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TRANSPORT OF α-AMINOISOBUTYRIC ACID IN *SACCHAROMYCES CEREVISIAE*

FEEDBACK CONTROL

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SUMMARY

The uptake of α-aminoisobutyric acid in baker's yeast proceeds at the expense of metabolic energy and does not reach a steady-state level if energy and substrate are provided. The uptake shows two components, one with a K_m of 5.4 mM and a V of 11 μmoles/g dry wt per min, the other with a K_m of 0.15 mM and a V of 0.5 μmole/g dry wt per min. α-Aminoisobutyric acid does not leave the cells under any conditions, except after treatment with nystatin. The uptake is *trans*-inhibited by a number of different amino acids, including α-aminoisobutyric acid itself, in a non-competitive manner, the K_i for α-aminoisobutyric acid *vs* α-aminoisobutyric acid uptake being 27 mM for the major component.

A model involving two forms of carrier and strictly unidirectional fluxes is described, suggesting a feedback control by the intracellular amino acid at the key step of uptake.

INTRODUCTION

Transport of amino acids in yeast cells has been studied both with exponential-phase cultures, either in the presence of nitrogen-containing compounds[1] or in a simple buffer[2], and with stationary cells[3]. Protein synthesis and, occasionally, amino acid metabolism has somewhat complicated kinetic calculations of transport so that inhibitors, like actidione[3] or arsenate[4], were employed with greater or lesser success. Studies performed in other cell types (*e.g.* refs 5–7) show the usefulness in this connection of analogues of natural amino acids, in particular α-aminoisobutyric acid. This amino acid is not incorporated into proteins, nor is it degraded metabolically in baker's yeast. However, it is transported readily by one or more of the transport agencies in the cell membrane.

Some kinetic data on its transport are reported and a transport model is advanced that attempts to account for the peculiarities of amino acid translocation in a number of microbial species.

MATERIALS AND METHODS

A pure strain of *Saccharomyces cerevisiae* isolated from yeast produced by the Kolín (Czechoslovakia) distillery was used. This is identical with the strain employed over years of work with both sugar and amino acid transport[3,8] and its constancy has been regularly checked by 23 tests of the genus *Saccharomyces* employing numerical taxonomy (Dr A. Kocková-Kratochvílová, Bratislava). It was maintained on wort agar slants and propagated in a medium containing (in 1 l): 2 g KH_2PO_4, 1 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 0.3 g trisodium citrate, 0.1 g $MgCl_2 \cdot 6\,H_2O$, 0.1 g $MnSO_4 \cdot 7\,H_2O$, 0.1 g $CaCl_2$, 0.1 g $ZnSO_4 \cdot 7\,H_2O$, 16 g D-glucose and 1 g Difco yeast extract, using a reciprocal shaker at 112 strokes/min at 30 °C. The cells were harvested after 20 h when less than 2% budding cells were present in the culture while the viability (using methylene blue) was in excess of 95%.

The washed suspension was then agitated using a magnetic stirrer for 3 h to reduce endogenous energy reserves[9].

Incubation was under aerobic conditions at 30 °C in a Dubnoff incubator, using a suspension of 4–6 mg dry wt/ml distilled water. Labelled amino acids and other components were added in distilled water. 1-ml suspension samples were withdrawn and filtered through a Synpor 6 filter (0.45 μm pore diameter; Synthesia, Czechoslovakia), washed with 1 ml distilled water and the filter used for further analysis. To determine the intracellular content of free amino acid, the filter was extracted for 30 min with 6% trichloroacetic acid, the sediment washed with the same acid and the extract (*plus* washing) analyzed.

Radioactivity was assayed either in a toluene scintillation liquid in a Mark I Nuclear Chicago spectrometer, or on aluminium planchets (with filters dissolved in dioxane and dried) in a Frieseke-Hoepfner 2π methane-flow counter

Chemicals

All the amino acids were of the L-configuration and were obtained from Lachema, Czechoslovakia (glycine, alanine, valine, leucine, isoleucine, cysteine, serine, threonine, phenylalanine, tyrosine, tryptophan, aspartic acid, glutamic acid), from Koch-Light, Great Britain (lysine, methionine, arginine, α-aminoisobutyric acid), from Merck, Germany (proline), from American Roland Co., U.S.A. (histidine) and from Calbiochem, U.S.A. (β-alanine, β-aminoisobutyric acid, α-aminobutyric acid, γ-aminobutyric acid, diaminobutyric acid). All sugars and inhibitors were from Lachema, Czechoslovakia, and were of reagent-grade purity.

All generally ^{14}C-labelled amino acids were from the Institute for Research, Production and Uses of Radioisotopes, Prague, with the exception of α-amino [^{14}C]-isobutyric acid which was from the Radiochemical Centre, Amersham.

RESULTS

Uptake constants

α-Aminoisobutyric acid is taken up by *Saccharomyces cerevisiae* at the expense of metabolic energy, the process being practically completely blocked by 2,4-dinitrophenol. On the other hand, the process is highly stimulated by preincubation with different sources of metabolic energy, like D-glucose, D-fructose, sucrose, maltose,

but much less with ethanol or acetate[9]. The source of energy appears to be a polyphosphate fraction not extractable with cold 6% trichloroacetic acid. For this reason, all subsequent experiments (unless stated otherwise) were done after a 2-h preincubation with 1% D-glucose and thorough washing.

The apparent concentration ratios (intracellular to extracellular) attained after suitable time intervals may reach values of well over 1000:1. In fact, as will be shown below, the intracellular concentration tends to rise indefinitely if a sufficient supply of amino acid is provided in the medium.

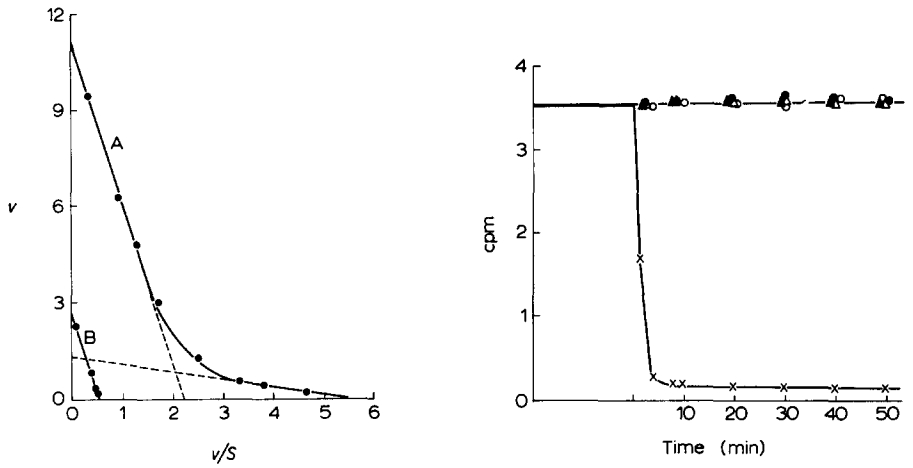


Fig. 1. A Hofstee plot of the initial rate of uptake of α-aminoisobutyric acid in baker's yeast. A, energy-rich cells; B, depleted cells. v is expressed in μmoles α-aminoisobutyric acid/g dry wt per min, S concn in mM.

Fig. 2. Efflux of ^{14}C-labelled α-aminoisobutyric acid from baker's yeast cells preloaded with 1 mM α-amino[^{14}C]isobutyric acid (initial radioactivity 23000 cpm/μg dry wt per ml). Transfer at time 0, to distilled water (○), 50 mM unlabelled α-aminoisobutyric acid (●), 0.5 mM 2,4-dinitrophenol (△), 0.5 mM uranyl nitrate (▲), 20 μg nystatin/ml (×).

As shown in Fig. 1, the initial rate of α-aminoisobutyric acid uptake shows two components in a reciprocal plot (here according to Hofstee[18]). The K_m of the "major" component was first estimated from a Lineweaver-Burk plot and the corresponding component subtracted from the "minor" one to obtain the approximate K_m of the minor component. Since the intersection of the curve on the y-axis in the Hofstee plot yields $V_1 + V_2$ and the intersection on the x-axis yields $V_1/K_{m1} + V_2/K_{m2}$, it was possible to calculate all the four values. Fitting them to the rate equation (with two Michaelis terms) gave results for v differing by less than 1% from the actual experimental values at both extremes of concentration. The values thus obtained were K_{m1}, 5.4 mM; V_1, 11 μmoles/g dry wt per min; and K_{m2}, 0.15 mM; V_2, 0.5 μmole/g dry wt per min. This is very similar to the situation with natural amino acids in yeast[8,10].

It will be seen that energy-depleted cells retain only the first component, although the maximum rate of uptake is reduced to about a quarter of the energy-rich form.

Efflux of α-aminoisobutyric acid

It has been observed previously[3,11] that the efflux of amino acids from baker's yeast cells is practically non-existent, although natural amino acids (glycine, aspartic acid, leucine, methionine and lysine were tested) can be released from yeast cells up to about 30% of the total amount. With α-aminoisobutyric acid, there is evidently no efflux at all (Fig. 2), whatever the conditions of experiment. The values of intracellular activity found after preloading with α-aminoisobutyric acid do not differ from those after transfer to an α-aminoisobutyric acid-free medium by more than ±2% (error of estimation) and no activity is found in the external medium beyond the background of counting. Only nystatin, which is known to increase non-specifically the permeability of plasma membranes, liberates 96–99% of intracellular activity within 4 min, which provides evidence for the free state of α-aminoisobutyric acid in the cell water.

The lack of efflux obviously prevents measurement of countertransport and of other types of interaction between unidirectional fluxes.

Trans-inhibition

It has been reported in several cell types (*Streptomyces hydrogenans*[12], *Neurospora crassa*[13], as well as *Saccharomyces cerevisiae*[11]) that intracellular amino acids inhibit the uptake of the same (and of other) amino acid species from the medium. This inhibition was termed *trans*-inhibition and is now generally ascribed to a feedback-type control from inside the cell. The transport of α-aminoisobutyric acid was shown to be markedly *trans*-inhibited in a similar fashion although two effects of preloading with an amino acid can be distinguished.

One effect (prevalent after preincubation in the absence of an energy source) tends to increase the subsequent uptake of labelled α-aminoisobutyric acid (Table I), perhaps due to an inductive synthesis of an α-aminoisobutyric acid carrier (this effect is completely abolished by cycloheximide although this inhibitor of protein synthesis has no influence on the uptake of α-aminoisobutyric acid itself[3]). The other effect

TABLE I

EFFECT OF PREINCUBATION WITH DIFFERENT AMINO ACIDS (10 mM) ON THE RATE OF SUBSEQUENT UPTAKE OF 1 mM α-AMINO[^{14}C]ISOBUTYRIC ACID

Preincubated with	*Initial rate (% of control)*	*Preincubated with*	*Initial rate (% of control)*
Water	100	Glutamic acid	136
Glycine	176	Lysine	138
Alanine	136	Arginine	176
Valine	220	Histidine	198
Leucine	210	Tryptophan	105
Isoleucine	226	Proline	165
Serine	183	α-Aminoisobutyric acid	199
Methylserine	138	β-Aminoisobutyric acid	147
Cysteine	82	α-Aminobutyric acid	122
Methionine	75	γ-Aminobutyric acid	147
Phenylalanine	157	β-Alanine	135
Tyrosine	132	Diaminobutyric acid	81
Aspartic acid	148		

which is much more pronounced (prevalent after preincubation with glucose when the amount of α-aminoisobutyric acid taken up may be 100 times greater than in impoverished cells) suppresses the subsequent rate of uptake of α-aminoisobutyric acid (Table II), apparently due to a powerful *trans*-inhibitory effect of the amino acid accumulated in the presence of glucose.

To assess the specificity of this *trans*-inhibition compared to competition, it had to be taken into account that preincubation with different amino acids resulted in different intracellular concentrations and hence no quantitative comparisons were permissible at this stage. For this reason, the intracellular pools of several selected amino acids were determined after suitable time intervals of preincubation. These concentrations were then used for estimating the competition of these amino acids with the uptake of α-aminoisobutyric acid (Table III). Hence it appears that the

TABLE II

EFFECT OF PREINCUBATION WITH DIFFERENT AMINO ACIDS (10 mM) IN 1% D-GLUCOSE ON THE RATE OF SUBSEQUENT UPTAKE OF 1 mM α-AMINO[^{14}C]ISOBUTYRIC ACID WITHOUT GLUCOSE

Preincubated with D-*glucose and*	*Initial rate* (% *of control with glucose alone*)	*Preincubated with* D-*glucose and*	*Initial rate* (% *of control with glucose alone*)
Water	100	Glutamic acid	21
Glycine	9	Lysine	8
Alanine	14	Arginine	8
Valine	25	Histidine	29
Leucine	23	Tryptophan	31
Isoleucine	25	Proline	59
Serine	18	α-Aminoisobutyric acid	52
Methylserine	32	β-Aminoisobutyric acid	3
Cysteine	2	α-Aminobutyric acid	13
Methionine	9	γ-Aminobutyric acid	17
Phenylalanine	31	β-Alanine	6
Tyrosine	21	Diaminobutyric acid	12
Aspartic acid	8		

TABLE III

COMPARISON OF COMPETITIVE AND *trans*-INHIBITORY EFFECTS OF VARIOUS AMINO ACIDS ON THE UPTAKE OF 1 mM α-AMINOISOBUTYRIC ACID

Values expressed as percent of control with water. Cells were preincubated with $1 \cdot 10^{-5}$ M amino acids and their free intracellular concentration estimated. This concentration was then used in parallel competition experiments with cells preincubated in water.

Amino acid	*Competition*	*Trans-inhibition*
113 μM glycine	4	13
235 μM alanine	72	9
63 μM valine	7	11
45 μM leucine	0	0
34 μM isoleucine	35	24
130 μM aspartic acid	18	0
71 μM phenylalanine	17	0
220 μM lysine	54	0
460 μM arginine	24	0
55 μM proline	38	3

specificity of competition for uptake differs from that of the *trans*-inhibition. Also, the efficiency of *trans*-inhibition appears to be lower than that of competition, glycine and valine being the possible exceptions to this rule.

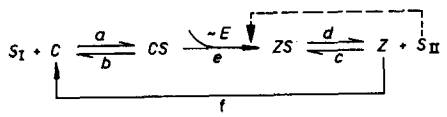
This is true also for the *trans*-inhibition by α-aminoisobutyric acid itself where an apparent K_{trans} of 20–35 mM was calculated (for the procedure see Discussion).

Fig. 3 shows that *trans*-inhibition only affects the maximum rate of uptake, *i.e.* either the mobility of the transmembrane movement or the effective amount of carrier, leaving the K_m intact. It is of interest that both components of α-aminoisobutyric acid influx are *trans*-inhibited by glycine in a similar way. The inhibition is formally non-competitive and one may calculate for the major component of α-aminoisobutyric acid influx a K_{trans} value for glycine of 14 mM (taking into account the intracellular concentrations reached after preincubation).

DISCUSSION

The transport of α-aminoisobutyric acid in *Saccharomyces cerevisiae* possesses two features that are characteristic for amino acid transport in yeasts and fungi, *viz.* a complete lack of efflux and a *trans*-inhibition of influx. Moreover, energy is obviously required for the uptake but not for maintaining the high intracellular concentrations (amino acid does not leave the cells even after a thorough depletion of energy reserves).

A kinetic model is presented that attempts to fit the observations. Let us assume a carrier C which combines on the outside with substrate S fo form CS. This complex then undergoes an energy-dependent change of conformation to ZS and, in the process, exposes its substrate-binding site to the inner pool where ZS can dissociate into Z and S. Z returns to the outer face, changing back to C in the process. The equilibria are shifted strongly in favour of ZS and C such that back reactions are negligible. Thus



Then

$$C_t = C + CS + Z + ZS \tag{1}$$

Assuming a steady state of the transport components (probably within fractions of a second after adding the transported substrate) one can write the following (the capital letters designating the concentrations of the various components):

$$dC/dt = -aC \cdot S_I + bCS + fZ = 0 \tag{2a}$$

$$dCS/dt = aC \cdot S_I - bCS - eCS = 0 \tag{2b}$$

$$dZ/dt = dZS - cZ \cdot S_{II} - fZ = 0 \tag{2c}$$

$$dZS/dt = -dZS + cZ \cdot S_{II} + eCS = 0 \tag{2d}$$

Manipulation of these equations to obtain CS, Z, and ZS in terms of C yields

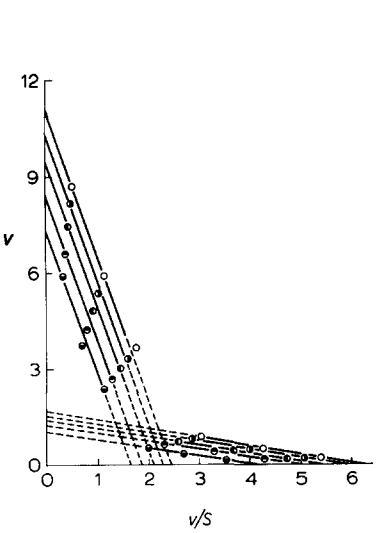
$$C_t = C\left\{1 + \frac{aS_I}{b+e}\left[1 + \frac{e}{f}\left(1 + \frac{cS_{II}+f}{d}\right)\right]\right\} = C \cdot Y \tag{3}$$

The flow of S is then expressed by

$$J_S = eCS = \frac{aeC_t \cdot S_I}{Y(b+e)} =$$

$$= \frac{eC_t}{1 + \frac{e}{f}\left(1 + \frac{cS_{II}+f}{d}\right)} \cdot \frac{S_I}{S_I + \frac{b+e}{a\left[1 + \frac{e}{f}\left(1 + \frac{cS_{II}+f}{d}\right)\right]}} = VS_I/(S_I + K_m) \quad (4)$$

It follows from Eqn 4 that S_{II} will depress the inward flow of S simply on the basis of the mass-action principle, but that the effect will be much less pronounced at low values of S_I, where it can be considered negligible. Also, S_{II} should increase both V and K_m of α-aminoisiobutyric acid influx in the same manner. However, it was repeatedly observed (see Fig. 4) that the effect of intracellular amino acid is as pronounced at extremely low as at high concentrations of S_I. Likewise, the effect of S_{II} on J_S was of a non-competitive nature, affecting only V (*cf.* Fig. 3). This suggested



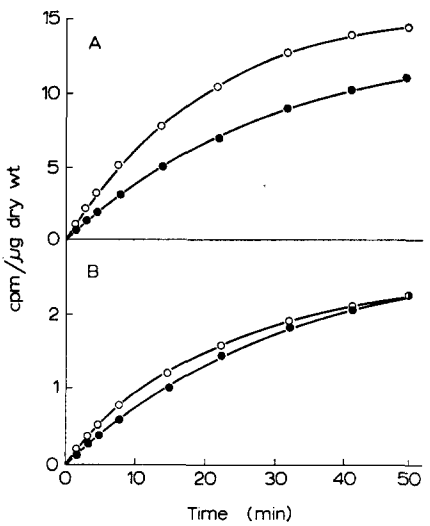


Fig. 3. Analysis of *trans*-inhibition of α-aminoisobutyric acid uptake. ○, no preloading; ◑, preincubation with 0.1 mM glycine; ◐, with 1 mM glycine; ◓, with 10 mM glycine; ◒, with 100 mM glycine. v in µmoles α-aminoisobutyric acid/g dry wt per min, S concn in mM.

Fig. 4. Uptake of ^{14}C-labelled α-aminoisobutyric acid (A, 0.01 mM α-aminoisobutyric acid; B, 10 mM α-aminoisobutyric acid) without (○) and after (●) preincubation for 2 h with 1 mM unlabelled α-aminoisobutyric acid. Initial radioactivity of medium was 96000 cpm/ml.

a distinct site of action of S_{II} on the transport reaction, *e.g.* at the $CS \rightarrow ZS$ step. Carried out rigorously, the last term of Eqns 2b and 2d would change to $eCS/(CS + K)(1 + S_{II}/K)_i$ where K is the half-saturation constant of the conversion of CS into ZS and K_i the inhibition constant of S_{II} in this reaction. This treatment would result in square equations that are practically intractable in this context, but it will serve us equally well if we assume that the step from CS to ZS is fully first-order so that its rate can be expressed by $eCS/K(1 + S_{II}/K_i)$ or $e'CS/i$. In this case, one can process

a set of differential equations analogous to Eqns 2a–2d to obtain for the rate of flow of S:

$$J_S = \frac{ae'C_tS_I}{iY(b + e'/i)} \tag{5}$$

where

$$Y = 1 + \frac{aS_I}{b + e'/i}\left[1 + \frac{e}{f}\left(1 + \frac{cS_{II} + f}{d}\right)\right]$$

If this expression is reduced to the Michaelis form we obtain

$$J_S = \frac{e'C_t}{i\left[1 + \frac{e}{f}\left(1 + \frac{cS_{II} + f}{d}\right)\right]} \cdot \frac{S_I}{\frac{b + e'/i}{a\left[1 + \frac{e}{f}\left(1 + \frac{cS_{II} + f}{d}\right)\right]} + S_I} \tag{6}$$

Although it is not essential for the argument, comparison of Eqns 4 and 6 will be simplified is one assumes (in analogy with processes of sugar transport in yeast[14] and in erythrocytes[15,16]) that the rate constants e (or $e' = e/K$) and f are much smaller than a, b, c and d, and that $b/a = K_{CS}$ and $d/c = K_{ZS}$. Let e/f also equal r. Then Eqn 4 becomes

$$J_S = \frac{eC_t \cdot S_I}{[1 + r(1 + S_{II}/K_{ZS})]\left[S_I + \frac{K_{CS}}{1 + r(1 + S_{II}/K_{ZS})}\right]} \tag{7}$$

and Eqn 6 becomes

$$J_S = \frac{eC_t \cdot S_I}{(1 + S_{II}/K_i)\,[1 + r(1 + S_{II}/K_{ZS})]\left[\frac{K_{CS}}{1 + r(1 + S_{II}/K_{ZS})} + S_I\right]} \tag{8}$$

Since it has been observed that K_m is not affected by S_{II} it follows that K_{ZS} is very large and, for practical purposes, Eqn 7 can be written as follows:

$$J_S = \frac{eC_t \cdot S_I}{(1 + r)\left(S_I + \frac{K_{CS}}{1 + r}\right)} \tag{9}$$

and Eqn 8 as

$$J_S = \frac{e'C_t \cdot S_I}{(1 + S_{II}/K_i)\,(1 + r)\left(\frac{K_{CS}}{1 + r} + S_I\right)} \tag{10}$$

Now, Eqn 9 does not predict any effect of S_{II} while Eqn 10 shows that V will decrease while the apparent K_m will remain unaffected. Hence, the feedback-control model is preferred to the mass-action one. This is also in agreement with the fact that the affinity spectrum of competition at the *cis*-side is different from the *trans*-inhibition pattern.

It is inherent in both models that uptake of S_I should actually stop only at an infinite concentration of S_{II}. Strange as this appears, it has been repeatedly shown that the uptake of α-aminoisobutyric acid does persist even after 10 h of incubation at an appreciable rate (Fig. 5) while in a reversible type of transport (whether mediated diffusion or active transport) a final intracellular level is generally reached within 10–20 min at comparable concentrations. The type of curve shown in Fig. 5 permits, at the same time, to assess the value of $K_t (= K_{trans}$, the *trans*-inhibition constant). Under conditions near saturation (here at $10 \times K_m$):

$$J_S \cong V = \frac{e' C_t}{(1 + S_{II}/K_t)(1 + r)} \tag{11}$$

If the concentration S_{II} is found when $J_S = V/2$ (in fig. 5 at 35 mM) it gives directly the value of K_t.

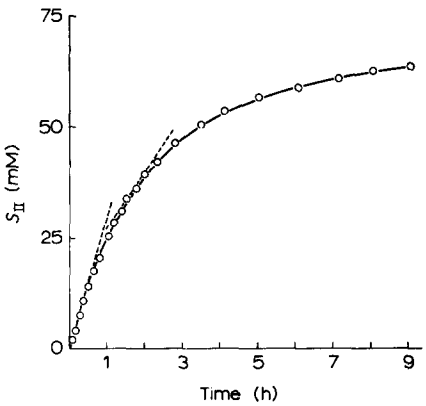


Fig. 5. Uptake of ^{14}C-labelled 50 mM α-aminoisobutyric acid over a prolonged period. Tangents at maximum and at half-maximum rates are shown.

It is generally observed that the apparent K_t values toward α-aminoisobutyric acid uptake are greater than the half-saturation constants of uptake of any of the amino acids tested, the average value obtained being 27 mM. However, a large error in this estimation, among other things, may be caused by intracellular compartmentation of the *trans*-inhibitory amino acid.

It is difficult to estimate the nature of the *trans*-inhibitory site but it is of interest that in some permease-defective mutants of baker's yeast, amino acids can be excreted from cells[17]. This might suggest that the genetically controlled transport protein is not the carrier C but rather the catalyst of the $CS \rightarrow ZS$ conversion (possibly the site of coupling with energy). In its absence or malfunction, the unidirectional character of this conversion is lost and the amino acid can cross the membrane both ways, using a non-energized carrier form.

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