CHAPTER II

EFFECT OF PHOSPHOLIPID ENRICHMENT ON NYSTATIN ACTION: DIFFERENCES IN ANTIBIOTIC SENSITIVITY BETWEEN IN VIVO AND IN VITRO CONDITIONS
INTRODUCTION

In this chapter in vitro studies were followed using liposomes to ascertain the action of nystatin. The term 'liposome' can be defined as an assemblage of phospho- and other lipids, sustaining a biomolecular configuration which does not require a mechanical support for its stability. Bangham and his co-workers reported the bilayer structure of liposomes and their ability to mimic the function of natural membranes (Bangham et al., 1965a, b, 1967, 1974; Szoka and Papahadjopoulos, 1980).

Liposomes can capture solutes during formation and release them later under suitable conditions at varied rate (Bangham et al., 1965a,b). In contrast to biological membranes, liposomes offer the advantage that the lipid composition can be varied under controlled conditions and influence of these variations on membrane permeability can be investigated. For example, liposomes can be prepared with varied fatty acid composition of the phospholipids (Papahadjopoulos and Miller, 1967), charged phospholipids (Montal and Muller, 1972) and with or without sterols (Kinsky et al., 1968;
Hopfer et al., 1984).

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 and non-electrolytes (Hsu Chen and Feingold, 1973a,b; Kruijff et al., 1974; Ohki et al., 1979; Oku et al., 1980; Cybulska et al., 1981). Liposomes containing sterols have been shown to bind and alter the ultraviolet absorption spectra of polyene antibiotics. Norman et al. (1972a) have demonstrated that filipin, lucensomycin, amphotericin B, nystatin and pimaricin are capable of binding to different sterol containing liposomes with subsequent change in UV absorption spectra. Drabikowski et al. (1973) and Strom et al. (1973) reported that fluorescence intensities of solutions of filipin and lucensomycin were enhanced in the presence of liposomes made from cholesterol and lecithin. Sessa and Weissman (1967, 1968) have studied the action of polyenes on cholesterol containing and cholesterol less liposomes by using rate of release of glucose, phosphate and chromate as an index of action. Kinsky et al. (1968) have concluded that polyene mediated release of trapped substance was much more rapid from cholesterol containing liposomes. It is well established that nystatin, amphotericin B, etruscomycin, and filipin could enhance the rate of release of various
encapsulated solutes (Sessa and Weissman, 1967; Kinsky et al., 1968; Sessa and Weissman 1968; Kinsky, 1970; Hsu Chen and Feingold, 1973a,b; Kruijff et al., 1974a,b; Hoogevest and Kruijff, 1978).

In order to ascertain how the altered lipid contents and other membrane components affect polyene action, the liposomal system was used. Liposomes were prepared from the lipid extracts of PC-or PE-enriched cells. Results of this chapter demonstrate the effect of nystatin on the leakage of Glu and Lys from the liposomes prepared from lipid extracts of cells having enriched levels of PC or PE. The significance of membrane components other than lipids which could affect polyene action is also discussed.
RESULTS

Lipid Composition of Liposomes

Since liposomes were prepared from the lipid extracts of different cells, the varied lipid composition used for liposomes was dependent on the supplementation during the growth of cells. Table 10 shows the summary of lipid composition taken from the first chapter. This table has been given for ready comparison with liposomal study of this chapter.

Characterization of Liposomes

Fig. 11 shows the elution pattern of the liposomes prepared from lipid extracts of S. cerevisiae (3059) from a sephadex G-25 column. The data based on the radioactivity of encapsulated amino acid, liposomal turbidity, contents of encapsulated bromophenol blue and amount of lipid phosphate in fractions 4 to 8 strongly suggest that an efficient encapsulation of the amino acid and bromophenol blue within the liposomes was possible under our experimental conditions (Fig. 11)(Naoi et al., 1977).
### TABLE 10

**LIPID COMPOSITION AND UPTAKE OF GLU AND LYS IN THE PRESENCE OF NYSTATIN IN SACCHAROMYCES CEREVISIAE CELLS**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium Supplemented</th>
<th>Amino acid uptake (^@) (% of inhibition)</th>
<th>Lipid Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glu</td>
<td>Lys</td>
</tr>
<tr>
<td>ade 5α(^a)</td>
<td>none</td>
<td>80</td>
<td>54</td>
</tr>
<tr>
<td>KA101 (chol)</td>
<td>choline (1 mM)</td>
<td>57</td>
<td>8</td>
</tr>
<tr>
<td>KA101 (chol)</td>
<td>ethanolamine (1 mM)</td>
<td>65</td>
<td>7</td>
</tr>
<tr>
<td>3059</td>
<td>none</td>
<td>70</td>
<td>41</td>
</tr>
<tr>
<td>3059</td>
<td>choline (20 mM)</td>
<td>71</td>
<td>13</td>
</tr>
<tr>
<td>3059</td>
<td>ethanolamine (20 mM)</td>
<td>66</td>
<td>2</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.

\(^@\) = data taken from Fig. 9 of 1st chapter
\(^a\) = wild type
\(^b\) = μmoles/g total lipid
* = ratio
Fig. 11: Elution pattern of liposomes prepared from lipid extract of yeast cells on sephadex G-25 column.

- Column size: 23.5x1 cms.
- Flow rate: 12 ml/hr.
- Elution buffer: Tris-HCl (100 mM, pH 7.4)
- Fractions: 2 ml

0-----0 Turbidity of liposomes at 400 nm

●● Encapsulated $^{14}$C-Glu

△△△ Phosphate content

□□□ Bromophenol blue content
Furthermore, the untrapped markers could be effectively removed as was evident from the elution of the free $^{14}$C-Glu and bromophenol blue in later fractions. The absence of lipid phosphorus or turbidity in later fractions indicated that all the liposomes were eluted in the void volume of the column. An identical pattern of encapsulation of $^{14}$C-Glu or $^{3}$H-Lys into liposomes was observed, when lipid extracts from different cells were used. It was therefore, possible to quantitatively encapsulate the amino acids into liposomes prepared from different lipid extracts (Bangham et al., 1974).

Liposomes prepared from the lipid extract of S. cerevisiae 3059 cells were subjected to nystatin by two procedures 1) incubating liposomes with nystatin 2) trapping the polyene during the preparation of liposomes. Since both procedures had given more or less same pattern of leakage of marker permeants, the incorporation of nystatin into liposomal membrane was preferred for further studies.

Encapsulation of Amino Acids by Liposomes

As can be seen from Table 11 the efficient encapsulation of both $^{14}$C-Glu and $^{3}$H-Lys was observed in the liposomes prepared from varied lipid composition. The efficiency of $^{14}$C-Glu encapsulation was increased in PC- or PE-enriched liposomes, when compared to normal liposomes.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium Supplemented</th>
<th>Glu</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-Nys</td>
<td>+Nys</td>
</tr>
<tr>
<td>ade 5αa</td>
<td>none</td>
<td>2.9</td>
<td>4.0</td>
</tr>
<tr>
<td>KA101 (chol)</td>
<td>choline (1 mM)</td>
<td>4.4</td>
<td>4.9</td>
</tr>
<tr>
<td>KA101 (chol)</td>
<td>ethanolamine (1 mM)</td>
<td>4.9</td>
<td>5.5</td>
</tr>
<tr>
<td>3059</td>
<td>none</td>
<td>3.2</td>
<td>3.8</td>
</tr>
<tr>
<td>3059</td>
<td>choline (20 mM)</td>
<td>5.4</td>
<td>6.8</td>
</tr>
<tr>
<td>3059</td>
<td>ethanolamine (20 mM)</td>
<td>8.0</td>
<td>7.7</td>
</tr>
</tbody>
</table>

* = encapsulation values were adjusted for phospholipid concentration in each case.
a = wild type
-Nys = absence of the nystatin
+Nys = presence of the nystatin
However, the $^3$H-Lys encapsulation was reduced (40 to 50%) in PC- or PE-enriched liposomes. The encapsulation of both $^{14}$C-Glu and $^3$H-Lys was higher in PE-enriched liposomes prepared from 3059 cells. Since the ergosterol content in the liposomes was not altered (Table 10), the difference in the encapsulation may be due to the varied molar ratios of PC or PE. It is interesting to observe that nystatin presence enhanced the encapsulation capacity of liposomes prepared from the lipid extracts of normal cells. It appears that the encapsulation of amino acids does not depend directly on the ergosterol content, but may be related to the packing of lipid molecules in the liposomes. The enhancement of encapsulation in the presence of nystatin may be due to the packing faults caused by nystatin.

**Effect of Nystatin on Liposomes**

As can be seen from the Figs. 12A and D, in the case of normal liposomes prepared from the lipids of wild type cells (ade 5\(a\)), the presence of nystatin elicited significant leakage of $^{14}$C-Glu and $^3$H-Lys. Figs. 12B and E and 12C and F show the leakage of $^{14}$C-Glu and $^3$H-Lys 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 5\(a\)), the differences in the leakage of $^{14}$C-Glu and $^3$H-Lys in the presence of
Fig. 12: Leakage of $^{14}$C-Glu and $^{3}$H-Lys from the liposomes prepared from the lipid extracts of ade 5α and PC- or PE-enriched cells (KA101) of Saccharomyces cerevisiae.

Leakage was followed at the indicated time intervals as described in 'Materials and Methods'.

- Leakage in the absence of nystatin
- Leakage in the presence of nystatin
nystatin, were not more than 20% (Figs. 12B and C; 12E and F). 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 $^{14}$C-Glu or $^3$H-Lys.

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-Glu and $^3$H-Lys was not very different in the presence or absence of the antibiotic (Figs. 13B and C; 13E and F). The leakage of $^3$H-Lys 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 (Figs. 13D and E).
Fig. 13: Leakage of $^{14}$C-Glu and $^{3}$H-Lys from the liposomes prepared from lipid extracts of normal and PC- or PE-enriched (choline- or ethanolamine-supplemented) cells of Saccharomyces cerevisiae (3059).

Leakage was followed at the indicated time intervals as described in 'Materials and Methods'.

- Leakage in the absence of nystatin
- Leakage in the presence of nystatin
DISCUSSION

Results of liposomes prepared from both supplemented cells or auxotrophic strain indicated that PC or PE enrichment affects the leakage of trapped amino acids. However, the data do not mimic the in vivo results where cells having enhanced levels of PC or PE demonstrated some selectivity towards polyene action (Table 10). In the auxotroph and in supplemented cells having enhanced PC or PE levels, the uptake of Glu remained sensitive to nystatin action while it had acquired resistance towards nystatin action in vitro. In the liposomes prepared either from PC- or PE-enriched lipid extracts, however, the leakage of both $^{14}$C-Glu and $^3$H-Lys became resistant and no selectivity was observed (Figs. 12 and 13).

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 other lipids 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. A probable explanation may be that nystatin, in the case of cells, might bind to the proteins and alters the membrane permeability by changing their conformation. Other explanation is that presence of proteins may cause phase separation of lipids in the bilayer and may bind to some lipids specifically. This specificity might be disturbed due to the external supplementation of choline or ethanolamine which resulted in increase of PC or PE levels. In addition, the protein-lipid interactions in cells may give rise to zones in the bilayer where the nystatin is having a solubility difference than that in the case of liposomes where no protein is present. This protein lipid interaction might be the possible reason for the resistance acquired by the PC- and PE-enriched cells.

It is also possible that the lipid asymmetry of the liposomal bilayer or the packing of the lipid molecules, may be different from the bilayer of intact cells which may
also influence the polyene action. The supplementation of choline and ethanolamine may cause the enrichment of PC or PE either on the outer side of membrane or inner side of the membrane, thereby causing asymmetrical arrangement of phospholipids. The resistance acquired by the cells may be due to the asymmetry of membrane lipids since it would amount difference in the binding/interaction of polyenes. In addition, the specific lipid protein interactions may also be responsible for the bilayer asymmetry which again determine the partitioning of nystatin. Similar asymmetry will not be present in the liposomes due to the absence of proteins. This probably could explain why the resistance acquired by PC- or PE-enriched cells is not found at the same level as in the case of liposomes?