CYTOLOGY OF *CANDIDA ALBICANS* AS INFLUENCED BY DRUGS ACTING ON THE CYTOPLASMIC MEMBRANE

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Received for publication 12 April 1963

ABSTRACT

GALE, GLEN R. (Veterans Administration Hospital, Durham, N.C.) Cytology of *Candida albicans* as influenced by drugs acting on the cytoplasmic membrane. J. Bacteriol. **86**:151–157. 1963.—An electron microscopic comparison was made of the cytological responses of *Candida albicans* to benzalkonium chloride, amphotericin B, and filipin, all of which are thought to exert their primary pharmacological actions on the cytoplasmic membrane of susceptible microorganisms. After 5 to 15 min of exposure to benzalkonium chloride, most of the cytoplasm became less dense, and intact nuclei, mitochondria, or intracytoplasmic membranes were not observed. The remaining dense cytoplasm contained numerous small holes. The polyene antifungal agents, amphotericin B and filipin, caused a reduction in cytoplasmic density, but had no observable effect on nuclei or mitochondria. The intervals before onset of changes induced by the polyene agents correlated with the known time-dependent binding of these drugs by cells, and the decreased electron scattering induced by all three agents was compatible with their known abilities to cause a loss of integrity of the cytoplasmic membrane with resultant leakage of cytoplasmic components. No consistent morphological effect of any of these drugs on the cytoplasmic membrane could be shown.

Polyene antifungal agents appear to exert their primary action at the cytoplasmic membrane of susceptible microorganisms. Evidence leading to this conclusion first appeared several years ago in investigations of the effects of nystatin on glycolysis and respiration of yeast and fungi (Lampen, Morgan, and Slocum, 1957; Bradley, 1958). Since drug concentrations inhibiting those aspects of metabolism approximated fungistatic concentrations, inhibition of gas exchange *per se* was thought to be a possible lethal mechanism. Gale (1960) found that amphotericin B, another polyene agent, had a similar effect on yeast, and suggested that the permeability of the cell was altered. Subsequent work with nystatin has shown that the primary site of action is at the cytoplasmic membrane, and that death of the cell is due to leakage of low molecular weight substances after a time-dependent binding of the drug to the membrane (Lampen, 1962). This time dependence seems to be a function of the physiological state of the cell, possibly owing to differences in cell-wall permeability of young cells as compared with old cells; stationary-phase cultures bind nystatin slowly, whereas log-phase cells bind it rapidly. The demonstration that heat-treated cells rapidly bind the drug (Lampen, 1962) excludes an enzymatic mechanism. All antifungal polyene compounds so far investigated cause a rapid loss of integrity of fungal protoplasts (Kinsky, 1962), but not of bacterial protoplasts (Shockman and Lampen, 1962).

Certain differences may exist in the mechanisms of antifungal actions of the various polyene agents. The inhibitory action of some (nystatin, amphotericin B) against yeast glycolysis can be overcome by the addition of potassium ion; that of others (pimaricin, filipin) cannot be so reversed. Reversal by K^+ appears to be via replacement of an ion necessary for enzymatic activity, and not through a drug-ion interaction (Lampen, 1962). Reversal by sterols of the fungistatic (Lampen, Arnow, and Safferman, 1960; Schneierson et al., 1962) and Leishmanicidal (Ghosh and Chatterjee, 1962) actions of polyene agents has been demonstrated. Lampen et al. (1960) showed no evidence of a metabolic interrelation between sterols and polyene agents; a physicochemical interaction between the two, similar to the well-known cholesterol-digitonin precipitation (Ransom, 1901), was considered a better explanation of the reversal of action. Phospholipids, which com-

pletely overcome the antimicrobial action of the synthetic surface-active detergents (Baker, Harrison, and Miller, 1941; Weinberg, 1961; Gale, Welch, and Hynes, 1962), do not reverse the action of polyenes (Ghosh and Chatterjee, 1962; Schneierson et al., 1962).

Since metabolic studies and investigations with protoplasts have thus indicated different mechanisms of action for various agents acting on the cytoplasmic membrane, this study was initiated to determine whether detectable cytological changes also differ in response to each drug.

MATERIALS AND METHODS

The organism used was a stock laboratory strain of *Candida albicans* maintained on Sabouraud agar slants. For each experiment, a loopful was transferred to 100 ml of Sabouraud broth and incubated stationary at 37 C for 16 hr. The polyene antibiotics, amphotericin B and filipin, were dissolved in dimethyl formamide and added to samples of the broth culture. Control samples and those receiving benzalkonium chloride contained the same final 0.8% concentration of dimethyl formamide as the samples receiving the polyene compounds.

Tube dilution assays showed the organism to be sensitive to benzalkonium chloride, amphotericin B, and filipin at concentrations of approximately 3.0, 0.7, and 1.2 μg/ml, respectively. Owing to the difference in cell density in the tube dilution assay as compared with the experimental situation, the drug concentrations were increased by about two orders of magnitude to 100 μg/ml. The adequacy of this concentration of each drug was tested by removing part of each sample prior to fixation for electron microscopy, washing it with a large excess of saline, and streaking the cells on Sabouraud agar plates. In four separate experiments, the time periods after which no viable microorganisms could be shown were 5 to 15, 30 to 60, and 60 to 120 min with benzalkonium chloride, filipin, and amphotericin B, respectively.

For reasons not at all clear, osmium tetroxide proved to be an unsatisfactory fixative for *C. albicans* when used by the method of Palade (1952) or Kellenberger, Ryter, and Séchaud (1958). Control cells, when treated by either method, showed large, electron-thin areas with no evidence of mitochondria or nuclei. It is interesting to speculate that the thick cell wall may be wholly or partly responsible. The formalin fixation method of Conti and Gettner (1962) was also used, with equally poor results. Permanganate (1.5%) in Michaelis buffer (Kellenberger et al., 1958) at either pH 6.1 or 8.0 failed to preserve cytoplasmic detail satisfactorily. Aqueous 1.5% potassium permanganate gave the most satisfactory results, aside from the quite marked granularity usually associated with this fixative, and it was used in all experiments. Duration of fixation was 1 hr at 0 C. After this, the cell pellets obtained by centrifugation of each suspension were dehydrated through a graded ethanol series, and polymerized at 50 C in methacrylate (30 parts ethyl to 70 parts butyl monomers) containing 0.2% uranyl nitrate to attempt to reduce polymerization damage (Ward, 1958). Sections were cut with a Porter-Blum microtome, picked up on carbon-stabilized, Formvar-coated 200-mesh grids, and examined in an RCA EMU3-F electron microscope using 100-kv accelerating voltage through a 30-μ objective aperture.

RESULTS

Control cells. Cells not exposed to any drug showed a consistent cytology over the 4-hr sampling period. A typical example is shown in Fig. 1. An ovoid cell roughly 3 by 4 μ was surrounded at its periphery by a relatively electron-dense area of the cell wall about 400 A in thickness; this was followed by a relatively electron-thin area of about 2,000 A. No structural detail of significance could be differentiated. The cytoplasmic membrane just within the cell wall appeared to be a three-layered unit membrane, with occasional invaginations into the cytoplasm. Even though the precise limits of this membrane system could seldom be followed around the entire periphery of the cytoplasm, probably owing to artifacts of fixation, its thickness was approximately 100 A. Depths of the invaginations were up to 1,500 A.

Within the cytoplasmic membrane were found the usual cytoplasmic organelles, the normal cytology of which did not differ meaningfully from the electron micrographs of *Saccharomyces cerevisiae* and *Rhodotorula glutinis* published by others (Hashimoto, Conti, and Naylor, 1959; Vitols, North, and Linnane, 1961; Thyagarajan, Conti, and Naylor, 1961, 1962), except that a definite nucleolus was not seen, and vacuoles were not seen as frequently as reported by Vitols et al. (1961). Cytoplasm was relatively homogeneously granular, as is usually unavoidable with permanganate fixation; granules ranged in size from

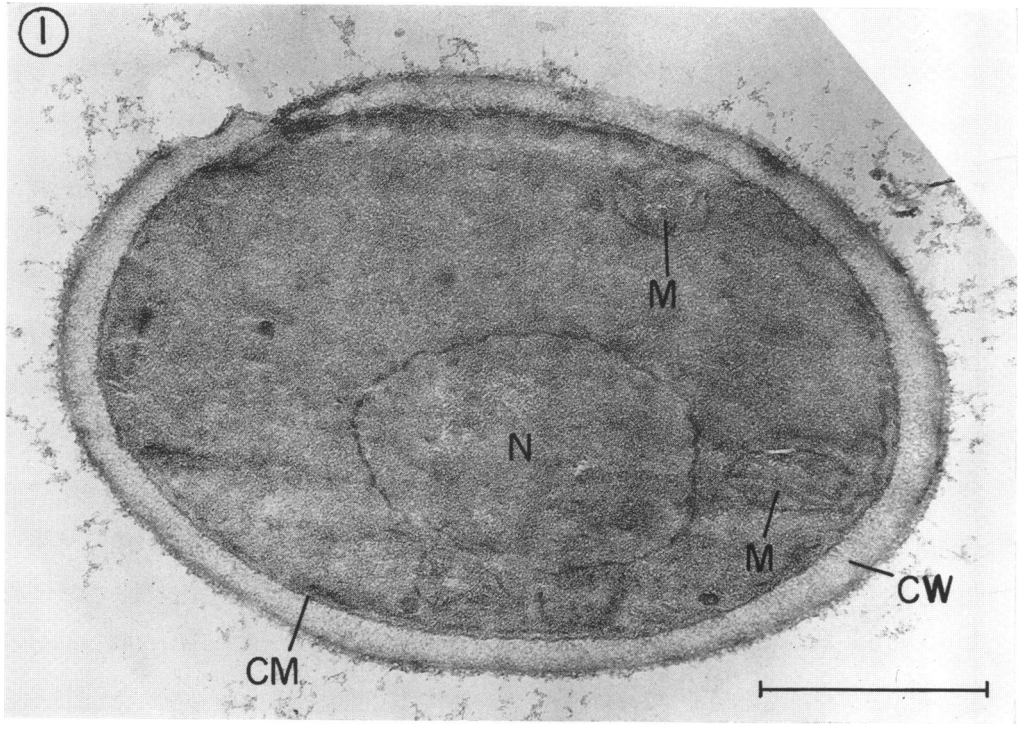

FIG. 1. *Electron micrograph of Candida albicans from 16-hr broth culture. This is a control cell, unexposed to any drug. The following abbreviations are used throughout: CW, cell wall; CM, cytoplasmic membrane; M, mitochondrion; N, nucleus; ETA, electron-thin area. The line in each figure represents 1.0 μ.*

about 50 A down to the lower limits of resolution of the microscope. Nuclei were slightly irregular in outline and frequently ovoid. Mitochondria were observed near the periphery of the cytoplasm and in close proximity to the nucleus. Occasional electron-dense intracytoplasmic bodies were seen, somewhat suggestive of Golgi bodies but failing to be resolved adequately for a definite answer. Intracytoplasmic membrane systems other than those surrounding the nucleus and mitochondria were observed occasionally.

Benzalkonium chloride-treated cells. The most rapid and marked alteration in cytology was observed in cells exposed to benzalkonium chloride. Within a period of 5 to 15 min, the changes shown in Fig. 2 had been incurred, consisting of the development of a rather large central area with relatively less electron-scattering properties, in which several smaller areas of approximately normal density were noted. Peripheral cytoplasm contained numerous small holes, ranging in size from about 100 to over 600 A in diameter. Nuclei, mitochondria, or intracytoplasmic membranes could not be demonstrated. The cytoplasmic membrane, when it could be visualized, was similar to that of control cells, but occasionally seemed to reveal more lamellations.

Polyene-treated cells. Changes in cytology of cells exposed to amphotericin B and filipin were similar, with the exception of time of onset. After 30 to 60 min, cells exposed to filipin showed areas of lower electron scattering in the cytoplasm, quite similar to those resulting from exposure to benzalkonium chloride. Nuclei and mitochondria appeared normal even after this change in the cytoplasm, with slight if any differences in characteristics as compared with control cells. The numerous small holes, so conspicuous in cells treated with benzalkonium chloride, were not present in the denser cytoplasm (Fig. 3). At this 30- to 60-min sampling period, a cytoplasmic membrane was noted at certain areas of the cytoplasmic periphery but, as with control cells, could seldom be seen in its entirety. When observed, it displayed no striking differences from the membrane of control cells.

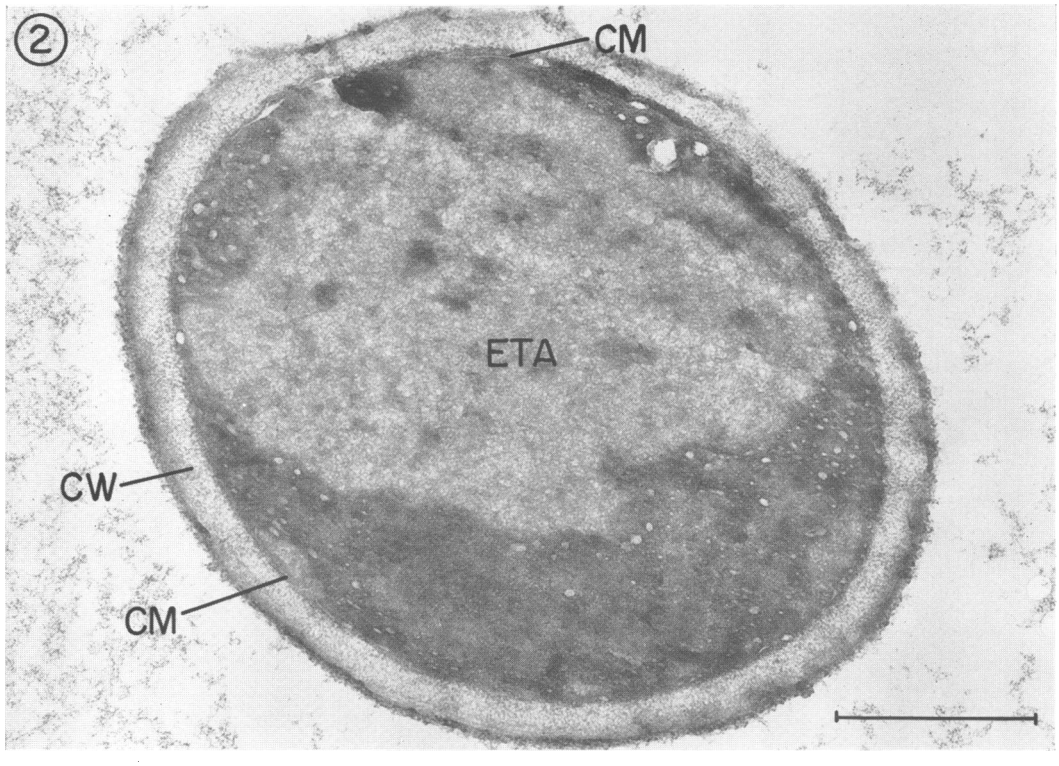

FIG. 2. *Electron micrograph of Candida albicans after exposure to benzalkonium chloride (100 μg/ml) for 15 min. Areas of cytoplasmic membrane are still evident. The electron-thin area of the cytoplasm is the predominant area; the remaining electron-dense cytoplasm shows numerous small holes. No nucleus or mitochondrion can be seen.*

Changes caused by amphotericin B were similar to those with filipin, but were noted after 1 to 2 hr of exposure to the drug (Fig. 4). More subtle differences may be inferred, but conclusions would be tenuous. There were suggestions of a better-maintained cytoplasmic membrane system than was obtained with filipin, and a possible difference in the texture of the electron-thin area induced in the cytoplasm. With both these polyene drugs, onset of changes correlated well with irreversible damage to the cells, as shown by the plating experiments described in Materials and Methods.

After 4 hr of incubation of cells with either filipin or amphotericin B, nuclei and mitochondria were seldom evident. However, since this sampling period was 2 hr or more past the time at which 100% of the cells had been damaged irreversibly by the drugs, it seems reasonable to assume that autolytic changes may have been in part responsible.

DISCUSSION

Chapman (1962), in his study of the effect of colistin sulfate on the cytology of *Escherichia coli*, discussed in part the necessary criteria for choosing representative cells in any comparative study. In the present study, the population represented older cells, some of which were a part of the original inoculum, and certainly a number of cells no more than a few minutes old. Despite such an age distribution, little, if any, cytological variation was observed in control preparations. However, when any drug is added to such a population, the response of the population as a whole will be in the form of a Poisson distribution, as concerns time of response and dosage at which response occurs. A certain number of cells will show a response to a low dosage in a relatively short time, regardless of the response being measured, whereas others will show a response only at higher drug concentrations and after a longer time inter-

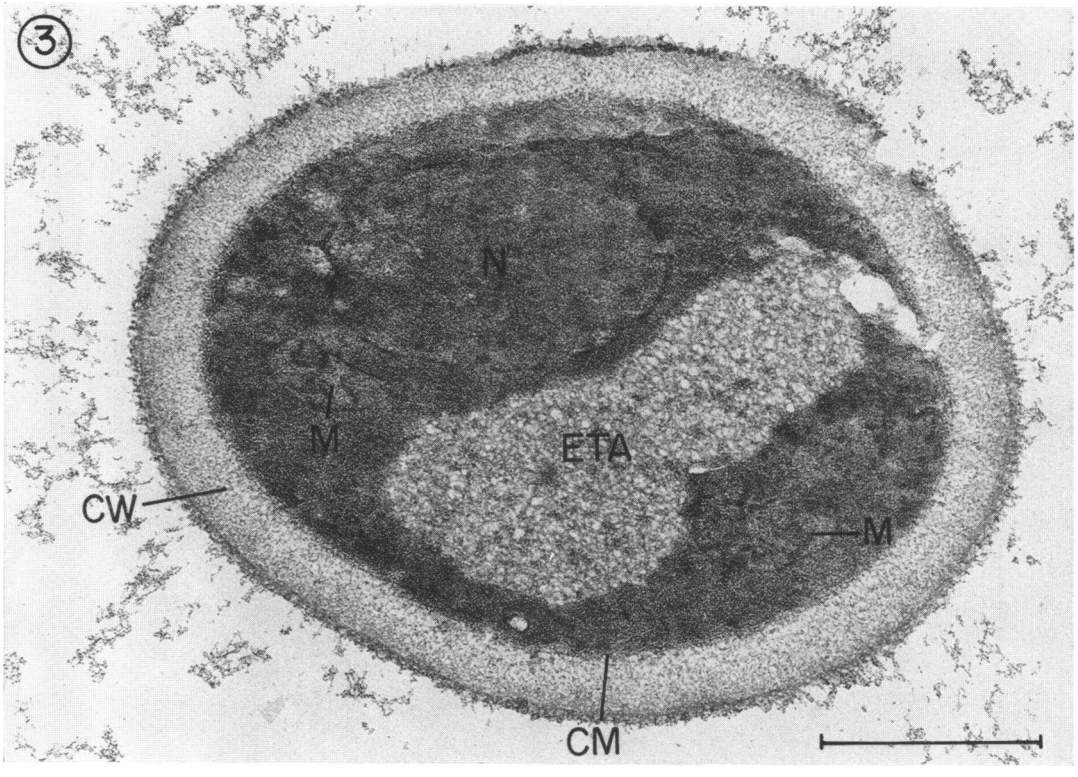

FIG. 3. *Electron micrograph of Candida albicans after exposure to filipin (100 μg/ml) for 60 min. Nucleus and mitochondria are evident; the discrete electron-thin area occupies a large part of the cytoplasm.*

val. The survival experiments reported herein show that dosage was not a limiting factor in response, since the drug concentrations employed were adequate to effect 100% killing of the cells after adequate exposure. Cytological response was therefore a function primarily of time and the more obscure inherent individual variations in degree of potential morphological change under the influence of the drugs investigated. There is no reason to believe that this change would be of the same degree in all cells within the population examined.

Being cognizant of these difficulties, every attempt was made to record the changes most representative of the cells in each experimental group. Replication of the experiment four times bolsters the likelihood that the reported changes are consistent under the experimental conditions employed.

The progress of damage to the cells correlates rather well with the biochemical data regarding the time dependence of the binding of polyene agents to susceptible cells (Lampen, 1962). Whereas benzalkonium chloride, known to be fungicidal and bactericidal within a few minutes, produced the maximal recordable change within 5 to 15 min, cells exposed to the polyene antibiotics appeared indistinguishable from control cells for up to 1 hr or more. It is to be regretted that fixation techniques did not result in better preservation of the cytoplasmic membrane, for it was thought that characteristic changes in it might be noted.

Of interest was the complete absence of intracytoplasmic organelles after 5 to 15 min of exposure to benzalkonium chloride. The original observation by Baker et al. (1941) that phospholipids antagonize the bactericidal surface-active compounds and the more recent demonstration that lecithin firmly binds certain such agents (Gale et al., 1962) suggest that these compounds may combine with virtually any phospholipid

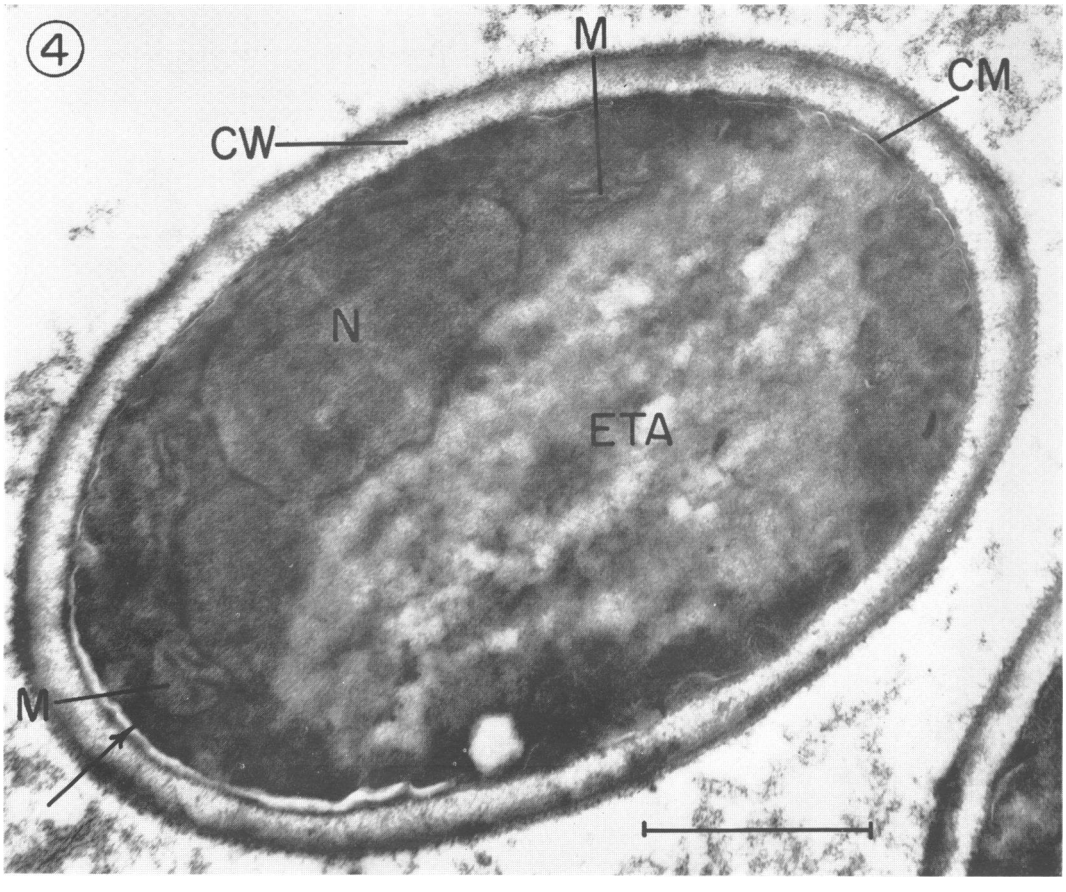

FIG. 4. *Electron micrograph of Candida albicans after exposure to amphotericin B (100 μg/ml) for 120 min. Nuclei and mitochondria are intact; electron-thin cytoplasm predominates. Note invaginations of cytoplasmic membrane into cytoplasm. Wide separation of cytoplasm from cell wall (arrow) is probably artifact of embedding, since this was noted occasionally even in control cells.*

component of the cell. If this is the case, injury to the cytoplasmic membrane by the surfactant may alter its selective permeability to the extent that the drug would be free to diffuse intracellularly to combine with phospholipid moieties of the nuclear and cytoplasmic membranes, leading to dissolution of these structures. The presence of intact nuclei and mitochondria in cells exposed to amphotericin B and filipin, even after cytoplasmic changes were evident, suggests a specificity of these agents for some component of the cytoplasmic membrane not present in the nuclear and mitochondrial membranes. Considering the evidence which has appeared regarding reversal of polyene inhibition (Lampen et al., 1960; Schneierson et al., 1962; Ghosh and Chatterjee, 1962), there seems every reason to believe that the receptor for the polyene drug will ultimately be shown to be a sterol of some type, and perhaps be limited to the cytoplasmic membrane.

Cytoplasmic changes observed in *C. albicans* are entirely compatible with chemical events associated with exposure of susceptible cells to each type of drug (Hotchkiss, 1946; Lampen, 1962). Decreased electron scattering can be explained only on the basis of decreased density of the cell; such a decrease in density can occur only through loss of material through a cytoplasmic membrane which has lost its selective permeability or energy-dependent membrane-transfer mechanisms. The more subtle differences in the mechanism of action of the two polyene agents were not resolvable with certainty under the conditions employed, except as pertains to time of onset.

ACKNOWLEDGMENTS

The competent technical assistance of Helen H. McLain and Sally Kendall is gratefully acknowledged. The crystalline amphotericin B was a gift of John T. Groel of the Squibb Institute for Medical Research. George M. Savage of The Upjohn Co. furnished the filipin. This work was aided by grant RG-9389 from the U.S. Public Health Service.

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