APPENDIX I

YEPD  A complex medium for routine growth

Difco - Yeast extract - 1%
Peptone - 2%
Dextrose - 2%
Agar - 2%

APPENDIX II

SCM  A synthetic complete medium with various constituents for routine growth

Difco-yeast nitrogen base without amino acids - 0.67%
Glucose - 2.0%
L-Amino Acids (mg/l)
Lysine - 20
Arginine - 10
Leucine - 10
Methionine - 10
Threonine - 60
Tryptophan - 10
Histidine - 10
Uracil - 10
Myo-inositol - 3 to 5

APPENDIX III

SGM  A synthetic growth medium for routine growth

KH$_2$PO$_4$ - 0.3%
(NH$_4$)$_2$SO$_4$ - 0.3%
CaCl$_2$ - 0.025%
MgSO$_4$ - 0.025%
Biotin - 0.001%
Glucose - 0.5%
APPENDIX IV

TOLUENE BASED SCINTILLATION FLUID

PPO - 4 g
POPOP - 0.2 g added to one litre of toluene

BRAY'S SCINTILLATION FLUID

PPO - 4 g
POPOP - 0.2 g
Napthalene - 60 g
Ethylene glycol - 20 ml
Methanol - 100 ml
1,4 Dioxan - To make up to 1000 ml
# APPENDIX V

## COMMON ABBREVIATIONS USED

<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>absorbance at 460 nm</td>
<td>$A_{460}$</td>
</tr>
<tr>
<td>cardiolipin</td>
<td>CL</td>
</tr>
<tr>
<td>minimum inhibitory concentration</td>
<td>MIC</td>
</tr>
<tr>
<td>phosphatidylcholine</td>
<td>PC</td>
</tr>
<tr>
<td>phosphatidylethanolamine</td>
<td>PE</td>
</tr>
<tr>
<td>phosphatidylinositol</td>
<td>PI</td>
</tr>
<tr>
<td>1,4-bis(2-(5 phenyloxazolyl) )-benzene phenyl-oxazolyl phenyl-oxazolyl-phenyl</td>
<td>POPOP</td>
</tr>
<tr>
<td>2,5-diphenyloxazole</td>
<td>PPO</td>
</tr>
<tr>
<td>phosphatidylserine</td>
<td>PS</td>
</tr>
<tr>
<td>uncharacterized</td>
<td>UC</td>
</tr>
</tbody>
</table>
PAPERS PUBLISHED
Phospholipid enrichment of *Saccharomyces cerevisiae* and its effect on polyene sensitivity

**T. VENUGOPAL RAO, AKHILESH TRIVEDI, AND RAJENDRA PRASAD**

*Membrane Biology Unit, School of Life Sciences, Jawaharlal Nehru University, New Delhi-110 067, India*

Accepted January 2, 1985


Sensitivity to polyene antibiotics, e.g., nystatin, amphotericin B, and filipin, was determined in phosphatidylcholine (PC) or phosphatidylethanolamine (PE) or phosphatidylserine (PS) enriched *Saccharomyces cerevisiae* cells, using glutamic acid, phenylalanine, glycine, and lysine transport as an index of polyene antibiotic action. As compared with normal cells, phospholipid-enriched cells acquired resistance towards different polyenes. However, the sensitivity of glutamic acid transport towards nystatin remained unaffected in PE-, PS-, or PE-enriched cells. In contrast to nystatin, the other two polyenes were more effective in checking the influx of amino acids. Results demonstrated that the specific enrichment of PC, PE, or PS could selectively protect *S. cerevisiae* cells from polyene antibiotic action.


La sensibilité des antibiotiques polyènes, comme le nystatine, l’amphotéricine B et lc filipin, a été déterminée à l’aide de cellules de *Saccharomyces cerevisiae* enrichie de phosphatidylcholine (PC) de phosphatidylethanolamine (PE), ou de phosphatidylserine (PS), utilisant comme index de l’action des antibiotiques polyènes le transport de l’acide glutamique, de la phénylalanine, de la glycine et de la lysine. Par comparaison aux cellules normales, les cellules enrichies de phospholipides ont acquis une résistance envers différents polyènes. Toutefois, la sensibilité du transport de l’acide glutamique face au nystatine n’a pas été affectée chez les cellules enrichies de PC, PE ou PS. Contrairement au nystatine, les deux autres polyènes ont été plus efficaces pour bloquer l’influx des acides aminés. Les résultats démontrent que l’enrichissement spécifique avec PC, PE ou PS pourrait protéger les cellules de *S. cerevisiae* contre l’action antibiotique des polyènes.

**Introduction**

Polyene antibiotics are effective antifungal agents which interact with membrane sterols. This interaction alters membrane permeability resulting in leakage of the cellular constituents and ultimately cell death (Lampen et al. 1960; Marini et al. 1961; Hamilton-Miller 1973; Gale 1974; Kruijff et al. 1974; Norman et al. 1976). There is now compelling evidence to demonstrate that lipids other than sterols also influence the overall sensitivity to the action of polyenes (Hickey 1953; Gale et al. 1975; Hammond and Kliger 1976; Iannitelli and Ikawa 1980; Danilenko and Stepanyuk 1982).

We have earlier demonstrated that *Candida albicans* cells, when grown on alkanes of different chain length, had significantly altered lipid composition. The results demonstrated that altered sensitivity to different polyenes could not be solely explained on the basis of ergosterol content. It was observed that other lipids of *C. albicans* could also influence the sensitivity of cells to various polyenes (Singh et al. 1979). However, owing to the gross lipid changes which occur in alkanegrown *C. albicans* cells, no specific role in mediating polyene antibiotic action could be envisaged for any individual amino acid (Singh et al. 1979).

To ascertain the effect of lipids other than ergosterol, an attempt has been made to follow the action of polyenes on cells of *Saccharomyces cerevisiae* enriched with phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS). In the present study, by using amino acid transport as an index of polyenes action, it is demonstrated that the specific enrichment of phospholipids of *S. cerevisiae* cells selectively affects the overall sensitivity of the cells to different polyenes.

**Materials and methods**

Nystatin, amphotericin B, amino acids, and cycloheximide were obtained from Sigma Chemical Company, U.S.A. Filipin was a gift from Upjohns Co. U.S.A. Radioactive amino acids were purchased from Bhabha Atomic Research Centre (B.A.R.C.), Bombay, India. All other chemicals were of analytical grade.

**Organisms and maintenance conditions**

The parental strain of *S. cerevisiae* (ade 5a) and mutant strains KA101 (a chl. inol. ben2. can1), requiring choline or ethan-alamine for growth, were obtained from Professor Susan A. Henry, Albert Einstein College of Medicine, Bronx, NY, U.S.A. Both the strains were first grown at 30°C in YEPD 1% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract) and transferred to synthetic medium. Synthetic complete medium contained 2% (w/v) glucose, 0.67% (w/v) Difco yeast nitrogen base without amino acids, and the following amino acids (in milligrams per litre): lysine (20), leucine (10), methionine (10), arginine (10), threonine (60), tryptophan (10), histidine (10), adenine (10), uracil (10), and myo-inositol (5).

Another strain of *S. cerevisiae* 3059 (haploid) was obtained from the National Chemical Laboratory, Pune, India. These 3059 cells were transferred from YEPD liquid medium to defined mineral salt solution containing 0.3% (w/v) KH₂PO₄, 0.3% (w/v) (NH₄)₂SO₄, 0.025% (w/v) CaCl₂, 0.025% (w/v) MgSO₄, 0.001% (w/v) biotin, and 0.5% (w/v) glucose as carbon source. Cells in the exponential growth phase were harvested by centrifugation at 15,000 × g for 10 min and washed thrice with sterile distilled water before further use.

**Lipid analysis**

The method of Folch et al. (1957) was used for total lipid extraction. Phospholipids were separated by two-dimensional thin-layer chromatography on silica gel G plates using chloroform–methanol–ammonia–water (66:27:3:0.8, by volume) and chloroform–methanol–acetic acid–water (32:4:5:1, by volume) solvent systems (A. Trivedi, 1983. Ph.D. thesis, Jawaharlal Nehru University, New Delhi).
Table 1. Lipid composition of S. cerevisiae cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium supplement</th>
<th>Concentration (mM)</th>
<th>Total lipid (mg/g dry cell weight)</th>
<th>Ergosterol (mg/g of total lipid)</th>
<th>Triglycerides (mg/g of total lipid)</th>
<th>PC/PE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (ade 5a)</td>
<td>None</td>
<td>—</td>
<td>120</td>
<td>20</td>
<td>135</td>
<td>2.6</td>
</tr>
<tr>
<td>KA101 (chol)</td>
<td>Choline</td>
<td>1</td>
<td>115</td>
<td>18</td>
<td>145</td>
<td>3.9</td>
</tr>
<tr>
<td>KA101 (chol)</td>
<td>Ethanolamine</td>
<td>1</td>
<td>110</td>
<td>18</td>
<td>75</td>
<td>1.8</td>
</tr>
<tr>
<td>3059</td>
<td>None</td>
<td>—</td>
<td>108</td>
<td>37</td>
<td>130</td>
<td>2.6</td>
</tr>
<tr>
<td>3059</td>
<td>Choline</td>
<td>20</td>
<td>108</td>
<td>37</td>
<td>150</td>
<td>4.3</td>
</tr>
<tr>
<td>3059</td>
<td>Ethanolamine</td>
<td>20</td>
<td>110</td>
<td>31</td>
<td>130</td>
<td>1.5</td>
</tr>
</tbody>
</table>


*Ratio in crude plasma membrane fraction.

University, New Delhi. The resolved phospholipids were identified by comparison with standard phospholipids, scraped from the plate, and phosphorus content was determined (Wagner et al. 1962). Ergosterol was estimated by direct extraction of the cells in boiling alcoholic KOH (10%) for 1 h (MacIntyre and Ralstan 1954). Triaclyglycerols were estimated by the method of Van Handel and Zilversmith (1957) using triolein as a standard. All the values produced in the results were an average of three to five separate determinations. Protein concentrations were determined by Bradford's method (1976).

Transport assay

Transport assay procedures were essentially similar to those described earlier (Jayakumar et al. 1978; Singh et al. 1978, 1979; Trivedi et al. 1982; Khare et al. 1982; Rao et al. 1983). The reaction mixture containing cells (250–350 μg protein/mL) was preincubated at 30°C for 10 min with cycloheximide (200 μg/mL) to inhibit protein synthesis. To see the effect of polypeptide antibiotics on amino acid transport, 10 μg/mL of various polypeptide antibiotics were added during preincubation of the reaction mixture. The reaction was initiated by the addition of radioactive amino acids (0.8 mM [14C]glutamic acid (1 μCi/μmol, 1 Ci = 37 GBq)) or 2 mM [3H]phenylalanine (1 μCi/μmol) or 0.55 mM [3H]glycine (4 μCi/μmol) or 1.66 mM [3H]lysine (4 μCi/μmol) to the assay mixture which was diluted 50-fold in chilled distilled water. The diluted suspension was then rapidly filtered through 0.45 μm filter discs (Maxflow, Bombay, India) and washed three with chilled water. The radioactivity retained on filter discs was determined in a Packard scintillation counter using a toluene-based scintillation fluid.

Results

Lipid composition

The auxotrophic mutant strain of S. cerevisiae KA101 (chol) has already been well characterized (Atkinson et al. 1980). It has been demonstrated that addition of choline (1 mM) or ethanolamine (1 mM) is essential for its continued growth, resulting in PC or PE enrichment (Atkinson et al. 1980). Our earlier detailed analysis of plasma membrane phospholipid contents revealed that PC or PE levels of KA101 (chol) and 3059 plasma membrane fractions were increased when those cells were grown in choline- or ethanolamine-supplemented media, respectively (Trivedi et al. 1982). Table 1 shows the lipid composition other than phospholipids of KA101 and 3059 and also includes the ratio between PC and PE, which was higher in choline-grown cells. However, as compared with normal cells, most of the other lipids, e.g., total lipid and ergosterol contents, remained unaffected in KA101 (chol), as well as in 3059 cells. Triacylglycerols exhibited some variation (T 100%). Thus, the auxotrophic mutants KA101 (chol) and 3059 exhibited a selective enrichment of PC or PE contents which was dependent on their growth conditions (Trivedi et al. 1982).

Nystatin action on PC- or PE-enriched cells

Since both influx and efflux of various nutrients is known to change as a result of polypeptide action (Kinsky 1971; Singh et al. 1979) the transport of four different amino acids was selected as an index of polypeptide sensitivity.

Figure 1A shows that the uptake of Glu, Phe, Lys, and Gly was significantly affected in wild-type (ade 5a) cells if nystatin was present during the transport assay. The inhibition in the total accumulation of all the amino acids varied between 50 and 80% (Fig. 1A).

The effect of nystatin was, however, significantly different in the auxotrophic strain KA101 (chol) when grown in medium containing either 1 mM choline or 1 mM ethanolamine. Almost complete resistance to the action of nystatin on amino acid (except Glu) transport was observed in PC- or PE-enriched KA101 (chol) cells (Fig. 1B). It is pertinent to mention here that in this auxotrophic strain, the ergosterol content did not change significantly and therefore, the resistance acquired by the cells towards nystatin action was probably due to PC or PE enrichment.

The results obtained with S. cerevisiae (3059) cells, grown in high concentrations of choline- or ethanolamine-supplemented medium, were similar to those obtained with the auxotroph. The effect of nystatin on the transport of all amino acids except Glu was not very significant in choline- or ethanolamine-supplemented cells (Fig. 2B). Nonsupplemented cells were, however, sensitive to nystatin as was evident from the 50 to 80% reduction in the transport of Phe, Gly, Lys, and Glu (Fig. 2A).

Amphotericin B and filipin action on PC- or PE-enriched cells

Other polyenes, e.g., amphotericin B and filipin, were able to reduce the influx of most of the amino acids between 2 and 40% in PC- or PE-enriched KA101 (chol) cells (Table 2). However, when compared with their effect on wild-type cells, both polyenes were not significantly effective in reducing the transport of most of the amino acids (Table 2). The resistance acquired to both the polyenes appears to be at a maximum in PC-enriched KA101 (chol) cells. It is evident from the data that as compared with nystatin (Figs. 1–2) the other two antibiotics were more effective in checking the influx of all the amino acids.

The effect of amphotericin B and filipin on choline- or ethanolamine-supplemented 3059 cells is given in Table 3. In contrast to PC- or PE-enriched auxotrophic strains, these supplemented cells remained sensitive to both the polyene.
Our earlier results have demonstrated that hydroxylamine, a known inhibitor of phosphatidylserine decarboxylase (EC 4.1.1.65), when added to the exponentially growing *S. cerevisiae* (3059) cells resulted in a two- to three-fold increase in the levels of PS. The preferential enrichment of PS was possible since the presence of hydroxylamine prevented the further conversion of PS to PE and PC (Trivedi et al. 1983). However, there was no apparent change in the composition of other lipids (data not shown).

To ascertain if the specific enrichment in PS content of *S. cerevisiae* would have any effect on polyene sensitivity, hydroxylamine-grown cells (with a higher PS level) were also tested. As can be seen from Table 4, PS enrichment resulted in an overall resistance towards the action of different polyenes, as judged from their effect on the uptake of amino acids. The uptake of Glu remained sensitive, as compared with the uptake of other amino acids, to various polyenes in spite of PS enrichment (Table 4).

**Fig. 1.** Uptake of Glu, Phe, Gly, and Lys in (A) ade 5a and (B) choline- or ethanolamine-grown KA101 (cho1) cells. ○, Uptake in the absence of nystatin; Δ, uptake in the presence of nystatin (10 μg/mL).

**Fig. 2.** Uptake of Glu, Phe, Gly, and Lys in (A) normal and (B) choline- and ethanolamine-supplemented *S. cerevisiae* (3059) cells. ○, Uptake in the absence of nystatin; Δ, uptake in the presence of nystatin (10 μg/mL).
the results that amphotericin B is more effective than nystatin, since the resistance acquired by \text{S. cerevisiae} toward various polyenes is not due to nonspecific factors, can influence the action of polyenes (Safe et al. 1977; Pierce et al. 1978; Johnson et al. 1978). We had earlier demonstrated that a change in lipid composition, other than sterols, significantly altered the polyene sensitivity of \text{C. albicans} cells (Singh et al. 1979). The present results further corroborate that lipids, other than sterols, have a significant role to play in determining the action of polyenes.

It has been shown that the action of certain polyenes on \text{S. cerevisiae} cells was antagonized by fatty acids, with longer chain fatty acids and more unsaturated fatty acids being the most effective (Iannitielli and Ikawa 1980). The enrichment of PC, PE, or PS of \text{S. cerevisiae} cells was also associated with an increased ratio of saturated versus unsaturated fatty acids (data not shown). This way one would have expected an overall increased sensitivity towards various polyenes. It seems that the resistance observed towards various polyenes is not due to fatty acid changes but may be due to the enrichment of PC, PE, or PS. It is interesting to note that the transport of Glu remained sensitive to nystatin, and resistance was not acquired even after PC, PE, or PS enrichment, suggesting that the effect of phospholipid enrichment on overall polyene action could result from selectivity to a different permease(s).

**Discussion**

In the present work it is demonstrated that specific enrichment of PC, PE, or PS selectively affected the overall sensitivity of \text{S. cerevisiae} cells to polyene antibiotics. It is also evident from the results that amphotericin B and filipin, under the conditions employed, are more potent polyenes, as compared with nystatin, since the resistance acquired by \text{S. cerevisiae} cells owing to PC, PE, or PS enrichment was maximum towards nystatin. Gale et al. (1975) have demonstrated that an increase in the neutral lipid fraction of the cell wall increases the nonspecific binding of polyenes and decreases the sensitivity of the organism as a whole. Evidence is now accumulating that lipid composition, and an array of environmental factors, can influence the action of polyenes (Safe et al. 1977; Pierce et al. 1978; Johnson et al. 1978).

**Acknowledgements**

We are grateful to Professor Susan A. Henry for providing the mutant strain KA101. T.V.G.R. acknowledges the Senior Research Fellowship award received from the Council of the Scientific and Industrial Research, India. This work was supported by a grant from the Indian Council of Medical Research (5/3-1 (8)/81-BMS).

**TABLE 2. Effect of amphotericin B and filipin on amino acid uptake in \text{S. cerevisiae} cells**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Glu ade</th>
<th>Phe ade</th>
<th>Gly ade</th>
<th>Lys ade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B (10 ( \mu )g/mL)</td>
<td>50</td>
<td>20</td>
<td>22</td>
<td>40</td>
</tr>
<tr>
<td>Filipin (10 ( \mu )g/mL)</td>
<td>60</td>
<td>2</td>
<td>26</td>
<td>NI</td>
</tr>
</tbody>
</table>

**TABLE 3. Effect of amphotericin B and filipin on amino acid uptake in \text{S. cerevisiae} (3059) cells**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Glu</th>
<th>Phe</th>
<th>Gly</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B (10 ( \mu )g/mL)</td>
<td>58</td>
<td>65</td>
<td>32</td>
<td>58</td>
</tr>
<tr>
<td>Filipin (10 ( \mu )g/mL)</td>
<td>65</td>
<td>32</td>
<td>20</td>
<td>NI</td>
</tr>
</tbody>
</table>

**TABLE 4. Effect of different polyenes on amino acid uptake in \text{S. cerevisiae} (3059) cells**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Glu</th>
<th>Phe</th>
<th>Gly</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>208</td>
<td>176</td>
<td>127</td>
<td>46</td>
</tr>
<tr>
<td>Nystatin (10 ( \mu )g/mL)</td>
<td>52</td>
<td>120</td>
<td>52</td>
<td>65</td>
</tr>
<tr>
<td>Amphotericin B (10 ( \mu )g/mL)</td>
<td>45</td>
<td>108</td>
<td>58</td>
<td>65</td>
</tr>
<tr>
<td>Filipin (10 ( \mu )g/mL)</td>
<td>130</td>
<td>147</td>
<td>53</td>
<td>48</td>
</tr>
</tbody>
</table>

*NOTE:* Hydroxylamine was added to the exponentially growing cells. Four hours after its addition, the cells were harvested as described earlier (Trivedi et al. 1983). W, without hydroxylamine; H, with hydroxylamine.

**TABLE 2. Effect of different polyenes on amino acid uptake in \text{S. cerevisiae} cells**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Glu ade</th>
<th>Phe ade</th>
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</thead>
<tbody>
<tr>
<td>Amphotericin B (10 ( \mu )g/mL)</td>
<td>50</td>
<td>20</td>
<td>22</td>
<td>40</td>
</tr>
<tr>
<td>Filipin (10 ( \mu )g/mL)</td>
<td>60</td>
<td>2</td>
<td>26</td>
<td>NI</td>
</tr>
</tbody>
</table>

*NOTE:* Amino acid uptake (10 min accumulation) in the absence of antibiotic was taken as 100% from which the percentage inhibition was calculated. N, none; Ch, choline; Ea, ethanolamine; NI, no inhibition.

**TABLE 3. Effect of amphotericin B and filipin on amino acid uptake in \text{S. cerevisiae} (3059) cells**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Glu</th>
<th>Phe</th>
<th>Gly</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B (10 ( \mu )g/mL)</td>
<td>58</td>
<td>65</td>
<td>32</td>
<td>58</td>
</tr>
<tr>
<td>Filipin (10 ( \mu )g/mL)</td>
<td>65</td>
<td>32</td>
<td>20</td>
<td>NI</td>
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</tbody>
</table>

**TABLE 4. Effect of different polyenes on amino acid uptake in \text{S. cerevisiae} (3059) cells**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Glu</th>
<th>Phe</th>
<th>Gly</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>208</td>
<td>176</td>
<td>127</td>
<td>46</td>
</tr>
<tr>
<td>Nystatin (10 ( \mu )g/mL)</td>
<td>52</td>
<td>120</td>
<td>52</td>
<td>65</td>
</tr>
<tr>
<td>Amphotericin B (10 ( \mu )g/mL)</td>
<td>45</td>
<td>108</td>
<td>58</td>
<td>65</td>
</tr>
<tr>
<td>Filipin (10 ( \mu )g/mL)</td>
<td>130</td>
<td>147</td>
<td>53</td>
<td>48</td>
</tr>
</tbody>
</table>

*NOTE:* Hydroxylamine was added to the exponentially growing cells. Four hours after its addition, the cells were harvested as described earlier (Trivedi et al. 1983). W, without hydroxylamine; H, with hydroxylamine.


Effect of phospholipid enrichment on nystatin action: differences in antibiotic sensitivity between in vivo and in vitro conditions

T. Venu Gopala Rao, Sudipto Das and Rajendra Prasad*
Membrane Biology Unit, School of Life Sciences, Jawaharlal Nehru University, New Delhi - 110 067, India
*Present address: School of Biological Sciences, University of Bath, Claverton Down, Bath BA2 7AY, Great Britain

Abstract
Polyene-nystatin had a selective effect on the transport of glutamic acid and lysine in phosphatidylcholine- or phosphatidylethanolamine-enriched cells of Saccharomyces cerevisiae. However, liposomes prepared from lipid extracts from the same cells did not mimic the results of in vivo studies. Our in vitro results demonstrated that factors other than sterols, e.g. phospholipid enrichment, protein lipid interactions and lipid bilayer asymmetry may also affect the overall susceptibility of polyene antibiotics.

Introduction
The polyene antibiotics are effective antifungal agents which interact with the sterols of the cell membrane to alter its structure and cause leakage of essential metabolites (Gale, 1974; Hamilton-Miller, 1973; Kinsky, 1967; Lampen et al., 1960; Norman et al., 1976). There are, however, reports to demonstrate that membrane lipids other than sterols also affect polyene sensitivity (Danilenko and Stepanyuk, 1982; Gale et al., 1975; Hammond and Kliger, 1976; Hickey 1953; Iannitelli and Ikawa, 1980; Johnson et al., 1978; Pierce et al., 1978).

In addition to intact cells, liposomes as model membranes have also been widely used to ascertain the mechanism of polyene action by following the binding of the antibiotic or the leakage of electrolytes or non-electrolytes (Kruijff et al., 1974; Ohki et al., 1979; Oku et al., 1980; Hsuchen and Feingold, 1973).

We had earlier observed that when C. albicans cells were grown on alkanes of different chain lengths, their lipid composition was altered, but ergosterol contents were not significantly changed. Such cells responded differently to polyene-nystatin, which indicated that lipids other than ergosterol could also affect the action of polyene (Singh et al., 1979).

Our present results demonstrate the effect of nystatin on the leakage of glutamic acid and lysine from the liposomes prepared from the lipids of cell extracts having enriched levels of phosphatidylcholine (PC) or phosphatidylethanolamine (PE). It was noted that polyene action on the liposomal membrane is different from its action on intact cells with similar lipid composition.
Materials and methods
Nystatin, glutamic acid and lysine were purchased from Sigma Chemical Company, U.S.A. 14C-glutamic acid and 3H-lysine were purchased from Bhabha Atomic Research Centre, Bombay, India. All other chemicals were of analytical grade.

Organism and growth conditions
The parental strain of *Saccharomyces cerevisiae* ade 5α and mutant strains KA101 (chol, inol, lys2, can1) requiring choline or ethanolamine for growth and MC13 (Cho⁺, inol, lys2, can1) (Atkinson et al., 1980), were obtained from Professor Susan A. Henry, Albert Einstein College of Medicine, Bronx, New York, U.S.A. Another strain, *S. cerevisiae* (3059) was obtained from the National Chemical Laboratory, Pune, India.

The growth conditions of these strains were essentially similar to those described by Trivedi et al. (1982). Cells of the exponential growth phase were harvested by centrifugation at 1,500 x g for 10 min and washed thrice with distilled water before suspending them finally for transport assay and lipid extraction.

Lipid extraction and estimation of ergosterol, triacylglycerols and phosphate
The method of Folch et al. (1957) was used for total lipid extraction from yeast cells grown under different conditions. Phospholipids were separated by thin layer chromatography using silica gel-G as an adsorbant (Wagner et al., 1962). Ergosterol was estimated by the direct extraction of the cells in boiling alcoholic KOH (10%) for 1 h (MacIntyre and Ralstan, 1954). Triacylglycerols were estimated by the method of Van Handel and Zilversmith (1957), using triolein as a standard. Lipid phosphorus was assayed according to the method of Eibl and Lands (1969).

Transport assay
Transport assay procedure was similar to that described earlier (Jayakumar et al., 1978; Singh et al., 1978, 1979; Khare et al., 1982; Rao et al., 1983; Trivedi et al., 1983).

Preparation of liposomes for permeability studies
One ml of lipid extract from different cells in chloroform containing 1 mg lipid was dried as a thin film in a flat bottom glass vial under a gentle stream of nitrogen. It was then vacuum desiccated to remove the last traces of the solvent. The film thus formed was vigorously vortexed for 2 min in a 20 mM solution of either 14C-glutamic acid or 3H-lysine in Tris-HCl buffer (100 mM, pH 7.4) in a nitrogen atmosphere (Bangham et al., 1974). Nystatin when required, was added to the lipid suspension prior to drying and mixed well to give a final concentration of 50 μg/ml.

For permeability studies, the liposomal suspension prepared from different lipid extracts was freed of untrapped markers by subjecting it to combined gel filtration and centrifugation on minicolumns as described by Fry et al. (1978). The resulting liposomal suspension was dialysed for 2 h against
500 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 10 mM NaCl. At indicated intervals of time, 10 μl aliquots were withdrawn and their radioactivity was measured using Bray's fluid in a Liquid Scintillation Counter (Packard Tricarb 3100, U.S.A.).

**Results and discussion**

We had earlier observed that *S. cerevisiae* (3059) cells when grown in media supplemented with a high concentration of choline (20 mM) or ethanolamine (20 mM), resulted in an increase of PC or PE levels respectively.

The auxotrophic mutants which require choline (1 mM) or ethanolamine (1 mM) for growth also showed a consistent increase in PC or PE levels (Trivedi et al., 1982). In order to assess if PC or PE enrichment would have any effect on polyene action, such cells were exposed to nystatin. Amino acid transport was used as an index of polyene action. It was observed that due to PC or PE enrichment of yeast phospholipids, polyene-nystatin had a selective effect on glutamic acid and lysine transport. The uptake of lysine became completely resistant, while glutamic acid uptake remained susceptible to nystatin action (Table 1).

In order to ascertain the effect of polyene antibiotic *in vitro*, liposomes were prepared from PC- or PE-enriched lipids, where the varied PC/ergosterol or PE/ergosterol lipid composition used for liposomes was dependent on the growth status of the cells (Table 1). Liposomes prepared from the lipid extracts of such cells were subjected to nystatin action either by incubating liposomes with it or by trapping the nystatin during the preparation of liposomes (Hoogeveen and Kruijff, 1978). Since both procedures had given a similar pattern of leakage of marker permeants (amino acids), the incorporation of nystatin into the liposomal membrane was preferred. Characterization of the liposomal preparation revealed that an efficient quantitative encapsulation of radioactive amino acids was feasible under our experimental conditions (data not shown).

**Effect of nystatin on liposomes**

In order to understand the effect of nystatin, the leakage of encapsulated 14C-glutamic acid and 3H-lysine was followed from the liposomes having different PC or PE levels. As can be seen from Figures: 1a and 1f, in the case of normal liposomes prepared from the lipids of wild type cells (ade 5a), the presence of nystatin elicited significant leakage of 14C-glutamic acid and 3H-lysine.

Figures 1b to 1e and 1g to 1j show the leakage of 14C-glutamic acid and 3H-lysine in the presence and absence of nystatin in liposomes prepared from the lipid extracts of PC- or PE-enriched cells. When compared to normal liposomes (ade 5a), the differences in the leakage of 14C-glutamic acid and 3H-lysine in the presence of nystatin, were not more than 20% (Figures 1b to 1e and 1g to 1j). Therefore, it appears that the PC- or PE-enriched liposomes became resistant towards nystatin action since the presence of polyene could not cause the leakage of encapsulated 14C-glutamic acid or 3H-lysine.
Figure 1 Leakage of $^{14}$C-glutamic acid and $^{3}$H-lysine from the liposomes prepared from lipid extracts of normal (ade 50) and PC- or PE-enriched cells (KA101 and MC13) of S. cerevisiae. Leakage was followed at the indicated time intervals as described in Materials and methods, in the presence (△) or absence (●) of nystatin. Figures 1a and 1f, ade 50; Figures 1b and 1g, KA101 choline-grown; Figures 1c and 1h, KA101 ethanolamine-grown; Figures 1d and 1i, MC13 choline-grown; Figures 1e and 1j, MC13 ethanolamine-grown.
Table 1 Lipid composition and uptake of glutamic acid and lysine in the presence of nystatin in *S. cerevisiae* cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium supplemented</th>
<th>Amino acid uptake (% of inhibition):</th>
<th>Lipid composition:</th>
<th>PE/ergosterol*</th>
<th>Ergosterol***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glu</td>
<td>Lys</td>
<td>PC/ergosterol*</td>
<td></td>
</tr>
<tr>
<td>ade 5n**</td>
<td>none</td>
<td>.80</td>
<td>54</td>
<td>2.03</td>
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<tr>
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<td>ethanolamine</td>
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<td>1.63</td>
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<tr>
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<td>chol</td>
<td>56</td>
<td>NI</td>
<td>2.72</td>
<td>1.42</td>
</tr>
<tr>
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<td>ethanolamine</td>
<td>62</td>
<td>NI</td>
<td>1.94</td>
<td>1.42</td>
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<tr>
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<td>70</td>
<td>41</td>
<td>1.06</td>
<td>0.40</td>
</tr>
<tr>
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<td>71</td>
<td>13</td>
<td>1.90</td>
<td>0.35</td>
</tr>
<tr>
<td>3059</td>
<td>ethanolamine</td>
<td>66</td>
<td>2</td>
<td>0.76</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Amino acids uptake in the absence of nystatin was taken as 100% from which the percentage inhibition was calculated. All values are an average of three to five separate determinations. NI, no inhibition.

* Ratio.
** Wild type.
*** µmoles/g total lipid.
**** MC13 is an inositol auxotroph. In contrast to 3059 cells, the addition of 1 mM of choline or ethanolamine to MC13, resulted in PC- or PE-enrichment. For 3059 cells to be enriched in PC or PE, however, higher concentrations (20 mM) of bases were required.
Figure 2. Leakage of $^{14}$C-glutamic acid and $^3$H-lysine from the liposomes prepared from lipid extracts of normal and PC- or PE-enriched (choline- or ethanolamine-supplemented) cells of S. cerevisiae (3059). Leakage was followed at the indicated time intervals as described in Materials and Methods, in the presence (△ — △) or absence (● — ●) of nystatin. Figures 2a and 2d, non-supplemented; Figures 2b and 2e, choline-supplemented; Figures 2c and 2f, ethanolamine-supplemented media.
In a similar manner to auxotrophic strains, the liposomes of PC- or PE-enriched lipids of supplemented cells (3059) also exhibited resistance towards nystatin, since the percentage leakage of both $^{14}$C-glutamic acid and $^{3}$H-lysine was not very different in the presence or absence of the antibiotic (Figures 2b and 2c, 2e and 2f). The leakage of $^{3}$H-lysine was somewhat enhanced by the presence of polyene in PC-enriched liposomes, but the percentage enhancement was not more than what was observed in the case of normal liposomes (Figure 2e).

Liposomes prepared from both supplemented cells or auxotrophic strains indicated that PC- or PE-enrichment affects the leakage of trapped amino acids. However, the data do not mimic the in vitro results where cells having enhanced levels of PC or PE demonstrated some selectivity towards polyene action (Table 1). In both the auxotrophs, and in supplemented cells having enhanced PC or PE levels, the uptake of glutamic acid remained sensitive to nystatin action whilst it had acquired resistance towards nystatin action in vivo. In the liposomes prepared either from PC- or PE-enriched lipid extracts, however, the leakage of both glutamic and lysine became resistant and no selectivity was observed (Figures 1 and 2).

Our earlier reports (Singh et al., 1979) as well as those from other laboratories (Hammond and Kliger, 1976; Gale et al., 1975; Hickey, 1953; Iannitelli and Ikawa, 1980) have demonstrated that factors other than sterols could also affect polyene action. Our present in vitro experiments further corroborate earlier results. It is evident that the efflux of both encapsulated amino acids became resistant towards nystatin.

Therefore, in contrast to intact cells which had an identical lipid composition, no selectivity of nystatin action was observed from in vitro results. This means that the presence of proteins and other membrane components may also be essential for polyene binding and interaction. It is also possible that the lipid asymmetry of the liposomal bilayer or the packing of lipid molecules may be different from the intact cells, which may also influence polyene action. However, this still needs to be further elucidated.

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References


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