Biochimica et Biophysica Acta, 394 (1975) 470–481

BBA 76988

ENERGY REQUIREMENTS FOR THE UPTAKE OF L-LEUCINE BY *SACCHAROMYCES CEREVISIAE**

E. H. RAMOS, L. C. DE BONGIOANNI, M. L. CLAISSE** and A. O. M. STOPPANI

Instituto de Quimica Biológica, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Buenos Aires (Argentina)

(Received January 3rd, 1975)

SUMMARY

(1) Substrates capable of activating mitochondrial electron transfer and oxidative phosphorylation, namely, pyruvate, acetate, propionaldehyde and butanol, stimulated the concentrative uptake (transport and accumulation) of L-[^{14}C]leucine by *Saccharomyces cerevisiae* (wild type strain 207, starved cells). Under adequate experimental conditions, the L-[^{14}C]leucine uptake versus the oxygen uptake ratio was almost the same with either pyruvate, acetate or D-glucose as energy sources. Substrate oxidation also increased L-[^{14}C]leucine incorporation into the cell protein.

(2) With *S. cerevisiae* D261 and D247-2 and propionaldehyde as an energy source, or with strain 207 and glucose as energy source, 2,4-dinitrophenol (50 μM) inhibited L-[^{14}C]leucine uptake, the inhibition being accompanied by stimulation of respiration. With *S. cerevisiae* 207 and propionaldehyde as energy source, 2,4-dinitrophenol inhibited both respiration and L-[^{14}C]leucine uptake, but with respiration being less affected than uptake. Displacement of accumulated L-[^{14}C] leucine was also inhibited by 2,4-dinitrophenol.

(3) In the presence of glucose, and for relatively brief incubation periods, anaerobically grown cells of *S. cerevisiae* 207 and of a ρ^- "petite" mutant of this strain incorporated L-[^{14}C]leucine with less efficiency than the original wild type strain 207, grown aerobically. With D-glucose as energy source, 2,4-dinitrophenol and iodoacetate inhibited alike L-[^{14}C]leucine uptake by the respiration competent cells.

(4) It is postulated that in respiration-competent yeasts, the mitochondrion contributes to L-[^{14}C]leucine uptake by supplying high-energy compounds required for amino acid transport and accumulation. Conversely, the promitochondrion in the anaerobically grown yeast, or the modified mitochondrion in the respiratory deficient mutant, competes for high energy compounds generated by glycolysis in the cytosol.

INTRODUCTION

Uptake of amino acids by *Saccharomyces* species is an energy-coupled process,

* Paper I in the series "Studies on amino acid uptake by yeasts".

** Permanent address: Laboratoire de Génétique Moléculaire du Centre National de la Recherche Scientifique, Gif-sur-Yvette, 91190, France.

as shown by its stimulation by suitable sources of metabolic energy, such as D-glucose and its inhibition by uncouplers of oxidative phosphorylation [1–5]. In the present study we have further investigated the nature of the energy sources capable of driving amino acid uptake in *Saccharomyces cerevisiae*. In that context, we have measured uptake of L-[^{14}C]leucine by: (a) wild type *S. cerevisiae* with low levels of endogenous substrates (starved cells); (b) the same yeast, energized by simultaneous oxidation of substrates capable of activating mitochondrial electron transfer and oxidative phosphorylation; (c) anaerobically grown, cytochrome-deficient and respiration-incompetent yeast [6] (starved, or energized by incubation with D-glucose) and (d) a cytochrome-deficient and respiration-incompetent [7, 8] ρ^- mutant, obtained from the wild type by the acriflavin method [9] (starved, or energized as above). Moreover, 2,4-dinitrophenol, a typical uncoupler of oxidative phosphorylation, and iodoacetate, a selective inhibitor of glycolysis, have been assayed on transport, accumulation and displacement of L-[^{14}C]leucine, with both the respiration-competent and the respiration-deficient yeasts.

MATERIALS AND METHODS

Yeasts and growth conditions

The yeasts used were: *S. cerevisiae*, diploid, wild type, strain 207 from the collection of the Institute of Agricultural Microbiology (Buenos Aires University); a cytoplasmatic ρ^- mutant, "petite colonie", isolated from the former strain by the acriflavine method [9]; diploid strain D261 (wild type yeast) and strain D247-2 (a nuclear mutant homozygote for the mutated gene cyc 1-1, that produces a partial deficiency in cytochrome *c* due to a complete absence of iso-1-cytochrome *c*, only iso-2-cytochrome *c* being synthesized [10, 11]), both from the Laboratoire de Genetique Physiologique, Gif-sur-Yvette (France).

The yeasts were grown in a Wickerham medium [12], in a New Brunswick 610 shaker placed in a constant temperature room, at 30 °C, until the early stationary phase was reached. The harvested cells were centrifuged at 4 °C, washed twice with distilled water and starved overnight as previously described [13]. Anaerobic cultures were performed in a 2-l flask provided with a three-way stopcock, a lateral safety valve with Hg and a magnetic stirrer. The medium contained: 100 g D-glucose, 70 g yeast extract (Difco), 5 g KH_2PO_4, 0.3 g Tween 20, 1.25 g Tween 80, 37.5 mg ergosterol and water to 1 l. After adding the inoculum, nitrogen (99.8 %) was bubbled through the medium for 2 h. The gas inlet was closed and the culture was allowed to continue, at 30 °C, with constant stirring. A nitrogen atmosphere was ensured through the culture medium until the stationary phase was reached. The yeast suspension was quickly poured into tubes containing ice and immediately centrifuged at 0 °C. The yeast was collected, suspended in distilled water and starved under nitrogen atmosphere. The absorption spectra of the yeasts employed is shown in Fig. 1. The presented spectra are in accordance with previous observations by other authors [7, 14]. Neither the ρ^- mutant nor the anaerobically grown yeast respired when oxygen uptake was assayed in the Gilson Oxygraph (model K).

L-[U-^{14}C]Leucine was purchased from the Radiochemical Centre, Amersham, U.K., through the Argentine Atomic Energy Commission and diluted with non-isotopic carrier as required.

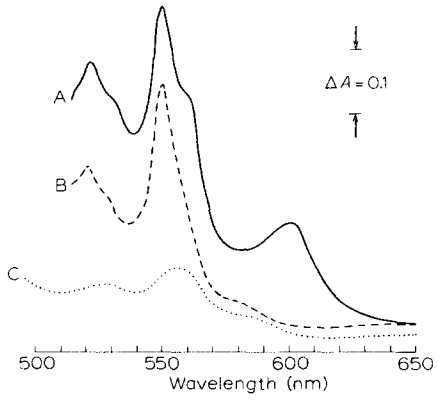

Fig. 1. Absorption spectrum of yeasts. A frame fixed near the phototube allowed the insertion of a 2 mm-thick sample of yeast paste in the sample beam and tracing paper in the reference beam. Other conditions were as described under Materials and Methods. A, wild-type yeast; B, anaerobically grown, wild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

Chemicals

L-Leucine and iodoacetic acid were purchased from Sigma Chemical Co. (iodoacetic acid was recrystallized from ethyl ether); 2,4-dinitrophenol was purchased from British Drug Houses Ltd, and D-glucose was purchased from Mallinckrodt. Other chemicals were of analytical grade.

Incubation techniques

Kinetics of L-[^{14}C]leucine uptake and displacement were carried out at 30 °C in a New Brunswick Gyratory Water Bath Shaker (model T.76) with 10 ml incubation mixture containing, unless otherwise stated, yeast (2 mg/ml), 20 mM phthalate buffer (K) pH 4.5 and additions as specified in each case. Anaerobic experiments were performed in specially designed vessels gassed with nitrogen (98.8%). Gassing was started 10 min before the beginning of the incubation. Oxygen uptake was measured by the Warburg direct method in manometers at 30 °C with air in the gas space.

Analytical methods

Determination of total intracellular L-[^{14}C]leucine was carried out by the following methods.

Method A. One ml of incubation mixture was filtered through a Millipore filter 25 ea, HA, 0.45 μm. The cells were immediately washed four times with 3–4 ml of 20 mM cold phthalate buffer, pH 4.5. Control experiments showed that cold (0 °C) washing did not extract intracellular ^{14}C-labelled compounds. The filtrates were discarded except in displacement experiments. The Millipore filters, after drying, were counted for ^{14}C. In kinetic experiments zero-time samples were taken and subtracted as the blank value.

Method B. One ml of incubation mixture was thoroughly mixed with 1 ml of 20 mM phthalate buffer (pH 4.5) at 0 °C and cells were washed twice in the centrifuge at 4 °C with the same phthalate buffer (1.0 ml). The washed cells were suspended in a 9 : 1 (v/v) methanol/water mixture (1.0–2.0 ml) and total suspension and the methanol

water-soluble ^{14}C-labeled material were counted directly. In order to establish possible conversion of L-[^{14}C]leucine into other substances, the methanol/water-soluble fraction was subjected to chromatography and radioautography in accordance with previously described methods [15]. As reported under Results, that conversion did not occur, at least not to a detectable extent, and therefore total ^{14}C values in cell suspensions or the methanol/water-soluble fraction were representative of their L-[^{14}C]leucine content. In one experiment (Table I, Expt 2) L-[^{14}C]leucine was eluted from the respective radioactive area in the chromatogram and counted. Radioactivity values in methanol/water extracts fit in well with those in water extracts from the same yeast. The water extracts were prepared by keeping 1–2 ml of yeast suspension (in distilled water) immersed in boiling water for 5 min, and after centrifugation, ^{14}C activity was measured in the supernatant.

After methanol/water extraction, the yeast residue was extracted twice with 5 % (w/v) trichloroacetic acid (1–2 ml) at 0–4 °C and, after centrifugation, the precipitated ^{14}C activity was measured; this is termed non-soluble- (or protein-) fraction radioactivity.

Displacement of intracellular L-[^{14}C]*leucine.* This was determined by measuring ^{14}C in the yeast cells after removal from the suspending medium by Millipore filtration (as described above). In some experiments ^{14}C activity in filtrates was measured.

Assay of radioactive samples. Total ^{14}C activity of samples in Millipore filters was counted in a Nuclear Chicago, Model 724 system with a scintillation mixture made of 2,5-diphenyloxazole (5 g) and *p*-bis-2-(5-phenyloxazolyl) (POPOP) (50 mg) in 1 l of toluene. ^{14}C activity in filtrates was counted by Bray's method [16].

Other methods. The cytochrome spectrum of yeast was determined as described by Cazzulo et al. [17] with a specially adapted Beckman DK-2 spectrophotometer, equipped with a xenon lamp PEX-75.

Expression of results. Unless otherwise stated, uptake of L-[^{14}C]-leucine by yeasts is expressed as μmol/g of cells. The concentration of yeast suspension is expressed by the weight after drying at 104 °C for 24 h.

RESULTS

Experiments with the wild type (aerobic) yeasts

Fig. 2a shows the general characteristics of L-[^{14}C]leucine uptake in the absence of exogenous substrates. The pH was 4.5 which was adequate for the subsequent assay of inhibitors such as 2,4-dinitrophenol and iodoacetate which permeate the cell membrane in the non-dissociated form [18, 19]. Maximal uptake rates were maintained during the first 10–30 min of incubation with 1–5 mM L-[^{14}C]leucine, and after 60 min incubation, an apparent steady-state was attained. Chromatographic and radioautographic analysis of the yeast soluble fraction showed that after incubation, L-leucine remained as the sole radioactive compound. Moreover, L-[^{14}C]leucine in the soluble cell fraction amounted to 98 % of total incorporated ^{14}C with the 30-min samples and to 95 % with the 90-min samples. Uptake was concentrative, as shown by the apparent concentration ratios (intracellular to extracellular) attained at the end of incubation (Fig. 2a, legend). Under the given conditions, displacement of accumulated L-[^{14}C]-leucine was very limited, since after 15 min reincubation with 1.0 mM external L-leucine, the efflux of internal L-[^{14}C]leucine amounted to 17 % of the initial value,

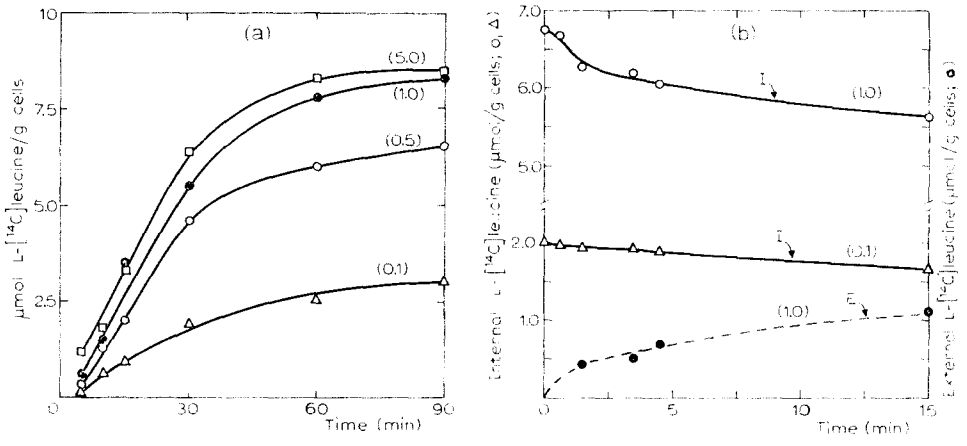

Fig. 2. (a) Kinetics of L-[^{14}C]leucine uptake by starved yeast. Cells suspended in 20 mM phthalate buffer (pH 4.5) containing L-[^{14}C]leucine in concentration (mM) as stated in parenthesis. Internal radioactivity was measured with Method B. Final intracellular concentration of L-[^{14}C]leucine (mM, with respect to cell water (60 % of wet weight [2])) was (in parenthesis, external initial concentration of L-[^{14}C]leucine): 2.0 (0.1); 4.3 (0.5); 5.5 (1.0) and 5.6 (5.0). (b) Displacement of accumulated L-[^{14}C]leucine by external L-leucine. Starved cells suspended in 15 ml of 20 mM phthalate buffer (pH 4.5) containing 0.1 or 1.0 mM L-[^{14}C]leucine. After 30 min incubation the cells were centrifuged, washed twice in the centrifuge with ice-cold distilled water (10 ml) and resuspended in 20 mM phthalate buffer (pH 4.5) containing 0.1 or 1.0 mM L-leucine. Internal (I; △, ○) and external (E; ●) radioactivities were measured as described in the text (in parenthesis, concentration of external L-leucine (mM) which was the same as used during preincubation with L-[^{14}C]leucine). Other conditions were as described under Materials and Methods.

matching the disappearance of intracellular L-[^{14}C]leucine with the amino acid appearance in the external medium (Fig. 2b). It should be noted that displacement values were smaller than those reported by other workers [2, 3], which may be due, firstly, to the more acidic pH employed in our experiments and secondly, to the absence of an energy source capable of driving the influx of the non-radioactive amino acid.

Table I shows the effect of different sources of metabolic energy for L-[^{14}C]-leucine uptake. It may be seen that, in addition to glucose, non-fermentable substrates capable of activating electron transfer and oxidative phosphorylation (pyruvate, acetate and butanol) increased L-[^{14}C]leucine uptake. Under the same experimental conditions, propionate, succinate, fumarate and glycine were ineffective. The concentrative nature of L-[^{14}C]leucine uptake was confirmed by the effect of the oxidized substrates, despite the relatively high external concentration of amino acid (5 mM). In this experiment, uptake was measured after 3 h incubation, in order to minimize the effect of the relatively slow rate of acetate and pyruvate oxidation by starved yeast [15]. An empirical correlation of L-[^{14}C]leucine uptake and oxygen consumtion yielded approximately the same value with D-glucose, pyruvate and acetate (Table I). Analysis of the incubated yeasts (soluble fraction) showed that despite substrate oxidation, L-leucine remained as the sole radioactive compound. Oxidation of endogenous substrates could also drive L-[^{14}C]leucine uptake, as results from the significant difference between the starved and non-starved yeast in Fig. 3.

TABLE I

EFFECT OF SUBSTRATE OXIDATION ON L-[^{14}C]LEUCINE UPTAKE BY *S. CEREVISIAE* (WILD TYPE)

Starved cells (4.8 (Expt 1) or 10.9 (Expt 2) mg/ml; strain 207) were incubated in Warburg-manometers in 20 mM phthalate buffer (pH 4.5) containing 5 mM L-[^{14}C]leucine (5.2 (Expt 1) or 1.1 (Expt 2) $\times 10^4$ counts/min), and addition as stated below; total volume, 3 ml; 3 h incubation. In Expt 1, uptake of L-[^{14}C]leucine was measured by Method B. In Expt 2, uptake of *p*-[^{14}C]leucine in the soluble fraction was measured by counting ^{14}C in the eluate of the L-leucine area in the chromatogram. Other experimental details were as described under Materials and Methods.

Addition (5 mM)	Expt 1				Expt 2	
	L-[^{14}C]leucine uptake (μmol/g of cells)		Total O_2 uptake $\left(\frac{\text{mmol}}{\text{g of cells}}\right)$ (*b*)	$\frac{(a)}{(b)} \times 10$	L-[^{14}C]leucine uptake in the soluble cell fraction $\left(\frac{\text{mmol}}{\text{l of cell water}}\right)$	Apparent internal vs external concentration ratio*
	Total (*a*)	Non-soluble fraction				
D-Glucose	357	45	3.51	1.01	105	21.0
Pyruvate	134	13	1.32	1.01	39	7.8
Acetate	87	7	0.95	0.91	37	7.4
Butanol	—	—	—	—	28	5.6
None	11	1	0.21(0.25)**	—	17	3.4

* Since the concentration of unlabelled (endogenous) L-leucine is neglected and the calculation is based on the initial (5 mM) concentration of external L-[^{14}C]leucine, ratios may be higher than stated (this applies also to values in Fig. 1 (legend)).

** L-[^{14}C]leucine omitted.

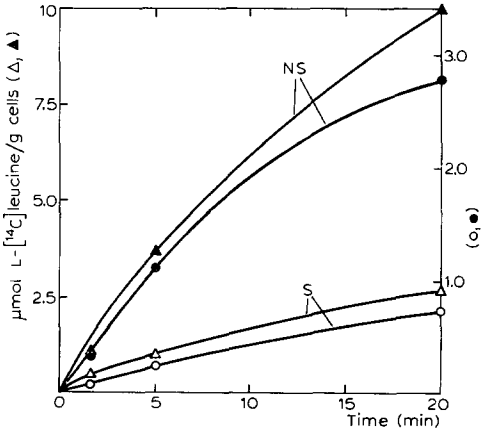

Fig. 3. Comparative uptake of L-[^{14}C]leucine by starved (S) and non-starved (NS) yeast. Cells suspended in 20 mM phthalate buffer (pH 4.5) containing 0.1 or 1.0 mM L-[^{14}C]leucine. Left ordinate, results with external 1.0 mM L-[^{14}C]leucine: (△), starved cells; (▲), non-starved cells. Right ordinate, results with external 0.1 mM L-[^{14}C]leucine: (○), starved cells; (●), non-starved cells. Other conditions were as described under Materials and Methods.

TABLE II

EFFECT OF PROPIONALDEHYDE AND 2,4-DINITROPHENOL ON L-[^{14}C]LEUCINE UPTAKE BY *S. CEREVISIAE* D261 and D247-2

Yeast D261, 5 mg; D247-2, 4.1 mg; 5 mM L-[^{14}C]leucine (4.1 · 10^4 counts/min); time of incubation, 1.5 h; other experimental conditions were as in Table I.

Propionaldehyde (mM)	2,4-Dinitrophenol (μM)	*S. cerevisiae* D261			*S. cerevisiae* D247-2		
		L-[^{14}C]leucine uptake (μmol/g cells)		Q_{O_2}	L-[^{14}C]leucine uptake (μmol/g cells)		Q_{O_2}
		Total	Non-soluble fraction		Total	Non-soluble fraction	
0	0	93	0	3.7	92	0	4.0
	50	91	0	25	119	0	21
5	0	194	11	13	252	16	17
	50	78	3	30	77	2	23

Table II shows the effect of propionaldehyde on L-[^{14}C]leucine uptake by yeasts D261 and D247-2. Propionaldehyde was oxidized to propionate by two aldehyde dehydrogenases (one exclusively NADP-requiring [20, 21]) and therefore, the increase of L-[^{14}C]leucine uptake depended on the oxidation of reduced pyridine nucleotides in mitochondria. 2,4-Dinitrophenol inhibited (59–69 %) the propionaldehyde-dependent uptake and also amino acid incorporation into the cell protein. Simultaneously, 2,4-dinitrophenol released the respiratory control and stimulated respiration in good agreement with its well known action on oxidative phosphorylation. With

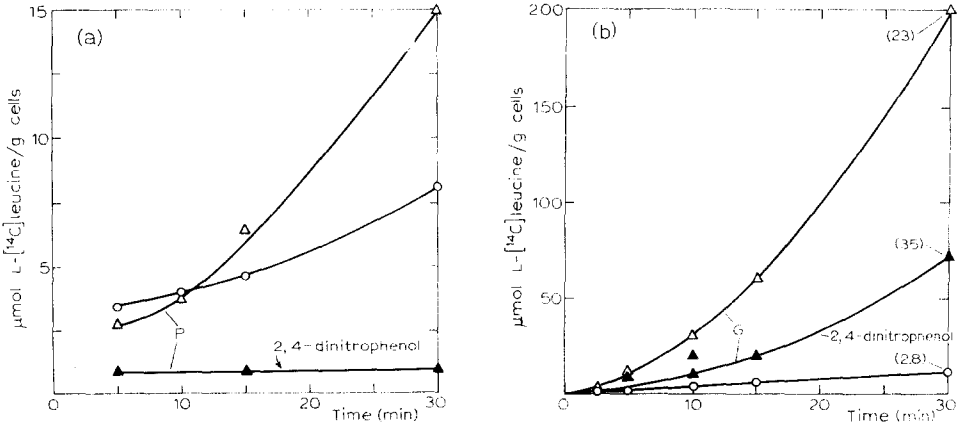

Fig. 4. (a) Effect of propionaldehyde (P) and 2,4-dinitrophenol on L-[^{14}C]leucine uptake. Starved cells suspended in 20 mM phthalate buffer (pH 4.5) containing 5 mM L-[^{14}C]leucine. Internal radioactivity was measured with Method B. (○), control; (△), same, plus 5 mM propionaldehyde; (▲), same, plus 5 mM propionaldehyde and 50 μM 2,4-dinitrophenol. (b) Effect of glucose (G) and 2,4-dinitrophenol on L-[^{14}C]leucine uptake. Unless otherwise stated conditions were as in (a). (○), control; (△), same, plus 160 mM glucose; (▲), same, plus 160 mM glucose and 60 μM 2,4-dinitrophenol. In parenthesis, oxygen uptake in μl/mg of cells at the indicated time. Other conditions were as described under Materials and Methods.

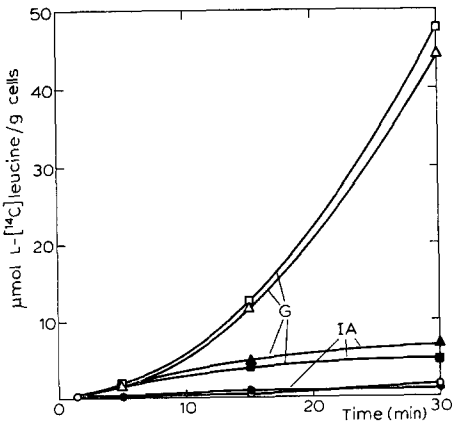

Fig. 5. Effect of glucose (G) and iodoacetate (IA) on L-[^{14}C]leucine uptake. Starved cells suspended in 20 mM phthalate buffer (pH 4.5) containing 1.0 mM L-[^{14}C]leucine with or without additions. Internal radioactivity was measured with method A. (○), control; (●), same, plus 1 mM iodoacetate; (△), same, plus 5 mM glucose; (▲), same, plus 5 mM glucose and 1 mM iodoacetate; (□), same plus 160 mM glucose; (■), same, plus 160 mM glucose and 1 mM iodoacetate. Other conditions were as described under Materials and Methods.

yeast 207 and propionaldehyde as substrate (Fig. 4a), 2,4-dinitrophenol inhibited uptake by 90 % and respiration by 70 % (not shown in the figure), the inhibition of respiration being in accordance with previous observations by Stoppani et al. [22]. This inhibition of respiration was not due to a direct action of 2,4-dinitrophenol on electron transfer, since, with the same yeast and glucose as substrate (Fig. 4b), the uncoupler, though inhibiting amino acid uptake by 65 %, increased the rate of respiration by 50 %.

Fig. 5 shows that iodoacetate, a typical inhibitor of glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase [23] inhibited about 90 % the glucose-dependent uptake. The effect of iodoacetate, and those of 2,4-dinitrophenol in Table II, Figs 4a and 4b must be interpreted as an inhibition of uptake (or accumulation) of L-[^{14}C]leucine rather than as an exaggerated displacement of the accumulated amino acid. In fact (Fig. 6), when cells loaded with L-[^{14}C]leucine were reincubated with the inhibitors, iodoacetate did not affect the displacement of intracellular L-[^{14}C]leucine while 2,4-dinitrophenol inhibited it by 45 % (at 2 min) and 55 % (at 5 min).

Experiments with respiration-deficient yeasts

Fig. 7a shows the L-[^{14}C]leucine uptake, under nitrogen atmosphere, by the anaerobically grown wild type yeast. Uptake was lower than that observed under air with the aerobically grown yeast (compare Figs 5 and 7a). Although lower than for the aerobic experiment, the amino acid uptake was still greatly increased by the addition of glucose. In this case, the initial rate of entrance doubled, while at 15 min incubation the amino acid incorporation was increased sixfold. 2,4-Dinitrophenol inhibited both the initial rate of entrance and total uptake of L-[^{14}C]leucine; at 15 min incubation, 53–55 % inhibitions were obtained in the presence of glucose and 60–70 % inhibitions were observed in the absence of added substrate (Fig. 7a, inset).

Fig. 7b shows the kinetics of L-[^{14}C]leucine uptake by the ρ^-, "petite" mutant, in the presence and absence of glucose. For comparative purposes, uptake values ob-

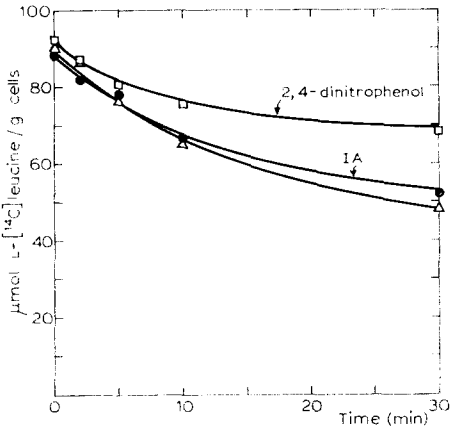

Fig. 6. Displacement of accumulated L-[^{14}C]leucine (effect of inhibitors). Yeast cells suspended in 20 mM phthalate buffer (pH 4.5) containing 5 mM L-[^{14}C]leucine and 160 mM glucose. After 30 min incubation the cells were washed in the centrifuge, suspended in 20 mM phthalate buffer (pH 4.5) and reincubated with the following additions: (△), 5 mM L-leucine; (●), 5 mM L-leucine plus 1 mM iodoacetate (IA); (□), 5 mM L-leucine plus 50 μM 2,4-dinitrophenol. Other conditions were as described in Fig. 2 (b) and under Materials and Methods. The points represent internal radioactivity.

tained in a parallel experiment with the original wild type yeast are included. In the absence of glucose the "petite" mutant took up less L-[^{14}C]leucine than the wild type yeast. In the presence of 160 mM glucose this was also verified for the shorter incubation periods, but at 15 min incubation the uptake of L-[^{14}C]leucine by the mutant was somewhat decreased with respect to the uptake by the wild type strain. (Similar results were obtained with 5 mM glucose.)

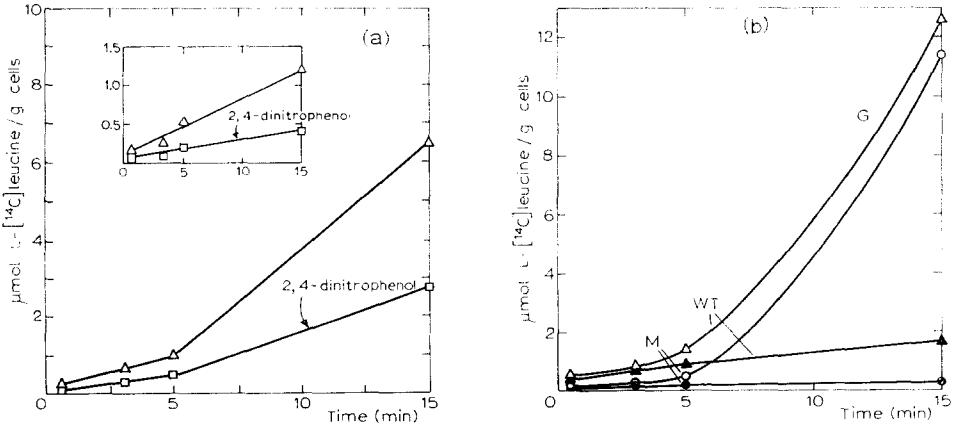

Fig. 7. (a) Effect of glucose (G) and 2,4-dinitrophenol on L-[^{14}C]leucine uptake by the anaerobically grown yeast. Starved cells suspended in 20 mM phthalate buffer (pH 4.5) containing 1 mM L-[^{14}C]leucine; nitrogen atmosphere. Internal radioactivity was measured with Method A. (△), control plus 160 mM glucose; (□), same, plus 50 μM 2,4-dinitrophenol. Inset: (△), control; (□), same, plus 50 μM 2,4-dinitrophenol. Other conditions were as described under Materials and Methods. (b) L-[^{14}C]leucine uptake by the ρ^- mutant (comparison with the wild type yeast). ρ^- mutant (M): (●), control; (○), same, plus 160 mM glucose. Wild type yeast (WT): (▲), control; (△), same, plus 160 mM glucose. Other conditions were as described under Materials and Methods.

With the ρ^- mutant, glucose increased the initial rate of entrance, the final total uptake and the incorporation of L-[^{14}C]leucine into the cell protein (15 % of total ^{14}C uptake). In contrast to the effect of glucose, 5 mM propionaldehyde inhibited uptake (experimental data omitted in Fig. 7b).

In accordance with the inhibitions observed with the wild-type yeast, 2,4-dinitrophenol and iodoacetate inhibited uptake of L-[^{14}C]leucine by the ρ^- mutant (Table III). However, 2,4-dinitrophenol did not inhibit the initial rate of entrance (uptake at 2.5 min incubation). On the other hand iodoacetate inhibited the stimulatory effect of glucose both on the rate of entrance (3 min incubation) and on total uptake (10 min incubation), the latter inhibition being about 66 %. Displacement of accumulated L-[^{14}C]leucine was irrelevant for 2,4-dinitrophenol and iodoacetate effects, since under conditions as described in Fig. 6, the inhibitors did not stimulate displacement of accumulated L-[^{14}C]leucine and moreover, at 30 min incubation, 2,4-dinitrophenol inhibited displacement by 32 %.

TABLE III

EFFECT OF GLUCOSE, 2,4-DINITROPHENOL AND IODOACETATE ON L-[^{14}C]LEUCINE UPTAKE BY THE "PETITE" MUTANT

Starved cells were suspended in 20 mM phthalate buffer (pH 4.5) containing 1 mM L-[^{14}C]leucine with additions as stated below. Samples were taken at the indicated time intervals and internal radioactivity was measured by Method A. Other experimental details were as described under Materials and Methods.

Expt	Glucose (mM)	Inhibitor	L-[^{14}C]leucine uptake (μmol/g cells)	
			2.5 min	10 min
1	0	None	0.164	0.220
		2,4-Dinitrophenol (50 μM)	0.188	0.252
	160	None	0.225	4.78
		2,4-Dinitrophenol (50 μM)	0.206	1.64
			3.0 min	10 min
2	0	None	0.145	0.142
	160		0.350	8.74
		Iodoacetate (1 mM)	0.230	3.46

DISCUSSION

Entrance (and accumulation) of amino acid in yeasts involves the operation of permeases [2, 3, 24, 25] and energy-yielding mechanisms [1–5]. The results presented here allow one to compare L-[^{14}C]leucine uptake by two types of yeasts essentially different in their energy supply mechanism, that is to say, one yeast endowed with a competent mitochondrial electron-transfer system (wild type, aerobically grown yeast) and two yeasts lacking several cytochromes, including the terminal oxidase (anaerobically grown and ρ^- mutant yeasts). Glucose increased the rate of entrance and accumulation of L-[^{14}C]leucine with all the assayed yeasts (Figs 4b, 5 and 7) in accordance with the view that glycolytically generated high-energy compounds energize the trans-

port (and accumulation) of amino acids. Furthermore, it is known [24] that high-energy compounds and amino acids resulting from glucose catabolism (aerobic and anaerobic) stimulate the rate of protein synthesis, which explains the glucose effect on L-[^{14}C]leucine incorporation into the non-soluble cell fraction in Table I. At a relatively high concentration of glucose (160 mM, Figs 7a and 7b) the uptake values for the wild type and the mutant yeasts were much alike (Fig. 7b), demonstrating that a normal mitochondrion was not essential for amino acid uptake. Nevertheless, the effects of pyruvate, acetate, butanol and propionaldehyde in Tables I and II show that metabolic energy originating in the mitochondrion was used, when necessary, to drive transport (and accumulation) of L-[^{14}C]leucine and also amino acid incorporation into the cell protein. In fact, the oxidation of those substrates compulsorily determined electron transfer and oxidative phosphorylation [13, 15, 22, 26] and moreover, in long-term incubation experiments, pyruvate and acetate were as effective as glucose (Table I) when their effectiveness was compared on the basis of oxygen consumption.

The results presented here lead us to postulate that the mitochondrion contributed to L-[^{14}C]leucine uptake by supplying high-energy compounds required for amino acid translocation (and accumulation) since direct coupling of amino acid translocation and electron transfer (as occurs in membrane preparations from *Escherichia coli* and other organisms [27]) was precluded by the absence of electron carriers in the yeast external membrane (plasmalemma) [28]. Reciprocally, the inhibition of L-[^{14}C]leucine uptake by 2,4-dinitrophenol (Tables II and Figs 4a and 4b) would reflect the depletion of high-energy phosphates at the translocating mechanism. This assumption is borne out by the effect of 2,4-dinitrophenol on yeast ATPase [29, 30] and also on the intracellular concentration of soluble nucleotide and inorganic polyphosphates [22]. With the respiratory-deficient yeasts, that contain a promitochondrial, uncoupler-activated ATPase [30], 2,4-dinitrophenol may stimulate the hydrolysis of glycolytically generated ATP, otherwise available for the energization of amino acid translocation. Accessibility of cytosol ATP to mitochondrial (or promitochondrial) energy-conserving mechanisms (that include the ATPase) was earlier postulated by Slater [31] and more recently by Groot et al. [32] and Subík et al. [33].

The effect of 2,4-dinitrophenol with the respiration-deficient yeasts (Figs 7a and 7b and Table III) is another instance of the uncoupler's ability to inhibit, at relatively low concentrations, energy-dependent anaerobic processes, for example protein synthesis [34] and the anaerobic operation of some reactions of the Krebs cycle [35] (in yeasts), or amino acid transport (in *Staphylococcus aureus* [36]).

Fig. 5 and Table III show that iodoacetate was an effective inhibitor of L-[^{14}C]-leucine uptake by aerobic and anaerobic yeasts, particularly after 15 min incubation (Fig. 5). Since iodoacetate inhibits glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase [23], limitation of energy supply seems to be a suitable mechanism for iodoacetate effect on L-[^{14}C]leucine uptake. Besides, iodoacetate inhibited amino acid incorporation into the cell protein (non-soluble fraction) with little change in the soluble pool and without increasing the displacement of internal L-[^{14}C]leucine to the external medium (Fig. 6). The inhibition of protein synthesis could indirectly exert a regulatory effect on the entrance process [37], mediated by unbound L-[^{14}C]-leucine.

ACKNOWLEDGEMENTS

This work was supported by grants of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Comisión Nacional de Energía Atómica. Mrs F. E. G. de Di Nasso gave able technical assistance. E. H. R. and A. O. M. S. are Career Investigators of CONICET.

REFERENCES

1 Halvorson, H. O., Fry, W. and Schwemmin, D. (1955) J. Gen. Physiol. 38, 549–573
2 Surdin, Y., Sly, W., Sire, J., Bordes, A. M. and De Robichon-Szulmajster, H. (1965) Biochim. Biophys. Acta 107, 546–566
3 Magaña-Schwenke, N. and Schwencke, J. (1969) Biochim. Biophys. Acta 173, 313–323
4 Eddy, A. A., Indge, K. J., Backen, K. and Nowacki, J. A. (1970) Biochem. J. 120, 845–852
5 Kotyk, A. and Rihová, L. (1972) Biochim. Biophys. Acta 288, 380–389
6 Criddle, R. S. and Schatz, G. (1969) Biochemistry 8, 322–334
7 Slonimski, P. P. (1949) Ann. Inst. Pasteur 76, 510–530
8 Slonimski, P. P. and Ephrussi, B. (1949) Ann. Inst. Pasteur 77, 47–63
9 Ephrussi, B., Hottinger, H. and Chimenes, A. M. (1949) Ann. Inst. Pasteur 76, 351–367
10 Sherman, F. and Slonimski, P. P. (1964) Biochim. Biophys. Acta 90, 1–15
11 Slonimski, P. P., Acher, R., Péré, G., Sels, A. and Somlo, M. (1965) in Régulations chez les Microorganismes, Colloque International du C.N.R.S., No. 124, pp. 435–461, Editions du C.N.R.S., Paris
12 Wickerham, L. J. (1951) U.S. Dept. Agr. Tech. Bull. No. 1029
13 Stoppani, A. O. M. and Ramos, E. H. (1964) Arch. Biochem. Biophys. 105, 470–482
14 Somlo, M. and Fukuhara, H. (1965) Biochem. Biophys. Res. Commun. 19, 587–591
15 Stoppani, A. O. M., Conches de Bongioanni, L., de Favelukes, S. L. S. and Sacerdote, F. L. (1958) Biochem. J. 70, 438–455
16 Bray, G. A. (1960) Anal. Biochem. 1, 279–285
17 Cazzulo, J. J., Claisse, L. M. and Stoppani, A. O. M. (1968) J. Bacteriol. 96, 623–628
18 Stoppani, A. O. M. (1949) Nature 164, 1096–1097
19 Aldous, J. G. (1948) J. Biol. Chem. 176, 83–88
20 Black, S. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 508–511, Academic Press, New York
21 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
22 Stoppani, A. O. M., Bennun, A. and De Pahn, E. M. (1964) Arch. Biochem. Biophys. 108, 258–265
23 Nord, F. F. and Weiss, S. (1951) in The Enzymes (Sumner, J. B. and Myrback, K., eds), Vol. 2, pp. 684–790, Academic Press, New York
24 Halvorson, H. O. and Cohen, G. N. (1958) Ann. Inst. Pasteur 95, 73–87
25 Grenson, M., Hou, C. and Crabeel, M. (1970) J. Bacteriol. 103, 770–777
26 Polakis, E. S. and Bartley, W. (1965) Biochem. J. 97, 284–297
27 Kaback, H. R. (1972) in The Molecular Basis of Biological Transport (Woessner, Jr, J. F. and Huijing, F., eds), pp. 291–319, Academic Press, New York
28 Fuhrmann, G. F., Wehrli, E. and Boehm, C. (1974) Biochim. Biophys. Acta 363, 295–310
29 Bennun, A., De Pahn, E. M. and Stoppani (1964) Biochim. Biophys. Acta 89, 532–539
30 Schatz, G. (1968) J. Biol. Chem. 243, 2192–2199
31 Slater, E. C. (1953) Nature 172, 975–978
32 Groot, G. S. P., Kovác, L. and Schatz, G. (1971) Proc. Natl. Acad. Sci. U. S. 68, 308–311
33 Subik, J., Kolarov, J. and Kovác, L. (1974) Biochim. Biophys. Acta 357, 453–456
34 Kovác, L. and Istenesová, A. (1964) Biochim. Biophys. Acta 82, 162–164
35 Stoppani, A. O. M., Bennun, A. and De Pahn, E. M. (1964) Biochim. Biophys. Acta 92, 176–178
36 Gale, E. F. (1951) Biochem. J. 48, 286–290
37 Grabeel, M. and Grenson, M. (1970) Eur. J. Biochem. 14, 197–204