BBA 75229

A PROLINE TRANSPORT SYSTEM IN *SACCHAROMYCES CHEVALIERI*

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(Received October 22nd, 1968)

SUMMARY

1. The properties of a proline accumulating system in nitrogen starved cells of *Saccharomyces chevalieri* are described. The system shows a Michaelis–Menten substrate dependence with an apparent K_m of $2.5 \cdot 10^{-5}$ M for L-proline and a K_i of $8.9 \cdot 10^{-5}$ M for D-proline.

2. The intracellular radioactivity accumulated corresponds to at least 85 % free proline, as indicated by chromatography, displacement of accumulated proline and bio-assay.

3. The uptake of proline is temperature dependent. Further, azide and 2,4-dinitrophenol inhibit the uptake.

4. Measurement of the initial velocity of proline influx shows that the uptake of proline is not inhibited by a number of natural amino acids. Only α-alanine is inhibitory. L-Hydroxyproline and other amino acids are poor inhibitors. Some amino acids, such as tryptophan, phenylalanine and tyrosine, stimulate uptake.

5. Among several structural analogs of proline, only sarcosine, L-thiazolidin-4-carboxylic acid, D- and L-azetidin carboxylic acid and 3,4-dehydro-DL-proline were found to be effective inhibitors of the system. All of these show a competitive type of inhibition.

6. The results presented are consistent with the idea that the proline transport system in yeast is very specific.

INTRODUCTION

Amino acid uptake by yeast cells has been reported in a number of papers to show a poor specificity (see reviews in refs. 1 and 2). Thus, it has been reported that the accumulation of a given amino acid in *Saccharomyces cerevisae* and *Neurospora crassa* may be inhibited by a number of structurally unrelated amino acids[3–11]. In addition, some mutations in fungi produce a generalized effect on amino acid uptake[9,12,13]. These observations suggest that the uptake of amino acids in fungi is also mediated by systems with low specificity.

In contrast, evidence has been also reported which points to the existence of specific amino acid transport systems in fungi[14–21]. Moreover, it has recently been observed that arginine, lysine and methionine are accumulated in *S. cerevisiae*, by transport systems which show a very high specificity[22–24], comparable to that currently found for bacterial "permeases" (ref. 25).

Also, it has been found that the derepression of the proline transport system in yeast does not change the uptake capacity for other amino acids[26].

This report describes the properties of a proline transport system for *Saccharomyces chevalieri* which is highly specific. The specificity of the system has been analysed by comparing the apparent affinity constant (K_m) of proline with the apparent inhibition constant (K_i) of the competitive inhibitor in question[27,28].

The proline transport system has been selected for extensive study mainly because, in our strain, proline does not appear to be utilized as a carbon source or in any manner other than for protein biosynthesis, a fact already reported for *Escherichia coli*[29] and *N. crassa*[30]. Furthermore, there exist a large number of non-amino acid structural analogs which can be tested as potential inhibitors of the system.

MATERIALS AND METHODS

Materials

Strains and growth conditions. Yeast strain and growth conditions were the same as described previously[26].

E. coli strain 55/1 requiring L-proline for growth was kindly provided to us by DR. J. CABELLO (Instituto de Química Fisiologica, Universidad de Chile, Santiago, Chile). This strain was cultivated and used to assay proline essentially as already described[32].

Radioactive compounds. DL-[*carboxy*-^{14}C]Proline (specific activity, 4 mC/mmole) was purchased from Calbiochem. Uniformly ^{14}C-labelled L-proline (specific activity, 168 mC/mmole) was a product of Schwarz Co.

Structural analogs. L- and D-azetidin carboxylic acid, betaine, creatinine, 4-hydroxymethylimidazol and sarcosine were obtained from Calbiochem; coumarilic acid, furan 2-carboxylic acid, α-picolinic acid, pirazol 3,5-dicarboxylic acid, pyridin 2-aldoxime, L-proline methyl ester and quinaldic acid were from Fluka AG; pipecolic acid, glycyl-L-proline, L-prolylglycine, L-prolylphenylalanine and poly-L-proline were from Mann Research Laboratories; 3-indol acetic acid was from Eastman Kodak; 4-thiazolidin carboxylic acid and *N*,*N*-dimethylglycine were from Nutritional Biochemicals; and nicotinic acid was obtained from Merck (Darmstadt)

3,4-Dehydro-DL-proline was kindly made available to us through the courtesy of DR. B. WITKOP (National Institutes of Health, Bethesda, Md., U.S.A.).

Other chemicals. All ingredients for media were obtained from Difco. D-Galactose was a Pfansthiel product.

Cycloheximide was a generous gift from DR. C. C. FENWICK of the Upjohn Co., Mich., U.S.A.

Methods

Uptake experiments. The assay of proline uptake was always run with cells collected during the exponential phase of growth and derepressed as previously described[26].

Initial velocity of uptake. Derepressed cells were adjusted to $2 \cdot 10^7$ cells per ml (hemocytometer) in 66 mM KH_2PO_4 (pH 5.5) *plus* 10 mM D-galactose. Usually 6 ml of the prepared cells were poured into 50-ml erlenmeyers and equilibrated at 30° in a

shaking water bath for 5 min; cycloheximide was added to a final conc. of $2.6 \cdot 10^{-3}$ mM, and phosphate–galactose buffer was added to bring the final volume to 9 ml after all the additions. At zero time the radioactive L-proline adjusted to a specific activity of 0.12 mC/mmole and the inhibitor if any, was added; 1-ml samples were taken immediately and at appropriate intervals, and filtered at once through Millipore filters (0.45 μ, pore size) then washed three times with 5-ml portions of water at room temperature (intracellular proline was not removed in the washing process). The filters were then dried and counted in a thin-window gas-flow counter (Nuclear Chicago Corp.). The efficiency of the counting method was 30%.

Accumulating capacity. The accumulating capacity was calculated by the ratio between internal and external concentration of L-proline at the steady-state level. The intracellular concentration of L-proline was calculated from the amount of radioactive L-proline accumulated by the cells at steady state, assuming that the intracellular water accounts for 47% of that total wet-volume of cells[33] and that L-proline is distributed throughout all the intracellular water.

Identification of the accumulated radioactivity. 4-ml aliquots of cell suspension, incubated as described with radioactive proline for 60 min, were centrifuged rapidly, and the cell pellets were resuspended in a water–acetone mixture (1:3, v/v). Nonradioactive proline was added, and the cell suspension was boiled for 7 min. The boiled cells were removed by centrifugation and the extract analysed by paper chromatography in the following solvents: I; phenol–water–NH_3(25%) (80:20:0.3, by vol.)[34]; II, ethanol–water–urea (80:20:0.5 g v/v/w)[35] and III; *n*–butanol–pyridine–water (315:175:240, by vol.)[36]

In each case a single radioactive spot was found which was located at the same place as the spot of unlabelled proline developed with ninhydrin. With all solvents a faint radioactive spot was found at the origin representing less than 10% of total radioactivity.

Bioassay. Derepressed cells were allowed to accumulate L-proline for 7 min at 30° in a rotatory shaker. After the accumulation period the cells were centrifuged, washed twice with 5 ml phosphate–galactose buffer and the pellets extracted with water–acetone mixture (1:1, v/v) for 10 min in boiling water. Aliquots of the cell-free extracts were tested for free L-proline with *E. coli* 55/1. This mutant has a block in the proline biosynthetic pathway at the level of γ-glutamic semialdehyde synthetase, and it is unable to grow unless supplied with L-proline.

In our hands this method allowed us to measure L-proline quantitatively from 0.01 to at least 0.25 μmole/ml.

RESULTS

Kinetics of labelled proline uptake

The uptake of DL-[^{14}C]proline is shown in Fig. 1. N-starved cells accumulate this substrate until a steady-state level is reached 60 min after zero time. At steady state the intracellular concentration of proline is at least 800 times that of the medium. D-Galactose at a final concentration of 10 mM enhances the accumulation capacity of the cells and initial velocity of uptake. 0.1 mM 2,4-dinitrophenol and 20 mM sodium azide effectively inhibit the accumulation of proline.

Effect of temperature and pH

The uptake of DL-[^{14}C]proline by N-starved cells is temperature dependent. At 30° the cells reached maximal internal proline concentration 60 min after zero time, whereas at the same time, the accumulation of proline by yeast cells incubated at 4° was only 4 % of this amount. There is a 20-fold difference in initial rate of entry at the two temperatures. The system is also pH dependent, with an optimal pH of 5.5 (Fig. 2).

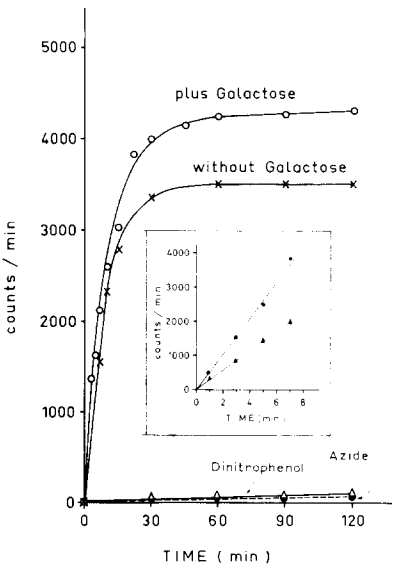


Fig. 1. Kinetics of proline uptake. The cells, resuspended in 66 mM KH_2PO_4 (pH 5.5), were incubated at 30° in the presence of 0.9 μM [^{14}C]proline. Samples were taken at appropriate intervals, and internal radioactivity was measured as described under MATERIALS AND METHODS. ×—×, without D-galactose; ○—○, *plus* 10 mM D-galactose; △—△, *plus* 10 mM D-galactose and 0.1 mM 2,4-dinitrophenol; ●---●, *plus* 10 mM D-galactose and 20 mM azide. In the inset, cells suspended in 66 mM KH_2PO_4 (pH 5.5) were incubated at 30° in the presence of 0.1 mM [^{14}C]proline. Samples were taken at the indicated intervals, and the internal radioactivity was measured as described under MATERIALS AND METHODS. ▲—▲, without D-galactose; ●—●, *plus* 10 mM D-galactose.

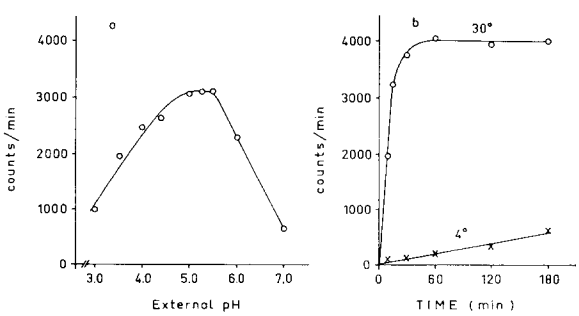


Fig. 2. a. Effect of pH on proline uptake. The cells were resuspended in 66 mM KH_2PO_4, previously adjusted to the indicated pH, *plus* 10 mM D-galactose, and the internal radioactivity was measured as described under MATERIALS AND METHODS. b. Effect of temperature on proline uptake. The cells were resuspended in 66 mM KH_2PO_4 (pH 5.5) *plus* 10 mM D-galactose and the kinetics of [^{14}C] proline uptake measured as indicated under MATERIALS AND METHODS. ○—○, at 30°; ×—×, at 4°.

Properties of the accumulated amino acid

Extraction of the internal radioactivity accumulated by the cells after 60 min of incubation can be accomplished with hot water. Only 5 % of the accumulated radioactivity remains associated with the insoluble residue. The extracted radioactivity was identified as 85–90 % free proline by paper chomatography in three different solvent systems and by bioassay.

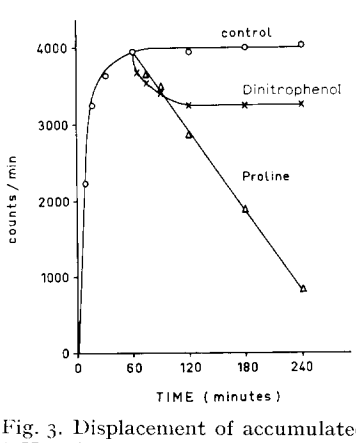
To determine if the uptake process affected the optical configuration of the proline accumulated, cells were allowed to accumulate L-proline, DL-proline or D-proline. Extracts prepared as described in MATERIALS AND METHODS were subjected to bioassay. Results given in Table I clearly demonstrate that L-proline is not transformed into the D-isomer by the accumulating system.

TABLE I

CONFIGURATION OF THE ACCUMULATED PROLINE DETERMINED BY BIOASSAY

Derepressed cells were allowed to accumulate L-proline, DL-proline or D-proline adjusted to a final concn. of $8 \cdot 10^{-4}$ M. After accumulation, cell extracts were prepared as described under MATERIALS AND METHODS, and 0.5-ml samples of the extracts were subjected to bioassay for free L-proline. Results are the means of two determinations and represent the amount of free L-proline found in the extracts.

Optical isomer	*Free proline (μmole/ml)*
None (control)	<0.01
L-Proline	0.21
DL-Proline	0.10
D-Proline	<0.01



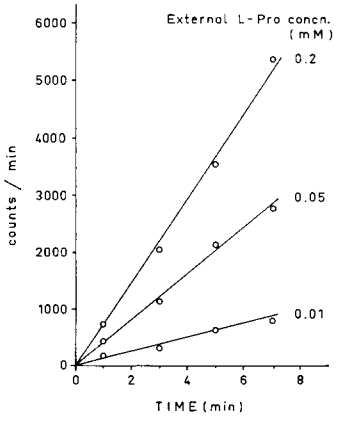


Fig. 3. Displacement of accumulated DL-[^{14}C]proline. Cells were suspended in 66 mM KH_2PO_4 (pH 5.5) *plus* 10 mM D-galactose and the kinetics of DL-[^{14}C]proline uptake was measured as indicated for Fig. 1. At steady state (60 min after zero time), the cell suspension was divided into three portions, and after the indicated additions, incubation was continued. The internal radioactivity was measured from samples taken at the indicated intervals. O—O, without additions; ×—×, *plus* 2,4-dinitrophenol at 0.1 mM final concn.; △—△, *plus* DL-proline, at 10 mM final concn.

Fig. 4. Initial velocity of L-[^{14}C]proline entrance. Cells were resuspended in 66 mM KH_2PO_4 *plus* 10 mM D-galactose (pH 5.5), and the kinetics of L-proline uptake were measured at the concentrations of L-proline indicated in the figure. Each point represents the mean of three different determinations.

Displacement of accumulated proline

The addition of excess nonradioactive DL-proline (10 mM) to a cell suspension which had accumulated DL-[^{14}C]proline up to the steady-state level resulted in a rapid decline of radioactivity in the cells. 180 min after the addition of nonradioactive DL-proline the intracellular radioactivity is almost completely displaced. 0.1 mM 2,4-dinitrophenol also displaces the internal radioactivity, but only to a limited extent after which no further radioactivity is lost from the cells (Fig. 3).

Initial velocity of L-[^{14}C]*proline influx*

As shown in Fig. 4, the kinetics of proline uptake by *S. chevalieri* cells are linear for at least the first 7 min after the addition of the labelled proline.

Effect of external L-[^{14}C]*proline concentration*

The transport system is highly specific for L-proline. As the external concentration of L-proline was increased, the system became saturated as shown in Fig. 5. The apparent affinity constant (K_m) for L-proline is $2.5 \cdot 10^{-5}$ M.

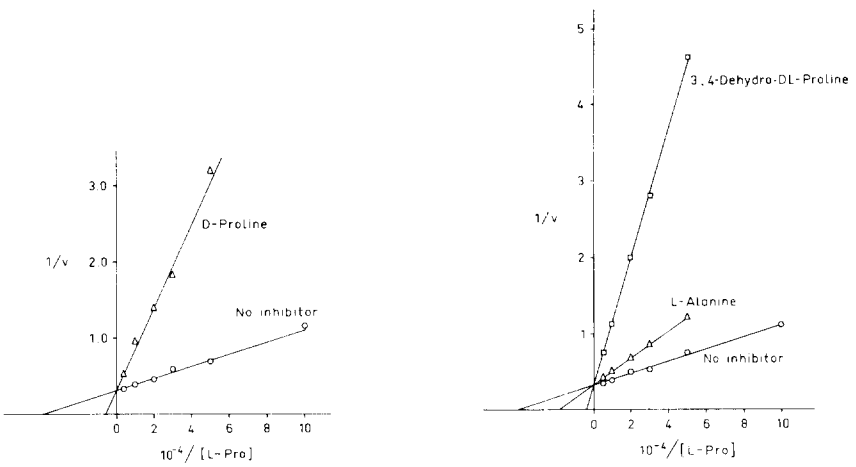


Fig. 5. Effect of external L-[^{14}C]proline concentration and inhibition of L-proline uptake by D-proline. Lineweaver–Burk plot ($K_m = 2.5 \cdot 10^{-5}$ M) showing competitive inhibition of L-proline uptake by D-proline (0.5 mM). Accumulated radioactivity was determined after 5 min of incubation as indicated in MATERIALS AND METHODS.

Fig. 6. Inhibition of L-proline uptake by L-alanine and 3,4-dehydro-DL-proline. Lineweaver–Burk plot showing competitive inhibition of L-proline uptake by L-alanine (0.1 mM) and 3,4-dehydro-DL-proline (0.2 mM). Accumulated radioactivity was measured after 5 min of incubation as indicated in MATERIALS AND METHODS.

Specificity of the system

The addition of amino acids at a concentration 100 times greater than that of the radioactive proline, showed that only D-proline and α-alanine produced a significant inhibition.

The kinetic analysis of initial velocity of uptake shows that both behave as competitive inhibitors for the L-proline accumulating system (Figs. 5 and 6). Hydroxy-proline is a poor inhibitor. Most of the other amino acids show only a small

TABLE II

INHIBITION OR ENHANCEMENT OF THE INITIAL VELOCITY OF INFLUX OF L-PROLINE BY AMINO ACIDS AT 100-FOLD HIGHER CONCENTRATION

Values given are the means of at least six separate experiments.

Amino acid (0.1 mM)	*Inhibition* (%)	*Enhancement* (%)
L-Alanine	48	
L-Arginine	12	
L-Asparagine		10
DL-Aspartic acid*		16
L-Cysteine		38
DL-Cysteine*	13	
L-Citruline	16	
Glycine	25	
L-Glutamic acid		13
L-Glutamine		24
L-Hydroxyproline	12	
L-Histidine	0	
L-Isoleucine		21
L-Leucine		15
DL-Lysine*	0	
DL-Methionine*		27
L-Ornithine	0	
L-Phenylalanine		56
D-Proline	42	
L-Serine	0	
L-Tyrosine		45
DL-Threonine*	0	
L-Tryptophan		80
DL-Valine*		28

* Assayed at 0.2 mM final concentration.

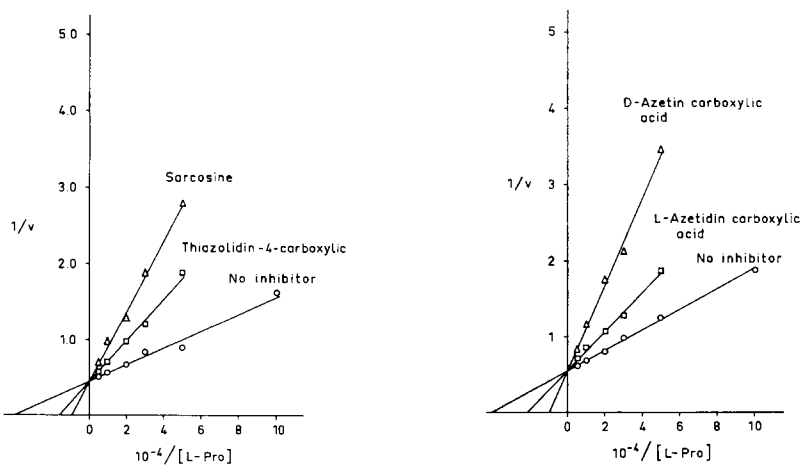


Fig. 7. Inhibition of L-proline uptake by sarcosine and L-thiazolidin 4-carboxylic acid. Lineweaver–Burk plot showing competitive inhibition of L-proline uptake by sarcosine (0.1 mM) and L-thiazolidin 4-carboxylic acid (0.1 mM). Accumulated radioactivity was measured after 5 min of incubation. For details see METHODS AND MATERIALS.

Fig. 8. Inhibition of L-proline uptake by D- and L-azetidin carboxylic acid. Lineweaver–Burk plot showing competitive inhibition of L-proline uptake by D- and L-azetidin carboxylic acids (0.2 mM). Accumulated radioactivity was measured after 5 min of incubation. For details see MATERIALS AND METHODS.

inhibitory effect whereas tryptophan, phenylalanine and tyrosine effectively enhance uptake. The results for all the amino acids tested are given in Table II.

A number of structural analogs inhibit only to a limited extent the uptake of proline. However, sarcosine, L-thiazolidin carboxylic acid, 3,4-dehydro-DL-proline, D- and L-azetidin carboxylic acid exhibit a significant inhibition. The nature of the inhibition mentioned above was further investigated by measuring initial velocity of uptake in the presence of the inhibitor. The results given in Figs. 6, 7 and 8 show that all of them behave as competitive inhibitors for the system.

TABLE III

INHIBITION OF INITIAL VELOCITY OF INFLUX OF L-PROLINE BY STRUCTURAL ANALOGS AT A 100-FOLD HIGHER CONCENTRATION

Values are the means of at least three separate experiments.

Analog (0.1 mM)	*Inhibition (%)*
D-Azetidin carboxylic acid	75
L-Azetidin carboxylic acid	56
Creatinine	7
Coumarilic acid	0
3,4-Dehydro-DL-proline	45
N-Methylglycine (sarcosine)	82
N,*N*-Dimethylglycine	26
N,*N*,*N*-Trimethylglycine (betaine)	8
Furan 2-carboxylic acid	22
Hydroxymethylimidazol	6
3-Indoleacetic acid	9
Nicotinic acid	8
α-Picolinic acid	5*
Pipecolic acid	23*
Pirazol 3,5-dicarboxylic acid	3
Piridin 2-aldoxime	0
L-Proline methyl ester	24
Quinaldic acid	6
L-Thiazolidin 4-carboxylic acid	69
Glycyl-L-proline	16
L-Prolylglycine	25
L-Prolylphenylalanine	8
Poly-L-proline	12

* These values were very poorly reproducible

TABLE IV

APPARENT AFFINITY CONSTANTS OF THE L-PROLINE UPTAKE SYSTEM FOR INHIBITORY STRUCTURAL ANALOGS

These constants were calculated from the experimental results shown in Figs. 7, 8 and 9.

Structural analog	$K_i \times 10^5$ (*M*)
L-Alanine	8.2
L-Azetidin carboxylic acid	20
D-Azetidin carboxylic acid	7.7
3,4-Dehydro-DL-proline	10
N-Methylglycine (sarcosine)	2.9
D-Proline	8.9
L-Thiazolidin 4-carboxylic acid	5.2

The apparent affinity constant (K_i) for the competitive type of inhibitors have been determined (Table IV). All of them have a K_i value larger than the apparent K_m of L-proline for its uptake system. From these values it appears that the affinity for the proline transport system decreases in the following order:
Sarcosine> thiazolidin 4-carboxylic acid >D-azetidin carboxylic acid >L-alanine >D-proline >3,4-dehydro-DL-proline >L-azetidin carboxylic acid.

DISCUSSION

The experiments reported here indicate that the proline transport system in *S. chevalieri* exhibits properties similar to those described for bacterial "permeases" (ref. 25). The quantity of L-proline accumulated, as measured by bioassay, may amount in some cases to 0.8 mol/l of intracellular water. This concentration can best be explained in terms of a catalytic transport mechanism, rather than by internal binding sites.

The bioassay and chromatographic analysis of the accumulated material indicate that it consists almost entirely of free proline. Also, nonradioactive proline rapidly displaces the internally accumulated radioactivity. Finally, no measurable change in the optical configuration of the L-proline by the accumulation process or by intracellular enzymes could be detected.

The observation that the initial velocity of proline uptake is enhanced in the presence of a source of energy, such as galactose, inhibited by 2,4-dinitrophenol or azide, together with the fact that the system is temperature dependent and operates against a concentration gradient, points to an energy requirement for the system.

The present work has shown that L-proline is concentrated by *S. chevalieri* cells by a very specific transport system. The fact that D-proline an L-alanine were the only amino acids found to be significant inhibitors is consistent with this idea. These results are also in accordance with the reported specificity of derepression of the proline transport system[26]. Further, structural analogs must have a very close similarity with the proline molecule in order to be effective inhibitors of the system.

From the results of inhibition studies it may be concluded that both the imino and carboxy group must be present as such because proline methyl ester, L-prolyl-phenylalanine, L-prolylglycine and glycyl-L-proline are poor inhibitors. Ring size modifications are also very limited, as can be seen from the results of inhibition with pipecolic acid and L-azetidin carboxylic acid. It is difficult to understand, however, why D-azetidin carboxylic acid is more inhibitory than L-azetidin carboxylic acid. Whether ring substitution may also affect affinity cannot be stated, since hydroxy-L-proline was the only case studied in the present investigation.

That the intramolecular distance between imino and carboxy groups is an indispensable factor in inhibition, rather than the ring itself, can be shown by the fact that sarcosine is the strongest of all inhibitors tested. On the other hand, *N*,*N*-dimethylglycine is a very poor inhibitor, thus further demonstrating the need of a free imino group.

The unexpected stimulatory effect of a number of amino acids may be due to several causes: (a) Dipeptide formation and uptake; (b) allosteric interactions between very close recognition sites for amino acids (permeases) at the membrane level; (c) "competitive stimulation" as described for ascites tumor cells[36,37]; (d) secondary effects

due to alteration of ion permeability. However, the present data cannot distinguish among these alternatives.

The proline transport system described here has many common characteristics with the arginine, lysine and methionine transport systems described by GRENSON *et al.*[22,23] and GITS AND GRENSON[24], thus confirming the general existence of very specific amino acid transport systems in yeasts.

ACKNOWLEDGEMENTS

This research was supported in part by the Rockefeller Foundation (Grant No. 2.107 4318) and the Facultad de Medicina, Universidad de Chile (Project 66-53), under a joint program, and by the Rockefeller Special Fund for Scientific Material under a joint program with the Organization of American States (O.E.A.).

We wish to express our gratitude to Dr. JULIO CABELLO of the Instituto de Química Fisiológica, Universidad de Chile, for the gift of a culture of *E. coli* 55/1 requiring L-proline.

The excellent technical assistance of Miss CARMEN L. VALENZUELA and Mrs. TERESA ARIAS is gratefully acknowledged.

REFERENCES

1 H. N. CHRISTENSEN, in A. KLEINZELLER AND A. KOTYK, *Membrane Transport and Metabolism*, Academic Press, London, 1960, p. 479.
2 J. T. HOLDEN, *Amino Acid Pools*, Elsevier Publishing Co., Amsterdam, 1962, p. 566.
3 E. S. TAYLOR, *J. Gen. Microbiol.*, 1 (1947) 86.
4 J. M. MATHIESON AND G. D. CATCHESIDE, *J. Gen. Microbiol.*, 13 (1955) 72.
5 J. SOBOREN AND J. F. NYC, *J. Bacteriol.*, 81 (1961) 20.
6 H. O. HALVORSON AND G. N. COHEN, *Ann. Inst. Pasteur*, 95 (1958) 73.
7 A. A. EDDY AND K. J. INDGE, *Biochem. J.*, 82 (1962) 14P.
8 H. E. BROCKMAN, *J. Gen. Microbiol.*, 34 (1964) 31.
9 Y. SURDIN, N. SLY, J. SIRE, A. M. BORDES AND H. DE ROBICHON-SZULMAJSTER, *Biochim. Biophys. Acta*, 107 (1965) 546.
10 P. ST. LAWRENCE, B. D. MALING, L. ATTWERGER AND M. RACHMEIER, *Genetics*, 50 (1964) 1383.
11 B. G. DE BUSK AND A. G. DE BUSK, *Biochim. Biophys. Acta*, 104 (1965) 139.
12 W. A. SORSOLI, K. D. SPENCE AND L. W. PARKS, *J. Bacteriol.*, 88 (1964) 20.
13 G. LESTER, *J. Bacteriol.*, 91 (1966) 677.
14 A. H. DOERMANN, *Arch. Biochem.*, 5 (1944) 373.
15 M. B. HOULAHAN AND H. K. MITCHELL, *Proc. Natl. Acad. Sci. U.S.*, 34 (1948) 465.
16 J. M. WIAME, J. BECHET, M. MOUSSET AND M. GRENSON, *Arch. Intern. Physiol. Biochim.*, 70 (1962) 766.
17 G. A. MAW, *J. Gen. Microbiol.*, 31 (1963) 247.
18 R. H. BAUERLE AND H. R. GARNER, *Biochim. Biophys. Acta*, 93 (1964) 316.
19 M. PETERSON AND D. R. STADLER, *Genetics*, 52 (1965) 466.
20 D. R. STADLER, *Science*, 150 (1965) 385.
21 W. R. WILEY AND W. H. MATCHETT, *J. Bacteriol.*, 92 (1966) 1698.
22 M. GRENSON, *Biochim. Biophys. Acta*, 127 (1966) 339.
23 M. GRENSON, M. MOUSSET, J. M. WIAME AND J. BECHET, *Biochim. Biophys. Acta*, 127 (1966) 325.
24 J. J. GITS AND M. GRENSON, *Biochim. Biophys. Acta*, 135 (1967) 507.
25 G. N. COHEN AND J. MONOD, *Bacteriol. Rev.*, 21 (1957) 169.
26 J. SCHWENCKE AND N. MAGAÑA-SCHWENCKE, *Biochim. Biophys. Acta*, 173 (1969) 302.
27 K. AHMED AND P. G. SCHOLEFIELD, *Can. J. Biochem. Physiol.*, 40 (1962) 1101.
28 N. BEGIN AND P. G. SCHOLEFIELD, *J. Biol. Chem.*, 240 (1965) 332.
29 R. J. BRITTEN, R. B. ROBERTS AND E. F. FRENCH, *Proc. Natl. Acad. Sci. U.S.*, 41 (1955) 863.
30 M. ZALOKAR, *Biochim. Biophys. Acta*, 46 (1961) 423.

31 H. J. VOGEL AND B.D. DAVIS, *J. Am. Chem. Soc.*, 74 (1952) 109.
32 R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
33 V. P. CIRILLO, *J. Bacteriol.*, 84 (1962) 485.
34 R. R. REDFIELD AND E. S. G. BARRON, *Arch. Biochem. Biophys.*, 35 (1952) 443.
35 B. ROTMAN AND R. GUZMAN, *Pathologie-Biologie, X^e Congr. Intern. Biol. Cell*, 9 (1961) 806.
36 J. A. JACQUEZ, *Biochim. Biophys. Acta*, 135 (1967) 751.
37 D. L. OXENDER AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 238 (1963) 3686.