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PHOSPHATE TRANSPORT IN *NEUROSPORA*

KINETIC CHARACTERIZATION OF A CONSTITUTIVE, LOW-AFFINITY TRANSPORT SYSTEM

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SUMMARY

Log-phase cells of *Neurospora crassa*, grown in standard minimal medium, possess an energy-dependent transport system for inorganic phosphate, with a $K_{\frac{1}{2}}$ (at pH 5.8) of 0.123 mM and a J_{max} of 1.64 mmoles/l cell water per min. Like the PO_4^{3-} transport system in yeast, the *Neurospora* system is stimulated by high intracellular K^+. In addition, it is inhibited by high extracellular salt concentrations, an effect which may be related to the known depolarization of the *Neurospora* plasma membrane at high salt concentrations.

The most striking property of the system is its strong dependence upon the extracellular pH. From pH 4.0 to pH 7.3, the J_{max} remains essentially constant but the $K_{\frac{1}{2}}$ increases nearly 400-fold, from 0.01 to 3.62 mM. The increase cannot be accounted for by a single system with a preference for $H_2PO_4^-$ (which would show only a 3-fold increase in apparent $K_{\frac{1}{2}}$ over this pH range) nor by two systems with different affinities and pH optima (which would display nonlinear double-reciprocal plots at intermediate pH values). It can be explained, however, by a model in which OH^- or H^+ is assumed to act as a modifier of the transport system, altering its affinity for substrate.

INTRODUCTION

In recent years, kinetic, biochemical, and genetic methods have been used to investigate the transport of inorganic phosphate in a variety of microorganisms: *Escherichia coli* [1–6], *Streptococcus faecalis* [7–8], *Staphylococcus aureus* [9–10], *Bacillus cereus* [11–12], *Saccharomyces cerevisiae* [13–19], *Chlorella* [20–23], and others. No such study has been carried out with *Neurospora crassa*, however, and because of the unique possibility of using microelectrodes to characterize the electrical properties of the plasma membrane in this microorganism [24–27] (properties important in under-

Abbreviation: HEPES, *N*-2-hydroxypiperazine-*N*-2-ethanesulfonic acid.

standing the movement of ions across the membrane) the present investigation was begun. This paper will discuss the kinetics of phosphate transport in wild-type cells grown in the standard minimal medium. Later papers (Lowendorf, H. S., Bazinet, Jr, G. F. and Slayman, C. W., unpublished results) will consider the derepression of a second phosphate transport system in P-starved cells, and the regulation of the second system by three genes which also control the synthesis of alkaline phosphatase [28].

A preliminary account of this work has already appeared [29].

METHODS

Growth

Wild-type strain RL21a of *N. crassa* was used throughout this work; the general methods of handling the cells have been described previously [30]. Logarithmic phase cells were grown in the minimal medium of Vogel [31] with 2 % sucrose, from an inoculum of 10^6 conidia/ml. The cultures were maintained at 25 °C and were aerated by constant shaking.

Flux experiments

For flux measurements, mid-log phase cells (15–16 h) were harvested by filtration, rinsed several times with distilled water, resuspended in buffer at a cell density of 0.2–0.4 mg dry wt/ml, and incubated at 25 °C with constant shaking. After preincubation for 25 min, $^{33}PO_4^{3-}$ and carrier PO_4^{3-} were added and, at intervals of approx. 1 min, cell samples were removed, harvested on Millipore filters (pore size 1.2 μm), and rinsed 3 times with 5–10-ml aliquots of distilled water. As in the case of K^+, Na^+, and Cl^- [30], one rinse was sufficient to remove more than 90 % of the extracellular isotope. Cell pellets (less than 30 mg dry wt) were extracted for 1 h at 100 °C in 3 ml of 0.1 M Na_3PO_4, a procedure which solubilized more than 99 % of the counts. Duplicate planchets were made from each extract and counted on a low-background gas-flow counter (Nuclear-Chicago). The counting efficiency of ^{33}P was not affected by the dry weight of the sample (below 30 mg) but was reduced by high PO_4^{3-} concentrations in the extract; for this reason, Na_3PO_4 was added to all samples of extracellular medium before counting to give a final concentration of 0.1 M.

Buffers

The incubation buffer used for most experiments was 3,3-dimethylglutaric acid (Eastman Organic Chemicals, Rochester, N.Y.) at 5 or 20 mM (depending on cell density), brought to pH 5.8 with NaOH. The buffer also contained 20 mM KCl, which served to maintain the intracellular K^+ concentration at its normal value near 180 mmoles/l cell water [30]. No carbon source was added. Control experiments showed that for phosphate, as for K^+ [30], 1 % glucose or 2 % sucrose had little effect on influx. The sugars did stimulate a slow, steady decline of total cell phosphorus, however: 1.64 mmoles/l cell water per min (measured in the absence of added extracellular phosphate), which represents a 5–6-fold stimulation of the loss observed without sugar.

Flux measurements at pH values other than 5.8 were made in the following buffer solutions: pH 4.0–6.4, 5 mM dimethylglutaric acid (titrated with NaOH)—

20 mM KCl, or P_i-free minimal medium (made by replacing the KH_2PO_4 in Vogel's medium with KCl) +20 mM dimethylglutaric acid, pH 7.3; 20 mM *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Calbiochem)+20 mM KCl, or P_i-free minimal medium+20 mM HEPES.

Isotopes

$^{32}PO_4^{3-}$ from several sources was used in early experiments but produced large amounts of scatter in the results. The isotope could not be completely diluted by carrier PO_4^{3-}, and initial rates of uptake by *Neurospora* were quite variable, depending on the batch of isotope and the length of time the batch had been stored in the laboratory. The scatter was not eliminated by boiling the isotope in HCl (to hydrolyze polyphosphates) nor by filtering (to remove particulate matter). Frequent reference has been made to contaminants in commercial $^{32}PO_4^{3-}$ [7, 12, 32], and apparently different batches contain different contaminants.

With $^{33}PO_4^{3-}$, most of the scatter disappeared, so that this isotope (obtained from Tracerlab or New England Nuclear) was used routinely throughout these experiments. As a precaution, the stock solution was always autoclaved (121 °C, 15 min) and filtered (Millipore, pore size 0.22 μm) before use.

Measurement of total cell phosphorus

Cell pellets (less than 40 mg dry wt) were digested in 2.2 ml of $HClO_4$ (60 %) in Kjeldahl flasks until colorless and were assayed for total phosphorus (as orthophosphate) by a modification of the method of Allen [33]. To use units consistent with previously reported intracellular K^+ and Na^+ concentrations, cellular P has been expressed throughout this paper in units of mmoles P/l cell water, even though it is recognized that most of the phosphorus does not occur as orthophosphate [34]. Calculations of intracellular concentration were made from the previously determined value (2.54, [30]) for the ratio intracellular water/dry weight, based on inulin estimates of the extracellular space.

Calculation of kinetic parameters

Michaelis–Menten parameters for phosphate fluxes were determined by computer, using a non-linear least-squares program devised by Hanson et al. [35]. Model parameters for the pH-dependent transport of phosphate were computed with the Marquardt algorithm [36], a more general non-linear curve-fitting procedure (IBM SHARE No. 3094). Both programs were run on the Yale Computer Center IBM 7094/7040 system. Fitted parameters are reported with confidence limits of ± 1 standard error; most other results are stated as the mean (of several observations) ± 1 S.E.M.

RESULTS

Growth experiments

As background for the main studies on phosphate transport, experiments were carried out initially to measure the total intracellular phosphorus content of *Neurospora*, and to see whether intracellular phosphorus remains constant during all portions of the growth cycle. Fig. 1 shows the results of one such experiment. At zero

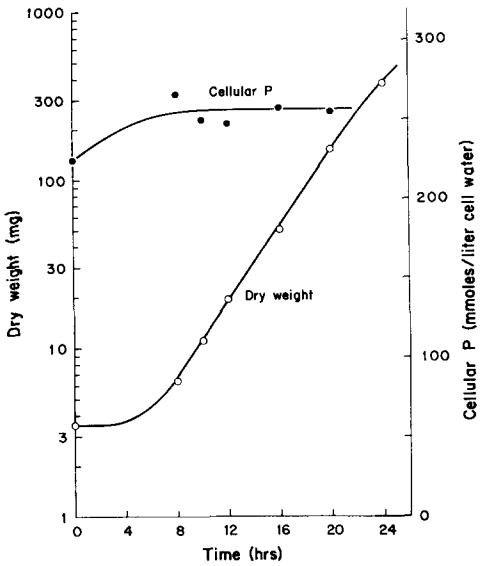

Fig. 1. Phosphate content of *Neurospora* during growth in Vogel's medium. Washed conidia were inoculated (10^6/ml) into medium and incubated on a reciprocal shaker (120 cycles/min) at 25 °C. At intervals cells from duplicate flasks were harvested, rinsed with distilled water, dried, weighed, and assayed for phosphorus. The doubling time in this experiment was 2.7 h.

time, conidia were inoculated into minimal medium and incubated with constant shaking at 25 °C. After a lag phase of about 4 h, the dry mass of the culture (left ordinate scale) increased exponentially with a doubling time of 2.6 h; this exponential phase lasted for 18–20 h, after which growth slowed and then stopped (not shown). During the lag phase of growth, intracellular phosphorus (right ordinate scale) increased from the conidial level of 219 mmoles/l cell water to a stable level in the range 248–266 mmoles/l cell water during exponential phase. Overall, the mean value for the 15–16-h cells routinely used in flux measurements was 271 ± 2 mmoles/l cell water (60 experiments).

This value is 30 % higher than that calculated from the earlier data of Harold [34] (205 mmoles/l cell water), a discrepancy which may arise either from the difference in strains between the two experiments (Em 5297a was used by Harold) or, more likely, from the fact that the higher value was measured directly on whole-cell digests, whereas the lower value was calculated after fractionation of the various phosphorus-containing compounds. The percentage distribution of phosphorus, as estimated by Harold [34], is also pertinent to the present experiments: only 4 % (corresponding to 8 mmoles/l cell water) appeared as orthophosphate; the largest fraction (42 %) was contained in nucleic acids, with 23 % in inorganic polyphosphate, 16 % in acid-soluble organic compounds, and 15 % in lipids.

PO_4^{3-} uptake

When mid log-phase cells were removed from growth medium and resuspended in buffer at the same pH (5.8), they were found to take up ^{33}P-labelled phosphate

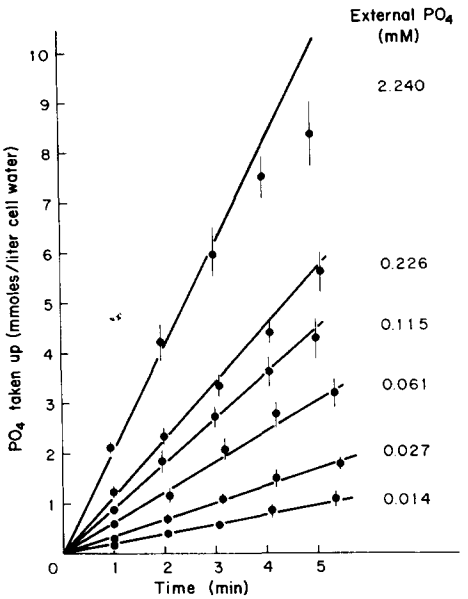

Fig. 2. Linearity of the initial phosphate entry. Cells were harvested from growth medium at 15–16 h, rinsed, resuspended in the standard dimethylglutaric acid buffer (pH 5.8) for 25 min; $^{33}PO_4^{3-}$ was then added (concentration shown at the end of each line). Each point plotted represents the average value for three determinations. All lines were fitted by the method of least squares, constrained to pass through the origin. Slopes and standard errors are as follows:

External PO_4^{3-} concn (mM)	Slope $\pm$ S.E. (mmoles/l cell water per min)	Total number of values
0.014	0.194 $\pm$ 0.010	15
0.027	0.334 $\pm$ 0.015	15
0.061	0.604 $\pm$ 0.022	15
0.115	0.889 $\pm$ 0.033	15
0.226	1.13 $\pm$ 0.04	15
2.24	2.05 $\pm$ 0.08	12 (first 4 points)

linearly with time for the first 5 min (as illustrated in Fig. 2), so that the initial fluxes could be determined directly from the early time points. At a saturating concentration of PO_4^{3-} (2 mM, see below), the initial rate of uptake averaged 1.60 $\pm$ 0.13 mmoles/l cell water per min. Separate control experiments (not illustrated), in which cells were grown in medium containing $^{33}PO_4^{3-}$ and then exposed to unlabelled PO_4^{3-}, indicated that most of this uptake represented a net flux of PO_4^{3-} into the cells. Very little PO_4^{3-} efflux occurred (at most, 20–30% of the influx), although *Neurospora* does show some loss of other P-containing compounds into the medium.

Energy dependence

As with all other transport systems which have been studied in *Neurospora*, the phosphate transport system appears to depend upon metabolic energy. Uptake is blocked both by agents which uncouple oxidative phosphorylation (e.g. 2,4-

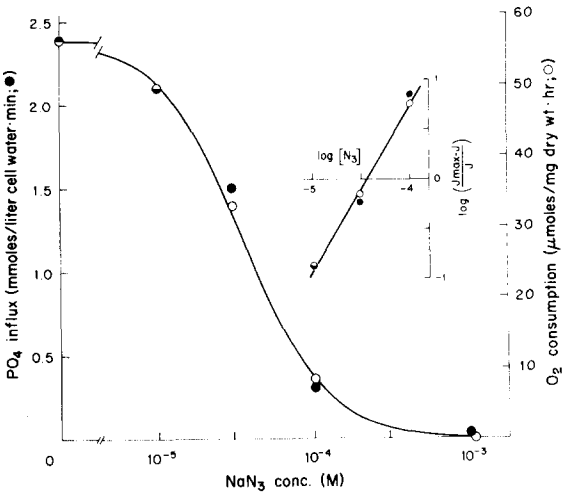

Fig. 3. Effect of NaN_3 on phosphate uptake and O_2 consumption. 15-h log-phase cells were harvested, washed, and preincubated in the standard dimethylglutaric acid buffer for 20 min. Then NaN_3 (pH 5.8) was added and, 5 min later, $^{33}PO_4^{3-}$ with 2 mM carrier phosphate. Phosphate fluxes were determined as in Fig. 2. Each point plotted is the average of two determinations. Rates of respiration are taken from Slayman and Tatum [45]. Curve is redrawn from the inset. Inset: a joint Hill plot of the respiration and flux data (J_{max} = 100 %). Least-squares estimates of K and n: $3.56 \cdot 10^{-8} \pm 0.41 \cdot 10^{-8}$, and 1.67 ± 0.08, respectively. $[N_3] = [N_3^-] + [HN_3]$.

dinitrophenol) and by agents which inhibit respiration. The clearest results have been obtained with NaN_3, and are shown in the dose-response curve of Fig. 3. Both phosphate influx (left ordinate scale) and O_2 consumption (right ordinate scale) decline along a single curve as the extracellular azide concentration is raised. The critical range of concentrations is 10^{-5}–10^{-4} M at pH 5.8, and both functions are half-maximal at $3.47 \cdot 10^{-5}$ M. The result is essentially identical to those obtained previously with potassium influx [37] and with membrane potential [25] in *Neurospora*.

(The curve drawn in Fig. 3 does not represent a simple titration curve for azide binding, but is too steep, indicating some kind of cooperativity. If the phosphate flux or respiration rate (J) is assumed to be proportional to the free concentration of a respiratory carrier to which azide $[N_3]$ binds, then

$$J = \frac{J_{max}}{1 + [N_3] \cdot \sqrt[n]{K}} \tag{1}$$

in which J_{max} is the maximal flux or rate of respiration (no azide binding), n is the degree of cooperativity in the binding reaction and $[N_3] \cdot \sqrt[n]{K}$ is the azide concentration at which carrier is half bound. This equation can be linearized by a log–log transformation:

$$\log \frac{J_{max} - J}{J} = n \log [N_3] - \log K, \tag{2}$$

and the data in Fig. 3 are plotted in this manner (Hill plot) in the figure inset. With J_{max} taken in both cases to be the experimentally observed value (2.38 mmoles

PO_4^{3-}/l cell water per min and 56.2 μl O_2/mg dry wt per h, in these experiments), least-squares estimates of the intercept and slope gave $K = 3.56 \cdot 10^{-8} \pm 0.41 \cdot 10^{-8}$ and $n = 1.67 \pm 0.08$. The same degree of cooperativity was obtained by Winzler [38] in a study of azide effects on yeast respiration, but does not appear in azide binding to extracted cytochrome oxidase [39]. Its origin probably lies, therefore, in multiple sites of action of azide in the respiratory chain.

Concentration dependence

When the initial velocity of PO_4^{3-} uptake was determined as a function of the PO_4^{3-} concentration in the medium, the results could be fitted well by the simple Michaelis–Menten equation (Fig. 4). The values of J_{max} and $K_{\frac{1}{2}}$, obtained by the least-squares procedure (see Methods), were 1.64 ± 0.09 mmoles/l cell water per min and 0.123 ± 0.022 mM, respectively.

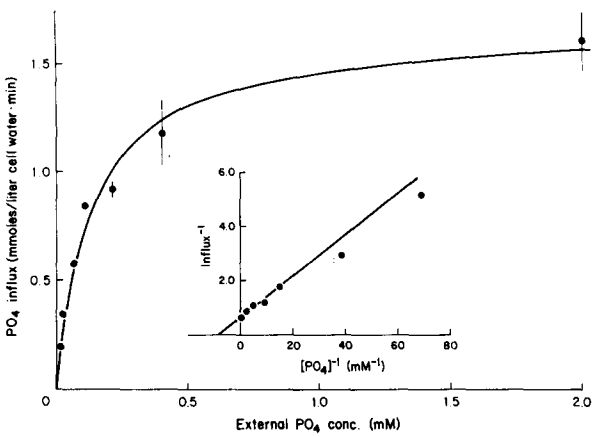

Fig. 4. Dependence of phosphate uptake on the external phosphate concentration. Data are taken from 5 separate flux experiments, all carried out with 15–16-h log phase cells in the standard dimethylglutaric acid buffer (pH 5.8). The curve was generated by computer fit [35] of the data to the Michaelis–Menten equation. Parameter estimates are as follows: $K_{\frac{1}{2}} = 0.123 \pm 0.022$ mM and $J_{max} = 1.64 \pm 0.09$ mmoles/l cell water per min. Inset: A double reciprocal plot of the data. Deviation of the fitted line and data at large reciprocal concentrations occurs because the fit was obtained by direct calculation, without the reciprocal transformation, and can be explained by the presence of a small amount of a second, high-affinity transport system [43] (Lowendorf, H. S., Bazinet, Jr, G. F. and Slayman, C. W., unpublished results).

Effects of K^+ on phosphate uptake

K^+ has been shown to influence phosphate uptake in at least four species of microorganisms: yeast (*Sacc. cerevisiae*) [13], *Strep. faecalis* [7], *E. coli* [1], *and B. cereus* [12], although the manner in which K^+ acts varies from species to species. In yeast, for example, maximal phosphate influx requires a high intracellular K^+ concentration, but K^+ need not be added to the extracellular medium [40]. By contrast, in *E. coli*, phosphate and K^+ influx appear to be coupled, at least under some circumstances [1], so that an electrically neutral net uptake of the two ions occurs; and a mutant strain has been reported which is simultaneously defective in the transport of both ions [2].

TABLE I

EFFECT OF K^+ AND Na^+ ON INFLUX OF PHOSPHATE

Influx parameters were obtained by fitting the Michaelis–Menten equation to plots similar to that of Fig. 4. Concentration range: 0.01–2.0 mM; 6 points for each curve. J_{max} plotted in Fig. 5. The clearest index of the difference in ionic composition between the two groups (upper 5, lower 3) is the ratio, $R_i = [Na^+]_i/[K^+]_i$, which is ≤ 0.64 (upper) and ≥ 1.71 (lower). Σd^2 represents the sum of the squared differences between the predicted values and the observed values.

External concns (mM)			Internal concns (mmoles/l cell water)			Parameters of phosphate influx (mmoles/l cell water per min)		
$[K^+]_o$	$[Na^+]_o$	Σ_o	$[K^+]_i$	$[Na^+]_i$	R_i	J_{max}	$K_{\frac{1}{2}}$ (mM)	Σd^2
0	6.3	6.3	128	69.6	0.54	1.84±0.18	0.143±0.046	0.102
10	6.3	16.3	166	19.2	0.12	1.92±0.11	0.161±0.025	0.080
20	6.3	26.3	170	12.9	0.08	1.85±0.04	0.125±0.008	0.004
10	26.3	36.3	106	67.4	0.64	1.67±0.20	0.137±0.053	0.121
20	25.2	45.2	136	46.7	0.34	1.15±0.08	0.086±0.021	0.023
0	25.2	25.2	65.6	119	1.81	1.46±0.12	0.214±0.048	0.121
10	26.3	36.3	56.6	116	2.05	1.35±0.10	0.082±0.024	0.040
20	25.2	45.2	71.3	122	1.71	1.05±0.02	0.067±0.006	0.003

Because of these apparent interactions between K^+ and phosphate in other organisms, a study was made of the possible K^+-dependence of the Michaelis–Menten parameters for phosphate influx in *Neurospora*. By preincubating cells in K^+-free buffer, it was possible to control the internal K^+ concentration as well as the external concentration; and phosphate flux measurements were consequently made on both low-K^+ and high-K^+ cells, at 0, 10, and 20 mM extracellular K^+. In all cases

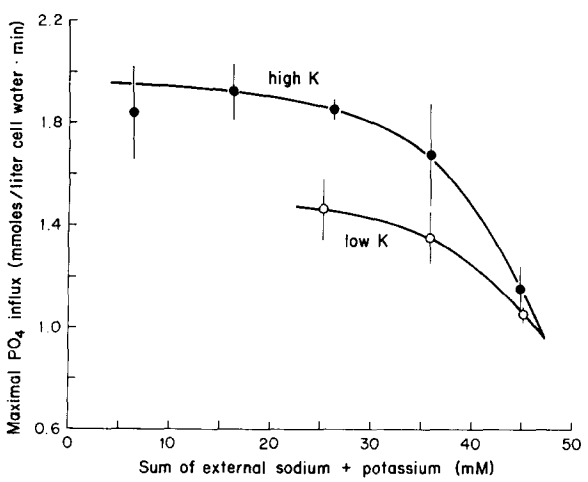

Fig. 5. Effect of external salt concentration on phosphate influx. Upper curve: cells with near normal internal ions; lower curve: cells depleted of K^+ and loaded with Na^+. Depleted cells were prepared by incubation for 25 min in K^+-free buffer. Buffers: 20 mM or 5 mM dimethylglutaric acid, brought to pH 5.8 with NaOH. Table I lists the Na^+ and K^+ contents of the cells and medium at the time of the phosphate flux measurements in this figure.

the plots of initial rate versus extracellular PO_4^{3-} concentration gave simple saturation curves and fitting the Michaelis–Menten equation to the data yielded the values of J_{max} and $K_{\frac{1}{2}}$ listed in Columns 7 and 8 of Table I. No systematic variation was evident in the values of $K_{\frac{1}{2}}$, but J_{max} appeared to be influenced both by the internal K^+ concentration (or the ratio $[Na^+]_i/[K^+]_i$) and by the total extracellular cation concentration ($[Na^+]_o + [K^+]_o$); these results are plotted in Fig. 5. At low extracellular salt concentrations (below approx. 30 mM), maximal phosphate influx in high-K^+ cells was about 25 % greater than in low-K^+ cells. At high salt concentrations (40–50 mM) phosphate influx was depressed in both types of cells, and the difference between the two types was abolished.

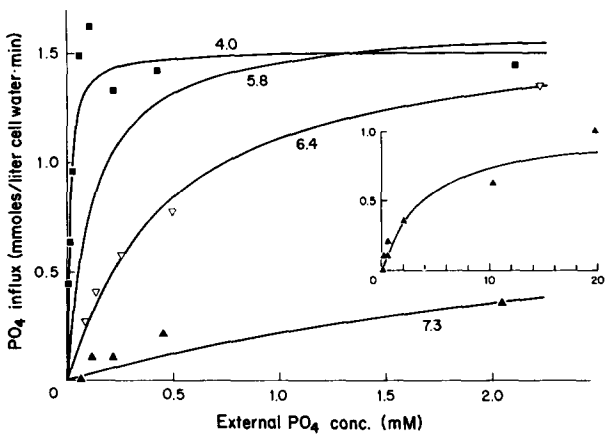

Fig. 6. pH-dependence of phosphate influx. Cells were grown at pH 5.8, harvested at 15–16 h, and transferred to buffer at the various pH's (see Methods). Flux measurements made as in Fig. 2. Plotted points are averages from 2–5 experiments. Curves are computer-fitted, as in Fig. 4. Inset: Extended plot of data at pH 7.3 to show why the least-squares curve passes below three of the low-concentration points. Fitting parameters listed in Table II. Columns 3 and 4.

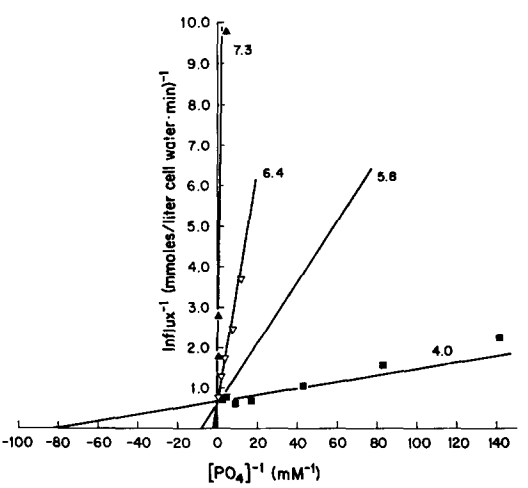

Fig. 7. Double-reciprocal plot of the data in Fig. 6.

Effect of pH

The most striking characteristic of phosphate influx in *Neurospora* was its strong dependence upon the extracellular pH. Cells were grown in the standard Vogel's medium at pH 5.8 and resuspended in buffer from pH 4.0 to 7.3; and at each pH, PO_4^{3-} influx was measured as a function of the extracellular phosphate concentration. Plots of influx versus $[PO_4^{3-}]_0$ gave simple saturation curves at all values of pH, as shown in Fig. 6 and in the double-reciprocal plots of Fig. 7. J_{max} fluctuated somewhat around a mean of 1.42 mmoles/l cell water per min without any systematic dependence upon pH. But the apparent values of $K_{\frac{1}{2}}$ rose nearly 400-fold from pH 4.0 to 7.3. Similar pH sensitivity of phosphate transport has been reported in yeast [13], bacteria [7, 8, 10], and algae [23], and possible mechanisms for it are considered in Discussion.

DISCUSSION

Net uptake of phosphate by wild-type *Neurospora*, harvested from exponential growth in Vogel's medium, can be described as a simple, energy-dependent, carrier-mediated transport process. It is blocked by low concentrations of respiratory inhibitors and uncouplers, and displays straightforward Michaelis–Menten kinetics, with a $K_{\frac{1}{2}}$ (at pH 5.8) of 0.123 mM and a J_{max} of 1.64 mmoles/l cell water per min. One can confirm that the measured rate of PO_4^{3-} entry is in fact sufficient to support the observed rate of exponential growth, by the following calculation: since the intracellular phosphorus concentration, $[P]_c$, remains constant during exponential growth (see Fig. 1), the total amount of cellular phosphorus must increase at the same rate as the cell mass. This means that the rate of PO_4^{3-} uptake by a unit mass of cells is given by $[P]_c/\tau$, where τ is the time-constant for the exponential growth process. Since τ is related to the mass-doubling time (t_D) by $\tau = t_D/\ln 2 = t_D/0.693$, it follows that

$$J = 0.693\ P_c/t_D. \tag{3}$$

With $[P]_c = 271$ mmoles/l cell water and $t_D = 2.6$ h $= 156$ min, $J = 1.20$ mmoles/l cell water per min or about 75 % of the rate calculated from the Michaelis–Menten equation (1.63 mmoles/l cell water per min) at pH 5.8, for the PO_4^{3-} concentration (37 mM) in Vogel's medium.

Two properties of the underlying transport system are of special note: (1) the dependence of J_{max} upon the cation content of both the cytoplasm and the extracellular solution, and (2) the very strong dependence of $K_{\frac{1}{2}}$ upon the extracellular pH. These two properties are discussed in order below.

The fact that J_{max} is greater in high-K^+ cells than in K^+-depleted cells was expected from the published results on yeast [40]; it could arise either directly, from interaction of intracellular K^+ (and/or Na^+) with the phosphate transport mechanism or indirectly, from depression of protein synthesis in the low-K^+ cells [41]. The inhibition of PO_4^{3-} transport at high salt concentrations, in both high-K^+ and low-K^+ cells, was not anticipated from the yeast data, and may have a specific and more interesting basis. Since high external salt concentrations are known to depolarize the plasma membrane of *Neurospora* [24], depressed phosphate uptake at high salt levels is consistent with the hypothesis [27, 42], that a variety of transport systems in *Neurospora* may be co-transport systems which are driven both by the trans-membrane

gradient of H^+ and by the membrane potential.

The strong dependence of $K_{\frac{1}{2}}$ for phosphate transport upon the external pH was again expected from experiments with other microorganisms, chiefly *S. faecalis*. In that organism it was suggested [7] to arise from the existence of two completely separate transport systems (one with a low $K_{\frac{1}{2}}$ and low pH optimum, and the other with a high $K_{\frac{1}{2}}$ and a high pH optimum), a model which was consistent with differential effects of arsenate at the pH extremes [8] and the isolation of a mutant defective in PO_4^{3-} transport at high pH but normal at low pH [7]. Trial calculations have enabled us to eliminate this kind of model for *Neurospora*, however, at least for wild-type cells grown under normal conditions. If the two putative systems were to have similar maximal velocities but $K_{\frac{1}{2}}$'s different by two orders of magnitude, then on a double-reciprocal plot ($1/J$ vs $1/S$) they should generate a conspicuously hyperbolic curve, concave downward. Fig. 7 demonstrates that this is not the case. At each pH, the data fall essentially on a straight line and can be fitted by a single set of Michaelis constants. (The slight bend in the double-reciprocal plot at pH 5.8 can be accounted for by the presence of a very small amount of a second phosphate transport system, which can be stimulated under conditions of phosphorus starvation [43] (Lowendorf, H. S., Bazinet, Jr, G. F. and Slayman, C. W., unpublished results)).

Consequently, we have considered several single-system models to describe the pH-dependence of PO_4^{3-} transport in *Neurospora*. The simplest of these is that only $H_2PO_4^-$, and not HPO_4^{2-}, is the substrate for the transport system, so that with rising pH the fractional concentration of substrate would fall, thus increasing the apparent value of $K_{\frac{1}{2}}$. This model can be ruled out directly on quantitative grounds: whereas the experimental $K_{\frac{1}{2}}$ increases 400-fold from pH 4.0 to 7.3, $H_2PO_4^-$ would decline only 3-fold (assume pK_2 for phosphate to be 7.0) over the same pH range. Intuitively, an enhancement of the effect might be expected by supposing, in addition, that HPO_4^{2-} is a competitive inhibitor of $H_2PO_4^-$ transport. But the actual kinetic equation for this model shows that both the apparent $K_{\frac{1}{2}}$ and J_{max} should decrease, and by the same factor, as pH increases [43]. This prediction is wholly incompatible with the data.

One class of single-system models which can describe the pH-dependence of phosphate transport in *Neurospora* is that in which H^+ or OH^- serve as modifiers of the transport system, altering its affinity for the substrate. Curve-fitting efforts with several forms of this model led us to a reduced version in which binding of substrate ($H_2PO_4^-$ or HPO_4^{2-}) is blocked by binding of OH^- (or enhanced by binding of H^+) at a modifier site. Kinetically, this version of the modifier model is indistinguishable from conventional competitive inhibition (by OH^-) at a single site, and the velocity equation is simply

$$J = \frac{J_{max}}{1 + \frac{K_s}{S}\left(1 + \frac{M}{K_m}\right)}, \tag{4}$$

where S is the external phosphate concentration, M is the modifier concentration, K_s is $K_{\frac{1}{2}}$ for $M = 0$, and K_m (corresponding to K_i in competitive inhibition) is the dissociation constant for the carrier–M complex. Fitting of this equation to the data plotted in Fig. 7 yields the following parameter values: $J_{max} = 1.45 \pm 0.05$ mmoles/l

TABLE II

KINETIC PARAMETERS OF PHOSPHATE INFLUX AT DIFFERENT VALUES OF pH_0

Michaelis–Menten parameters for the curves fitted in Fig. 6 (plus one curve not shown, for pH 4.98). Apparent values of $K_{\frac{1}{2}}$ and J_{max} for the modifier (competitive inhibition) model.

pH_0	$[OH^-]_0$ (mM)	Michaelis–Menten parameters		Calculated from Eqns 3 and 4	
		$K_{\frac{1}{2}}$ (mM)	J_{max} (mmoles/l cell water per min)	$K_{\frac{1}{2}}^{app}$	J_{max}^{app}
4.04	$1.07 \cdot 10^{-7}$	0.012 ± 0.003	1.51 ± 0.06	0.0093	1.45
4.98	$9.55 \cdot 10^{-7}$	0.005 ± 0.010	1.28 ± 0.10	0.0257	1.45
5.80	$6.31 \cdot 10^{-6}$	0.123 ± 0.022	1.64 ± 0.09	0.129	1.45
6.43	$2.69 \cdot 10^{-5}$	0.470 ± 0.169	1.64 ± 0.19	0.528	1.45
7.26	$1.82 \cdot 10^{-4}$	3.62 ± 1.73	1.01 ± 0.16	3.53	1.45

cell water per min; $K_s = 7.26 \cdot 10^{-6} \pm 2.71 \cdot 10^{-6}$ M, and $K_m = 3.75 \cdot 10^{-10} \pm 1.46 \cdot 10^{-10}$ M (which is equivalent to an acid pK of 4.57 for the modifier site).

At any given pH the apparent value of $K_{\frac{1}{2}}$ for a Michaelis fit to the flux data is

$$K_{\frac{1}{2}}^{app} = K_s \left(1 + \frac{M}{K_m}\right) \tag{5}$$

while $J_{max}^{app} = J_{max}$. The observed and calculated values of $K_{\frac{1}{2}}$ are listed in Table II, and are compared graphically in the semilogarithmic plot of Fig. 8. The match seems very satisfactory, especially at the higher values of pH, but the fact of a very high $K_{\frac{1}{2}}$ at pH 7.3 makes determination of the corresponding value of J_{max} difficult, so that the significance of the low value of J_{max} observed (1.01 mmoles/l cell water per min,

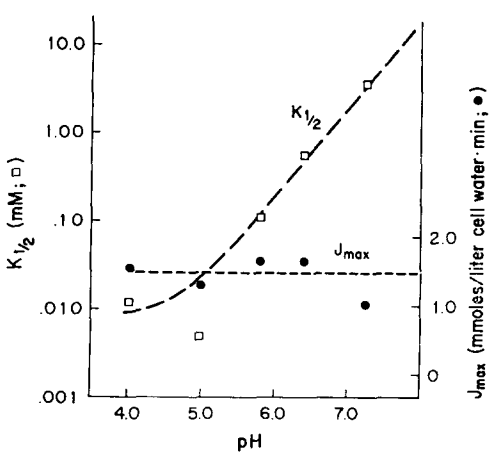

Fig. 8. Calculation of Michaelis–Menten parameters from the modifier (competitive inhibition) model. Points plotted are the values of $K_{\frac{1}{2}}$ and J_{max} calculated from individual fits of the Michaelis–Menten equation to the data in Fig. 6. Curves drawn were calculated from the three kinetic parameters in Eqn 4, fitted jointly to all of the data in Fig. 6. $J_{max}^{app} = J_{max}$; $K_{\frac{1}{2}}^{app} = K_s(1 + M/K_m)$. See also summary in Table II.

compared with that calculated from Eqn 4: 1.45 mmoles/l cell water per min) at pH 7.3 is not clear.

It is interesting to speculate about the overall significance of Eqn 4 and the modifier model in relation to the mechanism of PO_4^{3-} transport. As mentioned above, the simplest way to look at Eqn 4 is that OH^- compete with PO_4^{3-} at the binding sites for entry on a carrier. Alternatively, recent evidence from this laboratory (Slayman, C. W., Lowendorf, H. S., Gradmann, D. and Slayman, C. L., unpublished) suggests that the phosphate transport system in *Neurospora*, like the glucose transport system, is an H^+- and voltage-dependent cotransport system, so that the strong effect of pH on $K_{\frac{1}{2}}^{app}$ could arise as a kinetic consequence [44] of removing H^+ and net charge from the carrier at high pH. According to this view, the depression of transport at high external pH and at high external salt concentrations (discussed above) could both reflect the same underlying property of the transport system, although more is probably involved than simply a change in the electrical driving force since pH affects the $K_{\frac{1}{2}}$ for transport while salt affects the J_{max}.

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