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Proton- and Sodium-Coupled Phosphate Transport Systems and Energy Status of *Yarrowia lipolytica* Cells Grown in Acidic and Alkaline Conditions

R. Zvyagilskaya[1], O. Parchomenko[1], N. Abramova[2], P. Allard[3], T. Panaretakis[4], J. Pattison-Granberg[4,5], B.L. Persson[4,5]

[1]A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, Moscow 117071, Russia
[2]N.K. Koltsov Institute of Developmental Biology, Russian Academy of Sciences, ul. Vavilova 26, Moscow 117334, Russia
[3]Center for Structural Biochemistry, Novum, S-141 57 Huddinge, Sweden
[4]Department of Biochemistry and Biophysics, Wallenberg Laboratory, Stockholm University, S-106 91 Stockholm, Sweden
[5]School of Biosciences and Process Technology, Växjö University, S-351 95 Växjö, Sweden

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Abstract. In this study we have used a newly isolated *Yarrowia lipolytica* yeast strain with a unique capacity to grow over a wide pH range (3.5–10.5), which makes it an excellent model system for studying H^+- and Na^+-coupled phosphate transport systems. Even at extreme growth conditions (low concentrations of extracellular phosphate, alkaline pH values) *Y. lipolytica* preserved tightly-coupled mitochondria with the fully competent respiratory chain containing three points of energy conservation. This was demonstrated for the first time for cells grown at pH 9.5–10.0. In cells grown at pH 4.5, inorganic phosphate (P_i) was accumulated by two kinetically discrete H^+/P_i-cotransport systems. The low-affinity system is most likely constitutively expressed and operates at high P_i concentrations. The high-affinity system, subjected to regulation by both extracellular P_i availability and intracellular polyphosphate stores, is mobilized during P_i-starvation. In cells grown at pH 9.5–10, P_i uptake is mediated by several kinetically discrete Na^+-dependent systems that are specifically activated by Na^+ ions and insensitive to the protonophore CCCP. One of these, a low-affinity transporter operative at high P_i concentrations is kinetically characterized here for the first time. The other two, high-affinity, high-capacity systems, are derepressible and functional during P_i-starvation and appear to be controlled by extracellular P_i. They represent the first examples of high-capacity, Na^+-driven P_i transport systems in an organism belonging to neither the animal nor bacterial kingdoms. The contribution of the H^+- and Na^+-coupled P_i transport systems in *Y. lipolytica* cells grown at different pH values was quantified. In cells grown at pH values of 4.5 and 6.0, the H^+-coupled P_i transport systems are predominant. The contribution of the Na^+/P_i cotransport systems to the total cellular P_i uptake activity is progressively increased with increasing pH, reaching its maximum at pH 9 and higher.

Key words: *Yarrowia lipolytica* — Yeast — Plasma membrane — Phosphate transport — Phosphate regulation

Introduction

Phosphorus, an essential nutrient, is often present in low amounts in the environment [12], and yeasts, like other organisms, satisfy their demands for this indispensable element primarily by uptake of P_i by a number of plasma membrane transporters (for a recent review, *see* [20]), concentrating P_i against its thermodynamic gradient by cotransport with H^+ or Na^+. Among all eukaryotic non-animal cells, most of the available information on the P_i transport systems has been confined to the yeast *Saccharomyces cerevisiae*. In this fungus, three different systems have been proposed to be involved in uptake of P_i from the cultivation medium [20]. The so-called low-affinity system, with an apparent K_m for extracellular P_i of approximately 1 mM at its pH optimum of 4.5, has been suggested to be a constitutively expressed H^+/P_i cotransporter [4, 15, 27]. The two other systems are high-

Correspondence to: B.L. Persson
Bengt_P@dbb.su.se

affinity transporters which are derepressible by P_i starvation [20]. One of these transporters, a 65-kDa hydrophobic membrane protein, the product of the *PHO84* gene [7], specifies a high-affinity H^+/P_i cotransporter which is maximally active at pH 4.5 and has an apparent K_m value for phosphate of 1–15 μM [3, 5, 9, 23]. The other one, corresponding to the *PHO89* gene product [13], is a Na^+-coupled high-affinity P_i uptake system, active predominantly at pH 9.5, with a K_m for P_i in the order of 1 μM at pH 7.2 [13, 22]. These findings suggest that a high-affinity Na^+/P_i symport exists in yeasts. However, the rather low activity of the Pho89 Na^+-coupled transporter in *S. cerevisiae* cells expressed under suboptimal conditions casts some doubt on the physiological significance of this P_i transporter. Furthermore, the *S. cerevisiae* yeast is not the best model system for studying Na^+-coupled transporters. These transporters are active predominantly at alkaline conditions and *S. cerevisiae* thrives under acidic conditions with a limited growth at pH 7 or higher. In addition, there is currently no information available on a putative low-affinity Na^+/P_i-coupled transport in yeast cells grown at alkaline conditions. Clearly, our knowledge of yeast physiological phenomena has lagged behind that of yeast genetics and molecular biology. Further studies are needed to gain a more precise resolution of P_i transport mechanisms in yeast cells grown at different conditions than has been achieved so far. For this purpose, we have used in this study the recently isolated strain of *Yarrowia lipolytica*, remarkable by its high growth rate and, most importantly, for its unique capacity to grow over a wide range of pH values from 3 to 11 [1, 29]. This makes it an excellent model for characterization of H^+- and Na^+-coupled P_i transporters.

In this paper we report on the presence of several kinetically distinct novel H^+- and Na^+-dependent P_i uptake systems in *Y. lipolytica* cells grown at acidic and alkaline conditions and their contribution to the total P_i uptake activity in cells grown at different pH values. This paper is also focused on peculiarities of energy metabolism of *Y. lipolytica* cells grown at acidic and alkaline conditions. For this purpose, a method was elaborated for isolation of tightly coupled, well preserved mitochondria.

Materials and Methods

Yeast Strain and Culture Conditions

The *Yarrowia lipolytica* yeast strain used in this study was isolated from epiphytic microflora of salt-excreting leaves of arid plants in the Negev desert, Israel [1]. Cells were routinely grown at 30°C on complex low-phosphate (LP_i) YPD (1% yeast extract, 2% Bacto Peptone, 1% glucose, 2% agar) or high-phosphate (HP_i) YPD (1% yeast extract, 2% Bacto Peptone, 1% glucose, 0.2% KH_2PO_4, 2% agar) media, pH 4.5–10.0. For cells grown at pH 4.5, equivalent liquid LP_i-YPD and HP_i-YPD media were also used. LP_i-YPD medium was prepared as described previously [14] and contained about 250 μM P_i. Culture media were autoclaved, adjusted to the desired pH values with H_2SO_4 or KOH, and buffered with 50 mM Tris-succinate (pH 4.5–8.5) or CAPS-Tris (pH 9.0–10.0). Cultures were confluent in the same buffers and allowed to grow for 16–24 hr. Cell growth was monitored turbidometrically at 590 nm (OD_{590}).

Flow Cytometry

For the rapid assessment of yeast vitality and viability, cultures grown in HP_i or LP_i media at different pH values were harvested in early-exponential phase at an OD_{590} value of approximately 2, suspended in appropriate 25 mM buffers (Tris-succinate for pH 4.5–6.0 or CAPS-Tris for pH 9.5) supplemented with 2 mM $MgSO_4$ and 2% glucose, diluted in the same buffers to OD_{590} of 1.0, and exposed to the DNA-staining fluorescent probe propidium iodide at a final concentration of 50 μM, added at the moment of the analysis. The membrane potential generated across the plasma membrane or inner mitochondrial membrane was assayed with 20 or 2 nM $DiOC_6(3)$ cyanine dye (3,3′-dihexyloxacarbocynine iodide), respectively, at 30°C for 20 min in the dark [24]. Flow cytometric measurements were performed with a FACS Calibur (Becton Dickinson, Franklin Lakes, N.Y.) flow cytometer, equipped with a laser emitting at 488 nm. The obtained data from 15,000 cells were stored and analyzed on a logarithmic scale using the Cell Quest Software provided by Becton Dickinson.

^{31}P-NMR Spectroscopy

Cells grown in liquid LP_i medium at pH 4.5 were collected by centrifugation, washed with 25 mM Tris-succinate buffer, pH 4.5, suspended in the same buffer, and analyzed for intracellular changes in P_i and polyphosphates with a Varian Unity Plus 400 instrument, as described earlier [14].

Isolation of Mitochondria

Mitochondria were isolated as described earlier [2] with some modifications. Cells grown on solid HP_i and LP_i media, pH 4.5, or HP_i medium, pH 9.65, were collected and washed with ice-cold distilled water or with 50 mM Tris-HCl-buffer, pH 8.2, respectively. Then cells were incubated at room temperature for 30 min in 50 mM Tris-HCl buffer, pH 8.6–9.0, containing 10 mM dithiothreitol and washed twice with ice-cold distilled water (for cells grown at pH 4.5) or with 50 mM Tris-HCl buffer, pH 8.2 (for cells grown at pH 9.65). The weakened cells were incubated at 30°C under mild stirring for 15 to 20 min in 10 mM Hepes-EDTA buffer, pH 7.5 or 8.2 (for cells grown at pH 4.5 or 9.65, respectively), containing 1.2 M sorbitol and lytic enzymes (3 mg of Novozym 234 and 2–3 mg of Zymolase per 1 g of original cell, wet weight) to form spheroplasts. The spheroplasts were pelleted by centrifugation at 400 × g for 10 min, washed twice with 1.2 M sorbitol containing 0.4% (w/v) BSA (pH was adjusted to 7.5 or 8.2), and disrupted with a glass-glass Dounce homogenizer. The grinding medium contained 10 mM Tris-HCl buffer, pH 7.2, 0.4 M mannitol, 0.5 mM EDTA, and 0.4% BSA. The homogenate was mixed with an equal volume of the same buffer, except that 0.4 M mannitol was substituted for 0.6 M mannitol, centrifuged for 10 min at 3,200 × g and the supernatant was centrifuged at 7,500 × g for 20 min. The pellets from the second spin were gently resuspended in approximately 20 ml of washing medium, recentrifuged at 7,500 × g for 23 min and the pellets were

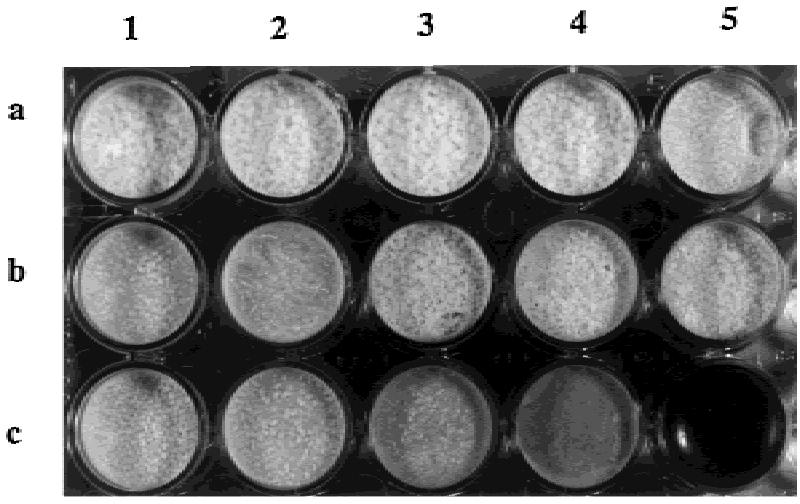


Fig. 1. Growth of *Y. lipolytica* cells on complex solid YPD media containing glucose (*a, c*) and glycerol (*b*). Cells grown on solid HP_i-YPD media at pH values ranging from 7 to 10.5 were aseptically collected in appropriate 50 mM buffers (Tris-succinate for pH 7–8, CAPS-Tris for pH 9–10, and CAPS-Tris-KOH for pH 10.5), spread on HP_i-YPD solid media buffered to pH 7.0, lane 1; pH 8.0, lane 2; pH 9.0, lane 3; pH 10.0, lane 4; pH 10.5, lane 5, allowed to grow for 72 hr. In panel *c,* media were supplemented with 5% NaCl.

resuspended in minimal volume of washing medium. The mitochondria thus obtained were fully active for at least 3 hr when kept on ice.

Oxygen Consumption

The oxygen consumption in mitochondrial suspensions was monitored polarographically with a Clark-type electrode in a medium containing (in mM) 600 mannitol, 2 Tris-phosphate, pH 7.2, 1 EDTA, 20 pyruvate, 5 malate, and mitochondria corresponding to 0.4 to 0.6 mg/ml of protein. Respiratory control and ADP/O ratios were calculated as recommended by Chance and Williams [8]. If needed, incubation media were supplemented by rotenone, an inhibitor of complex I of the respiratory chain, 2 mM KCN, an inhibitor of the cytochrome oxidase, or 2 mM SHAM, an inhibitor of the alternative (nonphosphorylative) oxidase.

Preparation of Plasma Membrane Vesicles

Isolation and purification of *Y. lipolytica* plasma membrane vesicles was performed by aqueous polymer two-phase partitioning method as described previously [11].

Phosphate Uptake Assay

Cells were separated from the culture medium by centrifugation at 2,300 × g for 15 min, washed once with ice-cold 25 mM Tris-succinate (pH 3.5–8.5), CAPS–Tris (pH 9.0–10.0) or CAPS–Tris–NH_4OH (pH 10.5–11.0) buffers, and suspended (30 μl, 0.546 mg of dry weight) in the same buffers supplemented with 3% glucose, 1 μl of [^{32}P]orthophosphate (0.18 Ci/μmol; 1 mCi = 37 Mbq; Amersham-Pharmacia Biotech, Sweden) and phosphate to a final concentration ranging from 1 μM to 2.5 mM. The suspension was blended and incubated for 1 to 5 min at 25°C. Phosphate uptake was terminated by addition of 3 ml of ice-cold dilution buffer. The samples were immediately filtered, the filters (Whatman GF/F) were washed once with the same cold solution, and the radioactivity retained on the filters was determined by liquid scintillation spectrometry. In all cases, the initial rate of P_i uptake was estimated from the first 1 min of accumulation.

ATPase Activity Assay

ATPase activity of isolated plasma membrane vesicles was determined spectrophotometrically as described [11].

ATP-Dependent H^+-Pumping

ATP-dependent H^+-pumping in isolated plasma membrane vesicles was measured by the acridine orange method [11].

Phosphate and Glucose Determination

Phosphate in the growth media was assayed spectrophotometrically at 850 nm essentially as described [17]. Glucose was determined amperometrically with glucose oxidase according to the protocol of Okuda and Miwa [18].

Abbreviations

CAPS, (3-[cyclohexylamino]-1-propanesulfonic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; MES, (2-[N-morpholino]ethanesulfonic acid); SHAM, salicyl hydroxamate.

Results and Discussion

Growth of the Model Strain

Y. lipolytica cells grew vigorously on glucose- or glycerol-containing solid HP_i-media at all pH values tested (pH 3.5–10.5), forming pronounced domes after 72 hr of subcultivation even at alkaline conditions (Fig. 1 *a, b*). It is even more astonishing that they can grow at alkaline conditions up to pH 10 in the presence of 5% NaCl (Fig. 1 *c*), thus reinforcing the notion that these cells are perfectly adapted to extreme growth conditions and therefore may be an exceptionally useful model system for

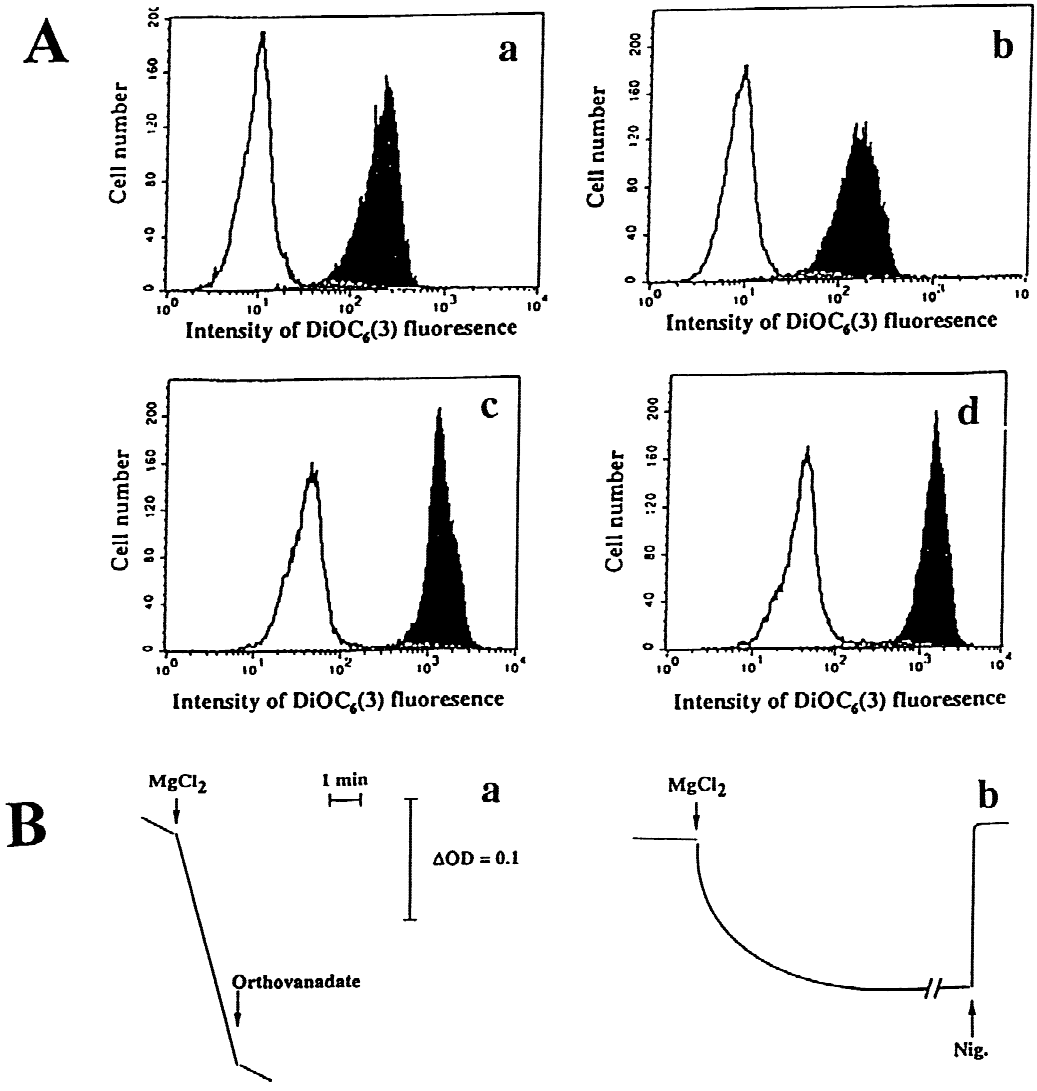


Fig. 2. Flow cytometric analyses of activity in the mitochondrial (*A, a, b*) and plasma membrane (*A, c, d*) compartments of *Y. lipolytica* cells grown in LP_i medium at pH 6.0 (*a, b*) and pH 10.0 (*c, d*); ATPase (*B, a*) and H^+-pumping activity (*B, b*) of isolated inside-out oriented plasma membrane vesicles isolated from *Y. lipolytica* cells grown in HP_i-YPD medium. The mitochondrial and plasma membrane potential were assayed in appropriate assay buffers with 2 or 20 nM $DiO_6(3)$, respectively, as described in Materials and Methods. In panel *B*, changes in NADH (trace *a*) and acridin orange (trace *b*) absorption were measured as described in Materials and Methods. The reactions were started by addition of 4 mM $MgSO_4$ and terminated by 100 μM orthovanadate (trace a) or 2 μM nigericin (Nig.; trace b), respectively.

clarifying phosphate metabolism in yeast cells grown under different pH conditions.

Viability and Energy-Coupling of Cells Grown at Acidic Conditions Is Independent of the External Phosphate Concentration

Our first step was to characterize phosphate transport systems in *Y. lipolytica* cells grown at acidic conditions (pH 4.5) in both phosphate-sufficient (HP_i) and phosphate-deficient (LP_i) media. Because growth of yeast cells in LP_i medium is not a trivial event, we examined the viability and energy status of LP_i-grown cells as compared to the HP_i-grown cells. Both phenotypes showed full viability as can be inferred from flow-cytometric measurements using the propidium iodide exclusion test (*not shown*). Therefore, the $DiOC_6(3)$ fluorescence observed (Fig. 2*A*, black peaks) can be considered as parameters of intact and viable cells. HP_i- and LP_i-grown cells exhibited similar high fluorescence activity in both the mitochondrial (Fig. 2*A*, *a, b*) and plasma membrane (Fig. 2*A*, *c, d*) compartments. 50 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler, totally

Table. Properties of respiration on pyruvate + malate of mitochondria from *Y. lipolytica* cells grown in different conditions

Medium, pH	Oxidation rate in state 3 respiration, ng-atom O/min per mg protein	RC	ADP/O	Inhibition by rotenone, %	Inhibition by KCN, %	Inhibition by (KCN + SHAM), %
HP_i, pH 4.5	625 ± 72	3.91 ± 0.19	2.96 ± 0.04	89.0 ± 1.0	88.1 ± 0.5	93.5 ± 0.7
LP_i, pH 4.5	432 ± 69	4.04 ± 0.23	2.97 ± 0.03	87 ± 0.1	82.5 ± 0.2	93.9 ± 0.6
HP_i, pH 9.65	369 ± 47	3.31 ± 0.39	2.82 ± 0.40	84.4 ± 5.0	79.2 ± 0.1	94.9 ± 2.1

Mitochondria were isolated and incubated as described in Materials and Methods.
Values are the average of 2 to 3 determinations from 4 to 6 independent experiments ± SD.

dissipated the $\Delta\Psi$-related fluorescence (Fig. 2*A*, white peaks). These results are in agreement with direct measurements of ATPase (Fig. 2*B, a*) and ATP-dependent proton-pumping (Fig. 2*B, b*) activities of inside-out oriented plasma membrane vesicles isolated from HP_i-grown cells, as well as with characteristics of mitochondria isolated from cells grown on HP_i- and LP_i-medium, pH 4.5 (Table). The H^+-ATPase activity of the isolated vesicles was typically 2 mmol/mg per min. Mitochondria from both cell types met all known criteria of physiological intactness as inferred from high respiratory rates upon oxidation of pyruvate plus malate, high respiratory control ratios, and ADP/O values, approaching the theoretically expected maxima. In the respiratory chain of these cells, all three points of energy conservation were functional and the bulk of electron flux was mediated through the cytochrome pathway with minor contribution of the alternative oxidase. A concerted action of KCN, an inhibitor of the cytochrome pathway, and SHAM, an inhibitor of the alternative oxidase, blocked the total respiration to almost zero.

Activation of High- and Low-Affinity H^+-Coupled Phosphate Uptake Is Dependent on the Phosphate Concentration in the Growth Medium and Its pH

In *Y. lipolytica* cells grown at different pH values, the accumulation of [^{32}P] displayed a distinctive time course that was reproducible between the cell preparations. The rate of P_i accumulation by both LP_i- and HP_i-grown cells were approximately linear for at least 15 sec (Fig. 3 *A, B*) with a tendency to increase at low concentrations and to decrease at high concentrations. In cells grown at pH 4.5, the CCCP-sensitive P_i uptake reaction (Fig. 3*A,* filled circles) was independent of the Na^+ concentration in the incubation medium as shown for cells grown in LP_i medium (Fig. 3*A,* open circles). Lineweaver-Burk plots (Fig. 4) revealed the presence of two distinct P_i transport systems in *Y. lipolytica* cells grown in LP_i- and HP_i media at pH 4.5 (Fig. 4, filled symbols) with apparent K_m values for P_i at pH 5.5 of 2–3 mM P_i (Fig 4*A, a*) and 12–18 μM (Fig. 4 *A, b*), respectively, closely matching the values reported for the P_i transporters in *S. cerevisiae* cells (*see* [20]).

The P_i uptake activity by *Y. lipolytica* cells grown at LP_i and HP_i conditions at pH 4.5 was clearly pH-dependent (Fig. 5). In P_i-starved cells, P_i accumulation was maximal at pH 5.5–6.5, half-maximal at pH 3.5 and 7.5, and very low at pH values above 8.5 (Fig. 5*A*, open squares). In cells grown in HP_i medium, the rate of P_i uptake peaked over a pH range extending from 4.5 to 5.5 and decreased 11-fold as the pH of the assay medium was increased to 8.5 (Fig. 5*B*, filled squares). These results suggest either a preferential uptake of $H_2PO_4^-$ over HPO_4^{2-} in both LP_i- and HP_i-grown cells or a reduction of the proton motive force above pH 5–6. Because the pH-sensitivity does not seem to be an absolute argument for proton-coupled cotransport, we examined the effect of the uncoupler CCCP on P_i transport in P_i-starved cells; it was found that CCCP inhibited the P_i uptake during the first min by more than 90% (Fig. 3*A*, filled circles), consistent with the view that the inward movement of P_i into cells grown at pH 4.5 is most likely driven by the H^+ gradient across the plasma membrane maintained by the plasma membrane H^+-ATPase. Moreover, the uptake of P_i was inhibited by KCN, an inhibitor of terminal cytochrome oxidase, by 85% and completely inhibited when also SHAM, an inhibitor of the alternative oxidase, was added (*not shown*), thus confirming the data on inhibitory analysis of mitochondrial respiration (Table).

Regulation of the H^+-Coupled High-Affinity Phosphate Uptake

To gain further insight into the regulation of the two kinetically distinct low- and high-affinity H^+-coupled P_i uptake systems in *Y. lipolytica* grown at pH 4.5, we measured phosphate uptake in cells following their growth in LP_i medium, pH 4.5 (Fig. 6*A*). We have previously shown that the rate of P_i accumulation catalyzed by HP_i-grown cells at different growth phases was very low, 0.35 μmol of P_i transported per min and g of cells (dry mass), and largely independent of the prevailing growth phase although transport decreased somewhat in

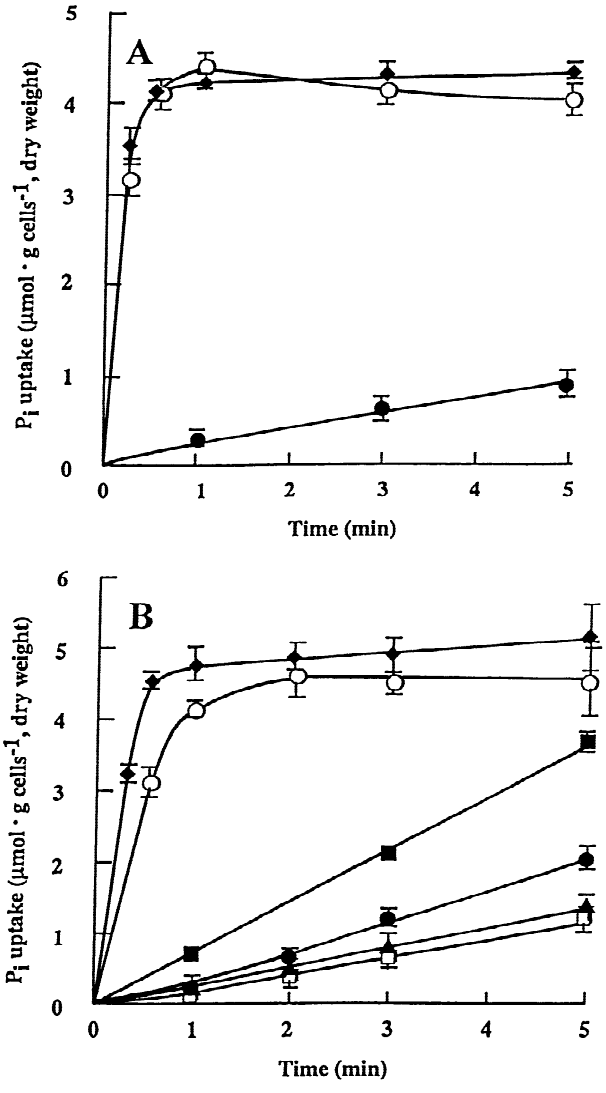


Fig. 3. Effect of alkali ions and CCCP on phosphate uptake by *Y. lipolytica* cells grown in LP_i medium at pH 4.5 (*A*) and pH 10.0 (*B*). (*A*) Cells grown in LP_i medium, pH 4.5, were incubated in the assay medium containing 25 mM Tris-succinate buffer, pH 5.5, and 3% glucose (♦). Where indicated, cells were treated with 25 mM NaCl (○) or 50 μM CCCP (●) in the assay medium. (*B*) Cells grown in LP_i medium, pH 10.0, were incubated in 25 mM CAPS-Tris, pH 9.5, 0.11 mM P_i and 3% glucose in the absence of any alkali ions (●) and in the presence of 25 mM Na^+ (♦), 25 mM K^+ (▲), 25 mM Li^+ (□), or 60 μM CCCP (○). Alkali ions were added as chloride salts. In the control experiment, the assay buffer was supplemented with 25 mM NaCl but glucose was omitted (■). Values are the average of four to five determinations using the same cell preparation; bars indicate SD.

cells approaching stationary phase [29]. However, in cells grown in LP_i medium containing approximately 250 μM phosphate (Fig. 6*B*), the initial rate of transport increased approximately 2.5-fold during the time course, reaching its maximum in the late exponential and stationary growth phases and showing a plateau of essentially unchanged activity between 5 and 9 hr of growth. This is in contrast to the P_i uptake pattern in *S. cerevisiae*, which revealed a monotonous increase in P_i uptake activity up to its maximum rate followed by a drastic decline in activity when the cells entered late-exponential growth phase [14, 21]. This prompted us to analyze the extracellular concentrations of P_i and glucose during the growth of *Y. lipolytica* cells. The cell growth was accompanied by an initial rapid consumption of external P_i from approximately 250 to 30 μM during the first 3 hr of growth, followed by a moderate (up to 150 μM) and transient increase in external P_i concentration with a subsequent slower rate of P_i utilization during the mid- and late-exponential growth phases (Fig. 6*B*). The transient increase in external P_i concentration was in phase with an unperturbed P_i transport activity, while both the initial and subsequent decreases in the external P_i concentration coincided with an increase of the P_i accumulation rate. The transient increase in external P_i concentration is a puzzling observation for which no definite explanation can be offered yet. P_i efflux phenomena have also been observed in a *S. cerevisiae* mutant lacking the Pho84 high-affinity proton-coupled P_i transporter [19] and in plant cells [10, 25]. It is plausible that this transient P_i increase in the incubation medium during the growth of *Y. lipolytica* cells may play a critical role in maintaining the functionality and/or preventing degradation of the P_i uptake system under conditions of severe P_i starvation, in line with the life strategy of this particular yeast species.

The highest P_i transport activity in *Y. lipolytica* cells was achieved when the extracellular P_i concentration was within the K_m value range (8–13 μM) of the high-affinity P_i transport system. This observation suggests that the derepression of the high-affinity P_i uptake system in the *Y. lipolytica* yeast is controlled by the availability of extracellular P_i. In cells grown in LP_i medium, we also measured changes in intracellular levels of free P_i and polyphosphates by ^{31}P-NMR (Fig. 6*C*). The samples analyzed were cells grown for 5, 7.5, 10 and 20 hr, corresponding to the situations of the initial rapid P_i consumption, the transient plateau in P_i uptake, and when the extracellular P_i concentration was close to zero, respectively. Cells grown in LP_i medium maintained much lower levels of phosphorous compounds than HP_i-grown cells, where the pools of free P_i and polyphosphates were largely unaffected by the prevailing growth phase [29]. Interestingly, in cells grown in LP_i medium under conditions of internal P_i efflux (7.5 hr) and subsequent extracellular phosphate deprivation (10 to 20 hr), the polyphosphate pool was diminished to almost zero. In contrast, the amount of intracellular free P_i was maintained at a level rather low, but significant enough to support the cell growth. Thus, it appears that under conditions when the cell meets no P_i limitations, an excessive amount of free P_i is predominantly stored in the form of polyphosphates, whereas during P_i starvation the cellular

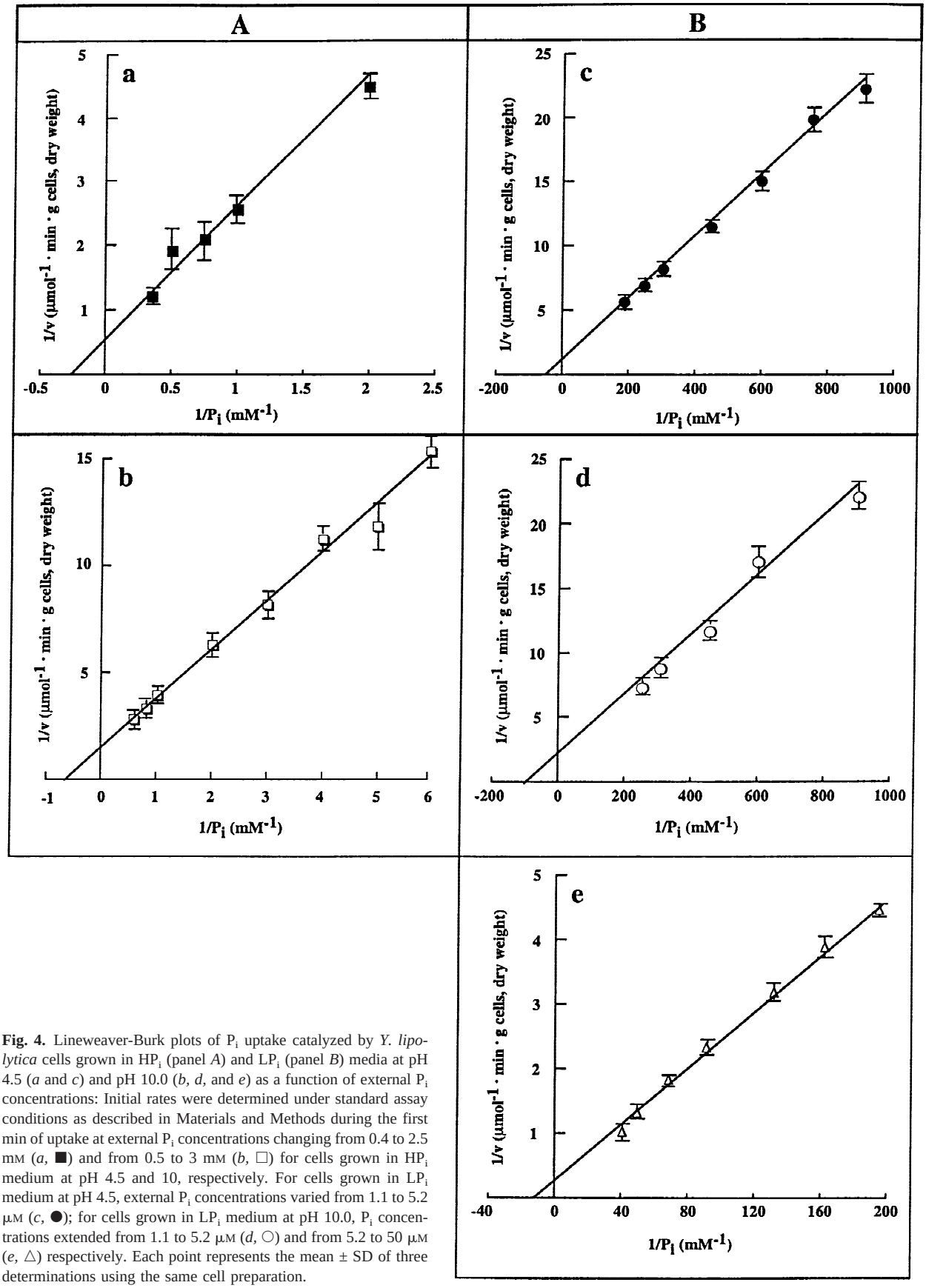


Fig. 4. Lineweaver-Burk plots of P_i uptake catalyzed by *Y. lipolytica* cells grown in HP_i (panel *A*) and LP_i (panel *B*) media at pH 4.5 (*a* and *c*) and pH 10.0 (*b*, *d*, and *e*) as a function of external P_i concentrations: Initial rates were determined under standard assay conditions as described in Materials and Methods during the first min of uptake at external P_i concentrations changing from 0.4 to 2.5 mM (*a*, ■) and from 0.5 to 3 mM (*b*, □) for cells grown in HP_i medium at pH 4.5 and 10, respectively. For cells grown in LP_i medium at pH 4.5, external P_i concentrations varied from 1.1 to 5.2 μM (*c*, ●); for cells grown in LP_i medium at pH 10.0, P_i concentrations extended from 1.1 to 5.2 μM (*d*, ○) and from 5.2 to 50 μM (*e*, △) respectively. Each point represents the mean ± SD of three determinations using the same cell preparation.

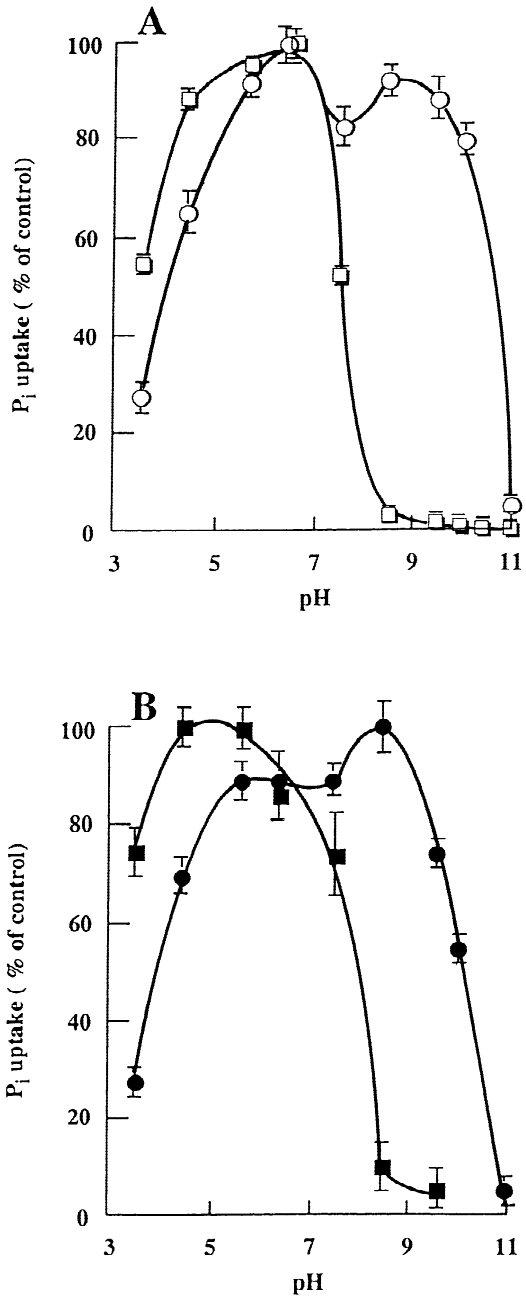


Fig. 5. pH-dependence of P_i uptake by *Y. lipolytica* cells. Cells were grown in LP_i (*A*) and HP_i (*B*) medium at pH 4.5 (□,■) and pH 10.0 (○,●). P_i transport activities were measured over a pH range of 3.5–11.0 in 25 mM assay buffer containing 3% glucose, 0.11 mM P_i during the first minute of uptake. The pH of the assay buffer was adjusted with Tris-succinate (pH 3.5–8.5), Tris–CAPS (pH 9.0–10.0) or Tris-CAPS-KOH (pH 10.5–11.0). For cells grown at pH 10.0, the assay buffers were supplemented with 25 mM NaCl. Values are the average of four to five determinations using the same cell preparation.

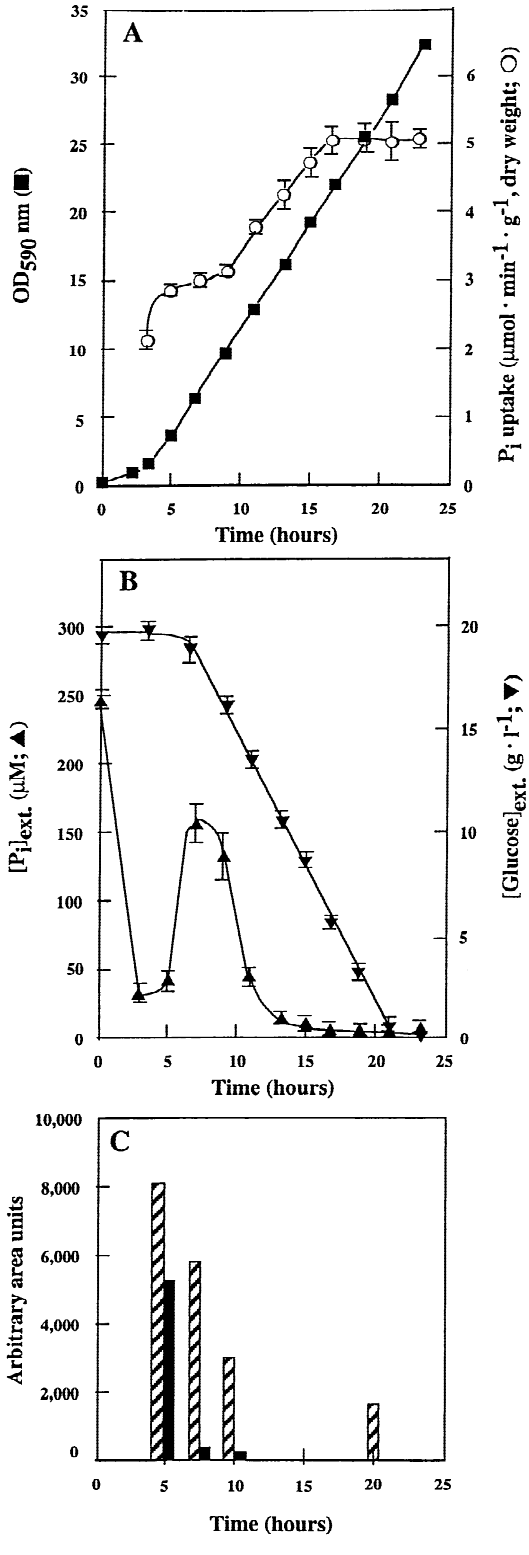
content of P_i declined, favoring a shift from organic to inorganic phosphates. Degradation of polyphosphates probably assists in the release of P_i into the medium. The obtained results strongly suggest that the derepression of the high-affinity P_i transporter in *Y. lipolytica* is maintained by the availability of extracellular P_i rather than the level of intracellular P_i. The fact that essentially all polyphosphate was depleted in cells showing the highest P_i transport activity, suggests, in line with earlier proposals [6, 14], that the P_i-sensitive regulation may also be mediated by the concentration of these phosphate polymers.

Thus, the results presented in this part of the paper clearly demonstrate that P_i is transported into *Y. lipolytica* cells grown at acidic pH conditions by two discrete systems, of which one is derepressible and operates at low external P_i concentrations and the other is most likely constitutive and active at high P_i concentrations. The derepression of the high-affinity P_i transport system may be under the control of extracellular P_i availability as well as the level of intracellular stores of polyphosphates. The two systems can operate in concert in suitably induced cells. The low K_m transporter would have the obvious benefit of being active under extreme phosphate shortage.

Cells Grown at Alkaline Conditions Exhibit a Na^+-Dependent Energy-Coupling of the Phosphate Uptake System

Our next step was to examine the characteristics of phosphate transport systems in *Y. lipolytica* cells grown at pH 9.5–10.0. Remarkably, cells grown in LP_i medium at pH 10.0 preserved, in spite of these severe conditions, full viability as judged by the propidium iodide test (*not shown*) and displayed a high $\Delta\Psi$-related fluorescence in both the mitochondrial (Fig. 7 *c*, black peak) and plasma membrane (Fig. 7 *d*, black peak) compartments, very similar to those for cells grown at pH 6.0 (Fig. 7 *a* and *b*, respectively, black peaks). In both types of cells the $\Delta\Psi$-fluorescence related to the mitochondrial potential was sensitive to the uncoupler CCCP (50 μM) (Fig. 7 *a, c*, shaded peaks), which is in harmony with direct measurements of oxidative and phosphorylative activities of high-quality mitochondria from cells grown at pH 6.0 [1] and pH 9.65 (Table). It is worthwhile to note that this is the first characterization of well preserved, tightly-coupled, fully competent mitochondria isolated from yeast cells grown at alkaline conditions. The plasma membrane potential was totally dissipated upon the addition of 50 μM CCCP in cells grown at pH 6.0 (Fig. 7 *c*, shaded peak). However, it was not significantly affected in cells grown at pH 10 even in the presence of 140 μM CCCP (Fig. 7 *d*, shaded peak), an observation suggesting that a hyperpolarization of the plasma membrane visualized with $DiO_6(3)$ in cells grown at pH 10 was not predominantly due to the proton electrochemical gradient.

The process of the P_i uptake by *Y. lipolytica* cells grown at pH 9.5–10.0 was energy-dependent as evident from the fact that substrate omission drastically diminished the rate of P_i accumulation (Fig. 3*B*, filled squares).



In contrast to *Y. lipolytica* cells grown at pH 4.5 where P_i accumulation was independent of the Na^+ concentration in the incubation medium (Fig. 3*A*), cells grown at pH 9.5–10.0 in LP_i and HP_i media containing the lowest attainable Na^+ concentration, exhibited an almost negligible P_i uptake (Fig. 3*B*, filled circles, Fig. 8). The activity was strongly promoted by the presence of Na^+, especially at low P_i concentrations (Figs. 3*B* and 8). At 10 mM Na^+, phosphate uptake was stimulated 6- to 10-fold (Fig. 8*A*). The effect of Na^+ was highly specific; other alkali ions (K^+ or Li^+) did not stimulate P_i uptake to the same extent (Fig. 3*B*). The estimated K_m for Na^+ was approximately 1.5 and 0.4 mM for LP_i- and HP_i-grown cells, respectively. These results suggest that P_i uptake in *Y. lipolytica* cells grown at alkaline conditions is mediated by transport systems differing from those engaged in P_i accumulation in cells grown at acidic conditions and is most probably driven by Na^+ symport.

The dramatic difference in the cellular P_i uptake rate observed in *Y. lipolytica* cells grown in LP_i- and HP_i-media at pH 9.5–10.0 (Fig. 8*A*, *B*) indicates that the Na^+-dependent P_i transport system responsible for P_i entry into the cell at alkaline conditions is under the control of the availability of the extracellular phosphate. In cells grown in LP_i medium at pH 9.5–10.0, the initial net uptake of [^{32}P]phosphate measured during the first 15 and 30 sec corresponded to internalization of approximately 55 and 80% of the total added radioactivity, respectively (Fig. 3*B*), considerably more than that reported in *S. cerevisiae* [13]. This is, to our knowledge, the first demonstration of a high-capacity P_i uptake system in yeast cells grown at alkaline conditions.

The P_i transport systems in *Y. lipolytica* cells grown in HP_i and LP_i media at pH 9.5–10.0 depended on the external pH, when P_i uptake was measured under conditions of constant Na^+ (20 mM) and P_i (0.11 mM) concentrations (Fig. 5*A*, *B*, circles). In cells grown at pH 9.5–10.0, the rate of P_i uptake showed a broad pH optimum extending from pH 5.5 to 9.5, decreasing to one-third its optimum at pH 3.5, and diminishing to almost zero at pH 11. The rather high transport activity seen at pH 6.5–8.5 in cells grown at alkaline conditions presumably indicates a very fast expression of the H^+-coupled phosphate transport system under appropriate conditions.

Fig. 6. Time course of [^{32}P]phosphate accumulation by *Y. lipolytica* cells. (*A*) Accumulation during growth in LP_i medium at pH 4.5. (*B*) Extracellular concentrations of phosphate and glucose. (*C*) Intracellular P_i levels and polyphosphates. Cells were aseptically separated from the cultivation medium by centrifugation as described in Materials and Methods and assayed for cell growth (■), P_i uptake (○), as well as for intracellular levels of P_i (striped bars) and polyphosphates (solid bars). The corresponding supernatants were used for glucose (▼) and phosphate (▲) determination. Phosphate transport activities were measured in 25 mM Tris-succinate buffer, pH 5.5, containing 3% glucose and 0.11 mM P_1 during the first minute of uptake. Values are the average of four to five determinations using the same cell preparation.

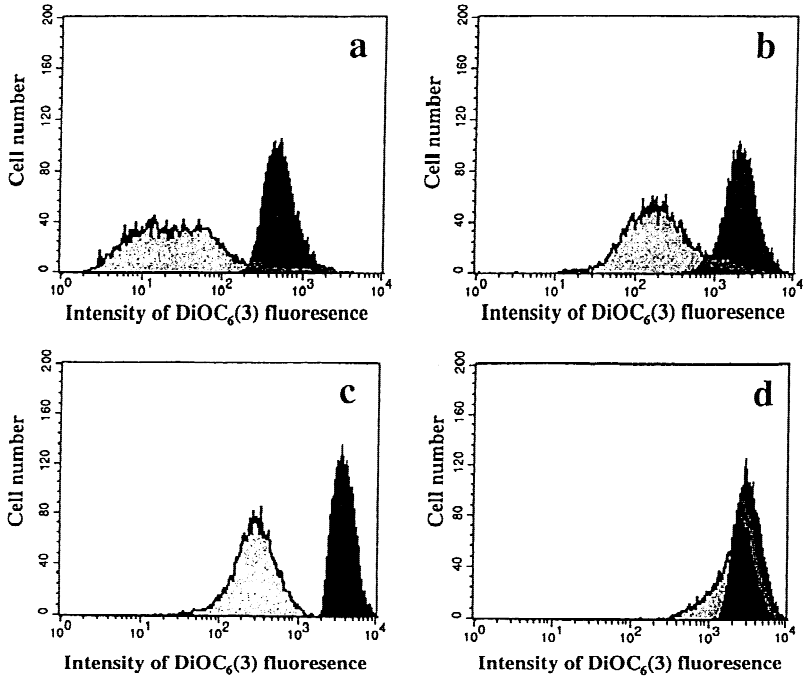


Fig. 7. Flow cytometric measurements. Activity in the mitochondrial (*a, b*) and plasma membrane (*c, d*) of *Y. lipolytica* cells grown in LP_i medium at pH 6.0 (*a, c*) and pH 10.0 (*b, d*). The mitochondrial and plasma membrane potentials were assayed in appropriate assay buffers with 2 or 20 nM $DiO_6(3)$, respectively, as described in Materials and Methods.

The high level of P_i internalization observed at optimal pH conditions in P_i-starved cells (Figs. 3*B* and 8*A*), but lacking in HP_i-grown cells (Fig. 8*B*), suggests the presence of at least two different Na^+-dependent carrier systems responsible for the active P_i uptake at alkaline conditions. To investigate this possibility, we assessed the kinetic properties of the P_i transport systems expressed in HP_i- and LP_i-grown cells by measuring the uptake activity over a wide concentration range of P_i (1 μM to 2 mM) (Fig. 4, open symbols). The uptake reaction measured in cells grown in HP_i medium at pH 9.5–10.0 in the presence of 20 mM Na^+ and various concentrations of P_i (0.167 to 2 mM) was clearly saturable with respect to substrate and could be described by simple Michaelis-Menten kinetics with an apparent K_m for monovalent orthophosphate of 0.6 mM (Fig. 4*A, b*). Because at pH 9.5 less than 5% of the phosphate is in the form of the monovalent anion [28], the apparent K_m derived from Fig. 4 (*A, b*) has to be corrected by a factor of 20 or more. The P_i accumulation by cells grown in LP_i-medium at pH 9.5–10.0 was also measured in the presence of 20 mM Na^+ and increasing P_i concentrations from 1.1 to 25 μM. Kinetics exhibited clearly discontinuous curves and Lineweaver-Burk plots of the data typically revealed two linear phases over the concentration range studied (Fig. 4*B, c, d*); the changes in linearity possibly indicate a multiphasic mechanism with different affinities of the transporter for phosphate, as well as different velocities. The term multiphasic in this context refers only to the shape of the reciprocal graphs, regardless of the mechanism of the uptake. Lineweaver-Burk plots for P_i concentrations ranging from 1.1 to 5.2 μM (Fig. 4*B, d*) and from 5.2 to 25 μM (Fig. 4*B, e*) indicate K_m values for P_i uptake of 10.7 μM and 87 μM, respectively, values that reflect an overestimation of at least 20-fold with respect to monophosphate. Previously, a multiphasic concentration-dependent uptake of ions and amino acids has been shown in higher plants [16, 26].

Thus, several novel discrete saturable Na^+-dependent P_i transport systems in *Y. lipolytica* cells grown at alkaline conditions have been characterized. One of these, a low-affinity Na^+-dependent transporter with a K_m value of 30 μM for monophosphate is most likely constitutively expressed at high P_i concentrations. This system is, to our knowledge, kinetically characterized here for the first time. The other two very active Na^+-dependent high-affinity systems are derepressible, operative under conditions of P_i-starvation, and represent the first examples of high-capacity Na^+-driven P_i-transport systems in an organism outside the animal and bacterial kingdom.

Phosphate Uptake into *Y. lipolytica* Is Regulated by pH-Dependent H^+- and Na^+-Coupled Transporters

The distinct characteristics of the P_i transport of *Y. lipolytica* cells grown at pH 4.5 and 10.0, i.e., at two extremes of the pH growth range for this yeast, prompted us to quantify the contribution of these two systems to P_i uptake in *Y. lipolytica* cells grown in buffered LP_i media at different pH values. P_i transport was assayed at pH 5.5 (optimal for H^+/P_i cotransport system) and pH 9.5 (op-

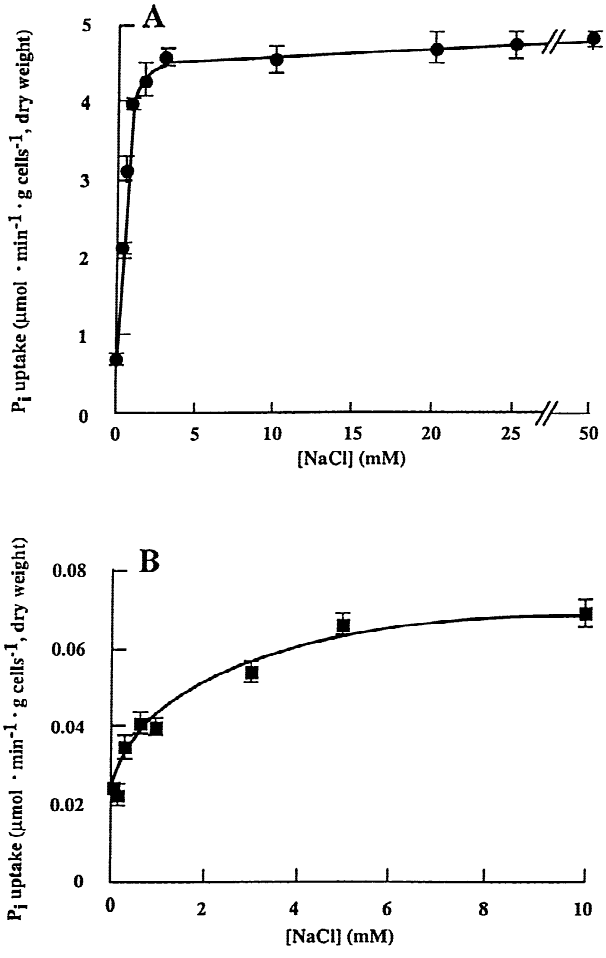


Fig. 8. Stimulatory effect of NaCl on P_i uptake by *Y. lipolytica*. Cells were grown at pH 10 in LP_i (*A*) and HP_i (*B*) medium. Uptake of P_i was determined in the assay buffer containing 25 mM CAPS-Tris, pH 9.5, 0.11 mM P_i and 3% glucose in the presence of various NaCl concentrations. Values are the average of four to five determinations using the same cell preparation ± SD.

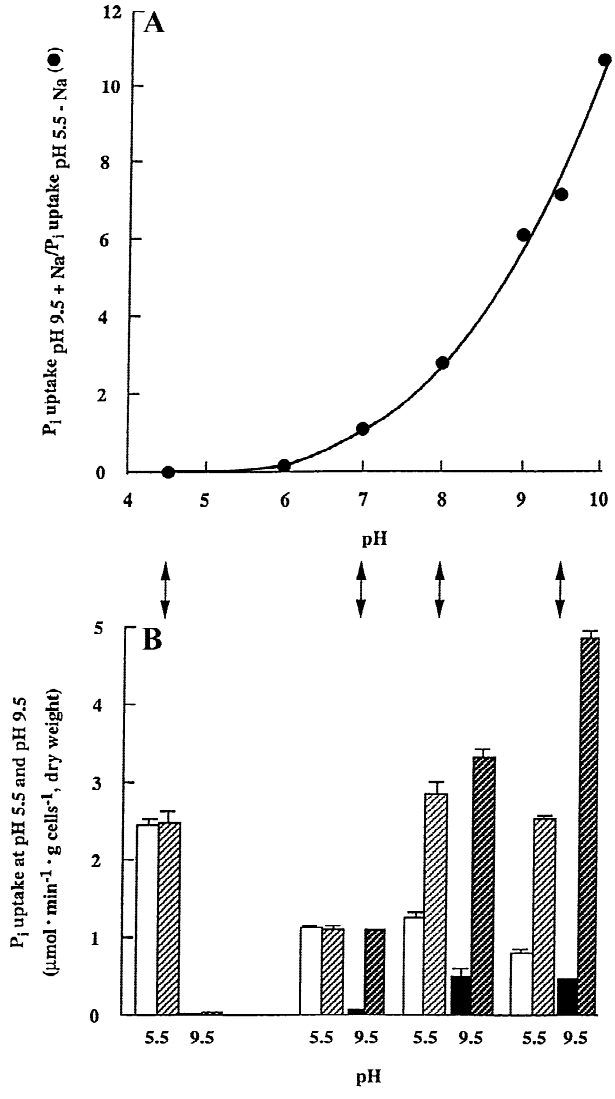


Fig. 9. Contribution of H^+- and Na^+-coupled P_i transport systems to the total P_i uptake by *Y. lipolytica* cells grown in LP_i medium at pH values ranging from 4.5 to 10.0. Cells were grown in buffered LP_i media at pH 4.5–10.0. For each aliquot of collected cells, uptake of P_i was measured for the first minute in 25 mM Tris-succinate assay buffer, pH 5.5, containing 0.11 mM P_i and 3% glucose, in the absence (open bars) or in the presence of 25 mM NaCl (striped bars), as well as in 25 mM CAPS-Tris assay buffer, pH 9.5, containing 0.11 mM P_i and 3% glucose, in the absence (black bars) or in the presence of 25 mM NaCl (striped shaded bars) (*B*). The P_i uptake at pH 5.5 in the absence of NaCl was taken as a measure of the H^+-coupled P_i transport system, while the P_i accumulation at pH 9.5 in the presence of 25 mM NaCl was taken as a measure of the Na^+-coupled P_i transport system(s). Shown in (*A*) is the ratio of the Na^+/H^+ P_i transport systems (●).

timal for Na^+/P_i cotransport system) in the absence and presence of 25 mM NaCl. Activity at pH 5.5 without NaCl was taken as a measure of the H^+/P_i cotransport system, while activity in the pH 9.5 buffer supplemented with 25 mM NaCl was taken as a measure of the Na^+/P_i-cotransport system. The results presented in Fig. 9 clearly show that the H^+-coupled P_i transport systems provide most, if not all, of the P_i uptake into *Y. lipolytica* cells grown at pH values of 4.5 and 6.0. The contribution of the Na^+/P_i cotransport systems to the total cellular P_i uptake activity progressively increased with increasing pH and reached its maximum at pH 9 and higher, where P_i accumulation was preferentially, if not exclusively, maintained through the Na^+/P_i cotransport systems. Nevertheless, H^+/P_i cotransport occurred even at pH 8.0, presumably as a consequence of a local pH gradient in the vicinity of the carrier in the plasma membrane. At pH 7.0, both H^+/P_i and Na^+/P_i cotransport systems were equally responsible for P_i uptake.

In summary, the findings presented in this paper show the advantage of *Y. lipolytica* as a model system in the study of phosphate transport as affected by large

fluctuations in pH values of the external medium. The utility of this fungus lies on its capacity of growing over a wide range of pH values, along with its fast growth and preservation of an efficient mitochondrial oxidative phosphorylation system even at extreme growth conditions. At pH 4.5, P_i transport by this yeast is primarily driven by the H^+ gradient generated across the plasma membrane, while at pH 9.5–10.0, P_i accumulation is essentially Na^+-dependent. The H^+- and Na^+-coupled P_i transport systems would thus possess overlapping but distinct biological roles in the acquisition of phosphate in *Y. lipolytica* cells at different growth conditions. Altogether, these results contribute to the general knowledge of phosphate metabolism and acquisition strategies of yeasts under different growth conditions.

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