EFFECT OF POLYENE ANTIBIOTICS ON PROTOPLASTS OF *NEUROSPORA CRASSA*[1]

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ABSTRACT

KINSKY, STEPHEN C. (Washington University, St. Louis, Mo.). Effect of polyene antibiotics on protoplasts of *Neurospora crassa*. J. Bacteriol. **83**:351–358. 1962.—The polyene antibiotics nystatin, amphotericin B, and filipin induced shrinkage of *Neurospora crassa* protoplasts stabilized in sucrose. At low concentrations of filipin (5 μg per ml), or higher concentrations of nystatin and amphotericin B (20 μg per ml), shrinkage was followed by swelling and lysis (bursting) of protoplasts. The effect of various parameters, such as metabolic inhibitors, protoplast concentration, and polyene concentration, was determined. The available evidence is consistent with the hypothesis that polyene antibiotics act by altering cellular permeability, possibly by reaction with the cell membrane, of sensitive organisms.

Previous reports from this laboratory have shown that polyene antibiotics produce a marked loss in the dry weight of mycelial mats of *Neurospora crassa*; this loss is accompanied by the appearance of various cytoplasmic constituents in the medium (Kinsky, 1961*a*, *b*). Mycelial atrophy, observed with low concentrations of all polyene antibiotics tested, began immediately and proceeded at a constant rate, and was not obtained with any other agent (metabolic inhibitors or nonpolyene antibiotics) which also inhibited growth of *Neurospora*. These results suggest that the polyene antibiotics exert their fungicidal activity by an alteration of cellular permeability, resulting in the leakage of an essential component(s), and have focused attention on the membrane of sensitive organisms as the site of action. In support of this view, the present communication provides evidence that these antibiotics induce rapid changes in shape and size of *Neurospora* protoplasts.

[1] Portions of this paper were presented orally at the Gordon Conference on Microbiological Deterioration, Tilton, N. H., July 18, 1961.

MATERIALS AND METHODS

Preparation of protoplasts. Neurospora protoplasts were prepared by modification of the method of Bachmann and Bonner (1959). A dense spore suspension (10^7 to 10^8 spores per ml) was obtained by washing a slant with distilled water and removing hyphal filaments by filtration through glass wool; 10 ml of the spore suspension served as the inoculum for a 2.5-liter Fernback flask containing 500 ml of minimal medium. Penicillin G (approximately 20,000 units) was added to minimize bacterial contamination. After 24 hr of growth as a standing culture at 30 C, the fragile mycelial mat was harvested by filtration in a Buchner funnel. Particular care was taken to maintain the mycelium moist at all times. The mat was subsequently washed by transfer, with the aid of a hooked stirring rod, to a 125-ml Erlenmeyer flask containing 25 ml of 20% sucrose in 0.05 M potassium phosphate, pH 6.8 (sucrose-phosphate buffer). The flask was slowly shaken for 2 min. This procedure was repeated twice and the wet mat was then added to a mixture of sucrose-phosphate buffer (4.5 ml), 10^{-1} M glutathione (0.05 ml), and centrifuged *Helix pomatia* extract (0.5 ml, see below). After 15 hr (overnight) incubation at 30 C, the tube contents were mixed by gentle inversion and the suspension filtered through glass wool to remove the major portion of hyphal filaments. Microscopic examination of the filtrate revealed a high concentration (10^6 to 10^7 per ml, occasionally 10^8 per ml) of the structures designated as *Neurospora* protoplasts by Bachmann and Bonner.

The few hyphal remnants, which were also observed, could be removed completely by low speed (1,500 × *g*) centrifugation in a model CL International clinical centrifuge. A purified preparation of *Neurospora* protoplasts was obtained

in this manner, but recovery was generally 25 to 50% of the original count because of settling of heavier protoplasts. Protoplasts were prepared just prior to each experiment, although they were apparently stable for periods as long as 36 hr (judging by microscopic appearance and osmotic sensitivity) when maintained at room temperature.

Antibiotics. Penicillin G (potassium salt) was obtained from Eli Lilly and Co., Indianapolis, Ind. Nystatin and amphotericin B were generous gifts of the Squibb Institute for Medical Research, New Brunswick, N. J. Filipin and cycloheximide were kindly donated by The Upjohn Company, Kalamazoo, Mich.

Stock solutions of the polyene antibiotics (nystatin, amphotericin B, and filipin) were made by dissolving appropriate quantities in pure dimethyl formamide to give a final concentration of 5 to 10 mg per ml. Subsequent dilutions (approximately 100-fold) were prepared, immediately before use, with 0.05 M potassium phosphate (pH 6.8) containing 20% sucrose. This was the same buffer employed in the preparation of protoplasts and, accordingly, no change in tonicity resulted when the antibiotics were added to the experimental cuvettes.

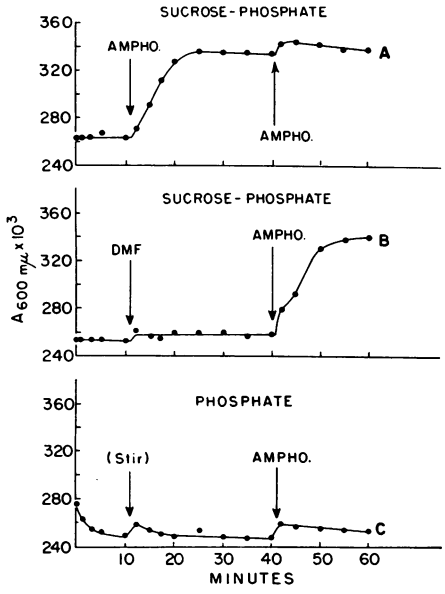

FIG. 1. *Effect of amphotericin B on the absorbancy of a suspension of Neurospora protoplasts. Each cuvette initially contained 0.25 ml of protoplasts. At zero time, 0.75 ml of 20% sucrose-0.05 M potassium phosphate buffer, pH 6.8 (curves A and B), or 0.75 ml of 0.05 M potassium phosphate buffer, pH 6.8 (curve C), were added. At 11 min, 0.1 ml of amphotericin B solution (50 μg per ml of sucrose-phosphate buffer containing 1% dimethyl formamide, abbreviated DMF) and 0.1 ml of 1% DMF (in sucrose-phosphate buffer) were added to cuvettes A and B, respectively, and all cuvettes were stirred. All cuvettes received an additional 0.1 ml of the amphotericin B solution at 41 min. The absorbancy was corrected for dilution after each addition.*

Miscellaneous. The *Helix pomatia* extract was purchased from L'Industrie Biologique Francaise, Gennevilliers (Seine), France, and was centrifuged for 30 min at 30,000 × *g*. This procedure resulted in considerable clarification of the extract, with no apparent loss in the ability to produce protoplasts. In a few experiments, this supernatant solution was further purified by precipitation of the active enzyme(s) between the limits of 20 and 80% saturated ammonium sulfate.

Microscopic examination of protoplast suspensions was performed with an AO-Spencer "Phasestar" microscope equipped with either bright-field or phase-contrast optics. The concentration of conidial spores or protoplasts was determined in a Petroff-Hausser bacterial counting chamber. All absorbancy measurements were made at 600 mμ in a Zeiss spectrophotometer.

RESULTS

The addition of amphotericin B to a suspension of protoplasts, diluted in sucrose-phosphate buffer, produced an immediate increase in absorbancy (Fig. 1, curve A). After the optical density had risen to a constant value, no further change occurred when more antibiotic was added. A solution of 1% dimethyl formamide in sucrose-phosphate buffer (the concentration of solvent present in the diluted antibiotic mixture) did not cause the absorbancy increase (Fig. 1, curve B). Curve C of figure 1 indicates that the absorbancy change due to amphotericin B required the presence of intact protoplasts, since prior lysis by dilution in 0.05 M potassium phosphate buffer (pH 6.8, sucrose absent) completely abolished the effect (Fig. 1, curve C).

Nystatin behaved like amphotericin B; addition of the antibiotic to a suspension of intact, but not lysed, protoplasts produced an increase in absorbancy, with no evidence of a lag phase, which rapidly reached a plateau. The optical

density changes induced by equivalent amounts of filipin were, however, different. The addition of this polyene to a suspension of protoplasts, diluted in sucrose-phosphate buffer, produced a transient increase in absorbancy which was followed by a rapid decline to a value significantly less than the initial reading (Fig. 2, curve A). Addition of more filipin, after the optical density decrease had ceased, caused no further change. These changes were not due to the solvent, dimethyl formamide, but occurred only upon subsequent addition of the antibiotic (Fig. 2, curve B). The effect of filipin was not observed if the protoplasts had been lysed by dilution in phosphate buffer before antibiotic addition (Fig. 2, curve C).

Stirring alone produced a very small increase in optical density, which quickly returned to the original value (curve C, Fig. 1 and 2). This artifact was due to contamination of the protoplast suspension by hyphal remnants which had settled to the bottom of the cuvettes, and was not observed with protoplasts purified by the procedure described above. It must also be emphasized that the absorbancy (optical density) changes described in this communication probably

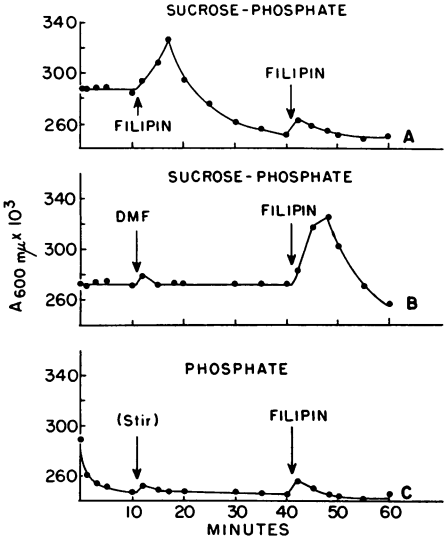

FIG. 2. *Effect of filipin on the absorbancy of a suspension of Neurospora protoplasts. Experimental procedure same as in Fig. 1, except that 0.1 ml of filipin solution (50 μg per ml sucrose-phosphate buffer containing 1% dimethyl formamide) was added to cuvette A at 11 min and to all cuvettes at 41 min.*

are not due to actual light absorption but may merely indicate changes in light scattering.

These differences in absorbancy changes observed with low concentrations of nystatin (or amphotericin B) and filipin are further exemplified by the experiments described in Fig. 3A. The addition of nystatin caused an immediate increase, which soon attained a constant value. Filipin caused a temporary increase in absorbancy, followed by a decrease; addition of nystatin at the conclusion of the filipin-induced decline had no significant effect (curve 2). However, filipin produced a rapid decrease in absorbancy when added to the protoplasts which had initially received nystatin (curve 1).

Nature of the absorbancy changes and correlation with microscopic observations. The polyene-induced absorbancy changes described in this paper were determined at 600 mμ. It must be emphasized that quantitatively identical changes were observed over the 400 to 750 mμ range, and a difference spectrum of *Neurospora* protoplasts (plus antibiotic, minus antibiotic) did not reveal either a peak or shoulder at any specific wavelength. Turbidity measurements below 400 mμ were difficult due to the high ultraviolet absorbance of the protoplast suspension.

In preliminary experiments designed to establish a physical basis for these absorbancy changes, samples of protoplast suspensions were removed for microscopic examination and protoplast count after the optical density had attained a constant maximum (induced by nystatin) or a constant minimum (induced by filipin). The decrease due to filipin was a consequence of almost complete protoplast destruction. The few (approximately 15% of the initial count) which could still be observed were swollen; doubling of the average diameter was not infrequent. The remaining protoplasts were also characterized by extremely large vacuoles and greater osmotic sensitivity. Control protoplasts were generally stable when the sucrose concentration of the suspension was diluted from 20 to 15%, whereas protoplasts treated with filipin were lysed almost immediately under these conditions. Thus, the decrease in absorbancy caused by filipin is superficially analogous to the lysis of protoplasts which followed dilution with hypotonic medium, and was also accompanied by a fall in optical density (curve C, Fig. 1 and 2).

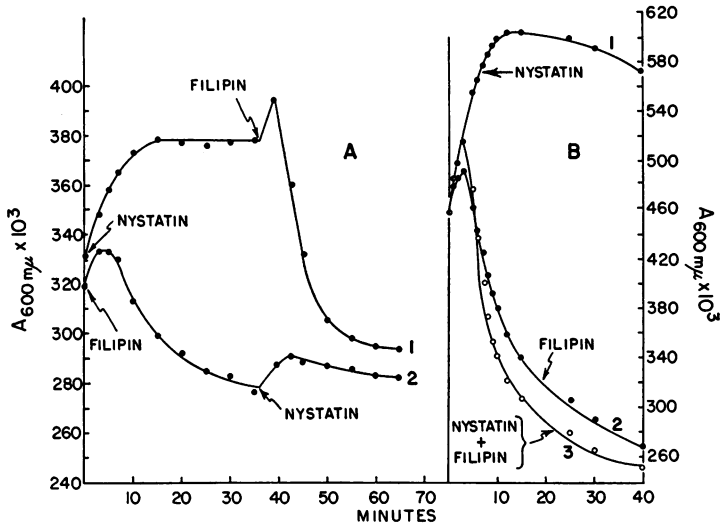

FIG. 3. (A) *Effect of sequential addition of nystatin and filipin on the absorbancy of a suspension of Neurospora protoplasts. Each cuvette initially contained 0.4 ml of protoplasts and 0.5 ml of 20% sucrose–0.05 M potassium phosphate buffer (pH 6.8). At zero time, 0.1 ml of nystatin solution (50 μg per ml of sucrose-phosphate buffer containing 1% dimethyl formamide) and 0.1 ml of filipin solution (also 50 μg per ml of sucrose-phosphate-dimethyl formamide mixture) were added to cuvettes 1 and 2, respectively. At 36 min, the additions were reversed and 0.1 ml of the filipin solution was added to cuvette 1, whereas cuvette 2 received 0.1 ml of the nystatin solution.* (B) *Effect of simultaneous addition of nystatin and filipin on the absorbancy of a suspension of Neurospora protoplasts. Each cuvette initially contained 0.2 ml of protoplasts and 0.7 ml of 20% sucrose–0.05 M potassium phosphate buffer (pH 6.8). At zero time, 0.1 ml of nystatin and filipin solutions were added to cuvettes 1 and 2, respectively, and 0.1 ml of a solution containing both nystatin and filipin at a final concentration of 50 μg per ml was added to cuvette 3. The protoplast preparation used in experiment A was not the same as employed in experiment B.*

Complete lysis of *Neurospora* protoplasts by either dilution or treatment with antibiotic never resulted in a decrease of optical density greater than 60% of the initial value. The residual absorption was due to the intense brown color of the *H. pomatia* extract present in the protoplast suspension.

There was no significant change in the protoplast count of suspensions which had received nystatin. However, these protoplasts were generally smaller than initially. Shrinkage of protoplasts was also characterized by a higher concentration of dense refractile inclusions within the cell and transformation from a spherical to an irregular, crenated form.

Although these spectrophotometric and microscopic observations suggest that nystatin (or amphotericin B) and filipin act differently, subsequent experiments have indicated that this conclusion is unwarranted. Figure 3B shows that the effects of nystatin and filipin are not additive. On the contrary, when both antibiotics are present the changes more nearly resemble those induced by filipin alone. These results suggest that both antibiotics effect a similar process and that filipin may be a more effective competitor for a common site.

The experiments described above were performed with the polyene antibiotics present at final concentrations of 5 μg per ml. (Assuming 100% antibiotic purity, this value would correspond to molar concentrations of 5.3×10^{-6}, 8.8×10^{-6}, and 5.4×10^{-6} for nystatin, filipin, and amphotericin B, respectively). The rate of absorbancy changes varied with the amount of polyene added, and appreciable changes were obtained with 0.5 μg of nystatin and 0.1 μg of filipin per ml (Fig. 4). These concentrations are of the same order of magnitude as those which inhibit formation or induce atrophy of *Neurospora* mycelial mats (Kinsky, 1961*b*).

Especially significant is the observation that

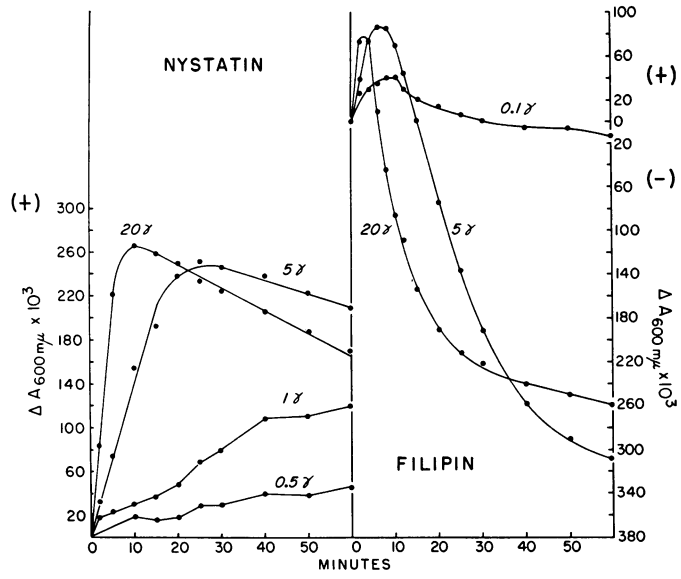

FIG. 4. *Effect of nystatin and filipin concentration on the absorbancy of a suspension of Neurospora protoplasts. Each cuvette initially contained 0.3 ml of protoplasts. At zero time, sufficient 20% sucrose–0.05 M potassium phosphate buffer (pH 6.8), containing the indicated amounts of nystatin or filipin, was added to give a final volume of 1.0 ml.*

higher concentrations of nystatin (20 μg per ml) produce absorbancy changes which are similar to those obtained with lower concentrations of filipin (5 μg per ml), that is, an optical density increase followed by a subsequent decrease. However, it should be noted that after 60 min, when the absorbancy in the presence of 20 μg filipin had fallen to an essentially constant value significantly less than the initial reading, the optical density in the presence of 20 μg of nystatin was still elevated, although declining. This supports the contention that all polyenes have a fundamentally identical mode of action and that the differences between nystatin (or amphotericin B) and filipin are quantitative and not qualitative.

Additional characteristics of the reaction between polyene antibiotics and Neurospora protoplasts. The characteristic effects of low concentrations of nystatin or filipin were neither duplicated nor modified by incubation of protoplasts with iodoacetamide (10^{-3} M), sodium azide (10^{-3} M), or cycloheximide (10^{-5} M), a nonpolyene antibiotic (Fig. 5). These are agents which completely inhibit growth of *Neurospora* mycelial mats but do not cause a weight loss (Kinsky, 1961*b*).

The optical density changes caused by nystatin and filipin depend on the amount of protoplasts present (Figs. 6 and 7). Similar results were obtained with amphotericin B. The final extent of absorbancy increase due to nystatin (measured by the maximal change in absorbancy observed), and also the rate of optical density increase (measured by the change in absorbancy observed during the first minute after antibiotic addition), were functions of protoplast concentration (Fig. 6). The final extent of absorbancy decrease produced by filipin was directly proportional to the amount of protoplasts initially added (Fig. 7).

In some experiments, especially those which employed protoplasts that had been standing at room temperature for approximately 24 hr, the rate of optical density increase did not show strict linear relationship to protoplast concentration. Under these conditions, the maximal absorbancy increase, induced by low concentrations of either nystatin or amphotericin B, did not remain constant but slowly declined. This is the situation obtained with fresh preparations of protoplasts treated with low concentrations of filipin or high concentrations of nystatin and suggests that the optical density changes reflect the relative rates of protoplast shrinkage and protoplast swelling.

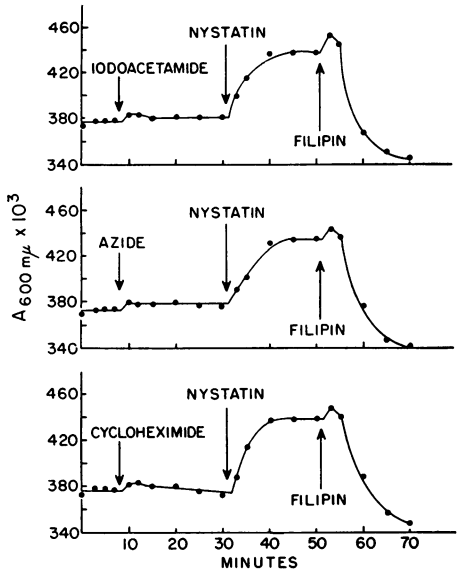

FIG. 5. *Effect of nystatin and filipin on the absorbancy of a suspension of Neurospora protoplasts in the presence of various inhibitors. Each cuvette initially contained 0.4 ml of protoplasts and 0.5 ml of 20% sucrose-0.05 M potassium phosphate buffer (pH 6.8). At 8 min, 0.1 ml of iodoacetamide (10^{-2} M), sodium azide (10^{-2} M), and cycloheximide (10^{-4} M) were added to different cuvettes. These inhibitor solutions were prepared with sucrose-phosphate buffer, so that no change in the tonicity of the incubation mixture resulted upon addition. All cuvettes received 0.1 ml of nystatin and 0.1 ml of filipin solution (see Fig. 3) at 31 and 51 min, respectively. Absorbancy was corrected for dilution after each addition.*

DISCUSSION

This investigation has shown that polyene antibiotics cause a rapid shrinkage, followed in some instances by swelling and lysis, of *Neurospora* protoplasts. Shrinkage can be demonstrated by an increased absorbancy which, if the subsequent rate of swelling (lysis) was slow, remained essentially constant. This situation was obtained with low concentrations of nystatin and amphotericin B.

In the particular case of filipin, protoplast shrinkage was rapidly followed by swelling and bursting. This explains not only the temporary increase in optical density followed by a decrease, but also accounts for the smaller magnitude of this increase when compared to the rise in absorbancy produced by an equivalent amount of nystatin (Fig. 3B).

The polyene-induced optical density changes, like the mycelial dry weight loss, were observed specifically with these antibiotics and were not altered by the presence of compounds which

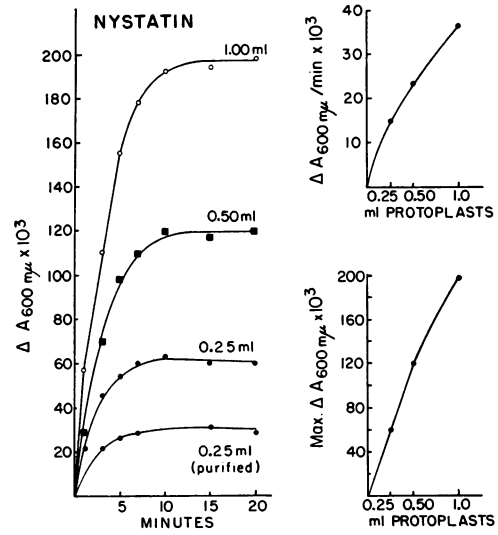

FIG. 6. *Effect of Neurospora protoplast concentration on absorbancy changes due to nystatin. Cuvettes initially contained 0.25, 0.50, and 1.0 ml of protoplasts and sufficient 20% sucrose-0.05 M potassium phosphate buffer (pH 6.8) to give a total volume of 1.0 ml. At zero time, 0.1 ml of nystatin solution (100 μg per ml of sucrose-phosphate buffer containing 1% dimethyl formamide) was added and the change in absorbancy at 600 mμ determined.*

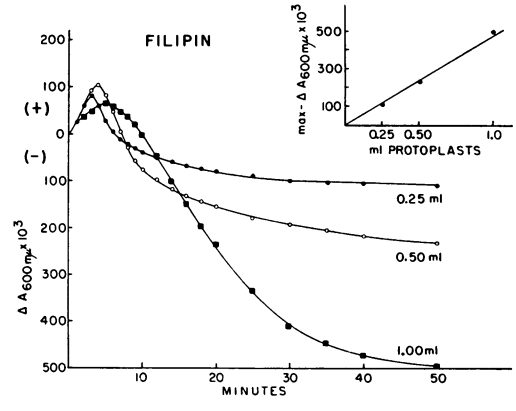

FIG. 7. *Effect of Neurospora protoplast concentration on absorbancy changes due to filipin. Experimental procedure same as in Fig. 6, except that 0.1 ml of filipin solution (100 μg per ml of sucrose-phosphate buffer containing 1% dimethyl formamide) was added at zero time.*

might be expected to interfere with synthetic processes (Fig. 5). These results suggest again that *de novo* membrane synthesis is not inhibited by the polyene antibiotics. The above experiments are consistent with the hypothesis that polyene antibiotics act directly on the cell membrane and ultimately cause death of sensitive organisms by facilitating loss of essential cytoplasmic constituents (Marini, Arnow, and Lampen, 1961; Kinsky, 1961*a*, *b*; Lampen, 1961). In the present experiments, the rate at which cytoplasmic constituents (consequently, water) leave the cell is probably faster than the rate at which the osmotic stabilizer, sucrose, can enter. This would account for the initial shrinkage of the protoplast. Eventually, however, sufficient sucrose enters the protoplast to produce the swelling and lysis observed with low concentrations of filipin and high concentrations of nystatin (Fig. 4).

Alterations in cellular permeability may follow if, by attachment to a component in the cell membrane, the polyene antibiotics so alter the structure of the membrane that it can no longer function as a selectively permeable barrier. These structural changes, if they indeed occur, may result more rapidly and extensively with filipin than with nystatin or amphotericin B. Thus, with low concentrations of filipin (5 μg/ml) the protoplasts undergo a transient shrinkage followed by swelling and lysis. However, protoplasts treated with nystatin, although smaller in size and crenated, nevertheless remain intact for considerable periods of time, as evidenced by microscopic observation and an increase in optical density which remains essentially constant. Structural integrity of nystatin-treated protoplasts is further indicated by the fact that subsequent addition of filipin produces a rapid fall in absorbancy, whereas nystatin causes essentially no change in suspensions of protoplasts which have been initially incubated with filipin and consequently undergone swelling and lysis (Fig. 3A).

Recent experiments by Lampen (1961) also suggest that filipin may cause more extensive damage to cell-membrane function than nystatin or amphotericin B. He has shown that inhibition of yeast-cell or protoplast glycolysis by low concentrations of either nystatin or amphotericin B was completely reversed by potassium or ammonium salts and concluded that glycolytic inhibition was due to the leakage of these essential cations. However, the inhibition produced by filipin was not reversed by these salts. The present experiments indicate that more rapid damage to the membrane by filipin might result in dilution (or inactivation) of glycolytic enzymes and cofactors by the medium and might lessen the chances to observe any reversal. This explanation is valid only if *Neurospora* and yeast (*Saccharomyces cerevisiae*) respond in an identical manner to polyene antibiotics. Lampen (*personal communication*) has shown that yeast protoplasts treated with these agents undergo a rapid swelling.

Since fungi and some algae (Lampen and Arnow, 1961), but not bacteria, are inhibited by the polyene antibiotics, it is tempting to speculate that the selective toxicity of these agents is due to a unique binding component in the membrane of sensitive organisms. This possibility is supported by preliminary experiments which show that lysozyme-prepared protoplasts of *Escherichia coli* are not affected by nystatin. However, there is no evidence that lysozyme has removed the entire bacterial cell wall. Therefore, an alternative possibility, that all organisms possess the same component in the cell membrane but that access of polyene antibiotics to this structure is limited (for instance by a portion of the bacterial cell wall), must still be considered.

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