Phosphate Transport in Yeast Vacuoles*

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The vacuole of the yeast Saccharomyces cerevisiae is a major storage compartment for phosphate. We have measured phosphate transport across the vacuolar membrane. Isolated intact vacuoles take up large amounts of added [32P]phosphate by counterflow exchange with phosphate present in the vacuoles at the time of their isolation. The bidirectional phosphate transporter has an intrinsic dissociation constant for phosphate of 0.4 mM. Exchange mediated by this carrier is faster than unidirectional efflux of phosphate from the vacuoles. The transporter is highly selective for phosphate; of other anions tested, only arsenate is also a substrate. Transport is strongly pH-dependent with increasing activity at lower pH. Similar phosphate transport behavior was observed in right-side-out vacuolar membrane vesicles.

Phosphate is an important nutrient, and phosphate metabolism in the yeast Saccharomyces cerevisiae has been extensively studied. This system has provided a model for understanding how a cell makes a coordinated response to environmental changes (1). Phosphate is often present in only low amounts in the environment (2), and as for other microorganisms, yeast has evolved complex mechanisms to deal with changes in phosphate availability.

One aspect of phosphate metabolism in yeast which has received substantial attention is the question of how the cell obtains phosphate from its surroundings. Several secreted phosphatases which release free phosphate in the extracellular space have been identified (3). Uptake of free phosphate from outside the cell is mediated by a number of plasma membrane transport systems. One has a high affinity for phosphate and is encoded by the PHO84 gene, whose expression is derepressed under conditions of phosphate starvation (4). Others include a sodium/phosphate cotransporter and a low affinity, constitutive transport system (5, 6).

Once phosphate has been taken up by the yeast cell, a second important consideration is its intracellular compartmentalization. In this respect the yeast vacuole plays a major role. The vacuole is the site of storage of large amounts of phosphate and polyphosphate, a linear polymer of phosphate in anhydrous potassium phosphate. 50 mM sodium citrate, pH 5.1. Spheroplasting was performed with lyticase (1700 units/g wet weight of cells). Osmotic stabilization was provided by 0.6 M sorbitol or sucrose buffered with 5 mM Tris-PIPES, pH 6.9. Vacuoles were purified from spheroplast lysate in two steps by density gradient centrifugation (28). The first gradient consisted of a bottom layer of buffered sucrose plus 2.5% Ficoll, a middle layer of a 2:1 mixture of buffered sorbitol and sucrose, and cell lysate on top. After centrifugation for 1 h at 75,000 × g the crude vacuoles were collected from the interface between the bottom two layers, diluted with 3 volumes of buffered sorbitol, then spun for 45 min at 5,000 × g over a 2.1 mixture of buffered sorbitol and sucrose. Purified vacuoles were obtained in the pellet and resuspended in a small volume of buffered sorbitol on ice. Vacuole suspensions were counted in a hemocytometer, then stored on ice overnight and used the next day.

Analysis of Vacuolar Contents—Phosphate was measured as described previously (27); polyphosphate was measured as orthophosphate released after boiling in 1 N H2SO4 for 10 min.

Vascular Transport Assays—We developed a new method for measuring transport in isolated intact vacuoles which we found more convenient than that described previously (25). Vacuoles were diluted to a concentration of 4 × 105/ml in ice-cold 0.6 M sorbitol, 5 mM Tris-PIPES, pH 6.5, shortly before the experiment. At time 0, the diluted vacuoles were added to an equal volume of 25 °C buffered sorbitol containing [32P]phosphate (10 μCi/ml) plus any additions and then incubated in a

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1 The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; NMR, nuclear magnetic resonance; MES, 2-(N-morpholino)ethanesulfonic acid; KP, potassium phosphate; DIDS, 4,4'-disothiocya-nostilbene-2,2'-disulfonic acid.
Vacuoles contain large amounts of polyphosphate (8), and also can contain high concentrations of orthophosphate (7). Phosphate may also be formed by hydrolysis of polyphosphate during purification of the vacuoles. Vacuoles isolated under isotonic conditions largely retain their contents during purification (30). Thus, the simplest explanation for the observed uptake of labeled phosphate is that it is mediated by a phosphate carrier in the vacuolar membrane, and is coupled to net efflux of phosphate from the vacuoles through this carrier.

We confirmed that there is indeed net movement of phosphate out of the vacuoles down its concentration gradient upon dilution of the vacuoles (Fig. 2A). The total amount of orthophosphate in the vacuole suspension also increases throughout the incubation, presumably due to hydrolysis of polyphosphate or other phosphorylated vacuolar constituents. Similar observations were made by Dürre et al. (31). Polyphosphate is present in much higher amounts than phosphate (Fig. 2B), so a decrease in vacuolar polyphosphate due to hydrolysis is difficult to observe. However, the high recovery of polyphosphate in the vacuoles throughout the incubation does serve to indicate that there is no substantial loss of vacuoles due to lysis.

As can be observed by comparison of Figs. 1 and 2, the total orthophosphate inside isolated vacuoles during incubation at 25 °C (Fig. 2) does not decline to as large an extent as would be expected from the shape of the downward phase of counterflow curves (Fig. 1). Thus, it appears that only a fraction of this internal phosphate is mobile. To follow both uptake of $^{32}$P-phosphate by counterflow and net efflux of mobile phosphate from the vacuoles the following experiments were performed in parallel. Vacuoles were equilibrated for 30 min at 25 °C with 40 mM potassium phosphate in buffered sorbitol, then the suspension was diluted 40-fold into buffered sorbitol lacking phosphate. In one set of experiments, tracer $^{32}$P-phosphate was added to the equilibration buffer (to follow efflux), while in the other it was added to the dilution buffer (to follow counterflow).

The results of the counterflow experiments are shown in Fig. 3A. Rapid uptake of $^{32}$P-phosphate is observed, leading to a 17-fold concentration of $^{32}$P-phosphate inside the vacuoles at the peak. After this point there is net release of radioactivity. The results of the efflux experiments are shown in Fig. 3B. In this case, movement of $^{32}$P-phosphate reflects the overall movement of phosphate out of the vacuoles. Over 90% of the radioactivity associated with the vacuoles is released upon dilution.

In principle, the counterflow exchange and the unidirectional efflux could be mediated by two distinct transporters; however, these data can be explained most simply by postulating a single transporter. This transporter can be described using a simple mobile carrier model in which a carrier exists in two distinct states corresponding to its binding of substrate at two faces of the membrane (28). An important parameter in this model is $K$, the intrinsic dissociation constant for the phosphate-carrier complex. The value of $K$ is readily obtained from the height of the peak of counterflow. The following relation can be derived (28),

$$K = \frac{P_1 - (S_1/S_2)P_2}{(S_1/S_2) - 1}$$

where $P_1$ is the concentration of phosphate inside the vacuoles at the peak of counterflow, $P_2$ is the concentration of phosphate outside, and $S_1/S_2$ is the ratio of the concentrations of $^{32}$P-phosphate inside and outside. From Fig. 3, $P_1$ is 25 mM, $S_1/S_2$ is 17, and $P_2$ is 1.1 mM. From these values, we obtain $K \approx 0.4$ mM. Theoretical curves based on this model are plotted through the experimental data points in Fig. 3.
We compared the rates of unidirectional efflux versus exchange of phosphate by adding unlabeled KP\(_i\) to the dilution buffer in the radioactive efflux experiments described above (Fig. 3B). The rate of exit of radioactivity increased dramatically in a concentration-dependent manner. From the model described above, we obtain a \(V_{\text{max}}\) for zero-trans efflux of phosphate from the vacuoles of at least 40 nmol/mg protein/min, while \(V_{\text{max}}\) for equilibrium exchange must be at least five times larger.

Uptake of \([^{32}\text{P}]\)phosphate by counterflow should depend on the presence of phosphate inside the vacuoles. Accordingly, no significant uptake of radioactivity is seen if the phosphate gradient across the vacuolar membrane is allowed to dissipate by diluting the vacuoles in buffered sorbitol and incubating them at 25 °C for 30 min before addition of \([^{32}\text{P}]\)phosphate (data not shown). As an additional control, vacuoles were equilibrated in a small volume of buffered sorbitol containing 40 mM KP\(_i\), 40 mM KCl, or no addition, then diluted into buffer containing \([^{32}\text{P}]\)phosphate (Fig. 4). Vacuoles equilibrated with phosphate gave a large peak of uptake of \([^{32}\text{P}]\)phosphate, while vacuoles preincubated without added phosphate gave only a small amount of uptake, presumably driven by the low levels of endogenous phosphate remaining inside the vacuoles after equilibration.

A prediction of the model is that addition of unlabeled phosphate to the exterior of the vacuoles during a counterflow experiment should lead to a decrease in the height of the peak of uptake of radioactivity and a shift of this peak to earlier times, due to the combined effects of competitive inhibition of uptake of \([^{32}\text{P}]\)phosphate and the increased rate of efflux of radioactivity described above. Addition of phosphate at different concentrations to the exterior of the vacuoles during counterflow experiments leads to the behavior predicted by the model (Fig. 5).

**Properties of Phosphate Transport**—Both uptake of \([^{32}\text{P}]\)phosphate by counterflow and efflux of preloaded \([^{32}\text{P}]\)phosphate are blocked at 0 °C (data not shown). Uptake of \([^{32}\text{P}]\)phosphate is not inhibited by either 100 μM DIDS, an inhibitor of the erythrocyte anion exchange protein band 3, or by carbonyl cyanide \(p\)-chlorophenylhydrazone, a protonophore,
or by 10 mM EDTA. The pH dependence of uptake was measured over a range of pH values from 5.5 to 7.3 (Fig. 6). It has previously been shown that isolated vacuoles are unstable outside of this range (17). The initial rate of uptake by counterflow increased substantially with decreasing pH.

Specificity—
The transporter appears to be very specific for phosphate. If a given ion is a substrate of the transporter, then its addition to the outside of the vacuoles should have an effect on [32P]phosphate counterflow uptake similar to that of addition of unlabeled phosphate (Fig. 5). If a substance is not a substrate for the transporter but is a competitive inhibitor of phosphate transport, its addition should lower the initial rate of uptake of [32P]phosphate. Addition of 2 mM arsenate to the external buffer during [32P]phosphate uptake experiments has the same effect as addition of 2 mM phosphate (Fig. 5), suggesting that arsenate is a substrate of the transporter. Arsenate is an analog of phosphate with a similar structure. Other inor-
conditions in which there is ATP-dependent uptake of $^{45}$Ca due to a pool of phosphate that provides a large although transient driving force for uptake via exchange.

In the yeast cell, the direction of net movement of phosphate across the vacuolar membrane depends on physiological conditions. When metabolic requirements for phosphate exceed what can be obtained from outside the cell, vacuolar polyphosphate pools are mobilized to replenish cytoplasmic phosphate (10, 11, 34). Several exopolyphosphatases have been identified in the vacuole (35, 36), where they can act to release phosphate from polyphosphate by hydrolysis. The released phosphate then moves out of the vacuole (10). This phosphate efflux may be mediated by the transporter that we have identified, although as previously noted, it is possible that the efflux and exchange activities we have observed are due to two distinct transporters. In the absence of any specific inhibitors of these activities, this possibility cannot be excluded.

Conversely, under conditions where phosphate and metabolic energy are available, and especially when phosphate is added to cells previously starved for phosphate, polyphosphate is synthesized (2, 37). However, the mechanism of polyphos-

![Graph](image1.png)

**Fig. 6. pH dependence of phosphate uptake.** $^{32}$PPhosphate uptake experiments were performed as described under "Experimental Procedures." Vacuoles were diluted into 0.6 M sorbitol buffered to different pH values with 5 mM Tris-PIPES for pH > 6.5 or 5 mM MES-Tris for pH < 6.5. Initial uptake rates were determined based on radioactivity taken up in 5 min, and are expressed as percent of uptake at pH 5.55. Points are the average of three independent experiments, ± S.E.

Next, we tested whether the counterflow and efflux behavior seen with intact vacuoles could be recapitulated in vesicles. Vesicles were equilibrated with buffer containing 50 mM phosphate, then diluted into buffer lacking phosphate. Tracer $^{32}$Pphosphate was added to either the equilibration buffer or the dilution buffer to follow efflux or counterflow, respectively (Fig. 8). Again, a pattern suggestive of counterflow is observed. In comparison with the results obtained with intact vacuoles, the peak of vesicle-associated $^{32}$Pphosphate is reached more rapidly. This can be explained by the smaller trapped volume inside the vesicles. The smaller signal due to this reduced trapped volume was probably also responsible for a variability in results between experiments that was not seen with intact vacuoles. Efflux proceeds on a time scale similar to counterflow (Fig. 8).

**Discussion**

We report here the characterization of a phosphate transporter in the vacuolar membrane of yeast. This transporter mediates bidirectional transport and has a millimolar affinity for phosphate. We were able to observe a large uptake of $^{32}$Pphosphate due to the presence in isolated yeast vacuoles of a pool of phosphate that provides a large although transient driving force for uptake via exchange.

Next, a number of phosphate-containing compounds were assayed for their ability to inhibit $^{32}$Pphosphate uptake. Neither pyridoxal phosphate, glucose 6-phosphate, nor ATP at 5 mM, nor pyrophosphate at 2 mM show any inhibition, although addition of unlabeled phosphate to these concentrations nearly abolishes uptake of $^{32}$Pphosphate. Furthermore, neither $^{32}$Ppyrophosphate nor [$\alpha$-$^{32}$P]ATP are taken up by vacuoles when added under the same conditions in which counterflow uptake of $^{32}$Pphosphate is observed (data not shown).

**Experiments with Vacular Membrane Vesicles—**Previous studies of vacuolar transport systems have used either isolated intact vacuoles or right-side-out vacuolar membrane vesicles formed by hypotonic lysis of vacuoles (32). We extended our investigation of vacuolar phosphate transport by also performing transport assays with such vesicles.

It has been shown that chloride ion can be transported into vacuolar membrane vesicles at the expense of the positive-inside membrane potential set up by the vacuolar H$^+$/ATPase (24). We tested whether a similar membrane-potential-driven uptake of $^{32}$Pphosphate into vesicles could be observed. Under conditions in which there is ATP-dependent uptake of $^{45}$Ca due to the action of the Ca$^{2+}$/H$^+$ exchanger (14) and in which fluorescence quenching of the dye Oxonol-V indicates that a membrane potential is formed (24), no substantial uptake of $^{32}$Pphosphate is observed (Fig. 7). Similarly, we saw no ATP-driven uptake of $^{32}$Pphosphate into intact vacuoles for which uptake by counterflow had been abolished by allowing the phosphate gradient across the vacuolar membrane to dissipate (data not shown). These results suggest that phosphate does not simply equilibrate with membrane potential as chloride appears to and argue against the existence of an electrical uniport for phosphate (33).

![Graph](image2.png)

**Fig. 7. Uptake of $^{45}$Ca$^{2+}$ or $^{32}$Pphosphate by vacuolar membrane vesicles.** Vesicles (100 µg of protein/ml) were incubated in buffer containing 20 mM MES-Tris, pH 6.7, 4 mM MgCl$_2$, 20 mM KCl, 0.6 mM ATP, and $0.25$ mM $^{45}$CaCl$_2$ (2 Ci/mol), 1 mM $^{32}$P$_2$K$_7$ (8.3 Ci/mol), or $0.1$ mM $^{32}$PiP$_7$ (93 Ci/mol). 100-µl aliquots were removed at time points, diluted in 4.5 ml of ice-cold wash buffer (10 mM MES-Tris, pH 6.7, 5 mM MgCl$_2$, 25 mM KCl), filtered on nitricellulose filters, and washed with 10 ml of wash buffer. $\bullet$, $^{45}$Ca; $\blacktriangle$, $^{45}$Ca + 2 µM bafilomycin A$_1$ (an inhibitor of the vacuolar H$^+$/ATPase); $\square$, 1 mM $^{32}$Pphosphate; $\Delta$, 0.1 mM $^{32}$Pphosphate.

Conversely, under conditions where phosphate and metabolic energy are available, and especially when phosphate is added to cells previously starved for phosphate, polyphosphate is synthesized (2, 37). However, the mechanism of polyphos-
phosphate synthesis and the vacuolar transport processes required for this synthesis are not clear. Polyphosphate synthesis requires a high energy phosphate donor rather than simply orthophosphate. This donor has not been definitively identified (38), but it presumably must be transported into the vacuole during periods of polyphosphate synthesis.

In vivo studies of phosphate metabolism in yeast conducted using $^{31}$P-nuclear magnetic resonance (NMR) (10, 11, 37), as well as studies using differential extraction techniques to distinguish vacuolar and cytoplasmic ion pools (7) have suggested that a large vacuole-to-cyttoplasm gradient of phosphate concentration can exist. However, we have found no evidence for active ATP-driven transport of phosphate across the vacuolar membrane similar to that seen for other substances located in the vacuole.

To satisfy conditions of electroneutrality, the efflux of phosphate from vacuoles that we observe must be accompanied by either cation efflux or inward movement of another anion. Since the only anion added to the outside of the vacuoles is the large buffer anion PIPES, the latter possibility seems unlikely. Rather, a vacuolar cation presumably moves out with phosphate, either through the same transporter or in parallel through a separate transporter, e.g. the cation channel previously identified in the vacuolar membrane (18, 19). The fact that we could not observe any membrane potential-driven phosphate uptake suggests that the phosphate carrier itself may perform electroneutral transport.

It should be noted that the vacuoles used in our experiments were all obtained from cells grown in medium containing ample free phosphate. Thus, the transporter does not appear to require phosphate starvation for its induction, like Pho84p, but rather is present constitutively.

 There is a striking stimulation of counterflow uptake as the pH is lowered from 7.3 to 5.5. This may suggest that the univalent form of phosphate is the substrate of this carrier, as is the case for the plasma membrane phosphate transporters (6).

Most of the transporters identified in the vacuolar membrane to date have been studied using vacuolar membrane vesicles. The majority of these transporters mediate active uptake driven by the H+-ATPase; this is probably a reflection of the fact that passive transport is difficult to observe in vesicles, given their small internal volume and the fact that they are largely devoid of vacuolar contents (32). With intact vacuoles, in contrast, passive transport systems can be discovered and analyzed more easily (25). Thus, using intact vacuoles we were able to observe and characterize a transporter which mediates passive transport of phosphate across the vacuolar membrane.

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