Nystatin-Induced Changes in Saccharomyces cerevisiae

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Nystatin (5 to 10 µg/ml) was fungicidal to Saccharomyces cerevisiae NCYC 361. There was an initial rapid uptake of nystatin by the yeast cells at 30 C, the uptake being pH dependent, with a maximum at low pH values (3 to 4). Photomicrographs indicated that nystatin-treated cells became granular. The effect of growth temperature on the subsequent sensitivity of the yeast cells to nystatin was investigated. Cells grown at 20 C were the most susceptible to all concentrations of nystatin tested; as the pretreatment growth temperature increased, the subsequent uptake of antibiotic by such cells decreased both in intensity and in overall extent, the most marked difference occurring between 20 and 25 C grown cells. Leakage of K+ and the optical density of suspensions treated with nystatin was similarly affected by growth temperature. Differences in the electrophoretic mobilities of cells grown at different temperatures were also observed. The total ergosterol content of 20 C grown cells was markedly higher than that of 40 C grown cells, with 30 C grown cells having an intermediate value.

Nystatin is an important antifungal antibiotic (3, 9) which alters the permeability of fungal cells (4, 9, 14–16) thereby causing the leakage of K+ and other essential metabolites. This alteration in cellular permeability is believed to be a consequence of the binding of a polyenic antibiotic to the sterols of the cell membrane (6, 14, 15). Resistance in Candida species has been shown to be associated with changes in the ergosterol content of the cells (1, 7, 8).

We previously described (19) some alterations in Saccharomyces cerevisiae cells upon treatment with nystatin. These included changes in the optical density of treated suspensions, and dehydrogenase activity. We have since observed that cells of this organism grown at different temperatures varied in their subsequent sensitivity to nystatin, and this effect was investigated in greater detail.

MATERIALS AND METHODS

Antibiotic. Nystatin, B.P., was kindly provided by E. R. Squibb, Ltd., Liverpool, England. Solutions of the antibiotic were prepared in dimethylformamide (DMF; British Drug Houses, Ltd., Poole, England) as follows. A 40-mg amount of nystatin was dissolved in DMF, and the volume was adjusted to 10 ml with DMF and then diluted with sterile water to give a desired concentration. Fresh solutions were prepared daily. DMF at the final concentration was not lethal to the cells.

Organism. S. cerevisiae NCYC 361 was grown into the stationary phase for 18 h in a 250-ml conical flask, containing 100 ml of double-strength Sabouraud liquid medium (Oxoid Ltd., London, England) in a shaking incubator, 100 oscillations/min, at 30 C. The culture was then centrifuged at 4,000 × g for 5 min, and the pellet was washed three times in sterile glass-distilled water. Suspensions were adjusted with sterile water, by reference to a standard curve, to give 2 mg (dry weight)/ml. Suspensions were stored at 4 C until required for use.

For experiments involving cultures grown at different temperatures, the organism was grown overnight at 30 C in 100 ml of Sabouraud liquid medium (Oxoid Ltd., London, England). A 0.5-ml amount of the culture was added to 100 ml of Sabouraud medium, prewarmed to the desired temperature, followed by incubation overnight at 20, 25, 30, 35, or 40 C. Such cells are referred to as 20 C cells, etc., as appropriate.

Antibiotic stability. Stability of antibiotic solutions was investigated in two ways. Solutions of 10, 15, 20, 25, and 30 µg of nystatin per ml were prepared and stored in the dark at 5, 30, 37, and 50 C. Samples were then removed at intervals and examined as follows: (i) chemically, by scanning on a Unicam SP800 ultraviolet spectrophotometer and 1-cm cells, using DMF in the reference cell; the maxima at 293, 306, and 320 nm were noted (see Fig. 1), and the extent of any instability was calculated by reference to a standard curve for freshly prepared nystatin solutions. (ii) Biologically, by carrying out a bioassay as described in the British Pharmacopoeia (18).

Uptake of nystatin by S. cerevisiae cells. Yeast cells (2 mg [dry weight]/ml) from cultures grown at various temperatures and double-strength nystatin
solutions were preincubated at 30 C. Equal volumes were mixed and held at 30 C. Samples were removed and centrifuged at 4,000 × g for 2 min, and the supernatant fluid was carefully pipetted off and transferred to a 1-cm cuvette. The remaining nystatin concentration was determined by spectrophotometric analysis at 306 nm. For periods up to 24 h, the ultraviolet absorbance at 306 nm was found to be equivalent to biological assay (as determined by bioassay), and hence to concentration of a nystatin solution (see below). The effect of pH on the uptake of nystatin after a 60-min contact period was determined as above, using Sorensen phosphate buffer. Spectrophotometric standard curves of nystatin were obtained at each pH.

Photographic technique. Photomicrographs of untreated (control) and nystatin-treated yeast cells were made at a magnification of ×900 under a Watson Microsystem 70 phase-contrast microscope.

Viability measurements, potassium (K⁺) loss, optical density changes. Optical density changes, viability measurements, and potassium (K⁺) loss were measured as described previously (19).

Sterol analysis. Sterol analysis of yeast cells grown at different temperatures was carried out by a technique similar to that described by Breivik and Owades (2). Washed suspensions of the cells were prepared by centrifugation and resuspension in sterile glass-distilled water (19); these were concentrated by centrifugation to give a suspension containing about 50 mg (dry weight)/ml. The dry weight was determined by drying 5-ml samples to constant weight at 105 C.

A 5.0-ml amount of the suspension was placed in a 25-ml round-bottom flask, and 2.5 g of potassium hydroxide was added and dissolved. Absolute ethanol (5 ml) was added, a reflux condenser was fitted, and the flask was heated at 85 to 90 C for 3 h. After 90 min, the walls of the flask were washed down with 2.5 ml of absolute ethanol. At the end of this alkaline digestion, the flask was cooled and stoppered, and the contents were shaken with 4 ml of distilled water and 10 ml of n-heptane. A blank without yeast cells was also prepared. After shaking, the flask contents were poured into tubes, and the layers were allowed to settle out, leaving the heptane as the supernatant fluid.

A 1-ml amount of the heptane layer was diluted to 10 ml with absolute ethanol, and the absorbances at 281.5 and 230 nm were measured with a Uvicrom H1620 spectrophotometer against a similar dilution of the blank. A calibration curve of concentration of pure ergosterol (British Drug Houses, Biochemicals, Ltd., Poole, England) against absorption at 281.5 nm confirmed that the E₁% for the compound was 290. Thus the equation given by Breivik and Owades (2) applied here, i.e., the percentage of ergosterols equals absorbance at 281.5 nm × F/290, where F is the factor for dilution of sample and weight of cells used. F is given by D × V/W, in which D is the dilution of extract prior to reading, V is the volume (milliliter) of heptane used in the extraction, and W is the dry weight (grams) of yeast cells used. The percentage of 24(28) dehydroergosterol was determined by the absorbance at 230 nm × F/518 or by percentage ergosterols × ratio (230 nm: 281.5 nm) × 0.56. The percentage of ergosterol is thus obtained from the equation: percentage ergosterol minus percentage 24(28) dehydroergosterol.

Changes in electrophoretic mobility of cells. A microelectrophoresis apparatus (Rank Brothers Ltd., Cambridge, England) at a controlled temperature of 25 C was used. This was calibrated using human erythrocytes, as previously described (17). Cells grown at 20, 30, and 40 C were suspended in Sorensen phosphate buffer, pH 7, after cell harvesting and washing in water in the usual manner. Distillation was such as to permit individual cells to be examined, while at the same time not impeding their growth by excess adjacent cells. Measurements were made at the stationary layer, as for erythrocytes, timings being made for migration of cells over 100 μm. Mobilities of cells at a range of pH values in the Sorensen phosphate buffer and of those treated for 60 min with nystatin or sodium dodecyl sulfate were also measured.

RESULTS

Uptake of nystatin. Before estimating the extent of nystatin uptake by treated suspensions, it was first necessary to devise a suitable assay procedure. Figure 1 describes the ultraviolet absorption spectrum of nystatin. There was a linear relationship between absorption at 306 nm (subsequently used), or 320 nm, and nystatin concentration. In the bioassay technique, there was a similar linear relationship between the zone of inhibition and the log₁₀ nystatin concentration. A comparison of the rates of loss of potency of various nystatin solutions was made at different temperatures, and an example of the results (with a 25 μg/ml concentration) is shown in Fig. 2a and b. The physical breakdown, based on the spectrophotometric evaluation (Fig. 2a), and the reduction of biological activity (Fig. 2b) do not bear any correlation after about 24 h of storage, and thus the spectrophotometric assay is not acceptable for prolonged studies. However, the uptake studies were carried out over a short contact period; thus the spectrophotometric technique afforded a rapid and accurate method for detecting residual antibiotic. In some experiments, this method was checked against the bioassay procedure, and the two methods were in close agreement. Of the drug taken up by the cells in 30 min at 30 C, two-thirds was removed from the solution in the first minute.

The pH of the mixture in each experiment was measured electrochemically and was 5.2 to 5.4. Since pH has a profound effect on nystatin activity and uptake (5), the pH of similar systems was adjusted by means of Sorensen
phosphate buffer, and the uptake of nystatin was measured after contact with the cells for 60 min at 30 C. The results indicated that the most marked change in uptake occurred between pH 4 and 7, with a flattening of the curve at the higher values. A repeat of this experiment at 4 C yielded negligible uptake at any pH.

Figure 3 shows the rates of uptake of nystatin at 30 C by S. cerevisiae cells previously grown at 20, 25, 30, 35, or 40 C. Two points in this figure are worthy of comment. (i) As the growth temperature increases, the rate and extent of uptake of nystatin by such cells decreases. (ii) The most marked difference occurs between 20 and 25 C cells, which suggests that alterations in cellular composition due to growth temperature could occur to the greatest extent between these points.

We have not detected any nystatin uptake to bacterial cells.

**Nystatin and viability.** Nystatin (5 to 10 μg/ml) is fungicidal to this strain of S. cerevisiae (19). Cells obtained from cultures grown at different temperatures, however, varied in their susceptibility at 30 C to the antibiotic. Cells grown at 20 C were considerably more susceptible to all concentrations of nystatin tested than were those grown at 30 or 40 C, e.g., percentage of viability after exposure of 20, 30, and 40 C cells to 5 μg/ml nystatin after 120 min were, respectively, <0.1, 8.9, and 25.6.

**Sterol contents of cells.** Table 1 shows the sterol content of cells grown at various temperatures. The total ergosterol content of the cells grown at 20 C is markedly higher than that of cells grown at 40 C, with 30 C grown cells giving an intermediate value. It must also be noted that the 24(28) dehydroergosterol content of 30 and 40 C cells is practically identical.

**Electrophoretic mobilities.** The electropho-
zero due to the ionogenic groupings on the surface being dissociated in such a manner that the overall net charge is zero) of cells grown at 20 or 30 C must lie below pH 3.5, showing the predominance of acidic groups on the surface. Yeast cells grown at 40 C, however, display a loss of mobility at about pH 6, suggesting totally different surface characteristics.

Twenty and 30 C cells maintained their charge over 300 min (Fig. 5a), and this was not significantly affected by nystatin uptake (Fig. 5b). However, 40 C cells appeared to lose their slight charge very rapidly (Fig. 5a), and indeed the polarity of some was reversed. The reason for this instability of electrophoretic mobility cannot be explained at present, but it is to be noted that nystatin (Fig. 5b) did not modify the effect to any great extent. Sodium dodecyl sulfate, a compound which can combine with surface lipid and affect the charge (13), was also tested. Here, a modification of the mobility is seen, with the electronegativity of the 20 and 30 C cells increasing to some extent. The cells grown at these lower temperatures are the most affected, although the charge loss of the 40 C cells makes comparison difficult.

![Graph](Fig. 5. Uptake of nystatin (initial concentration 20 μg/ml) at 30 C by S. cerevisiae cells, grown at different temperatures. Growth temperatures: ▲, 20 C; ■, 25 C; □, 30 C; ●, 35 C; Δ, 40 C.)

![Graph](Fig. 6. Influence of pH on the electrophoretic mobilities of S. cerevisiae cells grown at different temperatures. Growth temperatures: ■, 20 C; ●, 30 C; □, 40 C.)

**Table 1. Ergosterols content of S. cerevisiae cells grown at different temperatures**

<table>
<thead>
<tr>
<th>Growth temp (C)</th>
<th>Ergosterol (%)^a</th>
<th>24(28) Dehydroergosterol (%)^a</th>
<th>Total ergosterols (%)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.558</td>
<td>0.138</td>
<td>0.696</td>
</tr>
<tr>
<td>30</td>
<td>0.416</td>
<td>0.078</td>
<td>0.494</td>
</tr>
<tr>
<td>40</td>
<td>0.292</td>
<td>0.079</td>
<td>0.371</td>
</tr>
</tbody>
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^a As percentage of dry weight of organism.
VOL. 7, 1975

NYSTATIN-INDUCED CHANGES 125

cceptibility to nystatin in terms of viability changes, K⁺ loss, nystatin uptake, optical density changes, and electrophoretic mobility.

The total ergosterol content of 20°C cells is much higher than that of 40°C cells grown in otherwise similar conditions. This reference to “similar conditions” refers only to the physical conditions experimentally imposed upon the medium in which growth took place. It is acknowledged that a difference in temperature may bring about other variations within the system, such as modified solubility of gases. Hunter and Rose (12) found that at constant growth rate, maintained in a chemostat, cells grown at 15°C had a lower sterol content than those grown at 30°C. However, a lowering of growth rate increased the sterol content, and it is likely that in this case the decreased growth rate seen at the lower temperature is the prime factor affecting sterol yield. They also found that in batch-grown cultures, as in the present experiments, the total sterol content increased with a lowering of the incubation temperature and the ratio of ergosterol to 24(28) dehydroergosterol rose at lower temperatures. The 24(28) dehydroergosterol contents of 30 and 40°C cells are almost identical (Table 1). The growth rates of 30 and 40°C cells were found to be similar and differed greatly from cells grown at 20°C; cells grown at the two higher temperatures grew at a faster initial rate than those at 20°C. This suggests that the synthesis of 24(28) dehydroergosterol may be more dependent on growth rate than on the temperature at which it occurs. It is possible to calculate the change in the cellular sterol contents which occurs when the growth temperature is altered by 10°C. Table 2 shows that, whereas the alteration in ergosterol content occurs fairly consistently with growth temperature, the level of 24(28) dehydroergosterol changes considerably more between 20 and 30°C than between 30 and 40°C. It is this property which might account for the different uptake of nystatin, and hence for the other observed drug effects with the differentially grown cells.

Taking the molecular weight of nystatin as 948, and Avogadro’s number as $6 \times 10^{24}$, then 1 µg of the drug represents $6.33 \times 10^{14}$ molecules. On the basis of total counts revealing that 1 mg (dry weight) of cells is equivalent to about $6 \times 10^8$ cells, the cells grown at 20°C thus take up approximately $2.4 \times 10^8$ molecules of nystatin per cell in comparison to about $9.8 \times 10^8$ molecules per cell for 40°C cells. In different circumstances, Lampen et al. (16) calculated an uptake of as high as $7 \times 10^8$ molecules per cell.

Electrophoretic studies (Fig. 4, 5) obviously suggest that the ionogenic groupings on the

**Cellular changes.** When viewed under a light microscope, nystatin-treated yeast cells have a granular appearance (Fig. 6b). The photo-opaque material becomes more concentrated in discrete areas of the cell as the nystatin concentration increases (Fig. 6c,d) to very high levels in contrast to untreated yeast cells (Fig. 6a).

Cells grown at 20, 30, or 40°C were treated with nystatin (10 µg/ml) at 30°C, and changes in optical density at 500 nm were measured (Fig. 7). The greatest effect of the antibiotic is exerted on cells grown at the lowest temperature. The optical density increases most rapidly and to the greatest extent with 20°C cells. The comparatively small effect of nystatin on cells grown at 40°C suggests that the intracellular rearrangement which appears to occur with antibiotic treatment is much less significant in cells grown at the higher temperatures.

**Potassium loss.** The results of K⁺ leakage demonstrated that nystatin induced the greatest membrane damage in cells grown at 20°C, and the least in 40°C cells; as in previous experiments, intermediate results were obtained with 30°C cells. About twice as much K⁺ (µg/mg[dry weight] of cells) was released from 20°C as from 40°C grown cells.

**DISCUSSION**

_S. cerevisiae_ cells grown at different temperatures differ markedly in their subsequent sus-

**Fig. 5. Electrophoretic mobilities of _S. cerevisiae_ cells obtained from cultures grown at different temperatures. (a) Control (untreated) cells, (b) nystatin-treated cells, (c) sodium dodecyl sulfate-treated cells. Growth temperatures: ▲, 20°C; □, 30°C; ▪, 40°C.**

| Temperature | Optical Density
<table>
<thead>
<tr>
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<th></th>
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<tbody>
<tr>
<td>20°C</td>
<td>0.2</td>
</tr>
<tr>
<td>30°C</td>
<td>0.5</td>
</tr>
<tr>
<td>40°C</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**Fig. 6. Intracellular alterations in _S. cerevisiae_ cells.**

(a) Control (untreated) cells. (b) Nystatin-treated cells. (c) Sodium dodecyl sulfate-treated cells. Growth temperatures: ▲, 20°C; □, 30°C; ▪, 40°C.
Fig. 6. Photomicrographs of S. cerevisiae cells. (a) Untreated cells, showing regular appearance of cell contents. (b) Cells after treatment with 10 μg/ml nystatin for 60 min at 30 °C, the cytoplasm taking on a granular appearance. (c) As in b, but nystatin at 20 μg/ml. Photo-opaque material becomes concentrated in discrete areas of the cell. (d) As in b, but nystatin at 40 μg/ml. The effect noted in c becomes more pronounced.
Fig. 7. Changes in the optical densities at 30°C of nystatin-treated S. cerevisiae cells grown at different temperatures. Growth temperatures: □, 20°C; ○, 30°C; ▽, 40°C.

Table 2. Changes in the sterol fractions of yeast cells produced by altering the growth temperature by 10°C

<table>
<thead>
<tr>
<th>Temp change</th>
<th>Change in content as percentage of total</th>
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<tbody>
<tr>
<td>From</td>
<td>To</td>
</tr>
<tr>
<td>20°C</td>
<td>30°C</td>
</tr>
<tr>
<td>30°C</td>
<td>40°C</td>
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</table>

yeast cell surface do not participate in nystatin binding nor in subsequent activity. If, as has been shown (10, 11), the total volume of the cell decreases during nystatin treatment, it would be expected that the charge density would increase. There is a slight tendency for this to occur, repeatedly noted in duplicate experiments. The phenomenon is not displayed by 40°C cells which are more resistant to nystatin.

When treated with nystatin, yeast cell suspensions undergo physical changes characterized by an increase in optical density suggesting an overall rise in the light-scattering ability of the suspension. Nystatin treatment induced a granular appearance in yeast cells viewed under a light microscope. This could result from a rearrangement or precipitation (10, 11) of cytoplasmic constituents to produce a denser appearance.

ACKNOWLEDGMENT

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LITERATURE CITED