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Kinetic Characterization of the Two Phosphate Uptake Systems in the Fungus *Neurospora crassa*

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The kinetics of phosphate uptake by exponentially growing *Neurospora crassa* were studied to determine the nature of the differences in uptake activity associated with growth at different external phosphate concentrations. Conidia, grown in liquid medium containing either 10 mM or 50 μM phosphate, were harvested, and their phosphate uptake ability was measured. Initial experiments, where uptake was examined over a narrow concentration range near that of the growth medium, indicated the presence of a low-affinity (high K_m) system in germlings from 10 mM phosphate and a high-affinity (low K_m) system in germlings from 50 μM phosphate. Uptake by each system was energy dependent and sensitive to inhibitors of membrane function. No efflux of phosphate or phosphorus-containing compounds could be detected. When examined over a wide concentration range, uptake was consistent with the simultaneous operation of low- and high-affinity systems in both types of germlings. The V_{max} estimates for the two systems were higher in germlings from 50 μM phosphate than for the corresponding systems in germlings from 10 mM phosphate. The K_m of the high-affinity system was the same in both types of germlings, whereas the K_m of the low-affinity system in germlings from 10 mM phosphate was about three times that of the system in germlings from 50 μM phosphate.

Relatively little is known of the mechanisms whereby microorganisms, particularly fungi, achieve and regulate uptake of phosphate (13, 24). There are conflicting reports regarding the number and the kinetic properties of the uptake systems of the yeast *Saccharomyces cerevisiae* (2, 3, 8, 15). The differences may be due, in part, to differences in the culture conditions before assay, since, in studies with other fungi, phosphate uptake ability has been found to be increased after growth in low-phosphate media (4, 27, 28). The most extensive study of phosphate uptake by a filamentous fungus has been carried out recently with *Neurospora crassa* (18-20). This fungus was reported to possess two uptake systems, one of low affinity (high K_m) and one of high affinity (low K_m). Phosphorus starvation resulted in a dramatic increase in the activity of the high-affinity system.

These results are similar to those reported for the uptake of a variety of solutes in microorganisms where the activity of a high-affinity system either appears de novo or is increased after a period of starvation for the solute. Such reports suggest a mechanism by which an organism might adapt to different external solute concentrations. Namely, all uptake of the solute above that carried out by the low-affinity system present at its constitutive level is provided by the high-affinity system, derepressed to an appropriate extent. Quantitative evidence in support of such a control mechanism is lacking. We chose to test this hypothesis by studying the regulation of phosphate uptake activity in *N. crassa* growing exponentially at different phosphate concentrations. An essential part of the study has been the development of experimental techniques to describe accurately the uptake characteristics of the fungus at the instant of harvest. In this paper we report these techniques together with a detailed account of the uptake characteristics of germinating conidia (germlings) grown in medium containing phosphate at either a high or low concentration. In the accompanying paper (1), we examine the behavior of germlings grown at concentrations between these two extremes and discuss the extent to which the kinetic parameters of the uptake systems can account for the observed phosphate uptake rates of growing germlings.

MATERIALS AND METHODS

Our general method has been to grow *N. crassa* conidia for 2.5 h in a growth medium containing the appropriate concentration of phosphate and then

to transfer the germlings rapidly to fresh growth medium with $^{32}P_i$ at different concentrations to follow uptake. The technique of preincubating cells for a period in a simple buffer solution before assay, which has previously been used in phosphate uptake studies of *N. crassa* (16, 19), has been avoided. We saw no advantage of this technique for our studies and considered that such manipulations might alter the uptake characteristics from those existing during growth.

Where appropriate, values are given as the mean ±1 standard deviation.

Inoculum. The *N. crassa* wild-type strain, STA4 (St. Lawrence), was obtained from J. R. S. Fincham. Conidia were produced by growing the fungus on Vogel's medium N (32) supplemented with sucrose (20 g/liter), agar (15 g/liter), and, in the case of most uptake experiments, $^{35}SO_4^{2-}$ (2 μCi/ml). After 6 days at 25°C, the conidia and aerial mycelium were scraped from the agar surface and shaken in water. The resulting suspension was filtered through glass wool to remove mycelial fragments and centrifuged, and the pellet of conidia was resuspended in water. Appropriate volumes were then added to the different germination flasks of an experiment to give a final concentration of 10^6 conidia per ml. For technical reasons germination flasks could not always be inoculated at the same time, but storing the suspension for up to 3 h at room temperature had no effect on the subsequent germination or the uptake characteristics of the germlings.

Media for germination and uptake studies. The base medium (BM) contained: KNO_3, 2 mM; NH_4NO_3, 2 mM; $(NH_4)_2SO_4$, 3 mM; $MgSO_4$, 2 mM; $Ca(NO_3)_2$, 2 mM; citrate, 10 mM (1 M sodium citrate buffer, pH 6.4, 10 ml/liter); sucrose, 58 mM; ferric monosodium ethylenediaminetetraacetate, 54 μM; H_3BO_3, 46 μM; $MnCl_2$, 9 μM; $ZnCl_2$, 0.73 μM; $CuSO_4$, 0.32 μM; $(NH_4)_6Mo_7O_{24}$, 0.032 μM; *d*-biotin, 0.02 μM. BM was prepared at 2.5 times final strength, with the pH adjusted to 6.4. Phosphate solutions were prepared at twice final strength from KH_2PO_4 adjusted with KOH, such that when mixed with BM and diluted the final pH was 6.4. BM and phosphate solutions were autoclaved separately (121°C, 15 min). Radioactive phosphate and cycloheximide (final concentration, 14 μM; Upjohn Co., Kalamazoo, Mich.) were not autoclaved and were added directly to the appropriate sterilized solutions.

Germination and growth. Conidia were germinated by incubation at 30°C for 2.5 h in 100 to 500 ml of medium in a flask of four to five times the medium volume on a reciprocating shaker (thrust, 3 cm; 90 rpm). The medium was either BM + 10 mM phosphate (10 mM germlings) or BM + 50 μM phosphate (50 μM germlings). During incubation the pH of the medium remained between 6.3 and 6.4.

Uptake assays. Samples of 2.5-h germlings were harvested from the growth medium by vacuum filtration onto a cellulose nitrate filter (pore size, 1.2 μm). The pad of germlings was washed thoroughly with BM minus phosphate and dropped into resuspension solution (see below). The germlings were dislodged from the filter by swirling the flask, and the filter was then removed. In some experiments the resuspension solution was the final assay solution and contained BM + cycloheximide + $^{32}P_i$ in the same volume as the original germling sample. In most experiments the resuspension solution was twice final strength (BM + cycloheximide) at half the original sample volume. Subsamples (5 to 15 ml) were then removed and added to an equal volume of the appropriate $^{32}P_i$ solution at twice strength, to give correct final concentrations of germlings and medium. If germlings were left in twice strength (BM + cycloheximide) for longer than about 5 min, the subsequent phosphate uptake rate was slightly reduced, and so germlings were never left in this resuspension solution for longer than 3 min. Samples (1 ml) were removed at intervals and filtered by suction through glass-fiber filters (Whatman GF/A or GF/C). Each filter was washed four to five times with 10-ml volumes of 1 mM potassium phosphate buffer (pH 6.4) at room temperature (21 to 23°C) and then placed in a scintillation vial for radioactivity measurement.

Cycloheximide is known to inhibit growth (12) and prevent protein synthesis in *N. crassa* (11). This compound was included in assay media to prevent possible changes occurring in uptake characteristics due to the synthesis of new uptake activity during the course of the assay.

For inhibitor studies (see Table 1), germlings were suspended in BM + cycloheximide + $^{32}P_i$ for 10 min, and then inhibitor was added. The pH of the inhibitors was adjusted to keep the final medium pH at about 6.4. Nystatin (Mycostatin; E. R. Squibb & Sons, Princeton, N.J.) and 11-deoxycorticosterone (Sigma Chemical Co., St. Louis, Mo.) were dissolved in ethanol before addition. The final ethanol concentration was 1%, and by itself this did not affect phosphate uptake. For the 0°C treatment, germlings suspended in twice strength (BM + cycloheximide) were chilled on ice before addition of chilled, twice-strength $^{32}P_i$ solution.

Radiotracer. Radioactive sulfate and phosphate were obtained from the Radiochemical Centre, Amersham, England. We did not encounter the difficulties with using $^{32}P_i$ reported by Lowendorf et al. (19) in a similar study. One benefit of using $^{32}P_i$ was that a $^{32}P/^{35}S$ double-labeling technique could be used.

Measurement of radioactivity. Glass-fiber filter samples were prepared for counting by adding 1 ml of water plus 9 ml of scintillation fluid (based on the 2:1 toluene-Triton X-100 mixture of Patterson and Greene [23]). Aqueous samples were made to 1 ml with water, and scintillation fluid was added. Vials were capped, shaken thoroughly on a Vortex mixer, and left overnight. This prolonged soaking was necessary to obtain reproducible counting behavior of the samples. The vials were shaken again and counted by liquid scintillation spectrometry (Packard model 3320), using the channels ratio method for double-label counting (9). Quenched standards were prepared for each experiment, and

the data were quench-corrected using computer-generated curves. To maintain similar accuracy for all ^{35}S measurements, the ^{32}P overlap in any sample was not permitted to exceed 20% of the total ^{35}S counts. This was achieved by choosing appropriate specific activities for the $^{32}P_i$ solutions.

Use of ^{35}S labeling. Inclusion of $^{35}SO_4^{2-}$ in the agar medium when growing the fungus provided conidia that were radioactively labeled with ^{35}S. The ^{35}S content of the germlings in a given experiment (about 6×10^5 cpm/mg, dry weight) was then used to correct for slight differences in sample size. The ^{32}P counts in each sample were adjusted by the factor required to normalize the ^{35}S counts in that sample to the mean ^{35}S sample count for the experiment. In practice, corrections of up to about 10% were required.

Calculation of initial uptake rates. Sampling times were spaced such that at least five samples were taken during the linear period of uptake. In all instances, sampling was completed by 16 min. Uptake rates were calculated from lines fitted by least-squares analysis. Although it was not possible to determine the exact moment when germlings made effective contact with the $^{32}P_i$, because of the time taken for mixing, most calculated uptake lines passed through or close to the origin. In the case of 10 mM germlings assayed at high-phosphate concentrations, lines usually intercepted the ordinate above the origin (Fig. 1A). The concentration of phosphate in the uptake medium was taken as the concentration at the midpoint of the linear uptake period, thereby correcting for loss during this period. No correction was needed for phosphate solutions greater than 100 μM.

Uptake rates were first calculated on a per-milliliter basis. It was not practicable to measure germling dry weight in every experiment because of the large volumes of culture needed to obtain accurat' weights. Standard uptake rate values on a per-gram (dry weight) basis were measured directly for 10 mM germlings assayed at 1 mM phosphate (2.08 μmol/g [dry weight] per min) and for 50 μM germlings assayed at 50 μM phosphate (2.99 μmol/g [dry weight] per min). Similar assays were included in each experiment, and the ratio of the uptake rates of these assays (on a per-milliliter basis) to the standard uptake rate values was then used to convert the other measurements.

For comparative purposes we suggest that our uptake rate data may be approximately converted into other units as follows: to convert to uptake per milliliter (10^6 germlings), assume 10^6 2.5-h-old germlings weigh 38.6 μg; to convert to uptake per unit of cell water, assume the ratio of intracellular water to dry weight is 2.54 (31) or 2.3 (26); to convert to uptake per unit of surface area, assume the ratio of surface area to dry weight is 4.6 m^2/g; to convert to uptake per unit of fresh weight, assume the ratio of dry weight to fresh weight is 0.186.

Derivation of kinetic parameters. Phosphate uptake in *N. crassa* has been analyzed by assuming the simultaneous operation of two uptake systems each obeying Michaelis-Menten kinetics. Thus,

$$v = \frac{V_{max(LA)} \cdot s}{K_{m(LA)} + s} + \frac{V_{max(HA)} \cdot s}{K_{m(HA)} + s}$$

where v is uptake rate; s is phosphate concentration; $V_{max(LA)}$ and $V_{max(HA)}$ are the maximum uptake rates of the low-affinity and high-affinity systems, respectively; and $K_{m(LA)}$ and $K_{m(HA)}$ are the phosphate concentrations that give rise to half-maximum uptake rates for each system.

When uptake measurements were made over a wide phosphate concentration range, estimates of the kinetic parameters, $V_{max(LA)}$, $V_{max(HA)}$, $K_{m(LA)}$, and $K_{m(HA)}$, were derived by fitting the double-hyperbola equation to the data, using a computer-based method. Data were partitioned into two subsets, with each being repetitively solved for a single hyperbola, using the direct linear plot method (5, 6), after subtracting the calculated contribution of the hyperbola corresponding to the other subset. In all cases the first subset contained data from phosphate concentrations up to and including 10 μM. Measurements at higher concentrations comprised the second subset. Iteration of the fitting method was continued until successive estimates of each of the parameters differed by less than 0.1%. The merits of this method are discussed elsewhere (D. J. W. Burns and S. A. Tucker, Eur. J. Biochem., in press).

When uptake was followed over a restricted concentration range, the relationship between uptake rate and phosphate concentration was essentially linear on a Hofstee plot (10) and thus appeared to obey simple Michaelis-Menten kinetics. The two kinetic parameters of this "single" system were estimated by using a computer version of the direct linear plot method.

Parameters derived by single-hyperbola analysis are termed one-system estimates, whereas those derived from double-hyperbola analysis are termed two-system estimates.

RESULTS

Initial kinetic analysis of low- and high-affinity systems. In initial experiments we sought to find growth conditions, differing only in the external phosphate concentration, in which *N. crassa* was able to grow exponentially with either the low- or high-affinity system being primarily responsible for phosphate uptake. A 10 mM phosphate concentration was chosen as a maximum, since it would ensure virtual saturation of the constitutive low-affinity system [reported $K_{m(LA)}$ of 470 μM at pH 6.43; 19]. Similarly, a 50 μM phosphate concentration was chosen as a minimum since the activity of the low-affinity system presumably would be restricted (approximately 10% of $V_{max(LA)}$), whereas that of the high-affinity system would be saturated [reported $K_{m(HA)}$ of about 3 μM; 18]. Growth, measured as dry-weight increase, was exponential by 2.5 h and

remained identical for 50 μM and 10 mM germlings over the first 6 h (1).

When 10 mM germlings were examined at phosphate concentrations of 400 μM and above, uptake was linear with time (Fig. 1A). A Hofstee plot of the data gave a straight line (Fig. 1B), suggesting that uptake of phosphate from these solutions was due primarily to a single uptake system obeying Michaelis-Menten kinetics. The mean estimated one-system kinetic parameters of the single system were: K_m, 754 ± 98 μM; V_{max}, 3.59 ± 0.26 μmol/g (dry weight) per min (seven experiments). From the similarity in the respective K_m values, it was clear that this system corresponded to the low-affinity system previously described (19).

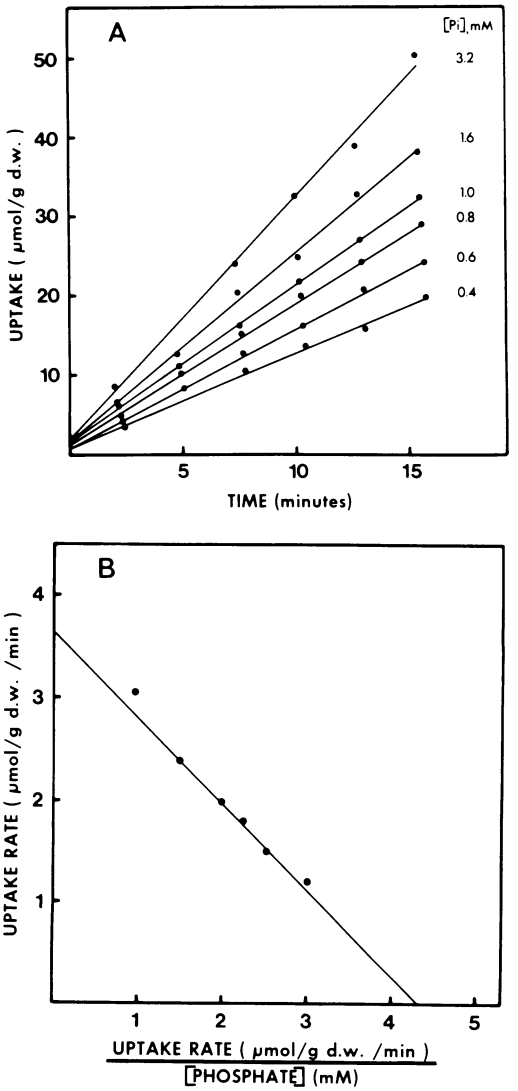

FIG. 1. *Phosphate uptake by 10 mM germlings over a narrow concentration range (400 μM to 3.2 mM). (A) Uptake with respect to time at different phosphate concentrations. (B) Hofstee plot of data. The kinetic parameters for a single hyperbolic function fitted to the data are: K_m, 843 μM; V_{max}, 3.64 μmol/g (dry weight [d.w.]) per min. This function is given by the solid line.*

Uptake by 50 μM germlings over the range 1 to 50 μM phosphate was also linear with time (Fig. 2A). The data obtained from 1 to 10 μM concentrations gave a linear Hofstee plot (Fig. 2B), indicating the predominant operation at dilute concentrations of a single uptake system obeying Michaelis-Menten kinetics. The mean estimated one-system kinetic parameters of this system were: K_m, 3.01 ± 0.29 μM; V_{max}, 2.59 ± 0.17 μmol/g (dry weight) per min (seven experiments). It was concluded that this system corresponded to the high-affinity system of Lowendorf et al. (18). The uptake rate at 50 μM phosphate was higher than that expected from the operation of the high-affinity system alone (see Fig. 2B) and suggested that there was a second uptake system contributing significantly to uptake at this phosphate concentration.

Metabolic properties of the uptake systems. Properties of the two systems were examined further by following uptake by germlings at a phosphate concentration at which uptake would be due largely to one of the two systems. Phosphate uptake by both systems was almost abolished by the respiratory inhibitors cyanide and azide and by lowering the temperature to 0°C (Table 1). Arsenate, which competes with phosphate in a number of biological systems including phosphate uptake in yeast (25), approximately halved the uptake rate when added in amounts equimolar to the phosphate present in the assay. Phosphate uptake was also affected by inhibitors of membrane function. The addition of nystatin, known to affect permeability of *N. crassa* cells (14), not only prevented further accumulation but also allowed leakage of accumulated radioactivity back into the medium. The steroid 11-deoxycorticosterone, which is suggested to be a general uptake inhibitor in *N. crassa* (17, 30), also prevented uptake and allowed leakage.

Efflux studies. Although in some uptake systems it is found that significant efflux of an ion occurs during uptake of that ion, we have not been able to demonstrate significant efflux of phosphate under our experimental conditions. In one experiment, 2.25-h 10 mM and 50 μM germlings were exposed, in the presence of cycloheximide, for 0.5 h to a $^{32}P_i$ solution at

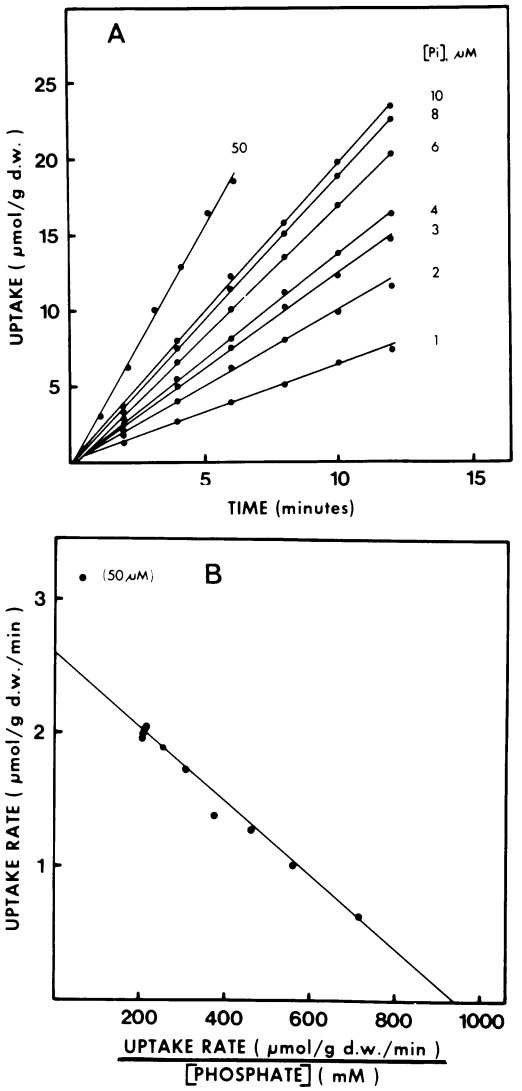

FIG. 2. *Phosphate uptake by 50 μM germlings over a narrow concentration range (1 to 50 μM). (A) Uptake with respect to time at different phosphate concentrations. (B) Hofstee plot of data, plus three further uptake rate determinations at 10 μM. The kinetic parameters for a single hyperbolic function fitted to the data (excluding the 50 μM value) are: K_m, 2.76 μM; V_{max}, 2.60 μmol/g (dry weight [d.w.]) per min. This function is given by the solid line.*

either 1 mM or 50 μM, respectively. They were then harvested and resuspended in medium lacking phosphate or medium containing nonradioactive phosphate at 50 μM or 1, 10, or 200 mM. The radioactivity present in the germlings 1.8 h after suspension was between 94 and 110% of the initial value. In another experiment, to avoid any effect of harvesting and resuspension, a "swamping" amount of nonradioactive phosphate was added during the course of radioactive phosphate uptake. Detectable uptake of $^{32}P_i$ ceased immediately, but no loss of accumulated radioactivity was observed subsequently (Fig. 3).

Detailed kinetic analysis of the uptake systems. The results of Lowendorf et al. (18, 19) and of some of our early kinetic experiments suggested that *N. crassa* germlings contained more than one uptake system. This was investigated further by following uptake over wide phosphate concentration ranges for each of the two types of germlings. These ranges (1 to 3,200 μM for 10 mM germlings, 1 to 1,600 μM for 50 μM germlings) were chosen, on the basis of preliminary experiments, to span the K_m regions of both systems in each case. Uptake was followed at 15 or 16 different concentrations within each range in three independent experiments. The data obtained for both types of germlings showed a curve when displayed on a Hofstee plot (Fig. 4 and 5). Using double hyperbola analysis, we derived two-system parameter estimates for the systems from these data. The curved lines shown in Fig. 4 and 5 were calculated by using the derived parameter values. These calculated curves pass close to the experimental data points, but it is difficult to judge the goodness of fit visually. A better assessment can be obtained from a direct comparison of the experimental data and the corresponding calculated values. Such a comparison showed that most pairs of values agreed to within 4%, with the worst agreement being

TABLE 1. *Effect of inhibitors and temperature on the phosphate uptake systems of N. crassa*

Treatment[a]	Uptake as % control[b]	
	10 mM germlings in 1 mM phosphate[c]	50 μM germlings in 50 μM phosphate[d]
NaN_3	3	3
NaCN	1	5
2,4-Dinitrophenol	5	0
Na_2HAsO_4	40	65
Nystatin	−11	−22
11-Deoxycorticosterone	−9	−8
0°C	7	3

[a] Inhibitors were present at 1 mM, except Na_2HAsO_4 (50 μM in 50 μM phosphate uptake treatment) and nystatin (7.5 μg/ml).

[b] Negative values indicate leakage of accumulated radioactivity. All uptake and leakage rates were essentially linear for 30 min.

[c] Low-affinity system contribution, 86% (Fig. 6).

[d] High-affinity system contribution, 75% (Fig. 6).

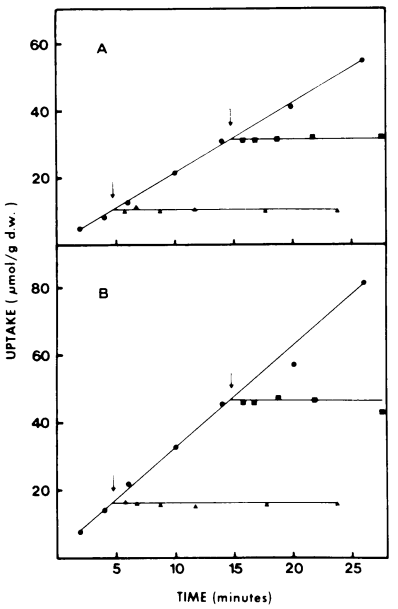

FIG. 3. *Lack of efflux of previously accumulated $^{32}P_i$ or other ^{32}P-labeled compounds in the presence of excess unlabeled phosphate. 10 mM germlings (A) or 50 μM germlings (B) were resuspended in BM + cycloheximide + $^{32}P_i$ at either 1 mM (A) or 20 μM (B). At times marked by arrows, 6 ml of the suspension was removed and added to 0.5 ml of 1 M nonradioactive phosphate.*

found for the 50 μM values (differences of 7.1 and 8.9% for Fig. 4 and 5, respectively). The average parameter estimates from the experiments (Table 2) show that $K_{m(HA)}$ is the only parameter not significantly different from its counterpart in the other type of germling.

From these parameter values the uptake rates of the germlings at the phosphate concentration of each growth medium can be calculated. The rate for 10 mM germlings is 3.37 μmol/g (dry weight) per min, and that for 50 μM germlings (at the 2.5-h phosphate concentration of 44 μM) is 2.88 μmol/g (dry weight) per min.

Relative contributions of the systems to uptake at different phosphate concentrations. Using the kinetic parameters of Table 2, we have calculated the relative contributions of each system to uptake over the concentration range 1 μM to 10 mM (Fig. 6). It can be seen that for 50 μM germlings the two systems contribute equally to uptake at 210 μM, whereas for 10 mM germlings equal contribution occurs at 88 μM.

DISCUSSION

Biochemical properties of the uptake systems. Preliminary kinetic assays indicated that phosphate uptake by 10 mM germlings during growth was due primarily to a low-affinity system, whereas that of 50 μM germlings was due primarily to a high-affinity system (Fig. 1 and 2). The different phosphate uptake behavior of the two types of germlings resulted from a physiological adaptation to the phosphate concentration in the medium rather than from changes in the growth rate, since dry matter increases were identical (1). The activity of both systems showed a similar dependence on continued metabolic energy production and on membrane integrity (Table 1). Lowendorf et al. (19) showed an energy dependence for the low-affinity system but did not report a similar study for the high-affinity system present in phosphate-starved cells (18). The energy requirement suggests that all phosphate uptake involves movement against an electrochemical gradient. This is supported by the observations that *N. crassa* hyphae are negatively charged with respect to the surrounding medium (29) and that inorganic phosphate levels in 2.5-h germlings are between 5 and 10 mM (unpublished observations).

We found no detectable efflux of phosphate or phosphorus compounds irrespective of which

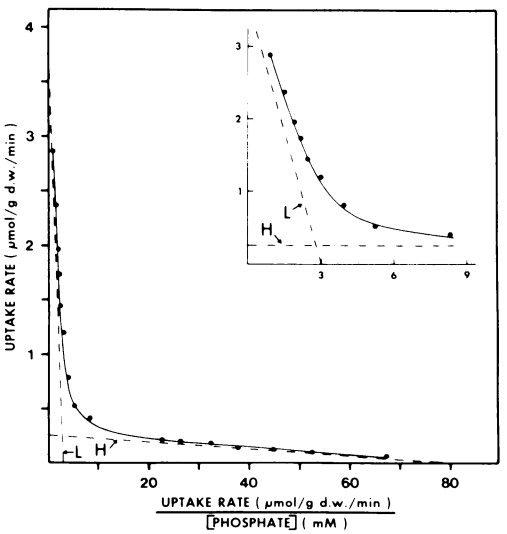

FIG. 4. *Hofstee plot for uptake data obtained over a wide phosphate concentration range (1 μM to 3.2 mM) using 10 mM germlings. The data were analyzed to give the kinetic parameters of the two component uptake systems. The high-affinity system (K_m, 3.19 μM; V_{max}, 0.26 μmol/g [dry weight, d.w.] per min) is shown by dotted line H, and the low-affinity system (K_m, 1,204 μM; V_{max}, 3.61 μmol/g [d.w.] per min) is shown by dotted line L. The solid line is the calculated sum of these two systems. The inset expands the portion of the plot close to the ordinate.*

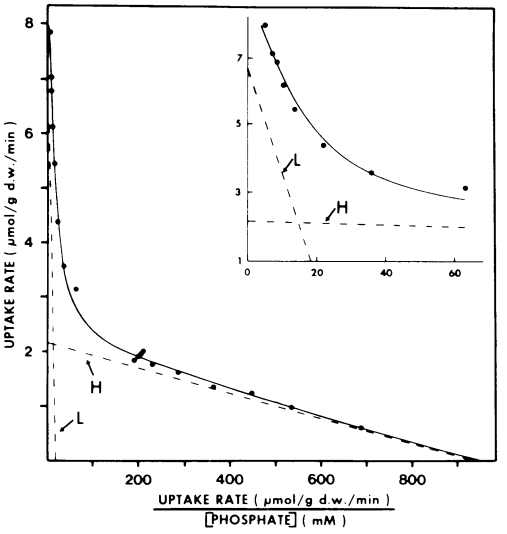

FIG. 5. *Hofstee plot for uptake data obtained over a wide phosphate concentration range (1 μM to 1.6 mM) using 50 μM germlings. The data were analyzed to give the kinetic parameters of the two component systems. The high-affinity system (K_m, 2.31 μM; V_{max}, 2.16 μmol/g [dry weight, d.w.] per min) is shown by dotted line H, and the low-affinity system (K_m, 366 μM; V_{max}, 6.66 μmol/g [d.w.] per min) is shown by dotted line L. The solid line is the calculated sum of these two systems. The inset expands the portion of the graph close to the ordinate.*

TABLE 2. *Comparison of two-system kinetic parameter estimates of the phosphate uptake systems of 10 mM and 50 μM germlings*[a]

System	K_m (μM)	V_{max} (μmol/g [dry wt] per min)
Low-affinity		
10 mM germlings	1,029 ± 153	3.41 ± 0.24
50 μM germlings	370 ± 25	6.33 ± 0.32
Difference[b]	S	S
High-affinity		
10 mM germlings	2.85 ± 0.31	0.28 ± 0.05
50 μM germlings	2.43 ± 0.10	2.33 ± 0.15
Difference[b]	NS	S

[a] Analyzed assuming that the two systems contribute significantly to uptake over the entire phosphate concentration range. Values are based on three independent experiments.

[b] Significant (S) or nonsignificant (NS) at the 1% level, using the *t* test.

system was mainly responsible for uptake (Fig. 3). In contrast, Lowendorf et al. (19) found efflux of both phosphate (up to 20 to 30% of the uptake rate) and other phosphorus compounds during uptake. We suggest that the efflux observed by these workers may be an artifact resulting from some abnormal alteration to the plasma membrane. This could result from the

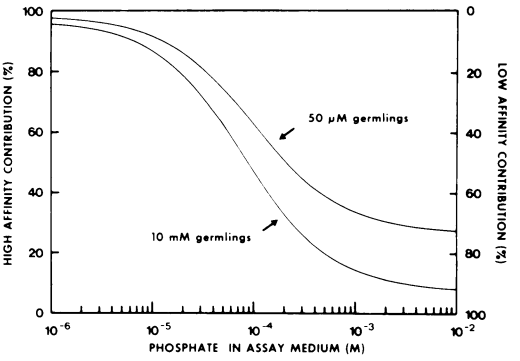

FIG. 6. *Diagram showing the relative contributions of the high- and low-affinity systems in 10 mM and 50 μM germlings to uptake over a wide phosphate concentration range. Curves were calculated with the parameter values listed in Table 2.*

assay system they used, as this involved resuspending mycelium for 25 min in a nongrowth buffer before adding radioactive phosphate. The occurrence of efflux complicates the derivation of kinetic parameters for influx (21) and may account, in part, for the differences between our parameter estimates and theirs (see below).

Kinetic interpretation over a wide concentration range. The markedly curved Hofstee plots for both 10 mM and 50 μM germlings, when uptake was measured over a wide concentration range (Fig. 4 and 5), are not consistent with the operation of a single Michaelis-Menten uptake system, but can be adequately explained by two uptake systems operating simultaneously across the plasma membrane. In our analysis of the uptake data, we allowed for the possibility that each kinetic parameter of the uptake systems might vary according to the physiological state of the fungus. For 50 μM germlings the values of $V_{max(LA)}$ and $V_{max(HA)}$ were increased about 1.9 and 8.3 times, respectively, over the corresponding values in 10 mM germlings (Table 2). The relative increase in uptake activity of each system caused by these changes would, in the absence of K_m changes, apply equally to all phosphate concentrations. However, whereas no difference was found between the $K_{m(HA)}$ values of the two sets of germlings, the $K_{m(LA)}$ value of 50 μM germlings was only 0.36 times that of 10 mM germlings. The effect of this change is to make the low-affinity system in the 50 μM germlings relatively more efficient at taking up phosphate from dilute solutions.

Despite the different experimental conditions, some comparisons can be made with the results of Lowendorf and his colleagues (18–20). Although these workers analyzed their

data in terms of two uptake systems, they assumed that the K_m values of the two uptake systems would be unaltered in fungus of different physiological status. This assumption meant that a reduction in $K_{m(LA)}$, such as we have found to be associated with a lowered external phosphate concentration, could not have been detected. Our $K_{m(LA)}$ of 1,029 μM at pH 6.4 for 10 mM germlings is similar to their $K_{m(LA)}$ value of 470 μM at the same pH obtained for mycelium grown at 37 mM phosphate. These workers reported that $K_{m(LA)}$ values were markedly influenced by assay pH. Slight differences in pH can be eliminated as a possible cause of the differences in $K_{m(LA)}$ that we have found, because our uptake assays for the two germling types were carried out in identical solutions prepared at the same time. Our $K_{m(HA)}$ values of 2.43 to 2.85 μM at pH 6.4 are similar to the values of 2.2 to 2.6 μM at pH 5.8 found by Lowendorf et al. (18, 20), who also reported that $K_{m(HA)}$ values were relatively unaffected by pH. These workers found that the major change after a period of phosphate starvation was a large increase in $V_{max(HA)}$, a result consistent with our finding that V_{max} values are higher in 50 μM germlings than in 10 mM germlings.

In the present work we have used the widely adopted dual-system interpretation to explain the nonconformity of the data with simple Michaelis-Menten kinetics. However, complex kinetics of a similar nature have also been explained on the basis of a single uptake system with properties of negative cooperativity (7, 22). Criteria for distinguishing between these two interpretations have been discussed by Glover et al. (7). The strongest criterion is the existence of mutants that lack one of the two systems. In the case of *N. crassa*, the *nuc-1* and *nuc-2* mutants have been shown to give phosphate uptake kinetics consistent with the operation of a single low-affinity system (16, 20). Furthermore, metabolic perturbations may reveal two systems through preferential effects on one of the components. In *N. crassa*, phosphorus limitation has differential effects on the kinetic parameters of the two systems. Also, the effect of pH on the systems is markedly different (see above). For these reasons, and because of its simplicity, we have chosen the dual-system approach but recognize that models based on negative cooperativity are not unequivocally excluded.

Relevance to uptake studies in general. Some of the results of the present study have general significance in uptake studies. When uptake is followed over a relatively narrow concentration range, data may be obtained that appear to obey simple Michaelis-Menten kinetics (e.g., Fig. 1 and 2). By assuming that only one system is operating, the kinetic parameters can be estimated, but it is important to realize that such one-system estimates are only of qualitative significance until it is shown that these kinetics are adhered to over a wide concentration range, or that the influence of other uptake systems is virtually negligible. For example, in 10 mM germlings the contribution of the high-affinity system to uptake in the $K_{m(LA)}$ region is very small, and there is no significant difference between one- and two-system estimates for the two uptake systems (data not given), but this clearly is not the case for 50 μM germlings (Table 3). Thus, in situations where two uptake systems are present, parameter estimates must be determined by double-hyperbola analysis.

TABLE 3. *Comparison of one- and two-system kinetic parameter estimates of the phosphate uptake systems of 50 μM germlings*[a]

System	K_m (μM)	V_{max} (μmol/g [dry wt] per min)
Low-affinity		
One-system	276 ± 14	8.98 ± 0.19
Two-system	370 ± 25	6.33 ± 0.32
Difference[b]	S	S
High-affinity		
One-system	2.77 ± 0.08	2.55 ± 0.19
Two-system	2.43 ± 0.10	2.33 ± 0.15
Difference[b]	S	NS

[a] The same data from three experiments were analyzed by one- and two-system analysis. For two-system analysis it was assumed that both systems contributed significantly to uptake over the entire concentration range. For one-system analysis the contribution of the high-affinity system to uptake at high concentrations, and that of the low-affinity system at low concentrations, was ignored. Thus, the high-affinity system analysis was based on measurements from 1 to 10 μM, and the low-affinity system analysis was based on measurements above 400 μM.

[b] Significant (S) or nonsignificant (NS) at the 1% level, using the *t* test.

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