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Contribution to the physiological characterization of glycerol active uptake in *Saccharomyces cerevisiae*

Fernanda Lages, Cândida Lucas *

Department of Biology, University of Minho, 4709 Braga Codex, Portugal

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Abstract

Evidence is presented here that in *Saccharomyces cerevisiae* IGC 3507, grown either on glycerol, ethanol or acetate, glycerol is transported by a high affinity uptake system of the electrogenic proton symport type, with K_m of 1.7 ± 0.7 mM, V_{max} 441 ± 19 $\mu mol\,h^{-1}\,g^{-1}$ dry weight and a stoichiometry of 1:1 proton per molecule of glycerol, at 30°C and pH 5.0. No competitors were found among other polyols and sugars. Glycerol maximum accumulation ratios followed p.m.f. with extracellular pH. CCCP prevented glycerol accumulation, and inhibited uptake. NaCl did not interfere with H^+/glycerol kinetics and energetics. This transport system was shown to be under glucose repression and inactivation. Glucose-grown cells presented, instead, a lower affinity permease for glycerol, probably a facilitated diffusion. Growth on glucose in the presence of NaCl did not induce the high affinity carrier. The stringent control of cell physiological condition over induction suggests for glycerol proton symport rather a physiological role connected with growth under gluconeogenic conditions.

Keywords: Glycerol transport; Glucose repression; Gluconeogenic induction; (*Saccharomyces cerevisiae*)

1. Introduction

Glycerol assimilation by *Saccharomyces cerevisiae* has long been considered to be a strain dependent phenomenon [1], probably the reason why it has been rather poorly explored up to the moment when attention has focused on the involvement of glycerol metabolism in osmoregulation. Since then, the generalised idea that glycerol, as a liposoluble compound, should walk freely through the lipid bilayer did no longer fit results. The compatible solute retention phenomena could possibly be better understood with the contribution of the activity of a mediated uptake for glycerol.

According to previous studies in other yeast species, more halotolerant than *S. cerevisiae*, like *Debaryomyces hansenii* [2], *Pichia sorbitophila* [3] or *Zygosaccharomyces rouxii* [4], glycerol was found to be actively transported, along with protons or sodium ions, thus establishing and maintaining a glycerol gradient in the presence of high concentrations of salt, counterbalancing glycerol's natural leakage. In *S. cerevisiae*, molecular biology evidence indicated that a specific permease could be involved in glycerol retention, connected with response to osmotic shock [5], produced either by high solute concentrations or by high ionic strength. The purpose of this work was to investigate on glycerol transport

* Corresponding author. Fax: +351-53-678980; E-mail: clucas@ci.uminho.pt

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across the plasma membrane of intact cells of a wild type strain of *S. cerevisiae*.

2. Materials and methods

2.1. Strains and growth conditions

S. cerevisiae IGC 3507 wild type strain was used. It was obtained from the Portuguese Yeast Culture Collection (PYCC), Gulbenkian Institute of Science (Portugal). It was maintained at room temperature in YEPDA (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar). Growth was performed in batch cultures, at 30°C and 160 rpm, in liquid mineral medium [6] or YEP, supplemented with 2% (w/v) glucose (YEPD), glycerol (YEPG) or ethanol (YEPE) as carbon sources.

2.2. Measurement of initial uptake rates

Cells were harvested in the early exponential growth phase ($A_{640\,nm} \approx 0.4$) by centrifugation, washed twice and resuspended to a final concentration of about 20–25 mg (dry weight) ml^{-1} in ice-cold distilled water. For estimating initial uptake rates of labelled glycerol, we used the method described earlier [3], using 100 mM Tris-citrate buffer pH 5.0 and aqueous solutions of [U^{14}C]glycerol (Amersham, 156 mCi/mmol, 50% ethanolic solution). The concentration of the final cell suspension was approximately 10 mg ml^{-1} dry weight. Sampling times for active glycerol uptake varied from 0 to 10 s (linearity of uptake was maintained up to 20 s), and for simple diffusion from 0 to 2 min. Concentration ranges up to 2.5 mM were used to measure saturable kinetics and from 3 to 20 mM to measure simple diffusion. Results were confirmed using [^{14}C]glycerol solutions with variable specific activity. The method used to estimate initial rates of proton uptake upon glycerol addition, in the absence or in the presence of several NaCl concentrations, was the same described earlier [2,3]. All the experiments were performed at 30°C.

The effect of other polyols or sugars over glycerol uptake was assayed incubating the cells for 20 s in 20 mM of each compound followed by a 10 s radiolabelled glycerol uptake experiment.

Kinetics of ethanol inhibition was determined incubating the cell suspension for 2 min in ethanol at crescent concentrations, from 5 to 15% (v/v). The effect of glucose was assayed incubating the cells in 20 to 100 mM glucose at different time intervals up to 30 min, after which glycerol uptake was assayed to determine the corresponding K_m and V_{max}. To assay glycerol transport eventual recovery after incubation in glucose, 400 μl cell suspension were incubated with 400 μl buffer containing 100 mM glucose (final concentration) for 30 min, quickly centrifuged, washed twice with ice-cold distilled water and re-incubated in buffer without glucose, with and without 10 μg ml^{-1} cycloheximide. Samples were taken at different time intervals and uptake was assayed as described above. Controls were performed before incubating in glucose and after 30 min incubation time.

2.3. Measurement of intracellular volume

The intracellular volume was measured as previously described [3,7,8]. Measurements were repeated in buffer with NaCl up to 1 M.

2.4. Measurement of [^{14}C]glycerol accumulation ratios

To measure [^{14}C]glycerol accumulation, the same method described before was used [3] with some modifications. The experiment was started by the addition of 10 mM [^{14}C]glycerol (ca. 700 dpm/nmol). Final cell concentration in each assay was approximately 15 mg dry weight per ml. Parallel experiments were performed adding, previous to the start of the reaction, 50 μM of CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone). The capacity to induce efflux of intracellularly accumulated labelled glycerol was assayed with 50 μM CCCP or 50 mM ''cold'' glycerol. The effect of extracellular pH on glycerol accumulation was tested over a pH range from 3 to 7, using the same buffer, in the absence and in the presence of 0.5 M NaCl [9].

2.5. Quenching controls

Quenching control of all experimental conditions involving the utilisation of scintillation counting was performed. Quenching effects, observed in the pres-

ence of high concentrations of NaCl, in experiments where neither filtering nor washing of the cells was performed, were avoided as mentioned by Lages and Lucas [3].

2.6. Estimation of intracellular and extracellular glycerol and glucose

Glucose and glycerol concentration measurements were performed by High Performance Liquid Chromatography (HPLC) analysis. (i) Growth media contents were analysed after incubation in ice for 30 min with 2% (v/v) perchloric acid, followed by a centrifugation at 12 000 rpm for 10 min at 4°C for protein precipitation. (ii) Intracellular contents were obtained with the following procedure. Culture samples were centrifuged and washed twice at 7000 rpm for 2 min at 4°C. The pellets obtained were incubated for 45 min at room temperature with trichloroacetic acid 5% (v/v) and shaken 15 min in a vortex, after which they were centrifuged at 3000 rpm for 15 min. Supernatants were kept in ice. Dilutions, when necessary, were performed with ultra-pure water obtained from a Permutit filtering device. Samples were mixed 1:1 with arabinose solution (10 $g\,l^{-1}$) as internal standard prior to injection. The HPLC used was a Gilson with a Merck Polyspher OA KC Cat. no. 51270 column, maintained at 50°C and using 0.05 N sulphuric acid in ultra-pure water as mobile phase at a flow rate of 0.5 $ml\,min^{-1}$.

2.7. Measurement of glycerol accumulation ratios by HPLC

To mimic experiments in which [^{14}C]glycerol accumulation was measured, 400 μl cell suspension were incubated at 30°C with 500 μl 100 mM Tris-citrate buffer pH 5.0 and 100 μl 100 mM glycerol were added to start the assay. The final cell suspension maintained the value indicated in Section 2.4. One such mixture was prepared to assay each desired incubation period of time. After a quick centrifugation, the supernatant, extracellular medium, was kept for control and the pellet was washed twice in ice cold distilled water and submitted to trichloroacetic acid treatment as described in Section 2.6. Controls were performed dosing glycerol in the supernatants of each washing.

2.8. Reproducibility of the results

All the experiments were repeated at least three times.

3. Results

3.1. Transport of glycerol by glycerol or ethanol-grown cells

S. cerevisiae presented very slow growth in mineral medium when glycerol was the sole carbon and energy source. Specific growth rate at 30°C was $0.10 \pm 0.03\ h^{-1}$ ($n = 5$), and lag phase lasted approximately 3 days. To overcome this difficulty, a starter of 0.2% (w/v) glucose was added. Glucose consumption preceded glycerol consumption and diauxy was observed. In YEPG, specific growth rate was $0.29 \pm 0.09\ h^{-1}$ ($n = 3$) and lag phase reduced to a few hours. Nevertheless, the velocity of glycerol disappearance from the medium, measured by HPLC, was 0.08 $g\,l^{-1}\,h^{-1}$ in mineral medium as in YEPG, an indication that growth in rich medium may, eventually, not be exclusively due to glycerol.

Early to mid-exponential cells in mineral medium containing either 2% (w/v) ethanol or glycerol, pre-

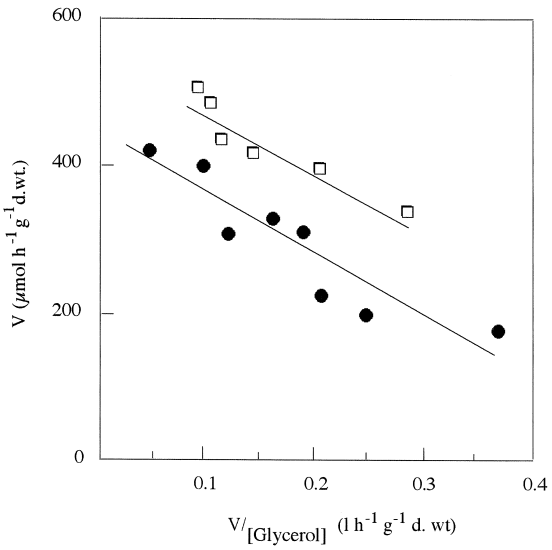

Fig. 1. Eadie–Hofstee plot of initial uptake rates at pH 5.0 of [^{14}C]glycerol (●) and protons upon glycerol addition (□) by cells of *S. cerevisiae* IGC 3507 grown in mineral medium supplemented with 2% (w/v) ethanol.

sented uptake for labelled glycerol following Michaelis–Menten kinetics (Fig. 1). The corresponding kinetic parameters, calculated from Eadie–Hofstee plots, are presented in Table 1. The uptake of labelled glycerol was totally inhibited by the protonophore CCCP [7,8] (not shown). Cells suspended either in water or 1 mM Tris-citrate buffer elicited transient extracellular alkalinization upon the addition of glycerol. Initial uptake rates for protons as a function of glycerol concentration followed Michaelis–Menten kinetics (Fig. 1). The corresponding parameters, calculated from Eadie–Hofstee plots, are also presented in Table 1. From both [^{14}C]glycerol and proton initial uptake rates, a stoichiometry of approximately 1 : 1 for a co-transport system of glycerol with protons was calculated both for glycerol or ethanol-grown cells. These results were confirmed in YEPG and YEPE (Table 1), as well as mineral medium with acetic acid 1% (v/v) as sole carbon and energy source (not shown).

To study the substrate specificity of the carrier, estimates of the initial uptake rates of labelled glycerol in the presence of several other polyols, sugars and non-metabolizable analogues of glycerol were obtained. Erythritol, D^{+}-arabitol, sorbitol, xylitol and D-mannitol as well as ethanediol, ethyleneglycol, 1,2-propanediol, 1,3-propanediol, polyethylenoglycol and dihydroxyacetone were used. Xylose and trehalose were also used. None of these compounds affected labelled glycerol uptake. Neither did they

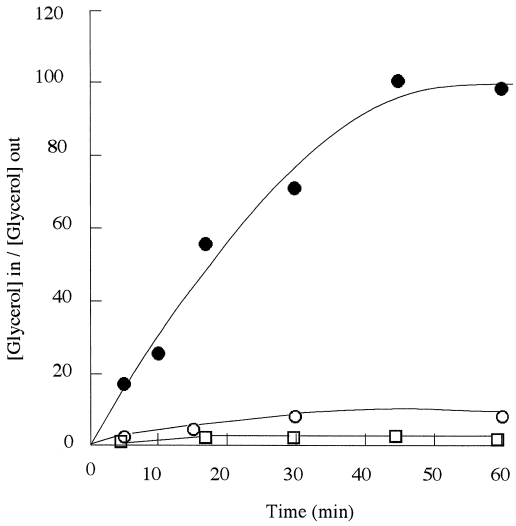

Fig. 2. Accumulation ratio of [^{14}C]glycerol in 100 mM Tris-citrate buffer pH 5.0, by cells of *S. cerevisiae* IGC 3507 grown in mineral medium supplemented with 2% (w/v) glycerol (●). Accumulation prevented by the addition of 50 μM CCCP (□). Accumulation ratio of glycerol measured by HPLC (○).

elicit extracellular alkalinization in cell suspensions assayed in the same conditions as mentioned above. Cold glycerol inhibited competitively [^{14}C]glycerol uptake (not shown). The effect of ethanol over glycerol proton symport was also studied. Exponential inhibition of V_{max} of [^{14}C]glycerol uptake was observed, while K_m was not affected significantly (not shown). From these results an exponential inhibition

Table 1
Kinetic parameters of [^{14}C]glycerol and proton uptakes at 30°C and pH 5.0 in cells of *S. cerevisiae* IGC 3507 grown in different carbon sources in either mineral or complete medium

Growth medium		Mineral medium		YEP	
Carbon source		Proton uptake upon glycerol addition	[^{14}C]Glycerol uptake	Proton uptake upon glycerol addition	[^{14}C]Glycerol uptake
Glucose	K_m (mM)	–	–	–	7.78 ± 2.20
	V_{max} (μmol h^{-1} g^{-1} d.w.)				91.1 ± 30.9
Glycerol	K_m (mM)	2.50 ± 1.19	1.40 ± 0.27		1.67 ± 0.12
	V_{max} (μmol h^{-1} g^{-1} d.w.)	472.5 ± 142.7	419.3 ± 24.2	+	421.4 ± 35.0
Ethanol	K_m (mM)	1.82 ± 0.84	1.14 ± 0.34	+	1.05 ± 0.25
	V_{max} (μmol h^{-1} g^{-1} d.w.)	435.7 ± 162.7	435.8 ± 21.6		309.8 ± 92.0

All kinetic parameters are medium values of at least three independent assays.
– No uptake detected.
+ Uptake detected, kinetic parameters not determinable due to strong background alkalinization in cell suspension.

constant of ethanol over glycerol uptake [10] of 0.737 M^{-1} was calculated.

In cells grown in mineral medium with glycerol or ethanol, accumulation of labelled glycerol against gradient could be observed (Fig. 2). CCCP prevented accumulation but, nevertheless, it did not elicit significant efflux of labelled glycerol when added after 60 min incubation time (not shown). Neither did 50 mM cold glycerol. Glycerol accumulation ratio, was, for this reason, determined by HPLC, in identical experiments performed using ''cold'' instead of radiolabelled glycerol. The results obtained (Fig. 2) showed that maximum accumulation ratio did not exceed 10 times. Glycerol intracellular molarity determined by HPLC was approximately 32 mM, while glycerol intracellular molarity, estimated with radiolabel achieved, after 50 min incubation time, a maximum of approximately 280 mM. The influence of extracellular pH on maximum glycerol accumulation ratio was studied over a pH range from 3 to 7. In/out ratios did not present significant variations in comparison with the value previously obtained at pH 5.0 and CCCP prevented glycerol accumulation over all the pH range tested (not shown).

3.2. Transport of glycerol by glucose-grown cells

S. cerevisiae was grown in a mineral medium with glucose and harvested throughout exponential phase. Glycerol uptake at 30°C and pH 5.0 did not follow saturation kinetics. Instead, a diffusion constant of $0.005 \pm 0.001\ l\,h^{-1}\,g^{-1}$ dry weight ($n = 5$) could be

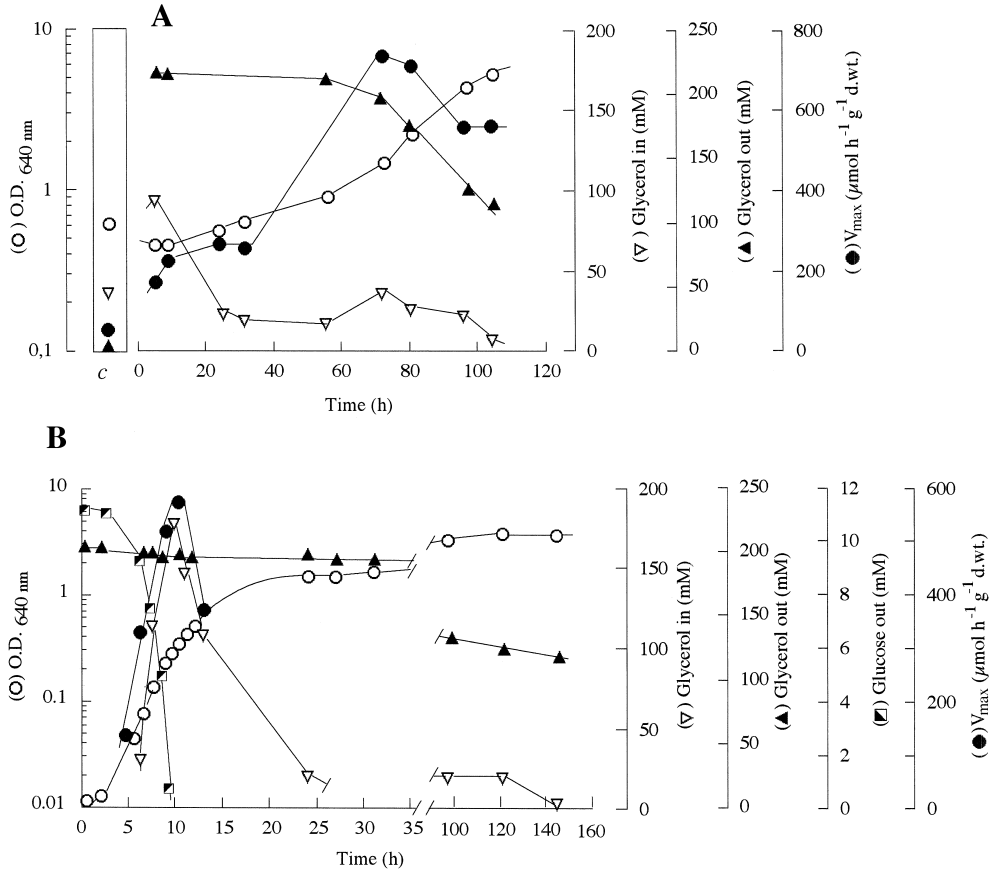

Fig. 3. (A) Induction of glycerol active uptake in cells of *S. cerevisiae* IGC 3507 transferred from mineral medium containing 2% (w/v) glucose to medium containing 2% (w/v) glycerol. *c*: Control values in cell suspension after recovery from glucose medium and before glycerol medium inoculation. (B) Induction of glycerol active uptake in cells growing in mineral medium supplemented with a 0.2% (w/v) glucose starter and 2% (w/v) glycerol. Glycerol concentrations were determined by HPLC and [^{14}C]glycerol uptake V_{max} was measured using 10 mM glycerol.

determined. Proton uptake upon glycerol addition to cell suspensions at pH 5.0 was also not detected. Neither was accumulation of labelled glycerol against gradient. To confirm if glycerol uptake was not detected because of any artefact due to the intracellular concentration of this substrate already present after recovery from glucose medium, cells, cultured in glucose, were centrifuged, washed and transferred to mineral medium with 2% (w/v) glycerol. Growth, intra and extracellular levels of glycerol and glucose were monitored. Besides, the kinetic parameters of labelled glycerol and proton uptakes were also monitored. Results, exemplified in Fig. 3A, show that the intracellular concentration of glycerol after growth on glucose was approximately 56 mM. Activity of H^+/glycerol symport was detected when glycerol consumption began. In the case when glucose was used as a starter for growth on glycerol in mineral medium, transport maximum activity was detected after glucose depletion (Fig. 3B). Starvation in mineral medium without glucose was also tried. Cells were starved for 1, 2, 4, 8 and 24 h in mineral medium without carbon source, after which they were centrifuged, washed and assayed as to proton or [^{14}C]glycerol uptake. No glycerol/H^+ uptake induction was obtained with this procedure.

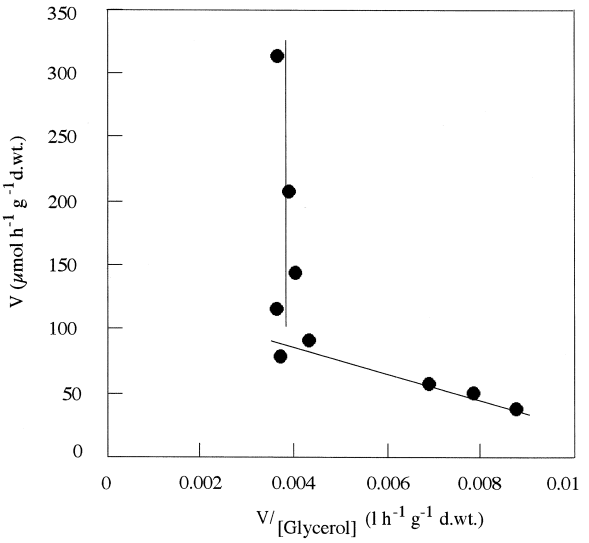

Fig. 4. Eadie–Hofstee plot of initial uptake rates at pH 5.0 of [^{14}C]glycerol by cells of *S. cerevisiae* IGC 3507 grown in YEPD (●). Two different methodologies were used to obtain the two sets of data (see Section 2).

On the other hand, cells grown in YEPD, collected from mid to late-exponential growth phase, during what is frequently named post-diauxic phase, behaved rather differently. Labelled glycerol uptake followed saturable kinetics of lower affinity (Fig. 4). No proton uptake upon glycerol addition could be detected (Table 1) and CCCP did not inhibit uptake. Accumulation did not exceed equilibrium, corresponding to an 1–2 in/out ratio (not shown). Assays, using labelled glycerol at concentrations above 10 mM, presented a second branch in Eadie–Hofstee plots characteristic of simple diffusion (Fig. 4). This was confirmed with second experimental approach for measuring initial uptake rates of labelled glycerol, as described in Section 2. The experimental values were then subjected to iteration and kinetic parameters of the saturable glycerol uptake are presented in Table 1. The simple diffusion constant thus obtained was 0.006 ± 0.001 $l\,h^{-1}\,g^{-1}$ dry weight ($n = 5$), identical to the one obtained in mineral medium.

3.3. Effect of glucose on glycerol / proton co-transport

Glucose effect over glycerol/proton co-transport was studied in ethanol-grown cells. Labelled glycerol accumulation in the presence of 100 mM glucose did not exceed a maximum ratio of 10 times. Glycerol accumulation controlled by HPLC reached an intracellular glycerol molarity higher than the one measured by radiolabel, corresponding to a maximum accumulation ratio of 17 times (Fig. 5). On the other hand, cells incubated for 20 s in 20, 80 and 100 mM glucose, were assayed as to labelled glycerol uptake. Results, exemplified in Fig. 6A, show no effects on [^{14}C]glycerol uptake K_m, while V_{max} increased in the presence of glucose, regardless to its concentration in the assay buffer. For higher incubation periods in 100 mM glucose, from 2 to 30 min, K_m was still not affected, but a significant increase (approximately 50%) in V_{max} was observable in the first 5–10 min incubation time, followed by a steep decrease (Fig. 6B). Control experiments were performed, monitoring intra and extracellular glycerol by HPLC which varied accordingly (not shown). The reversibility of this apparent slow disappearance of glycerol transport in the presence of glucose, was evaluated using cells

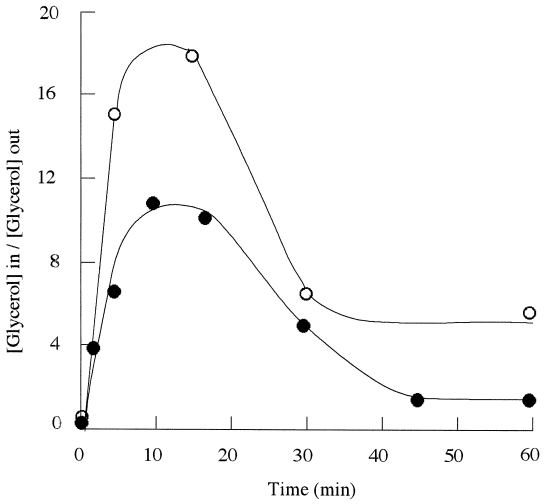

Fig. 5. Accumulation ratio of [^{14}C]glycerol in 100 mM Tris-citrate buffer pH 5.0 with 100 mM glucose, by cells of *S. cerevisiae* IGC 3507 grown in mineral medium supplemented with 2% (w/v) ethanol (●). Accumulation ratio of glycerol measured by HPLC (○).

incubated in 100 mM glucose for 30 min ($\approx 20\%$ V_{max}), washing them to remove glucose, and incubating again in buffer, without glycerol or ethanol (as inductors), at 30°C for different periods of time, in the absence and in the presence of cycloheximide. In either case, recovery of labelled glycerol V_{max}, after 2 h incubation, was still not observed.

3.4. The effect of salt over glycerol / proton co-transport

The possible induction of active transport by salt stress during growth was assayed. Cells grown on glucose in mineral medium in the presence of 0.2, 0.5 or 1 M NaCl were collected in early, mid and late exponential growth phases. The pellets were divided and washes were performed, in parallel, with water or water containing the same amount of salt from growth medium. Transport assays were performed in the presence of the same NaCl concentration from growth medium. No activity for glycerol proton symport was detected.

On the other hand, the action of NaCl over glycerol transport was also tested. Cells grown in either mineral or complete media, with either glycerol, ethanol or glucose as carbon sources, were incubated in buffer containing 1 M NaCl for 2 min prior to [^{14}C]glycerol or proton transport assays. Results, presented in Table 2, clearly indicate that no changes, either in kinetic parameters or in stoichiometry, were introduced by the presence of NaCl. In glucose-grown cells, simple diffusion assayed at 1 M NaCl presented a constant of 0.002 ± 0.0004 $l\,h^{-1}\,g^{-1}$ dry weight ($n = 3$), approximately 3 times lower than in the absence of salt.

Accumulation of labelled glycerol in the presence

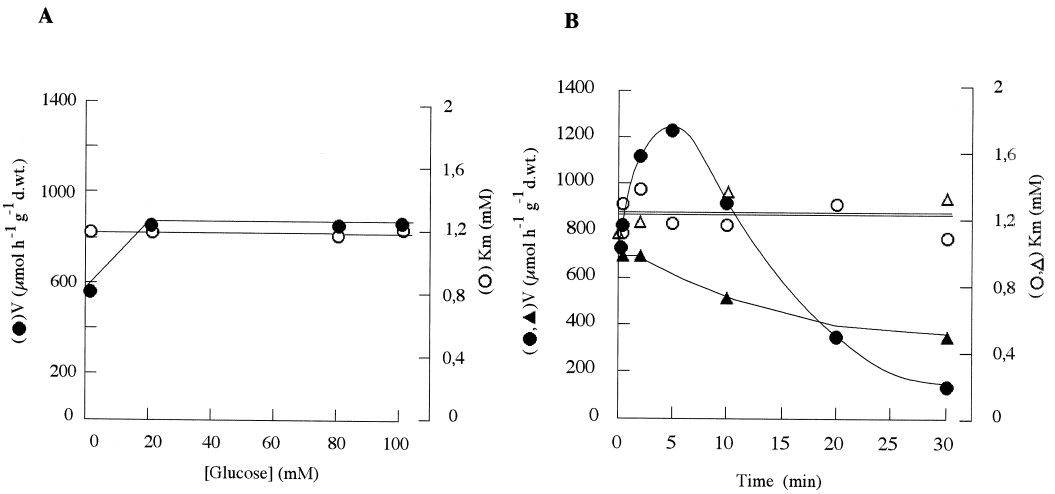

Fig. 6. (A) [^{14}C]Glycerol uptake kinetic parameters in cells of *S. cerevisiae* IGC 3507 grown in mineral medium supplemented with 2% (w/v) ethanol, and incubated in different glucose concentrations. (B) [^{14}C]Glycerol uptake kinetic parameters variation in cells incubated in 100 mM glucose for crescent periods of time (●, ○). Control in buffer without glucose (▲, △).

Table 2
Kinetic parameters of [^{14}C]glycerol and proton uptakes at 30°C and pH 5.0 in cells of *S. cerevisiae* IGC 3507 grown in mineral medium supplemented with different carbon sources assayed in the presence of 1 M NaCl

Carbon source		Proton uptake upon glycerol addition	[^{14}C]Glycerol uptake
Glucose	K_m (mM)	–	–
	V_{max} (μmol h^{-1} g^{-1} d.w.)		
Glycerol	K_m (mM)	1.91 ± 0.27	1.89 ± 0.65
	V_{max} (μmol h^{-1} g^{-1} d.w.)	465.4 ± 87.8	468.1 ± 59.9
Ethanol	K_m (mM)	1.61 ± 0.30	1.49 ± 0.78
	V_{max} (μmol h^{-1} g^{-1} d.w.)	550.2 ± 61.2	428.9 ± 73.4

All kinetic parameters are medium values of at least three independent assays.
– No uptake detected.

of several NaCl concentrations, 0.2, 0.5 or 1 M, was assayed in glycerol and ethanol-grown cells. No correlation could be established between maximum accumulation ratios obtained and the correspondent extracellular NaCl concentrations (Fig. 7A). The maximum glycerol intracellular molarity, reached at the several NaCl concentrations assayed, varied approximately 30% from control without salt. On the other hand, intracellular volume changed linearly with NaCl buffer concentrations (Fig. 7B), but standard deviation obtained from statistical treatment of all determinations was rather high, reaching 60%, introducing an experimental error factor eventually contributing to the changes in maximum accumulation ratios found for each NaCl concentration. CCCP prevented glycerol accumulation, but, this time, this drug did also

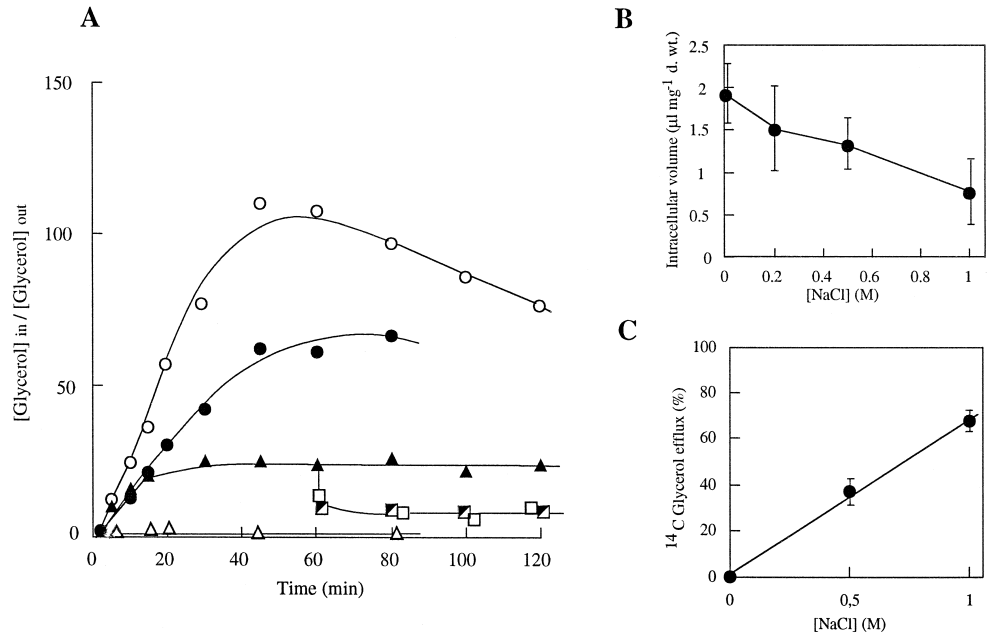

Fig. 7. (A) Accumulation of [^{14}C]glycerol by ethanol-grown cells of *S. cerevisiae* IGC 3507 in 100 mM Tris-citrate buffer pH 5.0 supplemented with different NaCl concentrations: 0 M (●); 500 mM (○); 1 M (▲). Efflux of radioactivity observed after the addition of 85 mM ''cold'' glycerol (◩) or 50 µM CCCP (□) to cells incubated in the presence of 1 M NaCl. Accumulation prevented by the addition of 50 µM CCCP (△) in cells incubated in 1 M NaCl. (B) Intracellular volumes determined incubating the cells for 30 min in buffer supplemented with different NaCl concentrations ($n = 9$ each). (C) Variation of the percentage of efflux of [^{14}C]glycerol with the NaCl external concentration. Efflux was obtained by the addition of 50 µM CCCP.

induce partial efflux of radiolabel. Efflux was also obtained by the addition of 50 mM ''cold'' glycerol. The amount of [^{14}C]glycerol extruded was directly proportional to the amount of NaCl in the assay buffer (Fig. 7C).

4. Discussion

Kinetic uptake data from glycerol- and ethanol-grown cells of *S. cerevisiae* in either mineral or complete media were consistent with the presence of a permease functioning as an electrogenic proton symport with a stoichiometry of one proton per glycerol molecule. Accumulation of glycerol against gradient was detected with radiolabelled glycerol. Nevertheless, due to the error that might be introduced in radiolabel determinations by glycerol metabolism during experimental time, and since CCCP and glycerol itself induced insignificant efflux, accumulation against gradient was confirmed by chromatography, not exceeding an in/out ratio of 10 times. This result is very similar to the case of H^+/glycerol symport in *P. sorbitophila* [3]. Chromatography, rather than experiments with radiolabelled substrate, should translate the true accumulation ratio for glycerol. On the other hand, the same in/out ratios were obtained with radiolabelled glycerol when the experiments were performed in the presence of glucose. In this case, the other way round, radiolabel should translate the true accumulation ratio for glycerol, since chromatography determinations do not exclude the possibility of glycerol being produced from glucose during experimental time, which is rather long. The action of CCCP preventing accumulation, as inhibiting uptake, suggested dependence on ΔpH and $\Delta\Psi$. This was consistent with the fact that glycerol maximum accumulation ratios followed p.m.f. over external pH [7,9], as expected for a substrate actively transported against gradient by an electrogenic proton symport. No inhibitor could be found between a rather broad number of compounds, indicating a very high substrate specificity of this transport system, similarly to glycerol active transport systems described in *P. sorbitophila* [3] and *Z. rouxii* [4].

Consistently with the activity of a permease, ethanol inhibited glycerol high affinity uptake exponentially, allowing the calculation of an exponential inhibition constant [10]. The value obtained was slightly lower than the ones published for other active transport systems in *S. cerevisiae* [10]. This could indicate an underneath stimulatory effect of ethanol over glycerol simple diffusion, according to its liposoluble character. Nevertheless, in cells grown in either glycerol or ethanol, we could not measure simple diffusion, probably below the level of detection with the technical approach utilised.

Glucose growing cells in mineral medium did not present evidence of mediated glycerol uptake, but, instead, of simple diffusion. Nevertheless, cells cultured in YEPD, presented glycerol uptake with a lower affinity than the one discussed above for glycerol- or ethanol-grown cells. The absence of proton uptake upon glycerol addition and the absence of CCCP inhibition over glycerol uptake, as well as a maximum in/out accumulation ratio that did not exceed equilibrium, led us to presume this to be a facilitated diffusion. On the other hand, taking into consideration the fact that this uptake is detected in YEPD in late exponential growth phase, after glucose exhaustion, we could argue it to be eventually under strict glucose repression, thus justifying the fact that it is not detectable in glucose mineral medium grown cells.

Results point to glycerol/H^+ symport being under glucose repression, just as glycerol consumption enzymes [11], and derepression alone is not enough to allow the appearance of transport activity. Induction is connected with growth under gluconeogenic conditions, using glycerol, ethanol or acetate as sole carbon and energy sources. This is a different situation from the constitutive active glycerol transport systems described in literature for *D. hansenii* [2], *P. sorbitophila* [3] or *Z. rouxii* [4]. Yet, in another yeast, *Candida intermedia* [12], a polyol/H^+ symport has been described, induceable by growth on sorbitol and repressed in the presence of glucose, and in the fungus *Fusarium oxysporum* [13], a facilitated diffusion for glycerol has been described, repressible by glucose and induced by glycerol or ethanol.

Transporters functional inactivation by glucose is usually the cause of steep decrease in uptake V_{max} after short incubation periods [14], which has not been the case with glycerol proton symport in *S. cerevisiae*. The other way round, a stimulation of high affinity glycerol uptake was observed, eventu-

ally the indirect consequence of the action of glucose present in the assay buffer over ΔpH, by stimulation of membrane proton ATPase activity. A similar situation has been described for maltose proton symport also in *S. cerevisiae* [15]. The slow disappearance of glycerol uptake V_{max}, observed after the initial stimulation, could be an indication of inactivation of the glycerol carrier by proteolytic activity, since no reversibility was observed after glucose removal.

NaCl did not interfere with glycerol proton symport, kinetic constants and stoichiometry being maintained in its presence. The variation on accumulation ratios observed, according to extracellular NaCl concentrations, were affected by the error introduced by experimental determination of intracellular volumes under these conditions. Significant differences were found though in the amount of intracellular ''free'' radiolabelled glycerol extruded by the addition of either ''cold'' glycerol or CCCP in the presence of different NaCl concentrations. The direct proportionality found between extracellular NaCl concentrations and the amount of radiolabel extruded suggests that the adaptation to salt shock during experimental time could involve the inhibition of glycerol consumption. These results are consistent with literature data, where the glycerol-3-phosphate pathway in *S. cerevisiae* has been considered to be osmoregulated by controlling both levels of glycerol-3P dehydrogenase, activated under osmotic stress [16,17] and glycerol kinase, inhibited under osmotic stress [16]. The inductive glycerol proton symport described here could have, according to the results, a physiological role connected with gluconeogenesis, but, apparently, no direct involvement in osmoregulation. This view is strengthened by the absence of induction of the high affinity glycerol carrier by growth on glucose under salt stress.

In conclusion, our results point out to the existence of two mediated transport systems for glycerol in *S. cerevisiae* IGC 3507. A low affinity transport system, present in glucose-grown cells, probably a facilitated diffusion, and a high affinity proton symport, induced by growth on gluconeogenic substrates. Results presented so far are consistent with the molecular evidence concerning the existence of a glycerol permease in glucose grown cells of *S. cerevisiae* [18]. A high molecular homology was found between the correspondent gene, *FPS1*, and the gene of the glycerol facilitator from *E. coli*, entitled *GlpF*, both genes included in the MIP family of channel proteins [18–20]. Nevertheless, the molecular structure of the *Fps1* protein and the similarities it presents with the glycerol facilitator protein from *E. coli* [18], suggest a type of mediated transport other than symport. Further investigation is being developed on the actual role of this constitutive permease and mediated low and high affinity glycerol uptake in correlation to *fps1* deletion.

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