MECHANISM OF PROTECTION BY STEROLS AGAINST POLYENE ANTIBIOTICS[1]

J. O. LAMPEN, PETER M. ARNOW, AND ROBERT S. SAFFERMAN[2]

Institute of Microbiology, Rutgers, the State University, New Brunswick, New Jersey

Received for publication December 14, 1959

Gottlieb and collaborators (1958, 1959) have recently reported that the antifungal action of the polyene antibiotics was prevented by sterols such as cholesterol or ergosterol. Only sterols with 27 to 29 carbon atoms were markedly effective. The magnitude of the protection varied with the different antifungals, filipin being one of the most sensitive to the presence of sterol, nystatin one of the least. It was also noted that the addition of cholesterol to a culture of *Saccharomyces cerevisiae*, which had been inhibited for 24 hr by filipin, resulted in resumption of growth. Thus a true reversal by the sterol of a pre-existing action of the antifungal agent appeared to have occurred. Since no direct reaction between sterol and polyene antibiotic was observed, a metabolic interrelation—possibly the inhibition by polyene of sterol biosynthesis—was proposed.

The information available on the mechanism of action of the tetraene antifungal agent nystatin (Lampen *et al.*, 1956, 1957, 1959; Lampen and Arnow, 1959; Scholz *et al.*, 1959) did not offer direct support for this hypothesis. The addition of nystatin to resting intact cells initiates a series of degradative changes which result in the destruction of a variety of enzymes and possibly of cellular structures. Reversal of this series of events, once initiated, has not been achieved, although it can be prevented or delayed by agents which impede the access of nystatin to the sensitive cell (Lampen and Arnow, 1959). We have, therefore, re-examined the antifungal effect of filipin, nystatin, and the tetraene antimycoin (Raubitscheck, Acker, and Waksman, 1952; Schaffner *et al.*, 1958), and particularly the nature of the interaction of the polyenes with sterols. Antimycoin was of special interest since the production of this antibiotic is stimulated by addition of mevalonic acid, a known precursor of the sterol molecule (Schaffner *et al.*, 1958).

MATERIALS AND METHODS

The antifungal agents employed in this study were: filipin (lot 4379-GBW-58) furnished by the Upjohn Company; antimycoin (lot B1766) prepared at this Institute; and either a commercial preparation of nystatin (2850 units per mg) or, where indicated, a highly purified preparation (HV-942; 5500 units per mg) obtained from the Squibb Institute for Medical Research. These materials were dissolved in dimethyl sulfoxide and diluted in pH 4.0 phthalate buffer or in pH 7.0 phosphate buffer. The final concentration of buffer in all test systems was 0.10 M. The various sterols were dissolved in ethanol or dimethyl sulfoxide as stated, and diluted in buffer. The maximum concentration of solvent in the tests was 0.53 per cent, unless otherwise stated.

Growth tests. S. cerevisiae (I. M. strain 216) was grown on a yeast glucose agar medium at 28 C. The organisms from one slant were suspended in 50 ml of sterile distilled water for use as an inoculum.

The data in table 1 are from a diphasic dilution assay devised by H. Lechevalier (*personal communication*). To a 250-ml Erlenmeyer flask were added 20 ml of an agar medium containing the following per liter: $(NH_4)_2SO_4$, 5 g; glucose, 5 g; yeast extract, 1 g; K_2HPO_4, 100 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $Fe_2(SO_4)_3 \cdot 9H_2O$, 10 mg; agar, 20 g. When this had solidified, 8 ml of the same medium without agar were added, 1 ml of the cell suspension and the indicated antibiotics and sterols in 1 ml. The flasks were incubated overnight at 27 C. After the incubation period, the liquid phase was mixed by swirling, and the turbidity was measured in a Klett colorimeter at 660 mμ. The maximal content of dimethyl sulfoxide in any flask (0.1 per

[1] Supported in part by a grant from the Squibb Institute for Medical Research, New Brunswick, New Jersey.

[2] Present address: Robert A. Taft Sanitary Engineering Center, U. S. Public Health Service, Cincinnati 26, Ohio.

cent) did not affect the rate of growth in control cultures.

Cell viability test. A culture of *S. cerevisiae* (I. M. strain 216) grown on a 1 per cent soya peptone-2 per cent glucose agar for 18 hr at 37 C was removed with water and diluted so that cell counts, as described in table 2, were obtained. The yeast cells were incubated at 28 C with antimycoin in pH 7.0 phosphate buffer or with nystatin in pH 4.0 phthalate buffer. After the incubation period the yeast cells were washed free of the unabsorbed antifungal and plated in triplicate on the soya peptone-glucose agar in the presence or absence of cholesterol. After incubation for 2 days at 28 C the colonies were counted. No additional colonies were observed after 7 days of incubation.

Manometric studies. These were carried out at 30 C using standard Warburg techniques. The gas phase was air. Each cup contained either phthalate buffer (pH 4.0) or phosphate buffer (pH 7.0) as stated, 2.5 mg dry weight of *S. cerevisiae* cells (commercial baker's yeast from Anheuser-Busch Inc., Old Bridge, New Jersey; washed four times with water), the antifungals and sterols as indicated in 0.1 ml of 1 per cent dimethyl sulfoxide, 60 μmoles of glucose, and water to 3.0 ml. The center well contained 0.2 ml of 10 per cent KOH solution. The glucose, antifungals, and sterols were added at 0 min from the sidearms.

Spectral data. The Cary recording spectrophotometer, model 14 M, was used in all tests.

TABLE 1

Effect of cholesterol on the inhibition of yeast growth by polyene antifungals

Cholesterol	Minimum Inhibitory Conc*			
	Nystatin	Actimycoin		Filipin
		Test A	Test B	
μg/ml	μg/ml	μg/ml	μg/ml	μg/ml
None	1.25	1.65	2.1	1.0
10	1.45	>10	—	—
20	1.55	>10	>83	17
30	1.80	>10	—	—

* Concentration of antifungal required for reduction of turbidity at 16 hr to 50 per cent of that in control cultures.

RESULTS

Protective effects for yeast growth. The concentrations of the several polyenes required to inhibit the growth of *S. cerevisiae* were increased in the presence of cholesterol. The effect observed with nystatin was small in comparison to the protection furnished by the sterol against antimycoin and filipin (table 1).

In tests employing a disc assay (Safferman, 1959) with *Aspergillus niger*, protection against antimycoin was obtained with cholesterol, ergosterol, stigmasterol, and β-sitosterol, but not with cholesterol acetate, Δ-4-cholestenone, sigmasterol acetate, cortisone, hydrocortisone, progesterone, testosterone, deoxycorticosterone, β-carotene, androstenediol, prednisone, prednisolone, Reichstein's compound S, squalene, or mevalonic acid. The antifungal agent and the steroids were employed at a concentration of 25 μg per ml of medium.

Gottlieb *et al.* (1958) found that C_{29} series steroids (sitosterol and stigmasterol) present in hexane extracts of carrots were highly effective in preventing the inhibitory activity of filipin on *Penicillium oxalicum*. In our laboratory, chloro-

TABLE 2

*Lack of sterol requirement by cells of Saccharomyces cerevisiae incubated with antimycoin or nystatin**

Antibiotic	Treatment Period	Viable Cells per ml		
		Initial	After antibiotic treatment	
			+Ch†	−Ch
	hr			
Antimycoin (100 μg/ml)	24	12×10^8	12×10^4	11×10^4
Nystatin (10 μg/ml)	48	8.6×10^8	32×10^4	34×10^4

* Details under Materials and Methods.

† Level of cholesterol (Ch) in antimycoin experiment was 100 μg/ml, in nystatin test 10 μg/ml.

form treatment of washed mycelium of *A. niger* produced extracts that could prevent the action of antimycoin on *A. niger*. The active component of these extracts was isolated and identified as ergosterol (Safferman, 1959).

The addition of cholesterol to cultures of *S. cerevisiae* inhibited by nystatin or antimycoin resulted in the appearance of growth after a lag period. Thus 25 μg of antimycoin per ml inhibited growth for more than 108 hr. When antimycoin and cholesterol (25 μg per ml) were added at 0 hr, growth occurred in 36 hr as compared to 22 hr for control cultures without polyene. Addition of cholesterol at 24 hr permitted growth to appear at 84 hr. Comparable results were obtained with 10 μg of nystatin per ml when cholesterol (20 μg per ml) was added at 0 or 15 hr. Since the absorption spectra, presented later, demonstrate that the sterol reduces the effective concentration of the antibiotic, an experiment was performed to learn if sterol addition was necessary after the cells had been washed free of unbound antibiotic. As is shown in table 2, more than 99.9 per cent of the cells had been killed during the 24 or 48 hr contact with the antibiotics. Nevertheless, some viable cells remained and grew readily once the polyene had been removed from the medium. Moreover, cholesterol was not required for the growth of these cells.

Protection of respiration by sterols. The inhibition of respiration produced by nystatin could be prevented in part by adding sterol to the test mixture either prior to or along with the nystatin (table 3). The best protection was observed with a sterol to nystatin ratio of 4:1, but even under these conditions the effect was slight. The addition of cholesterol to cells already inhibited by nystatin did not alter the effect of the polyene. Since absorption of nystatin by yeast appears essential to its inhibitory action (Lampen and Arnow, 1959) the uptake under the test conditions used for table 3 was determined. The addition of cholesterol (4:1 ratio) reduced the uptake of nystatin 25 to 30 per cent at 10 min and 30 to 45 per cent at 20 min. In view of the data (Lampen and Arnow, 1959) on the time sequence of inhibition by different concentrations of nystatin, this reduction in uptake appears sufficient to produce the partial protection afforded respiratory activity.

TABLE 3

*Protection of yeast respiration by cholesterol against nystatin action**

Nystatin	Cholesterol	O_2 Uptake	Metabolism Ceased
μg/ml	*μg/ml*	*μL60/ min*	*min*
0	0	460	>120
10	0	136	20
10	20	128	20
5	0	147	25
5	20	294	35
0	0	585	>120
2.5	0	405	45
2.5	10	495	65

* Purified nystatin sample; pH 4.0 phthalate buffer.

TABLE 4

*Effect of cholesterol on filipin action**

Filipin	Cholesterol	O_2 Uptake	Metabolism Ceased
μg/ml	*μg/ml*	*μL/60 min*	*min*
0	0	470	>120
5	0	186	30
10	0	160†	30
20	0	94	10
20	20	254	110
20	5	69	10

* Buffer: pH 7.0 phthalate.

† Cholesterol (20 μg/ml) was added 10 min after respiration had ceased. Oxygen uptake did not resume during the next 100 min. In a parallel series the addition of 10 μg/ml with the filipin protected respiration for at least 120 min.

The protection by sterols against filipin action is essentially complete and is markedly greater than that against nystatin (table 4). As with nystatin, the addition of cholesterol prevented the subsequent action of filipin but did not alter an existing inhibition. Measurement of the uptake of filipin by yeast could not be carried out by the usual procedures (Lampen *et al.*, 1959) since filipin is highly insoluble and sediments with the cells at 2500 × *g*.

Antimycoin, at a level of 50 μg per ml, inhibited respiration within 20 min, but had little effect at 30 μg per ml. The prior addition of 20 μg of cholesterol or ergosterol prevented inhibition; no reversal could be obtained once respiration had stopped.

Sensitivity of "high ergosterol" yeast. Yeasts of low and of high ergosterol content were prepared

by the procedure of Dulaney (1957). Cultures of *S. cerevisiae* (strain Y 2243) were obtained in this manner containing <0.1 per cent or 1.3 per cent of ergosterol. In the standard test for nystatin uptake (Lampen *et al.*, 1959) using 10 μg of nystatin per ml, the cells low in ergosterol absorbed 2.3 μg per mg of cells in 30 min, the others

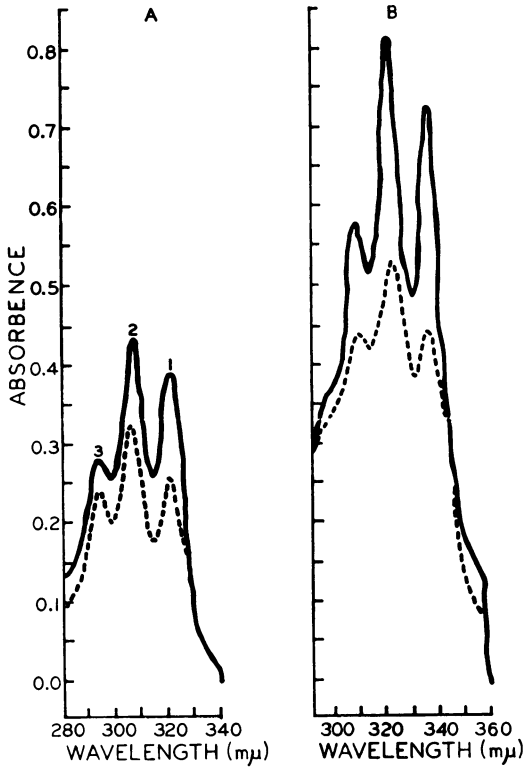

Figure 1. (*A*) Absorption spectra of nystatin (purified preparation), 10 μg/ml in pH 4.0 succinate-phosphate buffer after incubation for 2 hr at room temperature with (-----) and without (——) 20 μg of cholesterol/ml. (*B*) Absorption spectra of antimycoin, 50 μg/ml in pH 7.0 phosphate buffer after incubation for 80 min at room temperature with (-----) and without (——) 20 μg/ml cholesterol.

In (*A*) the nystatin and the steroid were dissolved separately in dimethyl sulfoxide and diluted with buffer to give a final solvent concentration of 0.3 per cent. Numbers over maxima refer to data of table 5.

The antimycoin (*B*) was dissolved in dimethyl sulfoxide and diluted in buffer to a final solvent concentration of 0.5 per cent. The cholesterol was dissolved in ethanol and diluted in buffer to a final alcohol concentration of 0.5 per cent.

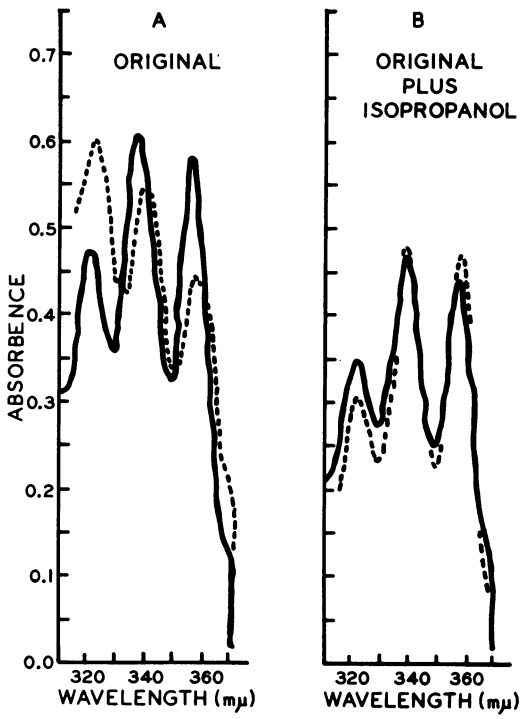

Figure 2. (*A*) Ultraviolet absorption spectra of filipin, 10 μg/ml in pH 4.0 succinate-phosphate buffer, after incubation at room temperature for 2 hr with (-----) and without (——) 20 μg of cholesterol/ml. (*B*) The absorption spectra of the same solutions after dilution with an equal volume of isopropanol.

The filipin and the cholesterol were dissolved separately in dimethyl sulfoxide and diluted with buffer so that the final solvent concentration was 0.3 per cent.

2.6 μg. With both cell types, effective inhibition of respiration and glycolysis at pH 4.0 required approximately 2 μg of nystatin per ml. These experiments offer no support for a metabolic interrelation between sterol and polyene, but do not eliminate this possibility.

Influence of sterols on the spectra of polyenes. When the polyene antibiotics were incubated in aqueous media containing sterols, their absorbence values decreased significantly. This is illustrated in figure 1 for nystatin and antimycoin and in figure 2 for filipin. The indicated changes were intensified on longer incubation. The sterols produced a general lowering of the absorbence values for antimycoin and nystatin without gross changes in the ratios between the several maxima

TABLE 5

*Effect of various steroids on the spectra of polyene antifungals**

Steroid Added (20 μg/ml)	Nystatin (10 μg/ml)		Antimycoin (50 μg/ml)		Filipin (10 μg/ml)	
	E_{m-1}	E_{m-1}/E_{m-3}	E_{m-1}	E_{m-1}/E_{m-3}	E_{m-1}	E_{m-1}/E_{m-3}
None	0.410	1.37	0.670	1.19	0.800	1.28
Cholesterol	0.345	1.23	0.380	1.31	0.550	0.87
Ergosterol	0.355	1.26	0.375	1.32	0.515	0.82
Cholesterol acetate	0.385	1.35	0.565	1.30	0.765	1.22
Testosterone	0.405	1.37	0.645	1.27	0.595	1.11
Progesterone	0.390	1.37	0.650	1.31	0.630	1.15
Δ-4-Cholestenone	0.400	1.23	0.575	1.26	0.680	1.13
Cortisone	0.400	1.36	0.590	1.25	0.710	1.23

* Antimycoin and filipin were incubated in pH 7.0 phosphate buffer at room temperature either alone or with steroid before the absorption spectra were determined. For nystatin, pH 4.0 succinate-phosphate buffer was used. The incubation period was 3 hr for antimycoin, 2 hr for filipin and nystatin. Absorbence values (E_{m-1} and E_{m-3}) are given for maxima 1 and 3 as indicated in figure 1.

(table 5), a result suggestive of a decrease in solubility of the polyene. With filipin there was a major shift in the character of the spectrum, and interaction between the sterol and the polyenic structure may actually have occurred. The possibility that the polyene antibiotics were destroyed in the presence of sterols could be eliminated, since the addition of an equal volume of isopropanol to the solutions in order to solubilize the polyene restored the absorbence of the sterol-polyene mixtures to that of the sterol-free controls (illustrated in figure 2 for filipin). In fact, with long incubation periods the sterols actually protected the polyenes against destruction (Safferman, 1959).

The structure required for the physicochemical interaction with the polyenes (table 5) appears similar to that necessary to prevent their antifungal action. Thus cholesterol and ergosterol were the most active, whereas such related substances as cholesterol acetate, Δ-4-cholestenone and progesterone showed weak or no activity. It should also be noted that cholesterol and ergosterol produced a continuing decrease in absorbence while the relative effect of the others became less with time.

DISCUSSION

To explain the apparent reversal by sterols of the inhibitory action of the polyene antifungals, Gottlieb *et al.* (1958, 1959) proposed that the polyenes may either prevent the biosynthesis of essential sterols by fungi or compete with sterols serving as co-factors for vital metabolic reactions. From the results presented here, it appears essential that other interpretations of the sterol-polyene interaction be given serious consideration as well. Thus from our results, based on spectral, growth, and respiratory measurements, one may suggest that purely physicochemical factors are involved in the action of sterols, and that sterols may be effective only in preventing inhibition, rather than in reversing an existing effect of the antifungal agent.

In the present study it was observed that (a) a direct interaction can occur between sterol and polyene antibiotic; (b) the structure required for this effect is similar to that needed to prevent the biological action of the polyenes; and (c) the addition of sterols will prevent the antifungal activity of the polyenes (i. e. will increase the concentrations required for inhibition). No evidence was obtained that the sterols altered an existing inhibition by the polyene. It appears probable, then, that the interaction of the sterol and polyene reduces the effective concentration of the antibiotic and in this manner increases the amount which must be added to inhibit growth or metabolic activity of the fungus. Any correlation between these changes can only be qualitative since there is no information on the antifungal activity of various molecular aggregates.

The addition of sterol to cultures whose growth had been inhibited for a period of time by a polyene antibiotic did initiate growth after a lag

period. One may suggest that the sterol reduced the effective concentration of antibiotic still present, permitting growth of the small number of surviving cells. It should be noted that when the excess polyene was removed by washing, the remaining viable cells were able to grow without the addition of sterol. Consistent with this explanation for the apparent reversal by sterols are the findings (a) that many aspects of the inhibitory action of nystatin appear irreversible (Lampen and Arnow, 1959), and (b) that after prolonged incubation on solid media containing nystatin, colonies of normal sensitivity to nystatin appear as the antibiotic gradually becomes inactivated (Donovick *et al.*, 1955).

The phenomenon of cholesterol-polyene antibiotic interaction strongly resembles that of the well known cholesterol-digitonin precipitation. When Ransom (1901) observed that cholesterol destroyed the hemolytic activity of plant saponins, considerable attention was attracted by this discovery that a normal physiological cell constituent possessed marked detoxifying properties. Cholesterol and the plant sterols were found to inhibit the hemolytic action of insect, reptilian, and bacterial poisons, as well as that of the plant saponins. Esters of cholesterol were inactive. Pascucci (1905) attributed the phenomenon to either chemical affinity or some solubility relationship. The question was not resolved until Windaus (1909) discovered that cholesterol was precipitated by the plant saponin, digitonin. It is interesting to note that digitonin and many plant saponins resemble the macrolide polyene antifungal antibiotics in some of their physical and chemical properties. They are all high molecular weight compounds, insoluble in ether, sparingly soluble in absolute alcohols but more soluble in aqueous alcohols. They possess numerous hydroxyl functions and contain both lipophilic and hydrophilic moieties. Of all the cholesterol-plant saponin complexes studied, the cholesterol-digitonin complex is the least soluble. The numerous other plant saponins produce complexes of greater solubility. How close the analogy may be between the interaction with sterols of the plant saponins and that of the polyene antibiotics remains to be clarified. However, our study has provided no direct evidence for a metabolic interrelation between sterols and the polyene antifungal agents, although this possibility cannot be ruled out. At present an interpretation of this phenomenon based on physicochemical effects seems attractive to explain the protection obtained.

Addendum

Since submission of this paper for publication it has been observed that nystatin causes a rapid loss of K^+ from the yeast cell with a resultant cessation of glycolysis (Marini, F., Arnow, P., and Lampen, J. O. 1960. Reversal by K^+ or NH_4^+ of the inhibition of glycolysis by nystatin. Bacteriol. Proc., **1960,** 160).

The addition of exogenous K^+ or NH_4 can reverse this inhibition under certain conditions. Nevertheless these ions do not prevent the K^+ loss or the fungicidal effect of nystatin, hence the action of the polyene still appears essentially irreversible.

SUMMARY

The activity of sterols in preventing the antifungal action of polyene antibiotics (filipin, nystatin, and antimycoin) has been confirmed. It was noted, however, that addition of a sterol did not relieve an existing metabolic inhibition. Also, in cultures inhibited by the antifungals for long periods of time, removal of the antibiotic by washing the cells was as effective as was the addition of sterol in producing a resumption of growth. The uptake of nystatin by yeast was reduced by cholesterol to an extent which appears adequate to account for the protective effects obtained.

The addition of sterol to an aqueous colloidal suspension of the polyene antifungals changes the absorption of the polyene, indicative of a lower solubility and thus a lower effective concentration. These changes were reversed when isopropanol was added to the mixtures to produce complete solution of the antibiotic. Thus sterol addition does not produce a destruction of the polyene; in fact, during long incubation, there is an actual protection.

Protection of yeast growth or metabolism was best afforded by those sterols and related compounds which produced the spectral shifts characteristic of lowered polyene solubility. There was also a rough correlation between the magnitude of the spectral changes and the biological effects.

These findings are discussed in relation to the mechanism by which sterols affect polyene action

and are compared with earlier reports on the detoxification of plant saponins by sterols.

REFERENCES

DONOVICK, R., PANSY, F. E., STOUT, H. A., STANDER, H., WEINSTEIN, M. J., AND GOLD, W. 1955 Some *in vitro* characteristics of nystatin (Mycostatin). In *Therapy of fungus diseases: An international symposium*, p. 176–185. Edited by T. H. Sternberg and V. D. Newcomer. Little, Brown and Co., Boston.

DULANEY, E. L. 1957 Preparation of ergosterol containing yeast. U. S. Patent No. 2,817,624, December 24.

GOTTLIEB, D., CARTER, H. E., SLONEKER, J. H., AND AMMANN, A. 1958 Protection of fungi against polyene antibiotics by sterols. Science, **128,** 361.

GOTTLIEB, D., CARTER, H. E., AND GAUDY, E. 1959 Sterol reversal of inhibitory action of filipin on the growth of fungi. Bacteriol. Proc., **1959,** 114.

LAMPEN, J. O. AND ARNOW, P. M. 1959 Significance of nystatin uptake for its antifungal action. Proc. Soc. Exptl. Biol. Med., **101,** 792–797.

LAMPEN, J. O., MORGAN, E. R., AND SLOCUM, A. C. 1956 Inhibition of sugar utilization by nystatin. Federation Proc., **15,** 295–296.

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. C., AND ARNOW, P. M. 1959 Absorption of nystatin by microorganisms. J. Bacteriol., **78,** 282–289.

PASCUCCI, O. 1905 Die Zusammensetzung des Blutscheibenstromas und die Hämolyse. Zweite Mitteilung. Die Wirkung von Blutgiften auf Membranen aus Lecithin und Cholesterin. Beitr. Chem. Physiol., **6,** 552–556.

RANSOM, F. 1901 Saponin und sein Gegengift. Deut. med. Wochschr., **27,** 194–196.

RAUBITSCHECK, F., ACKER, R. F., AND WAKSMAN, S. A. 1952 Production of an antifungal agent of the fungicidin type by *Streptomyces aureus*. Antibiotics & Chemotherapy, **2,** 179–183.

SAFFERMAN, R. S. 1959 Biosynthesis of polyene antibiotics with particular reference to the role of mevalonic acid in the production of antimycoin. Ph.D. thesis, Rutgers, the State University.

SCHAFFNER, C. P., STEINMAN, I., SAFFERMAN, R. S., AND LECHEVALIER, H. 1958 Role of inorganic salts and mevalonic acid in the production of a tetraenic antifungal antibiotic. Antibiotics Annual, **1957–1958,** 869–873.

SCHOLZ, R., SCHMITZ, H., BUCHER, TH., AND LAMPEN, J. O. 1959 Über die Wirkung von Nystatin auf Bäckerhefe. Biochem. Z., **331,** 71–86.

WINDAUS, A. 1909 Über die Entgiftung der Saponine durch Cholesterin. Ber. deut. chem. Ges., **42,** 238–246.