*Biochimica et Biophysica Acta*, 691 (1982) 144–150
Elsevier Biomedical Press

BBA 71356

# L-PROLINE TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

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(Received April 13th, 1982)

*Key words: L-Proline; Amino acid transport; (S. cerevisiae)*

**Transport of L-proline into *Saccharomyces cerevisiae* K is mediated by two systems, one with a $K_T$ of 31 $\mu$M and $J_{max}$ of 40 nmol·s$^{-1}$·(g dry wt.)$^{-1}$, the other with $K_T > 2.5$ mM and $J_{max}$ of 150–165 nmol·s$^{-1}$·(g dry wt.)$^{-1}$. The kinetic properties of the high-affinity system were studied in detail. It proved to be highly specific, the only potent competitive inhibitors being (i) L-proline and its analogs L-azetidine-2-carboxylic acid, sarcosine, D-proline and 3,4-dehydro-DL-proline, and (ii) L-alanine. The other amino acids tested behaved as noncompetitive inhibitors. The high-affinity system is active, has a sharp pH optimum at 5.8–5.9 and, in an Arrhenius plot, exhibits two inflection points at 15°C and 20–21°C. It is trans-inhibited by most amino acids (but probably only the natural substrates act in a trans-noncompetitive manner) and its activity depends to a considerable extent on growth conditions. In cells grown in a rich medium with yeast extract maximum activity is attained during the stationary phase, on a poor medium it is maximal during the early exponential phase. Some 50–60% of accumulated L-proline can leave cells in 90 min (and more if washing is done repeatedly), the efflux being insensitive to 0.5 mM 2,4-dinitrophenol and uranyl ions, to pH between 3 and 7.3, as well as to the presence of 10–100 mM unlabeled L-proline in the outside medium. Its rate and extent are increased by 1% D-glucose and by 10 $\mu$g nystatin per ml.**

## Introduction

The practically unidirectional transport of amino acids in baker's yeast, proceeding against considerable concentration gradients, is effected by at least ten systems with different specificities. Generally, yeasts possess two types of transport systems for amino acids: systems which are specific for only one amino acid or a family of structurally related amino acids are not inhibited by ammonium ions, and a general systems which is shared by a large number of amino acids and is inhibited by ammonia [1–6].

L-Proline transport into *Saccharomyces cerevisiae* is mediated by two systems with different kinetic parameters and substrate specificities [7]. This paper describes the kinetic properties of the high-affinity system that is exceptional in that it is highly specific but ammonia sensitive [5,7].

## Materials and Methods

*Microorganisms.* Most of the experiments were performed with aneuploid strain *Saccharomyces cerevisiae* K (CCY 21-4-60) derived from distillery yeast. *Saccharomyces cerevisiae* Σ 1278b (a kind gift of Dr. M. Grenson, Brussels) was used only for comparative experiments. Cell maintained either on wort-ager slopes (strain K) or on slants containing 10 g yeast extract, 10 g bactopeptone, 20 g D-glucose and 20 g agar in 1 l water (strain Σ 1278b) were grown in flasks on a reciprocal shaker at 28°C up to early stationary phase in a semisynthetic rich medium containing (g·l$^{-1}$): $KH_2PO_4$ 2,

$K_2HPO_4$ 1, L-proline 1 or $(NH_4)_2SO_4$ 2, $MgCl_2 \cdot 6H_2O$ 0.1, $CaCl_2$ 0.1, $MnSO_4 \cdot 7H_2O$ 0.1, trisodium citrate 0.3, yeast extract 3 (pH 5.4–5.8). The relatively poor medium used for comparison with Grenson's [2] work had the following composition ($g \cdot l^{-1}$) : $KH_2PO_4$ 2 L-proline 1 or $(NH_4)_2SO_4$ 1.2, $MgSO_4 \cdot 7H_2O$ 0.7, $CaCl_2$ 0.4, citric acid 10.5, KOH 9, NaCl 0.5, and 1 ml of a solution containing trace minerals (10 mg $H_3BO_3$, 2 mg KI, 0.8 mg $CaSO_4 \cdot 5H_2O$, 5 g $FeCl_3 \cdot 6H_2O$, 0.4 g $MnSO_4 \cdot 4H_2O$, 4 mg $Na_2MoO_4 \cdot 2H_2O$, 14 mg $ZnSO_4 \cdot 7H_2O$, 10 g citric acid, in 1 liter water). The pH was adjusted to 6.1, and 5 ml of a separately sterilized solution of vitamins (250 $\mu$g biotin, 100 mg thiamine, 1 g inositol, 100 mg pyridoxine, in 1 liter water) was added. Aliquots of 100 ml in 500-ml flasks were supplemented with 2 ml sterile 40% D-glucose solution prior to inoculation. Harvested cells were washed three times in tap water and aerated fro 2 h to deplete them of endogenous substrates. The cell pellet was left in a refrigerator at 4°C overnight and used on the following day.

*Incubation and L-proline uptake.* The suspension (5–10 mg dry wt. per ml) was incubated for 1 h with 1% D-glucose. Then it was washed and cells were resuspended in distilled water in Erlenmayer flasks at 30°C in a Dubnoff incubator. After 10 min of preincubation with cycloheximide (0.4 mM final concentration) at 30°C $^{14}$C-labeled L-proline was added to 0.1 mM and 0.2 ml samples were withdrawn at intervals, filtered through Synpor 5 (0.6 $\mu$m pore diameter) filters, washed twice with 1 ml of ice-cold water and the pellet with filter was transferred to a scintillation vial with a 7 ml toluene-plus-ethanol scintillation cocktail. During the experiment essentially no radioactivity was found in proteins, as determined by trichloroacetic acid precipitation.

Radioactivity was counted in a Beckmann liquid scintillation spectrometer 9000, programmed for quench correction.

*Chemicals.* All compounds were of the highest available purity. They included L-proline, amino acids and sarcosine (Koch-Light, U.K.), cycloheximide (Fluka, Switzerland), nystatin (Squibb, U.K.), L-azetidine-2-carboxylic acid, 3,4-dehydro-DL-proline, D-proline and 3-chlorophenylhydrazonomalononitrile (Calbiochem, Switzerland). The remaining compounds were obtained from Lachema (Czechoslovakia). Carrier-free $^{14}$C-labeled L-proline was obtained either from the Radiochemical Centre (U.K.) or from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia).

## Results

*Kinetic analysis of proline transport*

L-Proline uptake was linear with time for at least 5 min at 0.1 mM and proportional to cell concentration. Consequently we used the amount of L-proline accumulated in the cell in 2 min for computing the initial rate. The Lineweaver-Burk plot of $^{14}$C-labeled L-proline uptake into *Saccharomyces cerevisiae* is clearly biphasic, composed of a high-affinity component with a half-saturation constant $K_T$ of $31 \pm 1.2$ $\mu$M and maximum rate $J_{max}$ of 40 $nmol \cdot s^{-1} \cdot (g \text{ dry wt.})^{-1}$, and a low-affinity component with $K_T$ higher than 2.5 mM and $J_{max}$ of 150–165 $nmol \cdot s^{-1} \cdot (g \text{ dry wt.})^{-1}$. The second transport activity representing under our conditions less than the 10% of total activity was not studied further.

*Specificity of the high-affinity systems*

To screen the specific inhibitors of the high-affinity L-proline transport systems, the uptake of 0.1 mM L-proline was measured in the presence of amino acids and some L-proline structural analogs at 5 mM concentrations. The result, listed in Table I, show that all amino acids tested are inhibitors of L-proline uptake and that their inhibitory effects are in the range of 20–40% with some exceptions (glycine, L-alanine, L-aspartic and L-glutamic acids, L-cysteine). Except for L-alanine which was competitive all the amino acids proved to be noncompetitive inhibitors. All L-proline structural analogs were competitive inhibitors of L-proline uptake. As shown in Table II, their inhibitory constants ($K_i$) estimated from a reciprocal plot are greater than the apparent $K_T$ of L-proline for its high-affinity transport system in all cases. On this basis it may be concluded that the high-affinity system is specific for L-proline, L-alanine being the only other natural substrate. Common metabolic inhibitors, including uranyl ions, were only partly effective (Table I).

*Effects of temperature and pH*

The most readily interpretable temperature de-

TABLE I

INHIBITION OF THE INITIAL RATE OF ENTRY OF L-PROLINE BY AMINO ACIDS, STRUCTURAL ANALOGS OF L-PROLINE AT 50-FOLD HIGHER CONCENTRATION, AND SEVERAL METABOLIC INHIBITORS

The values given are the means of at least three separate experiments.

| Amino acid or analog | Inhibition (1%) |
|---|---|
| Glycine | 59 |
| L-Alanine | 74 |
| L-Leucine | 36 |
| L-Isoleucine | 21 |
| L-Valine | 38 |
| L-Serine | 24 |
| L-Threonine | 44 |
| L-Cysteine | 69 |
| L-Methionine | 36 |
| L-Asparagine | 22 |
| L-Glutamine | 27 |
| L-Aspartic acid | 82 |
| L-Glutamic acid | 76 |
| L-Proline | 100 |
| L-Arginine | 22 |
| L-Lysine | 21 |
| L-Histidine | 30 |
| L-Tyrosine | 27 |
| L-Tryptophan | 37 |
| L-Phenylalanine | 28 |
| 3,4-Dehydro DL-proline | 85 |
| D-Proline | 90 |
| L-Azetidine-2-carboxylic acid | 86 |
| Sarcosine | 97 |
| Sarcosine anhydride | 3 |
| Hydroxy-DL-/proline | 24 |
| 0.5 mM DNP [a] | 46 |
| 10 $\mu$M CCP [b] | 56 |
| 0.5 mM $UO_2(NO_3)_2$ | 71 |
| 0.5 mM IAA [c] | 53 |

[a] 2,4-Dinitrophenol. [b] 3-Chlorophenylhydrazonomalononitrile. [c] Iodoacetamide.

pendence in transport is that of $J_{max}$ since it involves only translocation rate constants, provided that the system reaches local equilibrium between the carrier and its substrate. Hence, the uptake of $^{14}$C-labeled L-proline was measured at a concentration 0.1 mM, which is 3-times higher than the $K_T$ of this imino acid. The Arrhenius plot, log $J_s$ vs. $1/T$, displayed two inflection points at temperatures $T_{m1}$ 15°C and $T_{m2}$ 20–21°C (Fig. 1.). The

TABLE II

APPARENT $K_i$ VALUES OF THE L-PROLINE TRANSPORT SYSTEM FOR INHIBITORY STRUCTURAL ANALOGS

| Structural analog | $K_i$ ($\mu$M) |
|---|---|
| 3,4-Dehydro-DL-proline | 71 |
| L-Azetidine-2-carboxylic acid | 120 |
| D-Proline | 105 |
| Sarcosine | 15.4 |
| L-Alanine | 72.5 |

apparent activation energies $E_A$ are 71, 105 and 71 kJ/mol in the three temperature intervals (I, II, III). The system is also pH dependent, with optimum at 5.8–5.9 (Fig. 2), only a little lower than the isoelectric point of L-proline (6.3).

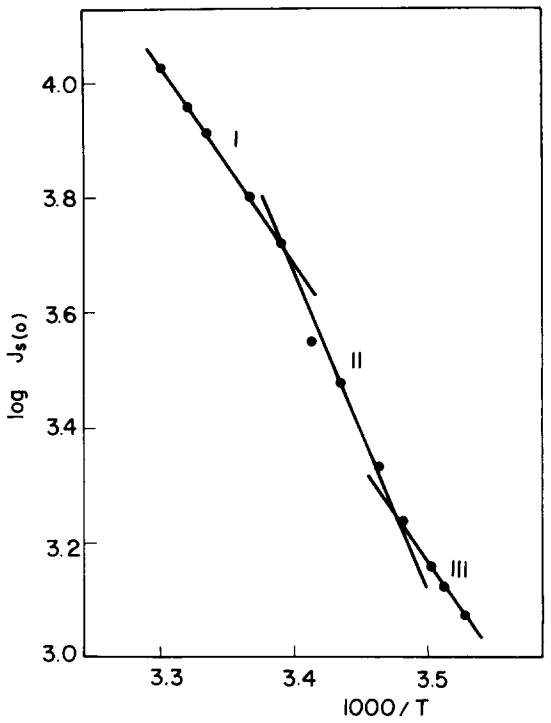


Fig. 1. Arrhenius plot of the effect of temperature on 0.1 mM L-proline uptake. The initial rate $J_{s(0)}$ is used, based on the average of two independent measurements. Transition temperatures were determined by the intersection of straight liness drawn through experimental points in such a way that the sum of their regression coefficients was maximum.

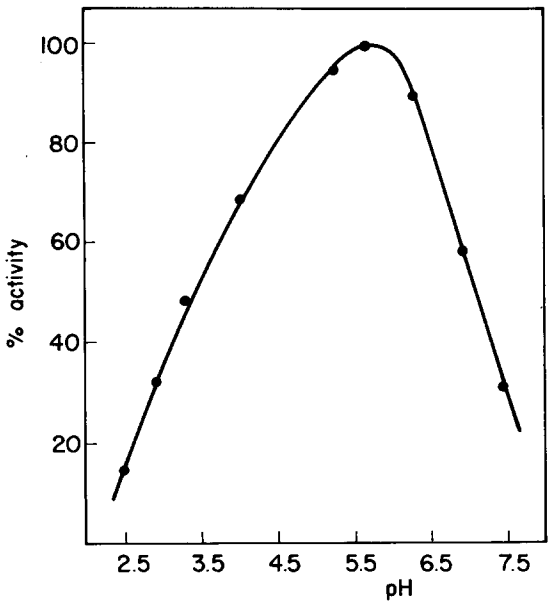


Fig. 2. Uptake of 0.1 mM L-proline as a function of pH. Uptake was measured at various pH values in citrate-phosphate buffer.

*Exit of accumulated proline*

The outward fluxes of amino acids from yeast cells are characterized by several peculiarities. Thus, cells never release more than a fraction (0–30%) of their total amino acid content after transfer to an amino acid-free medium. Moreover, repeated transfers of preloaded cells to that medium do not appreciably increase the amount of radioactivity lost from the cells [4,6]. This does not hold for the outward flux of L-proline accumulated within cells examined here. Transfer of cells loaded with $^{14}$C-labeled L-proline into water resulted in a rapid decline of intracellular radioactivity (typically 50–60% in 90 min) until a new equilibrium was attained (Fig. 3). Moreover, nearly all intracellular radioactivity was lost if such washing repeated (Fig. 4). The efflux was insensitive to 0.5 mM 2,4-dinitrophenol and uranyl nitrate, to pH between 3 and 7.3, as well as to the presence of 10–100 mM unlabeled L-proline in the outside medium. Its rate was increased by 1% D-glucose and, understandably, by nystatin (final concentration 10 μg per ml) which is known to increase nonspecifically the permeability of the plasma membrane.

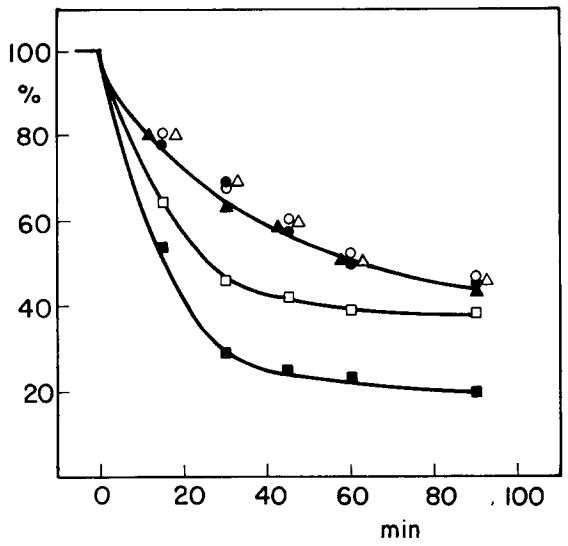


Fig. 3. Intracellular concentration (in %) of labeled L-proline during efflux from cells preinubated with 0.1 mM $^{14}$C-labeled L-proline as indicated in Materials and Methods. At steady state (60 min) the cells were washed with water, divided into six portions, the indicated additions plus cycloheximide (0.4 mM final concentration) were done and incubation was continued. Intracellular radioactivity was measured in samples taken at the indicated intervals. ○, Without additions ; ●, 0.5 mM 2,4-dinitrophenol; △, 0.5 mM uranyl nitrate; ▲, 100 mM L-proline; □. 1% D-glucose; ■, 10 μg nystatin/ml.

*Trans-inhibition*

It is known for several microorganisms, including *Streptomyces hydrogenans* [8], *Neurospora crassa* [9], *Penicillium chrysogenum* [10,11] and

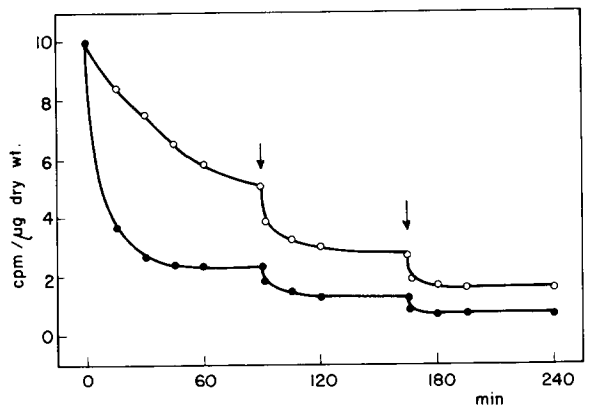


Fig. 4. Intracellular L-proline (cpm/μg dry wt.) during efflux from cells after repeated transfers (shown by arrows) of preloaded cells into water (○) or nystatin (●). All the other experimental conditions were as indicated for Fig. 3.

TABLE III

EFFECT OF 1-h PREINCUBATION WITH DIFFERENT AMINO ACIDS (5 mM) IN 1% D-GLUCOSE ON THE INITIAL RATE OF SUBSEQUENT UPTAKE OF 0.1 mM $^{14}$C-LABELED L-PROLINE WITHOUT GLUCOSE

The values given are the means of at least three independent experiments.

| Preincubated with glucose and | Initial rate (% control with glucose alone) | Preincubated with glucose and | Initial rate (% control with glucose alone) |
|---|---|---|---|
| Water | 100 | L-Glutamine | 59 |
| L-Alanine | 18 | L-Aspartic acid | 20 |
| Glycine | 88 | L-Glutamic acid | 9 |
| L-Leucine | 33 | L-Arginine | 71 |
| L-Isoleucine | 46 | L-Lysine | 71 |
| L-Valine | 56 | L-Histidine | 55 |
| L-Serine | 97 | L-Tyrosine | 27 |
| L-Threonine | 78 | L-Tryptophan | 35 |
| L-Cysteine | 9 | L-Phenylalanine | 30 |
| L-Methionine | 60 | L-Proline | 34 |
| L-Asparagine | 78 | 2-Aminoisobutyric acid | 96 |

*Saccharomyces cerevisiae* [4,12–14] that intracellular amino acids and/or their metabolites inhibit the transport of either the same or a different amino acid from the medium. This formally non-competitive inhibition was termed trans-inhibition and ascribed to a feedback control from inside the cell.

Transport of L-proline was shown to be markedly trans-inhibited by a number of different amino acids (Table III) but kinetic analysis of trans-inhibitory effects of the selected amino acids show that only the natural substrates of L-proline transport system, L-proline and L-alanine, behave as typical inhibitors in that they affect the $J_{max}$ of L-proline uptake while leaving the $K_T$ intact. On the contrary, L-leucine, L-cysteine, L-glutamate and L-arginine, exhibited some mixed-type trans-inhibition. It should be noted thath the reciprocal plots obtained in this connection possessed regression coefficients better than 0.99.

*Influence of growth stage and cultivation medium*

L-Proline transport system in *Saccharomyces cerevisiae* Σ1278b growing exponentially in a poor medium is fully expressed in the presence of L-proline (0.115 M) and completely inhibited by ammonium ions (2 mM) as the only sources of nitrogen [5]. On the contrary, our preliminary experiments show that in *S. cerevisiae* K grown in a rich medium containing yeast extract, the transport activity for L-proline is insensitive to the presence of ammonium ions and appears only in the stationary phase. To decide if such apparent discrepansies are caused by strain difference or by different growth media, we estimated L-proline transport activities in both *Saccharomyces cerevisiae* strains during their growth on various combinations of growth media and nitrogen

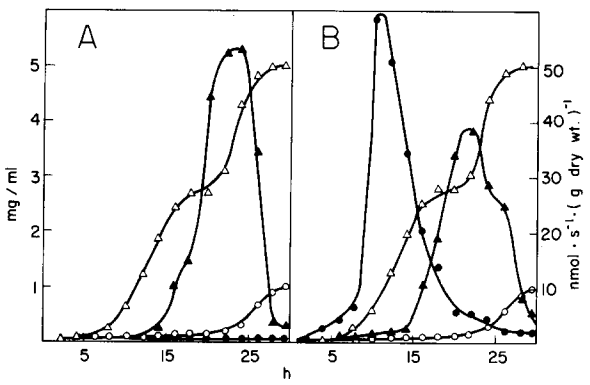


Fig. 5. Initial rate of L-proline uptake during growth (in h) in the presence of ammonium ions (A) or L-proline (B) as the source of nitrogen. Growth is expressed in mg dry wt. per ml suspension in a rich (△) and poor (○) medium. The rate of uptake (nmol per s per g dry wt.) was followed in samples removed at the indicated times from the rich (▲) or poor (●) medium.

sources. From the results presented in Fig. 5 for the *S. cerevisiae* K two main conclusions can be drawn. (1) During growth in a poor medium, maximum L-proline transport activity was reached at the beginning of the exponential phase no matter what strain was used. This activity was suppressed by ammonium ions and fell rapidly to a low level by middle exponential phase. (2) Transport in a rich medium reached a maximum either in the middle of the exponential phase (strain Σ 1278b) or in the stationary phase (strain K) and this activity was insensitive to the presence of ammonium ions in the growth medium.

## Discussion

In good agreement with previous observations by Lasko and Brandriss [7] on *Sacharomyses cerevisiae* MB1000, L-proline transport in *S. cerevisiae* K was formally in accordance with the operation of two different kinetic systems. The high-affinity system, detected also by Grenson at al. [5] and studied here in more detail, is very similar to the L-proline transport system of *Saccharomyses chevalieri* [15] at least in four respects. (1) It is inhibited competitively by a number of L-proline structural analogs, the sequence of their $K_i$ values being very similar. (2) L-Proline and L-alanine are the only natural substrates. (3) It has a pH optimum at 5.8–5.9. (4) It is inhibited noncompetitively by many other amino acids. Such noncompetitive inhibition which can only be due to allotopic binding (and inactivation) of either the carrier or the energy-transducing factor were also described in other lower eukaryotic systems, e.g., in *Sacharomycopsis lipolytica* for the basic amino acid transport system [16] or in *Aspergillus nidulans* for the acidic amino acid transport system [17].

The observation that L-proline transport by *Saccharomyces cerevisiae* K (like other amino acid transport systems) is stimulated by preincubation of the cells with a source of metabolic energy, such as D-glucose or ethanol [18], inhibited by metabolic inhibitors points to its energy requirements. Moreover, there is an obvious accumulation of L-proline in cells, attaining ratios near 100 : 1. These aspects of the system will be treated in detail in a subsequent paper.

Our experiments disclosed two peculiar features of the L-proline transport system. The first is the triphasic Arrhenius plot, the second is the existence of L-proline efflux from preloaded cells. In *Saccharomyces cerevisiae* the Arrhenius plots of transport systems including plasma membrane ATPase [19], L-lysine and L-asparagine transport in cells enriched in oleyl or linoleyl residues [20] and several other amino acid transport systems [21] were biphasic, the slope changing abruptly at an apparent transition temperature $T_m$ of 18–24°C, depending on the substrate and/or yeast strain used. The Arrhenius plot for L-proline transport is exceptional in that it is clearly triphasic in the temperature range used. The identity of activation energies below $T_{m1}$ and above $T_{m2}$ probably implies that the mechanism of L-proline transport process above and below these temperatures remains unchanged. The similar triphasic dependence of β-galactoside and β-glucoside [22] transport in *Escherichia coli* corresponded in both shape and temperature range to the ordered ⇌ fluid membrane lipid phase transition. Here the changes in slope at $T_{m1}$ and $T_{m2}$ were interpreted as the lower and upper ends of transition and the steep and relatively broad intermediate part as the region in which a change in the rate of transport is caused by the distribution of proteins between fluid and ordered lipid domains. Finally, according to Overath at al. [22] the ability to detect only biphasic Arrhenius plots of transport may be due to, for instance, by a broad fluid ⇌ ordered transition which ends close to or below 0°C, by very low transport activity in the ordered state or by much higher activation energy in the ordered than in the fluid state.

The insensitivity of the efflux of L-proline from preloaded cells to 2,4-dinitrophenol and uranyl ions, to pH between 3 and 7.3, as well as to the presence of unlabeled L-proline in the outside medium at high concentrations suggests simple diffusion as the most probable mechanism of L-proline efflux. While the positive effect of nystatin on the rate and extent of L-proline efflux is in accordance with its forming channels in the lipid membrane permeable to ions [23] and some low-molecular-weight compounds, the interpretation of the effect of D-glucose is more difficult. One possibility may be in the accelerated degradation of the

active transport protein(s) responsible for L-proline influx in the presence of this compound. This is disconcerting case of two opposite effects of glucose, one enhancing the synthesis of the L-proline transport system [18], the other increasing the rate of its proteolytic degradation (catabolic inactivation (cf. Refs 24 and 25).

The ability of a number of different intracellular amino acids to trans-inhibit L-proline uptake was unusual in that trans-inhibition in lower eukaryotes, including yeasts, was reported [4,13] to be connected always only with the particular system mediating unidirectional amino acid flux from the medium into cells, even if a polyvalent sensitivity to trans-inhibition in cells grown in the presence of ammonium ions was already reported before by our group [14]. In the case of L-proline uptake (Table III) there may be two classes of trans-inhibitors where only L-proline and L-alanine act in a typically noncompetitive manner.

Differences in L-proline uptake observed during cell growth on both media and nitrogen sources are not easily interpretable at the moment. It is important to note, however, that the repressive effect of ammonium ions is observed only on a poor medium. Whether the lack of repression by ammonium ions in the rich medium is due to binding of ammonium into an inactive compound or whether the involvement of glutamate dehydrogenase (postulated by Grenson and Hou [26]) is not crucial in the presence of some factors of the yeast extract is impossible to decide.

## Acknowledgement

The authors wish to thank Dr. A. Kotyk for many helpful discussions and for critical reading of the paper.

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