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EFFECT OF THE MEDIUM pH AND THE CELL pH UPON THE KINETICAL PARAMETERS OF PHOSPHATE UPTAKE BY YEAST

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Summary

1. Both the maximum rate of phosphate uptake and the K_m depend upon the pH of the medium in a complex way.

2. The effect of medium pH upon the maximum rate of uptake is mainly indirect and is correlated with changes in cell pH.

3. The K_m is affected by the medium pH both directly via an apparent competitive inhibition by hydroxyl anions and indirectly in a similar way as the maximum rate of uptake.

Introduction

According to Cockburn et al. [1] uptake of monovalent orthophosphate by yeast cells is accompanied by the influx of three protons and the efflux of two K^+. The influx of protons may be also apparent, being in fact an efflux of cellular OH^-. When a cotransport of monovalent phosphate and protons is involved one would expect that the uptake rate of orthophosphate depends upon the pH of the medium in such a way that this dependence reflects the simultaneous cotransport of three protons. One would expect that the kinetical parameters of phosphate uptake (the K_m and/or the maximum rate of uptake) depend upon the proton concentration of the medium. In case of a cotransport of three protons a third power relation between the kinetical parameters and the proton concentration may come to the fore [2]. A kinetical study of phosphate uptake may therefore give additional support to the hypothesis of Cockburn et al. [1]. Goodman and Rothstein [3] studied the effect of the medium pH upon the rate of phosphate uptake and showed that one of the main factors determining the pH dependence of phosphate uptake is, that only the monovalent form of phosphate is translocated through the yeast cell membrane. Knotková and Kotyk [4] found two maxima for the pH dependence of phosphate uptake.

These findings, however, refer to the amount of phosphate accumulated during an incubation period of about 1 h. As will be shown in this study, however, the cell pH decreases during phosphate uptake, by which the rate of uptake is affected seriously [5]. To avoid such indirect effects we have determined initial rates of phosphate uptake obtained from uptake experiments lasting less than 1 min.

Methods

The yeast, *Saccharomyces cerevisiae*, Delft 2, is aerated for one night at pH 4.5 to exhaust the internal substrate of the yeast. The cells are then washed by centrifuging and resuspending twice in citrate or succinate buffer of the desired pH. The buffers applied consist of a solution of either 100 mM citrate acid adjusted to the desired pH with Tris and 10 mM KCl or 100 mM Tris succinate without added KCl. After resuspending the cells 3% (w/v) glucose or 1% (v/v) ethanol is added. Nitrogen, or, in case of ethanol as a substrate, air is bubbled through the cell suspension for exactly 1 h at 25°C at a yeast concentration of 1% (w/v). By this procedure the rate of orthophosphate uptake is increased considerably [6].

Labelled phosphate ([^{32}P]phosphoric acid from Philips Duphar, The Netherlands, diluted with appropriate amounts of nonradioactive KH_2PO_4, when KCl was added, or Tris phosphate is added after the 1-h preincubation period. The yeast concentration is decreased to 0.9% (w/v) by this addition. Phosphate uptake is determined according to Borst-Pauwels and Jager [7]. Cell pH values are determined according to Borst-Pauwels and Dobbelman [8].

Results

We have determined initial rates of phosphate uptake after preincubating the cells at the desired pH of the medium. Phosphate uptake is followed for 40 s. During this time period the rate of uptake is almost constant except at the lower phosphate concentrations. When incubating the cells for a longer period the rates of uptake at relatively high phosphate concentrations increase considerably at pH_o (medium pH) values higher than 6.5. This increase is due to a decrease in pH_i (cell pH) as will be shown below.

Fig. 1 shows an Eadie plot of the initial rate of phosphate uptake found with the citrate buffer under anaerobic conditions. When the translocation process involved is described by Michaelis-Menten kinetics a straight line will be found on plotting the rate of uptake against the quotient of this rate and the phosphate concentration. Apparently phosphate uptake at $pH_o < 6.6$ is described by the Michaelis-Menten equation. At higher pH deviations from linearity occur. According to Hofstee [9] such deviations may point to the operation of two simultaneously occurring translocation processes each being described by Michaelis-Menten kinetics but with different K_m values. It was found earlier by us that at high pH_o a secondary phosphate translocation process comes to the fore, a process which is stimulated specifically by Na^+ [10]. In the medium used by us the Na^+ concentration amounts to 0.05 mM. This Na^+ is partly due to contamination of the glucose added and partly to leakage of Na^+ from the

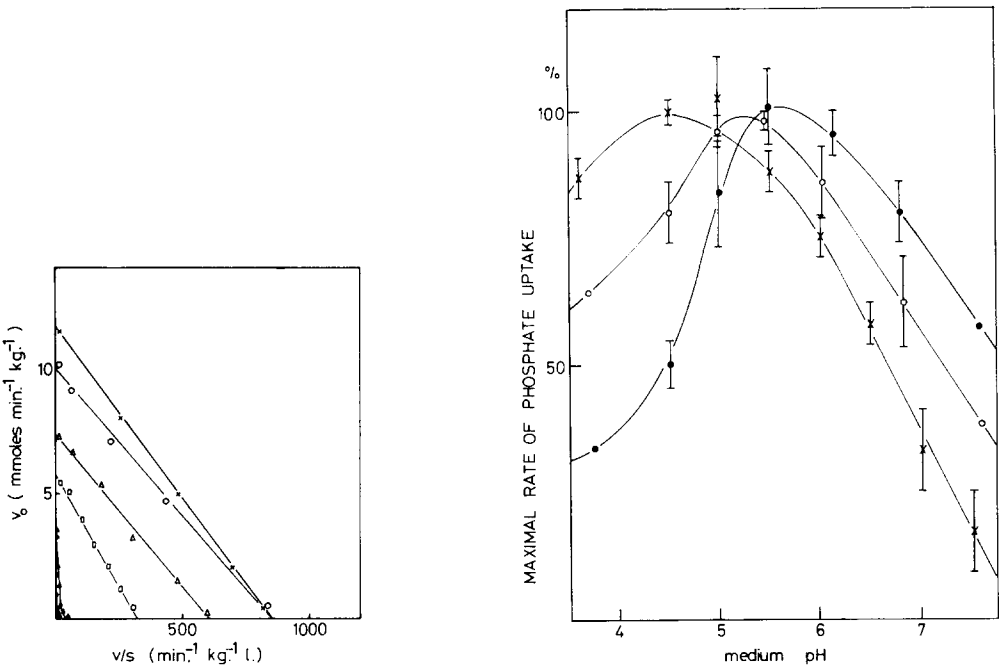


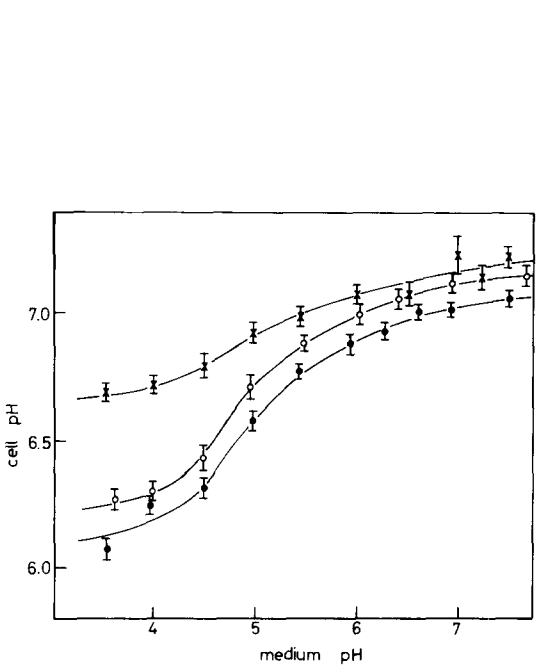
Fig. 1. Eadie plot of the initial rates of phosphate uptake (v_o) determined at varying medium pH values. The medium consists of 100 mM citrate (Tris) with 10 mM KCl and 3% (w/v) glucose under anaerobic conditions. X, pH 4.5; ○, pH 5.6; △, pH 6.1; □, pH 6.6, ▲, pH 7.2; ●, pH 7.5.

Fig. 2. Dependence of the maximum rate of phosphate uptake pH_o for various media (applied). The length of the bars denote standard errors of the mean X, 100 mM citrate (Tris) with 10 mM KCl, 3% (w/v) glucose, N_2; ○, 100 mM Tris succinate, without KCl, 3% (w/v) glucose, N_2; ●, 100 mM Tris succinate, without KCl, 1% (w/v) ethanol, air. The maximum rates of uptake are expressed as percents of the value found at the optimal pH concerned. The kinetical data refer to the Na^+ independent process. This applies also to Figs. 4–7.

yeast. This concentration is sufficiently high to account for the deviation from linearity observed in the Eadie plot at high medium pH (Blasco, F., Roomans, G.M. and Borst-Pauwels, G.W.F.H., ref. 16). The Na^+ dependent phosphate uptake process has a K_m of about 10^{-6} M with respect to the monovalent form of phosphate, whereas the K_m of the Na^+ independent phosphate translocation process is much higher.

We have calculated the values of the maximum rate of phosphate uptake and also the K_m values for the Na^+ independent transport process by correcting for the contribution of the Na^+ dependent process according to Cleland [11]. Fig. 2 shows that the maximum rate of uptake depends upon pH_o and that this dependence differs for the various media applied. The optimum pH_o is much lower for the citrate buffer with 10 mM KCl than for the Tris succinate buffer without added KCl. With ethanol as a substrate instead of glucose and applying the latter buffer a further shift to higher pH_o values is observed for the optimum pH_o.

We have now examined whether the differences in pH response observed with the various media applied may be traced to differences in pH_i. As is shown in Fig. 3 the dependence of pH_i upon pH_o differs greatly for the various media



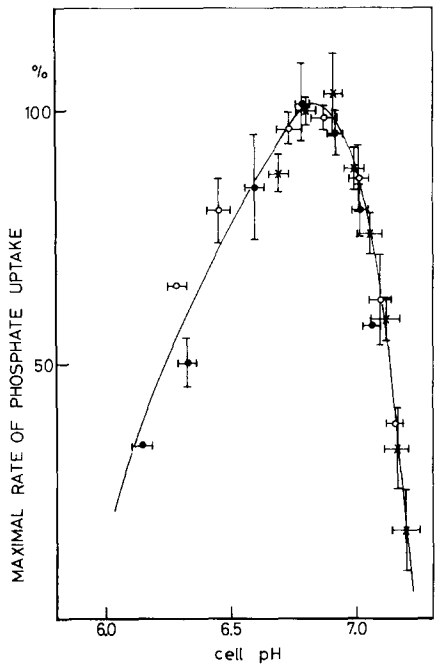


Fig. 3. Dependence of pH_i upon pH_o for the media applied in the experiments of Fig. 2. For the meaning of the symbols see also Fig. 2.

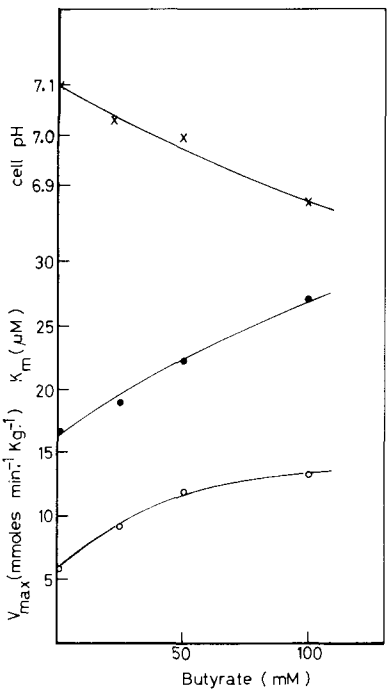
Fig. 4. Dependence of the maximum rate of phosphate uptake upon pH_i varied by pH_o. Same data as shown in Fig. 2.

applied. pH_i at a given pH_o is larger for the citrate buffer plus KCl than for the succinate buffer without added KCl, whereas under aerobic conditions with ethanol as substrate the cells have a still lower pH.

Fig. 4 shows that a single relation between the maximum rate of phosphate uptake and pH_i exists. The experimental points found with various media all lie approximately on a single curve. This indicates that pH_i rather than pH_o affects the rate of phosphate uptake.

The assumption that pH_i is probably the main factor determining the maximum rate of phosphate uptake is supported by experiments in which we varied the cell pH at a fixed medium pH by preincubating the cells with either butyrate or inactive orthophosphate. These preincubations lead to an acidification of the cells as is seen in Fig. 5 for the effect of butyrate upon phosphate uptake. Both the maximal rate and the K_m increase on decreasing the cell pH. This is also found at pH_o 6.72, 7.23 or 7.48. At a fixed pH_o 4.5 an increase in the maximum rate of phosphate uptake and in the K_m is found on decreasing pH_i to 6.8, whereas a further decrease in pH_i leads to a subsequent decrease in both parameters as has been shown earlier [5,12].

Fig. 6 shows that the experimental points found on varying pH_i at various fixed values of pH_o lie approximately on the same curve which has been found on varying pH_i by varying only pH_o. Only at the higher medium pH values applied is the maximum rate found after acidifying the cells somewhat



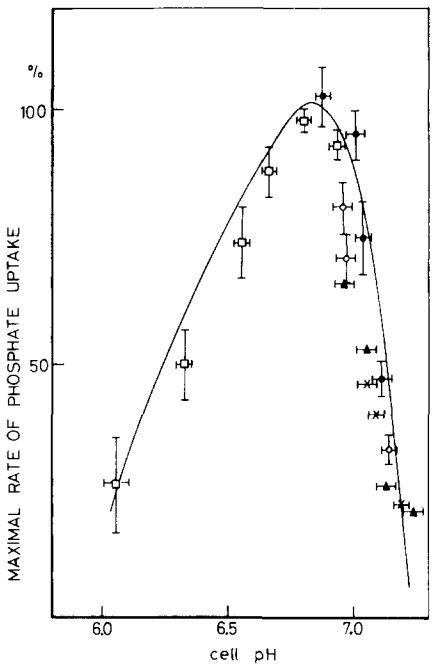


Fig. 5. Comparison of the effects of butyrate upon the K_m of phosphate uptake, the maximum rate of uptake and upon pH_i. Buffer: 100 mM citrate (Tris) and 10 mM KCl, 3% (w/v) glucose, N_2. The medium pH is 6.33. ○, maximal rate of uptake (V); ●, K_m; X, cell pH.

Fig. 6. Dependence of the maximum rate of phosphate uptake upon pH_i varied by preincubating the cells with butyrate or phosphate. pH_i is varied at various fixed values of pH_o by preincubating the cells for 6 min with varying amounts of Na^+ or Tris butyrate (25—100 mM) or inactive K^+ or Tris orthophosphate (0.8—3.0 mM) at the pH_o values indicated. Buffer: 100 mM citrate (Tris) and 10 mM KCl, 3% (w/v) glucose, N_2. □, pH 4.5; ●, pH 6.33; ○, pH 6.72; X, pH 7.23; ▲, pH 7.48. The line drawn is that of Fig. 4.

lower than expected according to the curve taken from Fig. 4.

The K_m of the Na^+ independent phosphate translocation process varies also with pH_o, see Fig. 7. Up to pH 5.5 the K_m decreases with increasing pH_o parallel with the decrease in the maximum rate of phosphate uptake. This is in accordance with the results discussed above and shown in Fig. 5, that is on varying pH_i both the maximum rate and the K_m either increase or decrease. This means that a correlation exists between both parameters supporting our earlier observation made at pH 4.5 [7], that the K_m is linearly related to the maximum rate of uptake according to eqn. 1.

$$K_m = K_{m,o} + aV \tag{1}$$

Above pH_o 5.5 the K_m increases, whereas the maximum rate of uptake still decreases on increasing pH_o. This increase in K_m is apparently due to a direct effect of pH_o upon this kinetical parameter. The direct effect of pH_o upon the K_m can be accounted for by replacing a in Eqn. 2 by a coefficient depending upon the medium OH^- concentration:

$$K_m = K_{m,o} + a(1 + OH/K)V \tag{2}$$

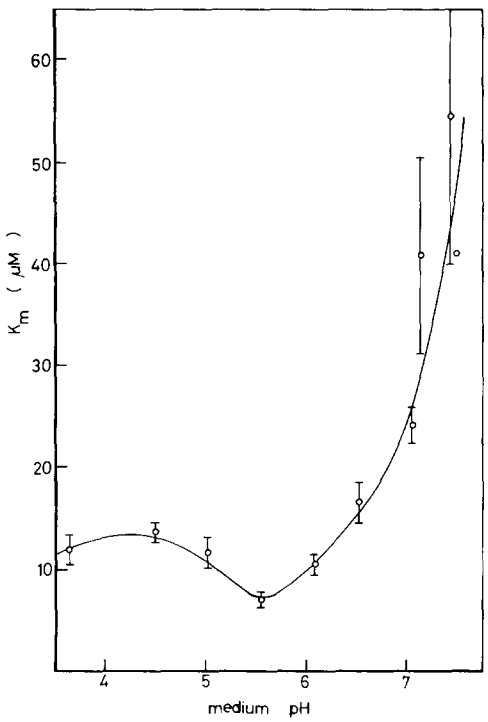


Fig. 7. Dependence of the K_m of monovalent phosphate uptake upon pH_o. Buffer: 100 mM citrate (Tris) and 10 mM KCl, 3% (w/v) glucose, N_2. The length of the bars denote the standard error of the mean.

TABLE I

KINETICAL PARAMETERS OF PHOSPHATE UPTAKE (Na^+ INDEPENDENT PROCESS)

The kinetical parameters expressed in moles per liter have been computed by means of a curve-fitting program fitting Eqn. 2. For the meaning of $K' = K_w/K$ we refer to the discussion. K_w is the dissociation constant of water. The dimensions of the parameters apply when V is expressed in mmol · min^{-1} · kg^{-1} dry weight, K_m in M and OH also in M.

	Value	Standard deviation
$K_{m,o}$	$1.40 \cdot 10^{-6}$ M	$2.26 \cdot 10^{-6}$
a	$0.69 \cdot 10^{-6}$ M kg min · $mmol^{-1}$	$0.21 \cdot 10^{-6}$
K	$1.21 \cdot 10^{-8}$ M	$0.40 \cdot 10^{-8}$
K'	$0.83 \cdot 10^{-6}$ M	$0.27 \cdot 10^{-6}$

In this equation K is a constant, and OH represents the concentration of the hydroxyl anions in the medium. The latter may be replaced by K_w/H expressing that a proton binding site is involved determining the value of the K_m. The constant K_w is the dissociation constant of water, whereas H means the concentration of protons in the medium. The values of $K_{m,o}$, a and K computed by means of a curve fitting program are tabulated in Table I.

Discussion

Apparently the initial rate of phosphate uptake is affected in a complex way by pH_o. It is shown that the effect of pH_o upon the maximum rate of Na^+ inde-

pendent phosphate uptake is mainly indirect and can be ascribed for the greater part to concomitant changes in pH_i. In this respect phosphate uptake resembles monovalent cation uptake by yeast cells. It has been shown by Ryan and Ryan [14] that the dependence of the rate of K^+ uptake upon pH_o is different for various media just as is found for phosphate uptake in this study. A single relation appeared to exist between the rate of K^+ uptake and pH_i showing that pH_i rather than pH_o determines the rate of K^+ uptake. It is not possible to make a direct comparison between the pH dependence of phosphate uptake and the pH dependence of K^+ uptake found by Ryan et al., because different methods for the determination of pH_i have been used. Using the method of this study, the pH optimum for monovalent cation by yeast appeared to be < 6.0 (Theuvenet, A.P.R., Roomans, G.M. and Borst-Pauwels, G.W.F.H., in preparation), whereas that for phosphate uptake is 6.8. This makes it improbable that a common pH sensitive factor is involved in the two translocation processes.

Also the K_m of Na^+ independent phosphate uptake depends upon pH_o. This dependence is even more complex than that found for the maximum rate of uptake. The K_m is affected in a similar way as the maximum rate of uptake by changes in pH_i. Keeping pH_o constant a linear relationship is found between the K_m and the maximal rate of phosphate uptake on varying pH_i, see Eqn. 2. It has been shown elsewhere [2] in a theoretical study of the kinetics of carrier mediated transport, that when the carrier is able to move freely across the cell membrane, the kinetical parameters of the translocation process are not constant any more, but depend upon the concentrations of those intracellular solutes which show affinity to the carrier involved. In addition the K_m will be linearly related to the maximum rate of uptake in such a case. Therefore one might hypothesize that phosphate uptake by yeast is a carrier mediated process, and that either intracellular protons or intracellular hydroxyl anions have affinity to the carrier.

The K_m of phosphate uptake is, when keeping pH_i constant see Eqn. 2, linearly related to the concentration of OH^- anions in the medium. As has been pointed out by Lowendorf et al. [14], such a linear relationship will be found when hydroxyl anions compete with orthophosphate for the phosphate binding sites and when protons bind to a modifier site affecting this affinity of phosphate to the phosphate binding sites. This shows that the condition, that either protons or hydroxyl anions have affinity to the phosphate carrier, is fulfilled. The dissociation constant of the proton modifier site complex would then be given by K', see Table I. Also phosphate uptake by *Neurospora crassa* is inhibited in an apparent competitive way by hydroxyl anions (or activated by protons) [14]. The apparent competitive inhibition constant for the hydroxyl anions is $3.75 \cdot 10^{-10}$ M far lower than the corresponding value calculated for yeast, namely $1.21 \cdot 10^{-8}$ M.

Our kinetical study does not give any indication that more than one proton or hydroxyl anion binding site on the phosphate carrier is involved. Therefore, from a kinetical point of view the finding of Cockburn et al. [1] that phosphate uptake by yeast cells is accompanied by a symport of three protons is not further supported. Of course it may be possible that two of the three protons are bound firmly to the phosphate transport system. It may also be possible, however, that an antiport of cellular hydroxyl anions is involved instead of

a cotransport of phosphate with protons, see also ref. 15. At this stage of progress in the elucidation of phosphate uptake mechanism we cannot distinguish between the two possibilities. It may be considered that only an apparent influx of protons with a stoichiometry higher than unity may be found with cells of which the metabolism is impaired, these being the type of cells used by Cockburn et al., and not with metabolizing cells as used in our study. Orienting experiments, however, indicate that at least 1.5 proton is cotransported with phosphate by our cells.

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