalemma" rather than membrane.

This membrane or plasmalemma was an undulating structure appressed to the cell wall over most of its surface. At certain points along the membrane, invaginations could be seen. Moore and McAlear (1961) referred to these structures as "lomasomes" and considered them unique to the fungi (Figure 5). The function and significance of these structures remains obscure. Similar structures, called mesosomes, are formed by bacterial membranes.

Cytoplasm. The cytoplasmic matrix contained most of the organelles seen in other yeasts. Ribosomes were infrequently observed throughout the cytoplasm. When they were present, the ribosomes appeared as small, dark, irregularly shaped particles. Generally, they are seen

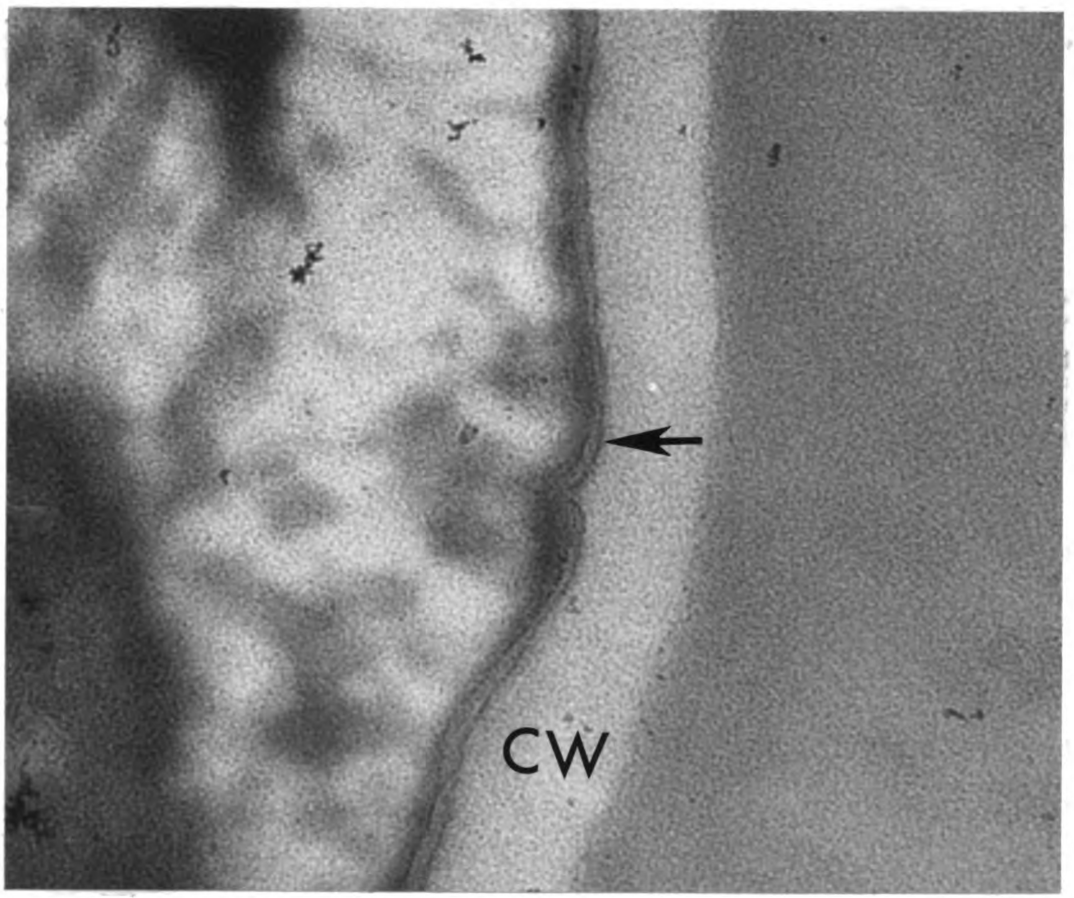


Figure 8. The cytoplasmic membrane, consisting of two dark outer layers and a lighter central one, is firmly attached to the inner surface of the cell wall. X160,000.

more often in younger than in older populations of cells. Ribosomes may also vary in size depending upon the nutritional status of the cell (Hawker, 1965). Vacuoles were usually bounded by a membrane and often looked homogeneous in the electron micrographs (Figure 9). Endoplasmic reticulum was apparent in many of the cells; however, continuity of these structures was indistinguishable. Structures resembling lipid or storage granules appeared as spherical, globose objects (Figure 10).

Mitochondria of various sizes and shapes were interspersed throughout the cytoplasm (Figure 11). The characteristic cristae pattern was lacking due to the presence of dense mitochondrial matrix. In general, there are fewer cristae patterns in fungal mitochondria than in those of higher organisms and they tend to be less rigidly arranged (Hawker, 1965).

Golgi bodies were not observed in any of the preparations. They are considered to be normally present in plant cells, but have rarely been identified in most yeasts and other fungi (Hawker, 1965).

Nuclear Membrane and Nucleus. The nucleus was surrounded by a nuclear membrane that appeared to be double-layered (Figure 12). Nuclear pores and cisternae, which are frequently observed in other yeasts (Robinow and Marak, 1966), were not visible. This might be the result of incomplete fixation. Also, continuity of the nuclear membrane (McAlear and Edwards, 1959) with membranes of the endoplasmic reticulum was not demonstrated with this technique.

The nucleus of *Torulopsis glabrata* was usually centrally located within the cell and there was no irregularity in its size and shape

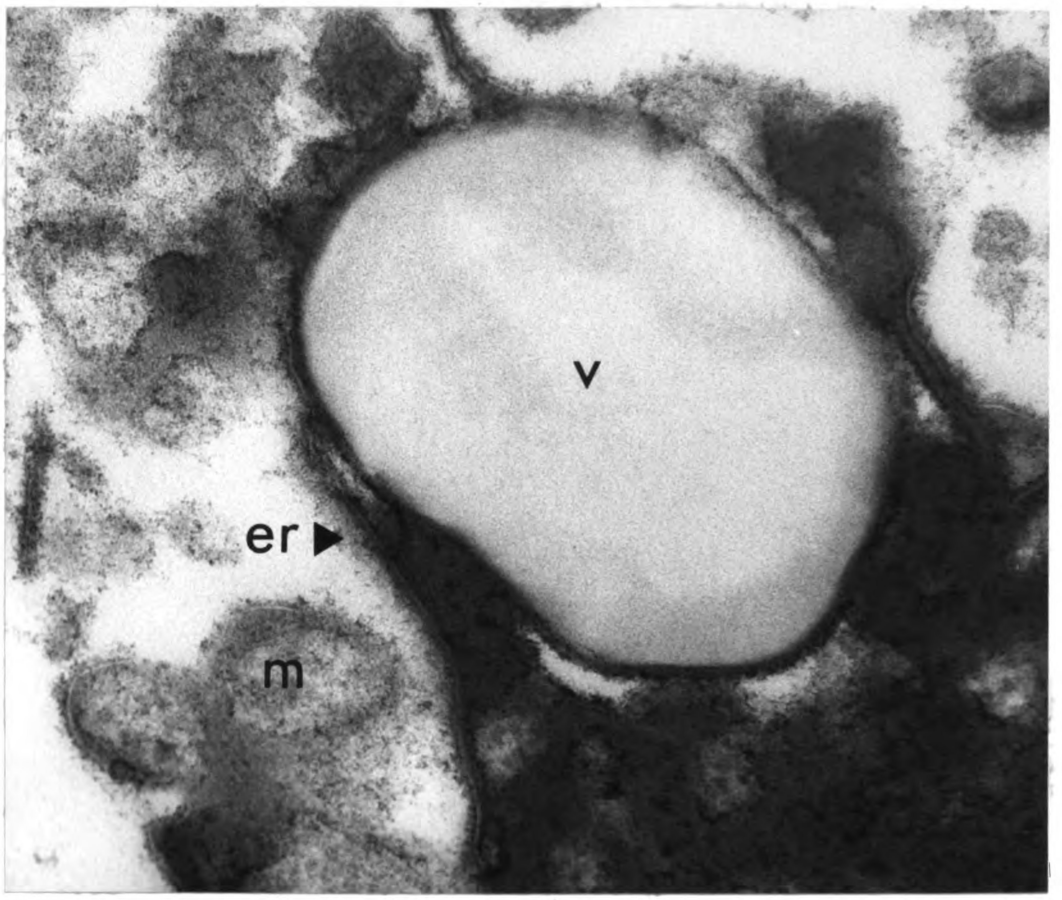


Figure 9. Vacuoles (v) were occasional features of the cytoplasm. They were usually bounded by a membrane and often looked homogeneous. X144,000.

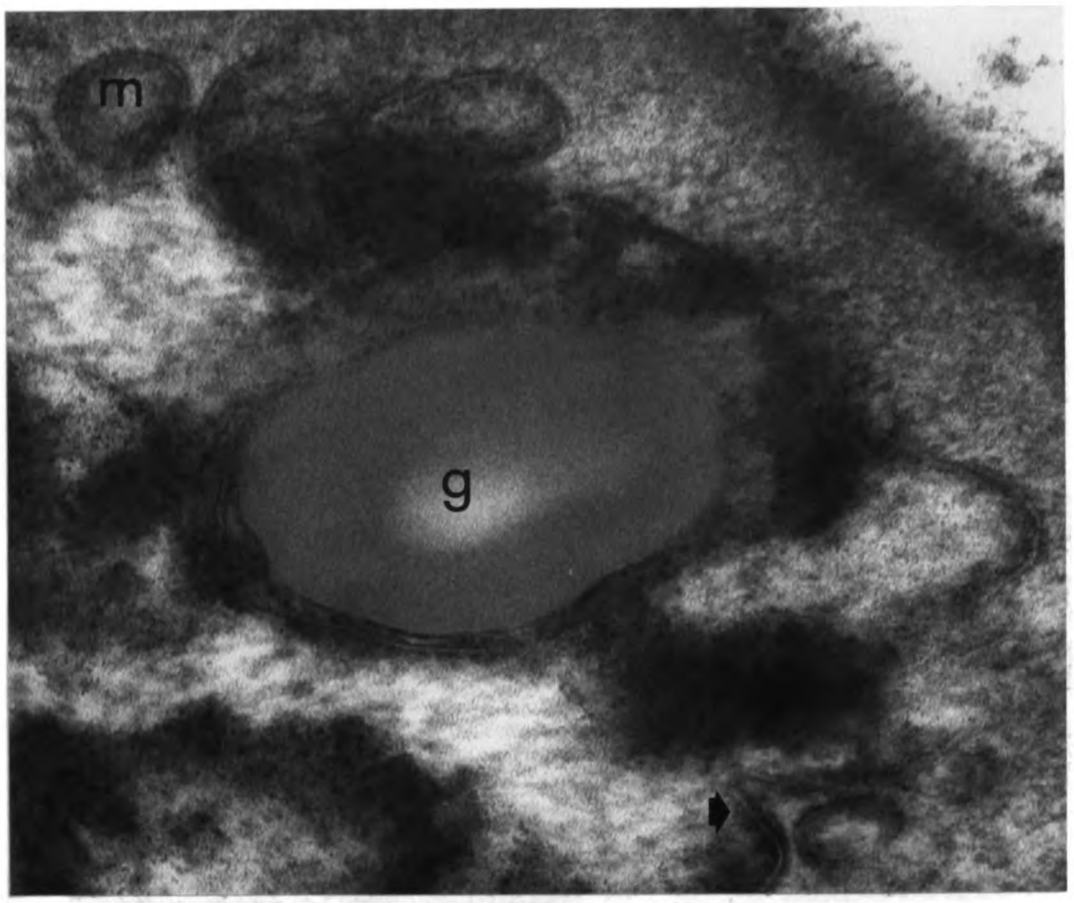


Figure 10. Storage granules (g) appeared as spherical globose structures. Remnants of the endoplasmic reticulum (arrow) and an occasional mitochondria were seen nearby. X180,000.

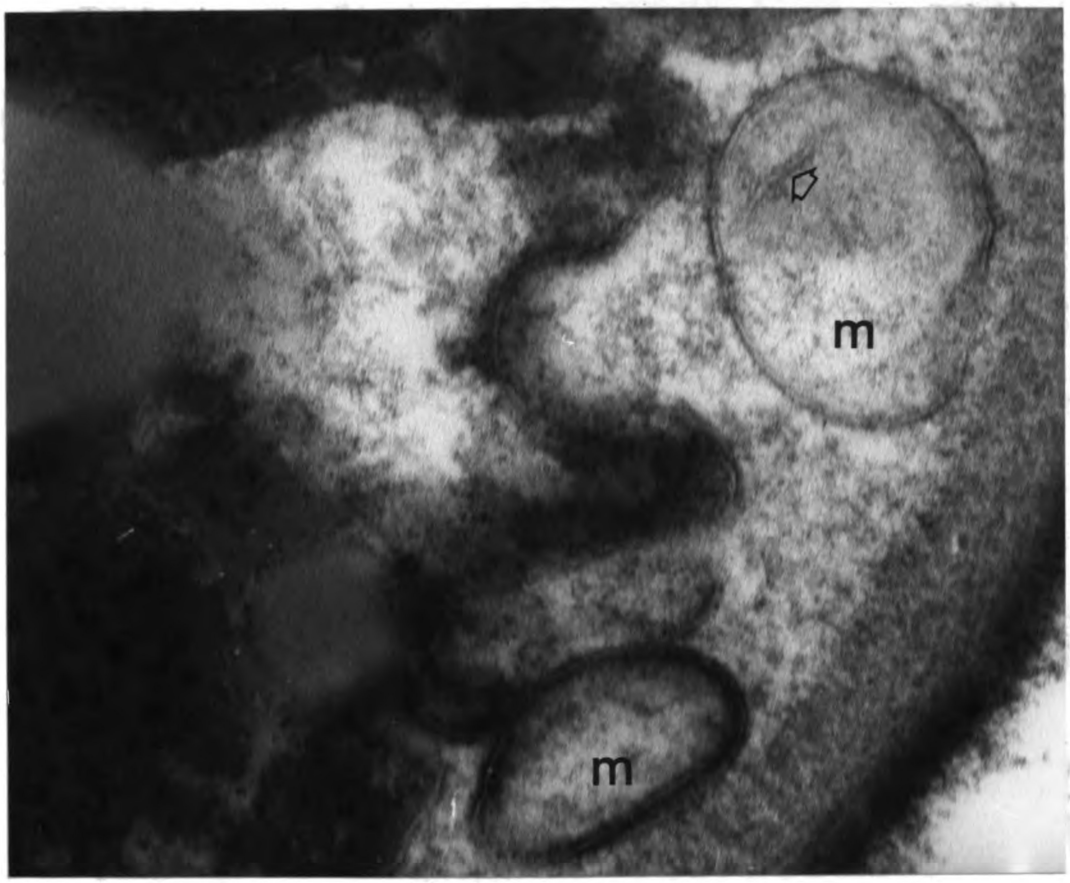


Figure 11. Mitochondria (m) were interspersed throughout the cytoplasm and appeared to be in different stages of development. Only partial cristae formations (arrow) were observed. Occasional mitochondria were attached to the cytoplasmic membrane and remnants of the endoplasmic reticulum. X180,000.

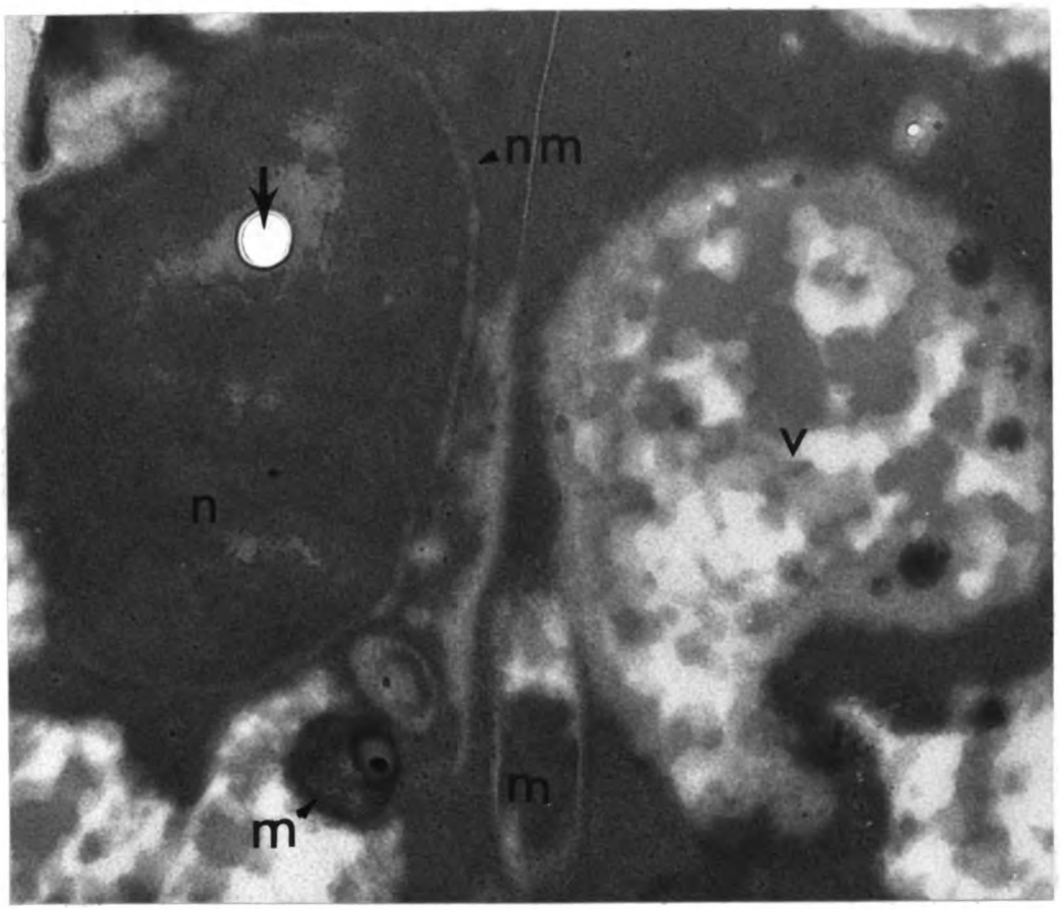


Figure 12. The nucleus (n) was surrounded by a double-layered nuclear membrane (nm). Other organelles, mitochondria (m) and vacuoles (v) are also present adjacent to the nucleus. Hole (arrow) represents artifact of dehydration. X72,000.

(Figure 12). Nucleoli, usually present within the nucleus, were not discernible. Occasional dark chromatin-like bodies were sometimes observed within the nucleus but these were not interpreted as nucleoli.

Treated Cell

Chapman (1962), in a study of the cytological effects of colistin sulfate on *Escherichia coli*, discussed the importance of selecting representative cells in any comparative study. In the present study, the population represented primarily older cells, some of which were a part of the original inoculum, and also a number of relatively young cells. Despite this age variation, little cytological differences were noted in the control preparations when the glutaraldehyde-osmium tetroxide mixture was used as the fixative.

It should be noted also that when a drug is added to such a population, the response of that population will be somewhat inconsistent or erratic. It can be represented in the form of a Poisson distribution curve, regarding time of response and the dosage at which the response occurs. Some cells respond quickly to a low dosage while others respond only at higher drug concentrations and after a longer period (Gale, 1963).

The viability studies reported here indicate that dosage was not a limiting factor in response, since the drug concentration used was adequate in killing all cells after adequate exposure; in this case, 48 hours. Cellular response was therefore a function of time. Nevertheless, it is unlikely that the degree of response would be

the same in all cells of this population. Replication of the procedure showed that the changes observed were relatively consistent under the conditions of the experiment.

Gale (1963) demonstrated that amphotericin B caused a reduction in cytoplasmic density, but that it had no effect on the nucleus and mitochondria of *Candida albicans*. Characteristic changes in the cytoplasmic membrane were undetected. This was attributed to incomplete fixation. These observations were made after 2 hours of exposure of *Candida albicans* to 100 mcg of amphotericin B per ml.

In the present study, 10 mcg of amphotericin B required 48 hours to "kill" *Torulopsis glabrata*. A 100 mcg concentration of the drug was inappropriate because of the precipitation problem. Although this presented no problem in the MIC procedure, the precipitate would have interfered with the electron microscopy study. Therefore, a 10 mcg concentration was used for 48 hours and found to be effective.

Amphotericin B had its most dramatic effect on the cytoplasmic membrane of *Torulopsis glabrata*. A separation and apparent shrinkage of the membrane from the cell wall was evident (Figures 13 and 14). The cell wall, on the other hand, appeared to be intact. Similar observations were made by Lane *et al.* (1972) when they exposed *Blastomyces dermatitidis* and *Histoplasma capsulatum* to amphotericin B for 6 hours. They also noted marked degenerative changes in these cells which eventually led to death of the organism.

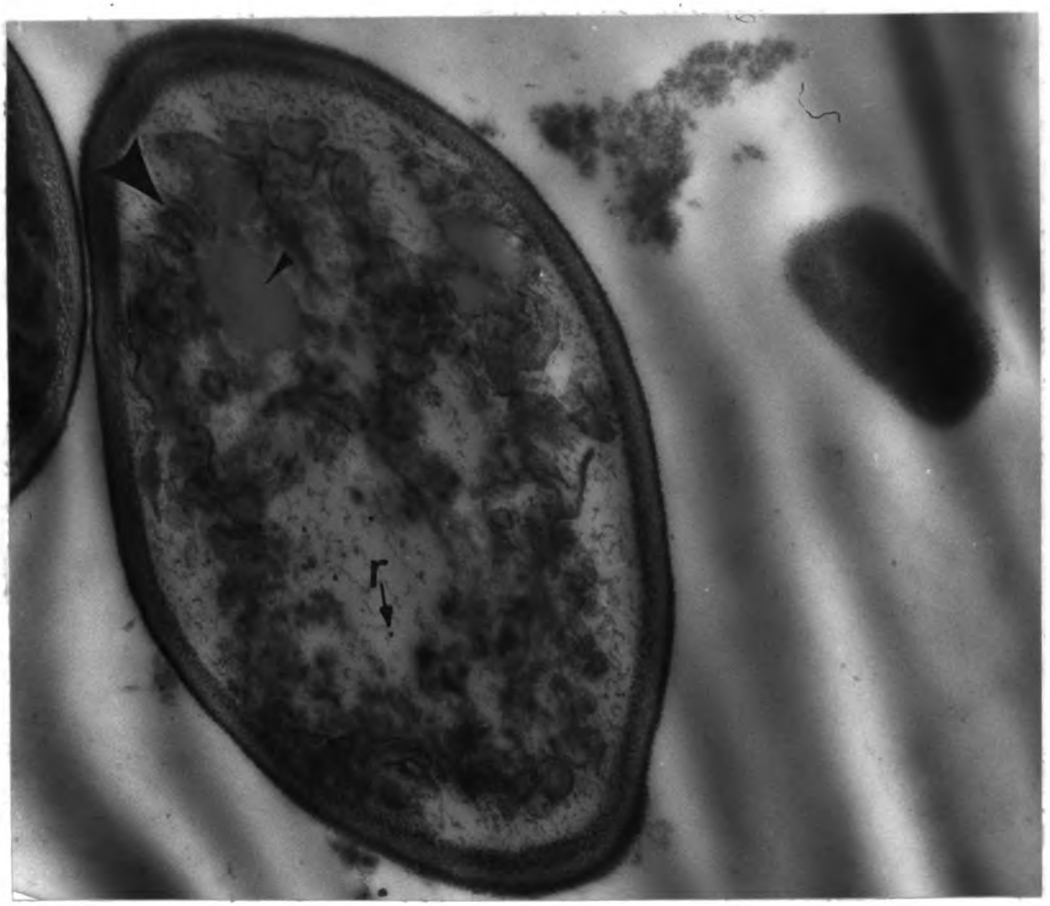


Figure 13. *Torulopsis glabrata* after 48 hours of exposure to amphotericin B. Note the separation of the cytoplasmic membrane from the cell wall (large arrow). Also, the cytoplasmic density is considerably diminished. Only occasional storage granules (small arrow) and freely dispersed ribosomes (r) could be discerned. X40,000.

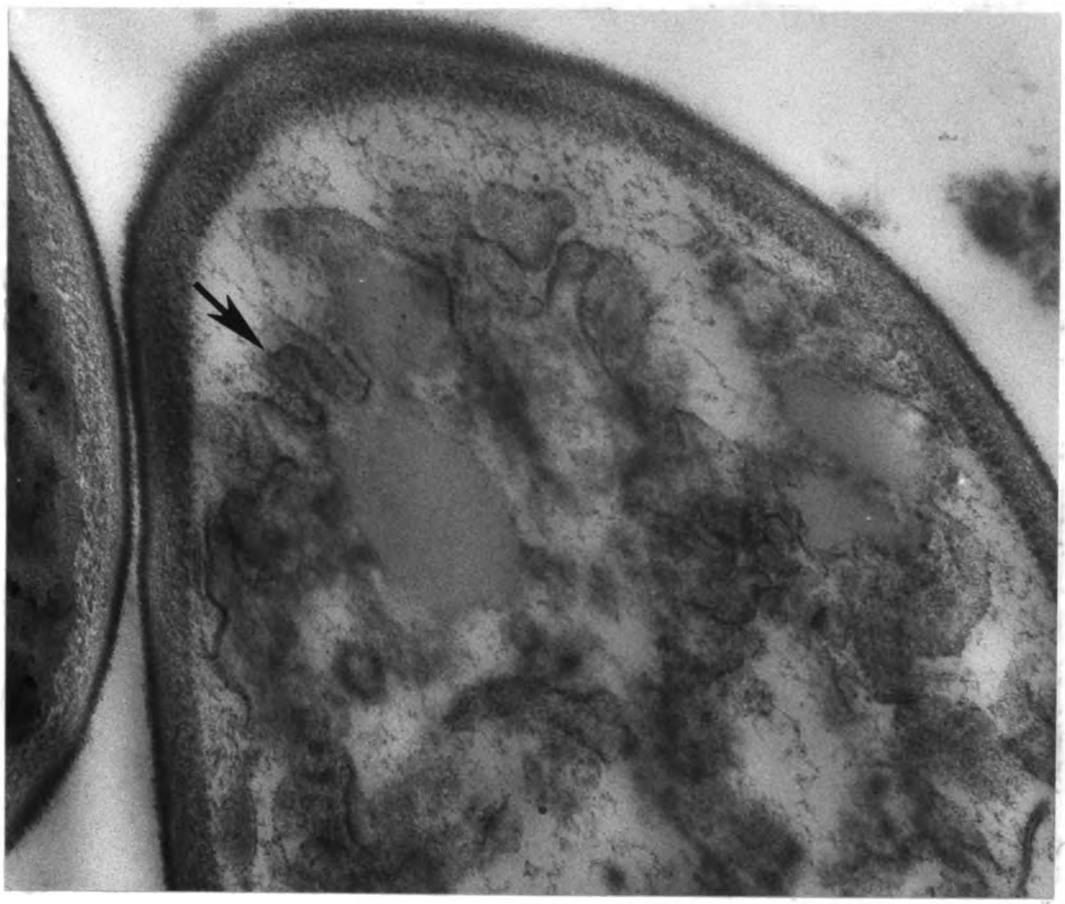


Figure 14. Higher magnification of Figure 13 showing the separation of the cytoplasmic membrane (arrow) from the cell wall. In addition to being separated, the membrane appears distorted. X72,000.

The overall density of *Torulopsis glabrata*, following exposure to the drug, was relatively "electron-thin" indicating a probable leakage of cytoplasmic material. Most of the cytoplasmic organelles were indiscernible with the exception of an occasional storage granule or lipid body. These results were most evident after 48 hours of exposure of the organism to amphotericin B.

When samples were withdrawn at 4, 8 and 12 hours, the cells showed no discernible ultrastructural variation from that of the control cell. The twenty-four hour sample of cells showed a slight detachment of the membrane from the cell wall, but the cytoplasmic density was maintained throughout the cell. As with the forty-eight hour sample, internal organelles were essentially indistinguishable (Figure 15).

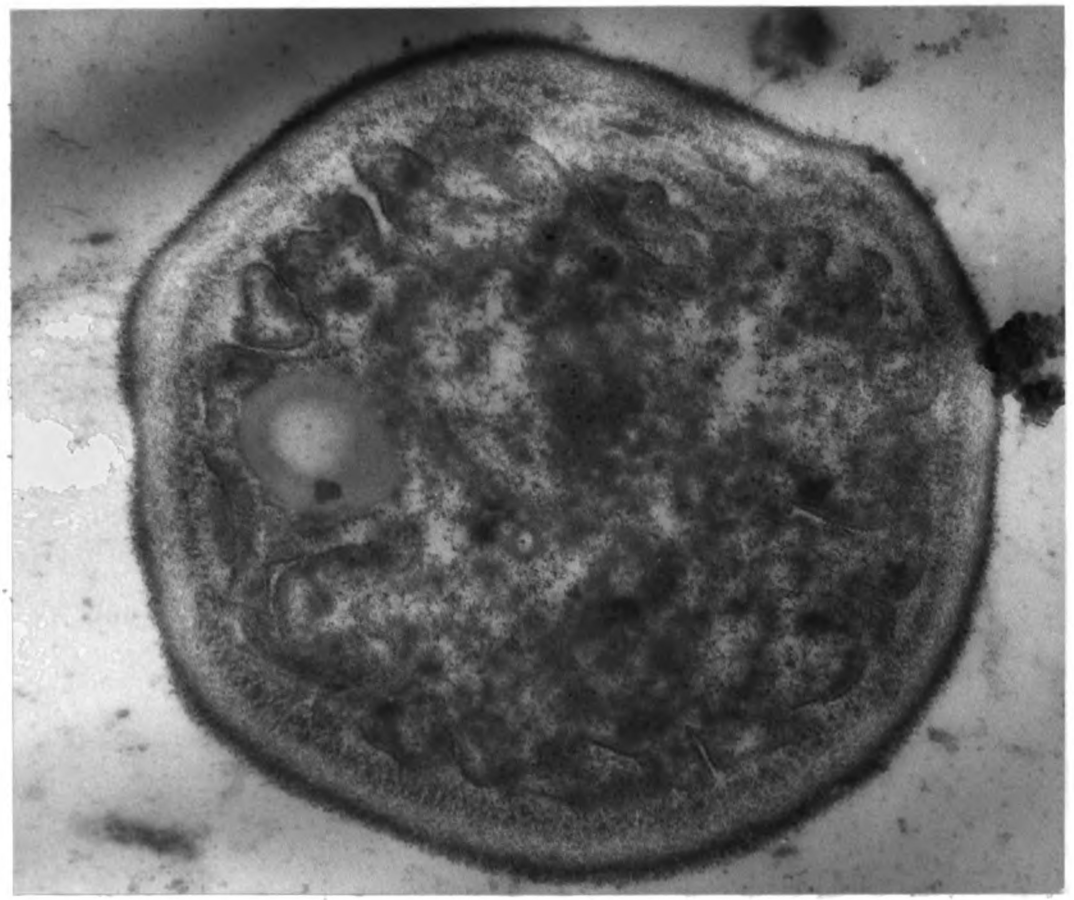


Figure 15. *Torulopsis glabrata* after 24 hours of exposure to amphotericin B. No substantial variation is apparent from that of the control cell; however, a slight detachment of the cytoplasmic membrane from the wall is evident. The cytoplasmic density appears to be consistent with that of the control cell. X90,400.

SUMMARY

The ultrastructure of *Torulopsis glabrata* and the cytological effects of amphotericin B, as seen in ultrathin sections, are described and illustrated with electron micrographs. Several fixatives and fixation techniques were employed. It was discovered that the ultrastructure of *Torulopsis glabrata* could best be demonstrated using a combination of glutaraldehyde and osmium tetroxide.

The ultrastructure was similar to that reported by authors who have studied other yeasts. A relatively thick cell wall (180 nanometers) and a double-layered cytoplasmic membrane were consistent features. Mesosomal-like appendages were frequently extended into the cytoplasm. Ribosomes, vacuoles, storage granules, mitochondria, and endoplasmic reticulum were also present within the cytoplasm.

The cytological effects of amphotericin B on *Torulopsis glabrata* were quite striking. The most dramatic effect of the drug was an alteration of the cytoplasmic membrane. Following 24 to 48 hours of exposure to amphotericin B, the cytoplasmic membrane of *Torulopsis glabrata* had separated from the cell wall but appeared intact.

Cytoplasmic density was reduced and was attributed to a leakage of intracytoplasmic material. Since decreased electron scattering can only be explained by a reduction in cell density, it follows that such a decrease in density can occur only through leakage of material across a permeable membrane.

REFERENCES

REFERENCES

Agar, H. D., and Douglas, C.: Studies on the Cytological Structure of Yeast: Electron Microscopy of Thin Sections. J. Bacteriol., 73, (1957): 365-375.

Anderson, H. W.: Yeast-like Fungi of the Human Intestinal Tract. J. Infect. Dis., 21, (1917): 341-386.

Bakerspigel, A.: Some Observations on the Cytology of *Candida albicans*. J. Bacteriol., 87, (1964): 228-230.

Bartner, E., Zinnes, H., Moe, R. A., and Kulesza, J. S.: Studies on a New Solubilized Preparation of Amphotericin B. In Antibiotics Annual 1957-1958, Medical Encyclopedia, Inc., (1958): 53-58.

Benham, R. W.: Cryptococci-Their Identification by Morphology and Serology. J. Infect. Dis., 57, (1935): 255-274.

Bennett, J. E.: Amphotericin B Toxicity: Review of Selected Aspects of Pharmacology. Ann. Int. Med., 61, (1964): 335-340.

Black, R. A., and Fisher, C. V.: Cryptococci Bronchopneumonia. Am. J. Dis. Child., 54, (1937): 81-88.

Block, E. R., and Bennett, J. E.: The Combined Effect of 5-Fluorocytosine and Amphotericin B in the Therapy of Murine *Cryptococcus*. Proc. Soc. Exp. Biol. Med., 142, (1973): 476-480.

Bonner, D., Mechlinski, W., and Schaffner, C. P.: Polyene Macrolide Derivatives. Biological Properties of Polyene Macrolide Ester Salts. J. Antibiot., 25, (1972): 261-262.

Bonner, D. P., Mechlinski, W., and Schaffner, C. P.: Stability Studies with Amphotericin B and Amphotericin B Methyl Ester. J. Antibiot., 28, (1975): 132-135.

Butler, W. T.: Pharmacology, Toxicity, and Therapeutic Usefulness of Amphotericin B. J.A.M.A., 195, (1966): 371-375.

Butler, W. T., Bennett, J. E., Alling, D. W., Wertlake, P. T., Utz, J. P., and Hill, G. J.: Nephrotoxicity of Amphotericin B. Early and Late Effects in 81 Patients. Ann. Int. Med., 61, (1964): 175-187.

Cass, A., Finkelstein, A., and Krespi, V.: The Ion Permeability Induced in Thin Lipid Membranes by the Polyene Antibiotics: Nystatin and Amphotericin B. B. J. Gen. Physiol., 56, (1970): 100-124.

Chapman, G. B.: Cytological Aspects of Antimicrobial Antibiosis. I. Cytological Changes Associated with the Exposure of *Escherichia coli* to Colistin Sulfate. J. Bacteriol., 84, (1962): 169-179.

Cooke, W. B.: Some Effects of Spray Disposal of Spent Sulphite Liquor on Soil Mold Populations. Proc. 15th Indiana Waste Conferences, Purdue University Eng. Bull., 45, (1961): 35-48.

Dennis, V. W., Stead, N. W., and Andreoli, T. E.: Molecular Aspects of Polyene- and Sterol-dependent Pore Formation in Thin Lipid Membranes. J. Gen. Physiol., 55, (1970): 375-400.

Dolan, C. T.: A Practical Approach to Identification of Yeast-like Organisms. Amer. J. Clin. Path., 55, (1971): 580-590.

Drutz, D. J., Spickard, A., Rogers, D., and Koenig, M. G.: Treatment of Disseminated Mycotic Infections. Amer. J. Med., 45, (1968): 405-418.

Dutcher, J. D.: The Chemistry of Nystatin and Related Antifungal Antibiotics. In Monographs on Therapy, Squibb Institute, New Brunswick, N.J., 2, (1957): 87-89.

Edebo, L., and Spetz, A.: Urinary Tract Infection with *Torulopsis glabrata* Treated by Alkalinization of Urine. Brit. Med. J., 2, (1965): 983-984.

Edwards, G. A., and Edwards, M. R.: The Intracellular Membranes of the Yeast-like Cells of *Blastomyces dermatitidis*. Am. J. Botany, 47, (1960): 622-632.

Edwards, M. R., Gordon, M. A., Lapa, E. W., and Ghiorse, W. C.: Micromorphology of *Cryptococcus neoformans*. J. Bacteriol., 94, (1967): 766-777.

Edwards, M. R., Hazen, E. L., and Edwards, G. A.: The Fine Structure of the Yeast-like Cells of *Histoplasma* in Culture. J. Gen. Microbiol., 20, (1959): 496-503.

Eichkoff, T. C.: Personal communication.

Feingold, D. S.: The Action of Amphotericin B on *Mycoplasma laidlawii*. Biochem. Biophys. Res. Commun., 19, (1965): 261-267.

Gale, G. R.: Cytology of *Candida albicans* as Influenced by Drugs Acting on the Cytoplasmic Membrane. J. Bacteriol., 86, (1963): 151-157.

Ganis, P., Avitable, G., Mechlinski, W., and Schaffner, C. P.: Polyene Macrolide Antibiotic Amphotericin B. Crystal Structure of the N-iodoacetyl Derivative. J. Amer. Chem. Soc., 93, (1971): 4560-4564.

Garriques, I. L., Sande, M. A., and Utz, J. P.: Combined Amphotericin B-Flucytosine Chemotherapy in Human Cryptococcosis. 13th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, D.C., American Society for Microbiology, 2, (1973): 239.

Gold, W., Stout, H. A., Pagano, J. F., and Donovick, R.: Amphotericins A and B, Antifungal Antibiotics Produced by a Streptomycete. I. *In vitro* Studies. Antibiot. Ann., (1955-1956): 579-586.

Grimley, P. M., Wright, L. D., and Jennings, A. E.: *Torulopsis glabrata* Infection in Man. Amer. J. Clin. Path., 43, (1965): 216-223.

Guze, L. B., and Haley, L. D.: Fungus Infections of the Urinary Tract. Yale J. Biol. Med., 30, (1958): 292-305.

Hahn, H., Condie, F., and Bulger, R. J.: Diagnosis of *Torulopsis glabrata* Infection. Successful Treatment of Two Cases. J.A.M.A., 203, (1968): 835-837.

Hamilton-Miller, J. M. T.: Chemistry and Biology of the Polyene Macrolide Antibiotics. Bacteriol. Rev., 37, (1973): 166-196.

Hasenclever, H. F., and Mitchell, W. O.: Antigenic Relationships of *Torulopsis glabrata* and Seven Species of *Candida*. J. Bacteriol., 79, (1960): 677-681.

Hasenclever, H. F., and Mitchell, W. O.: Pathogenesis of *Torulopsis glabrata* in Physiologically Altered Mice. Sab., 2, (1962): 87-95.

Hawker, L. E.: Fine Structure of Fungi as Revealed by Electron Microscopy. Biol. Rev., 40, (1965): 52-92.

Hofsten, B., and Hofsten, A.: Ultrastructure of a Thermotolerant Basidiomycete Possibly Suitable for Production of Food Protein. Appl. Microbiol., 27, (1974): 1142-1148.

Howarth, W. R., Tewari, R. P., and Solotorovsky, M.: Comparative *in vitro* Antifungal Activity of Amphotericin B and Amphotericin B Methyl Ester. Antimicrob. Agents Chemother., 7, (1975): 58-63.

Huppert, M., Sun, S. H., and Vukovich, K. R.: Combined Amphotericin B-Tetracycline Therapy for Experimental Coccidioidomycosis. Antimicrob. Agents Chemother., 5, (1974): 473-478.

Kagan, B. M.: *Antimicrobial Therapy*. W. B. Saunders Company, Philadelphia, Pa., (1970): 123.

Karnovsky, M. J.: A Formaldehyde-Glutaraldehyde Fixative of High Osmolality for Use in Electron Microscopy. J. Cell. Biol., 27, (1965): 137A.

Keim, G. R., Poutsiaka, J. W., Kirpan, J., and Keysser, C. H.: Amphotericin B: Comparative Acute Toxicity. Science, 179, (1973): 584-585.

Kinsky, S. C.: Effect of Polyene Antibiotics on Protoplasts of *Neurospora crassa*. J. Bacteriol., 83, (1962): 351-358.

Kinsky, S. C.: Polyene Antibiotics. *In* D. Gottlieb and P. D. Shaw (ed.) *Antibiotics*. Springer-Verlag, Berlin, 1, (1967): 122.

Kobayashi, G. S., Medoff, G., and Schlessinger, G.: Amphotericin B Potentiation of Rifampin as an Antifungal Agent Against the Yeast Phase of *Histoplasma capsulatum*. Science, 177, (1972): 709-710.

Kocan, R. M., and Hasenclever, H. F.: Normal Yeast Flora of the Upper Digestive Tract of Some Wild Columbids. J. Wildlife Dis., 8, (1972): 365-368.

Kreger-Van Rij, N. J. W., and Veenhuis, M.: A Comparative Study of the Cell Wall Structure of Basidiomycetous and Related Yeasts. J. Gen. Microbiol., 68, (1971): 87-95.

Lampen, J. O., Morgan, E. R., Slocum, A., and Arnow, P. M.: Absorption of Nystatin by Microorganisms. J. Bacteriol., 78, (1959): 282-289.

Lane, J. W., Garrison, R. G., and Johnson, D. R.: Drug-Induced Alterations in the Ultrastructural Organization of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. Mycopathol. Mycol. Appl., 48, (1972): 289-296.

Lees, A. W., Rao, S. S., Garret, J. A., and Eoot, P. A.: Endocarditis Due to *Torulopsis glabrata*. Lancet, 1, (Jan.-June 1971): 943-944.

Lodder, J., and DeVries, N. F.: Some Notes on *Torulopsis glabrata* (Anderson) nov. comb. Mycopath., 1, (1938): 98-103.

Lodder, J., and Kreger-Van Rij, N. J. W.: *The Yeasts. A Taxonomic Study*. Interscience Publishers, Inc., New York, N.Y. (1952): 408.

Manton, I.: Plant Cell Structure. In *Contemporary Botanical Thought*, ed. A. M. McLeod and L. L. Cobley, Edinburgh (1961).

Marks, M. I.: Personal communication.

Marks, M. I., and Eichkoff, T. C.: Application of Four Methods to the Study of the Susceptibility of Yeasts to 5-Fluorocytosine. Antimicrob. Agents Chemother., (1970): 491-493.

Marks, M. I., Langston, C., and Eichkoff, T. C.: *Torulopsis glabrata* - An Opportunistic Pathogen in Man. New Eng. J. Med., 283, (1970): 1131-1135.

Marks, M. I., and O'Toole, E.: Laboratory Identification of *Torulopsis glabrata*: Typical Appearance on Routine Bacteriological Media. Appl. Microbiol., 19, (1970): 184-185.

Marks, M. I., Steer, P., and Eichkoff, T. C.: *In vitro* Sensitivity of *Torulopsis glabrata* to Amphotericin B, 5-Fluorocytosine, and Clotrimazole (Bay 5097). Appl. Microbiol., 22, (1971): 93-95.

McAlear, J. H., and Edwards, G. A.: Continuity of the Plasma Membrane and Nuclear Membrane. Exptl. Cell Res., 16, (1959): 689-692.

McDowell, W. B.: Personal communication.

Mechlinski, W., and Schaffner, C. P.: Polyene Macrolide Derivatives. I. N-acylation and Esterification Reactions with Amphotericin B. J. Antibiot., 25, (1972): 256-258.

Mechlinski, W., Schaffner, C. P., Ganis, P., and Avitable, G.: Structure and Absolute Configuration of the Polyene Macrolide Antibiotic Amphotericin B. Tetrahedron Lett., 44, (1970): 3873-3876.

Medoff, G., and Kobayashi, G. S.: Amphotericin B - Old Drug, New Therapy. J.A.M.A., 232, (1975): 619-620.

Minkowitz, S., Koffler, D., and Zak, F. G.: *Torulopsis glabrata* Septicemia. Amer. J. Med., 34, (1963): 252-255.

Moore, R. T., and McAlear, J. H.: Fine Structure of Mycota. V. Lomasomes - Previously Uncharacterized Hyphal Structures. Mycologia, 53, (1961c): 194.

Oldfield, F. S. J., Kapica, L., and Pirozynski, W. J.: Pulmonary Infection Due to *Torulopsis glabrata*. Canad. Med. Ass. J., 98, (1968): 165-168.

Oroshnik, W., and Mebane, A. D.: The Polyene Antifungal Antibiotics. Prog. Chem. Org. Nat. Prod., 21, (1963): 17-79.

Pankey, G. A., and Daloviso, J. R.: Fungemia Caused by *Torulopsis glabrata*. Medicine (Baltimore), 52, (1973): 395-403.

Plaut, A.: Human Infection with *Cryptococcus glabratus*: Report of a Case Involving Uterus and Fallopian Tube. Am. J. Clin. Path., 20, (1950): 377-380.

Reynolds, E. S.: The Use of Lead Citrate at High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol., 17, (1963): 208-212.

Robinow, C. F., and Marak, J.: A Fiber Apparatus in the Nucleus of the Yeast Cell. J. Cell Biol., 29, (1966): 129-152.

Rose, H. D., and Heckman, M. G.: Persistent Fungemia Caused by *Torulopsis glabrata*. Treatment with Amphotericin B. Am. J. Clin. Path., 54, (1970): 205-208.

Shadomy, S.: Further *in vitro* Studies with 5-Fluorocytosine. Infec. Immun., 2, (1970): 484-488.

Stenderup, A., and Pederson, G. T.: Yeasts of Human Origin. Acta Pathol. Microbiol. Scand., 54, (1962): 462-472.

Sutton, D. D., Arnow, P. M., and Lampen, J. O.: Effects of High Concentrations of Nystatin Upon Glycolysis and Cellular Permeability in Yeast. Soc. Exp. Biol. Med., 108, (1961): 170-175.

Titsworth, E., and Gruneberg, E.: Chemotherapeutic Activity of 5-Fluorocytosine and Amphotericin B Against *Candida albicans* in Mice. Antimicrob. Agents Chemother., 4, (1973): 306-308.

Trump, B. F., and Bulger, R. E.: New Ultrastructural Characteristics of Cells Fixed in a Glutaraldehyde-Osmium Tetroxide Mixture. Lab. Invest., 15, (1966): 368-379.

Tsuchiya, T., Fukazawa, Y., and Kawakita, S.: Serological Classification of the Genus *Torulopsis*. Sab., 1, (1961): 145-153.

Tvedten, H.: Personal communication.

Utz, J. P., Bennett, J. E., Brandriss, N. W., Butler, W. T., and Hill, G. J.: Amphotericin B Toxicity. Combined Clinical Staff Conference at the National Institutes of Health. Ann. Int. Med., 61, (1964): 334-354.

Vandeputte, J., Wachtel, J. L., and Stiller, E. T.: Amphotericins A and B, Antifungal Antibiotics Produced by a Streptomycete. II. The Isolation and Properties of the Crystalline Amphotericins. Antibiot. Ann., (1955-1956): 587-591.

Vandevelde, A. G., Mauceri, A. A., and Johnson, J. E. III: 5-Fluorocytosine in the Treatment of Mycotic Infections. Ann. Intern. Med., 77, (1972): 43-51.

Van Uden, N., Sousa, L. D. C., and Farinka, M.: On the Intestinal Yeast Flora of Horses, Sheep, Goats, and Swine. J. Gen. Microbiol., 19, (1958): 435-445.

Van Uden, N.: The Occurrence of *Candida* and Other Yeasts in the Intestinal Tracts of Animals. Ann. N.Y. Acad. Sci., 89, (1960): 59-68.

Weber, M. M., and Kinsky, S. C.: Effect of Cholesterol on the Sensitivity of *Mycoplasma laidlawii* to the Polyene Antibiotic Filipin. J. Bacteriol., 89, (1965): 306-312.

Weissmann, G., Pras, M., and Hirschhorn, R.: A Common Mechanism for the Fungicidal and Nephrotoxic Effects of Amphotericin B. J. Clin. Invest., 45, (1966): 1084.

White, R. W., Lindsay, D. B., and Ash, R. W.: Ethanol Production from Glucose by *Torulopsis glabrata* Occurring Naturally in the Stomachs of Newborn Animals. J. Appl. Bact., 35, (1972): 631-646.

Wickerham, L. J.: Apparent Increase in Frequency of Infections Involving *Torulopsis glabrata*. Procedure for Its Identification. J.A.M.A., 165, (1957): 47-48.

Zygmunt, W. A.: Intracellular Loss of Potassium in *Candida albicans* after Exposure to Polyene Antifungal Antibio-ics. Appl. Microbiol., 14, (1966): 953-956.

APPENDICES

APPENDIX A

NOMENCLATURE

ANAEROBIC: A term used to describe an atmosphere free of molecular oxygen.

ASCOSPORE: A spore formed as a result of sexual reproduction developed in a sac-like cell known as an ascus.

ASCUS: A sac-like structure, characteristic of the ascomycetes, in which ascospores are produced.

ASPOROGENOUS: Lacking the ability to produce spores.

ASSIMILATION: A form of metabolism that involves a building up or synthesis of material by the cell.

BUDDING: A form of asexual reproduction typical of yeast, in which a new cell is formed as an outgrowth from the parent cell.

FERMENTATION: Anaerobic oxidation of carbohydrates by enzyme action of microorganisms.

FUNGEMIA: The presence of fungi or yeasts in the blood.

MYCELIUM(A): Mass of threadlike filaments, branched or composing a network, which constitute the vegetative portion of the fungus.

OXIDATION: The process of combining with oxygen, or the loss of electrons or hydrogen.

PHARMACODYNAMICS: The study of drug effects and the handling of drugs by the body.

PSEUDOHYPHAE: A false tubular-like structure formed by certain yeasts (e.g., *Candida albicans*) in the presence of human or fetal bovine serum.

SEPTATE: Possessing cross wells within the hyphal structure of certain fungi.

SPORE: A small reproductive unit or body, functioning like a seed, that is produced by the organism.

THERMOLABILE: Destroyed by heat at temperatures below 100 C.

THERMOSTABLE: Relatively resistant to heat (resistant to temperatures of 100 C).

APPENDIX B

TABLE OF MOST COMMON SOURCES AND ORGANISMS ISOLATED FROM THESE SOURCES

Organism	Percentage of Total from Source									
	Sputum (N=123)	Induced sputum (N=29)	Delayed induced sputum (N=10)	Gastric (N=49)	Mouth and throat (N=29)	Urine (N=130)	Vaginal (N=64)	Bronchial washings (N=15)	Stool (N=9)	Skin (N=8)
C. albicans	43	44	40	44	41	12	25	27	33	13
C. tropicalis	30	34	51	22	35	15	6	20	11	0
C. krusei	3	0	0	0	7	2	2	0	0	0
C. parapsilosis	7	0	0	0	3	9	3	0	22	63
Candida sp.	1	0	0	2	0	1	0	13	0	0
Geotrichum sp.	1	0	0	0	0	0	0	0	0	0
Torulopsis sp.	1	4	0	6	3	1	3	13	11	0
T. glabrata	15	18	10	24	10	61	59	27	22	25
Saccharomyces-like *sp.*	0	0	0	0	0	0	2	0	0	0

From C. T. Dolan, Amer. J. Clin. Path., 55, (1971): 584.

APPENDIX C

BATTERY OF BIOCHEMICALS FOR IDENTIFYING *TORULOPSIS GLABRATA*[*]

Organism	Assimilations[a]								Fermentations[b]						Miscellaneous Tests		
	D	M	L	S	G	C	X	T**	D	M	L	S	X	T	Urea	Pseudohyphae	Ascospore
Torulopsis glabrata	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-

[*]Results were recorded after 3-4 days of incubation at room temperature.

[**]Interpretation of symbols:

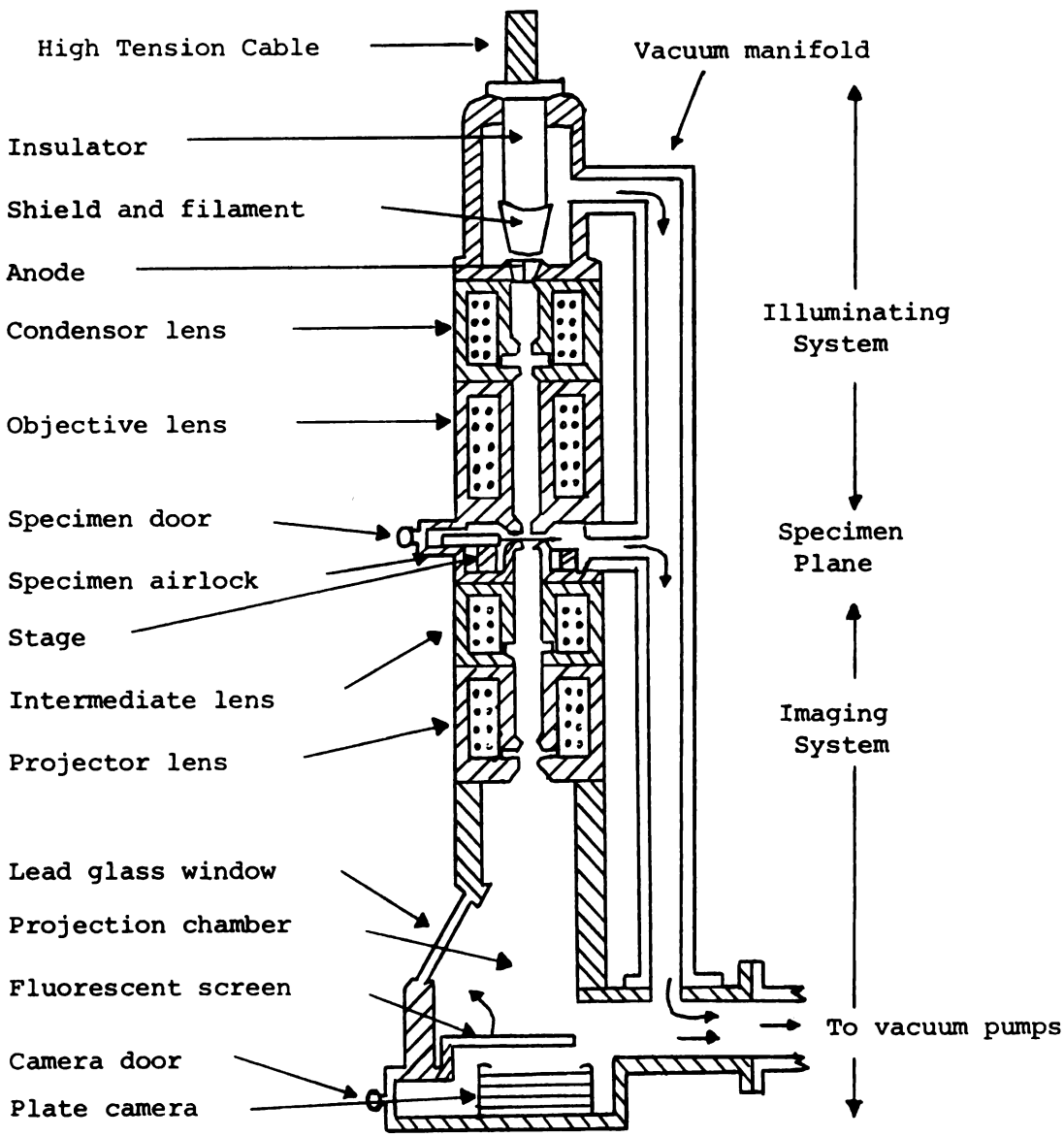
D = dextrose
M = maltose
L = lactose
S = sucrose
G = galactose
C = cellobiose
X = xylose
T = trehalose
\+ = positive
\- = negative

[a]Positive reaction based on acid production.

[b]Positive reaction based on acid and/or gas production.

APPENDIX D

CROSS SECTION OF A SINGLE-CONDENSOR ELECTRON MICROSCOPE



A simplified section through a simple single-condensor 4-lens medium performance electron microscope.*

---

*From G. A. Meek, *Practical Electron Microscopy for Biologists*. Wiley-Interscience Ltd., p. 98, 1971.

VITA

The author was born on September 18, 1947, in Mt. Clemens, Michigan. He began his undergraduate study in 1967 at Ferris State College. In 1972, he received the Bachelor of Science degree in Medical Technology. From June 1971 through June 1972, he served his medical technology internship at Edward W. Sparrow Hospital, Lansing, Michigan. He was employed at Sparrow Hospital following his internship and is still employed as a full time technologist working primarily in the Microbiology department. In September 1972, the author began pursuing a graduate degree in Clinical Laboratory Science at Michigan State University. He is currently a member of the American Society for Microbiology and is a past member of the American Society for Medical Technology. The author is married to the former Christine Ann Ezyk, a registered pharmacist presently employed at Lansing General Hospital, Lansing, Michigan.