OUKA
The University of Osaka
Institutional Knowledge Archive

Title	Morphological Studies on the Mode of Action of Nystatin on Candida albicans
Author(s)	Fujino, Tsunesaburo; Kamiki, Teruo; Akita, Yoshiya et al.
Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1962, 5(4), p. 233-238
Version Type	VoR
URL	https://doi.org/10.18910/83015
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

The University of Osaka

BIKEN JOURNAL 5, 233-238 (1962)

Morphological Studies on the Mode of Action of Nystatin on *Candida albicans*

TSUNESABURO FUJINO, TERUO KAMIKI, YOSHIYA AKITA AND MASASHI AKAGI

Department of Bacteriology, The Research Institute for Microbial Diseases, Osaka University, Osaka
(Received for publication, December 18, 1962)

SUMMARY

The action of nystatin on *Candida albicans* was studied morphologically. The results obtained were as follows:

1) When cells are treated with trichomycin or amphotericin B, like intact cells, they are gram positive, but when treated with nystatin they became gram negative.
2) Electron microscopy showed that the surfaces of the cells were affected by nystatin treatment and the nuclear apparatus especially was damaged severely.
3) Cell walls isolated from intact cells were severely damaged by nystatin treatment.
4) About 28 per cent of the total DNA was released from the cells by nystatin treatment.

These results show that not only the cell walls but also the internal structures of the cells are damaged by nystatin.

INTRODUCTION

Nystatin, one of a group of polyene antifungal antibiotics discovered by Hazen and Brown (1951), inhibits growth of a wide variety of fungi at concentrations of between 1 and 10 μg per ml, but does not affect growth of bacteria, rickettsia, viruses, or animal cells even at higher concentrations. The antibiotic is fungistatic at low concentrations and fungicidal at high concentrations. The mode of action of this antibiotic has not yet been clarified morphologically.

Lampen *et al.* (1957) and Bradley (1958) reported that, at about the same concentrations as those required to eliminate growth of various organisms, nystatin inhibited the endogenous respiration and utilization of various substrates including glucose by yeasts and other fungi. In 1959, Lampen *et al.* found that nystatin was absorbed from aqueous solution by sensitive fungi, but not by insensitive organisms and concluded that the absorption of nystatin by fungal cells was an essential step in the action of this drug. Recently, Marini *et al.* (1961), Sutton *et al.* (1961) and Kinsky (1961, 1962) described the changes occuring in nystatin treated cells

Most of this paper was given at the Annual Meeting of the Japanese Bacteriological Society on April 7, 1961 at Yokohama

and protoplasts of sensitive organisms, with particular emphasis to the indications of altered cell membrane permeability.

These interesting properties of the polyene antifungal antibiotic prompted us to a morphological investigation of its effect on pathogenic fungi. The work presented here is concerned with the effect of nystatin on *Candida albicans*.

MATERIALS AND METHODS

Organism

Candida albicans FIA 1048, isolated from a case of human candidiasis and stock-cultured in the Research Institute for Microbial Diseases, Osaka, Japan, was used for the present study.

Antibiotics

Trichomycin (commercial crystalline preparation with a potency of 3850 units per mg) was kindly supplied from the Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan. Nystatin (commercial crystalline preparation with a potency of 3770 units per mg) and amphotericin B (the commercial vials employed in this study, contained 50 mg of amphotericin B and approximately 41 mg of sodium desoxycholate, buffered with sodium phosphate) were obtained commercially from the E. R. Squibb & Sons Division, Olin Mathieson Chemical Corp., U. S. A. Stock solutions of these antibiotics were made by dissolving nystatin or trichomycin in 80 per cent n-propylalcohol and amphotericin B in 10 per cent dimethyl sulfoxide, to give a final concentration of 1 mg per ml. Subsequent dilutions were made with M/15 phosphate buffer (pH 7.0). The effect of the solvents at the concentrations used on the growth of *C. albicans* was negligible.

The sensitivities of this organism to the antifungal antibiotics in Sabouraud glucose broth (pH 7.0) are shown as follows:

Antibiotic	Nystatin	Trichomycin	Amphotericin B
Standard culture	2.2 μg/ml	0.3 μg/ml	2.5 μg/ml
Shaking culture	4.4 μg/ml	0.6 μg/ml	3.5 μg/ml

The minimal growth inhibitory concentrations of antifungal antibiotics for *Candida albicans* FIA 1048 in Sabouraud glucose broth at 35°C over a period of 72 hours.

Cell preparation

C. albicans FIA 1048 was cultured in Sabouraud glucose broth on a reciprocating shaker (120 strokes per minute) for 18 hours at 35°C. The cells were washed three times with M/15 phosphate buffer (pH 7.0) and before use were resuspended in the same buffer and adjusted to an optical density of the cell suspension of 0.24 at 550 mμ (Coleman spectrophotomer Junior type). This cell preparation contained 4.8×10^4 cells per ml.

Treatment of the organism with antifungal antibiotics for morphological studies

Two ml of stock solutions (containing one mg per ml) of these antibiotics were diluted with 78 ml of M/15 phosphate buffer (pH 7.0) and then 20 ml of the above cell preparation was added. The final concentration of these antibiotics was 20 μg per ml. The mixture was incubated at 35°C for 3 hours.

Cell staining

The method of Bartholomew and Mittwer (1952) was employed for Gram staining, and the method of Webb (1954) for cell wall staining. The Feulgen technique was carried out by the method of Rafalko (1946) with a slight modification.

Observation by electron microscopy

Cell fixation was performed in 1 per cent osmium tetraoxide or 0.6 per cent potassium permanganate buffered with 0.34 M isotonic veronal acetate buffer solution (pH 7.2), prepared accord-

ing to the method of Michaelis (1931), for one and half hours at room temperature. Specimens were dehydrated by serial passage through a graded ethyl alcohol series. Before each change of alcohol, the material was centrifuged at 1500 rpm for 5 minutes and between changes the centrifuge tube was agitated by hand to disperse the fixed cells. Partially polymerized butyl methacrylate containing 1 per cent benzoyl peroxide as initiator was used for embedding. The specimens were placed in gelatin capsules and polymerized at 55°C for 36 hours. Ultrathin sections were cut with a glases knife using a Porter-Blum microtome and micrographs were obtained with a JEM 5Y electron microscope (Japan Electron Opticus Laboratory Co., Ltd.). For morphological observation of the cell surface, the carbon replica method of Bradley modified as described by Suganuma (Suganuma, 1956) was used.

Isolation of cell walls

Washed cells were suspended in M/15 phosphate buffer (pH 7.0) and disrupted in a sonic disintegrator (Kubota 10 KC) for 6 hours. After the cell suspension had been centrifuged, the cell wall fraction was resuspended in trypsin solution (0.5 mg/ml) buffered with 0.05 M phosphate buffer (pH 7.6) and digested for 3 to 4 hours at 35°C.

Chemical determination of cell constituents

After nystatin treatment, the supernatant of the cell suspension was concentrated by lyophilization and the DNA, RNA and protein were estimated by the methods of Burton (1956), Dische and Schwartz (1937) and Folin's method modified by Lowry (1951), respectively.

RESULTS

1. *Cytochemical observations on nystatin-treated cells*

1) *Gram stain*

Intact cells and cells heated at 80°C for 30 minutes are Gram positive, but after nystatin treatment more than 90 per cent of the cells became Gram negative (Fig. 1A and B). No such change in Gram stainability could be observed either in trichomycin treated cells or in amphotericin B treated cells both of which remained Gram positive.

2) *Cell wall stain*

As shown in Fig. 1C, the cell walls of intact cells and cells heated at 80°C for 30 minutes, and even those of cells treated with trichomycin or amphotericin B stained well with crystalviolet. In cells treated with nystatin, the cytoplasm as well as the cell walls were homogenously stained by crystalviolet so that there was no sharply defined cell wall apparent (Fig. 1D).

3) *Observation of the nuclear site by the Feulgen technique*

The Feulgen reaction in intact cells showed that the nuclear site was laterally located in the cell. In nystatin-treated cells, however, no distinctive differences between the nuclear site and the cytoplasm would be distinguished.

These results suggest that the antifungal effect of nystatin is closely associated with damage to the cell wall and nuclear site. Thus the following observations were made by electron microscopy and the DNA, RNA and protein of the cells and supernatant measured.

2. *Electron microscopical observation of nystatin-treated cells*

1) *Carbon replica method*

Comparatively regular undulations were seen on the surface of untreated cells. But in nystatin-treated cells these undulations could not be seen. Moreover the surface of intact cells appeared to be smoother than that of nystatin-treated cells (Fig. 1E and F).

2) *Ultrathin sections*

In intact cells, the cell wall, cytoplasmic membrane, ergastoplasm-like structure and mitochondria-like membraneous structure could be distinguished. The nucleus surrounded by a definite membrane (width: 100 to 300 Å) could be seen and this seemed to correspond to the Feulgen positive site (Fig. 2A and B). On the other hand, in nystatin-treated cells, cell walls were thinner than those of intact cells and occasionally they showed defects. The cytoplasm was generally less electron dense than that of intact cells. Severe damage of the nuclear site was also observed (Fig. 2C, D, E).

3) *Effect of nystatin on isolated cell wall preparations*

Fig. 3 is an electron microgram of a cell wall preparation from nystatin treated cells after sonic disintegration and trypsin digestion. In the control figure (Fig. 3A), the cytoplasm of the cell appears to have just slipped out, causing only slight damage to the cell wall. However the cell wall preparation from nystatin treated cells appears severely damage. Similar damage was observed when an isolated cell wall preparation from intact cells was treated with nystatin.

3. *Chemical studies on the reaction mixture*

After nystatin treatment, the DNA, RNA and protein contents of the supernatant of the reacting mixture were estimated. Results were shown in Table 1 (see Materials and Methods).

Table 1. DNA, RNA and Protein in the Supernatant of the Reacting Mixture after Nystatin Treatment

	After nystatin treatment	Control
DNA	28.3 %	0 %
RNA	10.2 %	4.6 %
Protein	3.1 %	2.1 %

% : expressed as a percentage of the total content of the reaction mixture

About 28 per cent of the total DNA was released from the cells by nystatin treatment, while little RNA or protein was released.

DISCUSSION

Morphological studies on nystatin-treated fungal cells have not previously been reported.

Marini *et al.* (1961) concluded from a study on the inhibition of yeast metabolism by nystatin that this antibiotic directly damaged the cell membrane and thus produced a rapid increase in its permeability to small ions. Kinsky (1962) also suggests that the cell membrane of fungi is the site of action of antifungal antibiotics. The present study shows further evidence that one of the modes of action of nystatin is the specific damage of the cell wall. As shown above, almost 100 per cent of yeast cells of *Candida albicans* after heating at 80°C for 30 minutes (no living cells could be detected by this treatment) were Gram-positive like intact cells and those treated with trichomycin or amphotericin B. But of the three fungicidal antibiotics tested, only nystatin had any effect on Gram- and cell wall-stainability of this organism. Moreover, the cell walls of whole cells and isolated cell wall preparations were severely damaged by nystatin.

On the other hand, changes in the Feulgen reaction in nystatin-treated cells and release of about 28 per cent of the total DNA into the medium after nystatin treatment suggest that nystatin severely damages the nuclear unit of *Candida albicans*. Electron microscopy shows that destruction of intracellular structures and especially of the nuclear unit is caused by nystatin treatment. However, it is yet uncertain whether the damage of the nuclear unit by nystatin-treatment is due to the direct action of this antibiotic or only to a secondary effect due to cell wall destruction.

In Fig. 2A, organelles having a typical mitochondrial structure can be seen. This photograph was shown at the 34th Annual Meeting of the Japanese Bacteriological Society on April 7, 1961 at Yokohama, Japan. At this meeting Nagata reported similar results. This electron photograph is the first demonstration of the mitochondrial structure of *Candida albicans*.

ACKNOWLEDGEMENTS

We are greatly indebted to Drs. K. Fukai, M. Yoneda, J. Kawamata, T. Miwatani and M. Kondo of the Institute for Microbial Diseases, Osaka University, and to Dr. T. Terada for help with the electron microscope.

REFERENCES

Bartholomew, J. W. and Mittwer, T. (1952). The gram stain. *Bacteriol. Rev.* **16,** 1-29.

Bradley, S. G. (1957). Effect of nystatin on *Candida stellatoidea*. *Antibiotics & Chemotherapy* **8,** 282-286.

Bradley, S. G. (1958). Interactions between phosphate and nystatin in *Candida stellatoides*. *Proc. Soc. Exptl. Biol. Med.* **98,** 786-789.

Brown, R. N. and Hazen, E. L. (1957) Present knowledge of nystatin, an antifungal antibiotic. *Trans. N. Y. Acad. Sci.* **19,** 447-456.

Burton, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid. *Biochem. J.* **62,** 315-322.

Dische, Z. and Schwartz, K. (1937). *Mikrochim. Acta* **2,** 13.

Harman, J. W. and Masterson, J. G. (1957). The mechanism of nystatin action. *Irish J. Med. Sci.* **378,** 249-253.

Hazen, E. L. and Brown, R. (1951). Fungicin, an antibiotic produced by a soil actinomycete. *Proc. Soc. Exptl. Biol. Med.* **76,** 93-97.

Kinsky, S. C. (1961). Alterations in the permeability of *Neurospora crassa* due to polyene antibiotics. *J. Bacteriol.* **82,** 889-897.

Kinsky, S. C. (1962). Effect of polyene antibiotics on protoplasts of *Neurospora crassa*. *J. Bacteriol.* **83,** 351-358.

Lampen, J. O., Morgan, E. R. and Slocum, A. C. (1956). Inhibition of sugar utilization by nystatin. *Federation Proc.* **15,** 295.

Lampen, J. O., Morgan, E. R. and Slocum, A. C. (1957). Effect of nystatin on the utilization of substrates by yeast and other fungi. *J. Bacteriol.* **74,** 297-302.

Lampen, J. O., Morgan, E. R., Slocum, A. and Arnow, P. (1959). Absorption of nystatin by microorganisms. *J. Bacteriol.* **78,** 282-289.

Lampen, J. O. and Arnow, P. (1959). Significance of nystatin uptake for its antifungal action. *Proc Soc. Exptl. Biol. Med.* **101,** 792-797.

Lowry, O. H. and Rosebrough, N. J., Farr, A. L. and Randall, R. T. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265.

Marini, F., Arnow, P. and Lampen, J. O. (1961). The effect of monovalent cations on the inhibition of yeast metabolism by nystatin. *J. Gen. Microbiol.* **24,** 51-62.

Michaelis, L. (1931). Der Acetat-Veronal-Puffer. *Biochem. Z.* **234,** 139-141.

Nagata, A. (1961). Studies on the fine structure of *Candida albicans J. Yonago Med. Assoc.* **13,** 4-24.

Rafalko, J. S. (1946). A modified feulgen technic for small and diffuse chromatin elements. *Stain Technology* **21,** 91-93.

Shatkin, A. J. and Tatum, E. L. (1959). Electron microscopy of *Neurospora crassa mycelia*. *J. Biophys. Biochem. Cytol.* **6,** 423-426.

Suganuma, A. (1956). Morphological studies on Micrococcus pyogenes var aureus by carbon replica technique. *Medicine and Biology* **39,** 173-176.

Sutton, D. D., Arnow, P. M. and Lampen, J. O. (1961). Effect of high concentrations of nystatin upon glycolysis and cellular permeability in yeast. *Proc. Soc. Exptl. Biol. Med.* **108,** 170-175.

Webb, R. B. (1954). A useful bacterial cell wall stain. *J. Bacteriol.* **67,** 252-253.

EXPLANATION OF PHOTOGRAPHS

Fig. 1 A) Gram stain of intact cells of *Candida albicans* (×2,000) showing gram positive stainbility.
B) Gram stain of nystatin treated cells (× 2,000) showing a change in gram stanability.
C) Cell walls of intact cells (× 2,000).
The cell walls can be seen clearly.
D) Cell walls of nystatin treated cells (× 2,000).
The cell walls do not stain distinctly.
E) Electron micrograph of intact cells by carbon replica method.
F) Electron micrograph of nystatin treated cells by carbon replica method.

Fig. 2 A) Electron micrograph of an intact cell.
N=Nucleus, L=Lipoidal inclusion, CW=Cell wall
M=Mitochondria like structure, CM=Cytoplasmic membrane
B) Electron micrograph of intact cell showing nucleus and some filamentous structure.
C), D) and E) Electron micrograph of nystatin treated cell showing damages to subcellular structures especially in the nucleus.

Fig. 3 A) Isolated cell wall from intact cell.
B) Isolated cell wall from nystatin treated cell
C) Nystatin treated cell wall

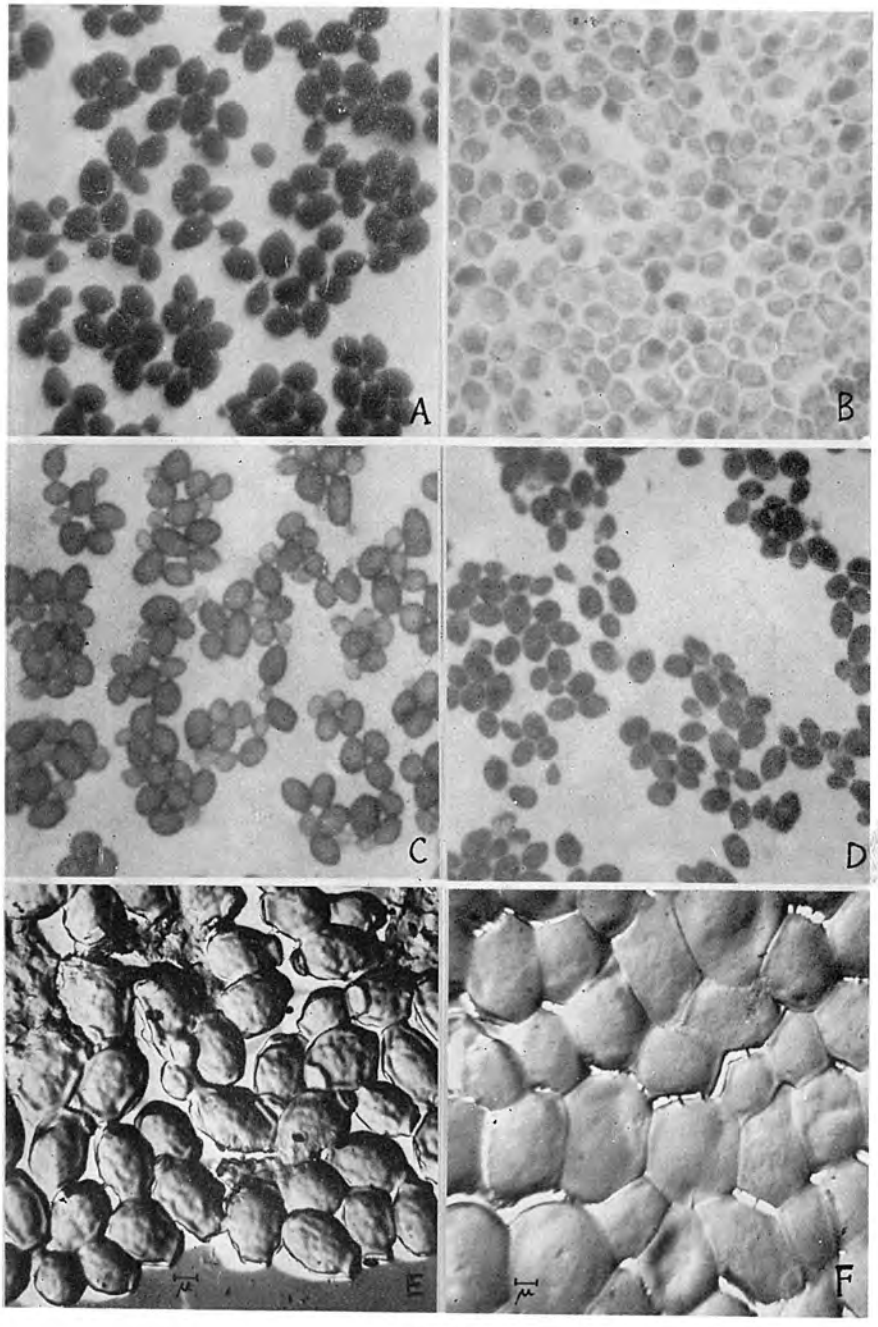


Fig. 1

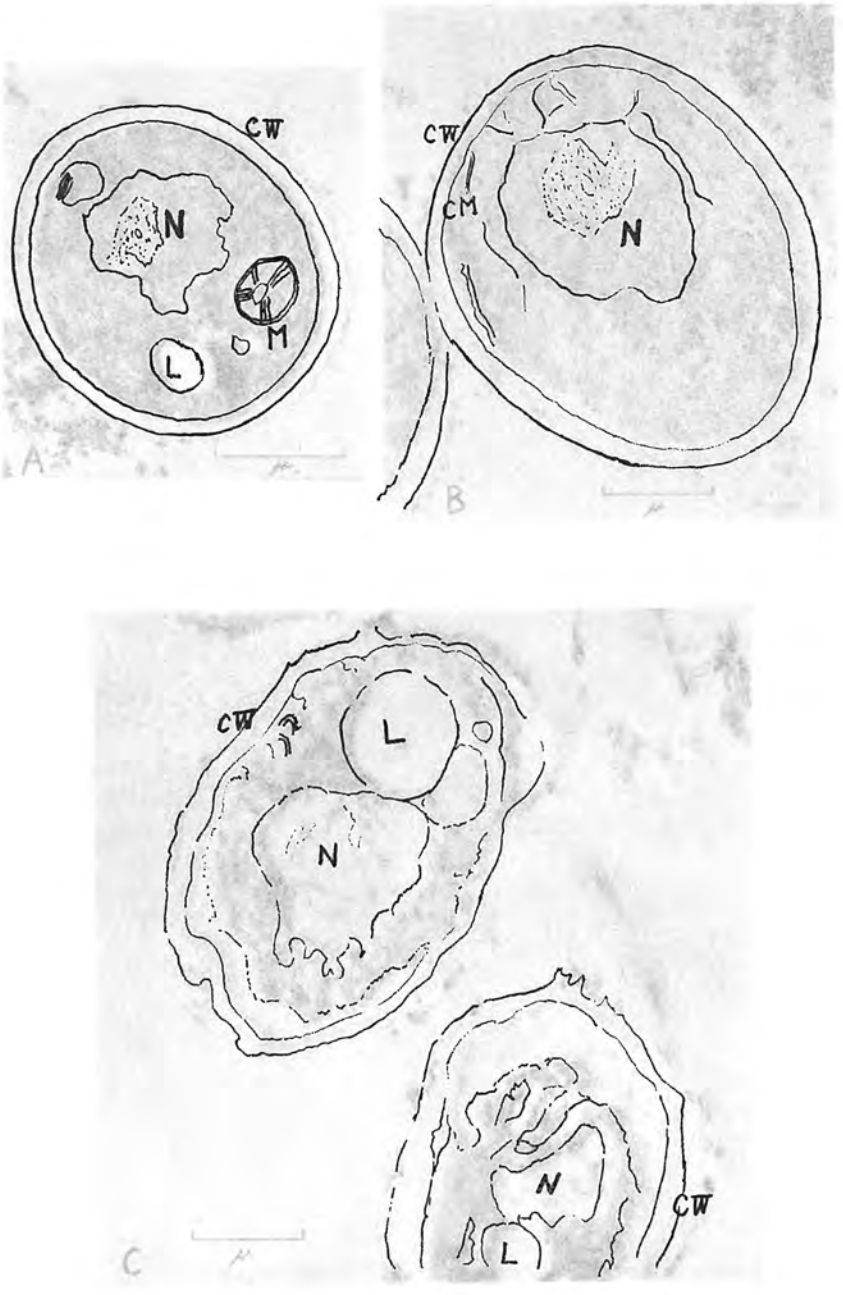


Fig. 2

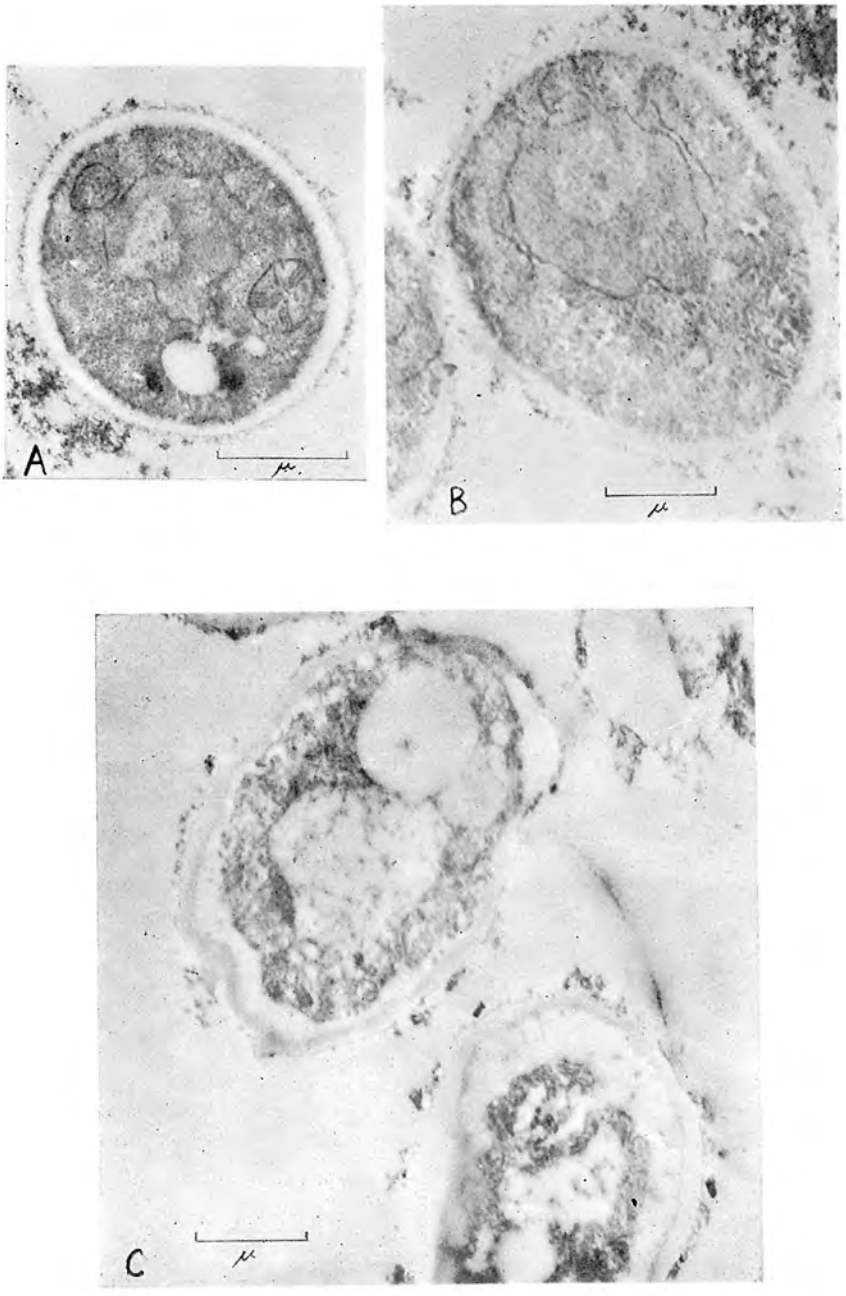
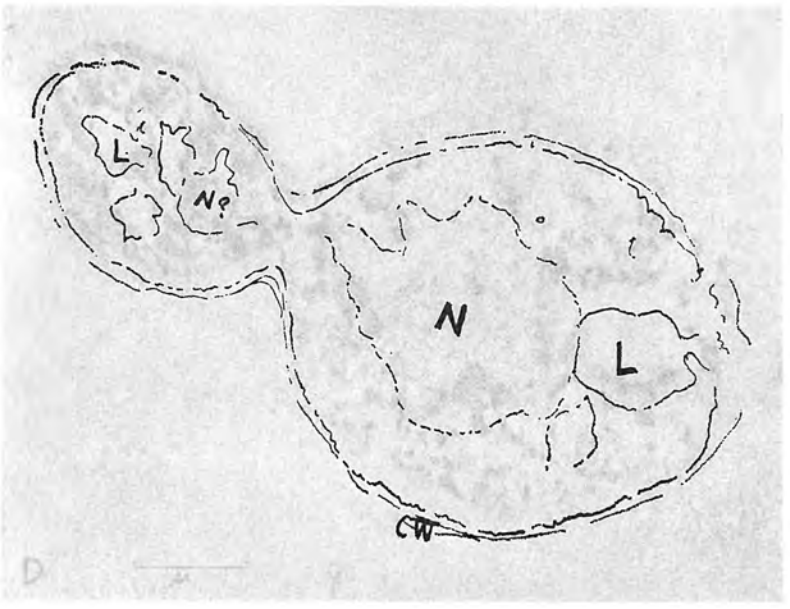


Fig. 2



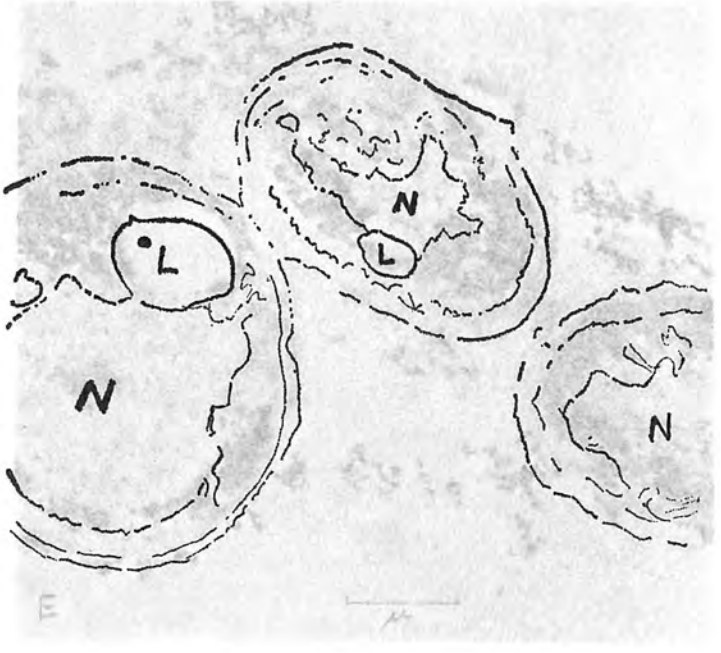
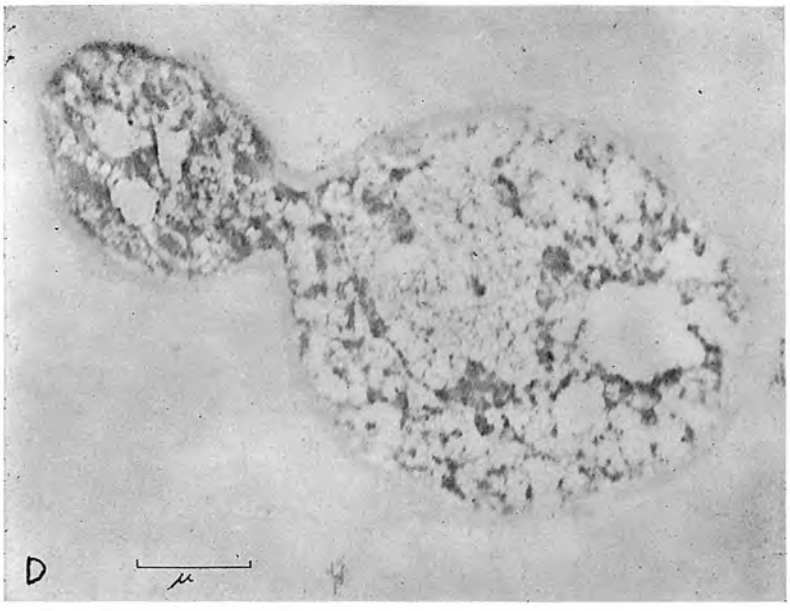


Fig. 2



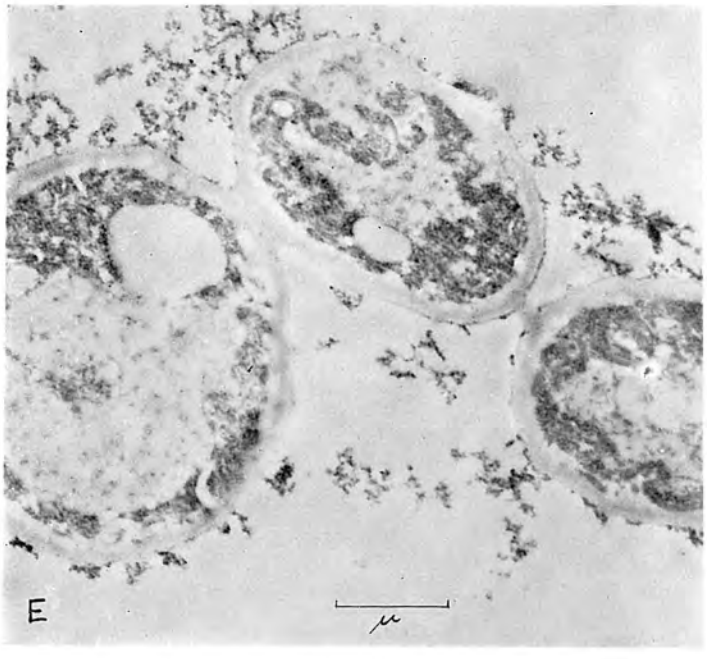
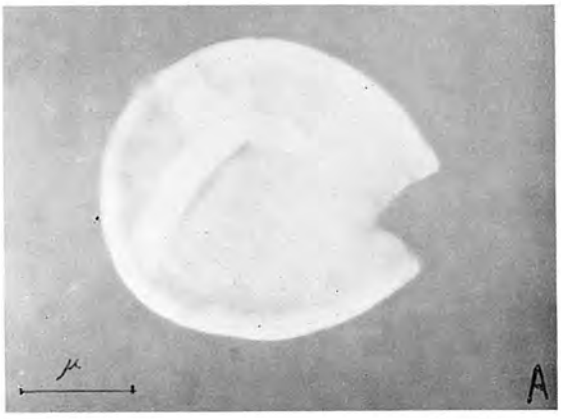


Fig. 2



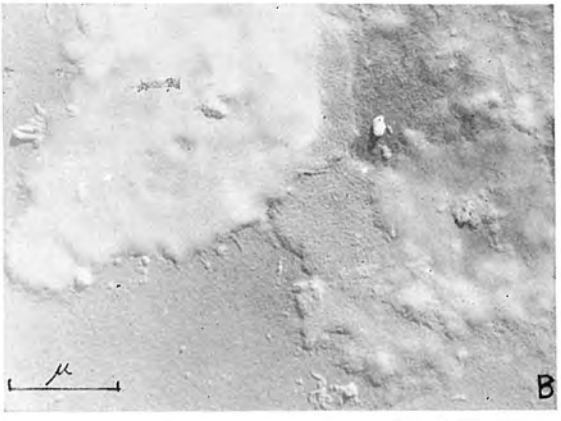




Fig. 3