STEROL REPLACEMENT IN SACCHAROMYCES CEREVISIAE. EFFECT ON CELLULAR PERMEABILITY AND SENSITIVITY TO NYSTATIN.

Francis KARST and Richard JUND

Laboratoire de Génétique Physiologique, I.B.M.C. du C.N.R.S.
15, rue R. Descartes, 67000 Strasbourg, France

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SUMMARY

More than 80 % of the cellular ergosterol can be replaced by cholesterol in a sterol requiring mutant strain of *Saccharomyces cerevisiae*. The effect of this replacement, as well as the effect of sterol starvation on the uptake and exit of cytosine and α-aminoisobutyric acid (α-AIBA) was studied in an attempt to elucidate the role of sterols in cellular permeability. Neither the exit of cytosine nor the exit of α-AIBA was affected by changes in the sterol content of the cell. Cells grown on cholesterol or on ergosterol had very similar rates of cytosine uptake, but a lower rate was found for sterol-starved cells. This difference may be a consequence of the cellular growth rate. However, nystatin induces a much slower exit of α-AIBA in cells grown on cholesterol than in cells grown on ergosterol. This strongly suggests that a change in membrane structure has taken place.

Ergosterol is the predominant sterol in aerobically growing *Saccharomyces cerevisiae* cells, comprising generally 90 % of the total sterol pool. Ergosterol is present in the yeast cytoplasmic membrane (1) and evidence has been presented to show that this sterol is required for the membrane synthesis (2). The study of the effect of sterols on the physical state of model membrane systems made of phospholipids has shown that sterols increase order and rigidity (3, 4). It is also well established that the permeability of artificial phospholipid membranes diminishes considerably after incorporation of cholesterol ; this effect has been demonstrated for the permeability of water (5), anions (6, 7), cations (8) and non electrolytes (6). Cholesterol present in plasma membranes is also thought to influence their permeability. Experiments performed on mycoplasma cells have demonstrated that cholesterol prevents the crystallisation of membrane lipids and diminishes considerably the non electrolyte permeability of this microorganism (9). Cobon and Haslam have demonstrated that changes

in ergosterol levels in yeast produce a concomitant change in the phase transition temperature of mitochondrial ATPase (10). An inverse relationship between the sterol concentration in the mitochondria and the transition temperature was established.

The present communication reports data obtained from the sterol requiring yeast mutant *erg 1*. In this strain, cholesterol was shown to replace efficiently the natural yeast sterol ergosterol. The effects of this replacement and of sterol starvation on cellular permeability to cytosine and α-aminoisobutyric acid were investigated. The polyene antibiotic nystatin was used as a probe to test structural differences of the cellular membrane in cells grown on different sterols.

MATERIAL AND METHODS

Yeast strains and genetic analysis. The mutant strain *FK erg 1* (ATCC 28382) and the parent strain *FL 100* (ATCC 28383) as well as the strain *RJ 16 sp2* carrying the *fcy 2-3* (permease less) and *fcy 1-1* (cytosine desaminase less) mutations are isogenic and have been previously described (11, 12). The genetic analyses were performed according to the methods of Mortimer and Hawthorne (13).

Media and culture methods. Yeast nitrogen base (Difco) 6.7 g/l, supplemented with 20 g/l glucose was the basic minimal medium. The sterols, 1.5 mg/l, were dispersed by 0.25 g/l Tergitol np40 (Sigma).

Sterol analysis. The cells growing in exponential phase were harvested and washed with distilled water containing 0.25 g/l Tergitol np40. The non saponifiable lipids were extracted and separated on silica gel G plates as previously described (14). The sterol fraction was acetylated by using radioactive $|^{14}C|$ acetic anhydride (15). Ergosterol and cholesterol acetates were separated on silica gel G plates impregnated with silver nitrate, developed overnight in a cyclohexane-benzene (3-2 V/V) solvent system. The respective amounts of radioactivity of different spots were counted in presence of 10 ml of a mixture of toluene diphenyloxazole 5 g/l which allowed the measurement of the absolute amount of each compound.

Uptake and efflux measurement. All experiments were carried out at 30°C on exponentially growing cells in minimal medium. Uptake and efflux of cytosine were measured as previously described (12). For the measurement of the efflux of α-aminoisobutyric acid (α-AIBA), the cells were incubated for 45 min. in the presence of $|^{14}C|$ labelled substrate, then filtered through a membrane filter (1.2 μ pore size), washed once with fresh medium and resuspended in fresh medium. At different times during the experiment, 1 ml samples were removed, filtered and washed with 2 x 10 ml ice cold water. The filters were then dried and their radioactivity counted in toluene plus 5 g/l diphenyloxazole (Intertechnique liquid scintillation spectrometer SL 30).

RESULTS

Sterol analysis.

Whereas the sterol content of the wild type strain *FL 100* is 0.9% (w/dry weight), the sterol content of the *erg 1* mutant strain was never greater than 0.15%, whether culture medium was supplemented with ergosterol (1 mg/l) or cholesterol (1 mg/l). On the other hand, a mean value of 14 % residual ergosterol (vs the amount of total sterols) was present in the mutant strain after 8 generations of growth in minimal medium supplemented with cholesterol. This data suggests that a leaky synthesis of ergosterol occured in the *erg 1* mutant strain, or that cholesterol was partially converted to ergosterol. This latter hypothesis was eliminated on the basis of following experiment. The mutant strain was grown 8 generations in minimal medium supplemented with 4.9 mg/l of $|^{14}C|$ cholesterol (40 mCi/mM). The sterols were extracted and separated after acetylation (see Material and Methods). $|^{14}C|$ radioactivity was associated with acetyl cholesterol only (less than 1/1000 of the counts were found in acetyl ergosterol).

Active transport of cytosine in the strain *erg 1 fcy 1-1*.

A recombinant strain *erg 1 fcy 1-1* was used to bypass the metabolism of cytosine (inactivation of cytosine desaminase by the *fcy 1-1* mutation). Initial velocities of cytosine uptake were measured in this strain after growth on cholesterol or on ergosterol, or after 18 hours of sterol starvation (corresponding to twice the doubling time). The Km values as calculated from the double reciprocal plots (fig. 1) were not significatively different, but the Vmax were lowered in the mutant in comparison to the wild type strain. The observed values (mM internal concentration • 30 sec^{-1}) are 2 mM for the wild type strain, 0.53 and 0.33 for the mutant strain cultured on cholesterol and ergosterol respectively, and 0.15 for the mutant strain after sterol starvation. Initial rates of cytosine uptake were measured at different temperatures from 3° to 36° in the recombinant strain *erg 1 fcy 1-1* grown on cholesterol or ergosterol. The

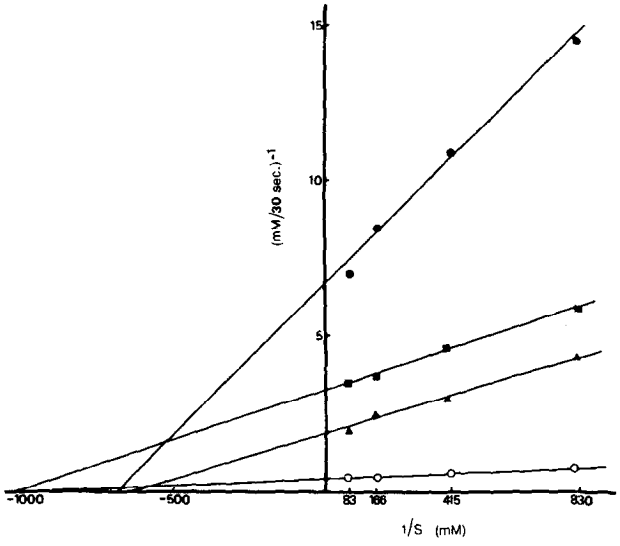

Figure 1. Double reciprocal plots of initial velocities of cytosine uptake in strain *erg 1 fcy 1-1* as a function of external concentration. Cytosine concentration in cells is expressed in mM of cell water per 30 sec. incubation. - ~ - culture medium supplemented with 2 µg/ml ergosterol (Vmax 0.328, doubling time 240 min.) ; -▲- medium supplemented with 2 µg/ml cholesterol (Vmax 0.535, doubling time 195 min.) ; -●- 18 hours sterol starvation (Vmax 0.148, doubling time 400 min.) ; -O-O- wild type strain (Vmax 1.57, doubling time 140 min.).

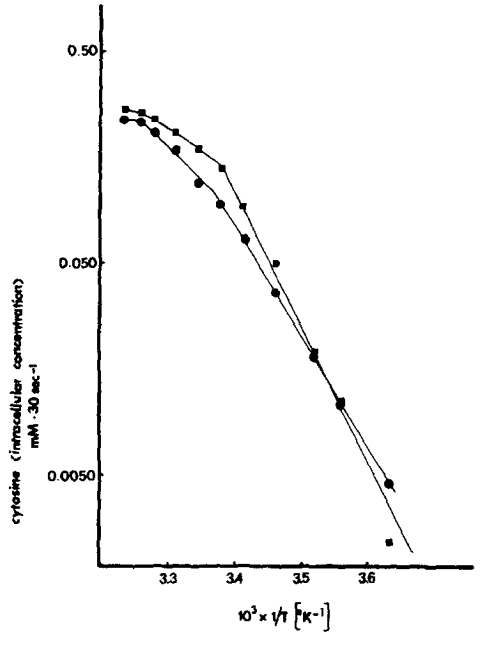

Figure 2. Arrhenius plots of initial velocity of cytosine uptake as a function of temperature. 1 ml samples of the *erg 1 fcy 1-1* cells grown on cholesterol (-■-■-), or ergosterol (-●-●-) are preincubated for 5 min. at the different temperatures before uptake measurement.

corresponding Arrhenius plots (fig. 2) show biphasic kinetics with the same transition temperature at 23°.

Efflux of $|^{14}C|$ cytosine and $|^{14}C|$ α-aminoisobutyric acid (α-AIBA).

α-aminoisobutyric acid (α-AIBA) is a non metabolized compound, which enters the yeast cell by active transport (16). Extracellular cytosine is also concentrated and accumulated in *Saccharomyces cerevisiae* cells having the cytosine desaminase-less mutation (*fcy 1-1*). In a cytosine desaminase-less cytosine permease-less (*fcy 2-3*) strain, cytosine enters and leaves the cell by free diffusion (12). The two compounds, α-AIBA and cytosine were therefore chosen for testing cellular permeability in *erg 1* cells grown on cholesterol, ergosterol and in cells starved for sterols. The assays for cytosine permeability were carried out on two recombinant strains carrying the mutations *erg 1*

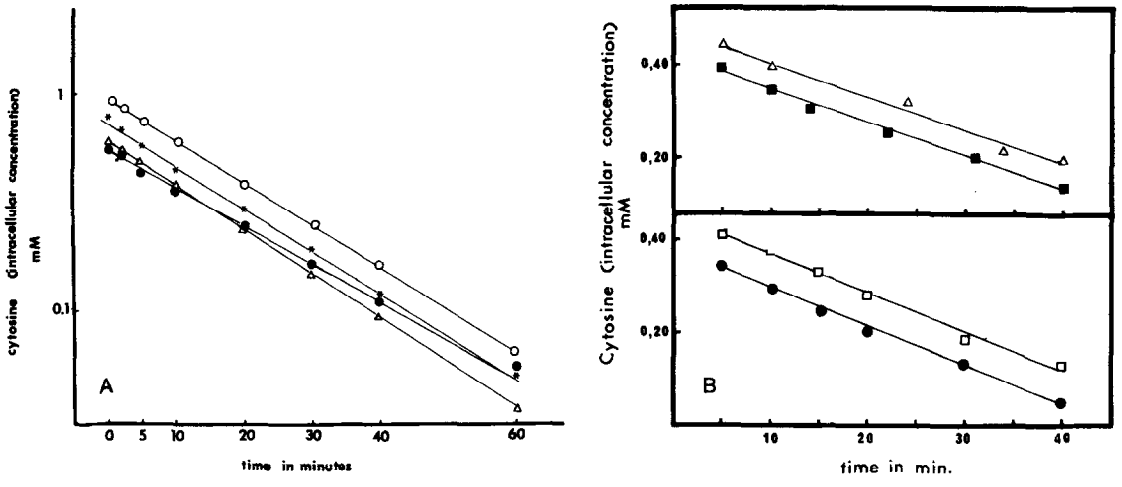

Figure 3. The cells are incubated for 45 min. in minimal medium plus 0.50 mM of $[^{14}C]$ cytosine, then washed and transfered in fresh medium without cytosine, at time zero. The exit is measured as described in Methods.
3A : Cytosine efflux from strain *fcy 1-1 fcy 2-3* grown without sterol (-△-△), and from strain *erg 1 fcy 1-1 fcy 2-3* grown on cholesterol (○-○), ergosterol (*-*-*), and after a 15 hours period of sterol starvation (●-●).
3B : Cytosine efflux from strain *erg 1 fcy 1-1 fcy 2-3* in the presence of nystatin. Nystatin is added at time zero at a final concentration of 10 µg/ml.
- Cells grown on cholesterol ; efflux measured in presence (■-■) and absence (△-△) of nystatin.
- Cells grown on ergosterol ; efflux measured in presence (●-●) and absence (□-□) of nystatin.

fcy 1-1 fcy 2-3, or *fcy 1-1 fcy 2-3*. It was observed that cytosine exit in strain *erg 1 fcy 1-1 fcy 2-3* follows first order kinetics with the same rate constant (k min. ≃ 0.040) whether the strain was grown in the presence of cholesterol, ergosterol or in sterol depleted medium (fig. 3A). Moreover the initial intracellular cytosine concentration was always higher in cholesterol grown cells than in ergosterol grown cells. The kinetics of cytosine exit in strain *erg 1 fcy 1-1 fcy 2-3* are therefore very similar to that observed in strain *fcy 1-1 fcy 2-3* (fig. 3A). When the wild type strain *FL 100* and the *erg 1* mutant strain were preloaded with $|^{14}C|$ α-AIBA, no significant exit of $|^{14}C|$ label was detected, whether the growth medium had been supplemented with ergosterol or cholesterol (fig. 4).

Effect of nystatin on the exit of cytosine and α-AIBA.

The polyene nystatin is highly effective in inhibiting the growth of yeasts and other fungi. Several studies have indicated that the crucial step in the antifungal action of this substance is its binding to membrane sterols (17). The study of yeast mutants resistant to nystatin has shown that these cells are unable to synthesize ergosterol and thus accumulate ergosterol precursors (18). Moreover, ergosterol requiring mutants are more resistant to nystatin if ergosterol is replaced by cholesterol in the culture medium (14). The effect of addition of nystatin (10 mg/l) on the efflux of cytosine and α-AIBA was therefore investigated on *erg 1* cells growing in the presence of cholesterol or ergosterol, and on the wild type cells. Nystatin had no significant effect on cytosine free diffusion in the cytosine permease-less cells (fig. 3B). In contrast, a very fast exit of α-AIBA was induced by the polyene in the wild type strain grown in minimal medium whether or not it was supplemented with ergosterol or cholesterol (fig. 4B). A rapid exit of α-AIBA also occured in the presence of nystatin in *erg 1* cells cultured on ergosterol, but it was greatly decreased when the cells were cultured on cholesterol (fig. 4A).

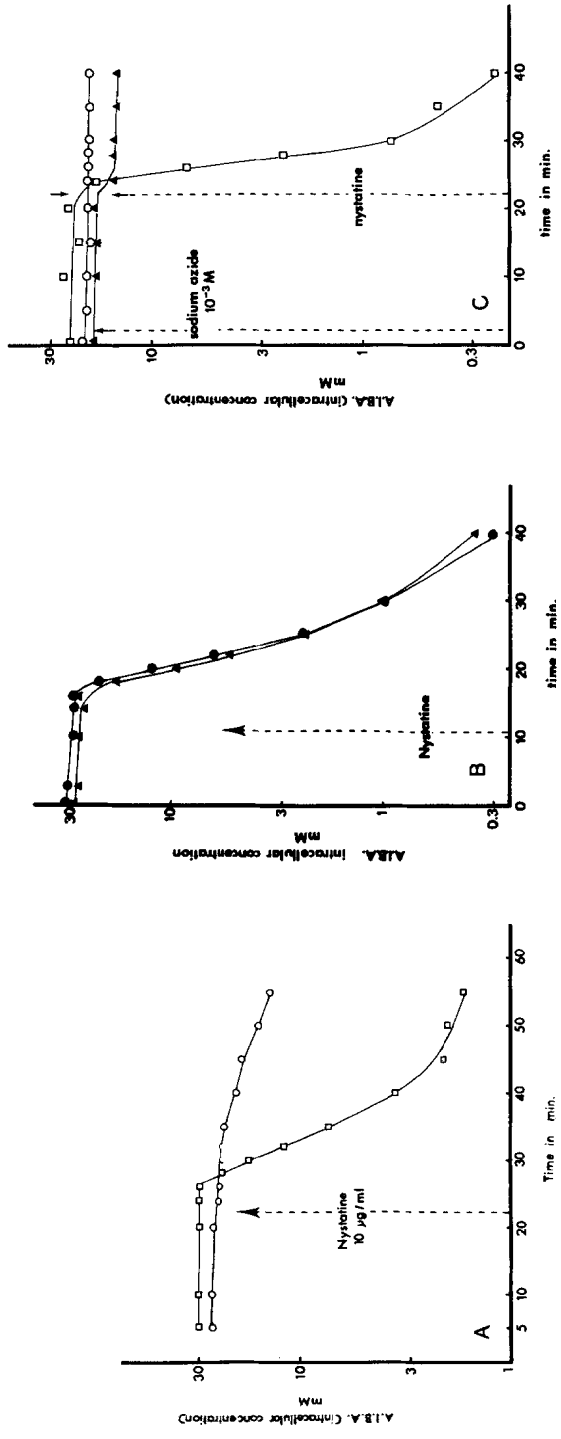

Figure 4. α-aminoisobutyric acid (α-AIBA) exit induced by nystatin, from strain *FL 100* and *erg 1*. The cells are incubated for 45 min. in minimal medium plus 0.32 mM of [^{14}C] α-aminoisobutyric acid. The cells are then washed and transfered in fresh medium without α-AIBA (time zero), and the exit is measured as described in Methods.

4A : Exit measured in *erg 1* cells cultured on ergosterol (□—□), and cholesterol (○—○). Nystatin (10 µg/ml) is added at time 22 min.

4B : Exit measured in wild type strain after growth in minimal medium supplemented with ergosterol (▲—▲) and cholesterol (●—●). Nystatin is added at time 12 min.

4C : Exit measured from wild type strain.
Cells grown in standard medium (□—□) ; cells grown on proline 4 mg/ml as sole ammonium source (▲—▲) ; cells grown in standard medium and pretreated with 10^{-3}M sodium azide during 19 min. before addition of nystatin (○—○).

DISCUSSION

It can be concluded from the above data that the replacement of approximately 80 % of the cellular ergosterol by cholesterol has no stricking effect on the cellular permeability of compounds such as cytosine or α-aminoisobutyric acid (α-AIBA). The differences observed in initial velocities of cytosine uptake (fig. 1) in cells grown on ergosterol, cholesterol and after sterol starvation are more likely linked to the general physiology of the cells as reflected by the differences in growth rates. The kinetics of the down-hill exit of cytosine and α-AIBA are very similar for both sterols as well as after sterol starvation. It is significant that similar exit kinetics were found for a compound like cytosine which leaves the cell rapidly (half life 20 min.) anf for α-AIBA which is not released by the cell. The fact that nystatin induces a significantly lower exit of α-AIBA from cells which have been grown on cholestetol than from those grown on ergosterol, points to the structural integration of these sterols in the cell membrane (fig. 4A). This difference of sensitivity cannot be explained by a protective effect of cholesterol against nystatin by fixation on the outside of the cellular membrane (19), since the wild type strain always presents the same sensitivity to the polyene, whether the cells are grown on ergosterol, cholesterol or in absence of sterols. Furthermore the fact that cholesterol promotes a better growth of *erg 1* mutant than ergosterol rules out the possibility that cellular energetics is the primary target of nystatin action, since nystatin is a more efficient inhibitor of fast growing cells than of slow growing cells. This was demonstrated by the fact that there is no release of α-AIBA in yeast cells pretreated with 10^{-3}M sodium azide (fig. 4C), or in cells grown with proline as sole ammonium source. Acceleration of α-AIBA exit by nystatin, in contrast to its lack of effect on the exit of cytosine is in agreement with other data on the selective loss of metabolites induced by nystatin (17). Experiments with mycoplasma (20) and yeast mitochondria (10) as well as the *in vitro* measurement of diffusion of liposomes (6) point to

an important role of sterols in permeation and diffusion. However our present experiments, as well as the data reported on pig erythrocytes (19) point to a different conclusion. One way to conciliate these two sets of data would be to admit that a very small amount of sterol is sufficient to endow its specific properties on the membrane. In our experiments, then, the residual ergosterol content would be responsible for the basic membrane properties. The isolation of completely blocked mutants would permit probing of this hypothesis.

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REFERENCES

1. Longley R.P., A.H. Rose and B.A. Knights (1968) Biochem. J. 108, 401-412.
2. Mopurgo G., G. Serlupi-Crescenzi, G. Tecce, F. Valente and D. Venettacci (1964) Nature (London) 24, 897-899.
3. Butler K.W., I.C.R. Smith and H. Schneider (1970) Biochim. Biophys. Acta, 219, 514-517.
4. Boggs J.M. and J.C. Hsia (1972) Biochim. Biophys. Acta, 290, 32-42.
5. Finkelstein A. and A. Cass (1967) Nature, 216, 717-718.
6. Demel R.A., K.R. Bruckdorfer and L.L.M. Van Deenen (1972) Biochim. Biophys. Acta, 255, 321-330.
7. Papahadjopoulos D. and J.C. Watkins (1967) Biochim. Biophys. Acta, 135, 639-652.
8. Vanderkooi J.M. and A. Martonosi (1971) Arch. Biochem. Biophys. 147, 632-645.
9. De Kruyff B., R.A. Demel and L.L.M. Van Deenen (1972) Biochim. Biophys. Acta, 255, 331-347.
10. Cobon G.S. and J.M. Haslam (1973) Biochem. Biophys. Res. Commun. 52, 320-325.
11. Karst F. and F. Lacroute (1974) Biochem. Biophys. Res. Commun. 59, 370-376.
12. Chevallier M.R., R. Jund and F. Lacroute (1975) J. Bacteriol. 122, 629-641.
13. Mortimer R.K. and D.G. Hawthorne (1966) Genetics, 53, 165-178.
14. Karst F. and F. Lacroute (1973) Biochem. Biophys. Res. Commun. 52, 741-747.
15. Benveniste P., M.J.E. Hewlins et B. Fritig (1969) Eur. J. Biochem. 9, 526-536.
16. Kotyk A. and L. Rihova (1972) Biochim. Biophys. Acta, 288, 380-389.
17. Kinsky S.C. (1971) Antibiotics, 1, 122-141, Springer, New-York.
18. Bard M. (1972) J. Bacteriol. 111, 649-657.
19. Deuticke J. and C.H.R. Zöllner (1972) Biochim. Biophys. Acta, 226, 726-731.
20. Rottem S., V.P. Cirillo, B. De Kruyff, M. Shinitzky and S. Razin (1973) Biochim. Biophys. Acta, 323, 509-519.