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THE EFFECT OF ALTERED ERGOSTEROL CONTENT ON THE TRANSPORT OF VARIOUS AMINO ACIDS IN *CANDIDA ALBICANS*

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Summary

Candida albicans cells have low levels of ergosterol when grown in ascorbic acid-supplemented media. When cells are grown in hydroquinone-supplemented media, the ergosterol levels became higher as compared to normal cells. The uptake of lysine, glycine, glutamic acid, proline, methionine and serine is reduced in hydroquinone-supplemented cells. In contrast to hydroquinone-supplemented cells, the rate and level of accumulation of these amino acids are higher in ascorbic acid-supplemented cells. Nystatin-resistant isolates of *C. albicans* with low ergosterol contents also exhibit an increased rate and level of accumulation of these amino acids. The uptake of phenylalanine and leucine remained unaffected by such a change in ergosterol levels brought about by different supplementation of the media. The results demonstrate a correlation between ergosterol levels and amino acids uptake. Contrary to various reports, the rate of K^+ efflux does not seem to correlate with the amino acid uptake in *C. albicans* cells.

Introduction

Ergosterol is the predominant sterol of aerobically growing yeast cells [1—3] and is mainly present in the cytoplasmic membrane [4]. The study of the effect of sterols on the physical state of various artificial membrane systems has revealed that sterol increases order and rigidity of such membranes [5,6]. The permeability of model membranes for water, anions, cations and non-elec-

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trolytes has also been shown to be reduced significantly after the incorporation of cholesterol [7—11]. Furthermore, in vitro incorporation or replacement of sterol into various cellular membranes was found to affect the permeability of the cells [12—15].

Yeast cells are known to exhibit altered sterol contents when grown on media supplemented with various compounds e.g. ascorbic acid, hydroquinone, methylene blue and potassium persulfate [16]. Various polyene antibiotics e.g. nystatin and amphotericin-B have also been used to get resistant strains [17,18] having low levels of sterols. The effect of altered sterol levels and their relation to amino acid transport is yet to be explored. In the present study, the ergosterol levels of *Candida albicans* cells were altered by above-mentioned approaches and the uptake of various amino acids was followed. Our results demonstrate a relationship between the amino acid uptake and the ergosterol level in *C. albicans* cells.

Materials and Methods

Materials. Nystatin, ergosterol, bovine serum albumin, sodium azide and arsenate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All ^{14}C-labelled amino acids were from Bhabha Atomic Research Centre, Bombay, India. All other chemicals were obtained from commercial sources and were of the highest purity available.

Organism and growth conditions. C. albicans strain 3100 was grown in a synthetic media as described earlier [19]. Hydroquinone and ascorbic acid were supplemented at a final concentration of 3 mg/ml and 5 mg/ml, respectively. Nystatin-resistant cells were obtained according to Hebeka and Solotorovsky [17,18] where cells were grown in the presence of different concentrations of nystatin. For cells grown in various supplemented media the following terms are used: cells grown in hydroquinone-supplemented media, Hy cells; cells grown in ascorbic acid-supplemented media, As cells; and cells grown in nystatin-supplemented media, Nys cells.

Maintenance of nystatin-resistant strain. The sensitive parent of *C. albicans* and resistant strains were maintained on agar slopes of complete media at 5—7°C and were recultured at intervals of 3—4 weeks. In order to prevent reversion to the resistant strain, antibiotics were incorporated into slants of the complete agar medium according to their resistance capacity.

Transport assay. Reaction mixture containing normal cells or cells grown on different supplemented media (150—200 μg protein/ml) were preincubated at 30°C for 10 min after the addition of cycloheximide (200 μg/ml) to inhibit protein synthesis. The reaction was initiated by the addition of ^{14}C-labelled amino acid. At indicated time intervals, 0.1 ml aliquots were taken out in 5 ml chilled distilled water. The diluted suspension was rapidly filtered through 0.45 μm millipore filter and radioactivity retained was counted in a Packard scintillation counter using a toluene-based scintillation fluid.

Lipid extraction and estimation. The method of Folch et al. [20] was used to extract lipids from different cells. The chloroform/methanol (2 :1, v/v) extract collected after three times extraction from yeast residue, was washed with 0.9% NaCl solution to remove non-lipid contaminants. The lipid con-

taining lower organic phase was evaporated to dryness in a rotary evaporator under reduced pressure and temperature. The residue was suspended in chloroform and stored at −20°C under N_2 atmosphere. Total lipid content was estimated gravimetrically and phospholipid according to Wagner et al. [21].

Sterol extraction and estimation. Harvested cells were boiled in 10% alcoholic KOH for 1 h. The hydrolysate were cooled and extracted three times with petroleum ether. The ether layer was evaporated to dryness and the residue was dissolved in known volumes of chloroform. Suitable aliquots were used for colorimetric estimation of sterols by MacIntyre and Ralston's method using ergosterol as a standard [22]. The ether extract of ergosterol was also analyzed spectrophotometrically by taking absorbance at 282 nm [23].

Protein concentrations were determined by Lowry's method [24].

K^+ release measurements. For the estimation of K^+, different types of cells were suspended at a final concentration of protein (200 μg/ml) in sterile distilled water or 0.05 M Tris-HCl buffer, at 25°C and constantly stirred throughout the course of measurement. The K^+ concentration was measured using a K^+ selectrode ® (Radiometer A/S, Copenhagen, Denmark) which was connected to a potentiometric recorder (Riken Penshi Co. Ltd., Japan). When not in use the electrode was kept in 0.1 M KCl and the 'salt bridge' filled a fresh before use. The instrument was calibrated against standard solution of KCl and could be used effectively to measure K^+ over the range of $2 \cdot 10^{-7}$—10^{-4} M. The release of K^+ was initiated from different cells by the addition of cold amino acid (lysine 1.66 mM, proline 1 mM, glutamic acid 0.83 mM, glycine 0.55 mM, phenylalanine 2 mM, leucine 2 mM, methionine 1.5 mM and serine 0.25 mM) or nystatin (30 μg/ml). The release was recorded till the recorder showed a constant level of release which was attained within 6—10 min.

The percent of K^+ release was calculated from the following expression used by Chen et al. [25]:

$$\frac{K^+ \text{ release in presence of amino acid or nystatin} - \text{control}}{\text{Total intracellular amount of } K^+ - \text{control}} \times 100$$

Here control designates, the amount of K^+ present in the cell suspension prior to the addition of amino acid or nystatin. The amount of intracellular K^+ was obtained by heating the cells in boiling water bath for 5 min.

Nystatin solution. Stock solution (2 mg/ml) was prepared in dimethylformamide, stored below 0°C, and used within a week. Immediately before use, portions were diluted in water. At the maximum concentration of organic solvent in the medium (0.5%), the physiology of cells was not detectably altered. Antibiotic concentrations are expressed as pure compound.

Results

Lipid composition of normal, Hy and As cells

There was a 26% increase in the total ergosterol level when cells were grown in hydroquinone-supplemented media (Table I). However, the total ergosterol content was reduced by 16—17% in As cells. The ergosterol values were compared with the normal glucose-grown cells and the changes were found to be

TABLE I

LIPID COMPOSITION IN NORMAL, Hy AND As *C. ALBICANS* CELLS

Lipids, phospholipids and ergosterol contents were estimated as described in Materials and Methods. Hy cells, cells grown in hydroquinone-supplemented media; As cells, cells grown in ascorbic acid-supplemented media.

Cells	Lipid composition (mg content/g protein)		
	Total lipid	Phospholipid	Ergosterol
Normal	100.0 ± 7.05	26.0 ± 2.65	15.0 ± 0.32
Hy cells	98.0 ± 4.80	26.0 ± 3.08	19.0 ± 0.58
As cells	100.0 ± 3.26	27.0 ± 2.18	12.5 ± 0.36

statistically very significant (Table I). It is pertinent to mention here that beside ergosterol, there was no significant change in total lipid and phospholipid levels of both Hy and As cells (Table I).

Amino acids uptake in normal, Hy and As cells

The transport of lysine, glycine, phenylalanine, leucine, glutamic acid, proline, methionine and serine has been shown to be an active process in *C. albicans* cells [19]. In order to investigate if the changes in ergosterol contents observed with Hy and As cells had any effect on the characteristics of uptake of these amino acids, the transport rate and level of accumulation of different amino acids was followed. The uptake of lysine, glycine, glutamic acid, proline, methionine and serine was reduced significantly (25—40%) in Hy cells (having high ergosterol levels). However, the uptake of these amino acids was more in As cells (having low ergosterol levels) (Fig. 1A and B). In contrast to the transport of lysine, glycine, glutamic acid, proline, methionine and serine in As and Hy cells, the uptake of leucine and phenylalanine remained more or less the same in both kinds of supplemented cells (Fig. 1A and B).

Kinetics of uptake of various amino acids in normal, Hy and As cells

The changes observed in the uptake of different amino acids in cells having low and high levels of sterol could also be due to a change in the affinity of the carrier(s) for these amino acids. The kinetic data revealed that the apparent K_m values for all of these amino acids in As and Hy cells were found to be same to that of normal cells, (0.33 mM for lysine, 0.11 mM for glycine, 0.16 mM for glutamic acid, 0.25 mM for proline, 0.16 mM for methionine, 0.14 mM for serine, 0.30 mM for phenylalanine and 0.20 mM for leucine) (Fig. 2A and B). But, there was a decrease in V values in cells having high content of ergosterol and an increase in V values in cells having low content of ergosterol. For instance, the V value (μmol/mg protein per min) for lysine uptake in normal cells was 0.33 ± 0.01 while it increased in As cells to 0.40 ± 0.03 and reduced to 0.25 ± 0.01 in Hy cells (Table II). However, the V value (μmol/mg protein per min) for phenylalanine (0.83 ± 0.03) and leucine (0.36 ± 0.02) remained unchanged in Hy and As cells (Table II).

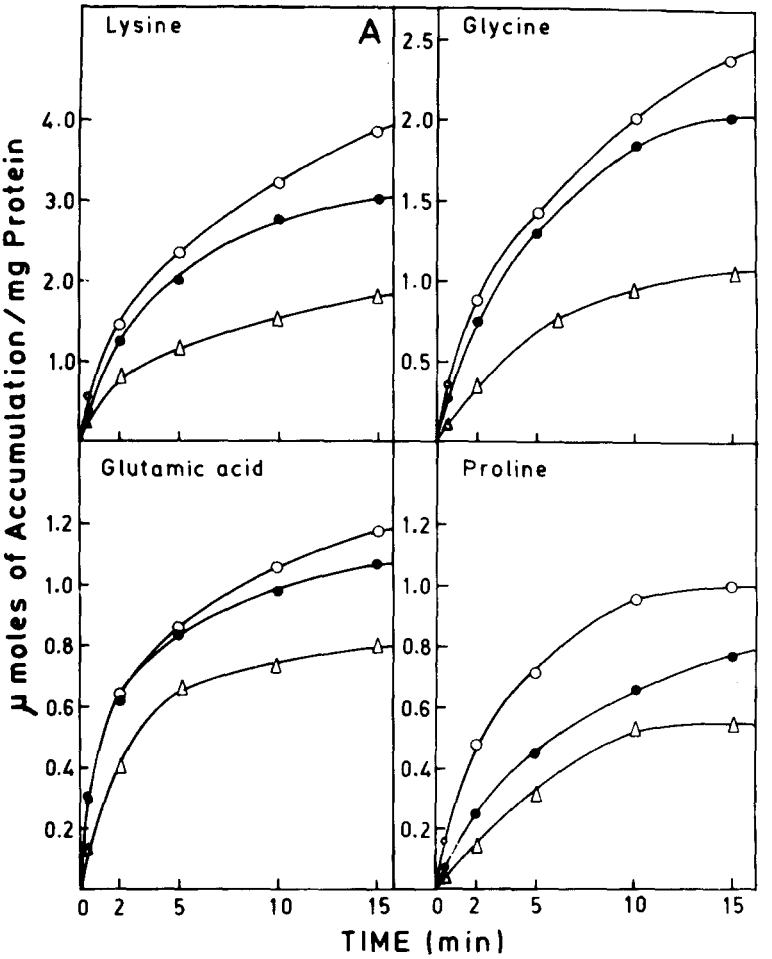

Fig. 1A.

Release of K^+ during the uptake of different amino acids in normal, Hy and As cells

The percent release of K^+ during amino acid uptake was found to be different for different amino acids. However, the change in ergosterol content brought about by different supplementation (Hy and As cells did not have any significant effect on the release of K^+ (Table III).

The sensitivity of nystatin, which is known to cause a rapid efflux of K^+ [25—31] was checked in these normal, Hy and As cells. It was observed that the cells having more ergosterol had a more rapid rate of K^+ efflux compared to the cells with less ergosterol (Fig. 3). It seems that the efflux of K^+ depends on the extent of binding of the antibiotic with the available sterols.

Ergosterol content of Nys cells

There was a gradual reduction in total ergosterol contents in cells grown in media supplemented with different concentrations of nystatin (Table IV). There was a significant reduction in total ergosterol content at lower concentra-

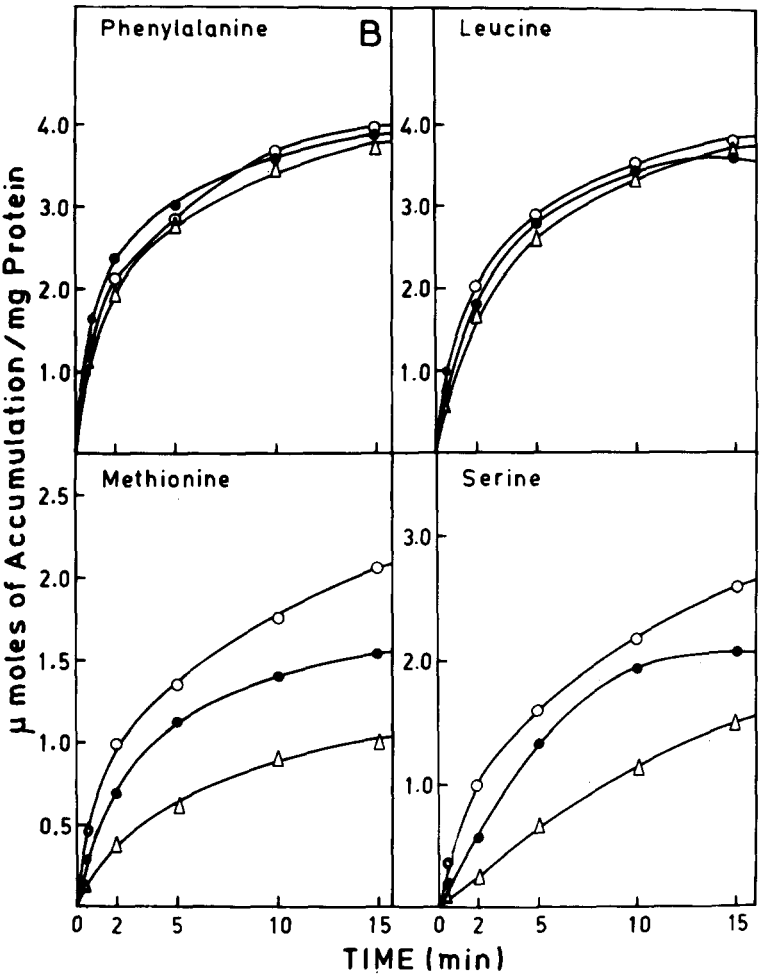

Fig. 1. (A, B) Transport of lysine, glycine, glutamic acid, proline, phenylalanine, leucine, methionine and serine in normal, Hy (cells grown in hydroquinone-supplemented media) and As (cells grown in ascorbic acid-supplemented media) *C. albicans* cells. Cells (150—200 μg protein/ml) were preincubated for 10 min with cycloheximide (200 μg/ml) and the uptake was initiated by the addition of ^{14}C-labelled amino acids (lysine 1.66 mM, proline 1 mM, glutamic acid 0.83 mM, glycine 0.55 mM, phenylalanine 2 mM, leucine 2 mM, methionine 1.5 mM and serine 0.25 mM) to the assay mixture. At indicated time intervals 0.1 ml aliquots were taken out, diluted, filtered and radioactivity retained in cells was counted. The changes shown are statistically significant since the *P* values range from <0.002 to <0.05. The uptake of amino acids in normal cells (●———●); in Hy cells (△———△) and in As cells (○———○).

tions of nystatin as compared to higher concentration of the antibiotic. The total reduction of ergosterol as compared to normal cells ranged from 10 to 28% (Table IV).

Amino acids uptake of Nys cells

When the cells were grown in media supplemented with lower concentrations of nystatin (0.25 and 0.5 μg/ml) there was an increased level of accumulation of proline, lysine, serine, methionine, glutamic acid and glycine but as the concentration of nystatin was raised in the media, the uptake of above amino

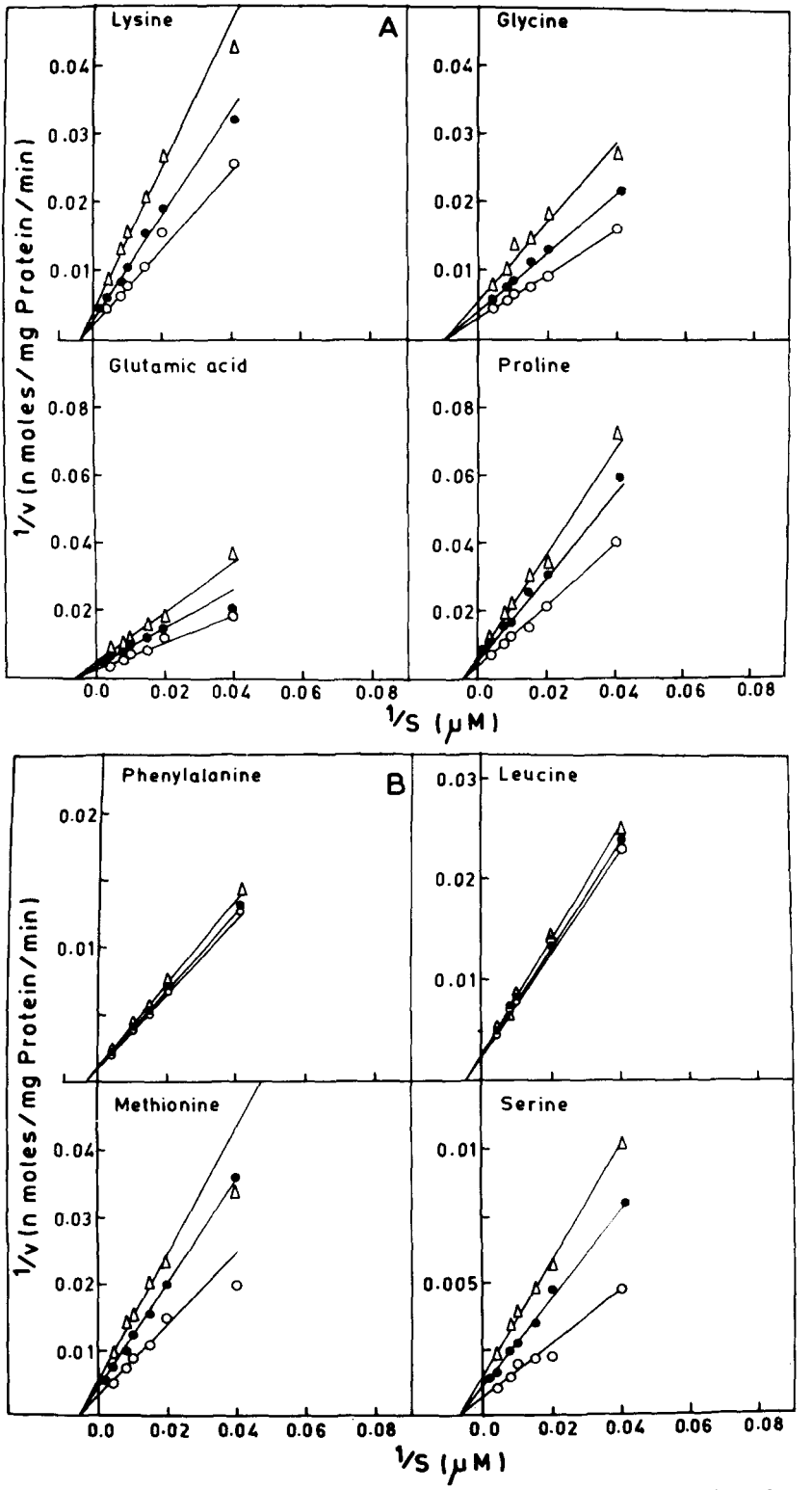

Fig. 2. (A, B) Lineweaver-Burk plots of lysine, glycine, glutamic acid, proline, phenylalanine, leucine, methionine and serine uptake in normal, Hy and As *C. albicans* cells. Assay conditions were similar to those described for Fig. 1. The reaction was terminated after 30 s. Symbols are as used in Fig. 1. The changes shown are statistically significant since P values range from <0.01 to <0.05.

TABLE II

K_m AND V VALUES OF AMINO ACIDS IN NORMAL, As AND Hy CELLS

K_m values of an amino acid in all type of cell has been found to be same. The changes observed in V are statistically significant since P values range from <0.01 to <0.05.

Amino acid	K_m (mM)	V (μmol/mg protein per min)		
		Normal cells	Hy cells	As cells
Lysine	0.33 ± 0.005	0.33 ± 0.010	0.25 ± 0.018	0.40 ± 0.030
Glycine	0.11 ± 0.003	0.25 ± 0.025	0.18 ± 0.020	0.33 ± 0.035
Glutamic acid	0.16 ± 0.011	0.33 ± 0.020	0.22 ± 0.030	0.36 ± 0.026
Proline	0.25 ± 0.023	0.22 ± 0.017	0.16 ± 0.030	0.28 ± 0.008
Phenylalanine	0.30 ± 0.025	0.83 ± 0.035	0.83 ± 0.028	0.83 ± 0.028
Leucine	0.20 ± 0.008	0.36 ± 0.028	0.36 ± 0.014	0.36 ± 0.036
Methionine	0.16 ± 0.008	0.23 ± 0.016	0.21 ± 0.013	0.33 ± 0.032
Serine	0.14 ± 0.010	0.80 ± 0.010	0.66 ± 0.005	0.13 ± 0.033

acids was reduced gradually (Fig. 4A and B). The uptake of phenylalanine and leucine in nystatin-grown cells, however, remained more or less the same except that there was some reduction in leucine uptake at higher concentrations of nystatin (Fig. 4A).

Kinetics of uptake of various amino acids in Nys cells

The kinetic results with Nys cells demonstrated that the variation in concentration of the antibiotic in the medium did not have any effect on the apparent K_m values for different amino acids (the only concentrations of nystatin 0.5 and 10 μg/ml are plotted in Fig. 5A and B, since the picture remained the same with the other concentrations of the antibiotic). The V values were, however, effected significantly. For instance, the V value (μmol/mg protein per min) for lysine uptake in normal cell was 0.33 ± 0.01 and for Nys cells (0.5 and 10 μg/ml) were 0.66 ± 0.02 and 0.25 ± 0.03 (Table V). Like Hy and As cells the V value of phenylalanine (0.83 ± 0.03) remained unaffected in Nys cells (Table V). It should be pointed out that the concentration

TABLE III

RELEASE OF K^+ IN NORMAL, Hy AND As *C. ALBICANS* CELLS DURING AMINO ACID UPTAKE

K^+ was estimated as described in Materials and Methods.

Amino acid	% of K^+ release		
	Normal cells	Hy cells	As cells
Proline	15 ± 1.8	16 ± 2.3	16 ± 2.8
Glycine	25 ± 2.3	25 ± 3.1	26 ± 2.8
Glutamic acid	12 ± 0.8	13 ± 2.0	12 ± 1.5
Lysine	20 ± 1.8	20 ± 2.7	20 ± 0.5
Phenylalanine	6 ± 1.3	7 ± 1.6	6 ± 0.8
Serine	23 ± 2.1	25 ± 1.6	25 ± 1.6
Methionine	12 ± 1.9	12 ± 1.9	13 ± 2.0
Leucine	16 ± 1.8	13 ± 1.2	15 ± 3.1

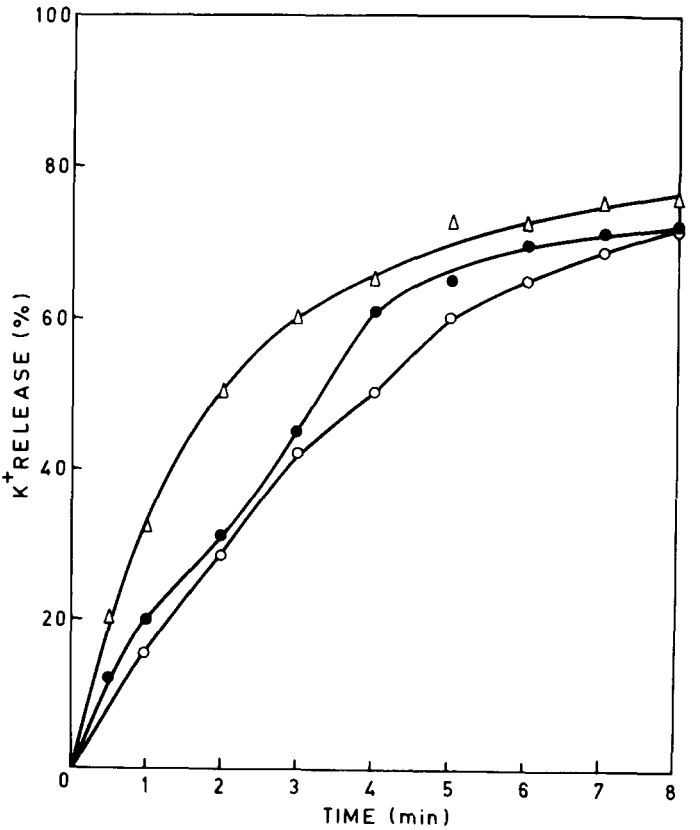

Fig. 3. Rate of K^+ efflux by nystatin in normal, Hy and As *C. albicans* cells. K^+ was estimated as described in Materials and Methods, with the addition of 30 μg/ml nystatin. The changes shown an statistically significant since *P* values range from <0.02 to <0.05. K^+ efflux in normal cells (●———●), in Hy cells (△———△) and in As cells (○———○).

TABLE IV

TOTAL ERGOSTEROL CONTENT IN *C. ALBICANS* CELLS RESISTANT TO DIFFERENT NYSTATIN CONCENTRATIONS

Ergosterol extraction and estimation was done as described in the Materials and Methods.

Cells	Nystatin concn. (μg/ml)	Total ergosterol (mg ergosterol/g protein)	Decrease (%)
Normal	—	15.0 ± 0.32	—
Nys	0.25	13.5 ± 0.45	10.0
Nys	0.50	12.3 ± 0.26	18.0
Nys	2.00	11.5 ± 0.18	23.3
Nys	4.00	11.2 ± 0.25	25.3
Nys	8.00	11.0 ± 0.15	26.6
Nys	10.00	10.8 ± 0.25	28.0

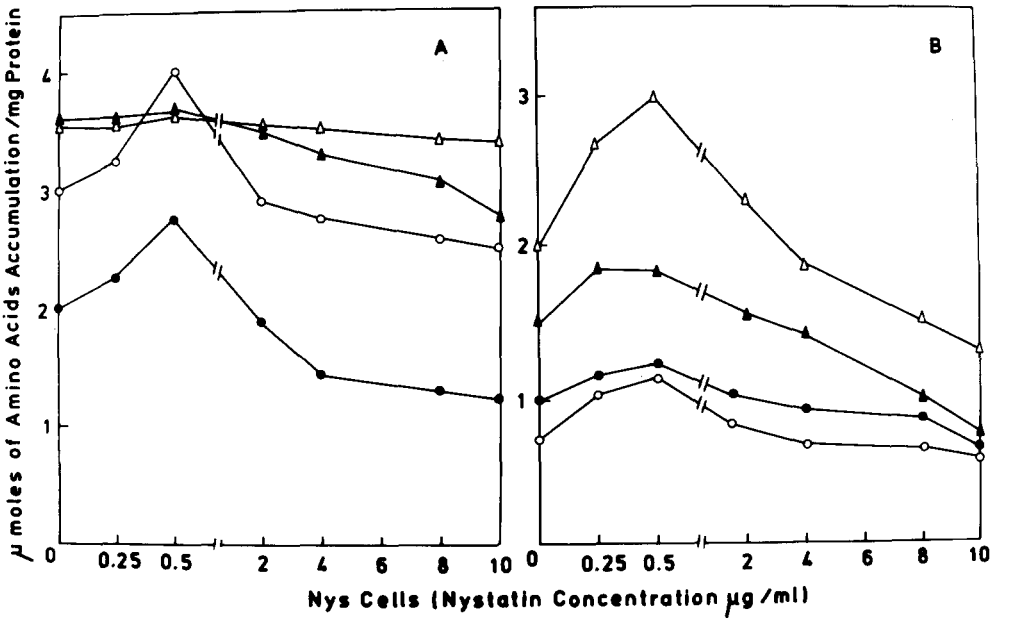

Fig. 4. (A, B) Accumulation of lysine, glycine, glutamic acid, proline phenylalanine, leucine, methionine and serine in normal cells and in cells grown on different nystatin concentration (Nys cells). Assay conditions were similar to those described for Fig. 1. Each value represents 15 min accumulation of an amino acid. The changes in most of the cases are statistically significant since P values range from <0.02 to <0.05; (A) ○——○, lysine; ●——●, glycine; △——△, phenylalanine; ▲——▲, leucine; (B) ○——○, proline; ●——●, glutamic acid; △——△, serine; ▲——▲, methionine.

of nystatin where an increased level of accumulation was demonstrated also corresponded with increased V values and vice versa (Fig. 5A and B).

Release of K^+ during the uptake of amino acids in Nys cells

The efflux of K^+ in Nys cells during the uptake of various amino acids remained the same as compared to normal cells (data not shown).

TABLE V

K_m AND V VALUES OF AMINO ACIDS IN NORMAL AND Nys CELLS

Amino acid	K_m (mM)	V (μmol/mg protein per min)		
		Normal cells	Nys cells	Nys cells
Lysine	0.33 ± 0.005	0.33 ± 0.010	0.66 ± 0.025	0.25 ± 0.031
Glycine	0.11 ± 0.003	0.25 ± 0.025	0.40 ± 0.056	0.14 ± 0.030
Glutamic acid	0.16 ± 0.011	0.33 ± 0.020	0.40 ± 0.036	0.20 ± 0.026
Proline	0.25 ± 0.023	0.22 ± 0.017	0.28 ± 0.032	0.14 ± 0.012
Phenylalanine	0.30 ± 0.025	0.83 ± 0.035	0.83 ± 0.036	0.83 ± 0.050
Leucine	0.20 ± 0.008	0.36 ± 0.028	0.36 ± 0.018	0.28 ± 0.013
Methionine	0.16 ± 0.008	0.23 ± 0.016	0.28 ± 0.023	0.15 ± 0.008
Serine	0.14 ± 0.010	0.80 ± 0.050	1.00 ± 0.028	0.57 ± 0.060

K_m value of an amino acid in all type of cell has been found to be same. The changes observed in V are statistically significant since P values range from <0.01 to <0.05.

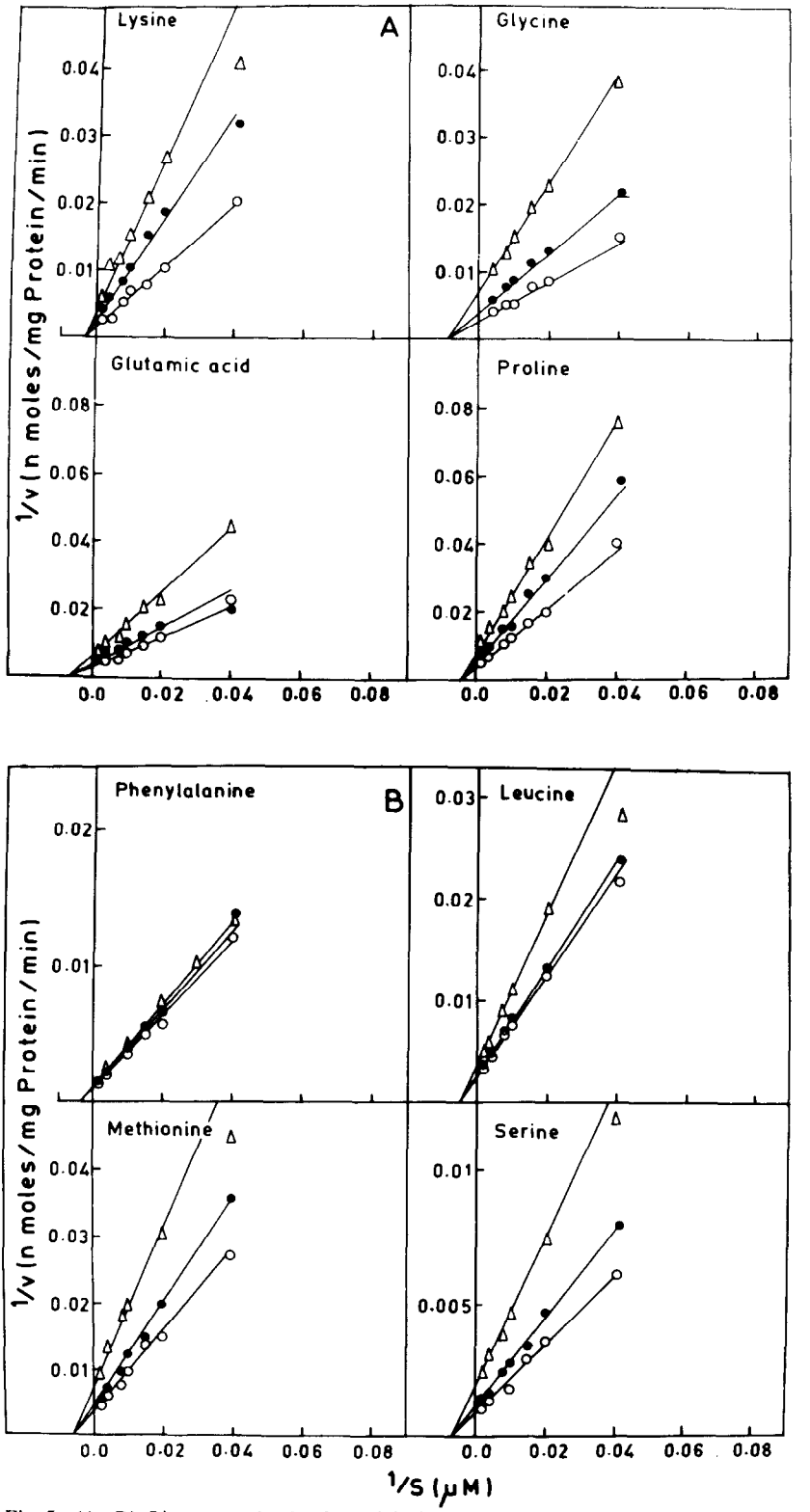

Fig. 5. (A, B) Lineweaver-Burk plot of lysine, glycine, glutamic acid proline, phenylalanine, leucine, methionine and serine uptake in normal and in Nys *C. albicans* cells. Assay conditions were similar to those described for Fig. 1. The reaction was terminated after 30 s. The changes shown are statistically significant since P value ranges from <0.01 to <0.05. The uptake of amino acids in normal cells (●——●); 0.5 μg/ml Nys cells (○——○) and 10 μg/ml Nys cells (△——△).

Discussion

The transport of lysine, glycine, proline, glutamic acid, phenylalanine, leucine, methionine and serine has been shown to be an active process in *C. albicans* cells [19,32]. The results with various respiratory inhibitors, uncouplers and ionophores revealed that there are no major differences in the mode of energy coupling for the uptake of these amino acids. However, as a consequence of altered lipid composition by growing *C. albicans* cells in various hydrocarbons (alkanes of different chain length) these amino acid transport systems responded differently [19,32]. Since the change in the lipid composition observed in alkane-grown cells was a gross change, no specific role of individual lipid component could be assessed. In the present work, however, an attempt has been made to elucidate the involvement of ergosterol in the uptake of various amino acids in *C. albicans* cells. Ergosterol has been identified as the major sterol in *C. albicans* cells, which account over 90% of the total sterols [3].

Our results have demonstrated that the low level of ergosterol in the cells grown in ascorbic acid-supplemented media (As cells) resulted in significant increase in lysine, glycine, proline, glutamic acid, methionine and serine uptake. On the other hand, a 26% increase in the ergosterol levels in cells grown in hydroquinone-supplemented media (Hy cells) resulted in a significant reduction in the uptake of afore-said amino acids (Fig. 1A and B). The kinetic data demonstrated that the inhibition was not due to a change in the apparent K_m values. The uptake rate (V) for these amino acids, were, however, different in Hy and As cells as compared to normal cells (Fig. 2A and B). These results are in agreement with the earlier findings with artificial and various cellular membranes, where the addition or removal of sterol molecules have been shown to be involved with a simultaneous change in membrane permeability [7—15].

In another approach ergosterol levels were altered by isolating nystatin-resistant *C. albicans* cells. The polyene antibiotic resistance has already been shown to be associated with the low level of sterol [33,34]. A significant resistance towards polyene antibiotic was achieved by a gradual supplementation of higher concentration of antibiotic [17,18] and such resistant isolates have been found to have low levels of ergosterol (Table IV). The drop in ergosterol content was more rapid at lower concentration (0.25—0.5 μg/ml) than at higher concentrations of the antibiotic. Like in As cells the uptake of lysine, glycine, proline, glutamic acid, methionine and serine was also increased in cells grown at lower concentration of nystatin, but on the contrary, at higher concentration of the antibiotic (4—10 μg/ml) the transport of these amino acids was reduced (Fig. 4). It would appear that the reduction in amino acid uptake in Nys cells grown at higher concentration of nystatin was due to some unknown side effects as has been envisaged by Solov'eva et al. [35].

In yeast cells, a simultaneous efflux of K^+ has been demonstrated during the uptake of various amino acids [36,37]. In mouse ascites carcinoma cells the increased velocity of glycine uptake has been related to elevated intracellular level of K^+ [38]. Our results have demonstrated that the extent of release of K^+ was different for different amino acids. The change in ergosterol contents brought about by different supplmentation of the media did not influence the

observed release pattern of K^+ for any of the studied amino acid (Table III). However, the addition of nystatin to Hy cells (having more ergosterol) caused a rapid efflux of K^+ compared to As cells (having low ergosterol) (Fig. 3). This is because of the fact that the efflux of K^+ would depend upon the extent of binding of the antibiotic to available ergosterol [29—31]. Similar to Hy and As cells, the nystatin-resistant isolates (having low ergosterol), also had similar level of K^+ efflux upon the addition of different amino acids. Contrary to various reports [36,37], it would appear that there is no apparent correlation between the K^+ efflux and in the increase or decreased rate of amino acids uptake in *C. albicans* cells (Fig. 3, Table III).

The transport of lysine, glycine, glutamic acid, proline, methionine and serine increased in As and Nys cells (lower concentration), but Hy cells demonstrated a reduction in the transport rate and level of accumulation of these amino acids. The uptake of phenylalanine and leucine remained unaffected in As, Nys and Hy cells. These observed changes in amino acid uptake are probably due to the change in the ergosterol levels, since the level of other lipid components remained unaltered in all kinds of supplemented cells. Therefore, it would appear that the different amino acids behave differently to the changed environment brought about by altered ergosterol levels.

Acknowledgements

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