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KINETICS OF L-[^{14}C]LEUCINE TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

EFFECT OF ENERGY COUPLING INHIBITORS *

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Summary

1. L-[^{14}C]Leucine transport into *Saccharomyces cerevisiae* involves a high-affinity, low-velocity system (system 1) and a low-affinity, high-velocity system (system 2). These systems are characterized by the different values of the kinetic parameters K_T and J_{max}, and are both capable of concentrative transport. The general amino acid permease is assumed to be a part of the high-affinity system.

2. The kinetics of L-[^{14}C]leucine entrance show an initial rapid phase (the 'very early uptake') before reaching the steady-state rate. The contribution of the very early uptake to total entrance values affects the values of K_T and J_{max}, especially when the steady-state rate is relatively slow, as with starved yeast, and then negative K_T and J_{max} values may result. The very early uptake is increased by pretreatment of starved yeast with D-glucose, this latter effect being counteracted by iodoacetate.

3. After energization of starved yeast by pretreatment with D-glucose or propionaldehyde, the apparent $K_{T,2}$ value greatly decreases whilst the $K_{T,1}$ value decreases to a much more limited extent, or does not vary. With the energized yeast, $K_{T,2}$ decreases throughout incubation whilst $K_{T,1}$ variation is insignificant. Energization increases $J_{max,1}$ and $J_{max,2}$ several-fold and with the energized yeast at the steady-state phase, $J_{max,2} \geqslant 4J_{max,1}$. Variation of K_T and J_{max} values as a function of the metabolic state of yeast cells may be explained

* Paper III in the series: Amino acid uptake by yeasts.
Abbreviations: DCCD, dicyclohexylcarbodiimide; CCCP, carbonylcyanide 3-chlorophenylhydrazone; FCCP, carbonylcyanide 4-trifluoromethoxyphenylhydrazone.

in terms of variation of rate constants k_{-1}, k_{+1} and k_{+2} for each transport system.

4. Dicyclohexylcarbodiimide, quercetin and diethylstilbestrol inhibit transport at 0.05 mM L-[^{14}C]leucine, in good agreement with a function of the plasmalemma ATPase for the operation of system 1. Dio-9, propionic and isobutyric acids, pentachlorophenol, carbonylcyanide 3-chlorophenylhydrazone and carbonylcyanide 4-trifluoromethoxyphenylhydrazone, which affect the proton gradient and/or the membrane potential inhibit L-[^{14}C]leucine uptake at all the assayed amino acid concentrations.

5. The polyene antibiotic, nystatin, which forms channels in membranes permeable to K^+ and H^+, inhibits systems 1 and 2 activity but enniatin (also a K^+ ionophore) does not.

Introduction

The occurrence of multiple transport components for the accumulation of amino acids has been observed in yeast and fungi [1—16]. Ramos and coworkers [10,11] described in *Saccharomyces ellipsoideus* two kinetic systems for transport of L-[^{14}C]leucine, one with high affinity and low transport activity and the other with low affinity and high transport activity. The kinetic parameters, K_T and V, of these systems were dependent on the energization state of the yeast cells and it was suggested that the high-affinity system involves the general amino acid 'permease' [10,11] *.

It is generally accepted [16] that the uptake of amino acids by yeasts may be energized by the influx of protons across the plasmalemma. The resulting influx of positive charge is neutralized by ejection of protons from the cell interior, protons being expelled by the proton pump, in which the plasmalemma ATPase [17—19] may play an essential role [16,20]. There is evidence in *Neurospora* [16] that the plasmalemma ATPase is involved in amino acid transport and protonophores inhibit amino acid absorption by yeasts [10—12,16, 21—23], an inhibition that fits in well with the idea of the essential function played by the proton gradient in transport processes. However, coupling of metabolic energy and transport across the cell membrane is less documented in yeasts than in other microorganisms, and the biological mechanisms for the generation of an electrochemical potential across the yeasts' plasmalemma are still unknown [16].

In order to extend our previous observations on energy coupling in amino acid transport in *Saccharomyces* [10,11,21,22], in the present study with *Saccharomyces cerevisiae*, the kinetics of L-[^{14}C]leucine transport have been examined with regard to (a) variation of intracellular levels of metabolic energy and proton concentration, (b) the effect of inhibitors of the plasmalemma ATPase, (c) the effect of proton conductors, and (d) the effect of ionic channel formation in the cell membrane.

* In paper II of this series, L-[^{14}C]leucine transport systems were termed systems A and B. In order to avoid confusion with secondary kinetic parameters those systems are now termed system 1 and system 2.

Materials and Methods

The yeasts employed were *S. cerevisiae*, diploid, strain 207 wild type; the cytoplasmic *rho*$^-$ mutant, 'petite colonie', and *S. cerevisiae* var. *ellipsoideus*, strain 208, wild type. 'Starved' yeast means that before incubation the yeast was suspended in distilled water and aspirated with air for 18—20 h at 20—25°C under sterile conditions. 'Energized' yeast means starved yeast pretreated with 5 mM D-glucose or 5 mM propionaldehyde, in 20 mM phthalate buffer (pH 4.5), for 10—15 min before L-[^{14}C]leucine addition. Characteristic features of yeasts and culture conditions were as described earlier [10,11,22,24].

Diethylstilbestrol, iodoacetic acid and quercetin (Sigma Chemical Company, St. Louis, MO), DCCD (Koch and Light Co., Colnbrook, U.K.), pentachlorophenol (K and K Laboratories, Plainview, NY), Dio-9 (Gist-Brocades N.V., Delft, The Netherlands), propionic acid (Baker Analyzed Reagent) and dimethylformamide (E. Merck, Darmstadt) were purchased from the sources indicated in parentheses. Enniatin was supplied by Dr. Yu. A. Ovchinnikov (U.S.S.R. Academy of Sciences, Moscow, U.S.S.R.) and nystatin, by E.R. Squibb and Sons (Argentina). CCCP and FCCP were supplied by Dr. P.G. Heytler (E. Dupont de Nemours, Wilmington, DE). Isobutyric acid was a redistilled sample (b.p. 153—155°C). Other reagents were as described earlier [10, 11,22,24].

Incubation techniques and assay of radioactive samples. The kinetics of L-[^{14}C]leucine uptake were carried out at 30°C, in a New Brunswick Gyratory Water Shaker (Model T-76). The incubation mixture (2—5 ml) contained yeast (2 mg/ml), 20 mM potassium phthalate buffer (pH 4.5) and additions as specified in each case (standard experimental conditions). Inhibitors were added in the solvent indicated in each case. Control samples were added to the corresponding volume of pure solvent. Other experimental conditions were as described in Refs. 10, 11, 22 and 24.

Analytical methods. Assay of radioactive samples was performed after filtration of incubation mixtures through a Millipore filter 25 ea, HA 0.45 μm (method A in Ref. 22). Intracellular amino acid concentration was determined using a Technicon Amino acid Analyzer (type I) as described by Spackman et al. [25]. Other experimental conditions were as described earlier [10,11,22, 24].

Expression of results. Unless otherwise stated, uptake of L-[^{14}C]leucine by yeast is expressed as μmol/g cells (dry weight). The concentration of yeast suspensions is expressed by the weight after drying at 104°C for 24 h. The water content of the cells is assumed to be 60% of the wet weight [2].

Results

Kinetics of L-[^{14}C]leucine uptake

Knowledge of very early events is essential for understanding the kinetics of L-[^{14}C]leucine uptake. Fig. 1 shows a typical representation of L-[^{14}C]leucine entrance into starved *S. cerevisiae* as a function of incubation time and amino acid concentration. It is to be seen that at short incubation periods the kinetics involves two phases which will be termed the 'very early' and the 'steady-state'

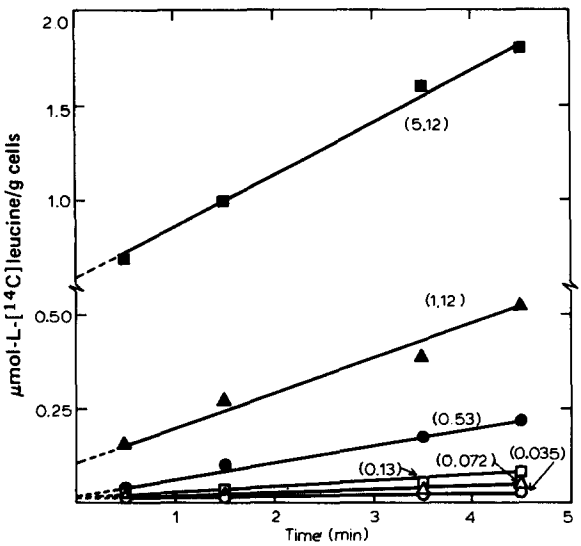

Fig. 1. Kinetics of L-[^{14}C]leucine entrance into starved yeast. Cells were suspended in 20 mM phthalate buffer (pH 4.5) containing L-[^{24}C]leucine at concentrations (mM) as indicated by the figures in parentheses. At the times indicated on the abscissa, samples were taken and internal radioactivities were measured. Other conditions as in Materials and Methods.

phase, respectively. Extrapolation of straight lines to zero time (T_0) allows one to calculate the very early uptake value. These values increased with the amino acid concentration and with 5.1 mM L-[^{14}C]leucine, the very early uptake represented 45% of total L-[^{14}C]leucine entrance after 4.5 min incubation (Fig. 1). Table I shows that the very early uptake is an energy-dependent process. In fact, preincubation with D-glucose significantly enhanced L-[^{14}C]leucine entrance in the 0—10 s incubation period, whilst addition of iodoacetate to the preincubation mixture prevented the effect of D-glucose.

TABLE I

ENERGY REQUIREMENT FOR THE VERY EARLY UPTAKE OF L-[^{14}C]LEUCINE BY *S. CEREVISIAE*

Starved cells were suspended in 20 mM phthalate buffer (pH 4.5). 1.0 mM L-[^{14}C]leucine was added at T_0; 5 mM D-glucose and 1 mM iodoacetate were added at T_0 — 15 min. With 'blank' samples, the yeast suspension was filtered through a Millipore filter, L-[^{14}C]leucine solution was layered on top of the cake and sucked through immediately. The yeast was then washed with 20 mM phthalate buffer (pH 4.5) and L-[^{14}C]leucine entrance was measured. Other conditions as in Materials and Methods.

Yeast	Additions	L-[^{14}C]Leucine uptake (μmol/g cells)			
		Blank	T_0	T_0 + 5 s	T_0 + 10 s
Wild type	D-glucose	3.50	3.42	3.59	—
	none	0.31	0.48	0.54	—
Rho$^-$ mutant	D-glucose	1.79	1.71	—	2.20
	D-glucose + iodoacetate	0.98	1.16	—	1.10
	none	0.99	0.97	—	0.72

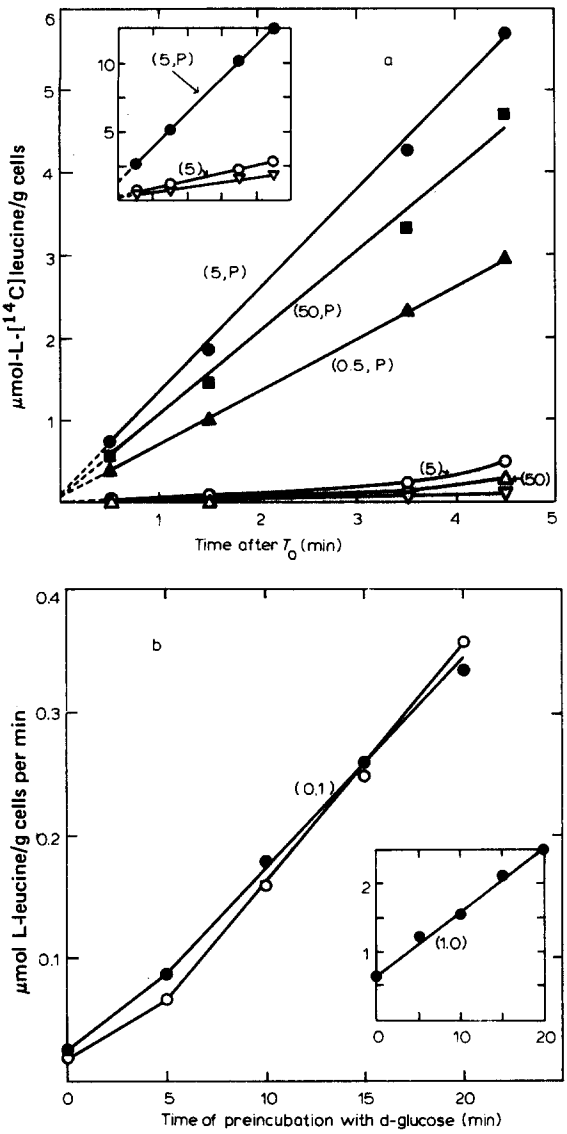

Fig. 2. (a) Effect of preincubation with D-glucose on L-[^{14}C]leucine entrance. Starved cells were suspended in 20 mM phthalate buffer (pH 4.5) and 0.1 mM L-[^{14}C]leucine was added at T_0. ▽, control sample; P, cells preincubated with D-glucose for 10 min before T_0 (●,■,▲) otherwise, D-glucose was added at T_0 (○,△); D-glucose concentration is indicated by the figures in parentheses (mM). At the times indicated on the abscissa, samples were taken and internal radioactivities were measured. Inset: same conditions, except for 1.0 mM L-[^{14}C]leucine. (b) Effect of time of preincubation with D-glucose on the rate of L-[^{14}C]leucine entrance. Cells were preincubated with 5.0 mM D-glucose for the time indicated on the abscissa and then L-[^{14}C]leucine was added (at T_0), at the concentrations indicated by the figures in parentheses. ● and ○, samples incubated with L-[^{14}C]leucine for 5 or 1.5 min after T_0, respectively. Other conditions as in Materials and Methods.

In order to establish optimal conditions for kinetic studies, the effect of the energy load of the yeast cells on the rate of L-[^{14}C]leucine entrance was examined. Fig. 2a shows that preincubation of yeast with D-glucose increased

L-[^{14}C]leucine entrance rate as a function of D-glucose concentration. With the energized yeast, the kinetics of L-[^{14}C]leucine entrance were linear and the contribution of the very early uptake to total uptake was relatively small. Since displacement of intracellular L-[^{14}C]leucine was negligible [10,11,22], it may be assumed that entrance values measured the amino acid influx across the yeast cell plasmalemma. It can be seen from Fig. 2a that 0.5 and 50 mM D-glucose activated uptake less than 5 mM D-glucose and accordingly, this latter concentration was subsequently used to energize yeasts when D-glucose was the energy source. Similar results were obtained with 1.0 mM L-[^{14}C]leucine, although at this concentration the very early uptake effect was greater than at 0.1 mM L-[^{14}C]leucine (Fig. 2a, inset). Fig. 2b shows that the rate of L-[^{14}C]leucine uptake increased lineally as a function of time of preincubation with D-glucose, in accordance with the metabolic nature of the D-glucose effect.

Characterization of L-[^{14}C]leucine transport systems

The uptake kinetics for L-[^{14}C]leucine transport show a deviation from simple Michaelis-Menten kinetics. Since saturation isotherms were similar to those previously reported with *S. cerevisiae* [22] and *S. ellipsoideus* [10,11], the primary data are omitted. Fig. 3 shows the Hofstee [26] plot for entrance values obtained with (a) the wild-type yeast, energized by pretreatment with propionaldehyde [10,11,22], and (b) the *rho*$^-$ mutant energized by pretreat-

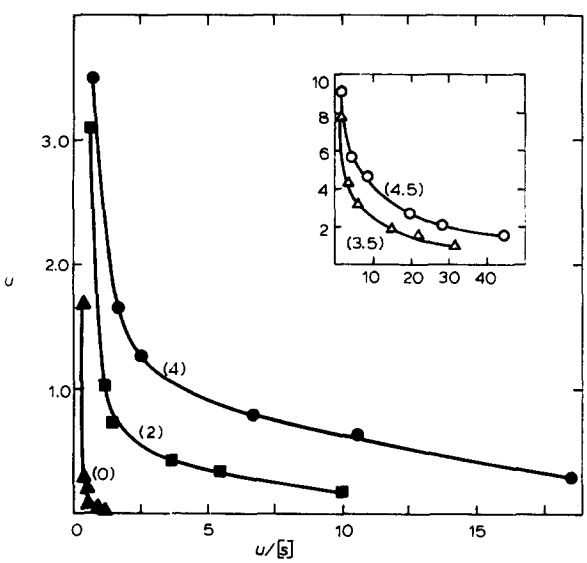

Fig. 3. Effect of L-[^{14}C]leucine concentration on the amino acid uptake (u). Starved cells (wild-type) were suspended in 20 mM phthalate buffer (pH 4.5) containing 5.0 mM propionaldehyde. After 15 min incubation, L-[^{14}C]leucine was added (at T_0) at concentrations corresponding to the values stated on the abscissa. After T_0, samples were taken at the times (min) indicated by the figures in parentheses and internal radioactivities were measured. Other conditions were as described under Materials and Methods. Hofstee representation [26] of entrance values. Inset: same conditions, except for yeast which was the *rho*$^-$ mutant energized by 15 min pretreatment with 5.0 mM D-glucose. u, μmol L-[^{14}C]leucine/g cells; s, L-[^{14}C]leucine (mM).

ment with D-glucose (inset). With the former yeast, the stimulation of L-[^{14}C]-leucine uptake involved the utilization of energy rendered available by the oxidation of reduced pyridine nucleotides in the yeast mitochondrion, whilst with the *rho*$^-$ mutant, energy was made available by glycolysis [10,11,22]. Entrance rates are represented by total uptake values (u) at fixed times of incubation. The concave-upwards curves reveal the existence of two kinetic systems, irrespective of the substrate used to energize L-[^{14}C]leucine transport. These kinetic systems, which henceforth will be termed system 1 and system 2 are characterized by parameters J_{max} and K_T. J_{max} expresses the maximum flux that the yeast cells can exhibit towards the amino acid and K_T formally expresses the substrate concentration at which the flux is one half the limited flux J_{max}. K_T and J_{max} are often represented as K_m and V in transport studies. The rate equations for each system then become:

$$v = u/\Delta t = \frac{J_{max} \cdot s}{K_T + s} \tag{1}$$

or

$$v = u/\Delta t = J_{max} - K_T \frac{v}{s} \tag{2}$$

where v is the entrance velocity, u the L-[^{14}C]leucine uptake at incubation time Δt, s the L-[^{14}C]leucine concentration and K_T and J_{max} are the kinetic parameters defined above. Eqn. 1 can be rearranged to the Lineweaver-Burk form, which can be used for data analysis.

J_{max} and K_T values were computed from sets of influx data, using the Lineweaver-Burk form of Eqn. 1, or the graphical method of Hofstee [26]. L-[^{14}C]-Leucine concentrations were in the range 0.010—5.5 mM. Fig. 4 show the variation of K_T and J_{max} as functions of incubation time and the metabolic state of yeast cells. K_T values are represented by their reciprocals, which may be considered as indicative of the amino acid affinity for the transport system. In order to calculate J_{max} values for the initial samples, the time of processing of these samples was taken as 6 s. It is worth recalling that the results presented in Fig. 4 were obtained with the same strain of yeast after a 4 year interval, a circumstance that stresses the constancy of $K_{T,1}$ and $K_{T,2}$ displaying different behaviour. Concerning J_{max} values, a remarkably rapid initial decay was observed with the energized yeast and a similar, though much smaller, variation was observed with the starved yeast. That decay was maximal when high external concentrations of L-[^{14}C]leucine were used, which points to the assumption that the very early uptake effect was largely responsible for the time-dependent variations of J_{max} and $K_{T,2}$.

Table II shows representative values for K_T and J_{max}, calculated by the procedure of Hofstee [26] (results obtained with the Lineweaver-Burk procedure are included for comparative purposes). It can be seen that: (a) with the energized yeast, $K_{T,1}$ was more than one order of magnitude smaller than $K_{T,2}$; (b) $K_{T,1}$ for the starved yeast was approximately twice $K_{T,1}$ for the energized yeast but in other experiments [25] the variation was not significant; (c) $K_{T,2}$ for the energized yeast was smaller than $K_{T,2}$ for the starved yeast when the very early uptake effect was accounted for; (d) with the energized yeasts, $J_{max,1}$ values were 20—30% of the corresponding $J_{max,2}$ values; (e) with the wild-type

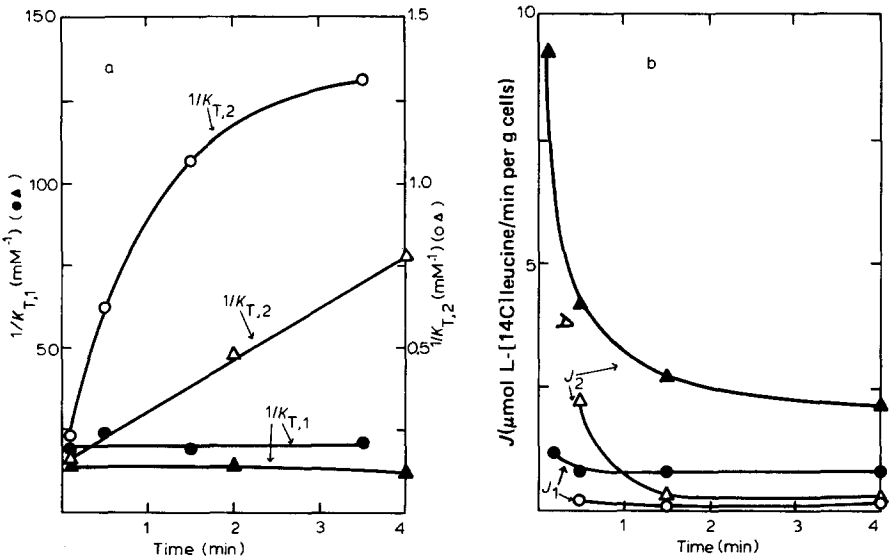

Fig. 4. Influence of incubation time and metabolic load of yeast on kinetic parameters K_T (a) and J_{max} (b). Wild-type yeast energized with 5 mM D-glucose, for 15 min before T_0. L-[^{14}C]Leucine was added at T_0 and the time of incubation after T_0 was as stated on the abscissa. L-[^{14}C]Leucine concentrations were as in Fig. 3. Other conditions were as described in the text. (a) Results with two different samples of energized yeast (○,● and △,▲). (b) Results with the same samples of yeast; ▲ and △, energized, and ● and ○, starved.

yeast, energization determined a 30- or 5-fold increase of the $J_{max,1}$ or $J_{max,2}$ values, respectively; (f) with the *rho*$^-$ mutant, the increase in J_{max} values resulting from energization was much higher than with the wild-type yeast, since L-[^{14}C]leucine uptake by the starved mutant (not shown) was insignificant [10,

TABLE II

KINETIC PARAMETERS OF L-[^{14}C]LEUCINE TRANSPORT IN ENERGIZED AND STARVED *S. CEREVISIAE*

Yeast was energized by pretreatment with 5 mM D-glucose for 15 min. Exp. A was performed 4 years before B (same strain of yeast). Experimental conditions were as described in Figs. 3 and 4 and in the text. r_1 and r_2 are correlation coefficients.

Experiment	Yeast	Time of incubation (min)	r_1	$K_{T,1}$ (mM)	$J_{max,1}$ (μmol/min/g cells)	r_2	$K_{T,2}$ (mM)	$J_{max,2}$ (μmol/min/g cells)
A	wild-type	3.5	1.00	0.055	0.79	1.00	0.92	2.34
	(energized)		1.00 *	0.047	0.75	0.99	0.76	2.18
		4.5—3.5	0.86	0.030	0.63	0.96	0.33	1.64
	wild-type	3.5	0.99	0.140	0.03	1.00	—12.7	—1.49
		4.5—3.5	0.77	0.058	0.02	0.93	4.47	0.26
B	wild-type	2	1.00	0.067	1.24	0.97	3.78	12.20
	(energized)	4	1.00	0.081	1.23	0.99	2.17	7.13
	Rho$^-$ mutant	2	0.99	0.074	1.36	0.93	4.84	10.0
	(energized)	4	0.97	0.046	0.96	0.98	3.04	11.3

* Calculated with the Lineweaver-Burk form of Eqn. 1; for other calculations, the procedure of Hofstee [26] was used.

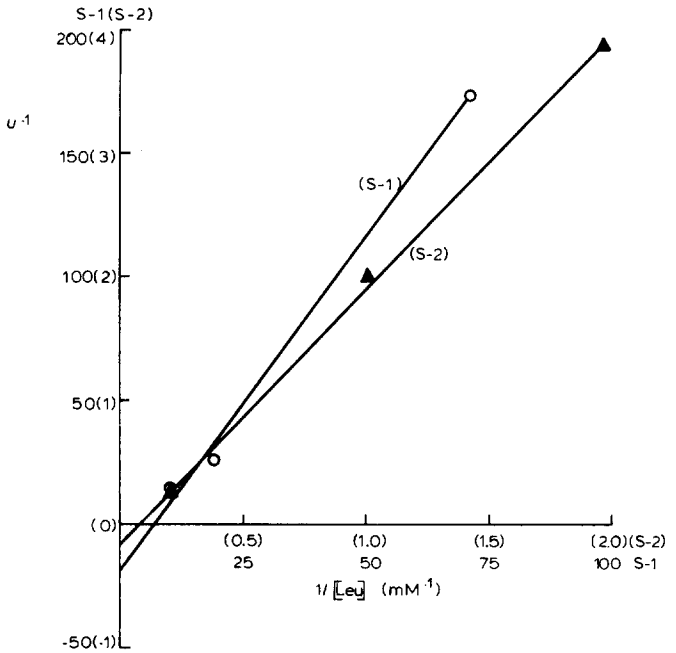

Fig. 5. Kinetics of L-[^{14}C]leucine transport in starved yeast (very early uptake). L-[^{14}C]Leucine was added at T_0 at concentrations corresponding to the values states on the abscissa; samples were taken during the 0—5 s incubation time. Other conditions as in Materials and Methods. S-1, system 1; S-2, system 2.

11,22]; (g) negative J_{max} values were observed with starved yeast under conditions in which the very early uptake contributed significantly to total uptake. The very early uptake was the cause of negative kinetic parameters, since, after subtracting the corresponding value from total entrance, positive J_{max} (and K_T) values were obtained (Table II). The occurrence of a negative intercept on the ordinate axis is illustrated in Fig. 5, which depicts typical uptake kinetics for starved yeast at the T_0 + 6 s time of incubation. Summing up, in good agreement with the our observations on *S. ellipsoideus* [10,11], system 1 may be defined as a high-affinity, low-velocity transport system whilst system 2 is a low-affinity, high-velocity system. With system 2, energization of yeast cells affected both transport velocity (J_{max}) and affinity (K_T), whilst with system 1, energization apparently affected solely velocity. In terms employed by Alvarado and Mahmood [27], energization caused an essentially *V* effect on system 1, and a mixed (*V* plus *K*) effect on system 2. Similar results were obtained with propionaldehyde-energized yeast although J_{max} values were somewhat smaller than with D-glucose as source of energy. For example, at T_0 + 4 min, the values obtained were: $K_{T,1}$, 0.045; $J_{max,1}$, 0.28; $K_{T,2}$, 1.03; $J_{max,2}$, 1.03 (units as in Table II, primary data omitted).

Assuming that L-[^{14}C]leucine transport involves two simultaneously occurring processes, the rate equation then becomes [28]:

$$v = \frac{J_{max,1} \cdot s}{K_{T,1} + s} + \frac{J_{max,2} \cdot s}{K_{T,2} + s} = \frac{A \cdot s + B \cdot s^2}{C + D \cdot s + s^2} \quad (3)$$

TABLE III

SECONDARY COEFFICIENTS *A—D* FOR ENERGIZED AND STARVED *S. CEREVISIAE*

Primary parameters were taken from Table II (Expt. A, wild-type yeast). The figures are the average of two typical experiments. *A*, mM · μmol · min^{-1} · g^{-1}; *B*, μmol · min^{-1} · g^{-1}; *C*, mM^2; *D*, mM.

Yeast	Incubation time (min)	*A*	*B*	*C*	*D*	$(A/B + BC/A)$	$4C$	D^2
Energized	4.5—3.5	0.70	2.27	0.01	0.36	0.34	0.04	0.13
Starved	4.5—3.5	0.58	0.28	0.26	4.53	2.20	1.04	20.5

where v is the total entrance rate, s is L-[^{14}C]leucine concentration, $K_{T,1}$, $K_{T,2}$, $J_{max,1}$ and $J_{max,2}$ are primary kinetic parameters, and coefficients A—D are secondary kinetic parameters defined as follows: $A = K_{T,1}J_{max,2} + K_{T,2}J_{max,1}$; $B = J_{max,1} + J_{max,2}$; $C = K_{T,1}K_{T,2}$ and $D = K_{T,1} + K_{T,2}$. Table III shows representative values for coefficients A—D, as obtained with the wild-type yeast. It can be seen that coefficients A—D were affected by the metabolic state of the yeast cells, particularly C and D, which after energization decreased by more than one order of magnitude. In all cases $A/B + BC/A < D$ and $4C < D^2$, which is in accordance with the operation of a dual-transport mechanism [28,29]. Similar results were obtained with the *rho*$^-$ mutant (experimental data omitted).

Eqn. 3 allows calculation of the uptake rate as a function of the amino acid concentration and resulting values may be compared with the experimental

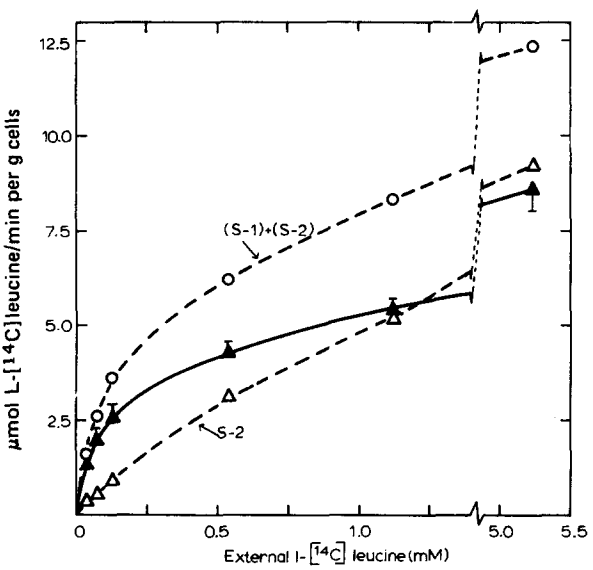

Fig. 6. Comparison of total uptake values (▲) with computed activities for the (S-1) + (S-2) (○) and S-2 (△) systems, at several L-[^{14}C]leucine concentrations. S-1 + S-2 activity was calculated with Eqn. 3 and S-2 activity was calculated with Eqn. 1. The K_T and J_{max} values used for computation of uptake values were: $K_{T,1}$, 0.043; $J_{max,1}$, 0.74; $K_{T,2}$, 1.43; $J_{max,2}$, 2.58 (units as in Table II; 4.5 min incubation). The bars on the experimental points indicate the deviation of experimental values with respect to the mean (duplicate measurements). S-1, system 1; S-2, system 2.

ones. Fig. 6 shows that within the 0—0.1 mM L-[^{14}C]leucine concentration range, the measured uptake values approached those calculated from Eqn. 3. However, at the higher L-[^{14}C]leucine concentrations, the observed values approached those calculated from Eqn. 1 for system 2. Experimental data in Fig. 6 allow one to calculate intracellular L-[^{14}C]leucine concentration with respect to cell water (60% of wet weight [2]). If uptake values are converted into concentration values (mmol/l cell water; mM) and the endogenous L-leucine concentration (approx. 6.0 mM) is summed, the total intracellular L-[^{14}C]-leucine concentration ($[Leu]_{in}$) at each external L-[^{14}C]leucine concentration ($[Leu]_{out}$) is obtained. The data in Fig. 6 lead to the conclusion that the $[Leu]_{in}/[Leu]_{out}$ ratio was always above unity, thus proving the concentrative nature of L-[^{14}C]leucine transport, whatever the kinetic system involved may be.

Effect of inhibitors of plasmalemma ATPase

DCCD and quercetin inhibit the solubilized yeast plasmalemma ATPase [17,18] and DCCD, quercetin and diethylstilbestrol inhibit *Neurospora* plasmalemma ATPase [19]. In order to establish whether these inhibitors affect L-[^{14}C]leucine transport, they were assayed as described in Table IV. The *rho*$^-$ mutant was used for these experiments since this yeast lacks an active mitochondrial ATPase [30] the inhibition of which would complicate the interpretation of results. The yeast was preincubated with D-glucose for 15 min before T_0, to energize transport, and inhibitors were added 5 min before T_0

TABLE IV

EFFECT OF INHIBITORS OF PLASMALEMMA ATPase ON L-[^{14}C]LEUCINE UPTAKE BY *S. CEREVISIAE* AND *S. ELLIPSOIDEUS* (*Rho*$^-$MUTANTS)

Starved cells were suspended in 20 mM phthalate buffer (pH 4.5). L-[^{14}C]Leucine, 5 mM D-glucose and inhibitors were added at T_0, $T_0 - 15$ and $T_0 - 5$ min, respectively. DCCD, quercetin and diethylstilbestrol were added in dimethylformamide (60 μl/ml). Values in parentheses indicate percentage inhibition of L-[^{14}C]leucine entrance. Where not indicated, the difference from the control value is not deemed significant. Other conditions as in Materials and Methods.

Experiment	Yeast	External L-[^{14}C]-leucine (mM)	Inhibitor (μM)	Entrance of L-[^{14}C]leucine (μmol/g cells)		
				T_0	T_0 + 2 min	T_0 + 4 min
A	*S. cerevisiae*	0.05	none	0.10	0.65	1.22
			DCCD (100)	0.06	0.45 (31)	0.72 (41)
		1.00	none	0.60	3.50	6.03
			DCCD (100)	0.60	3.50	6.73 (—12)
B	*S. cerevisiae*	0.05	none	—	0.66	1.14
			quercetin (500)	—	0.47 (29)	0.76 (33)
			diethylstilbestrol (50)	—	0.49 (26)	0.73 (36)
		1.00	none	—	3.64	5.26
			quercetin (500)	—	3.68	5.62 (—7)
			diethylstilbestrol (50)	—	3.17 (13)	4.66 (12)
C	*S. ellipsoideus*	0.05	none	0.12	0.83	1.66
			DCCD (200)	0.11	0.38 (54)	1.28 (23)
		1.00	none	0.75	3.73	6.99
			DCCD (200)	0.83	4.22 (—13)	8.37 (—20)

TABLE V

EFFECT OF DIO-9 ON L-[^{14}C]LEUCINE UPTAKE BY *S. CEREVISIAE* (*Rho*$^-$MUTANT)

L-[^{14}C]Leucine, 5 mM D-glucose and Dio-9 were added at T_0, $T_0 - 15$ and $T_0 - 5$ min. Dio-9 was added in ethanol (13 μl/ml). Other conditions were as in Table IV. From entrance values for T_0 + 2 min were subtracted the corresponding T_0 values.

External L-[^{14}C]-leucine (mM)	Dio-9 (μg/ml)	Entrance of L-[^{14}C]leucine (μmol/g cells)	
		T_0	T_0 + 2 min
0.05	none	0.13	0.91
	2.5	0.11 (14)	0.70 (23)
	10	0.05 (65)	0.28 (69)
	50	0.03 (74)	0.15 (83)
1.00	none	0.87	4.51
	2.5	0.76 (10)	4.38
	10	0.66 (21)	2.55 (43)
	50	0.57 (32)	1.15 (74)

in order to ensure enough time for penetration and reaction with energy coupling mechanisms. The effects observed are presented in Table VI and deserve the following comments: (a) DCCD inhibited uptake with 0.05 mM L-[^{14}C]leucine but did not inhibit, or even increased it, with 1.0 mM L-[^{14}C]-leucine; (b) quercetin inhibited uptake only with 0.05 mM L-[^{14}C]leucine; (c) diethylstilbestrol inhibited at both 0.05 and 1.0 mM L-[^{14}C]leucine, this latter inhibition being, however, smaller than with 0.05 mM L-[^{14}C]leucine. DCCD was also assayed with *S. ellipsoideus* (Table IV) the inhibitions being similar to those observed with *S. cerevisiae*.

As opposed to the effect of DCCD and quercetin, Dio-9, which is also a plasmalemma ATPase inhibitor [18,19,31,32], prevented uptake at both 0.05 mM and 1.0 mM external L-[^{14}C]leucine (Table V). Dio-9 inhibitions are in good agreement with observations by Foury et al. [31] on *Schizosaccharomyces pombe* and L-[^{14}C]leucine, and Roon et al. [32] on *S. cerevisiae* and amino acids other than L-[^{14}C]leucine.

Effect of carboxylic acids

Short-chain carboxylic acids are known to affect transport processes in yeasts, mainly through acidification of cells caused by proton translocation [33]. Propionic and isobutyric acids were therefore assayed on L-[^{14}C]leucine transport using the *rho*$^-$ mutant. Table VI shows that both acids inhibited L-[^{14}C]leucine entrance, the greater inhibition being obtained with 1.0 mM L-[^{14}C]leucine. Since carboxylic acids decrease the cellular pH without affecting glycolysis [33] or the concentration of ATP [34], the inhibition of L-[^{14}C]-leucine uptake can be attributed to modification of the intracellular H^+ concentration.

Effect of proton conductors

Pentachlorophenol, CCCP and FCCP inhibited L-[^{14}C]leucine uptake by the *rho*$^-$ mutant irrespective of amino acid concentration (Table VII). Interestingly

TABLE VI

CARBOXYLIC ACID INHIBITION OF L-[^{14}C]LEUCINE UPTAKE BY *S. CEREVISIAE* (*Rho*$^-$ MUTANT)

L-[^{14}C]Leucine, 5 mM D-glucose and carboxylic acids were added at T_0, $T_0 - 15$ and $T_0 - 5$ min, respectively. Carboxylic acid solutions were adjusted to pH 4.5. Other conditions were as described in Table IV. From entrance values for T_0 + 4 min were subtracted the corresponding T_0 values.

External L-[^{14}C]-leucine (mM)	Carboxylic acid (10 mM)	Entrance of L-[^{14}C]leucine (μmol/g cells)	
		T_0	T_0 + 4 min
0.05	none	0.13	1.97
	propionic	0.12	1.49 (24)
	isobutyric	0.11	1.63 (17)
1.00	none	0.75	9.01
	propionic	0.55 (26)	4.78 (46)
	isobutyric	0.53 (29)	6.11 (32)

TABLE VII

EFFECT OF PROTON CONDUCTORS ON L-[^{14}C]LEUCINE UPTAKE BY *S. CEREVISIAE* (*Rho*$^-$MUTANT)

L-[^{14}C]Leucine, 5 mM D-glucose and proton conductors were added at T_0, $T_0 - 10$ and $T_0 - 1$ min, respectively. Other conditions were as described in Table IV. From entrance values for T_0 + 4 min were subtracted the corresponding T_0 values. PCP, pentachlorophenol.

External L-[^{14}C]-leucine (mM)	Proton conductor (μM)	Entrance of L-[^{14}C]leucine (μmol/g cells)	
		T_0	T_0 + 4 min
0.05	none	0.26	2.29
	PCP (50)	0.18 (28)	0.50 (78)
	CCCP (10)	0.24 (5)	1.52 (34)
	FCCP (10)	0.28	1.77 (23)
1.00	none	1.43	9.57
	PCP (50)	1.00 (30)	1.04 (89)
	CCCP (10)	1.31 (8)	4.74 (50)
	FCCP (10)	1.19 (17)	5.42 (43)
5.00	none	2.56	7.64
	PCP (50)	1.57 (38)	2.73 (64)

TABLE VIII

EFFECT OF PENTACHLOROPHENOL ON KINETIC PARAMETERS OF L-[^{14}C]LEUCINE TRANSPORT

Experimental conditions were as in Table VII and Fig. 3. Values in parentheses indicate percentage inhibition of L-[^{14}C]leucine entrance.

Pentachlorophenol (μM)	$K_{T,1}$ (mM)	$J_{max,1}$ (μmol/min per g cells)	$K_{T,2}$ (mM)	$J_{max,2}$ (μmol/min per g cells)
0	0.068	0.55	1.58	3.39
25	0.052	0.43 (22)	1.00	2.54 (25)

TABLE IX

EFFECT OF NYSTATIN AND ENNIATIN ON L-[^{14}C]LEUCINE UPTAKE BY *S. CEREVISIAE* (*Rho*$^-$ MUTANT)

L-[^{14}C]Leucine, 5 mM D-glucose and inhibitors were added at T_0, $T_0 - 10$ and $T_0 - 5$ min, respectively. Inhibitors were added in dimethylformamide (13 μl/ml). Other conditions were as described in Table IV. From entrance values for T_0 + 4 min were subtracted the corresponding T_0 values.

External L-[^{14}C]-leucine (mM)	Inhibitor (μg/ml)	Entrance of L-[^{14}C]leucine (μmol/g cells)	
		T_0	T_0 + 4 min
0.05	none	0.12	1.47
	nystatin (5)	0.08 (54)	0.43 (71)
	nystatin (10)	0.01 (91)	0.10 (93)
	enniatin (5)	0.14 (—16)	1.90 (—29)
	enniatin (10)	0.12	1.61 (—9)
1.00	none	0.93	8.72
	nystatin (5)	0.75 (19)	2.03 (76)
	nystatin (10)	0.24 (74)	0.86 (90)
	enniatin (5)	1.16 (—24)	8.59
	enniatin (10)	1.03 (—10)	7.51 (13)

enough, with CCCP and FCCP the very early uptake was less inhibited than total uptake at T_0 + 4 min. When assayed on the kinetic parameters for L-[^{14}C]-transport no evidence for competitive inhibition of transport could be obtained since pentachlorophenol decreased J_{max} but did not increase K_T (Table VIII). The reported decrease in K_T is not significant since with other yeast samples these values were not affected by pentachlorophenol or other uncouplers (experimental data omitted).

Effect of nystatin and enniatin

Nystatin, a polyene antibiotic, in a complex with sterol, forms channels in a lipid membrane permeable to ions, in particular to K^+ and H^+ [35—37]. Table IX shows that nystatin decreased L-[^{14}C]leucine entrance irrespective of the amino acid concentration. As opposed to these results, enniatin, a cyclodepsipeptide which also increases K^+ permeability of biological membranes [38], did not inhibit, or rather increased uptake (Table IX). The different behaviour of nystatin and enniatin suggests that the mode of interaction with the cell membrane is essential for these antibiotics' effect.

Discussion

In good agreement with previous observations by Ramos and coworkers [10,11] on *S. ellipsoideus*, L-[^{14}C]leucine transport in *S. cerevisiae* is formally in accordance with the operation of two kinetic systems. The interaction of L-[^{14}C]leucine with each system may be represented in a simple form by reaction 4.

$$S_e + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} (SC) \xrightarrow{k_{+2}} S_i + C \qquad (4)$$

where S_e and S_i are the permeant L-[^{14}C]leucine at the *cis* (external) and *trans* (internal) surfaces of the plasmalemma, respectively; C is the unloaded carrier (permease); SC is the amino acid-carrier complex; k_{+1} and k_{-1} are association and dissociation rate constants, respectively, and k_{+2} may be regarded as representing the activity of the energizing mechanisms.

The kinetic parameters of amino acid transport systems of yeast and fungi have been repeatedly measured in order to characterize such systems and, particularly, the corresponding permeases [1—5,8,13—16]. These determinations were performed on the assumption that K_T values are essentially binding constants, equivalent to K_s in enzyme kinetics. However, K_T values for L-leucine transport systems in *S. ellipsoideus* [10,11] and *S. cerevisiae* (Table II and Ref. 25) vary significantly as a function of the metabolic state of the yeast cells, especially $K_{T,2}$. Variation of K_T values can be explained in terms of the corresponding rate constants k_{+1}, k_{-1} and k_{+2}, since according to elementary Michaelis-Menten kinetics, for each transport system:

$$K_T = (k_{-1} + k_{+2})/k_{+1} \tag{5}$$

Therefore, the decrease in $K_{T,2}$ as a consequence of cell energization (Table II) may be due to the increase in k_{+1}, a condition leading to a higher concentration of the L-leucine-carrier complex in the cell membrane. Incidentally, a similar variation of $K_{T,2}$ was reported with *S. ellipsoideus* [10,11]. On the other hand, $K_{T,1}$ variations are either relatively small (Table II) or quite insignificant [24], which leads one to believe that the increase in k_{+2} that follows energization of system 1 is compensated by a decrease in k_{-1} and/or an increase in k_{+1}. The constant, k_{-1}, represents the rate of decomposition of the SC complex towards the *cis* side of the membrane and k_{+1} is the rate of formation of the SC complex (Eqn. 4). Accordingly, a simultaneous variation of rate constants can increase the velocity of L-[^{14}C]leucine influx without significant modification of $K_{T,1}$. An exhaustive explanation of the dependence of kinetic parameters on metabolism must take into account the fact that the availability of the carrier on the medium side of the cell plasmalemma can be affected by the concentration of internal and external ions, particularly, proton concentration. Consequently, specific information concerning the dependence of kinetic parameters on ion concentration (H^+, K^+) and membrane potential [23,28,29] is essential in understanding the variation of K_T and J_{max} in accordance with the metabolic conditions in the yeast cell. In contrast to the kinetic parameters of phosphate uptake [39], that information is not available for amino acids, but the effect of carboxylic acids as shown in Table VI strongly suggests dependence of K_T and J_{max} on cellular proton concentration.

Values calculated for the coefficients, A—D, satisfy the inequalities $A/B + BC/A < D$ and $4C < D^2$ (Table III) which are valid for dual mechanisms of transport via two independently operating single-site transport systems. Rate equations for the concentration-dependence of the influx rate of substrate by a two-carrier transport process are similar, however, to the equations for a two-site, one-carrier transport system [28]. When this latter process occurs, one of the J_{max} (or V values) might become negative if, by varying the concentrations of cotransported ions, coefficient A becomes either too small or too large [28]. In the present study that possibility has not been explored, but the kinetics of

L-[^{14}C]leucine uptake by starved yeast for short incubation times yield negative K_T and J_{max} values (Table II and Fig. 5). These negative values may be interpreted, however, as kinetic artifacts due to the incidence of the very early uptake effect on total entrance values and consequently, negative parameters must not be taken as an evidence for the two-site, one-carrier transport mechanisms. Assuming that uptake values at 1.0 mM L-[^{14}C]leucine largely represent system 2 activity, L-[^{14}C]leucine transport systems could be further differentiated by their responses to inhibitors, such as DCCD and quercetin (Table IV), Dio-9 (Table V), carboxylic acids (Table VI), and CCCP and FCCP (Table VII). In all probability, the high-affinity system is carrier-mediated and involves the general amino acid permease [6]. This system would function effectively when L-[^{14}C]leucine concentration is relatively small and energy sources are easily available.

DCCD, quercetin, diethylstilbestrol and Dio-9 effectively inhibit L-[^{14}C]-leucine transport at 0.05 mM (Tables IV and V). Since inhibition of mitochondrial ATPase can be ruled out [30], the observed effects may involve: (a) inhibition of the plasmalemma ATPase; (b) variation of the membrane potential by exchange of K^+ for protons [16,20,23]; (c) a general permeabilizing effect on the cell plasmalemma [32], or (d) combination of these mechanisms. The inhibition of the plasmalemma ATPase is borne out by the fact that DCCD and quercetin concentrations effective on L-[^{14}C]leucine transport are well above those inhibiting the solubilized ATPase [17—19,32]. The same conclusion may be valid for diethylstilbestrol, since concentrations at which it causes generalized perturbation of artificial lipid films [40] largely exceed those affecting both L-[^{14}C]leucine transport (Table IV) and the plasmalemma ATPase [18]. Unlike DCCD and quercetin, Dio-9 inhibits transport at 0.05 and at 1.0 mM L-[^{14}C]leucine (Table V). Dio-9 inhibits the solubilized plasmalemma ATPase [18] and this inhibition supports the enzyme function for system 2 operation. However, Dio-9 inhibition may also be explained by (a) the increase in the proton gradient that follows K^+ efflux [31]; (b) a general permeabilizing action on the cell membrane [32], or (c) both. Since nystatin (Table IX) provides evidence for transport inhibition as a consequence of increased membrane permeability (10 μg/ml nystatin does not inhibit the yeast plasmalemma ATPase [17] but 5 μg/ml nystatin increases membrane permeability to K^+ and H^+ [35,36]) it is worth questioning whether plasmalemma ATPase inhibition really plays a role in Dio-9 inhibition of system 2. A negative answer to this point is suggested by the fact that with *Sch. pombe*, the Dio-9-induced K^+ efflux is not secondary to the inhibition of the plasmalemma ATPase activity [31]. Nevertheless, the inhibition of the plasmalemma ATPase cannot be ruled out.

Uncouplers of oxidative phosphorylation, like 2,4-dinitrophenol and carbonylcyanide phenylhydrazone derivatives, increase the conductance of protons across artificial phospholipid membranes [40] and bacterial plasma membrane [41], but, as exemplified by pentachlorophenol and analogues, uncouplers can also inhibit amino acid transport competitively, by interacting at specific sites for the transport mechanism [42]. The results in Table VII strongly support the first alternative since pentachlorophenol, CCCP and FCCP were more effective at 1.0 mM than at 0.05 mM [^{14}C]leucine and moreover,

pentachlorophenol produced significant inhibition even at 5.0 mM L-[^{14}C]-leucine. The effect of pentachlorophenol on K_T (Table VIII) is also against competition for L-[^{14}C]leucine binding sites. These results indicate that both the high- and low-affinity L-[^{14}C]leucine transport systems depend on the proton gradient for activity. The effect of proton conductors in Table VII is in accordance with the previously described 2,4-dinitrophenol inhibition of L-[^{14}C]leucine transport in *rho*$^-$ mutants of *S. cerevisiae*, *S. ellipsoideus* [10, 11,23], *Sch. pombe* [12] and *Saccharomyces carlsbergensis* [43].

Summing up, the observations here reported demonstrate the function of the proton gradient and the plasmalemma ATPase for the operation of L-[^{14}C]leucine high-affinity transport system. In all probability this system involves the general amino acid permease [6] and therefore, the mechanism energizing L-[^{14}C]leucine transport may be valid for most of the amino acid utilized by yeasts for growth and metabolism. On the other hand, the very nature of the low-affinity system is by no means clear and its elucidation requires further investigation.

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