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Proton/Phosphate Stoichiometry in Uptake of Inorganic Phosphate by Cultured Cells of *Catharanthus roseus* (L.) G. Don

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ABSTRACT

Upon absorption of phosphate, cultured cells of *Catharanthus roseus* (L.) G. Don caused a rapid alkalinization of the medium in which they were suspended. The alkalinization continued until the added phosphate was completely exhausted from the medium, at which time the pH of the medium started to drop sharply toward the original pH value. Phosphate exposure caused the pH of the medium to increase from pH 3.5 to values as high as 5.8, while the rate of phosphate uptake was constant throughout (10–17 micromoles per hour per gram fresh weight). This indicates that no apparent pH optimum exists for the phosphate uptake by the cultured cells. The amount of protons cotransported with phosphate was calculated from the observed pH change up to the maximum alkalinization and the titration curve of the cell suspension. Proton/phosphate transport stoichiometry ranged from less than unity to 4 according to the amount of phosphate applied. At low phosphate doses, the stoichiometries were close to 4, while at high phosphate doses, smaller stoichiometries were observed. This suggests that, at high phosphate doses, activation of the proton pump is induced by the longer lasting proton influx acidifying the cytoplasm. The increased H^+ efflux due to the proton pump could partially compensate protons taken up via the proton-phosphate cotransport system. Thus, the $H^+/H_2PO_4^-$ stoichiometry of the cotransport is most likely to be 4.

It is well known that various nutrients are cotransported with protons across the plasmalemma of plant cells (9, 10). Ullrich-Eberius *et al.* (14, 15) demonstrated with *Lemna*, that addition of inorganic phosphate induced a depolarization of eletrical membrane potential (E_m) of plasmalemma, the degree of which was dependent on the pool size of endogenous inorganic phosphate. The depolarization was limited to the electrogenic component of E_m. Based on their results and other kinetic considerations, they concluded that inorganic phosphate uptake is energized by the electrochemical proton gradient and proceeds by a 2 $H^+/H_2PO_4^-$ cotransport mechanism. However, the proposed stoichiometry, 2 $H^+/H_2PO_4^-$, was derived solely from kinetic considerations of phosphate uptake; the quantity of protons transported with phosphate was not determined.

In this paper, I show direct evidence that phosphate uptake by the cultured cells of *Catharanthus roseus* (L.) G. Don proceeds by a proton cotransport mechanism with a possible stoichiometry of 4 $H^+/H_2PO_4^-$.

MATERIALS AND METHODS

Plant Materials

Cultured cells of *Cathananthus roseus* were obtained from the stock cultures of Dr. Ashihara of Ochanomizu University, and cultured in Murashige and Skoog basal medium (8) supplemented with 4.5 μmol of 2,4-D and 3% sucrose as described previously (11). Cells cultured for 6 to 10 d after transfer to fresh medium were collected on a filter paper under suction and washed with 10 mM $CaCl_2$. Cells were weighed and suspended at the density of 5 g fresh weight in 50 mL of incubation medium consisting of 10 mM $CaCl_2$, 10 mM KCl, and 1% glucose in a 100 mL Erlenmeyer flask. The flask was stoppered with a sleeve-ventilating plastic cap to which a glass pH electrode (TOA Electronics, GST-5211C) was fixed to measure the medium pH. The flask was shaken at 140 rpm at room temperature (25 ± 2°C). Under these conditions, the oxygen concentration of the medium (about 130 μM) was well above the critical oxygen pressure of the cell (about 95 μM); thus, the cells were in an aerobic state. The external pH, determined with the glass electrode, connected to a TOA Electronics HM-16S pH meter, was recorded with a pen recorder (TOA Electronics, INR-231A).

Titration Curve of Cell Suspension

A titration curve of the cell suspension was obtained with a portion of the same cell suspension. The cell suspension (5.5 mL) was pipetted into a small vial and its pH was adjusted to between pH 6 and 7 with 0.1 N NaOH. This was titrated with 0.01 N HCl until the medium pH attained 3.2 using an automatic titrator (TOA Electronics, AUT-301). Although the titration was carried out before the experiment, the profile of the curve did not change when obtained after experiment. The titration curve was prepared with every batch of cell suspension used.

Proton Uptake

A series of 0.5 M phosphate solutions with a pH range of 3.2 to 6.2 (pH interval about 0.5) were prepared from a solution of disodium phosphate by titrating with HCl. When phosphate was to be added, a solution with nearest pH to that of the cell suspension was used. The small pH difference of the added phosphate solution from that of the suspension (usually 0.1–0.3), in most cases caused negligible pH shift when pH of the suspension was below 4 and the amount of

phosphate added was small (final concentration in the medium outside the cells: 0.01–0.8 mM). However, a small but rapid pH shift immediately after the application of phosphate solution was discounted in the calculation of proton uptake. Proton uptake (or excretion) corresponding to an observed pH shift was calculated from the titration curve. Buffer capacity of the added phosphate was neglected in the calculation since, in addition to its low concentration, the pH range worked in the present study (3.5–5.8) was far from its p*ka*s ($pKa_1 = 2.12$, $pKa_2 = 7.21$).

Phosphate Uptake

Phosphate uptake was determined by the decrease of inorganic phosphate in the medium. After the addition of phosphate, 0.3 mL of the cell suspension was withdrawn from the flask at intervals, and put into a microcentrifuge tube with a membrane filter (Millipore, Ultrafree C3HK). It was centrifuged at 15,000 rpm for 15 s and inorganic phosphate in the filtrate was determined by the method of Bencini *et al.* (1).

RESULTS AND DISCUSSIONS

Titration of Cell Suspension

Figure 1 shows the titration profile of the cell suspension of *Catharanthus roseus*. Above pH 4, buffering capacity of the cell suspension was weak. In contrast, it was strong below pH 4. This suggests that the cell wall components such as pectic substances contribute to this strong buffering action. Presence of cell wall-fixed groups ($pKa = 3.2$) has been reported in corn roots (12).

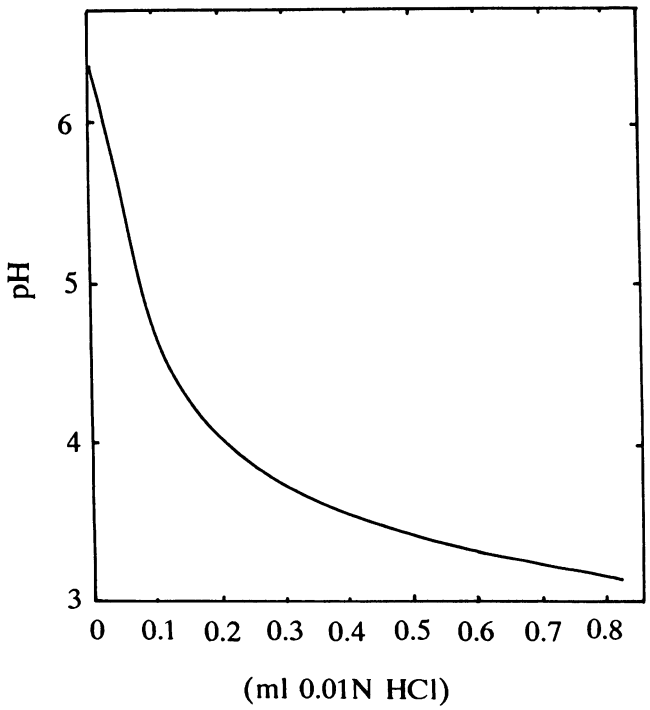

Figure 1. Titration curve of cell suspension. Five and one-half mL of cell suspension (0.5 g fresh weight cells + 5 mL incubation medium) was titrated with 0.01 N HCl using an automatic titrator (TOA Electronics, AUT-301). One injection volume of the titrant was set between 2.5 to 25 μL.

Proton Flux during Phosphate Absorption

When cells were washed with $CaCl_2$ and transferred to the unbuffered, simple incubation medium, the medium pH dropped rapidly from an initial value between 5 and 6 to as low as below 4 after approximately 8 h. This acidification of the medium was dependent on the exchange of K^+ with H^+ at a stoichiometry of $K^+/H^+ = 1$, and the final pH attained was lower when the KCl concentration in the medium was higher (our unpublished data). Usually, the cell suspension was allowed to incubate for 6 to 8 h before the experiment until the medium pH stabilized in the range 3.5 to 4.

Figure 2, a and b, shows the profiles of pH shift of the medium upon application of inorganic phosphate to the cell

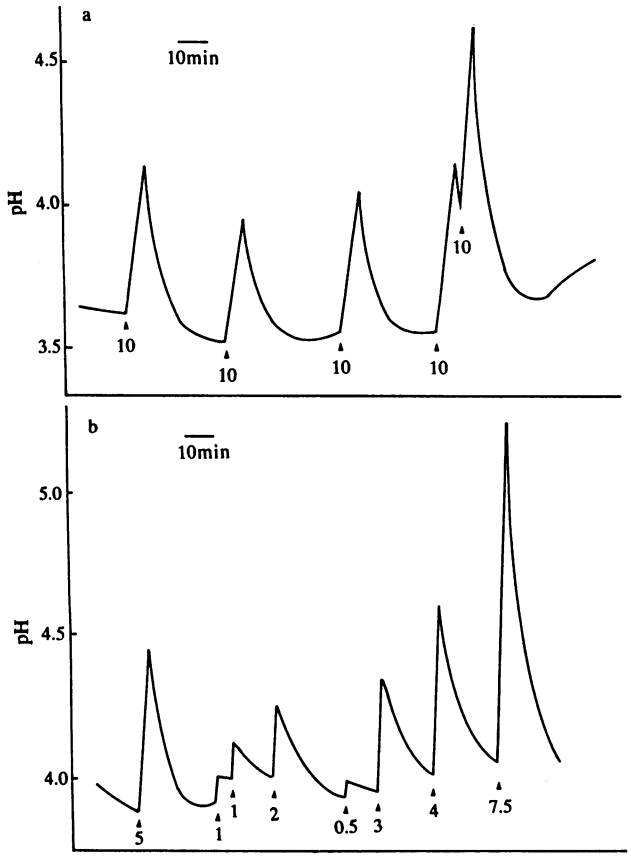

Figure 2. Profiles of pH shift in response to phosphate administration. a, A 10 μmol of phosphate solution (pH 3.62) was added successively to the cell suspension at the time marked by arrowheads. Note that the time required to attain the peak is always the same (about 7 min). b, pH response to different amount of phosphate. One-half to 7.5 μmol of phosphate (pH 4.01) were added at the time indicated by arrowheads. The numbers under the arrowheads mean μmol of phosphate added. Note that the first 1 μmol administration caused a normal alkalinization but no successive acidification, and also that time required to attain the maximum alkalinization is a linear function of time.

suspension. Several seconds after phosphate addition, a rapid and almost linear increase in pH was observed. This continued until when the shift abruptly turned into a exponential decrease. These pH shifts could be repeated more than 10 times in the same cell suspension, and could be observed even when the medium pH was returning toward the initial acidic pH value (Fig. 2a). The larger the amount of added phosphate, the greater the alkalinization of the medium. It should be noted that the initial rate of pH increase was independent of the amount of phosphate applied, but the time required to attain the maximum alkalinization was a linear function of phosphate dose. Interestingly, when the phosphate dose was small (1 μmol or less), the decreasing phase of pH was not always observed (Fig. 2b, for the first 1 μmol application).

The rapid alkalinization was found to be coupled to the uptake of phosphate (Fig. 3). Disappearance of phosphate from the medium was a linear function of time (10.7 μmol/h/g fresh weight) and the phosphate exhaustion from the medium corresponded exactly to the endpoint of the alkalinization, *i.e.* the peak time of the pH shift (Fig. 3). On the other hand, the kinetics of proton uptake as calculated from pH shift was not a linear function of time: the initial rate of proton uptake was the fastest (22.8 μmol/h/g fresh weight, for the first 5 min) and the rate decreased gradually until phosphate was completely exhausted (9.6 μmol/h/g fresh weight, for the last 5 min). In the subsequent acidification phase, proton excretion (16.8 μmol/h/g fresh weight for the first 5 min) from the cells was independent of phosphate flux. These results are consistent with the idea that phosphate is taken up by a proton cotransport mechanism.

Vanadate, an inhibitor of the plasmalemma proton pump, also caused a similar pH shift, but the rates of both alkalinization and acidification were very slow as compared with phosphate (Fig. 4). In addition, the final pH attained in the acidification process was higher than the initial pH before addition of vanadate. These results indicate that the plasmalemma proton pump was involved in the acidification process and was inhibited by vanadate at least partially. The fact that the subsequent addition of phosphate released the inhibition by vanadate (Fig. 4) is consistent with the results reported by González de la Vara and Medina (6).

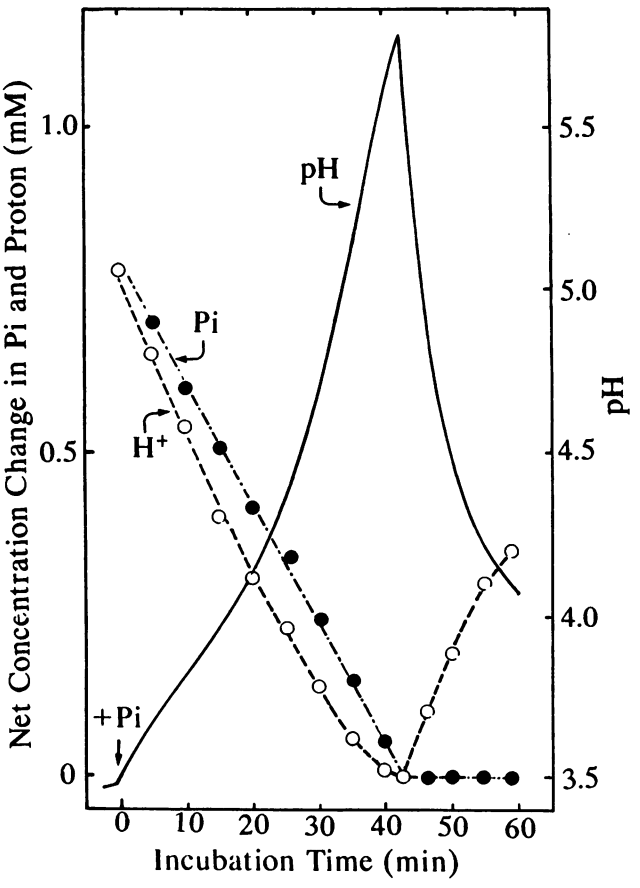

Figure 3. Time courses of proton and phosphate uptake by the cultured cells of *C. roseus*. Forty μmol of phosphate (pH 3.5) was added at time zero and the phosphate remaining in the medium was determined at 5 min intervals. Net changes in the concentrations of proton and phosphate are shown as their changes at 42 min (peak time of pH shift) zero. Note that uptake of phosphate proceeded linearly but that of proton did not.

Stoichiometry of Proton/Phosphate Cotransport

Figure 5 shows the stoichiometry of proton/phosphate cotransport as expressed in $H^+/H_2PO_4^-$ ratio as a function of phosphate dose. In calculating the ratio at a given phosphate dose, the total amount of proton transported into the cell via phosphate cotransport system was estimated from the pH shift of the medium (up to the maximum alkalinization at which phosphate uptake was complete as shown in Fig. 3) with the aid of the titration curve of the cell suspension (Fig.

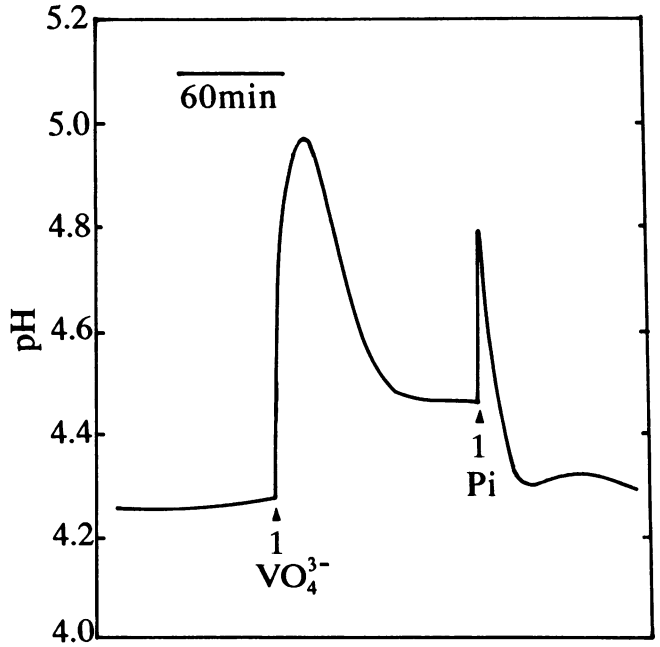

Figure 4. Shift of the medium pH in response to vanadate addition (1 μmol) to cell suspension. Note that the medium pH after acidification was higher than the initial (partial inhibition of proton pump) and release of the inhibition by the subsequent addition of phosphate (1 μmol).

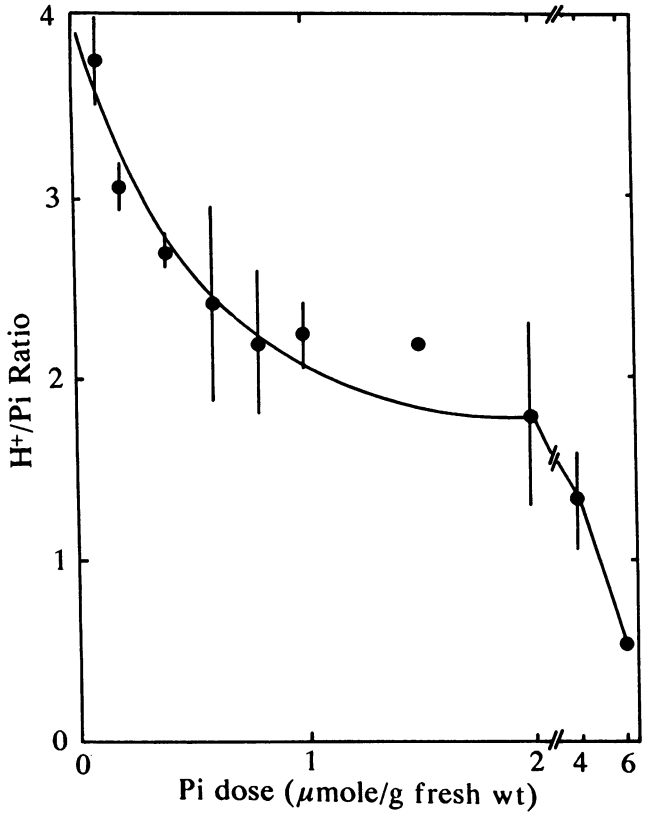

Figure 5. Stoichiometry of proton/phosphate cotransport as expressed in $H^+/H_2PO_4^-$ ratio. Each point with vertical bar (standard deviation) represents the average of 2 to 10 determinations.

1). The ratio, obtained by dividing the total amount of proton absorbed by the phosphate dose that caused the proton absorption, ranged from less than unity to 4. The lower the phosphate dose, the greater the $H^+/H_2PO_4^-$ ratio. Using yeast cell suspension, Cockburn *et al.* (4) obtained similar results: they observed a rapid increase of the medium pH upon addition of phosphate (although not as sharp as those of present study), from which they calculated the amount of proton cotransported with phosphate. Their calculated $H^+/H_2PO_4^-$ ratio ranged from 3.16 to 1.76, the lower the phosphate dose, the greater the ratio.

These results strongly suggest that proton pump of the plasmalemma is involved not only in acidification of the external medium but also in the alkalinization process that is coupled to phosphate uptake. According to the proton cotransport mechanism, phosphate uptake should depolarize E_m of plasmalemma and acidify the cytoplasm, which in turn, could stimulate the plasmalemma proton pump. The rapid acidification of the medium after phosphate exhaustion from the medium clearly indicates that this is the case. Although the rates of phosphate and proton uptake are the same for low and high phosphate doses, acidification of cytoplasm could occur with high doses because both protons and phosphate could be absorbed for a longer period of time. Absence of the acidification phase when phosphate dose was low (Fig. 2b) may indicate that the coupled proton influx was too small to activate the pump.

The transient depolarization of membrane potential as observed in *Lemna* (14, 15), sunflower (2), and white clover (3, 5) indicate an initial rapid entry of protons into the cell (causing depolarization) and the subsequent stimulation of the electrogenic proton pump (causing repolarization). Thus, the reason for the lower $H^+/H_2PO_4^-$ ratio at higher phosphate doses may be due, if not all, to the activation of proton pump during uptake of phosphate. This would, in effect, recirculate the protons to the external medium, resulting in an apparent lower proton/phosphate stoichiometry. Involvement of the proton pump in the proton/phosphate cotransport has been suggested also in corn root tissues (13).

However, it is also possible that this was caused by the changes in the fluxes of other substances (such as glucose, K^+, Ca^{2+}, Cl^-, organic acids, and so on) via other cotransport systems, mediated by the phosphate-induced depolarization of E_m. Since the depolarization takes place immediately upon phosphate addition, this should apply even at the lowest doses of phosphate that would not activate the proton pump. This might affect the apparent proton flux. Therefore, although the results shown in Figure 5 suggest the most likely stoichiometry ratio for proton/phosphate cotransport is 4, this stoichiometry is still, in the strict sense, left open, and its correctness should be examined in future studies.

In addition, the buffering action of phosphate added as transport substrate, which was neglected throughout the present experiments to avoid complexities, may also affect proton/phosphate stoichiometry when its dose is high; its buffering action may reduce the pH change especially at the early stage of transport when much of the added phosphate remains in the medium. Therefore, the rate of proton uptake calculated for the first 5 min (22.8 μmol/min/g fresh weight) in the experiment shown in Figure 3 is more or less underestimated as compared with those at later stages.

pH Optimum for Phosphate Transport

In the present study, no effort was made to buffer the cell suspension. This is because, in part, the cell suspension itself had a strong buffering capacity in the acidic range (Fig. 1). In addition, when the pH of the suspension was modified with HCl or NaOH, it spontaneously returned toward the original pH at an appreciable rate. This indicates that any manipulation of the proton concentration in the medium would affect proton balance across the plasmalemma and cause proton flux. Therefore, bufferization of medium at a fixed pH value may bring about a pH stress to the cells and complicate the analysis of proton flux during phosphate transport.

Under this condition, the net proton flux across the plasmalemma was calculated from the change in the medium pH with the aid of the titration curve. The pH rise started within a second or two after addition of phosphate and continued for a number of minutes. For example, under the standard conditions, application of 10 μmol phosphate caused a pH increase lasting 7 to 10 min. Thus, equilibration of protons between the external medium and the local vicinity of phosphate uptake system in the plasmalemma was rapid enough, and the time observed for the pH shift was long enough, to allow quantification of transported protons on the basis of pH shifts in the medium.

It should be noted that no apparent pH optimum for the phosphate transport system was observed. The medium pH shifted from 3.5 to 5.8 upon addition of phosphate, but the rate of phosphate uptake was constant throughout until phosphate was completely taken up by the cells (Fig. 3). The linearity of phosphate uptake was always observed irrespective of phosphate dose (5–100 μmol in the standard assay), although the rate differed with age of cells after subculture (10–17 μmol/h/g fresh weight).

The absence of pH optimum in phosphate uptake by *C. roseus* is quite unusual. In other plant materials, distinct pH optimums have been determined using various pH buffers in the medium; corn roots, pH 3 (12); tobacco protoplast, pH 4.5 (7); *Lemmna*, pH 6 (14). In these studies, however, the pH at the close vicinity of the transport system might be different from that of the buffered medium: it could be more acidic when the proton pump is operating (13), but more alkaline when proton/phosphate cotransport initiates (see below). In addition, as discussed above, these observed pH optimums could be, in part, a reflection of pH stress caused by buffering the medium. Thus, pH optimum for phosphate transport needs further studies.

Ionic Form of Phosphate in Transport

In the proton/phosphate cotransport mechanism, the protons required for transport are assumed to be provided by the external medium and the proton pump. When a small amount (1 μmol or less; Fig. 2b) of external phosphate is provided, the total amount of proton incorporated into the cytoplasm would be small and the proton pump is not activated. In this case, protons required for cotransport may be provided exclusively by the medium. But when larger amounts of phosphate are applied, acidification of the cytoplasm could occur, because more phosphate and, therefore, more protons would be absorbed by the cell since more phosphate is available to the cell. This, in turn, would activate the proton pump. However, if the activated proton pump cannot compensate all of the protons transported into the cytoplasm via the cotransport system, alkalinization of the external medium would occur. Therefore, we may reasonably assume that the pH of the close vicinity of transport system is always more alkaline than that of the external medium during phosphate transport.

In addition, the maximum alkalinization in response to phosphate administration was up to pH 7.2 in an experiment with repeated addition of phosphate (nine consecutive applications with 20–250 μmol at each time, data not presented). This indicates that the pH in the close vicinity of the transport system was equal to or higher than 7.2, *i.e.* the affinity of the system for protons is extraordinary high. However, it should be noted that, mechanistically, OH^- efflux (OH^-/Pi antiport) instead of H^+ influx (H^+/Pi cotransport) cannot be excluded.

Although the above considerations favor the idea that the milieu of the transport system during phosphate uptake is around neutrality, the stoichiometry 4 $H^+/H_2PO_4^-$ suggests, on the contrary, a very acidic pH at the site of phosphate transport. Thus, because the *in situ* pH at the site of phosphate transport is hard to estimate, ionic form of phosphate transported by the system cannot be accurately determined.

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