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Net phosphate transport in phosphate-starved *Candida utilis*: relationships with pH and K^+

Roger M. Bourne

Department of Microbiology, University of Queensland, St. Lucia (Australia)

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Phosphate transport was studied in phosphate-starved *Candida utilis* using ^{31}P-NMR and in situ pH and K^+-specific electrodes. A transient efflux of K^+ was associated with the initial net influx of orthophosphate across the plasma membrane and decrease of both the plasma membrane pH gradient and the cytosol pH. Recovery of cytosol pH, and the plasma membrane pH gradient after phosphate uptake, was slow when extracellular K^+ was depleted, but rapid when K^+ was added to the suspension either with orthophosphate or after phosphate uptake. Net phosphate transport into the vacuole occurs against its concentration gradient and is associated with an increase of the tonoplast pH gradient. It is proposed that transport of orthophosphate into the vacuole is mediated by an electrical uniport and driven by the tonoplast membrane potential.

Introduction

Phosphate uptake from the environment Is an activity of critical importance to all living cells. In most cells phosphate uptake occurs against its concentration gradient and is consequently dependent on, and intimately associated with, energization of the cellular membranes.

In yeasts phosphate uptake across the plasma membrane from a medium at pH 4.5–5.0 occurs mainly via an H^+/P_i symport which transports the monovalent orthophosphate anion with stoichiometry of 2–3 H^+ per $H_2PO_4^-$ [1]. Transport of phosphate against its chemical potential gradient is energized by the electrochemical potential gradient of H^+, or proton motive force (pmf = $\Delta\Psi$–60 ΔpH mV, where $\Delta\Psi$ is the transmembrane electrical potential difference, and ΔpH the trans-membrane pH gradient), created by the plasma membrane H^+-ATPase [2]. Simultaneous measurements of cytosol and medium pH changes associated with phosphate uptake are consistent with phosphate transport across the plasma membrane via such a H^+/P_i symport [3].

Phosphate uptake is enhanced in the presence of K^+ at low medium pH – an effect which is thought to indirectly result from the increasing of intracellular pH by K^+ [2,4]. In P-starved *C. utilis* uptake of phosphate is accompanied by a net influx of $3H^+$ and an efflux of $2K^+$ [5]. When energy metabolism is inhibited the uptake of H^+ and efflux of K^+ continue for the duration of phosphate uptake. In the absence of inhibitors H^+ influx and K^+ efflux occur for only approx. 0.3 min before the fluxes of H^+ and K^+ are reversed. At the conclusion of phosphate uptake there is a net uptake of K^+ and release of H^+ [5]. The transient efflux of K^+ accompanying phosphate uptake has been assumed to reflect depolarization of the plasma membrane by the influx of an excess of H^+ with the monovalent orthophosphate anion and consequent release of K^+ down is concentration gradient via an electrical uniport [4,5].

Yeast cells maintain orthophosphate concentration gradients across their vacuole membranes to the extent that the orthophosphate concentration in the vacuole may be up to 25-times higher than in the cytosol [3,6], however, the mechanism of phosphate accumulation in the yeast vacuole is unknown. The mechanism must necessarily be different from that which maintains the plasma membrane orthophosphate concentration gradient because, as Nicolay et al. [6] noted, a lower pH in

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Et_3N, triethanolamine; NMR, nuclear magnetic resonance; MeP, methylphosphonate; P_i, orthophosphate; mV, millivolts; pmf, protonmotive force.

Correspondence (current address): R.M. Bourne, Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, U.K.

the vacuole than in the cytosol would tend to favour the accumulation of orthophosphate in the cytosol if an H^+/P_i symport were active. In contrast with the situation observed in mitochondria, the concentration gradient of orthophosphate across the tonoplast is not in equilibrium with the pH gradient [6].

The studies reported here concern the relationships between net phosphate transport, extracellular K^+ levels and K^+ fluxes, and the pH gradients across the plasma membrane and tonoplast of *Candida utilis* recovering from phosphate starvation. The use of in situ pH and K^+ electrodes in the NMR apparatus has enabled the continuous measurement of extracellular pH and K^+ concentration – important experimental parameters which are difficult or impossible to measure with similar accuracy or time resolution by NMR methods. With this experimental apparatus it has been possible to correlate results previously obtained in separate NMR [3,6] and bench experiments [2,4,5] with P-starved yeasts.

Materials and Methods

Cultures of *Candida utilis* (UQMCC-23Y) were grown in a glucose mineral salts medium and phosphate-starved as described previously [3]. The P-starved cells were then washed twice by centrifugation and resuspension in cold (4 °C) reverse-osmosis water. The final pellet was resuspended at approx. 60% w/v (intracellular volume ≈ 30% total volume [3]) in cold 20 mM succinate buffer adjusted to pH 5.0 with triethanolamine base (Sigma), and stored at 0–4 °C until used (0–5 h). Storage of the cells for 0–5 h did not result in any observed variation in phosphate transport behavior.

The yeast suspension was aerated and recirculated in a chamber similar to that described previously [3,7], except that an in situ K^+-specific electrode (Phillips IS561) was included together with the pH electrode. The internal reference of the combination pH electrode also served as the reference for the K^+ electrode. Leakage of KCl from the reference cell into the sample is too slow to affect the calibration of the K^+ electrode during the course of an experiment. 30 ml of the cell suspension + 200 µl antifoam (1% glycerol monooleate) + 250 µl 1 M methylphosphonic acid (adjusted to pH 5.0 with triethanolamine) + 50 µl 2 M KCl was maintained at 30 °C and aerated with 95% O_2/5% CO_2 at approx. 70 ml min^{-1} in the sample chamber. Before the commencement of experiments the suspension was aerated (approx. 10 min) until the extracellular pH stabilized at 4.8–5.0.

Experiments were performed at the Research School of Chemistry, Australian National University, Canberra. The instrument used was a Varian VXR300S with 16 mm broadband probe. Protons were decoupled during acquisition by the 'Waltz-16' broadband method. The temperature was maintained at 28 ± 1 °C. The spectrometer was operated in the Fourier transform mode at 121.4 MHz. Transients were averaged using 4k of memory over a spectral width of 15 kHz. 60° (40 µs) pulses were applied with 1 s recycle delay and free induction decays were accumulated in 30 s blocks except from $t = -40$ to 0 min in Expt. 1 (2 min/spectrum). Chemical shifts were referenced relative to an external standard of methylene diphosphonic acid contained in an in situ capillary and assigned a chemical shift of 16.72 ppm [8].

Cytosol pH was calculated from the chemical shift of the intracellular methylphosphonate resonance [3]. Vacuole pH was calculated from the chemical shift of the vacuole orthophosphate resonance [9]. Titration of the chemical shifts of orthophosphate and methylphosphonate was performed by stepwise addition of HCl to a solution containing 10 mM $(NH_4)_2HPO_4$, 2.7 mM NaCl, 10 mM methylphosphonic acid, and KCl + KOH to make pH = 10 and $[K^+] = 150$ mM. Extracellular pH and $[K^+]$ were measured continuously with the in situ pH and K^+-specific electrodes which were connected to TPS pH/mV meters (Model 1852, TPS Electronics, Brisbane). Outputs of the pH/mV meters were recorded on a two channel chart recorder. Extracellular pH and $[K^+]$ were calculated from the chart at the times corresponding to the middle of the period of acquisition of each spectrum (block of free induction decays). Further calculations of pH and $[K^+]$ were made during periods when the time interval between successive spectra was insufficient to indicate rapid changes. In calculations of $[K^+]$ no correction was made for the response time of the electrode. Fluxes of H^+ and K^+ across the plasma membrane were calculated assuming the extracellular volume was 70% of the sample volume. The buffer capacity of the extracellular medium (18.8 mM equiv./pH in Expt. 1) was determined at the conclusion of the experiment by addition of aliquots of 1 M NaOH and HCl.

Orthophosphate concentrations in Expt. 1 (Fig. 1) were calculated as described previously [3]. Saturation corrections were not determined for Expt. 2 so the intensity of the vacuole orthophosphate resonance in Fig. 4a is expressed in arbitrary units.

Phosphate was added either as 0.5 M or 1 M KH_2PO_4, or as 0.5 or 0.2 M H_3PO_4 adjusted to pH 5.0 with triethanolamine (P_i/Et_3N). Methylphosphonate was added as 1 M methylphosphonic acid (Aldrich) adjusted to pH 5.0 with triethanolamine (MeP). These solutions were injected into the sample suspension via a peristaltic pump at approx. 0.37 ml min^{-1}.

Additions were made to the sample suspension commencing as follows:

Expt. 1. $t = -20$ min, 50 µl 2 M KCl; $t = 0$, 1.0 ml 0.5 M P_i/Et_3N (addition interrupted by bubble); $t =$

16.5, 500 μl 0.2 M KCl; t = 25.5, 500 μl 0.2 M KCl; t = 38, 1.0 ml 0.5 M P_i/Et_3N; t = 56.25, 100 μl 1 M $MgCl_2$; t = 71.25, 600 μl 1 M KH_2PO_4. Each addition was washed in with 250 μl distilled water.

Expt. 2. t = 4 min, 500 μl 0.5 M P_i/Et_3N; t = 20, 1.0 ml 0.2 M P_i/Et_3N; t = 35, 500 μl 0.5 M P_i/Et_3N; t = 55, 250 μl 1 M MeP; t = 80, 1.0 ml 0.5 M P_i/Et_3N. Each addition was washed in with 250 μl distilled water.

Results

Two experiments are described in this paper. The results are representative of those found in several similar experiments. ^{31}P-NMR spectra obtained during phosphate uptake by the P-starved *C. utilis* suspension were similar to those illustrated previously [3].

Experiment 1

Cells used in this experiment were grown on complete medium and then P-starved until the pellet mass had increased 4.7 times.

Fig. 1A shows the intensities of the medium (P_{ex}) cytosol (P_{cyt}), and vacuole (P_{vac}) orthophosphate resonances expressed as millimolar concentration in the particular compartments. Fig. 1B shows the intensity of the polyphosphate resonance (polyP) and the sum of P_{ex}, P_{cyt}, P_{vac} and polyP, expressed as millimolar concentration in the total sample volume. (The phosphomonoester resonance was of very low and essentially constant intensity throughout both experiments). The approximately constant value of the sum of the intensities indicates that there was no net loss of phosphate into NMR-invisible pools [3]. In general the compartmentation of accumulated phosphate was very similar to that found in earlier experiments with P-starved *C. utilis* [3].

Fig. 2A shows measured and calculated pH values in the medium (pH_{ex}), cytosol (pH_{cyt}) and vacuole (pH_{vac}). The absence of MeP accumulation in the

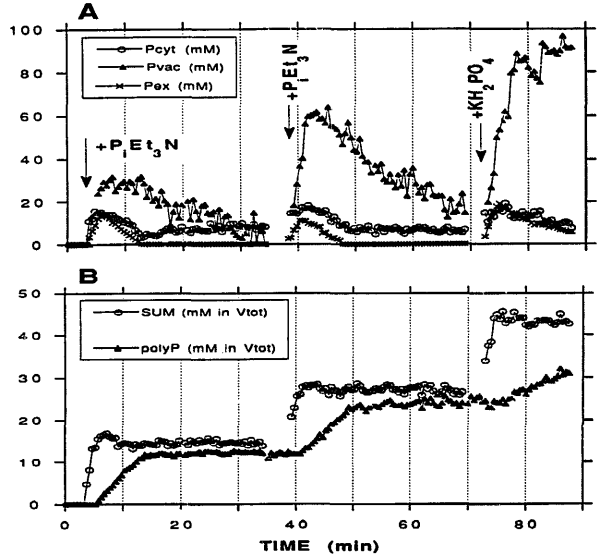

Fig. 1. Compartmentation of orthophosphate and synthesis of polyphosphate (Expt. 1). (A) Calculated millimolar orthophosphate concentrations in the medium (P_{ex}), vacuole (P_{vac}), and cytosol (P_{cyt}). Orthophosphate was added to the yeast suspension at the times indicated. (B) Intensity of the polyphosphate resonance (polyP) and the sum of the P_{ex}, P_{vac}, P_{cyt}, and polyP resonances (SUM) expressed as millimolar phosphate concentration in the total sample volume (V_{tot}).

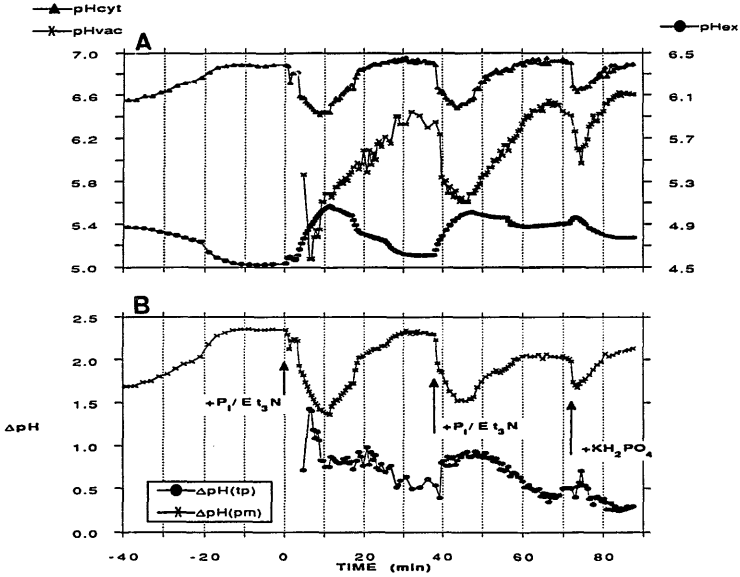

Fig. 2. Changes in cytosol, vacuole, and medium pH, and in plasma membrane and tonoplast pH gradients during phosphate accumulation (Expt. 1). (A) pH in the medium (pH_{ex}), cytosol (pH_{cyt}), and vacuole (pH_{vac}). (B) pH gradients across the plasma membrane (Δ_1H(pm)) and tonoplast (ΔpH(tp)) calculated from the data in (A) above.

vacuole precludes the determination of pH_{vac} before the appearance of the P_{vac} resonance. The pH changes associated with phosphate uptake were also similar to those observed previously [3], although the shorter accumulation time (30 s) for each spectrum used in the present study provides a considerably more detailed record of intracellular pH changes. As expected, phosphate uptake led to an increase of pH_{ex} and a decrease of pH_{cyt} consistent with the transport of phosphate across the plasma membrane via an H^+/P_i symport.

In order to obtain a clearer indication of the relationship between phosphate transport and the pH gradients across the plasma membrane (ΔpH_{pm}) and tonoplast (ΔpH_{tp}), ΔpH_{pm} and ΔpH_{tp} were calculated from the data shown in Fig. 2A and are illustrated in Fig. 2B. Whereas net orthophosphate transport across the plasma membrane resulted in a decrease of ΔpH_{pm}, the initial influx of orthophosphate into the vacuole was associated with an increase in ΔpH_{tp}. This increase was small and quickly reversed when extracellular $[K^+]$ was high ($t = 74$ min).

Fig. 3A shows the measured concentration of extracellular K^+ (K_{ex}). The log scale is intended to emphasize changes when K_{ex} is low. The transient efflux of K^+ observed at $t = 1$, 3, and 39 min which is associated with the commencement of phosphate uptake is in good agreement with earlier observations in P-starved *C. utilis* [5]. It should be noted that the real magnitude of the transient K^+ efflux must be significantly greater than indicated by Fig. 3A but is attenuated by the relatively slow response time of the K^+ electrode compared with the duration of the transient K^+ efflux.

Fig. 3B shows calculated H^+ and K^+ fluxes across the plasma membrane. Because of the slow response time of the K^+ electrode calculations of the K^+ flux, and the sum of H^+ and K^+ fluxes, are imprecise during periods of rapid change of K_{ex}. Addition of KCl to the suspension at $t = -20$, 16.5, and 25.5 min led to an approximately stoichiometric exchange of intracellular H^+ for extracellular K^+.

It can be seen in Fig. 2B and Fig. 3 that the development of ΔpH_{pm} and its recovery after phos-

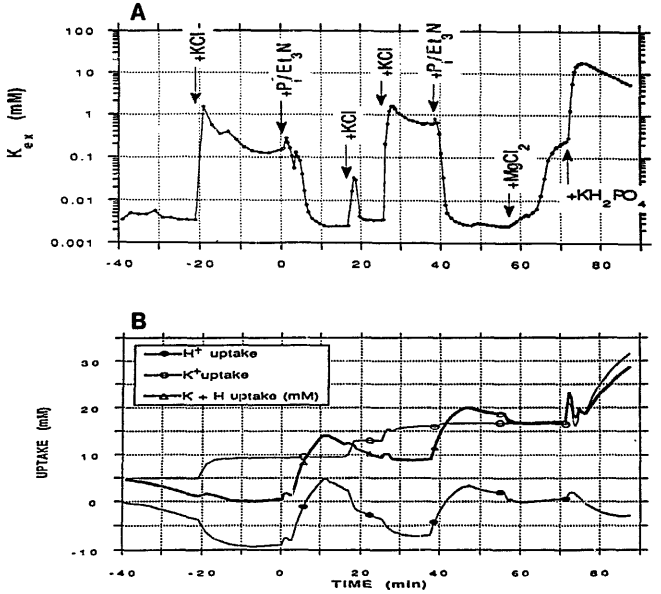

Fig. 3. Concentration of extracellular K^+, and calculated fluxes of H^+ and K^+ across the plasma membrane during phosphate uptake (Expt. 1). (A) Extracellular K^+ concentration (K_{ex}) measured with the in situ K^+-specific electrode. The log scale is intended to emphasise small changes when K_{ex} is low. Additions were made as indicated. (B) Uptake of H^+ and K^+ across the plasma membrane, and the sum of these fluxes (bold line). For clarity the individual data points shown in (A) above are omitted.

phate uptake was relatively slow when K_{ex} was low but fast when KCl was added to the suspension.

Experiment 2

This experiment was performed with a different culture of P-starved *C. utilis* from that used in Expt. 1. Cells were P-starved until the wet pellet mass had increased 3.3-times.

Fig. 4A shows changes in the intensity of the P_{vac} resonance during orthophosphate and methylphosphonate uptake. Fig. 4B shows the corresponding changes in ΔpH_{pm} and ΔpH_{tp}. Uptake of both orthophosphate and methylphosphonate caused a decrease of ΔpH_{pm} suggesting that both are transported by the H^+/P_i symport. However, addition of MeP (and its uptake across the plasma membrane) has no effect on P_{vac} and the sudden increases in ΔpH_{tp} associated with the initial influx of orthophosphate into the vacuole did not occur after addition of MeP. It has been shown previously that MeP is not transported into the vacuole [3]. These observations indicate that the sudden increases in ΔpH_{tp} are indeed the result of orthophosphate influx into the vacuole.

The similarity of observed pH changes and phosphate compartmentation in this experiment, Expt. 1 above, and previous experiments with P-starved *C. utilis* [3], indicate that the degree of P-starvation does not critically affect the gross changes in these experimental parameters.

Discussion

Plasma membrane transport

In order to simplify the study of the ion fluxes associated with phosphate uptake, experiments were conducted in the simplest possible medium consisting

of succinate buffer, triethanolamine, and KCl. By adding phosphate as P_iEt_3N rather than the potassium salt it was possible to observe phosphate uptake in the absence of large additions to the extracellular K^+ pool and thus to gain insights into the possible mechanism of the previously observed stimulation of phosphate uptake by K^+ [2]. The sodium salt of phosphate was not used in order to reduce unmeasured background fluxes of ions.

It is evident from Fig. 2 that the pH changes associated with the initial stages of phosphate uptake are very rapid. The onset of phosphate uptake was associated with a decrease of ΔpH_{pm}. When the addition of P_iEt_3N commencing at $t = 0$ was interrupted by a small bubble in the injection tube ΔpH_{pm} began to recover rapidly with concommitant rapid uptake of K^+ from the medium. When the bubble had passed ($t = 3$ min) ΔpH_{pm} again fell rapidly when phosphate uptake resumed and continued to fall until the exhaustion of P_{ex} at $t = 11$ min. There was a steady net uptake of H^+ across the plasma membrane until phosphate influx ended (Fig. 3B). Similarly, when P_iEt_3N was added a second time ($t = 38$–48 min) ΔpH_{pm} again decreased continually and there was net H^+ influx until the end of phosphate uptake. In contrast, when KH_2PO_4 was added at $t = 71$ min ΔpH_{pm} began to recover, with net efflux of H^+, within 3 min of its initial fall – long before the cessation of phosphate uptake. This rapid recovery of pH_{cyt} after addition of KH_2PO_4 has been observed previously [3]. In general, recovery of ΔpH_{pm} was slow when K_{ex} was depleted but rapid when K^+ was added with phosphate or following phosphate uptake. From these observations it is concluded that the rate of recovery of ΔpH_{pm} is dependent on the availability of extracellular K^+ to exchange for H^+ extruded by the plasma membrane H^+-ATPase. In the absence of K^+ to exchange for extruded H^+ the major component of the plasma membrane pmf would be

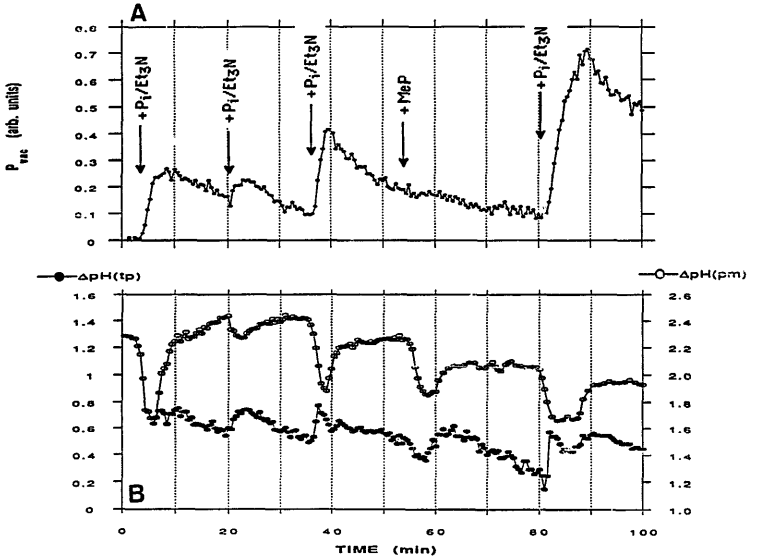

Fig. 4. Effect of orthophosphate and methylphosphonate uptake on plasma membrane and tonoplast pH gradients (Expt. 2). (A) Orthophosphate accumulation in the vacuole. The intensity scale is arbitrary (see Materials and Methods). Additions were made as indicated. (B) Changes in the plasma membrane ($\Delta pH(pm)$) and tonoplast ($\Delta pH(tp)$) pH gradients.

$\Delta\Psi$. These results suggest that the K^+-enhancement of phosphate uptake may be related to the ability of the cell to recover from the effects of phosphate uptake on cytosol pH and ΔpH_{pm}, rather than the existence of a high cytosol pH prior to phosphate uptake as suggested previously [2].

When K_{ex} was very low (e.g. $t = 5$–17, and 40–60 min in Expt. 1) ΔpH_{pm} and pH_{cyt} recovered slowly. This recovery necessitates fluxes of an ion other than K^+ to balance the charge of H^+ extruded by the plasma membrane H^+-ATPase [4]. The most likely process is the efflux of Cl^- or bicarbonate from the cytosol. Blatt and Slayman [10] have shown that the Cl^- permeability of the *Neorospora* plasma membrane is significant and can account for the slow dissipation of $\Delta\Psi$.

Associated with the initial influx of orthophosphate there was a transient efflux of K^+ at $t = 1$, 3, and 39 min. The profiles of the K^+ fluxes were very similar to those previously reported for P-starved *C. utilis* [5] and *S. cerevisiae* [11]. The transient efflux of K^+ has been attributed to depolarization of the plasma membrane by the influx of 2–3 H^+ with each $H_2PO_4^-$ anion, and consequent release of K^+ down its concentration gradient [4,5]. The results presented here lend further support to this hypothesis and show that these fluxes of K^+ across the plasma membrane are closely associated with changes of ΔpH_{pm} and pH_{cyt}.

The transient K^+ release may occur via a voltage-gated K^+ channel [4,12], and serve to balance the charge fluxes across the plasma membrane. Uptake of K^+ can be indirectly attributed to activity of the plasma membrane H^+-ATPase. The pmf developed by the ATPase may drive K^+ uptake through an electrical uniport, or an H^+/K^+-symport [4,5,13]. Alternatively, the ATPase may directly exchange H^+ from the cytosol for extracellular K^+ [14]. There is evidence that K^+ influx and efflux occur through separate channels [4].

Tonoplast transport

Fig. 1A shows that phosphate transport into the vacuole occurred against a significant concentration gradient. Such transport against a chemical potential gradient requires an 'external' driving force. Since the trans-tonoplast pmf is positive-inside it is unlikely that this phosphate accumulation is mediated by an H^+/P_i symport. Nicolay et al. [15] demonstrated the accumulation of orthophosphate in the acidic matrix of both in situ and isolated peroxisomes in several yeast species. Phosphate was not accumulated in the peroxisomes if they were incubated in the absence of ATP or in the presence of the H^+-ATPase inhibitor DCCD. It thus appears that a pmf is necessary for the transport of phosphate across the peroxisomal membrane and it is conceivable that this process is mediated by a phosphate uniport and driven by $\Delta\Psi$. This may also be the mechanism of orthophosphate transport into yeast vacuoles. If this were the case then phosphate transport would be expected to dissipate $\Delta\Psi$ and stimulate activity of the tonoplast H^+-ATPase. Continued phosphate uptake across the tonoplast would then result in steady acidification of the vacuole and an increase in ΔpH_{tp} until an equilibrium was established where ΔpH was the major component of the tonoplast pmf. If vacuolar H^+ were then exchanged for a cation, say K^+, from the cytosol then ΔpH would be dissipated, the ATPase would increase $\Delta\Psi$ accordingly, and futher phosphate uptake would be possible. Several H^+/cation antiports, including one for K^+, have been found in the yeast tonoplast [16]. This mechanism of phosphate accumulation in the vacuole would be dependent on the availability of a suitable cation in the cytosol to exchange for H^+ from the vacuole and thus prevent the potentially limiting buildup of ΔpH_{tp}. From Figs. 1–3 it is evident that the increase of ΔpH_{+p} associated with phosphate uptake was smallest, and the peak of P_{vac} was greatest, in the presence of excess extracellular K^+, suggesting that K^+, entering the cytosol from the medium so as not to deplete K^+ levels in the cytosol, may act to dissipate ΔpH_{+p} in the way just proposed. According to this hypothesis phosphate uptake across the plasma membrane occurring without simultaneous phosphate transport into the vacuole would be expected to cause acidification of the cytosol without a parallel acidification of the vacuole and consequently a decrease of ΔpH_{tp}. This is exactly what is observed in Fig. 4B where MeP was added to the P-starved cell suspension at $t = 55$ min.

The tonoplast membrane potential is probably sufficiently large to maintain observed orthophosphate gradients via an electrical uniport. Isolated vacuolar vesicles of *S. cerevisiae* were found to have a $\Delta\Psi$ of 75 mV (inside positive) when $\Delta pH = 0.7$ units [17]. Assuming a very simple model of phosphate accumulation where the monovalent anion equilibrates with $\Delta\Psi$, a value of $\Delta\Psi_{tp} = 60$ mV would be sufficient to account for a 10:1 concentration gradient of $H_2PO_4^-$ across the tonoplast. The real situation, involving an equilibrium of both mono- and dianions, is likely to be more complex [18].

Dietz et al. [19] reported that the uptake of anions by isolated plant vacuoles was stimulated by excess Mg^{2+}. This stimulation was not due to increased activity of the tonoplast ATPase. Excess Mg^{2+} has a similar effect on vacuolar phosphate accumulation in P-starved *C. utilis* (results not shown). If the transport of phosphate and other anions across the tonoplast is mediated by electrical uniports, then the stimulatory effect of Mg^{2+} could be attributed to a decrease of the tonoplast surface potential [2]. This effect may be responsible for the slowing of the rate of decrease of P_{vac} after $MgCl_2$ is added at $t = 56$ min in Expt. 1.

That Mg^{2+} uptake did occur was indicated by the downfield shift of the polyphosphate resonance following $MgCl_2$ addition (not shown) [20].

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