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KEYWORDS: diamidine compound, *Candida albicans*, fungistatic, membrane disruption.

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ANTIMYCOTIC ACTION OF A DIAMIDINE COMPOUND, 1, 4-BIS-(M, M'-AMIDINOPHENOXYMETHYL)-CYCLOHEXANE DILACTATE, ON CANDIDA ALBICANS

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Abstract. The antimycotic action of 1, 4-bis-(m, m'-amidinophenoxyethyl)-cyclohexane dilactate (MAC), a synthetic diamidine compound, on *Candida albicans* was studied. The minimum inhibitory concentration (MIC) ranged from 3.31 to 6.25 $\mu\text{g/ml}$ against both standard and clinically isolated strains. MAC was fungistatic at MIC and weakly fungicidal at the concentration of 100 $\mu\text{g/ml}$. MAC did not affect the cell wall or cause cell lysis. Intracellular constituents, such as 260 nm and 280 nm absorbing materials, were released from the cells by treatment with MAC indicating that MAC affected membrane permeability. The release of 260 nm absorbing material was inhibited by the presence of Ca^{2+} and Mg^{2+} . Acidic phospholipids such as cardiolipin and phosphatidylglycerol inhibited the anti-*Candida* activity of MAC, but sterols and lecithin were not inhibitory, indicating that MAC interacted with acidic phospholipids of the cell membrane. Freeze-fracture electron microscopy showed that MAC caused aggregation of membrane particles and patch formation on the P face, which may indicate that MAC is a membrane disrupting agent. It appeared that MAC affected *C. albicans* at the cell membrane by interacting with acidic phospholipids and caused disorganization of the membrane structure resulting in the release of intracellular constituents without lysis.

Key words : diamidine compound, *Candida albicans*, fungistatic, membrane disruption.

β -Lactam antibiotics and other useful antibacterial agents have been actively developed and have made a great contribution to the treatment of infectious bacterial diseases. However opportunistic infections such as candidiasis and cryptococcosis have tended to increase as a result of the use of antibacterial agents. For the treatment of these mycoses, 5-fluorocytosine (1) and imidazole compounds such as clotrimazole (2, 3), miconazole (4, 5, 6) and econazole (6, 7, 8) have been used along with the development of polyene antibiotics such as amphotericin B and nystatin.

1, 4-bis-(m, m'-amidinophenoxyethyl)-cyclohexane dilactate (MAC) (Fig. 1), which was found in the process of screening the anti-*Candida* activity of diamidine compounds, showed the strongest effectiveness against the *Candida* species and also some effect against Gram-positive bacteria and *Trichomonas vaginalis* *in vitro* (9).

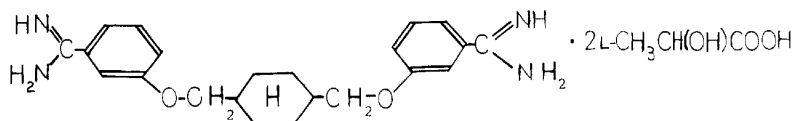


Fig.1 Chemical structure of 1, 4-bis-(m,m'-amidinophenoxymethyl)-cyclohexane dilactate.

The chemotherapeutic effectiveness of diamidine compounds against bacteria, fungi, *Trypanosoma* and *Pneumocystis carinii* has been widely examined (10-16). However, the mechanism of the action of these compounds on fungi (15, 23) has not been studied extensively (17-22).

This study was attempted to make clear the mode of action of MAC on *C. albicans*.

MATERIALS AND METHODS

Organisms. Stock strains and clinical isolates were maintained on Sabouraud glucose agar slants. *Candida albicans* AHU 3656 was used as a test organism throughout the study.

Chemicals. Sterols and phospholipids were purchased from Sigma Co. (U.S.A.), and all reagents used were of special grade. MAC was kindly supplied by Eisai Co., Ltd. (Tokyo).

In vitro anti-Candida activity. Each concentration of MAC solution was prepared with Sabouraud glucose agar medium by the twofold serial dilution method. Inoculum was prepared in 10 ml sterile saline by suspending two loopsful of *C. albicans* cultured on Sabouraud glucose agar slants at 37°C for 20 h. One loopful of the inoculum suspension was streaked on the surface of the test medium, and the minimum inhibitory concentration (MIC) was determined after incubation at 37°C for 48 h.

Effect on growth. (A) 0.2 ml of inoculum suspension in saline (2.5×10^5 cells/ml), which was prepared by suspending four loopsful of *C. albicans* cultured on Sabouraud glucose agar slant at 37°C for 18 h, was added to 7 ml of Sabouraud glucose liquid medium in the culture vessel of a Biophotometer. Then, 0.8 ml of MAC dissolved in water to give the required concentration was put into the culture vessel. Each of the test vessels was incubated at 37°C with gentle agitation and the growth curves were recorded turbidimetrically using a Biophotometer (JASCO BIO-LOG II). (B) The experiment was carried out under the same conditions as described in experiment (A), except for the inoculum concentration (1.25×10^7 cells/ml, final) and the addition of MAC at the middle of the exponential growth phase. (C) One ml of inoculum suspension was added to 18 ml of Sabouraud glucose liquid medium and the incubation was started. After 4.5, 7.5 and 10 h, 1 ml of MAC was added to each culture Monod tube to give a final concentration of 12.5 µg/ml. The growth was measured turbidimetrically at 650 nm.

Viable cell count. One ml of inoculum suspension, which was precultured at 37°C for 18 h, was added to 18 ml of Sabouraud glucose liquid medium in a Monod tube. Next, 1 ml of MAC dissolved in water was added to give a final concentration of 6.25 and 100 µg/ml. The test tubes were incubated at 37°C with gentle shaking. After incubation for 2 and 4 h, a 0.2 ml sample from each tube was diluted with 1.8 ml of sterile saline. Cell suspensions were serially diluted with sterile saline and 0.1 ml of each diluent was spread on Candida GS agar plates (Eiken). After incubation at 37°C for 48 h, colony numbers were counted to calculate the viable cell numbers per ml of suspension.

Measurement of cell lysis. Cells cultured in Sabouraud glucose liquid medium for 21 h were

harvested by centrifugation, washed twice with deionized water, then suspended in 0.02 M citrate buffer (pH 5.8) to give a cell concentration of about 0.3 in absorbance at 650 nm. MAC was added to the suspension to give a final concentration of 6.25 and 100 $\mu\text{g/ml}$. Each sample was incubated at 37°C with gentle shaking and the absorbance at 650 nm was measured at appropriate intervals. The lytic activity was expressed as the percentage of the decrease in absorbance compared with the initial absorbance.

Leakage of intracellular constituents. The harvested cells were suspended in 0.02 M citrate buffer (pH 5.8) to give a viable cell number of *C. albicans* of about 10^8 cells/ml. MAC was added at the final concentration of 6.25, 12.5 and 25 $\mu\text{g/ml}$ and the mixture was incubated up to 2 h at 37°C. An aliquot was withdrawn every 30 min, centrifuged at 6,000 rpm for 20 min, and the absorbance at 260 nm and 280 nm of the supernatant was measured. The same concentration of MAC was added to the blank to avoid the effect of MAC around the absorbance wave length. When the effect of Ca^{2+} or Mg^{2+} was examined, CaCl_2 or MgSO_4 was added to the buffer at the final concentrations of 1, 2.5, 5, 10 and 20 mM and MAC was added at a final concentration of 100 $\mu\text{g/ml}$.

Effect of phospholipids. The effect of phospholipids and sterols on the anti-*Candida* activity of MAC was examined using the agar-disc plate method. The disc (Toyo Roshi, 8 mm in diameter) containing 40 or 50 μg of MAC was prepared. Phospholipids in various concentrations (dissolved in 50 μl chloroform or chloroform-methanol, 3:1 v/v) or sterol (dissolved in 50 μl ethanol) was absorbed into the disc. MAC is soluble in water but not in the solvents. The solvents used here did not decompose MAC. The discs were dried at 37°C for 1 h and placed on glucose agar in which *C. albicans* had been inoculated beforehand. The inoculated agar was prepared as follows: *C. albicans* was cultured on a Sabouraud glucose slant overnight. Four loopsful of the culture were suspended in 10 ml of saline. Sixty μl of the suspension was mixed with 20 ml of Sabouraud glucose agar just before solidification of the agar.

The inhibitory zone of cell growth around the disc was measured after incubation at 37°C for 18 h and the diameter of the zone was compared among the discs containing different concentrations of lipids. The effectiveness was expressed as a decrease of the diameter (mm) compared with the control.

Electron microscopy. The pelleted cells were immediately frozen on a specimen holder by immersion into liquid nitrogen. Freeze-fracture was performed by a FD-2 (Hitachi) at -110°C. The replicas were washed with diluted sodium hypochlorite solution and the specimens were examined under a Hitachi H-500 electron microscope at an accelerating voltage of 75 kV.

RESULTS

Anti-Candida activity of MAC. The minimum inhibitory concentration (MIC) of MAC against seven strains of *C. albicans* is shown in Table 1. Anti-*Candida* activity of MAC was comparatively strong and there was no difference in MICs between stock strains and clinical isolates.

Effect of MAC on growth. The effect of MAC on the growth of *C. albicans* is shown in Fig. 2. When it was added to the medium before inoculation, the growth rate was inhibited at a concentration of 6.25 $\mu\text{g/ml}$ (MIC). Maximum growth was attained at this concentration but it took longer than the control. This pattern of inhibition is similar to the penicillin effect on cocci at MIC. At the concentration of 25 $\mu\text{g/ml}$, growth was inhibited completely (Fig. 2A). When it was added

at the middle logarithmic growth phase, as shown in Fig. 2B, MAC inhibited the growth to some extent at the concentration of 25 $\mu\text{g/ml}$ and completely at the concentration of 100 to 200 $\mu\text{g/ml}$. When MAC (12.5 $\mu\text{g/ml}$) was added at each growth phase as shown in Fig. 3, it was more effective when added at an earlier growth phase than at a later phase. MAC added at the middle logarithmic growth phase at the concentration of 6.25 $\mu\text{g/ml}$ (MIC) failed to inhibit the growth rate.

TABLE 1. MINIMUM INHIBITORY CONCENTRATION OF MAC ON *C. ALBICANS*

Strains	MIC ^a ($\mu\text{g/ml}$)
<i>Candida albicans</i> AHU 3656	6.25
<i>C. albicans</i> Yu-1200	3.13
<i>C. albicans</i> IFM 4020	3.13
<i>C. albicans</i> E7*	3.13
<i>C. albicans</i> E8*	6.25
<i>C. albicans</i> E9*	6.25
<i>C. albicans</i> E10*	6.25
<i>C. albicans</i> E11*	6.25

* Clinically isolated strains

^a MIC (minimum inhibitory concentration) of MAC in Sabouraud glucose agar medium. Inoculated plates (detailed under methods) were examined to determine the extent of growth after incubation at 37°C for 48 h.

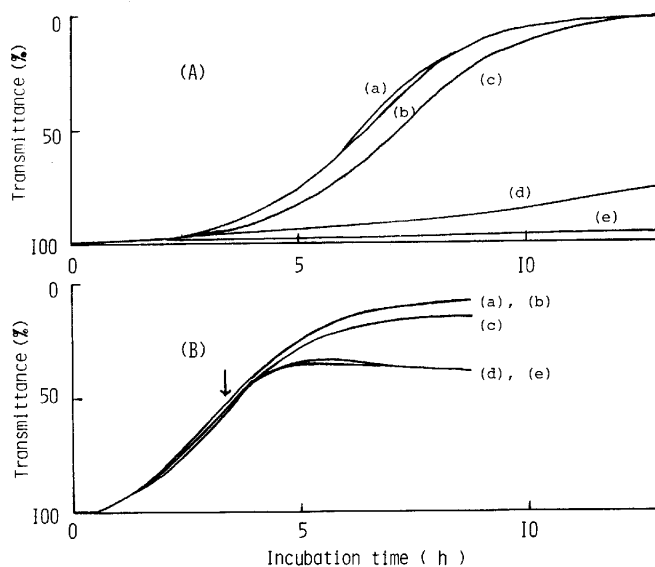


Fig. 2. Effect of MAC on the growth of *Candida albicans* in Sabouraud glucose liquid medium. (A) MAC was added to the medium before the inoculation of the cells. (a) control, (b) 3.13 $\mu\text{g/ml}$, (c) 6.25 $\mu\text{g/ml}$, (d) 12.5 $\mu\text{g/ml}$ and (e) 25 $\mu\text{g/ml}$. (B) MAC was added to the culture media at the middle of the log phase (arrow). (a) control, (b) 6.25 $\mu\text{g/ml}$, (c) 25 $\mu\text{g/ml}$, (d) 100 $\mu\text{g/ml}$, (e) 200 $\mu\text{g/ml}$.

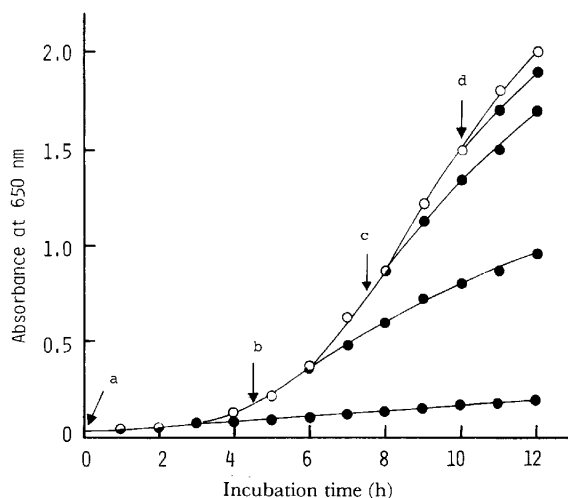


Fig. 3. Effect of MAC on each growth phase of *Candida albicans*. MAC (12.5 µg/ml, final) was added at times indicated by arrows. The drug was added at (a) lag phase, (b) early log phase, (c) middle log phase, (d) late log phase and (e) control. Symbols : ○, control ; ●, 12.5 µg/ml.

Effect on the viability. MAC inhibited the proliferation of *C. albicans*, as monitored by the change in optical density (Figs. 2 and 3). However, the optical density does not always coincide with the viable cell number in the medium. The viable cell count did not decrease by treatment with MAC at the concentration of 6.25 µg/ml for 4 h. At 100 µg/ml, the viable cell count decreased slightly but not markedly. The result indicates that MAC acts fungistatically at the concentration of 6.25 µg/ml (MIC) and as a weak fungicide at the concentration of 100 µg/ml when the cell density was less than 1.2×10^5 /ml (Table 2).

Effect on cell lysis. Fig. 4 shows the effect of MAC on cell lysis of *C. albicans* suspended in 0.02 M citrate buffer (pH 5.8). The percentage of the decrease in absorbance after 120 min incubation with various concentrations of MAC was as follows : 4.5 % without MAC, 7.9 % with 6.25 µg MAC/ml, and 15.7 % with

TABLE 2. EFFECT OF MAC ON THE VIABILITY OF *CANDIDA ALBICANS*

Incubation time (h)	Viable cell count per ml		
	Control	MAC Concentration ^a	
		6.25 µg/ml	100 µg/ml
0	1.2×10^5	1.2×10^5	1.2×10^5
2	1.2×10^5	1.2×10^5	3.5×10^4
4	2.7×10^5	1.5×10^5	2.1×10^4

^a MAC (1,4-bis-(m,m'-amidinophenoxy)methyl)-cyclohexane dilactate) concentration in Sabouraud glucose liquid media. Viable cell number was measured as described under methods.

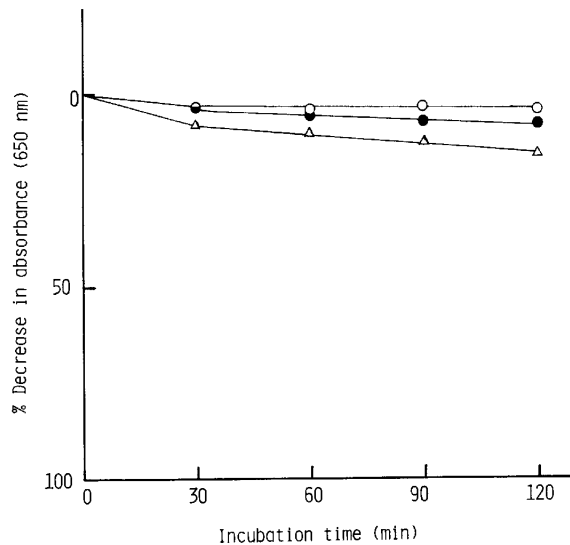


Fig. 4. Effect of MAC on the lysis of *C. albicans* in 0.02 M citrate buffer. (pH 5.8)
 Symbols : ○, control; ●, 6.25 µg/ml; △, 100 µg/ml.

100 µg MAC/ml (Fig. 4). This result, together with the data in Table 2, demonstrates that MAC did not cause cell lysis or induce autolysis of *C. albicans*.

Effect on cell permeability. The harvested and washed cells were suspended in 0.02 M citrate buffer (pH 5.8), then incubated at 37°C in the presence of various concentrations of MAC. During incubation, samples were taken and centrifuged. The resulting supernatants were examined for soluble substances released from the cells. Fig. 5 shows the changes in absorbance at 260 and at 280 nm of the supernatant. The absorbance increased both at 260 and 280 nm with the increase of the incubation period. Higher concentrations of MAC released more absorbing materials, indicating that the drug affected cell permeability and caused leakage of the intracellular components in proportion to the drug concentration. The finding coincided with the result of growth inhibition.

The effect of Ca^{2+} and Mg^{2+} on permeability was examined assuming that these divalent cations acted as membrane stabilizers. Both Ca^{2+} and Mg^{2+} clearly inhibited the release of 260 nm absorbing materials (Fig. 6). (The release of 280 nm absorbing materials showed the same pattern as 260 nm absorbing materials.) Ca^{2+} inhibited the release at a lower concentration than Mg^{2+} . The higher potency of Ca^{2+} remains to be explained. The result suggested that both Ca^{2+} and Mg^{2+} protected the membrane from the effect of MAC.

Effect of sterols or phospholipids on fungistatic activity. Various concentrations of sterols and phospholipids were mixed with MAC on paper discs as mentioned in the methods section, and the discs were tested on the inoculated agars to see if any inhibition of the growth of *C. albicans* was observed. This method is based on

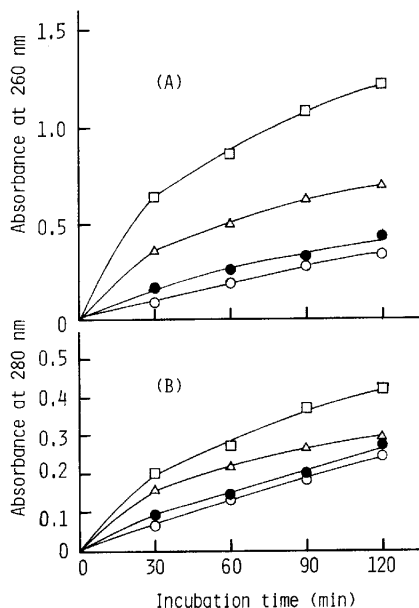


Fig. 5. Effect of MAC on the leakage of 260 nm absorbing materials (A) and 280 nm absorbing materials (B) from *Candida albicans*. Conditions are described in the text. Symbols : ○, control; ●, 6.25 μg/ml; △, 12.5 μg/ml; □, 25 μg/ml.

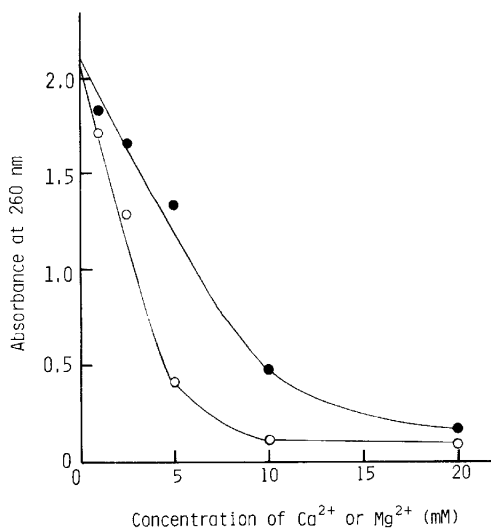


Fig. 6. Effect of Ca^{2+} or Mg^{2+} on the leakage of 260 nm absorbing materials from *Candida albicans*. Symbols : ○, CaCl_2 ; ●, MgSO_4 .

the assumption that MAC diffuses into the agar because it is water soluble, but cannot diffuse into the agar if it binds to lipids because lipids cannot penetrate into the agar.

Ergosterol and cholesterol did not interfere with the activity of MAC, but phosphatidylglycerol and cardiolipin showed some inhibition (Table 3). Phosphatidylcholine did not show any effect suggesting that only acidic phospholipids have

TABLE 3. EFFECT OF STEROLS AND PHOSPHOLIPIDS ON THE FUNGISTATIC ACTIVITY OF MAC

Sterols and phospholipids ($\mu\text{g}/\text{disc}$) ^a	Inhibitory zone (mm) ^b	Decrease (mm)
Ergosterol		
0	15.2	—
10	15.2	0
100	15.2	0
1000	15.2	0
Cholesterol		
0	17.8	—
10	17.8	0
100	17.8	0
1000	17.8	0
Phosphatidyl glycerol ^c		
0	17.8	—
19	17.3	0.5
56	17.0	0.8
167	16.0	1.8
500	8.0	Complete inhibition
Cardiolipin ^d		
0	17.7	—
6	17.7	0
19	17.1	0.6
59	16.6	1.1
175	14.1	3.6
Lecithin ^e		
0	15.8	—
10	15.8	0
20	15.8	0
40	15.8	0
80	15.8	0

^a The concentration of MAC was 50 $\mu\text{g}/\text{disc}$, except lecithin in which the concentration of MAC was 40 $\mu\text{g}/\text{disc}$.

^b The data shows the average of duplicated experiments.

^c from egg ^d from bovine heart ^e from bovine liver

an affinity with MAC. The result indicates that MAC may affect the membrane of *C. albicans* by virtue of interacting with membrane acidic phospholipids.

Electron microscopic study. The effect of MAC on the cell membrane of *C. albicans* was examined by electron microscopy using a freeze-fracture method.

The intact cell membrane showed some small invaginations and ripple like surface structures on both the P and E faces (Photo. 1a and 1c). The membrane particles of the P face (Photo. 1b) showed a dense and uniform distribution. The E face, on the contrary, showed sparsely distributed particles on the surface (Photo. 1d).

The MAC treated cells showed marked changes compared with the intact. The membrane particles on the P face aggregated and particle free areas (patches) appeared randomly (Photo. 2). The density of the particles seemed to decrease and, in some areas, the particles were arranged in a crystal-like regular pattern (Photo. 2d). The change on the E face was not clear because the particles were so sparse. These changes were common among 60 to 70 % of the cells which were exposed to MAC. The number of cells showing these changes increased with the incubation period at higher concentrations of MAC.

These changes in the membrane structure may be brought about by the rearrangement of the lipid bilayer and membrane proteins caused by the interaction of MAC with the membrane phospholipids.

DISCUSSION

The mode of action of diamidine compounds with regard to bacteria has been studied by many investigators (17-22), but very few have reported on the mode of action on fungi (15, 23). In this report, the author attempted to make clear the mode of action of a diamidine compound, 1, 4-bis-(m, m'-amidino-phenoxy-methyl)-cyclohexane dilactate (MAC), on *Candida albicans*. MAC did not show any marked effect on the viability of the cell, but it acted as a fungistatic agent at the concentration of 6.25 $\mu\text{g}/\text{ml}$ and as a weak fungicide at the concentration of 100 $\mu\text{g}/\text{ml}$. MAC did not affect the cell wall or cause any cell lysis. It acted on the cell membrane probably by interacting with membrane phospholipids and altered the permeability of the membrane by disorganization of the membrane structure resulting in the leakage of intracellular components.

Some antimicrobial agents have been reported to affect the cell membrane of fungi. Among them are polyene antibiotics (amphotericin B and nystatin (25, 26)), azalomycin F (27), pyrrolnitrin (28), imidazole antimycotics (miconazole (30, 31) and clotrimazole (24, 32)), polypeptide antibiotics (polymyxin B and colistin (29)) and hexachlorophene (33). Polyene antibiotics interact with sterols of susceptible fungal cell membranes. Intracellular low molecular weight substances (electrolytes, glucose etc.) start to leak first, followed by high molecular substances (protein, nucleic acid etc.) (25, 26). During the leakage of potassium and sugar, intracellular pH becomes acidic by replacement of potassium with hydrogen ion. Subsequently various hydrolase enzymes, especially ribonuclease, are activated resulting in the progression of autolysis. Pentamidine isothionate inhibits RNA and protein synthesis of *C. albicans* but does not act as a fungicide (23). Clotrimazole inhibited the synthesis of protein, RNA and DNA and at the same time altered the membrane permeability to cause the activation of RNase in a manner similar to polyene (24). The mode of action of MAC resembled polyene antibiotics which bound to sterols in the membrane, except that MAC seemed to bind to acidic phospholipids in the membrane.

The antagonistic effect of phospholipids on membrane disruption by antimicrobial agents such as diamidine compounds (15, 22), polyene antibiotics (34), clotrimazole and miconazole (38, 39, 40), mycobacillin (35, 36), synthetic detergents (37) and azalomycin F (38) have been studied. The fungistatic (15) and bacteriostatic actions (22) of propamidine were antagonized by soya lecithin. The anti-*Candida* activity of clotrimazole or miconazole was inhibited by phospholipids with unsaturated fatty acid chains (40). These reports suggest that the drugs mentioned above interact with phospholipids preferentially. MAC activity on *C. albicans* was antagonized by phosphatidylglycerol and cardiolipin, but not by lecithin. This indicates that MAC has a higher affinity for acidic phospholipids than other phospholipids which may explain why MAC is not strongly fungicidal against *C. albicans*.

Ca^{2+} or Mg^{2+} protected MAC induced leakage of intracellular materials from *C. albicans*. These ions inhibited the effects of miconazole on *C. albicans* (44), candidin on *Saccharomyces* sp (43) and so on. The role of these cations has not been made clear, but the author suspects that cations protect the membrane from attack by the membrane disrupting agents.

Disorganization of the membrane structure of *C. albicans* by MAC was visualized by freeze-fracture electron microscopy. The major structural changes in the membrane were aggregation of the membrane particles and formation of particle free areas (patches). Similar observations have been reported for the effect of econazole on *Saccharomyces* sp. (41) and the effect of amphotericin B on *Epidermophyton floccosum* (42). Patch formation is one common feature of cell membranes disrupted by various factors. MAC may be such a membrane disrupting agent which interacts with the membrane and then modifies its structure. Consequently, the cell membrane may be made permeable to intracellular macromolecules.

In conclusion, MAC acted on *C. albicans* as a fungistatic agent or as a weak fungicide, and the activity was affected by acidic phospholipids and divalent cations. The mode of action of MAC was postulated to be the disruption of the membrane.

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LEGENDS TO PHOTOS

- Photo. 1. Electronmicrographs of freeze-fractured replica of intact *C. albicans*.
- 1-a. P-face of the fractured replica. Small invaginations and ripple-like surface structure are evident. The inserted Bar indicates 1 μ m.
 - 1-b. High magnification micrograph of the P-face, showing uniform and dense distribution of membrane particles. Bar : 0.2 μ m.
 - 1-c. E-face of the fractured replica. Small invaginations show complementary convexed structures of the P face. Bar : 1 μ m.
 - 1-d. High magnification micrograph of the E face showing a sparse distribution of the particles. Bar : 0.2 μ m.
- Photo. 2. Electron micrographs of freeze-fracture replica of *C. albicans* exposed to MAC for 4h at 37°C.
- 2-a. P face of the fractured replica of the cell exposed to MAC at the concentration of 100 μ g/ml. The surface is irregular. Bar : 1 μ m.
 - 2-b. High magnification micrograph of P face of the cell exposed to 6.25 μ g MAC/ml. The distribution of membrane particles is less dense than shown in Photo. 1-b and many particle free areas are seen. Bar : 0.2 μ m.
 - 2-c. P face of the cell exposed to 100 μ g MAC/ml. The membrane particle aggregation and apperance of particle free areas are more distinct than those seen in Photo. 2-b. Bar : 0.2 μ m.
 - 2-d. P face of the cell treated with MAC 100 μ g/ml. A crystalline-like pattern of the particles is shown.

