STUDIES ON THE MODE OF ACTION OF FILIPIN ON *SACCHAROMYCES CEREVISIAE*

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Filipin is an antifungal agent produced by *Streptomyces filipinensis*. It has been characterized as a conjugated polyene. Previous papers have described the isolation, purification, and properties of the antibiotic and have shown it to be a conjugated polyene (Ammann, *et al.*, 1955, and Whitfield, *et al.*, 1955). Filipin inhibits the growth of fungi, but not that of any of the bacteria tested. Therefore, it appears that the antibiotic involves some mechanism that is specific in the fungi. Gottlieb, *et al.* (1960) have indicated that filipin caused certain morphological changes such as swelling with subsequent bursting in *Aspergillus flavus*. Abnormal budding and clumping were noted in *S. cerevisiae*. Accompanying these phenomena were permeability changes that resulted in the appearance of various metabolites in the external medium (Sloneker, 1958). These phenomena suggest that the cell surface may be involved. Marini and collaborators (1961) also concluded that the polyene antibiotic nystatin damages the cell membrane and thus produces a rapid increase in permeability of small ions. The resulting depletion of cellular K^+ halts glycolysis. Filipin has been shown to inhibit respiration in whole cells of *S. cerevisiae* but cell free extracts were not inhibited (unpublished data).

Certain sterols have been shown to reverse the inhibition of growth of *S. cerevisiae* by filipin (Gottlieb, *et al.*, 1959, 1960, and Lampen, *et al.*, 1960). However, the antagonistic action of sterols toward the action of filipin has not been elucidated.

This paper describes some of the effects of filipin on leakage from the yeast cell under different growth conditions as well as the leakage and uptake of substances in filipin treated cells in the presence and absence of sterol.

Materials and Methods

The basic medium used in these studies was Difco dehydrated yeast nitrogen base with 1 per cent glucose. The medium was prepared with 6.7 g yeast nitrogen base, 10 g glucose, and 1000 ml of distilled water. Inocula for the experiments were obtained by growing *S. cerevisiae* (F-1) in 20 ml of the basic medium on a reciprocal shaker in 125 ml flasks. The yeast was allowed to grow until the inoculum measured 5 per cent transmission on the Lumetron using a 530 mμ filter. Two successive transfers were made before the yeast was used as inoculum in the experiments. The experimental tubes were inoculated with 0.05 ml of this suspension.

The leakage of amino acids and inorganic phosphate from filipin treated yeast cells was determined in a 0.1 M sodium citrate buffer (pH 6.0) or the basic medium minus the nitrogen source. Actively grow-

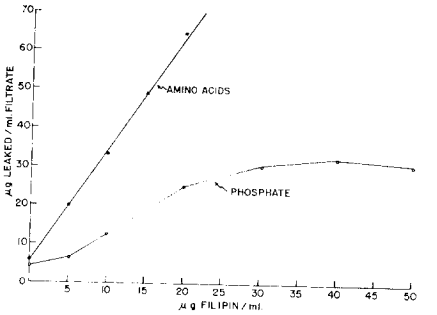

Fig. 1.—Leakage of amino acids and phosphate from filipin treated yeast cells at different concentrations of filipin after 30 minutes incubation.

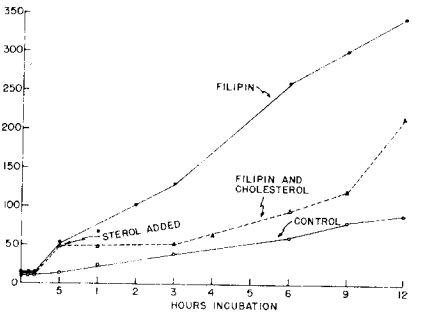

Fig. 3.—Leakage of amino acids from filipin treated yeast cells suspended in a citrate buffer after the addition of cholesterol.

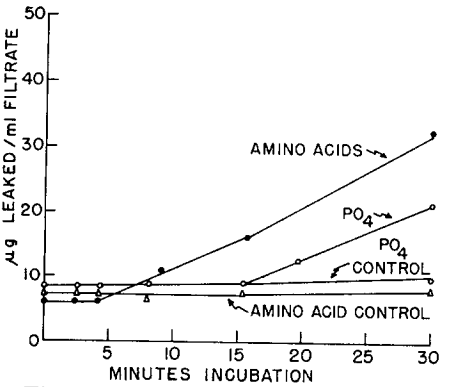

Fig. 2.—Leakage of amino acids and phosphate from filipin treated cells in a citrate buffer as a function of time.

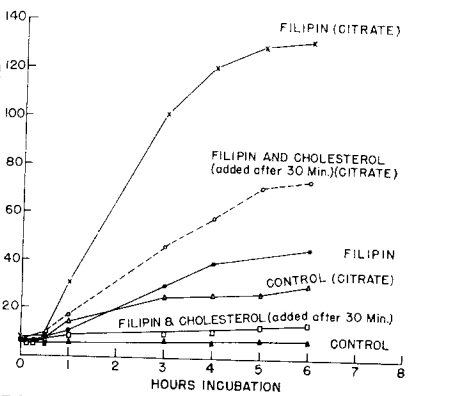

Fig. 4.—Leakage of amino acids from filipin treated yeast cells suspended in the basic medium minus nitrogen.

ing yeast cells were centrifuged, washed three times with distilled water and then suspended (1 g wet wt/100 ml) in the buffer or basic medium minus nitrogen. Filipin was added so that 10 μg/ml was supplied in 0.02 ml of reagent grade methanol. The effect of cholesterol on the leakage of amino acids and inorganic phosphate from filipin treated cells was determined by adding reagent grade cholesterol at a ratio of 4:1, 30 minutes after the addition of filipin. Aliquots (5 ml) were taken at intervals, passed through an ultrafine Sintered filter and the filtrate was analyzed for amino acids and inorganic phosphate. Amino acid leakage from filipin treated cells was determined by the ninhydrin method. The Fiske and Subbarow method as given by Umbreit, *et al.* (1957, p. 272) in "Manometric Techniques" was used for the assay of inorganic phosphate.

To obtain yeast with labelled metabolites for which leakage could be determined even in a complete nu-

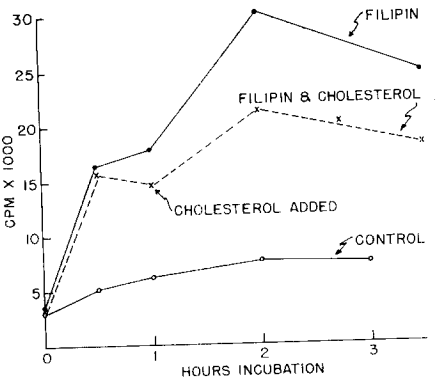

Fig. 5.—Leakage of radioactive C^{14} materials from filipin treated yeast cells in the basic medium.

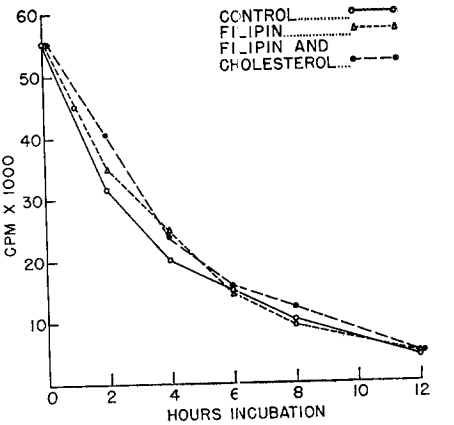

Fig. 6.—Uptake of uniformly labelled C^{14} glucose by filipin treated yeast cells in the basic medium.

trient medium, glucose-U-C^{14} was used. Thus leakage could be determined under growing conditions. The cells were harvested as described above and suspended in 25 ml (1 g wet wt/100 ml) of the basic medium. Filipin was added at the rate of 50 μg/ml. After 1 hour incubation with filipin, cholesterol was added to one group of flasks at the rate of 100 μg/ml. Aliquots (5 ml) were taken at various intervals, filtered and the activity of the filtrate counted by scintillation counting. The amino acids which leaked from the cells were identified by paper chromatography. The filtrate was evaporated to 1 ml on a rotary evaporator. Fifty lamda fractions were examined on Whatman No. 1 filter paper with a butanol-acetic acid-water solvent system (4:1:1). The positions of the amino acids were determined by spraying with ninhydrin reagent. Dried filter paper strips were placed in a 14 ml scintillation solution and then counted.

Results

Leakage of amino acids and phosphate from filipin treated cells. In the citrate buffer, the leakage of amino acids and inorganic phosphate was directly related to the concentration of filipin (Fig. 1). At 25 μg filipin ml, the amino acid concentration was increased to the point that measurement by the ninhydrin method was impossible without prior dilution. Inorganic phosphate leakage was also related to the concentrated of filipin. However, the amount leaked was much less than the amino acids even at concentrations of 50 μg/ml. Since the amount of leakage which occurred with the addition of 10 μg/ml was easily measured, that concentration was used in further experiments.

Efforts were made to determine when leakage began after the addition of filipin. The leakage of amino acids could be detected between 5-10 minutes after the addition of filipin. Inorganic phosphate could not be

detected until after 15-20 minutes incubation. The controls showed no detectable amino acid or phosphate after 30 minutes incubation (Fig. 2).

Leakage of amino acids in presence of cholesterol. Certain sterols reverse the inhibition of growth by filipin. Leakage of amino acids was measured in the citrate buffer before and after the addition of cholesterol. The cholesterol was not added until after 30 minutes incubation with filipin to make certain that leakage had begun. Leakage of amino acids began after approximately 10 minutes and continued up to 12 hours. The addition of cholesterol reduced leakage immediately but did not completely stop it (Fig. 3). After 3 hours approximately 50 μg more amino acids per mililiter filtrate had leaked from the filipin treated cells than from the cells with filipin plus cholesterol. The rapid increase in leakage in both treatments after approximately 6 hours could not be readily explained. The possibility exists that the large loss of amino acids from cells was due to autolysis caused by an unbalance of nutrients. Therefore, it was possible that an energy source might reduce the leakage. The basic medium minus a nitrogen source was used in the same type experiment as explained above. Amino acid leakage was significantly lower in filipin treated cells in the basic medium minus nitrogen (Fig. 4). Approximately 3 times more leakage occurred in the citrate buffer as in the basic medium minus nitrogen. No leakage occurred in the control in the basic medium. When cholesterol was added after 30 minutes incubation with filipin, leakage was very similar to the control.

Leakage of C^{14} labelled materials from cells in a complete growth medium. Since amino acid leakage due to filipin in the basic medium minus nitrogen was greatly reduced compared to citrate buffer, the phenomenon might not take place under normal growing conditions. In order to determine the extent of leakage in a growth medium, yeast cells uniformly labelled with C^{14} were used. The labelled yeast cells were suspended in the basic medium and aliquots taken at intervals. The curves in Figure 5 show that leakage of labelled materials in a complete growth medium was significantly higher in filipin treated cells than in the controls. The addition of cholesterol to the filipin treated cells reduced the rate of leakage but did not completely stop it. These results are similar to those found in the experiments using citrate buffer and the basic medium without a nitrogen source.

The amino acid constitutents of the filtrate from filipin treated yeast were identified by paper chromatography. After 3 hours incubation, a greater number of amino acids could be detected in the filtrate from filipin treated cells than from the control (Table 1). Nine amino acids could be detected in the filtrate from filipin treated cells, while 7 could be detected in the control. The concentration as shown by the intensity of radioactivity on the paper chromatograms of the amino acids from the filipin treated cells was higher than that of the control. The addition of cholesterol after 1 hour did not change the type or number of amino acids from the filipin treated cells. The concentrations of these

amino acids were similar to the treatment with filipin alone. The amino acids which are commonly found in the amino acid pool were generally in highest concentrations in the medium. The high activity of cysteine and lysine is probably due to other materials which had the same Rf value. These data indicate a general leakage of amino acids from the filipin treated cells.

TABLE 1.—Leakage of Labelled Amino Acids from Filipin Treated Yeast Cells after 3 Hours Incubation in the Basic Medium.

Amino Acid	Control C.P.M.	Filipin C.P.M.
cysteine	78	1217
lysine	..	517
aspartic	11	352
glycine	18	267
glutamic	35	291
threonine	..	156
alanine	19	128
valine	43	133
leucine	12	48

Uptake of Glucose by Filipin Treated Cells. Previous experiments have indicated that leakage of vital materials could be important in the inhibition of growth of yeast by filipin. The possibility exists that the uptake of certain materials may also be inhibited. The uptake of uniformly labelled C^{14} was tested in yeast cells in the basic medium. Uptake of glucose-U-C^{14} was measured by determining the disappearance of C^{14} activity after various intervals. Filipin was added in concentrations which were inhibitory to growth (30μg filipin/mg dry wt). The uptake of glucose was not impeded by the presence of filipin (Fig. 6).

Reversal of Inhibition of Filipin by Cations. Marini, *et al.* (1961) have reported that glycolysis and respiration were inhibited by the polyene nystatin at pH 5.8 or above but this effect could be prevented by adding NH_4^+ or K^+ (.004 - 012 M). They concluded that nystatin directly damages the cell membrane and thus produces a rapid increase in permeability of small ions. The cationic reversal of yeast growth inhibition by filipin was studied. Na^+, K^+, NH_4^+, Mg^{++} and Ca^{++} ions were added as chloride salts to the basic medium (pH 6) in concentrations from 0.004 to 0.02 M. The inhibition of growth by filipin (5 μg/ml) was not reversed with any of the cations tested.

DISCUSSION

The leakage of amino acids from filipin treated yeast cells takes place under many conditions. Amino acid and inorganic phosphate leakage was greater in a sodium citrate buffer than in the basic growth medium minus a nitrogen source. The decreased leakage in the growth medium without nitrogen was probably due to utilization of the amino acids in other processes. Leakage of C^{14} labelled materials from C^{14} labelled cells under the influence of the antibiotic indicated that leakage also occurs when the yeast are under growing conditions. Some of the leakage of C^{14} compounds in the complete growth medium consisted of C^{14} labelled amino acids. The total activity of all the amino acids does not equal the total activity of leaked materials. Therefore, it appears that other substance leaked from the cell in the presence of filipin.

Cholesterol was shown to reduce leakage either in a buffer or growing medium. The mechanism of the reversal of growth by filipin is not understood. Lampen (1960) has suggested that there is a physicochemical effect similar to that of cholesterol-digitonin complex. If such an interaction occurs between sterol and filipin, the decreased leakage with the addition of sterol could possibly be due to the inactivation of filipin by complex formation, but data now in press indicate that a vital phenomenon might be involved in this reversal.

The reversal of nystatin inhibition of yeast glycolysis by cations (Marini, *et al.*, 1961) could not be demonstrated for the growth inhibition by filipin. Thus the same mechanism for producing leakage of metabolites might not be involved in growth inhibition as in glycolysis. Supporting this difference is the fact that sterols did not prevent nystatin inhibition of growth but do prevent the inhibition caused by filipin (Gottlieb, *et al.*, 1955).

The uptake of C^{14} labelled glucose was not reduced by filipin, indicating that the vital permeability characteristics of the cell were not completely destroyed. It is possible that the uptake of certain materials is not affected, whereas the ability to retain certain materials in the cell is destroyed. Although it cannot be stated with certainty that the primary effect of filipin is on the cell surface, these data indicate that leakage of vital materials may be important in the inhibition of growth of yeast by filipin.

Summary

Experiments with the antibiotic filipin have indicated that one site of action is probably the cell membrane under growing conditions. A rapid increase of leakage of amino acids and inorganic phosphate could be detected in yeast cells after 20 minutes in the presence of filipin in 0.1 M sodium citrate buffer. The quantity of materials which leaked from the cell increased with the concentration of filipin. Uniformly C^{14} labelled yeast cells actively growing in a complete Wickerham's medium showed a much higher rate of leakage of C^{14} labelled materials in the presence of filipin than in its absence. Under growing conditions, the quantity and number of amino acids which could be detected in the supernatent from filipin treated cells were greater than in the control. The addition of cholesterol reduced leakage in filipin treated cells but did not stop this effect. Concentrations of filipin which were inhibitory to growth did not impede the uptake of glucose. Cations did not reverse the inhibitions of growth by filipin.

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