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IMINO ACID TRANSPORT IN YEAST: THE UPTAKE OF SARCOSINE

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SUMMARY

1. The properties of a sarcosine uptake system in nitrogen-starved cells of *Saccharomyces chevalieri* are described. The intracellular radioactivity accumulated corresponds to free sarcosine as indicated by radioautography.

2. Internally accumulated [^{14}C]sarcosine does not outflow when cells are resuspended in fresh buffer, but slowly exchanges with externally added [^{12}C]-sarcosine, L-[^{12}C]proline and D-[^{12}C]proline.

3. Sarcosine uptake is temperature dependent and it is strongly inhibited by 20 mM azide and 1 mM 2,4-dinitrophenol.

4. Initial velocity of sarcosine uptake depends on external sarcosine concentration. The system shows a Michaelis–Menten substrate dependence with an apparent K_m of $3.1 \cdot 10^{-5}$ M. L-Proline, D-proline, DL-azetidin carboxylic acid and L-thiazolidin carboxylic acid were found to competitively inhibit the sarcosine uptake and their K_i were calculated. Among a number of amino acids only L-alanine and glycine effectively inhibit the sarcosine uptake.

5. These experiments strongly suggest that the accumulation of L-proline and sarcosine in yeast cells are mediated by the same uptake system. Moreover, kinetics results agree with our proposition that the uptake of all imino acids in yeast is mediated by the same transport system.

INTRODUCTION

Previous studies from our laboratory have demonstrated that L-proline uptake in *Saccharomyces chevalieri* is mediated by a very specific[1] and repressible[2] transport system. However, the fact that various imino acids were able to competitively inhibit the uptake of proline suggested the possibility that this may be mediated by a general imino acid uptake system. This idea has received strong support from our recent report which demonstrates that, when *S. chevalieri* cells were subjected to the nitrogen-starvation conditions previously described[2], the uptake of L-proline, D-proline, DL-azetidin carboxylic acid and sarcosine were also simultaneously derepressed[3], while the uptake of leucine, isoleucine and phenylalanine were not.

In this communication we report kinetic evidence which indicates that sarcosine and proline uptakes are mediated by the same transport system. In addition, kinetic data are presented which support the hypothesis that, in *S. chevalieri* the uptake of

imino acids is mediated by a common transport system. A preliminary report of this work has been presented[4].

MATERIALS AND METHODS

Materials

Strains and growth conditions. Yeast strain and growth conditions were those previously described[2].

Radiochemicals. [*carboxy*-^{14}C]Sarcosine was purchased from Amersham. Its original spec. act. of 12.9 Ci/mole was adjusted at 0.12 Ci/mole with unlabeled sarcosine.

Compounds. Sarcosine, betaine, L- and D-azetidin carboxylic acid and all the amino acids were obtained from Calbiochem. L-Proline methyl ester was a Fluka product. Glycyl-L-proline and L-prolylglycine were from Schwarz/Mann. L-4-Thiazolidin carboxylic acid and *N*,*N*-dimethyl-glycine were purchased from Nutritional Biochemicals. 3,4-Dehydro-DL-proline was a kind gift from Dr B. Witkop (N.I.H., Bethesda, Md., U.S.A.). Cycloheximide was obtained through the courtesy of Dr C. A. Fenwick of the Upjohn Co., Mich., U.S.A. Ingredients for media were obtained from Difco. D-Galactose was purchased from Pfansthiel (U.S.A.). KH_2PO_4 was either Mallinckrodt product No. 7100 or the Merck product No. 4871.

Methods

All the experiments were done with cells previously derepressed as described before[2].

Uptake experiments. The assay for sarcosine uptake was run as described[3]. The specific radioactivity of [^{14}C]sarcosine was adjusted to 0.12 Ci/mole with unlabeled sarcosine. Because the dry weight of $1 \cdot 10^7$ cells is 0.200 mg, our results expressed as nmoles/10^7 cells may be easily converted to the expression nmoles/mg dry weight.

Accumulating capacity. The maximal intracellular concentration of sarcosine was calculated assuming that intracellular water represents 47% of the total wet volume of the cells[5], and that sarcosine distributes homogeneously in the intracellular water.

Identification of the accumulated radioactivity. 10-ml aliquots of a $2 \cdot 10^7$ cells/ml suspension in 66 mM KH_2PO_4 (pH 5.5) containing 10 mM D-galactose (phosphate–galactose buffer), were incubated at 30 °C in a shaker with [^{14}C]sarcosine in the presence of 0.7 μg/ml cycloheximide. After 40 min of incubation, the cells were rapidly centrifuged, resuspended in 1 ml of a water–acetone mixture (1:3, v/v) and boiled for 7 min at 100 °C. The boiled cells were removed by centrifugation. The supernatant was analyzed by paper chromatography with the addition of enough unlabeled sarcosine to allow visual detection with polychromatic developer[6]. The solvent systems used were: ethanol–water–urea (80:20:0.5, v/v/w) [7]and *n*-butanol–acetic acid–water (50:25:25, by vol.). The dried chromatograms were cut every 5 mm and the strips counted by liquid scintillation (Nuclear Chicago) in vials containing 4 g/l Omnifluor (N.E.N.) in toluene. After counting, the chromatograms were carefully reassembled and developed with polychromatic developer[6]. Finally, the chemical and radiochemical chromatographic patterns were compared.

Initial velocity of uptake. Initial velocity of uptake was run according to the method published by Magaña-Schwencke and Schwencke[1].

RESULTS

Kinetics of labeled sarcosine uptake

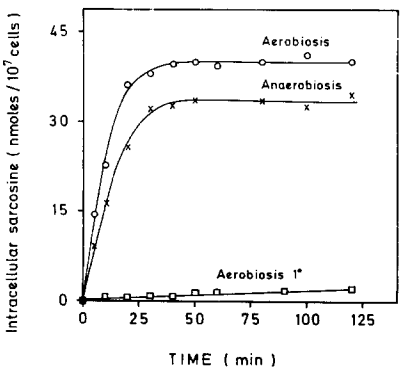
The uptake of [^{14}C]sarcosine is shown in Fig. 1. It may be seen that a plateau is approached after about 40 min of incubation. The uptake under anaerobic conditions is only slightly diminished. This result is similar to that reported for the uptake of carbohydrates in yeast[8]. At the plateau level the intracellular sarcosine concentration is at about 1000 times that of the medium reaching values of 50 mM.

Effect of temperature and metabolic inhibitors

The uptake of [^{14}C]sarcosine is temperature dependent (Fig. 1). At 30 °C sarcosine reached maximal internal concentration after 40 min whereas at this time, the sarcosine accumulation at 1 °C represents only 4.2% of this amount. There is a 100-fold difference in the initial rate of entry at the two temperatures. The system is also strongly inhibited by metabolic inhibitors such as 2,4-dinitrophenol and azide (Table I).

Properties of the accumulated radioactivity

The results of radioautography of the internal radioactivity indicate that 100% of it corresponds to sarcosine (Fig. 2). About 4% of the accumulated radioactivity remains associated with the insoluble residue after the water–acetone extraction. The appearance of glycine, *N,N*-dimethylglycine or *N,N,N*-trimethylglycine would have been clearly detected by using the *n*-butanol–acetic acid–water solvent.



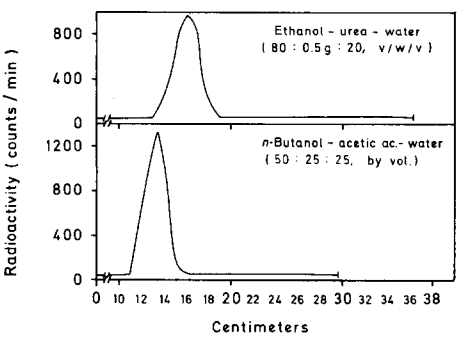


Fig. 1. Kinetics of sarcosine uptake. The cells, resuspended in phosphate–galactose buffer, were incubated at 30 °C (unless otherwise indicated) in the presence of [^{14}C]sarcosine. Samples were taken at intervals and internal radioactivity was measured. Anaerobiosis conditions were obtained by resuspending the cells in degassed phosphate–galactose buffer and covering it with deoxygenated vaseline. For further details see under Materials and Methods.

Fig. 2. Radiochromatographic analysis of intracellular radioactivity. Chromatograms obtained from the extracted radioactivity after 40 min of sarcosine uptake were cut into 5-mm pieces and separately counted. The dried pieces were reassembled after counting and developed with ninhydrin polychromatic developer. For a description of the detailed process, see Materials and Methods.

TABLE I

EFFECT OF METABOLIC INHIBITORS ON SARCOSINE UPTAKE

Inhibitor and [^{14}C]sarcosine were added at zero time and the accumulation assay run as described under Materials and Methods. Values are the mean of at least three separate determinations.

Inhibitor	*Inhibition* (%)		*Inhibition* (%)	
2,4-Dinitrophenol	0.5 mM	88	1.0 mM	97
Azide	10.0 mM	90	20.0 mM	97
Na_2HAsO_4	0.1 mM	1	1.0 mM	1
KF	1.0 mM	0	10.0 mM	0

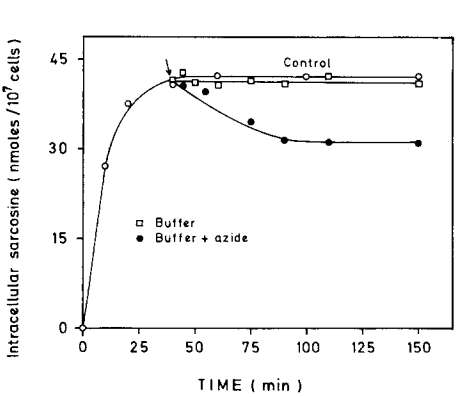
Outflow of accumulated [^{14}C]sarcosine

When cells, which have accumulated [^{14}C]sarcosine up to the plateau level, are resuspended in fresh buffered incubation medium, without labeled sarcosine, no exit of the intracellular [^{14}C]sarcosine can be observed (Fig. 3). However, when 33 mM azide is added to the fresh phosphate–galactose buffer, a slow [^{14}C]sarcosine outflow is produced reaching a new plateau level. Only 26% of the intracellular sarcosine is lost by this treatment. Very similar results can be observed from Fig. 4, when azide was added to the incubation medium in the presence of external [^{14}C]sarcosine.

The addition of excess nonradioactive sarcosine, L-proline or D-proline, causes a slow exit of internally accumulated [^{14}C]sarcosine.

Concentration dependence of the [^{14}C]sarcosine uptake

Initial velocity of sarcosine uptake depends on the external sarcosine concentra-



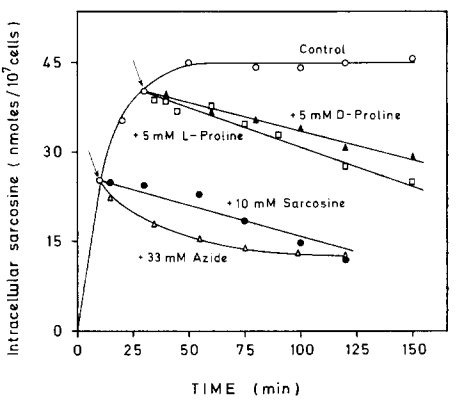


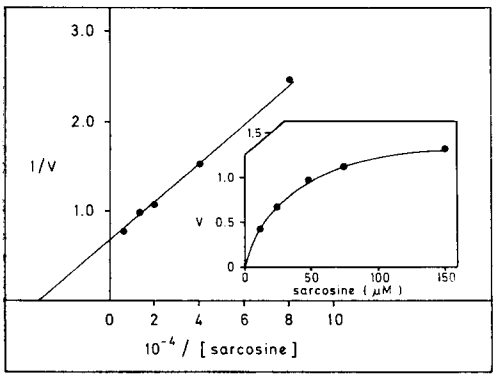
Fig. 3. Outflow of intracellular sarcosine. The cells were allowed to accumulate up to 40 min and then, the cell suspension was divided into three portions and centrifuged; after resuspension as indicated (azide is 33 mM), incubation was continued. The internal radioactivity was measured from samples taken at the indicated intervals.

Fig. 4. Displacement of intracellular sarcosine. Cells resuspended in phosphate–galactose buffer were incubated and the kinetics of [^{14}C]sarcosine uptake was measured as indicated in Fig. 1. At times indicated by the arrows, sarcosine, azide, D-proline and L-proline were each added to different portions of the cell suspension and incubations continued. Samples were taken to measure intracellular radioactivity.

tion. The uptake system follows Michaelis–Menten kinetics (Fig. 5) with an apparent K_m for sarcosine of about 31 μM.

Specificity of the system

Among a number of amino acids, at 10-fold excess final concentration with respect to sarcosine, only L-proline, D-proline, L-alanine and glycine produced a significant inhibition of sarcosine uptake (Table II). The results in the table also show



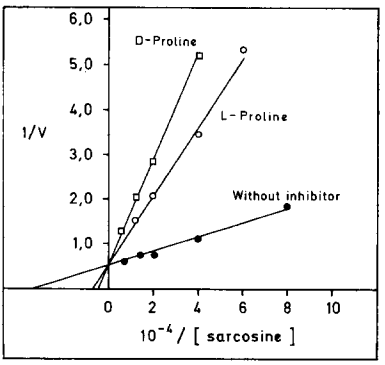


Fig. 5. Effect of external [^{14}C]sarcosine concentration. Lineweaver–Burk plot (K_m 31 μM). The uptake of [^{14}C]sarcosine for 3 min was measured as indicated in Materials and Methods. v is expressed in arbitrary units. In the inset, the direct substrate concentration curve plot.

Fig. 6. Inhibition of sarcosine uptake by D-proline and L-proline. Lineweaver–Burk plot showing competitive inhibition of sarcosine uptake by D-proline (0.5 mM) and L-proline (0.1 mM). Accumulated radioactivity was measured after 3 min of incubation. For details see Materials and Methods.

TABLE II

INHIBITION OF INITIAL UPTAKE VELOCITY OF SARCOSINE BY AMINO ACIDS AND STRUCTURAL ANALOGS

Inhibitor and [^{14}C]sarcosine were added at zero time and the accumulation assay run as described under Materials and Methods. Values are the mean of at least three separate determinations

Amino acid	*1 mM inhibition* (%)	*Analog*	*1 mM*	*5 mM*
			inhibition (%)	
L-Alanine	93	D-Azetidin carboxylic acid	50	96
L-Cysteine	3	L-Azetidin carboxylic acid	31	96
L-Phenylalanine	5	3,4-Dehydro-DL-proline	18	—
Glycine	73	*N,N*-Dimethylglycine	11	6
L-Glutamic acid	10	*N,N,N*-Trimethyl-glycine	6	36
DL-Hydroxyproline	9	L-Thiazolidin 4-carboxylic acid	59	99
D-Proline	78	L-Proline methyl ester	18	90
L-Proline	94	Glycyl-L-proline	0	4
L-Ornithine	10	Prolylglycine	2	+4
L-Serine	8			
L-Tryptophan	11			

that non-protein imino acids effectively inhibit sarcosine uptake although all of them have a cyclic structure.

The pattern of sarcosine uptake inhibition by analogs and amino acids is almost identical to that found for L-proline uptake[1].

The nature of the inhibition already mentioned was further analyzed by measuring the initial velocity of sarcosine uptake in the presence of the inhibitor. The results given in Figs 6 and 7 indicate that all of them behave as competitive inhibitors for the system. Results with L-alanine and glycine will be communicated elsewhere.

The apparent affinity constant (K_i) for the competitive inhibitors of sarcosine uptake are shown in Table III. For comparison the K_i values, previously reported for the same compounds as inhibitors of L-proline uptake[1], are also included. There is an

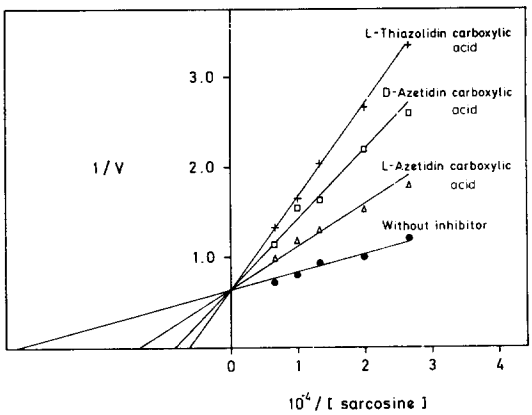


Fig. 7. Inhibition of sarcosine uptake by analogs. Lineweaver–Burk plot showing competitive inhibition of sarcosine uptake by D-azetidin carboxylic acid (0.2 mM), L-azetidin carboxylic acid (0.2 mM) and L-thiazolidin carboxylic acid (0.4 mM). Accumulated radioactivity was measured after 3 min of incubation as indicated in Materials and Methods.

TABLE III

APPARENT AFFINITY CONSTANTS OF THE SARCOSINE AND L-PROLINE UPTAKE SYSTEM

The constants for the sarcosine uptake system were calculated from the experimental results shown in Figs 6 and 7. The constants for the L-proline uptake system were taken from our previous work[2].

Structural analog	$K_i \times 10^5$ (*M*)		$K_m \times 10^5$ (*M*)
	Sarcosine uptake	*Proline uptake*	
L-Azetidin carboxylic acid	16.4	20.0	
D-Azetidin carboxylic acid	7.3	7.7	
N-Methylglycine (sarcosine)	—	2.9	3.1
L-Proline	2.7	—	2.5
D-Proline	8.6	8.9	
L-Thiazolidin-4-carboxylic acid	4.0	5.2	

excellent agreement between K_m for sarcosine and its K_i when studied as an inhibitor for L-proline uptake. Also the K_m for L-proline and its K_i as competitive inhibitor of sarcosine uptake are essentially the same. Moreover, the K_i of all the competitive inhibitors for either L-proline or sarcosine uptake are closely the same.

DISCUSSION

Sarcosine transport in *S. chevalieri* is mediated by a transport system with restricted affinity for imino acids. This system, hereafter named the imino acids transport system, allows the accumulation of L-proline[1] and sarcosine. Also, the D-proline and DL-azetidin carboxylic acid uptakes are mediated by the same system[3].

L-Proline (K_m 25 μM) and sarcosine (K_m 31 μM) show, so far, the highest affinity for the imino acid transport system. Only L-alanine and glycine, among the amino acids, are recognized and accumulated by the imino acids transport system. This may be due to the fact that these amino acids are the smaller ones and may "mimic" the imino acid structure. However, it is also possible that both amino acids may be simultaneously concentrated by another transport system[5] or may show another type of interaction as reported for the renal transport activities for glycine, proline and hydroxyproline[9]. Conversely, this does not rule out the possibility that some of the imino acids may pass *via* some amino acid uptake system. The specificity of the imino acids transport system makes it unlikely that sarcosine and L-proline uptakes could be enhanced by a general transport system developed by nitrogen starvation[10], as the one described for fungi. Moreover, it is noteworthy that, *N*-methyl amino acids (imino acids) do not inhibit the already mentioned nonspecific amino acid permease of fungi.

The results shown above open the possibility of using any of the nonmetabolizable imino acids to study the uptake of L-proline in cells which actively metabolize this imino acid. However, the approach may present a number of problems[11,12]. Our data coincide with previous findings that cyclic and noncyclic imino acid transport and amino acid transport are biologically distinct in eucaryotic cells[13,14]. Finally, the fact that the uptake of L-proline, D-proline, sarcosine and DL-azetidin carboxylic acid are all, simultaneously derepressed by nitrogen starvation[3], is consistent with our hypothesis that there exists a common specific transport system for imino acids in yeast. The close agreement between the K_i values of competitive inhibitors independently calculated for L-proline or sarcosine uptakes strongly supports this idea.

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