*NYSTATIN BINDING BY PROTOPLASTS AND A PARTICULATE FRACTION OF NEUROSPORA CRASSA, AND A BASIS FOR THE SELECTIVE TOXICITY OF POLYENE ANTIFUNGAL ANTIBIOTICS**

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Previous reports have shown that mycelial mats of *Neurospora crassa* rapidly decreased in dry weight when incubated with low concentrations of polyene antibiotics.[1, 2] Mycelial atrophy was accompanied by the appearance of various cytoplasmic constituents in the medium. These results suggested that polyene antibiotics exerted their primarily fungicidal effect by an alteration of cellular permeability causing loss of essential components from the cell. Further support for this hypothesis was provided by the demonstration that low concentrations of polyene antibiotics promoted crenation and shrinkage of sucrose-stabilized Neurospora protoplasts, indicating loss of cytoplasmic components at a rate greater than the entrance of osmotic stabilizer.[3] More recently, Slayman and Slayman have shown that nystatin rapidly abolished the membrane potential of Neurospora hyphae, indicating marked alteration in the permeability of ions responsible for the potential difference.[4]

Since the structural unit mainly concerned with the maintenance of selective permeability is the cell membrane, it seems plausible to assume that this might be the principal site of polyene antibiotic action. The data in this paper demonstrate that protoplasts, and a particulate fraction obtained by differential centrifugation of a Neurospora extract, bind appreciable amounts of nystatin and other polyene antibiotics. The available evidence suggests that the binding particles may have originated from the cell membrane. These results are consistent with the hypothesis that binding of polyene antibiotics produces alterations which inhibit the ability of the fungal cell membrane to function as a selectively permeable barrier. Moreover, the absence of any polyene binding by protoplasts of the polyene-insensitive bacterium, *Bacillus megaterium*, indicates that the selective toxicity of these agents is probably due to the existence of a unique binding component in the membrane of sensitive organisms.

Materials and Methods.—Chemicals: The sources of the antibiotics and the preparation of stock solutions were described previously.[2, 3] The author is particularly indebted to R. Thomas and R. E. Bennett of the Squibb Institute for Medical Research, New Brunswick, New Jersey, for a gift of radioactive nystatin. N-acetyl glucosamine (GNAc), uridinediphospho-N-acetyl glucosamine (UDP-GNAc), flavin mono-nucleotide (FMN), and lysozyme were purchased from the Sigma Chemical Company, St. Louis, Missouri. Radioactive UDP-GNAc was kindly donated by S. Nathenson and J. L. Strominger of this department.

Preparation of Neurospora extract and isolation of particulate fraction: Lyophilized 48-hr mycelial mats were pulverized in a mortar. Extracts were prepared by homogenizing the mycelial powder at 2° with 0.05 *M* potassium phosphate, pH 6.8 (hereafter referred to as "buffer") in a Lourdes Multimixer operating at 60 volts for 5 min. Fifty ml of buffer were used per gram of dry weight Neurospora. The resulting suspension was centrifuged for 30 min at 22,000 × *g*. The supernatant solution was carefully removed by aspiration to avoid disturbing the pellet. This residue was again homogenized after resuspension in half the volume of buffer used above and once

more centrifuged. The combined supernatant solutions were then spun for 1 hr at 100,000 × g. The pellet was dispersed in 0.25 volumes of buffer and stored at −17°.

Preparation of protoplasts and the effects of nystatin: Neurospora protoplasts were prepared from 24 hr old mycelial mats by the method previously described[3] with a minor modification. To reduce the absorbancy of the protoplast suspension at 306 mμ (see assay for nystatin below), the incubation mixture consisting of protoplasts and hyphal remnants was first centrifuged for 7 min at 2,000 × g. The pellet was then resuspended in the initial volume of sucrose-phosphate buffer (20% sucrose in 0.05 M potassium phosphate, pH 6.8) and filtered through glass wool to remove the major portion of hyphal remnants. The action of nystatin on Neurospora protoplasts (and protoplasts of *Bacillus megaterium*) was followed spectrophotometrically at 600 mμ. It was shown in a previous paper that increased absorbancy at this wavelength was due to shrinkage and crenation of sucrose-stabilized protoplasts.[3] Protoplasts of *Bacillus megaterium* (ATCC strain 9885) were prepared with lysozyme.[10]

Nystatin determination: Nystatin exhibits sharp absorption maxima at 293, 306, and 321 mμ in aqueous solution (Fig. 3*B*). Lampen *et al.*[5] have studied the uptake of the antibiotic by determining the decrease in optical density at 321 mμ which occurred when cells of different microorganisms were incubated with nystatin and subsequently removed by centrifugation. This procedure was not applicable to either Neurospora mycelia or protoplasts since, in the presence of the antibiotic, compounds which had a small but, nevertheless, significant absorption at 321 mμ (also 293 and 306 mμ) were liberated into the medium. However, a quantitative method for nystatin was developed based on the observation of Zondag *et al.*[6] that the polyene, pimaricin, was rapidly decomposed by visible light in the presence of riboflavin. This phenomenon was studied in greater detail and led to the development of a spectrophotometric assay which was not influenced by the presence of a lysate prepared from Neurospora protoplasts and could therefore be used for the determination of nystatin without any interference from other ultraviolet-absorbing compounds. Figure 1*A* shows that there was a rapid decrease in optical density at 306 mμ

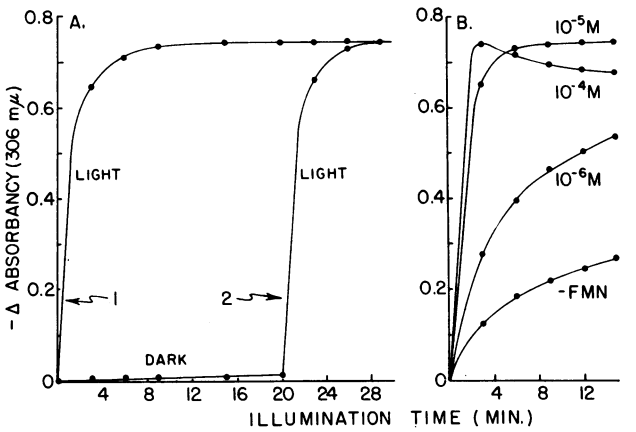

FIG. 1.—*A*. Photolytic decomposition of nystatin. The complete system contained 20 μg of nystatin and $10^{-5}M$ FMN in 1 ml of potassium phosphate buffer, pH 6.8. Tubes were illuminated by placing them between two parallel General Electric fluorescent light fixtures equipped with "cool-white" bulbs; distance between bulbs and tubes was 1.5 cm on either side. *B*. Effect of FMN concentration on photolytic decomposition of nystatin. Same conditions as in A except that the FMN concentration was varied.

when nystatin was mixed with FMN and illuminated (Curve 1). No reaction was observed in the dark (Curve 2). Although there was a slow optical density decrease in the absence of FMN, photodecomposition was markedly stimulated by this compound (Fig. 1*B*). The rate was exceedingly rapid in the presence of 10^{-4} M FMN, but the fall in optical density did not remain constant and slowly declined. This was due to the conversion of FMN to lumichrome which resulted in an *increased* absorption at 306 mμ. No significant interference by this reaction was obtained with lower concentrations of FMN. On the basis of these experiments, nystatin was rou-

tinely determined by removing duplicate 1 ml aliquots from the incubation medium (see below). The absorbancy of one sample was determined immediately and the absorbancy of the other was measured after addition of 0.01 ml of $10^{-3}M$ FMN and 30 min illumination. The decrease in optical density was linearly dependent on nystatin concentration over a wide range. The validity of this assay was further confirmed by experiments which demonstrated that antibiotic binding determined by the photolytic procedure gave results in excellent agreement with those obtained using radioactive nystatin as described below.

Binding of nystatin by protoplasts and particles: All experiments were performed in a darkened room to minimize destruction of nystatin. Binding by protoplasts was determined by mixing appropriate quantities of the protoplast suspension with buffer containing 20% sucrose. Nystatin was added to zero time to give a final volume of 3 ml. The tubes were incubated at 25° for 20 min without shaking. The contents were then centrifuged for 30 min at 22,000 × *g* and the nystatin remaining in the supernatant was determined. Internal controls consisted of tubes from which the protoplasts first had been removed by centrifugation before addition of the antibiotic.

Binding experiments with particles were performed in an essentially similar manner, but the final volume of particulate fraction, potassium phosphate buffer (sucrose omitted), and nystatin was 6.5 ml, and the reaction mixture was centrifuged at 140,000 × *g* for 45 min. When C^{14}-labelled nystatin was employed, binding of the antibiotic was measured by determining the decrease in the radioactivity of the supernatant solution. Aliquots were dried at 50° for 45 min before counting in a Nuclear-Chicago low background counter. It must be emphasized that in the experiments with protoplasts and particles, centrifugation was not the ideal method for terminating the binding reaction. Although the results presented below are recorded as the amount of antibiotic disappearing in 20 min, it is realized that the actual time of contact between protoplasts (or particles) and nystatin was longer.

Chitin synthetase: Chitin synthetase activity was determined by the method of Glaser and Brown.[7]

Results.—Binding of nystatin by protoplasts: Lampen *et al.*[5, 8] have demonstrated that only cells of nystatin-sensitive organisms bind appreciable quantities of the antibiotic and this observation was confirmed in preliminary experiments which showed a rapid disappearance of nystatin from the medium in the presence of Neurospora mycelial mats. These experiments *per se* do not justify the conclusion that the selective toxicity of polyene antibiotics is due to a unique component in the membrane of senive sitorganisms (molds, fungi, and some algae[9]). It is also possible that all organisms possess the same component but that access of the antibiotic is limited, for instance, by the bacterial cell wall. To decide between these alternatives, the effect of polyene antibiotics on bacterial protoplasts was examined.

Nystatin at a concentration of 100 μg/ml had no effect on the growth of *B. megaterium* (Fig. 2*A*). This should be compared with the minimum concentration of 2 μg/ml sufficient to completely inhibit Neurospora mycelial mat formation.[2] Figure 2*B* shows that nystatin at a concentration of 100 μg/ml also had no effect on protoplasts of *B. megaterium*, whereas 5 μg/ml produced an immediate shrinkage and crenation of sucrose-stabilized Neurospora protoplasts.[10, 11] Although these experiments are consistent with the concept of a special binding component in the membrane of sensitive organisms, it is also possible that *B. megaterium* protoplasts absorbed the antibiotic but that any subsequent effect was not seen (due, for instance, to rapid inactivation of the antibiotic). The data of Table 1 indicate, however, that no nystatin was bound by protoplasts of *B. megaterium*. On the other hand, Neurospora protoplasts at a thousandfold greater dilution bound a significant amount of the antibiotic.

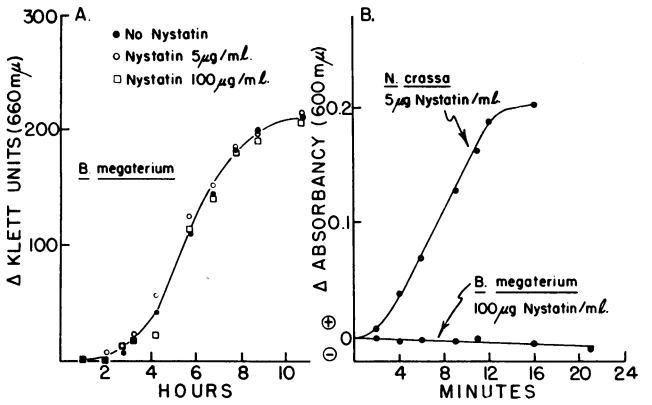

FIG. 2.—*A*. Effect of nystatin on growth on *B. megaterium*. *B*. Effect of nystatin on Neurospora and *B. megaterium* protoplasts. The complete system contained: 0.2 ml of Neurospora protoplast suspension, 0.01 ml of nystatin solution (500 μg/ml), and 0.8 ml of sucrose-phosphate buffer (Curve 1); 0.03 ml of *B. megaterium* protoplast suspension, 0.02 ml of nystatin solution (5 mg/ml), and 0.97 ml of sucrose-phosphate buffer (Curve 2). The reaction was started at zero time by addition of the antibiotic. Absorbancy values were corrected, when necessary, by changes in identical cuvettes to which dimethylformamide was added to give a concentration of solvent equivalent to that of the antibiotic solution.

TABLE 1

NYSTATIN BINDING BY PROTOPLASTS

Organism	ml protoplast suspension*	μg nystatin dis appearing in 20 min
Neurospora crassa	0.5	6.66
	1.0 (10^5–10^6/ml)	11.10
Bacillus megaterium	0.5	0
	1.0 10^8–10^9/ml)	0

* The values in parentheses represent the approximate protoplast concentration determined in a Petroff-Hausser counting chamber. Tubes contained the amount of protoplast suspension indicated, nystatin (final concentration, 20 μg/ml), and sucrose-phosphate buffer, pH 6.8, to give a total volume of 3 ml.

Binding of polyene antibiotics by a particulate fraction obtained from Neurospora: In preliminary experiments designed to test for possible sources of error in the spectrophotometric determination of nystatin, it was observed that concentrated Neurospora extracts, prepared as above, caused a significant decrease in the optical density of the antibiotic at 306 mμ. Subsequent fractionation of the extract revealed that this effect was obtained with the particles sedimenting in 1 hr at 100,000 × *g*. A typical experiment is illustrated in Figure 3*A*. Although there was a slight decrease in the absorbancy of the diluted particulate suspension, the decline was initially faster when the particles were incubated with nystatin. Furthermore, the calculated sum of absorbancies due to the particles and nystatin alone was appreciably greater than actually observed in cuvettes containing both components, and, as indicated by the shaded area, this deviation increased with time. This phenomenon was obtained over a wide range of the particle:nystatin ratio. Moreover, the difference spectrum depicted in Figure 3*B* shows that the decline was not limited to the peak at 306 mμ but also occurred at 293 and 321 mμ. No shift in the nystatin spectrum was observed.

The above experiments suggested that interaction between nystatin and the particles had occurred, and the possibility that this represented actual binding of the antibiotic was tested by the method described above. Nystatin did disappear from

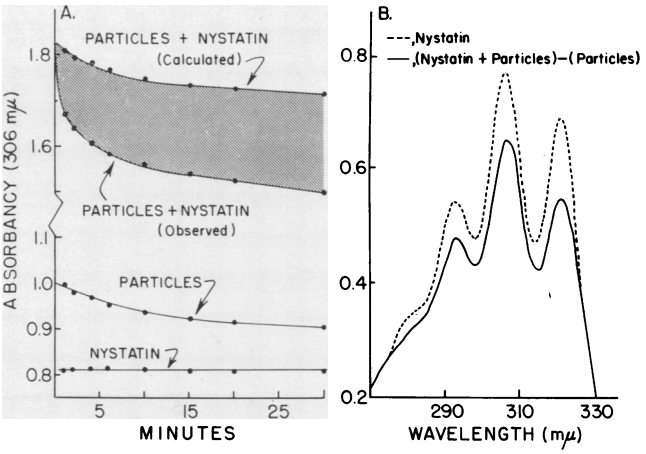

FIG. 3.—*A*. Effect of particles on absorbancy of nystatin. The complete system contained: 0.25 ml of particulate fraction, 0.04 ml of nystatin (500 μg/ml), and 0.71 ml of potassium phosphate buffer, pH 6.8. Reaction was started by addition of the particles. When particulate fraction or nystatin was omitted, the volume was brought to 1 ml by addition of buffer. *B*. Spectrum of nystatin in presence and absence of particulate fraction. A mixture of 0.2 ml of particulate fraction, 0.04 ml of nystatin (500 μg/ml), and 0.76 ml of buffer was incubated 30 min at room temperature in the dark. The spectrum was then recorded (Cary Model 14 Spectrophotometer) against a reference cuvette consisting of particles incubated without nystatin. Control cuvette contained 20 μg nystatin per ml buffer incubated without particles.

solution when incubated with the particles in a reaction which was dependent on the amount of particulate fraction added (Fig. 4*A*). Extent of binding was markedly influenced by the nystatin concentration although absorption of the antibiotic could readily be demonstrated at the lowest concentration tested, 5 μg/ml (Fig. 4*B*). Binding of other polyene antibiotics by the particulate fraction was indicated in experiments which showed that preincubation with filipin and amphotericin B inhibited the uptake of radioactive nystatin (Table 2). These results suggest that

TABLE 2

EFFECT OF AMPHOTERICIN B, FILIPIN, AND UNLABELLED NYSTATIN ON BINDING OF RADIOACTIVE NYSTATIN

Variation of complete system	Total CPM in supernatant	μg radioactive nystatin bound	Per cent inhibition radioactive nystatin binding
(1) Particles omitted	9,360	0	—
(2) None	5,280	52.3 (48.8)*	—
(3) + Amphotericin B	6,660	34.6	33.8
(4) + Filipin	7,980	17.6	66.2
(5) + Unlabelled nystatin	6,720	33.8	35.3

* The value in parentheses was determined by spectrophotometric assay. The complete system, containing 1 ml of particles and 4.94 ml of potassium phosphate buffer, pH 6.8, was incubated 20 min at 25° before the addition of 0.06 ml of radioactive nystatin solution to give a final concentration of 20 μg/ml. After an additional 20 min incubation, the nystatin remaining was determined as described in the text. Amphotericin B, filipin, and unlabelled nystatin were each present at a final concentration of 20 μg/ml during the first incubation period in experiments 3, 4, and 5.

the antibiotics compete for a common site on the particles. This conclusion is consistent with the hypothesis that all polyene antibiotics have an identical mode of action since all which have been tested induce Neurospora mycelial atrophy and protoplast shrinkage.[2, 3] In the case of filipin, it was previously shown that protoplast shrinkage was rapidly followed by swelling and lysis, and it was implied that

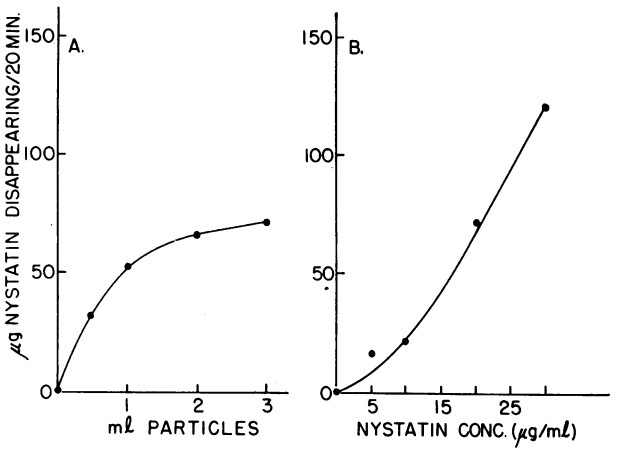

FIG. 4.—*A*. Effect of particle concentration on nystatin binding. Tubes contained the amount of particulate fraction indicated on the abscissa, potassium phosphate buffer, pH 6.8, and nystatin (final concentration, 20 μg/ml) to give a total volume of 6.5 ml. *B*. Effect of nystatin concentration on binding by particles. Tubes contained 3 ml of the particulate fraction used in *A*, varying concentrations of nystatin as indicated on the abscissa, and buffer to give a total volume of 6.5 ml.

this antibiotic might be more tightly bound causing more extensive "cell membrane damage." Therefore, the contention that it is the particulate fraction which contains the "site" of polyene antibiotic action gains additional support from the observation that filipin was the more effective inhibitor of radioactive nystatin uptake.[12]

There are several lines of evidence which indicate that these results are due to binding of the antibiotic by the particles and not transformation to a form unresponsive in the spectrophotometric assay (Table 2). (1) The amount of nystatin which disappeared was the same when determined spectrophotometrically or by radioactive measurement. (2) Pre-incubation with unlabelled nystatin inhibited the disappearance of radioactive nystatin, suggesting that some of the binding sites had already been occupied. (3) A spectrum of particles which had been incubated with the antibiotic and then recovered by centrifugation showed the characteristic nystatin maxima at 293, 306, and 321 mμ. Additional experiments have shown that bound nystatin could not be removed by repeated washing of the particles with 0.05 *M* potassium phosphate, pH 6.8. The antibiotic could, however, be extracted from the particles by either acetone or ethanol.

The ability of the particulate fraction to bind nystatin was not diminished either by 15 hr dialysis against buffer, pretreatment of the particles with crystalline ribonuclease and trypsin, or heating the particles at 100° for 5 min. Extraction of the particles with an ethanol-acetone solution resulted, however, in a complete loss of antibiotic binding activity (Table 3). This activity was almost fully restored by addition of the material(s) which had been extracted. Ergosterol could partially replace the ethanol-acetone extract. This sterol was tested because Gottlieb *et al.*[13] have observed that cholesterol and ergosterol can antagonize the inhibitory action of filipin and other polyene antibiotics toward fungi, and Lampen *et al.*[14] have indicated that cholesterol can combine in solution with nystatin.

Chitin synthetase: Recent evidence indicates that the Neurospora cell wall con-

TABLE 3

RECONSTITUTION OF ETHANOL-ACETONE EXTRACTED PARTICLES

Particle treatment	Addition to particles	μg Nystatin Bound/0.4 ml Particles/hr Initial Nystatin Concentration (μg/ml)	
		10	20
None	None	13.31	26.92
Extracted	None	0	0
Extracted	Extract	10.08	21.13
Extracted	Ergosterol	6.81	8.52

Six ml of the particulate fraction were extracted by stirring for 10 min at 20° with 24 ml of a solution consisting of equal volumes of absolute ethanol and acetone. After centrifugation for 10 min at 2,000 × *g*, the particles were resuspended in 6 ml of distilled water. Particles were "reconstituted" by adding to 2 ml aliquots of the resuspended material either 8 ml of the ethanol-acetone supernatant or 500 μg of ergosterol dissolved in 0.5 ml of acetone. The solvents were removed by aeration of the tubes followed by lyophilization. Control tubes containing 2 ml of unextracted particles or 2 ml of extracted particles, to which no further addition was made, were also lyophilized. The dried particles were then resuspended in 2 ml of 0.05 *M* potassium phosphate, pH 6.8. Binding was measured in the usual manner except that the final reaction volume, consisting of 0.4 ml particles, buffer, and the indicated amounts of nystatin, was 2.75 ml and the incubation time was 1 hr.

sists of a "glucose polymer" and chitin.[15] Glaser and Brown[7] have shown that a particulate fraction obtained from Neurospora synthesizes chitin from UDP-GNAc in a reaction which requires endogenous (particle-bound) primer and is stimulated by GNAc. An attempt was made to demonstrate chitin synthetase activity in the particulate fraction used in the present studies, since it seemed plausible to assume that enzymatic activity associated with the formation of a major structural component of the cell wall might be localized in particles derived from the cell membrane. Table 4 shows that there was significant conversion of

TABLE 4

CHITIN SYNTHETASE ACTIVITY IN PARTICULATE FRACTION

Variation of complete system	Total CPM in insoluble precipitate	Per cent UDP-GNAc incorporation
None	8980	25.9
+ 20 μg/ml nystatin	9140	26.4
Zero time control	50	0.2

The complete system contained per 1.25 ml final volume: 1.0 ml of particles, 0.5 μmoles of unlabelled UDP-GNAc, 2.83 mμmoles of C^{14}-acetyl-labelled UDP-GNAc (34,700 cpm). Buffer: 0.05 *M* Tris-0.001 *M* $MgCl_2$, pH 7.7. Incubation time: 2 hr at 25°.

labelled UDP-GNAc to an insoluble product when incubated with particles. Nystatin did not inhibit this reaction.

Discussion.—The present investigation was based on the assumption that the permeability changes which polyene antibiotics induce in Neurospora[2, 3] and yeast[16, 17] result from binding of these agents to the cell membrane. The experiments described in this paper have shown a rapid absorption of the polyene, nystatin, by protoplasts and a particulate fraction of Neurospora consistent with this assumption. The evidence that these particles are related to the cell membrane is only circumstantial and based mainly on the demonstration of chitin synthetase activity. Attempts to prepare purified membranes ("ghosts") by osmotic lysis of protoplasts were generally unsuccessful. The existence of a cell membrane in Neurospora has been demonstrated by the electron micrographs of Shatkin and Tatum.[18]

Although this investigation has been concerned primarily with the particles isolated in 1 hr at 100,000 × *g*, it should be emphasized that this fraction does not account for all the nystatin bound by the mycelial mats. The amount bound by this fraction varies considerably and, depending on the preparation, may account

for as much as 80 per cent or as little as 20 per cent of the mycelial binding capacity. In the latter case, the residual binding activity is found in the pellet obtained from the initial centrifugation at 22,000 × *g*. Subsequent experiments have shown that this binding activity can be "transformed" into particles sedimentable only at high speed (100,000 × *g*) by repeated homogenization of the 22,000 × *g* pellet. These results indicate that an appreciable portion of the cell membrane may be bound to the cell wall present in the low speed fraction.

The inability of *B. megaterium* protoplasts to bind nystatin suggests that the selective toxicity of polyene antifungal antibiotics is due to a unique component in the membrane of sensitive organisms. The present experiments indicate that this component may involve a sterol. Sterols have not been found in bacteria although they occur in many yeasts, molds, and algae.[19]

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[10] McQuillen has recently reviewed the evidence which indicates that lysozyme completely removes the cell wall of *B. megaterium* (in *The Bacteria*, ed. I. C. Gunsalus and R. Y. Stanier (New York: Academic Press, 1960), vol. 1, p. 249).

[11] J. O. Lampen has also observed that bacterial protoplasts are not affected by nystatin (personal communication).

[12] The validity of this statement is, of course, contingent on the proof of structure and molecular weight determination of the polyene antibiotics and establishment of criteria for absolute purity.

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