Biochimica et Biophysica Acta, 467 (1977) 220—237

BBA 77704

SOME PROPERTIES OF L-[^{14}C]LEUCINE TRANSPORT IN *SACCHAROMYCES ELLIPSOIDEUS* *

E.H. RAMOS, L.C. DE BONGIOANNI, M.C. CUESTA CASADO and A.O.M. STOPPANI

Instituto de Química Biológica, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121 Capital Federal (Argentina)

(Received December 8th, 1976)

Summary

(1) Propionaldehyde, a substrate capable of activating electron transfer and oxidative phosphorylation, stimulated the concentrative uptake (entrance and accumulation) of L-[^{14}C]leucine by starved *Saccharomyces ellipsoideus*. Under adequate experimental conditions the ratio of L-[^{14}C]leucine uptake to oxygen uptake was higher with propionaldehyde than with D-glucose or endogenous substrates as energy sources.

(2) With D-glucose as energy source, antimycin significantly inhibited L-[^{14}C]leucine entrance and accumulation but the inhibition was less than that of respiration. With propionaldehyde as the energy source, cycloheximide did not affect L-[^{14}C]leucine uptake despite the almost complete inhibition of amino acid incorporation into the cell protein.

(3) Preincubation of starved yeast with D-glucose or propionaldehyde before L-[^{14}C]leucine addition significantly increased the amino acid entrance values. The effect of energization on amino acid entrance increased with the time the yeast was preincubated with the oxidizable substrates. Energization of a *S. ellipsoideus* rho$^-$ mutant with D-glucose, caused less L-[^{14}C]leucine entrance than with the wild type yeast.

(4) Addition of 2,4-dinitrophenol to the D-glucose or propionaldehyde-energized *S. ellipsoideus* brought about a significant inhibition of L-[^{14}C]leucine entrance, but the D-glucose-energized yeast was much less sensitive to the uncoupler than its propionaldehyde-energized counterpart. 2,4-Dinitrophenol also inhibited L-[^{14}C]leucine entrance in the D-glucose-energized rho$^-$ mutant, to the same extent as with the D-glucose-energized wild type yeast. Preincubation of yeast with 2,4-dinitrophenol prevented to about the same extent the energization of L-[^{14}C]leucine transport either by D-glucose or by propionaldehyde.

* Paper II in the series: studies on amino acid uptake by yeasts.

(5) The kinetics of L-[^{14}C]leucine transport as a function of amino acid concentration indicate the existence of two apparently distinct transport systems, namely, one with high affinity and low transport activity (System A) and the other with low affinity and high transport activity (System B). The kinetic parameters K_T and V characterizing these systems were dependent on the energization state of the yeast cells since K_{TA} (starved) < K_{TA} (energized); K_{TB} (starved) > K_{TB} (energized), while V (starved) < V (energized), (for both systems). Systems A and B could be further differentiated by the effect of pH and temperature.

(6) Ammonium ions significantly inhibited L-[^{14}C]leucine entrance in D-glucose energized yeast when added simultaneously with D-glucose, but stimulated the very early entrance when added after energization had occurred. With the glucose-energized rho$^-$ mutant, ammonium ions increased L-[^{14}C]leucine entrance irrespective of the time addition.

(7) Preincubation of yeast with cyclic AMP caused an increased rate of L-[^{14}C]leucine entrance. The effect (a) was related to the nucleotide concentration, and (b) was higher with 1.0 mM than with 0.1 mM L-[^{14}C]leucine. This difference indicates that L-[^{14}C]leucine transport System B was the more responsive to cyclic AMP.

Introduction

It is currently accepted that the mechanism by which amino acids are transported into yeast cells involves a succession of operations comprising: the recognition of the molecule to be transported by a relatively specific receptor-site (binding); its translocation through the permeability barrier; energetic interactions (coupling), and the release of the molecule inside the yeast cells. Balance between influx and efflux accounts for the amino acid accumulation inside the yeast cell. Moreover, the possibility of the existence of regulatory mechanisms common to a class of molecules must be considered [1]. There are reasons to believe that amino acid entrance is driven by a spontaneous influx of protons coupled to the efflux of an equivalent number of K^+, the subsequent ejection of protons from the yeast depending on energy metabolism [2]. Accordingly, energy coupling is an essential step in the amino acid transport mechanism [3—7]. In Paper I of this series [8] we presented evidence supporting the view that in respiration-competent *Saccharomyces cerevisiae* the mitochondrion can effectively contribute to L-[^{14}C]leucine uptake by supplying high-energy compounds required for amino acid transport and accumulation, and conversely, the promitochondrion in the anaerobically grown yeast, competes for high-energy compounds generated by glycolysis in the cytosol. The observations described below with *Saccharomyces ellipsoideus* confirm and extend those with *S. cerevisiae*.

Materials and Methods

Yeast and chemicals. The yeasts used were *S. cerevisiae* var. *ellipsoideus*, diploid, wild type, strain 208, isolated by Professor N. Palleroni (formely at

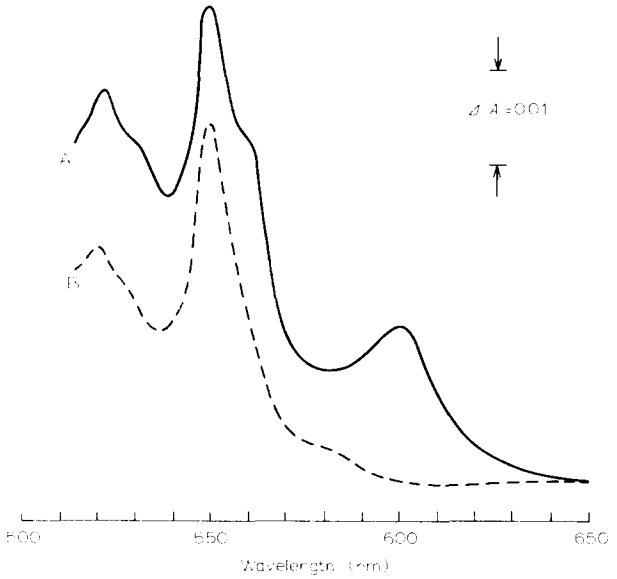

Fig. 1. Absorption spectrum of yeasts. A frame fixed near the phototube allowed the insertion of a 2 mm-thick yeast paste in the sample beam and tracing paper in the reference beam. Other conditions were as described under Materials and Methods. A, wild type yeast; B, rho$^-$ ("petite") mutant. The absorption maxima of cytochrome $a + a_3$, b and c are 603, 562 and 549.5 nm.

the Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina) and a cytoplasmic rho$^-$ mutant "petite colonie" isolated from the wild type strain by the acriflavin method [9]. The absorption spectra of the yeast employed are shown in Fig. 1. The rho$^-$ mutant did not respire when oxygen uptake was assayed in the Gilson Oxygraph model K.

Antimycin and cyclic AMP were purchased from Sigma Chemical Company, St. Louis, Mo. Other reagents were obtained from sources previously indicated.

Incubation techniques. Unless stated otherwise the kinetics of L-[^{14}C]leucine uptake and displacement were carried out at 30°C, in a New Brunswick Gyratory Water Shaker (Model T-76) with 10 ml incubation mixture containing yeast (2 mg/ml), 20 mM phthalate (K) buffer pH 4.5 and additions as specified in each case (standard experimental conditions). Oxygen uptake was measured by the Warburg direct method in manometers at 30°C with air in the gas space.

Analytical methods. Unless otherwise stated, determination of the cytochrome spectrum of yeast as well as the determination of total intracellular L-[^{14}C]leucine were performed as described in ref. 8. In most cases, assay of radiactive samples was performed after filtration of incubation mixtures through a Millipore filter 25 ea, HA 0.45 μm (Method A [8]).

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 [5].

Results

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

The kinetics of L-[^{14}C]leucine uptake by starved yeast, as a function of the

TABLE I

EFFECT OF SUBSTRATE OXIDATION ON L-[^{14}C]LEUCINE UPTAKE BY *S. ELLIPSOIDEUS*

Starved cells (4.8 mg/ml) were incubated in Warburg manometers in 20 mM phthalate buffer pH 4.5 containing 5 mM L-[^{14}C]leucine and additions as stated below; total volume, 3 ml; 3 h incubation. Total L-[^{14}C]leucine uptake was measured by Method B [8]. Other conditions were as described under Materials and Methods.

Addition (5 mM)	L-[^{14}C]Leucine uptake (μmol/g cells)		Apparent internal vs. external concentration ratio *	Total oxygen uptake (mmol/g cells) (c)	(a/c) × 10
	Total (a)	Non-soluble fraction (b)			
D-Glucose	401	37.6	48.4	4.29	0.93
Propionaldehyde	221	13.6	27.6	1.54	1.44
None	23.8	0.4	3.2	0.24	0.99

* This ratio was calculated on the basis of free intracellular L-[^{14}C]leucine (a—b) and the initial (5 mM) concentration of external L-[^{14}C]leucine. The concentration of unlabelled (endogenous) L-leucine (about 1—2 μmol/g of cells) is neglected. Therefore, ratios may be higher than stated.

external concentration of L-[^{14}C]leucine and the time of incubation resembled those reported previously with *S. cerevisiae* [8]. Initial rates were maintained during the first 15 min and after 60 min an apparent steady state was reached with 0.1 and 0.5 mM L-[^{14}C]leucine, but not with the higher amino acid concentrations. Analysis of the 90 min incubated yeast showed that soluble L-[^{14}C]leucine amounted to 95% of the total incorporated ^{14}C. On the other hand, displacement of accumulated L-[^{14}C]leucine was very limited, since after 3.5 min reincubation with 1.0 mM external L-leucine the maximal efflux of L-[^{14}C]leucine amounted to 5% of the initial value, while after 15 min reincubation the corresponding value was 20% (experimental details omitted).

Effect of energy sources

Table I shows that oxidation of D-glucose and propionaldehyde increased the uptake of L-[^{14}C]leucine by *S. ellipsoideus*. Incubation was for 3 h, which means that uptake values involved both entrance and accumulation of L-[^{14}C]-leucine. Analysis of the soluble fraction of the incubated yeast showed that despite the relatively long-term incubation, L-[^{14}C]leucine was the sole radiactive compound. The concentrative nature of L-[^{14}C]leucine uptake was confirmed by the effect of substrate oxidation on the internal vs. external amino acid concentration ratio. An empirical correlation of L-[^{14}C]leucine uptake and oxygen consumption shows that, in the given experimental conditions, propionaldehyde was more effective than glucose (or the endogenous substrates) as energy source for driving L-[^{14}C]leucine transport. Since propionaldehyde was oxidized to propionate by two aldehyde dehydrogenases [10,11] and propionate oxidation was limited [12], the stimulation of L-[^{14}C]leucine uptake brought about by propionaldehyde oxidation involved the utilization of energy made available by the oxidation of reduced pyridine nucleotides in the yeast mitochondrion. This presumption is borne out by the effect of antimycin, a highly selective inhibitor of electron transfer and coupled phosphorylation in yeast [13,14]. In fact, antimycin inhibited the glucose-stimulated accumulation

TABLE II

EFFECT OF ANTIMYCIN ON L-[^{14}C]LEUCINE UPTAKE BY *S. ELLIPSOIDEUS*

Starved yeast (20 mg) suspended in 20 mM phthalate buffer (pH 4.5) containing 5 mM L-[^{14}C]leucine ($2.36 \cdot 10^5$ cpm) and antimycin as stated below. Final vol. 3.0 ml. Incubation for 3 h in Warburg manometers at 30°C. ^{14}C incorporation was measured by Method B [8]. Other conditions were as described under Materials and Methods.

Glucose (mM)	Antimycin (μg)	L-[^{14}C]Leucine uptake (cpm/mg cells)	Q_{O_2}
5	0	5850	46.9
	5	1520 (74) *	4.6 (90)
0	0	470	6.8
	5	330	3.7

* Inhibition of L-[^{14}C]leucine uptake (%)

of L-[^{14}C]leucine by 74% while respiration was inhibited by 90% (Table II). The difference between these two inhibitions may be partly accounted for by L-[^{14}C]leucine uptake driven by anaerobically generated high-energy compounds.

Since preincubation of yeast with an energy source may increase the biosynthesis of protein carriers involved in amino acid transport, it seemed necessary to establish whether this mechanism accounted for the effect of energy sources as shown in Table I. Accordingly, yeast cells were subjected to long-term incubation with L-[^{14}C]leucine and propionaldehyde, in the presence of cycloheximide, an inhibitor of protein biosynthesis in *S. ellipsoideus* [15]. The results given in Table III indicate that the antibiotic scarcely affected the energization of L-[^{14}C]leucine transport by propionaldehyde, despite a 98—99% inhibition of amino acid incorporation into the non-soluble cell fraction. Since this inhibition reflects that of protein biosynthesis [15], the effect of propionaldehyde on L-[^{14}C]leucine transport can be attributed to generation of high energy compounds rather than to stimulation of permease biosynthesis.

TABLE III

ENERGIZATION OF L-[^{14}C]LEUCINE UPTAKE BY PROPIONALDEHYDE IN THE PRESENCE OF CYCLOHEXIMIDE

Starved cells suspended in 10 ml of 20 mM phthalate buffer (pH 4.5) containing 5.0 mM L-[^{14}C]leucine. Propionaldehyde (5.0 mM) and cycloheximide (4.4 μg/ml) were added as indicated below. After incubation, samples were taken and total ^{14}C incorporation in whole cells and the non-soluble fraction was measured. Other conditions were as described under Materials and Methods and ref. 8.

Additions	L-[^{14}C] Leucine uptake (μmol/g cells)			
	1 h incubation		3 h incubation	
	Total	Non-soluble fraction	Total	Non-soluble fraction
None	38	0.8	62	2.2
Cycloheximide	40 (—3) *	0.2 (75)	84 (—35)	0.2 (91)
Propionaldehyde	142	7.7	300	25.3
Propionaldehyde + cycloheximide	132 (7)	0.1 (99)	258 (14)	0.2 (99)

* Inhibition of L-[^{14}C]leucine uptake (%)

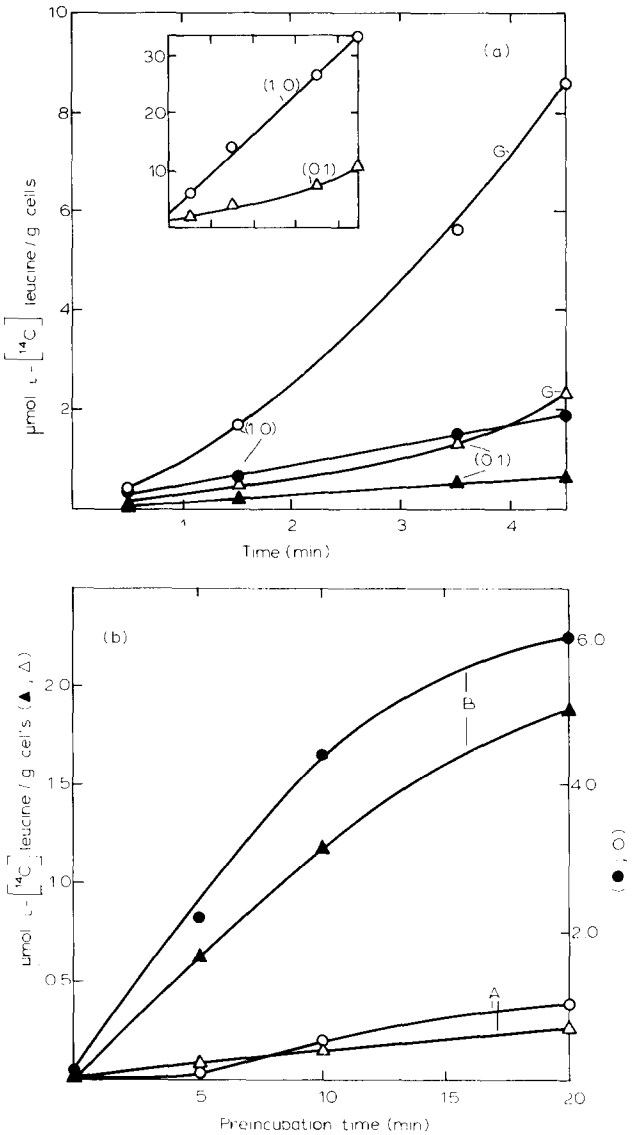

Fig. 2. Effect of D-glucose on L-[^{14}C]leucine entrance into starved yeast. (a) Cells were suspended in 20 mM phthalate buffer pH 4.5 containing 5 mM D-glucose (where indicated by G) and L-[^{14}C]leucine at the concentration (mM) indicated by the figures in parenthesis. Inset: same experimental conditions except that the cells were preincubated for 10 min with 5 mM D-glucose before L-[^{14}C]leucine addition. L-[^{14}C]Leucine concentration is indicated by the figures in parenthesis. (b) Effect of the time of preincubation of yeast with D-glucose on L-[^{14}C]leucine entrance. Starved cells were suspended in 20 mM phthalate buffer pH 4.5 containing 5 mM D-glucose. After the time indicated in the abscissa, 0.1 or 1.0 mM L-[^{14}C]leucine was added. After 10 s (A) and 2 min (B) incubation, samples were taken and internal radioactivities were measured. Left ordinate, entrance values with 0.1 mM L-[^{14}C]leucine; (○), after 10 s; (▲), after 2 min. Right ordinate, entrance values with 1.0 mM L-[^{14}C]leucine; (○), after 10 s; (●), after 2 min. Other conditions were as described under Materials and Methods.

Fig. 2a shows the effect of D-glucose on L-[^{14}C]leucine uptake for relatively short incubation times (amino acid entrance). When D-glucose and the amino acid were simultaneously added to the yeast suspension, the presence of D-glu-

cose brought about a 4-fold increase in the rate of L-[^{14}C]leucine entrance into the yeast cells. On the other hand, when the cells were preincubated with glucose for 10 min before the amino acid addition, the rate of amino acid entrance increased 10-fold with 0.1 mM L-[^{14}C]leucine and 16-fold with 1.0 mM L-[^{14}C]leucine (Fig. 2a, inset). It is remarkable that with the glucose-energized yeast, extrapolation of entrace values at zero time incubation gave a positive intercept. This phenomenon, henceforth termed the "very early" amino acid entrance, could be directly confirmed by measuring uptake at the shortest possible time of incubation ($\leqslant$ 10 s). The concentrative nature of the uptake process was enhanced by preincubating the yeast with D-glucose, as shown by the corresponding intracellular vs. extracellular concentration ratios, namely, 68 and 21, respectively (Fig. 2a, inset), against 4 obtained with the non-energized yeasts. Both the "very early" and 2 min entrance values increased as a function of the time the yeast was preincubated with glucose (Fig. 2b), which indicates that the former was a metabolically dependent process and not a contamination of yeast with traces of the incubation medium.

Fig. 3a shows that 2,4-dinitrophenol prevented the energization of L-[^{14}C]-leucine transport by D-glucose and propionaldehyde. With D-glucose, the inhibition was 80 % while with propionaldehyde, 2,4-dinitrophenol decreased uptake to below the level of the endogenously driven uptake. Almost identical results were obtained with 0.1 mM L-[^{14}C]leucine (experimental data omitted). These results resemble those obtained with *S. cerevisiae* 207, but differ to some extent from those with *S. cerevisiae* D261 and D-247-2 (ref. 8). In the same experimental conditions, 2,4-dinitrophenol also prevented the D-glucose-dependent uptake of L-[^{14}C]leucine by the respiratory deficient yeast (Fig. 3b), but the inhibition occurred only with 1.0 mM L-[^{14}C]leucine and was relatively small as compared with that of the wild type yeast (Fig. 3a). Incidentally, it must be recalled that the presence of D-glucose was essential for the entrance and accumulation of L-[^{14}C]leucine by the rho$^-$ mutant and, furthermore, that with this yeast D-glucose could not be replaced by a non-fermentable source of energy, such as propionaldehyde (Fig. 3b).

Table IV shows the effect of pretreating the yeast with D-glucose, propionaldehyde and 2,4-dinitrophenol on L-[^{14}C]leucine entrance at different amino acid concentrations and times of incubation. In order to simplify the description of this experiment the following convention is adopted. T_0 is the time of addition of L-[^{14}C]leucine; $T_0 - t$ is the time of addition of the energy source and/or 2,4-dinitrophenol; $T_0 + t$ is the time when samples of incubation mixture were taken for measuring L-[^{14}C]leucine entrance. L-[^{14}C]Leucine entrance was measured either at T_0 + 10 s or at T_0 + 5 min. The T_0 + 10 s samples served to measure the "very early" entrance values. The results given in Table IV may be summarized as follows: (a) with the wild type yeast and 0.1 mM L-[^{14}C]leucine, glucose and propionaldehyde were almost equally effective in driving transport, but with 1.0 mM amino acid, D-glucose was nearly twice as effective as propionaldehyde. The effect of substrate oxidation revealed itself by the variation of both the very early entrance and translocation values; (b) energization of the rho$^-$ mutant with D-glucose caused entrance values equivalent to 22—26% of those obtained with the wild type yeast; (c) addition of 2,4-dinitrophenol at $T_0 - 1$ min to the energized, wild type yeast determined inhibition of

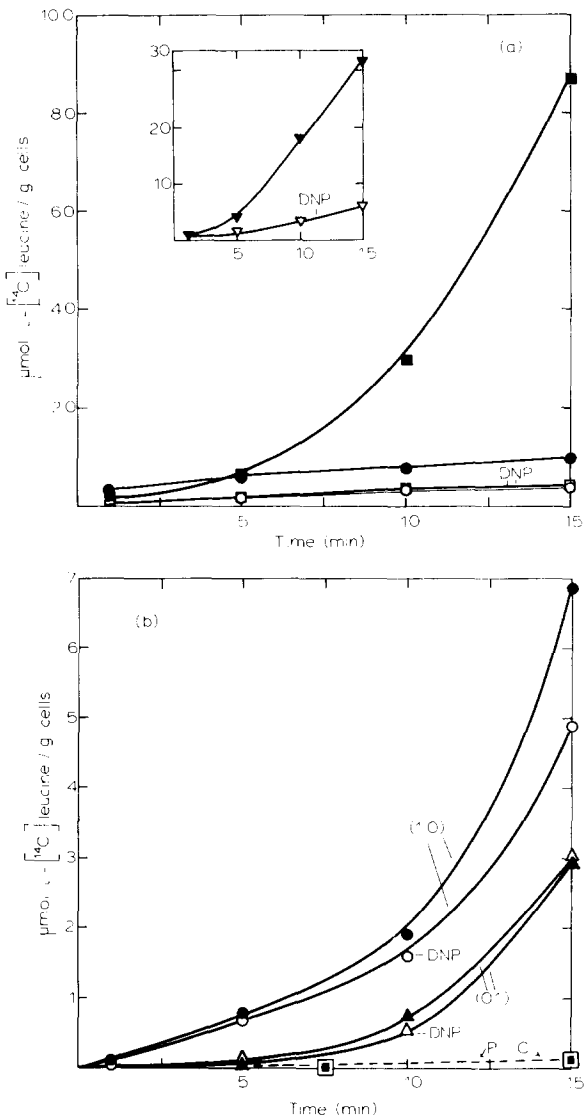

Fig. 3. Effect of 2,4-dinitrophenol (DNP) on L-[^{14}C]leucine entrance into starved wild type yeast and the rho$^-$ mutant. (a) Wild type yeast cells were suspended in 20 mM phthalate buffer pH 4.5 containing 1.0 mM L-[^{14}C]leucine and the following additions; (●), none; (○), 50 μM 2,4-dinitrophenol; (■), 5.0 mM propionaldehyde; (□), 5.0 mM propionaldehyde plus 50 μM 2,4-dinitrophenol. At the times indicated in the abscissa samples were taken and internal radioactivities were measured. Inset: Same conditions; (▼), 5.0 mM D-glucose; (▽), 5.0 mM D-glucose plus 50 μM 2,4-dinitrophenol. (b) rho$^-$ mutant cells were suspended in 20 mM phthalate buffer pH 4.5 containing L-[^{14}C]leucine (concentration (mM) as indicated by the figures in parenthesis) and 5 mM D-glucose. (▲), 0.1 mM L-[^{14}C]leucine; (△), same plus 50 μM 2,4-dinitrophenol; (●), 1.0 mM L-[^{14}C]leucine; (○), same plus 50 μM 2.4-dinitropehnol. P (■), 5 mM propionaldehyde in place of D-glucose; C (□), control. Other conditions were as described under Materials and Methods.

both "very early" and entrance values, but, in contrast with the results presented in Fig. 3a, the D-glucose-energized yeast was significantly less sensitive to the uncoupler than its propionaldehyde energized counterpart. Finally, in the

TABLE IV

EFFECT OF PREINCUBATION WITH ENERGY SOURCES AND 2,4-DINITROPHENOL ON L-[^{14}C]-LEUCINE ENTRANCE INTO *S. ELLIPSOIDEUS*

Starved cells were suspended in 20 mM phthalate buffer (pH 4.5). L-[^{14}C]leucine was added at T_0; glucose (5.0 mM), propionaldehyde (5.0 mM) and 2,4-dinitrophenol (50 μM) were added at the times indicated below. Other experimental conditions were as described in the text.

Yeast	L-[^{14}C] Leucine (mM)	Addition, and time of addition (min)	Entrance of L-[^{14}C]leucine (μmol/g cells)	
			T_0 +10 s	T_0 +5 min
Wild type	0.1	Glucose (T_0 — 15)	0.59	8.38
		Propionaldehyde (T_0 — 15)	0.34	7.38
		None	0.08	1.07
	1.0	Glucose (T_0 — 15)	1.50	31.3
		Glucose (T_0 — 15) + 2,4-Dinitrophenol (T_0 — 1)	1.38 (8) *	23.2 (26)
		Propionaldehyde (T_0 — 15)	0.96	17.5
		Propionaldehyde (T_0 — 15) + 2,4-Dinitrophenol (T_0 — 1)	0.37 (61)	2.22 (88)
		None	0.31	2.18
rho$^-$ mutant	0.1	Glucose (T_0 — 15)	0.13	2.36
	1.0	Glucose (T_0 — 15)	0.71	6.83
		Glucose (T_0 — 15) + 2,4-Dinitrophenol (T_0 — 1)	0.71 (0)	4.86 (29)

* Inhibition of L-[^{14}C]leucine entrance (%)

same experimental conditions, addition of 2,4-dinitrophenol to the glucose-energized rho$^-$ mutant inhibited the amino acid entrance to about the same extent as in the D-glucose-energized, wild type yeast.

Kinetic parameters of L-[^{14}C]leucine transport with starved and energized yeasts

Fig. 4a shows the effect of L-[^{14}C]leucine concentration on the amino acid entrance by starved *S. ellipsoideus*. Representation of initial velocities as a function of amino acid concentration yielded a hyperbolic curve which indicates that the transport system was saturable. Since displacement was negligible it may be assumed that entrance values (v) measured L-[^{14}C]leucine influx across the cell plasmalemma. v is defined by Eqn. 1

$$v = \frac{[S] \cdot V}{K_T + [S]} \quad (1)$$

where [S] is the external concentration of L-[^{14}C]leucine, V expresses the maximum flux that the yeast cells can exhibit toward the amino acid, and K_T, often represented as K_m in transport studies, formally expresses the substrate concentration at which the flux is exactly one half the limiting flux V. K_T and V may be complex functions of several possible factors that include some type of "affinity" of the amino acid for the system, pH, the ionic composition of the medium and temperature [16]. The Lineweaver-Burk procedure for enzymological data-analysis may be applied and Eqn. 1 can be rearranged to give

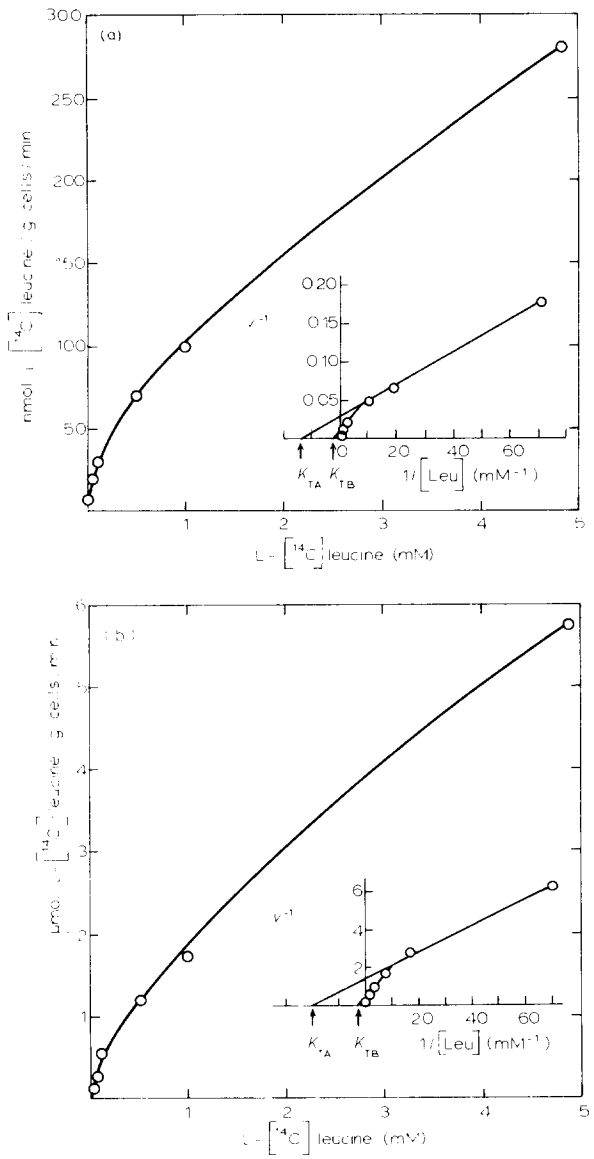

Fig. 4. Effect of L-[^{14}C]leucine concentration on the amino acid entrance into starved yeast. (a) Cells were suspended in 20 mM phthalate buffer pH 4.5 containing L-[^{14}C]leucine at the concentration stated in the abscissa. Inset: double reciprocal plot of entrance values. Other conditions were as described under Materials and Methods. (b) Same as in (a) except that cells were preincubated for 15 min with 5 mM D-glucose before L-[^{14}C]leucine addition.

Eqn. 2

$$\frac{1}{v} = \frac{K_T}{V} \cdot \frac{1}{[S]} + \frac{1}{V} \qquad (2)$$

Representation of $1/v$ as a function of $1/[S]$ gives a straight line from which V and K_T values can be calculated. The double-reciprocal plot presented in the

inset in Fig. 4a shows, however, a deflection, thus suggesting the existence of two apparently distinct transport systems. Fig. 4b shows the result of a similar experiment using yeast cells energized by 10 min preincubation with D-glucose. The double-reciprocal plot (inset) confirms the existence of two kinetically different systems and furthermore, similar results were obtained with the D-glucose energized rho$^-$ mutant yeast (experimental data omitted). L-[^{14}C]Leucine transport systems, henceforth termed A and B, respectively, can be characterized by the different values of K_T and V. Measurement of K_T and V, performed with two samples of the same yeast strain (wild type) at a six-month interval, yielded the following values (K_T, mM; V, μmol/g cells per min; in parenthesis, the average of each pair of values). Starved yeast; K_{TA}, 0.05, 0.05 (0.05); V_A, 0.10, 0.12 (0.11); K_{TB}, 3.0, 2.8 (2.9); V_B, 0.37, 0.25 (0.31). Energized yeast: K_{TA}, 0.19, 0.08 (0.14); V_A, 3.6, 2.7 (3.2); K_{TB}, 0.59, 0.38 (0.49); V_B, 6.5, 5.6 (6.1). Since an imperfect reproducibility of the kinetic constants may be expected [17], the variations are not abnormally large. It is noteworthy that (a) the K_{TA} for the starved yeast was lower than the K_{TA} for the energized yeast; (b) the K_{TA} for the starved yeast was nearly two orders of magnitude smaller than the K_{TB} for the same yeast; (c) the K_{TB} for the energized yeast was significantly smaller than the K_{TB} for the starved yeast; (d) energization determined a 30- or 20-fold increase of the V_A (or V_B); (e) V_A values were about 30—40% of the corresponding V_B values. Summing up, System A was a high affinity, low transport activity system while system B was a low affinity, high transport activity system.

Effect of ammonium ions

Ammonium ions are known to inhibit amino acid uptake by *S. cerevisiae* during the logarithmic phase of growth [17]. The results presented in Table V show that, under adequate experimental conditions, a similar inhibition may be obtained with resting *S. ellipsoideus*. Thus, ammonium ions were added to the wild type yeast (starved and energized) and the rho$^-$ mutant (energized) either at the same time as glucose, or shortly before L-[^{14}C]leucine. It can be seen that: (a) with the wild type yeast, simultaneous addition of ammonium ions and glucose (at $T_0 - 25$ min) inhibited both the amino acid very early entrance and translocation (as compared with the glucose-treated yeast), the inhibitions being greater with the lower amino acid concentration; (b) addition of ammonium ions to the glucose energized wild type yeast (at $T_0 - 0.25$ min) stimulated the very early entrance of L-[^{14}C]leucine but inhibited (to some extent) the amino acid translocation, irrespective of the amino acid concentration; (c) considering the inverse variation of very early entrance values after addition of ammonium ions at $T_0 - 25$ (condition (a)) and $T_0 - 0.25$ min (condition (b)), the inhibitory effects of ammonium ions in condition (a) may be estimated as 61 and 51% for 0.1 and 1.0 mM L-[^{14}C]leucine, respectively; (d) in the absence of an energy source, addition of ammonium ions to the starved wild type yeast did not significantly modify L-[^{14}C]leucine entrance (experimental data omitted). In contrast with the results obtained with the wild type yeast, ammonium ions apparently stimulated very early entrance and translocation of L-[^{14}C]-leucine into the rho$^-$ mutant, regardless of the time of incubation and the amino acid concentration (Table V).

TABLE V

EFFECT OF AMMONIUM IONS ON L-[^{14}C]LEUCINE ENTRANCE INTO *S. ELLIPSOIDEUS*

Starved cells were suspended in 20 mM phthalate buffer pH 4.5; L-[^{14}C]leucine was added at T_0; 5 mM glucose and 20 mM $(NH_4)_2SO_4$ were added at the times indicated below. Other conditions were as described in the text.

Yeast	L-[^{14}C] Leucine (mM)	Addition, and time of addition (min)	Entrance of L-[^{14}C]leucine (μmol/g cells)	
			T_0 +10 s	T_0 +5 min
Wild type	0.1	Glucose (T_0 — 25)	0.74	15.1
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 25)	0.44 (—41) *	6.77 (—55)
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 0.25)	0.91 (+21)	13.5 (—10)
		None	0.04	0.57
	1.0	Glucose (T_0 — 25)	2.74	40.5
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 25)	2.07 (—24)	26.8 (—34)
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 0.25)	3.58 (+27)	37.3 (—8)
		None	0.12	1.29
rho$^-$ mutant	0.1	Glucose (T_0 — 25)	0.22	2.76
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 25)	0.28 (+27)	3.99 (+45)
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 0.25)	0.29 (+32)	3.96 (+44)
	1.0	Glucose (T_0 — 25)	0.85	7.67
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 25)	1.34 (+58)	9.75 (+17)
		Glucose (T_0 — 25) + NH_4^+-(T_0 — 0.25)	1.07 (+20)	9.59 (+15)

* Variation of L-[^{14}C]leucine entrance (%): (—), diminution; (+), increase

Effect of cyclic AMP

A peculiar feature of some strains of yeast (*Schizosaccharomyces pombe; S. cerevisiae*) in conditions of glucose starvation is the stimulation of amino acid uptake by cyclic AMP [18]. *S. ellipsoideus* seems to belong to the same group of yeasts, as exemplified by L-[^{14}C]leucine. Fig. 5 shows the effect of cyclic AMP on entrance values for 0.1 mM L-[^{14}C]leucine (System A), and for 1.0 mM L-[^{14}C]leucine, less the 0.1 mM L-[^{14}C]leucine values (System B). It is seen that pretreatment of yeast with cyclic AMP increased L-[^{14}C]leucine entrance through System B as a function of the nucleotide concentration, the maximal increase being 41% (with 5.0 mM cyclic AMP), while entrance of L-[^{14}C]leucine through System A was significantly less stimulated (11%, with 5.0 mM cyclic AMP). The effect of cyclic AMP introduces an additional difference between Systems A and B.

Effect of pH

Fig. 6 shows the effect of pH on the entrance of 0.1 (Fig. 6a) and 1.0 (Fig. 6b) mM L-[^{14}C]leucine into the starved yeast. Incubation was for 2.5 and 10

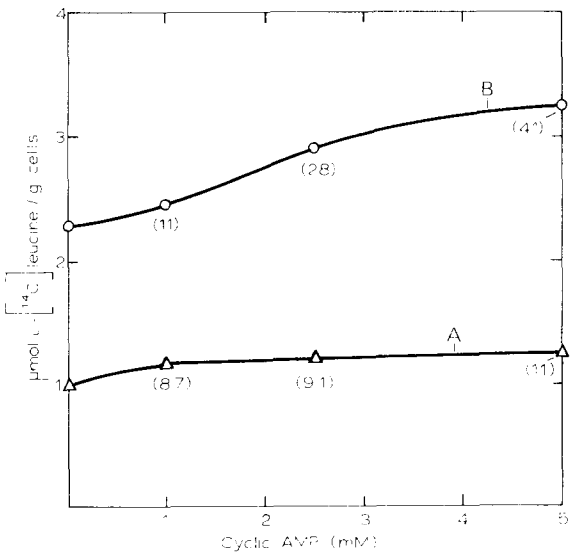

Fig. 5. Effect of cyclic AMP on L-[^{14}C]leucine entrance. Starved cells were suspended in 20 mM phthalate buffer pH 4.5 containing cyclic AMP as indicated in the abscissa. After 30 min of incubation, L-[^{14}C]leucine was added and entrance at 5 min was determined. A, entrance values for 0.1 mM L-[^{14}C]-leucine; B, same, for 1.0 mM L-[^{14}C]leucine less the corresponding A values. Other conditions were as described under Materials and Methods. The figures in parenthesis indicate the increase (%) of L-[^{14}C]-leucine entrance.

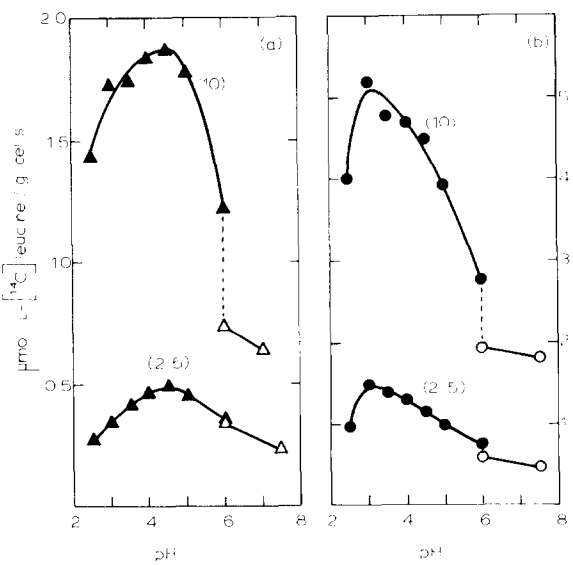

Fig. 6. Effect of pH on L-[^{14}C]leucine entrance. Starved cells were suspended in either 20 mM phthalate buffer pH 2.4–6.0 or 20 mM KH_2PO_4 buffer pH 6.0–7.5 containing 0.1 (a) or 1.0 (b) mM L-[^{14}C]leucine. At the times (min) indicated in parenthesis, samples were taken and internal radioactivities were measured. (▲, ●), phthalate buffer; (△, ○), phosphate buffer. Other conditions were as described under Materials and Methods.

TABLE VI

EFFECT OF TEMPERATURE ON L-[^{14}C]LEUCINE ENTRANCE AT DIFFERENT L-[^{14}C]LEUCINE CONCENTRATIONS

Starved cells were suspended in 20 mM phthalate buffer pH 4.5. L-[^{14}C] Leucine concentration and temperature were as indicated below; 5 min incubation. Other conditions were as described under Materials and Methods. In order to calculate temperature coefficients and energy activation values for System B, uptake values for 1.0 mM L-[^{14}C]leucine were subtracted those for 0.1 mM L-[^{14}C]leucine (System A).

L-[^{14}C] Leucine (mM)	Temperature (°C)	L-[^{14}C] Leucine uptake (μmol/g cells)	Temeprature coefficient for 10° C interval (Q_{10})	Energy of activation (kJ/mol)
0.1	10	0.03		
	20	0.36	12.0	172
	30	0.55	1.5	31.3
1.0	10	0.43		
	20	0.68	11.6	32.6
	30	0.92	1.4	24.7

min. It is seen that with 0.1 mM L-[^{14}C]leucine, the curve shows a maximum between pH 4.0 and pH 5.0 while with 1.0 mM concentration, uptake rates continuously increase to a maximum at about pH 3.0. At pH 6.0, uptake values were lower with phosphate buffer, as compared with those obtained with phthalate buffer, thus suggesting an inhibitory action of phosphate ions. Assuming that values at 0.1 mM and 1.0 mM L-[^{14}C]leucine mainly reflected translocation and accumulation through Systems A and B, respectively, the results in Fig. 6 support the assumption of two kinetically different transport systems, with different pH optima. The same pH optima were observed after 2.5 and 10 min incubation, thus implying that the pH variation essentially affected L-[^{14}C]leucine translocation.

Effect of temperature

Table VI shows the variation of uptake rates as a function of temperature, with 0.1 and 1.0 mM L-[^{14}C]leucine. It may be seen that in the 10—20° C range, the temperature coefficient and energy of activation of System A were much higher than with System B.

Discussion

The observations described here demonstrate that *S. ellipsoideus* is capable of an energy-dependent, essentially unidirectional, concentrative transport of L-[^{14}C]leucine. The function of the mitochondrion in the energization of L-[^{14}C]leucine transport is clearly evidenced by (a), the effect of propionaldehyde in Table I, III and IV and Fig. 3a, since aldehydes activate mitochondrial electron transfer and oxidative phosphorylation, and (b) antimycin inhibition of L-[^{14}C]leucine transport (Table II) since antimycin is a specific inhibitor of mitochondrial electron transfer and the coupled phosphorylation [13,14]. However, a normal mitochondrion is not essential, as shown by the results,

with the rho⁻ mutant yeast (Fig. 3b and Tables IV and V). Furthermore, after short-term incubation, glycolysis activated L-[^{14}C]leucine transport more effectively than mitochondrial respiration and phosphorylation (Fig. 3a and Table IV). Energy coupling may involve the operation of the γ-Glutamyl cycle whose enzymes are present in Saccharomyces [19].

The inhibition of L-[^{14}C]leucine transport by 2,4-dinitrophenol deserves special comment. Comparison of results presented in Fig. 3a and Table IV indicates that the uncoupler effect varied according to (a) the nature of the substrate employed to energize transport; and (b) the moment at which 2,4-dinitrophenol was added to the yeast. With regard to point (a), the higher inhibitions were observed when propionaldehyde was the energy source, or, in other words, when transport was energized from the mitochondrion. Under these conditions, 1 min incubation with the uncoupler was enough to decrease by 61% the "very early" entrance and by 88% the amino acid translocation values. On the other hand, with D-glucose as energy source, the uncoupler effect varied widely. In fact, when 2,4-dinitrophenol was added before energization (as shown in Fig. 3a) the inhibition was 80%, but when added after energization (as shown in Table IV), the inhibition was either negligible (very early entrance) or limited (the T_0 + 5 min measurement). Furthermore, nearly identical, weak inhibitions were obtained when 2,4-dinitrophenol was added either to the D-glucose-energized wild type or to the respiration incompetent rho⁻ mutant (Fig. 3 and Table IV). In this connection, it is worth recalling that with an ATP depleted, antimycin inhibited *S. carlsbergensis*, 50 μM 2,4-dinitrophenol inhibited about 15% both proton and glycine uptake [20], and similar inhibitions are known to occur with anaerobic *S. cerevisiae* and non-respiring *Schizosaccharomyces pombe* [18]. Consequently, the limited effect of 2,4-dinitrophenol with the D-glucose energized rho⁻ mutant in Table IV may well reflect an increased proton conductance at the plasmalemma [20] since rho⁻ mutants do not show promitochondrial ATPase activity [21]. On the other hand, with the D-glucose energized wild type yeast, 2,4-dinitrophenol inhibition may involve (a) the above-mentioned effect at the plasmalemma and (b) an exaggerated hydrolysis of glycolytically generated ATP by the mitochondrial ATPase [22,23]. Finally, the much more significant inhibitions after long-term incubation with the uncoupler (Fig. 3a) can be essentially attributed to inhibition of oxidative phosphorylation [13].

A peculiar feature of L-[^{14}C]leucine translocation by the glucose-energized yeast is the abnormally high initial values (the so-called "very early" entrance values) in Fig. 2a. Possible explanations of that effect may be (a) the existence of a steep proton gradient at the cell surface, determined by glucose catabolism in the absence of the permeant amino acid [24]; (b) the binding of L-[^{14}C]-leucine to a specific carrier protein [25], and (c) a combination of these. Since "very early" values may be significant, as compared with those in the 0—5 min-incubation period ignorance of the former may lead to erroneous calculation of initial velocities of entrance.

The inhibitory effect of ammonium ions in Table V can be explained by (a) decreased production of high energy compounds at the mitochondrion due to reduced operation of the trycarboxylate cycle [26]; (b) transinhibition [27] of the general amino acid permease [17] by amino acids resulting from glucose

catabolism (a process involving the anabolic NADP-dependent glutamate dehydrogenase [28]); (c) direct inhibition of the general amino acid permease by ammonium ions as occurs with *S. cerevisiae* [17] and *Aspergillus nidulans* [29] and (d) a combination of these. The operation of mechanism (a) is strongly supported by the fact that ammonium ions inhibited L-[^{14}C]leucine translocation only with the respiratory competent wild type yeast and when added together with D-glucose. In other words, the inhibition took place under conditions in which ammonium ions were able to shift glucose catabolism from a highly effective energy yielding process, namely, glycolysis plus the tricarboxylate cycle, to a less effective one, namely, the pentose pathway [30]. Otherwise, addition of ammonium ions stimulated (instead of inhibiting) L-[^{14}C]-leucine translocation which is at variance with the direct inhibition of the permease [17,29]. The mechanism by which ammonium ions stimulated L-[^{14}C]-leucine translocation, particularly with the rho$^-$ mutant, remains to be established.

A good indication of the existence of distinct sites for amino acid transport in cells is provided by the observation of saturation kinetics from which the apparent affinity constant K_T can be determined. It is recognized that if an amino acid utilizes more than one transport system, the corresponding kinetic constants may be different. Accordingly, the measurement of affinity constants for amino acid transport systems has been extensively used to characterize such systems for yeast [1,5–7,31,32]. The results presented in Fig. 4 and text indicate the interaction of L-[^{14}C]leucine with two different transport systems. Systems A and B may be represented by reaction 3

$$S_e + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (SC) \xrightarrow{k_{+2}} S_i + C \tag{3}$$

where S_e and S_i are the permeant L-[^{14}C]leucine at the cis(external) and trans-(internal) surface 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 considered to describe the activity of the energizing mechanisms. In this scheme, the strict formalism of the Briggs-Haldane treatment of enzyme kinetics is preserved, but the equilibrium at the trans face of the membrane is ignored [23]. Then, the term K_T is given by Eqn. 4

$$K_T = (k_{-1} + k_{+2})/k_{+1} \tag{4}$$

and the term V is given, similarly, by Eqn. 5

$$V = 0.5\, Ck_{+2}k_{-1}/(k_{+2} + k_{-1}) \tag{5}$$

The system is typically vectorial and resembles the one proposed by Kotyk and Říhová [27] to account for α-amino isobutyric acid transport in *S. cerevisiae*, except that in Eqn. 3 the reaction on the right involves the energy-dependent change of SC conformation postulated by Kotyk and Říhová [27].

The dependence of kinetic parameters on the metabolic conditions of the yeast cells was suggested by Grenson et al. [17] and demonstrated by Kotyk and Říhová [27] in connection with α-amino isobutyric acid transport in *S. cerevisiae*. The results presented in this paper, which extend those reported by

the latter authors, admit the following interpretation. With the starved yeast k_{-1} (or k_{+1}) >> k_{+2}, and accordingly $K_T \simeq k_{-1}/k_{+1}$, K_T being essentially a binding constant equivalent to K_s in enzyme kinetics [33,34]. Assuming that energization increased the value of K_{+2}, it follows that $K_T = K_s + k_{+2}/k_{+1}$, and consequently, K_T (energized) > K_T (starved). This is what actually happens with respect to System A. On the other hand, with System B, the reverse occurs, since K_{TB} (energized) < K_{TB} (starved), which may be interpreted on the assumption that energization significantly increases the value of K_{+1}, in other words, the rate of the amino acid binding to the specific carrier. The different responses of Systems A and B to energization (or deenergization) may be considered characteristic features of these systems. Other characterizing features are (a) the activation of System B by cyclic AMP (Fig. 5); (b) the different pH curves (Figs. 6); and (c) the relatively high temperature coefficient of System A in the 10—20°C range (Table VI).

The occurrence of multiple transport components for the accumulation of amino acids has been repeatedly observed in yeasts and fungi [1,5,6,31,32,35—37]. With regard to *S. cerevisiae*, Grenson et al. have presented kinetic and genetic evidence in support of the existence of a general amino acid permease [17] and also for specific permeases for arginine [1], lysine [31], methionine [32] and dicarboxylic acids [35]. The general amino acid permease is a good candidate for being a part of one of the systems (A) that transport L-[^{14}C]leucine in *S. ellipsoideus*.

Acknowledgements

This work was supported by grants of Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET), Comisión Nacional de Energía Atómica (Argentina) and the Multinational Program of Biochemistry, Organization of the American States, Irene B. de López gave able technical assistance. E.H.R. and A.O.M.S. are Career Investigators of CONICET and M.C. Cuesta Casado is Research Fellow of CONICET.

References

1 Grenson, M., Mousset, M., Wiame, J.M. and Bechet, J. (1963) Biochim. Biophys. Acta 127, 325—338
2 Cockburn, M., Earnshaw, P. and Eddy, A.A. (1975) Biochem. J. 146, 705—712
3 Halvorson, H.O., Fry, W. and Schwemin, D. (1955) J. Gen. Physiol. 38, 549—573
4 Stoppani, A.O.M., Ramos, E.H. and Widuczinski, I. (1960) Nature 188, 1188—1189
5 Surdin, Y., Sly, W., Sire, J., Bordes, A.M. and De Robichon-Schulmajster, H. (1965) Biochim. Biophys. Acta 107, 546—566
6 Magaña-Schwencke, N. and Schwencke, J. (1969) Biochim. Biophys. Acta 173, 313—323
7 Kotyk, A. and Říhová, L. (1972) Folia Microbiol. 17, 353—356
8 Ramos, E.H., Bongioanni, L.C. de, Claisse, L.M. and Stoppani, A.O.M. (1975) Biochim. Biophys. Acta 394, 470—481
9 Ephrussi, B., Hottinger, H. and Chimenes, A.M. (1949) Ann. Inst. Pasteur 76, 351—367
10 Black, S. (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 1, pp. 508—511, Academic Press, New York
11 Seegmiller, J.E. (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 1, pp. 511—516, Academic Press, New York
12 Ramos, E.H., Bongioanni, L.C. de and Stoppani, A.O.M. (1969) Biochim. Biophys. Acta, 177, 671—673
13 Stoppani, A.O.M., Bennun, A. and Pahn, E.M. de (1964) Arch. Biochem. Biophys. 108, 258—265

14 Stoppani, A.O.M., Pigretti, M.M., Ramos, E.H. and de Favelukes, S.L.S. (1963) Biochim. Biophys. Acta 74, 132—135
15 Widuczynski, I. and Stoppani, A.O.M. (1965) Biochim. Biophys. Acta 104, 413—426
16 Alvarado, F. and Mahmood, A. (1974) Biochemistry, 13, 2882—2890
17 Grenson, M., Hou, C. and Crabeel, M. (1970) J. Bact. 103, 770—777
18 Foury, F. and Goffeau, A. (1975) J. Biol. Chem. 250, 2354—2362
19 Mooz, E.D. and Wigglesworth, L. (1976) Biochim. Biophys. Res. Commun. 68, 1066—1072
20 Seaston, A., Carr, G. and Eddy, A.A. (1976) Biochem. J. 154, 669—676
21 Groot, G.S.P., Kováč, L. and Schatz, G. (1971) Proc. Natl. Acad. Sci. U.S. 68, 308—311
22 Slater, E.C. (1953) Nature, 172, 975—978
23 Subik, J., Kolarov, J. and Kováč, L. (1974) Biochim. Biophys. Acta 357, 453—456
24 Eddy, A.A. and Nowacki, J.A. (1971) Biochem. J. 122, 701—711
25 Bussey, H. and Umbarger, H.E. (1970) J. Bact. 103, 277—285
26 Holzer, H. and Witt, I. (1958) Biochem. Z., 330, 545—554
27 Kotyk, A. and Říhová, L. (1972) Biochim. Biophys. Acta 288, 380—389
28 Grenson, M. and Hou, C. (1972) Biochem. Biophys. Res. Commun. 48, 749—756
29 Robinson, J.H., Anthony, C. and Drabble, W.T. (1973) J. Gen. Microbiol. 79, 65—80
30 Holzer, H. and Witt, I. (1960) Biochim. Biophys. Acta 38, 163—164
31 Grenson, M. (1966) Biochim. Biophys. Acta 127, 339—346
32 Gits, J.J. and Grenson, M. (1967) Biochim. Biophys. Acta 135, 507—516
33 Dawson, A.C. and Widdas, W.F. (1964) J. Physiol. Lond. 172, 107—122
34 Stein. W.D. (1967) The Movement of Molecules across Cell Membranes, p. 175, Academic Press, New York
35 Joiris, C.R. and Grenson, M. (1969) Arch. Int. Physiol. Biochem. 77, 154—156
36 Wolfinbarger, L. Jr. and DeBusk, A.G. (1972) Biochim. Biophys. Acta 290, 355—367
37 Hunter, D.R. and Segel, I.H. (1971) Arch. Biochem. Biophys. 144, 168—183