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Phosphate uptake in *Lemna gibba* G1: energetics and kinetics

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Abstract. Phosphate uptake was studied by determining [^{32}P]phosphate influx and by measurements of the electrical membrane potential in duckweed (*Lemna gibba* L.). Phosphate-induced membrane depolarization (ΔE_m) was controlled by the intracellular phosphate content, thus maximal ΔE_m by 1 mM $H_2PO_4^-$ was up to 133 mV after 15 d of phosphate starvation. The ΔE_m was strongly dependent on the extracellular pH, with a sharp optimum at pH 5.7. It is suggested that phosphate uptake is energized by the electrochemical proton gradient, proceeding by a $2H^+/H_2PO_4^-$ cotransport mechanism. This is supported also by the fusicoccin stimulation of phosphate influx. Kinetics of phosphate influx and of ΔE_m, which represent mere plasmalemma transport, are best described by two Michaelis-Menten terms without any linear components.

Key words: *Lemna* (phosphate uptake) – Membrane potential – Proton/phosphate cotransport.

Introduction

The electrochemical proton gradient at the plasmalemma was recently proposed to be the primary driving force for phosphate uptake (Ullrich-Eberius et al. 1981). The mechanism was assumed to be a $2H^+/H_2PO_4^-$ cotransport along the electrochemical proton gradient. In this paper further evidence is presented for $H^+/H_2PO_4^-$ cotransport as the energy-providing mechanism by the correlation between phosphate influx and phosphate-induced membrane depolarization, pH dependence, dependence on the intracellular phosphate status and the effect of fusicoccin.

Proton/phosphate cotransport is localized most likely at the plasmalemma, not at the tonoplast. This is supported by several strong arguments. i) The maximum membrane depolarization, as analyzed here in phosphate-starved duckweed, is attained within 1 min or less, whereas accumulation in the vacuole under similar conditions becomes apparent only after 2 h (Bieleski and Ferguson 1983; Rebeille et al. 1983). ii) Proton/phosphate cotransport into the vacuole, driven by an electrochemical proton gradient, is not possible, since transport of protons through the tonoplast into the positively charged and acidic vacuole (Rona and Cornel 1979) by itself requires energy. iii) Moreover, in phosphate-starved cells the downhill electrochemical gradient for phosphate is directed towards the vacuole. An energy-free influx of phosphate into the vacuole would thus result in hyperpolarization of the membrane potential (E_m) and even reduce the E_m depolarization observed in duckweed instead of generating it. The kinetics of the phosphate-induced rapid E_m changes thus provides a useful tool for reanalyzing phosphate uptake kinetics as was done for glucose (Novacky et al. 1978; Ullrich-Eberius et al. 1978).

The kinetics of active phosphate uptake was investigated in order to determine whether the amount of energy required for transport processes and different uptake mechanisms, i.e. H^+/solute cotransport or cation uniport, might be reasons for differences in the kinetic pattern obtained with differently charged substrates (Thellier and Ayadi 1968; Thellier et al. 1981; Grünsfelder and Simonis 1973; Epstein 1976; Nissen 1973; Nissen and Nissen 1983; Komor and Tanner 1975; Jung and Lüttge 1980; van Bel et al. 1982; Kochian and Lucas 1982).

Abbreviations: E_m = electrical membrane potential difference; ΔE_m = phosphate-induced, maximal membrane depolarization; FW = fresh weight

Material and methods

Plant material. Lemna gibba L., strain G1 (obtained from the *Lemna* collection of Professor R. Kandeler, Vienna, Austria) was grown axenically under short-day conditions (28° C/8 h day to 23° C/16 h night) at an energy fluence rate of 25 W m^{-2}. The nutrient solution contained as macronutrients, 3.96 mM KNO_3, 5.47 mM $CaCl_2$, 1.22 mM $MgSO_4$, 1.47 mM KH_2PO_4, and 29 mM sucrose at an initial pH of 4.8.

For phosphate starvation, the plants were kept in a phosphate- and sucrose-free culture medium (−P) for 9 d under long-day conditions (28° C/16 h day to 23° C/8 h night) and for additional 3–5 d in the dark. Prior to the experiments the plants were transferred to standard experimental perfusion solution (Higinbotham et al. 1964) without phosphate for 15 h (1 mM KCl, 1 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, pH 5.7).

Electrophysiological measurements. Lemna plants were mounted in a vertical 4-ml Plexiglas chamber. The chamber was constantly perfused with experimental solution at a rate of 16 ml min^{-1}. The pH of the solution was adjusted with HCl and NaOH and, throughout the experiments, was simultaneously controlled in the perfusion chamber by a pH microelectrode (MI-410; Microelectrode Inc. Grenier Industrial Village, Londonderry, N.H., USA). Measurements of E_m were performed using Ag-AgCl electrodes in glass micropipettes (tip diameter less than 0.5 µm) containing 3 M KCl, inserted from the lateral side of the plant into the pallisade parenchyma. The E_m was recorded by an electrometer amplifier (Keithley 604, Munich, FRG) and a chart recorder. Further details were as described by Novacky et al. (1978). Experiments were performed in complete darkness at 25° C.

Phosphate uptake. For each sample, 100 mg fresh weight (FW) of plants were floated on 5 ml standard experimental perfusion solution (−P), buffered to pH 5.7 with 1 or 5 mM N-2-hydroxyethypiperazine-N′-2-ethanesulfonic acid (Hepes). The incubation was carried out in a photo-Warburg apparatus (VL 166; Braun, Melsungen, FRG), at 25° C. In light experiments, irradiance was 140 W m^{-2}. Plants were preincubated in the experimental solution for 1 h in light or dark. The uptake was started by injection of ^{32}P-labeled (Radiochemical Centre, Amersham, UK) phosphate solution adjusted to pH 5.7. After an incubation time of 10 min the plants were washed five times with 10 ml experimental solution containing unlabeled phosphate for 15 min at 0° C. The ^{32}P content was determined by measuring the Cerenkov radiation in the solution after digesting the plant material with concentrated H_2SO_4, H_2O_2, and $HClO_4$. Fusicoccin was used at a final concentration of 15 µM in 0.1% ethanol.

Calculations. The procedure of curve-fitting of the kinetic data of phosphate uptake and phosphate-induced membrane depolarization (ΔE_m) to one or to the sum of two Michaelis-Menten terms was carried out by non-linear least-squares regression analysis as described by van Bel et al. (1982).

Results

Effect of phosphate starvation. The rate of phosphate influx was strongly dependent on the intracellular phosphate content, which rapidly decreased in plants growing on a phosphate-deficient nutrient medium (Ullrich-Eberius et al. 1981). Figure 1 shows the strong effect of phosphate starvation on the phosphate-induced E_m depolarization, confirming a causal correlation of phosphate with phosphate uptake and with ΔE_m.

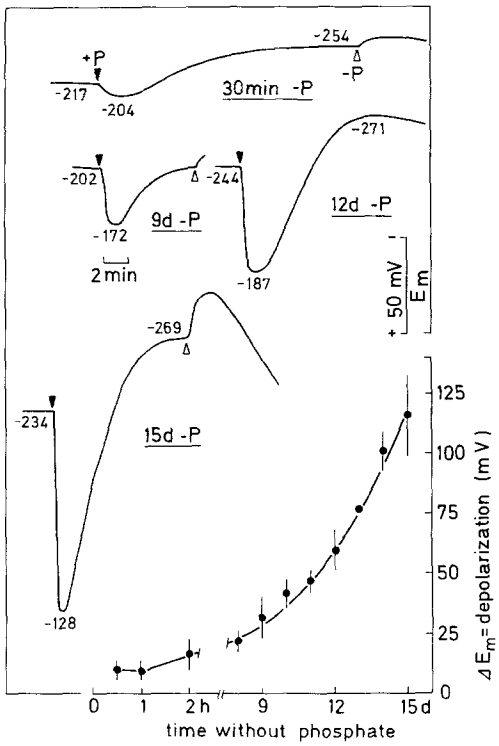

Fig. 1. Effect of phosphate starvation on phosphate-induced ΔE_m in *Lemna gibba*. Measurements in darkness with 1 mM $H_2PO_4^-$ at pH 5.7. Numbers at the traces denote recorded mV. *Solid arrowheads* indicate addition and *open arrowheads* removal of phosphate from the perfusion solution. Bars indicate standard deviation ($n = 3$ to 53 experiments)

pH. Since phosphate uptake rapidly decreased with decreasing extracellular H^+ concentration an investigation was initiated to determine if the phosphate-induced E_m depolarization would respond similarly. Figure 2 shows that the E_m between pH 5.6 and 8.3 was about −260 mV on average, but decreased below pH 5.6. In contrast to maximal ΔE_m at pH 5.6 (115 mV), no depolarization by phosphate occurred at pH 8 (Figs. 2, 3). However, in the dark at pH 8 there was still [^{32}P]phosphate influx, 34% of the maximum rate.

Dependence of ΔE_m on E_m. The magnitude of the membrane potential in duckweed varies depending on the length of the dark period after the phosphate-starvation period under long-day conditions. Thus plants with different membrane potentials were available without applying inhibitors or changing K^+ concentrations (further details see Ullrich-Eberius et al. 1983). Figure 4 shows that

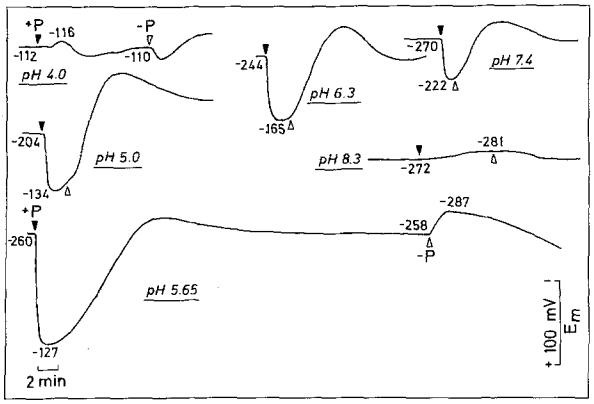

Fig. 2. pH-Dependence of phosphate-induced ΔE_m. Measurements in darkness with 1 mM $H_2PO_4^-$. Plants were phosphate-starved for 15 d. Numbers at the traces denote recorded mV. *Solid arrowheads* indicate addition and *open arrowheads* removal of phosphate from the perfusion solution

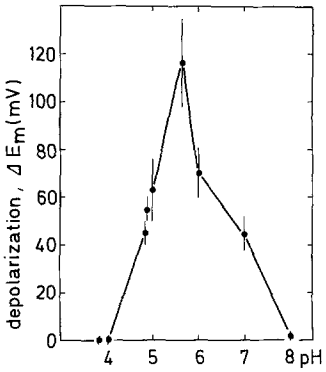

Fig. 3. pH-Dependence of ΔE_m. Measurements in darkness with 1 mM $H_2PO_4^-$. Bars indicate standard deviations ($n=4$ to 7 experiments)

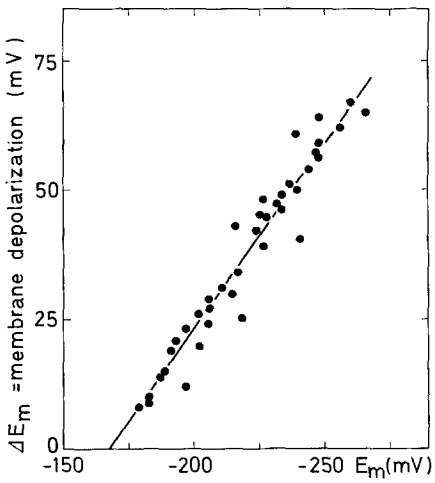

Fig. 4. Dependence of phosphate-induced (1 mM $H_2PO_4^-$) ΔE_m on E_m in darkness at pH 5.7. $r=0.96$

the phosphate-induced ΔE_m was strongly dependent on the magnitude of the E_m, increasing with higher E_m. Correspondingly phosphate uptake at low E_m was only 267 nmol g^{-1} FW h^{-1} but 1,403 nmol g^{-1} FW h^{-1} at high E_m (Ullrich-Eberius et al. 1981).

Effect of fusicoccin. Fusicoccin, known to stimulate the H^+-extrusion pump at the plasmalemma and hence to hyperpolarize the membrane (Marrè 1979; Böcher et al. 1980), increased the rate of phosphate uptake in duckweed, in the light and in the dark, independent of phosphate starvation prior to the experiments (Table 1).

Kinetics of phosphate uptake. [^{32}P]Phosphate uptake was studied after 9 to 12 d of phosphate starvation, i.e. when the increase in phosphate uptake by starvation was strongest. At external concentrations between 1 µM and 1 mM, phosphate uptake showed discontinuous uptake isotherms in the light and in the dark (Fig. 5). Eadie-Hofstee plots revealed that two uptake functions might be involved (Fig. 5). Apparent transport constants were in the range of 6.1 to 8.4 µM phosphate (K_{T_I}) and 65 to 76 µM ($K_{T_{II}}$), without differences between light and dark conditions (Table 2). Little dependence on the time of phosphate starvation was obvious between 9 and 12 d (Table 2). In contrast, V_{max} of uptake increased with the period of phosphate starvation and was strongly increased by light (Table 2).

Kinetics of ΔE_m. The effect of increasing phosphate concentration on E_m was studied in plants starved for phosphate for 12 d. Figure 6 shows the increase in ΔE_m with increasing phosphate concentration, with the typical fast depolarization, spontaneous repolarization and hyperpolarization still in the presence of phosphate, and the typical transient hyperpolarization after removal of phosphate.

Table 1. Stimulation of [^{32}P]$H_2PO_4^-$ uptake by 15 µM fusicoccin (FC) in 0.1% ethanol. Uptake period 10 min, 5 mM Hepes-NaOH at pH 5.7. In experiments with +P plants phosphate concentration was 0.1 mM, with −P plants 1 mM phosphate. −P plants were kept without phosphate for 12 d. Mean values, number of experiments in brackets, ±SD

Plants		Control	FC	%
Phosphate uptake (nmol $H_2PO_4^-$ g^{-1} FW·h^{-1})				
+P	Light	164 (5) ±4.2	184 (7) ±5.8	112
+P	Dark	122 (5) ±4.2	179 (8) ±5.3	147
−P	Dark	1,270 (9) ±25.2	1,549 (15) ±65.2	122

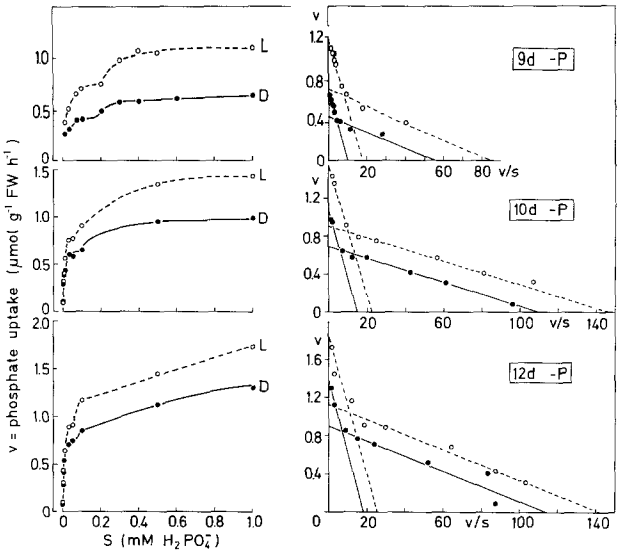

Fig. 5. Concentration dependence of $[^{32}P]H_2PO_4^-$ uptake in light (*L*) and darkness (*D*) at pH 5.7. Plants were phosphate-starved for 9, 10 or 12 d. *Left*, linear plot, *right*, Eadie-Hofstee plot. Mean values of single experiments are plotted

Table 2. Transport parameters for kinetics of phosphate uptake and phosphate-induced ΔE_m, obtained from Eadie-Hofstee plots. K_T values are expressed in µM phosphate and V_{max} values in nmol $H_2PO_4^- \cdot g^{-1}$ FW $\cdot h^{-1}$ and ΔmV (membrane depolarization)

				K_{T_I}	V_{max_I}	$K_{T_{II}}$	$V_{max_{II}}$
Uptake	9 d	−P	Light	8.4	718	65	1,170
			Dark	8.3	455	68	715
	10 d	−P	Light	6.1	900	68	1,530
			Dark	6.2	680	71	1,060
	12 d	−P	Light	7.9	1,120	74	1,850
			Dark	7.9	900	76	1,375
Membrane depolarization	12 d	−P	Dark	3.8	37	39	60

Plotting ΔE_m against the extracellular concentration (Fig. 7) again shows a discontinuous dependence on the extracellular concentration (Fig. 7, insert). Both constants of half maximum depolarization were somewhat lower than those for ^{32}P influx with $K_{T_I} = 3.8$ µM and $K_{T_{II}} = 39$ µM (Table 2). The discrepancies between the kinetics of phosphate influx and ΔE_m may be explained by the fact that ΔE_m values reflect minimum H^+ influx values, because ΔE_m is the sum of an H^+ influx and an H^+ efflux component.

Curve-fitting analysis. Additional iterative fitting of the kinetics of phosphate uptake and of phosphate-induced ΔE_m to one Michaelis-Menten or the sum of two Michaelis-Menten terms confirmed

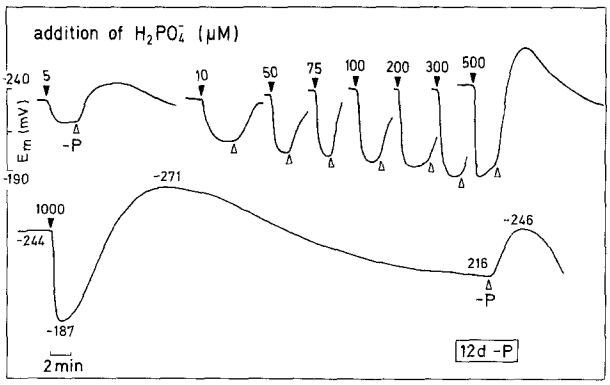

Fig. 6. Concentration dependent, phosphate-induced ΔE_m. Measurements in the dark at pH 5.7. *Solid arrowheads* indicate addition of phosphate, *open arrowheads* removal of phosphate from the perfusion solution. Numbers at the traces denote recorded mV

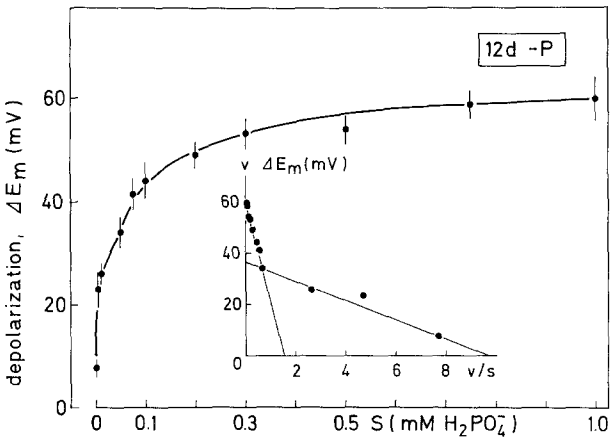

Fig. 7. Concentration-dependent, phosphate-induced ΔE_m, linear and Eadie-Hofstee plot. Bars indicate standard deviation of $n = 4$ to 53 experiments

that both kinetics could better be described by two than by one Michaelis-Menten function (Table 3). The residual fault is lower in the case of two Michaelis-Menten terms and the number of patches is higher.

Differing values between those obtained by the graphical analysis in the Eadie-Hofstee plot and those by iterative fitting to two Michaelis-Menten functions are caused by the fact that the plots give parameters of two Michaelis-Menten functions in sequence and the fitting is based on the assumption of two mechanisms operating simultaneously or overlapping each other. The only clear result we can obtain from both kinetic-analysis methods is that the kinetics of both phosphate uptake and phosphate-induced ΔE_m cannot be described by one single Michaelis-Menten function and that in the used concentration range no linear component appears.

Table 3. Transport parameters for kinetics of phosphate uptake and phosphate-induced ΔE_m, calculated by iterative fitting to the functions: $v = V_{max} \cdot \frac{S}{(K_T + S)}$ and $v = V_{max_I} \cdot \frac{S}{(K_{T_I} + S)} + V_{max_{II}} \cdot \frac{S}{(K_{T_{II}} + S)}$; K_T values are expressed in μM phosphate, and V_{max} values in nmol $H_2PO_4^- \cdot g^{-1}$ FW $\cdot h^{-1}$ and ΔmV (ΔE_m) (rf = residual fault, P = patches, L = light, D = dark)

Plants			One Michaelis-Menten term				Two Michaelis-Menten terms					
			K_T	V_{max}	rf	P	K_{T_I}	V_{max_I}	$K_{T_{II}}$	$V_{max_{II}}$	rf	P
Uptake												
9 d	−P	L	22	981	$2.45 \cdot 10^{-2}$	3/18	5.8	526	250	762	$1.3 \cdot 10^{-2}$	9/18
		D	14	550	$2.12 \cdot 10^{-2}$	3/17	0.26	265	170	458	$2.9 \cdot 10^{-3}$	12/17
12 d	−P	L	12	1,358	$2.1 \cdot 10^{-2}$	4/18	10.0	1,175	740	6,480	$1.5 \cdot 10^{-2}$	5/18
		D	9.1	1,001	$2.5 \cdot 10^{-2}$	6/17	8.0	894	840	6,529	$8.4 \cdot 10^{-3}$	5/17
$\frac{\Delta E_m}{12\text{ d}}$	−P	D	10	50.4	$3.89 \cdot 10^{-2}$	5/11	1.1	14.4	55	45.7	$1.11 \cdot 10^{-2}$	9/11

Discussion

Evidence for H^+/phosphate cotransport. From the present experiments it became quite obvious that phosphate-induced membrane depolarization is linked to phosphate uptake, which is controlled by the intracellular inorganic phosphate concentration (Ullrich-Eberius et al. 1981). Phosphate starvation induced an increase in phosphate uptake and simultaneously it increased the amplitude of ΔE_m at the onset of phosphate transport (Fig. 1). If phosphate uptake were energized in a primary ATP-dependent uniport, the membrane would hyperpolarize as the result of an intracellular increase in negative charges. Assuming a phosphate/OH^- countertransport or a neutral ATP-triggered KH_2PO_4 uptake, no E_m change should be observed. Since, however, E_m is strongly depolarized at the onset of phosphate influx, by up to 133 mV (Fig. 2), we take this as strong evidence for the operation of an H^+/phosphate-cotransport mechanism. Only the actively maintained portion of the E_m is transiently depolarized by phosphate, not the diffusion potential, which in duckweed is identical with the K^+ equilibrium potential (E_{K^+}) (Ullrich-Eberius et al. 1983). Also in the absence of extracellular K^+, in a solution of Na^+, Ca^{2+}, and SO_4^{2-}, E_m is depolarized by phosphate (Ullrich-Eberius et al. 1981), thus making a K^+/phosphate cotransport improbable. In addition, Gibb's free energy (ΔG^o) for K^+ influx along the electrochemical K^+ gradient would yield only 9.4 kJ mol^{-1} (at an E_m of −220 mV), whereas the energy requirement for phosphate uptake under the standard culture conditions is at least 22.2 kJ mol^{-1}.

The energy requirement results from the phosphate distribution in non-starved and phosphate-starved *Lemna gibba* and *Spirodela*, according to Bieleski (1968), Bieleski and Ferguson (1983), and Ullrich-Eberius et al. (1981). In non-starved plants the total phosphate content amounts to 41 μmol g^{-1} FW; 70% of it is inorganic phosphate (P_i). Ten per cent of the P_i is estimated to be located in the cytosol. Assuming the cytoplasmic volume to be 10% of the cell volume, the cytosolic P_i concentration is about 28.7 mM. The concentration of the monovalent P_i species would be 2.2 mM at pH 8 or 11.5 mM at pH 7.4. At standard culture conditions with an extracellular P_i concentration of 1.47 mM at pH 4.8 the energy requirement for phosphate uptake ranges between 22.2 and 26.3 kJ mol^{-1}, depending on the cytosolic pH.

In phosphate-starved plants the total phosphate content is 4 μmol g^{-1} FW of which only 3% is P_i. Ninety per cent of all P_i is located in the cytosol, maintaining a cytosolic concentration of 0.083 mM $H_2PO_4^-$ at pH 8 or 0.43 mM at pH 7.4. The energy requirement for phosphate uptake between 5 μM and 1 mM extracellular phosphate would then range between 15.1 and 32.1 kJ mol^{-1}.

The assumption of H^+/phosphate cotransport as the uptake mechanism is further supported by the strong dependence of ΔE_m on the extracellular pH. Beyond pH 8 the E_m is no longer visibly affected by phosphate (Figs. 2, 3).

In an apparent contradiction, at pH 8 net phosphate uptake still proceeds with 34% of the maximum rate (Ullrich-Eberius et al. 1981). For thermodynamic reasons this influx cannot be explained by passive diffusional influx. It might be explained by the following consideration. In Fig. 4 it was shown that ΔE_m was strongly dependent on the magnitude of E_m. But according to the second term of the Nernst equation for phosphate ($E_P = RT \ln \frac{[P]_i}{[P]_o} + zF E_m$) the energy requirement with

increasing E_m increases simultaneously with the energy available from the electrochemical proton gradient ($\Delta\tilde{\mu}H^+$). Since $\Delta\tilde{\mu}H^+$ is defined as: $RT \ln \frac{[H^+]_i}{[H^+]_o} + zF E_m$, only ΔpH must be the crucial determinant for the rate of phosphate uptake and hence for phosphate-induced ΔE_m. As the experiments were performed at a constant extracellular pH of 5.7, simultaneously with the change in E_m the intracellular pH, i.e. the pH of the cytosol in the vicinity of the plasmalemma, must have changed. This must be caused by a change in carbohydrate availability after the plants had been transferred from the long-day culture to permanent dark culture. Similarly, a greater sensitivity to pH_i or ΔpH than to $\Delta\tilde{\mu}H^+$ was found for H^+/phosphate cotransport in *Saccharomyces (Borst-Pauwels and Peters 1977)*, for H^+/glucose cotransport in *Chlorella* (Komor et al. 1979), for H^+/Cl^- cotransport in *Chara* (Sanders 1980), and for H^+/glucuronic acid cotransport in *Rhodotorula* (Niemietz and Höfer 1984). At pH 8, in the absence of any ΔpH (Köhler 1982), the energy requirement for H^+ extrusion is reduced to 20.3 kJ mol^{-1}. Therefore, H^+ extrusion might exceed H^+/phosphate influx, thereby making H^+/phosphate influx undetectable by mere E_m measurements. Nevertheless, H^+/phosphate cotransport may occur even at pH 8 as a consequence of a local pH gradient in the vicinity of the carrier in the plasmalemma.

Similarly to the apparent, but not real, dependence of phosphate uptake on E_m the effect of fusicoccin may be explained. Fusicoccin enhances the H^+-extrusion pump, resulting in increased E_m (by up to 150 mV in duckweed) and in increased ΔpH (Marrè 1979; Böcher et al. 1980). Since an increased E_m would not stimulate anion uptake, it must be the increased ΔpH which enhances phosphate uptake. The much more energy-requiring uptake of sulfate is strongly enhanced by fusicoccin even at pH 8 (Lass and Ullrich-Eberius 1984). This altogether is regarded as not being in contradiction to a H^+/phosphate-cotransport mechanism.

Biphasic uptake kinetics. Since H^+/phosphate cotransport is most likely the mechanism for phosphate uptake, membrane potential changes, which are easily measured at the onset of phosphate influx, can be taken as a very rapid reflection of phosphate uptake.

Phosphate uptake was revealed to proceed biphasically with two saturating isotherms. Eadie-Hofstee transformation yielded two apparent transport constants of K_{T_I} = 6 to 8 µM and $K_{T_{II}}$ 65 to 76 µM phosphate, without difference between light or dark or by the extent of phosphate starvation. The values of V_{max_I} were 0.455 to 1.20 and $V_{max_{II}}$ values 0.715 to 1.85 µmol phosphate g^{-1} FW h^{-1}. The V_{max} values were higher in light and increased with the time of phosphate starvation (Table 2). Correspondingly, membrane depolarization was dependent on the extracellular phosphate concentration and could also be best described by two Michaelis-Menten functions without a linear component (Tables 2 and 3). Whether these two functions correspond to two mechanisms operating in sequence, as Nissen (1973) and Nissen and Nissen (1983) suggested or are overlapping mechanisms, as assumed by Borstlap (1981) and van Bel et al. (1982) cannot be decided by our experiments.

The absence of any linear component for phosphate uptake in *Lemna* is in agreement with the thermodynamic consideration. Uptake of the monovalent phosphate anion into starved duckweed would require energy up to 0.4 to 2.3 M extracellular phosphate, depending on the intracellular phosphate dissociation. A linear, energy-independent influx component can be expected only above this concentration. Such changes in kinetics at high extracellular phosphate concentration, explainable by the electrochemical phosphate potential, were indeed clearly shown in *Elodea* leaves (Grünsfelder and Simonis 1973).

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