*Biochimica et Biophysica Acta*, 413 (1975) 95–103


BBA 77106

# GENETIC REGULATION OF PHOSPHATE TRANSPORT SYSTEM II IN *NEUROSPORA*

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(Received June 9th, 1975)

## SUMMARY

Phosphate transport system II, previously shown to be responsible for high-affinity phosphate uptake under conditions of phosphorus starvation, is regulated by at least three genes: *pcon-nuc*-2, *preg*, and *nuc*-1. *nuc*-1 and *nuc*-2 mutants cannot be derepressed for phosphate transport system II, while *pcon*$^c$ and *preg*$^c$ mutants are partially constitutive.

## INTRODUCTION

Previous work from this laboratory has shown that *Neurospora crassa* possesses two transport systems for inorganic phosphate [1, 2]. The first (system I) is formed constitutively, and is the dominant system in cells grown in the usual minimal medium; it has a $J_{max}$ of about 1.5 mmol/l cell water per min and a $K_{\frac{1}{2}}$ that increases from 12 $\mu$M to 3.6 mM as the extracellular pH is raised from 4.0 to 7.3. During phosphorus starvation, a second transport system (system II) is derepressed; it is an active, high-affinity system with a $J_{max}$ of about 5.2 mmol/l cell water per min and a $K_{\frac{1}{2}}$ of about 3 $\mu$M (independent of the extracellular pH).

It is reasonable to suppose that the synthesis of system II is under the control of one or more regulatory genes, and in fact evidence exists for parallel regulation of this system along with a whole family of enzymes involved in phosphorus metabolism. Lehman et al. [3] have reported that mutants, altered in the ability to synthesize alkaline phosphatase, show similar changes in high-affinity phosphate transport (defined as the ratio of phosphate uptake at two extracellular concentrations, 0.1 and 2 mM, at pH 7.5) Two mutants, *nuc*-1, mapping on linkage group I, and *nuc*-2, on linkage group II [4], do not form alkaline phosphatase and transport phosphate poorly at 0.1 mM, even under conditions of phosphorus limitation. By contrast, two other kinds of mutants, *pcon*$^c$, which is extremely closely linked to *nuc*-2, and *preg*$^c$, which maps about 1.5 centimorgans away, are constitutive, forming alkaline phosphatase and transporting phosphate well at 0.1 mM even when grown on high-phosphate medium.

In the present paper, we have verified that the high-affinity phosphate transport

measured by Lehman et al. [3] is mediated by system II In addition, we have made a quantitative determination of the activities of systems I and II under normal and phosphorus-limited conditions in *nuc*-1, *nuc*-2, *pcon*$^{c}$ and *preg*$^{c}$ mutants

## METHODS

*Strains.* Mutant strains *nuc*-1 (T28-M1, from FGSC No. 1994), *nuc*-2 (T28-M2; from FGSC No 1998), *pcon*$^{c-6}$, (T)*pcon*$^{c-2}$ and *preg*$^{c-2}$ were obtained from Dr R. Metzenberg. (T)*pcon*$^{c-2}$ was isolated in strain T(II → I)NM177, in which a portion of linkage group II containing *pcon* has been translocated to linkage group I, just to the left of the mating type locus. Wild-type strain 74-OR8-1a (FGSC No 988) was also obtained from Dr R Metzenberg, and was the wild type used in all experiments unless otherwise mentioned In a few instances, for comparison with previous work, a second wild-type strain (RL21a) was also employed

*Flux measurements* Two types of cells were routinely used in flux experiments "normal" (high-P) cells, prepared by inoculating conidia ($10^6$/ml) into Vogel's minimal medium plus 2 % sucrose and incubating on a reciprocating shaker (120 strokes/min) at 25 °C for 15 5 h; and P-starved cells, prepared by growth in the same medium for 14 h followed by transfer to P-free minimal medium plus 2 % sucrose plus 20 mM 3,3-dimethylglutarate buffer, pH 5.8 [1], for an additional 3.5 h For flux measurements, cells were harvested by filtration, washed with distilled water, resuspended in P-free minimal medium (no sucrose) plus 20 mM dimethylglutarate buffer, pH 5.8, and maintained in a 25 °C water-bath with constant shaking. After preincubation for 10–45 min, $^{33}PO_4^{3-}$ (at the desired concentration, from 0.006 to 10 mM) was added, and cell samples were collected, extracted and counted as described previously [1]. In all cases, uptake was linear for at least 5 min, so that initial rates could be obtained directly from the early time points Kinetic parameters were determined for systems I and II by computer fit of the data to the sum of two Michaelis curves [1, 2]

## RESULTS

*Initial characterization of the mutants*

As previously reported by Lehman et al [3], *nuc*-1 and *nuc*-2 strains are easily distinguished from *pcon*$^{c}$ and from wild-type *Neurospora* by their inability to grow in stationary cultures on low concentrations of inorganic phosphate at high pH. Fig. 1 illustrates that the same distinction can be made under the conditions of our experiments (Vogel's minimal medium instead of Fries, dimethylglutarate buffer instead of morpholinopropane sulfonate buffer). At pH 6.9, both the wild type and *pcon*$^{c-6}$ grew significantly at phosphate concentrations as low as 0 05–0 1 mM, while *nuc*-1 required 1 mM phosphate and *nuc*-2, 1–3 mM phosphate.

At lower pH values, the differences in growth rate among the strains are less striking. Even at pH 5.8, however, a routine comparison of the ability of the various strains to grow in shaking cultures in the standard Vogel's minimal medium (37 mM phosphate) plus 2 % sucrose revealed an interesting difference (Table I): although all of the strains grew with approximately the same doubling time (2 53–2 99 h), *nuc*-1 and *nuc*-2 maintained substantially lower concentrations of total cell phosphorus

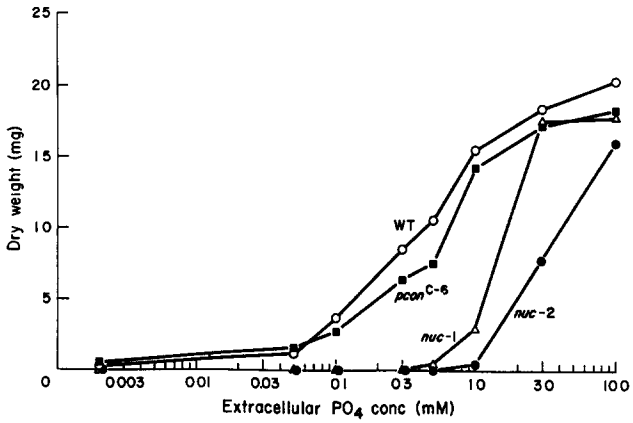


Fig 1 Growth as a function of the extracellular phosphate concentration at pH 6 9. Conidia were inoculated at $10^4$/ml into 5 ml of P-free minimal plus 2 % sucrose plus 100 mM dimethylglutarate buffer, pH 6.9, plus 0–100 mM phosphate, and incubated at 25 °C for 7 days The final pH was 6 3–6 9, depending on the amount of growth Each point is the average of two determinations.

TABLE I

GROWTH RATES AND CELL PHOSPHORUS IN VOGEL'S MEDIUM

Conidia were inoculated at $10^6$/ml into 1750 ml of Vogel's medium plus 2 % sucrose in a 3-l Florence flask, and incubated with vigorous aeration at 25 °C. 100-ml aliquots were harvested at intervals, washed, dried, weighed, and assayed for cell phosphorus [1] Each value for cell phosphorus is the mean (±S E.) of five determinations

| Strain | Doubling time (h) | Cell phosphorus (mmol/l cell water) |
|---|---|---|
| RL21a (wild type) | 2.75 | 293±3 |
| 74-OR8-1a (wild type) | 2 99 | 283±5 |
| *nuc*-1 | 2 67 | 215±19 |
| *nuc*-2 | 2 53 | 234±6 |
| *pcon*$^{c-6}$ | 2 70 | 342±8 |

(215 and 234 mmol/l cell water), and *pcon*$^{c-6}$, a substantially higher concentration (342 mmol/l cell water), than did wild-type *Neurospora* (283 mmol/l cell water for 74-OR8-1a).

*Derepression of phosphate transport*

Most of the growth properties mentioned so far can be accounted for by assuming that *nuc*-1 and *nuc*-2 are unable to derepress transport system II for inorganic phosphate, and that *pcon*$^{c-6}$ is constitutive for this system [3]. At high extracellular pH, where system I has an extremely high $K_{\frac{1}{2}}$ [1], mutants lacking the ability to form system II should be severely restricted in growth on low-phosphate medium. At low pH, on the other hand, system I should be able to support rapid growth in all of the strains, although the additional presence of system II in constitutive mutants (such as *pcon*$^{c-6}$) might lead to an elevated steady-state level of cell phosphorus. (The depression of cell phosphorus in *nuc*-1 and *nuc*-2 is harder to account for in terms of the

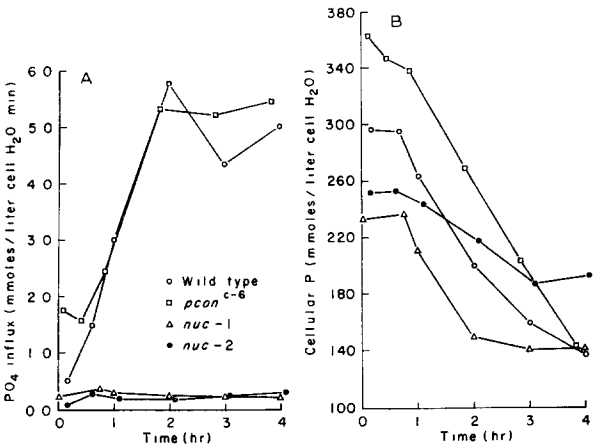


Fig 2 Time course of derepression of phosphate transport during phosphorus starvation. Cells were grown for 14 h in Vogel's minimal plus 2 % sucrose, washed, and resuspended in P-free minimal plus 2 % sucrose plus 20 mM dimethylglutarate buffer, pH 5 8 At intervals, aliquots of cells were tested with 50 $\mu$M $^{33}PO_4^{3-}$ to determine the initial rate of uptake. Samples were also taken for the assay of total cell phosphorus [1]

simple transport hypothesis just outlined, and will be discussed below )

In order to test this hypothesis directly, changes in phosphate transport were followed in the various strains during incubation under derepressing conditions. Previous work had shown that wild-type strain RL21a, grown in Vogel's minimal plus 2 % sucrose and transferred to P-free minimal plus 2 % sucrose (pH 5.8), exhibits a 10-fold increase in the initial rate of uptake of 50 $\mu$M phosphate over a 2-h period, as system II is derepressed [2] Fig. 2A illustrates nearly identical results for wild-type strain 74-OR8-1a, in which phosphate influx increased about 11-fold, from 0.5 to 5 75 mmol/l cell water per min, and then stabilized at 4.5–5.0 mmol/l cell water per min. The mutants responded quite differently. Both *nuc*-1 and *nuc*-2 took up phosphate extremely slowly under the control conditions (0 1–0 2 mmol/l cell water per min) and, even more strikingly, failed to show a significant increase in phosphate uptake during incubation in P-free medium. On the other hand, *pcon*$^{c-6}$ (one of the presumed constitutive mutants) took up phosphate rapidly under the control conditions (1 74 mmol/l cell water per min), and then increased still further to a rate slightly above that shown by the wild-type strain during incubation in P-free medium (about 5.4 mmol/l cell water per min).

Changes in cell phosphorus during the same experiment are illustrated in Fig 2B. As before, there were characteristic differences in initial cell phosphorus among the various strains, with *nuc*-1 and *nuc*-2 containing significantly lower concentrations (235 and 250 mmol/l cell water) and *pcon*$^{c-6}$, a significantly higher concentration (365 mmol/l cell water) than wild-type strain 74-OR8-1a (295 mmol/l cell water). After transfer to P-free minimal plus 2 % sucrose, all strains showed a short lag and then a decrease in cell phosphorus to about 180 mmol/l cell water in *nuc*-2 and, somewhat further, to 140 mmol/l cell water in the other three cases. (The decreases in cell phosphorus result from increases in dry weight in the absence of phosphate uptake [2].)

*Kinetic characterization of systems I and II*

To make a quantitative determination of the activities of system I and system II in the various strains under repressing and derepressing conditions, phosphate uptake was measured over a range of external phosphate concentrations for cells grown in

TABLE II

MICHAELIS-MENTEN PARAMETERS FOR SYSTEMS I AND II IN NORMAL AND PHOSPHORUS-STARVED CELLS

Normal cells were grown 15.5 h in Vogel's medium plus 2 % sucrose, P-starved cells were grown 14 h in this medium, followed by an additional 3.5 h in P-free medium plus 2 % sucrose For flux measurements, cells were washed and transferred to P-free medium (no sucrose), pH 5.8, and $^{33}PO_4^{3-}$ was added to the desired final concentration. The initial rates of $^{33}PO_4^{3-}$ uptake were fitted by computer to the sum of two Michaelis-Menten curves. Data for strain RL21a are from ref. 2 Data for all other strains were obtained by holding the $K_{\frac{1}{2}}$ values of systems I and II in common among the strains, while allowing the $J_{max}$ values to vary

| Strain | | System I | | System II | |
|---|---|---|---|---|---|
| | | $K_{\frac{1}{2}}$ (mM) | $J_{max}$ (mmol/l cell water per min) | $K_{\frac{1}{2}}$ (mM) | $J_{max}$ (mmol/l cell water per min) |
| RL21a | normal | 0 166±0 052 | 1 52±0.26 | 0 00263±0 00077 | 0 159±0 184 |
| | P-starved | 0 166±0 052 | 1 19±0 40 | 0 00263±0 00077 | 5 19 ±0 30 |
| 74-OR8-1a | normal | 0.174±0 058 | 1.77±0 39 | 0 00216±0 00083 | 0 023±0 25 |
| | P-starved | 0.174±0 058 | 0 52±0 44 | 0.00216±0 00083 | 4 38 ±0 29 |
| *nuc*-1 | normal | 0 174±0 058 | 1 41±0 36 | 0 00216±0.00083 | 0.00 ±0 26 |
| *nuc*-2 | normal | 0 174±0 058 | 0 28±0 37 | 0 00216±0.00083 | 0 00 ±0 25 |
| | P-starved | 0.174±0.058 | 1 54±0.33 | 0.00216±0 00083 | 0.00 ±0 19 |
| $pcon^{c-6}$ | normal | 0 174±0 058 | 2 92±0.36 | 0 00216±0 00083 | 1 36 ±0 26 |
| (T)$pcon^{c-2}$ | normal | 0 174±0 058 | 2 43±0 37 | 0 00216±0 00083 | 2.07 ±0 26 |
| $preg^{c-2}$ | normal | 0 174±0.058 | 1 42±0.36 | 0 00216±0 00083 | 2 66 ±0 23 |
| | P-starved | 0 174±0 058 | 0 00±0.49 | 0 00216±0 00083 | 5 84 ±0 33 |

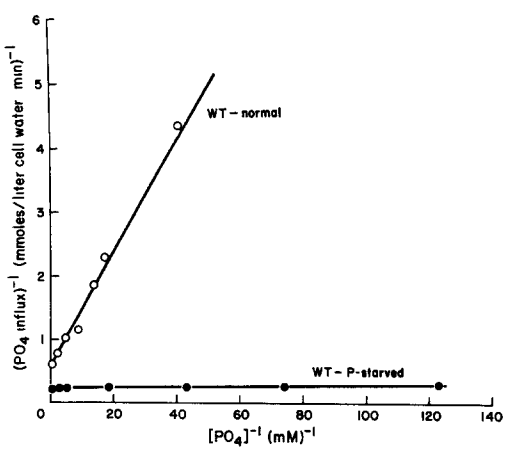


Fig 3. Lineweaver-Burk plots of phosphate transport in normal and P-starved wild-type strain 74-OR8-1a. Points are averages of up to six determinations; curves are computer fits using the parameters from Table II.

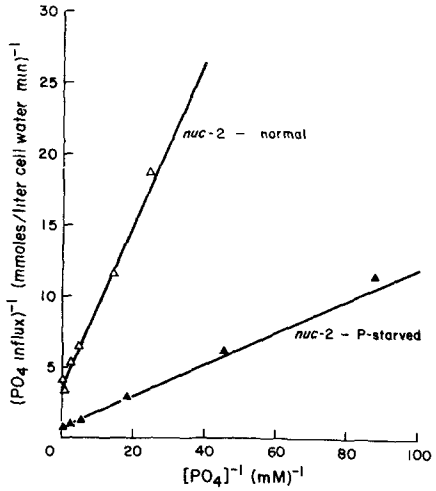


Fig 4 Lineweaver-Burk plots of phosphate transport in normal and P-starved *nuc*-2 As in Fig 3, but note change of scale

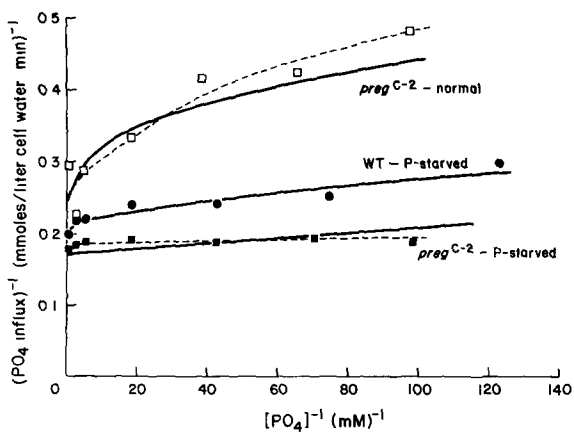


Fig 5 Lineweaver-Burk plots of phosphate transport in normal and P-starved $preg^{c-2}$ (For comparison, the curve of P-starved wild-type strain 74-OR8-1a is also replotted in this graph.) The solid curves for $preg^{c-2}$ are computer fits using the parameters from Table II (joint fit of all strains) Because of the evident deviation from the experimental fits, the data for $preg^{c-2}$ were also fit separately (dashed curves) The parameters in this case were as follows·

| | | $K_{\frac{1}{2}(I)}$ | $J_{max(I)}$ | $K_{\frac{1}{2}(II)}$ | $J_{max(II)}$ |
|---|---|---|---|---|---|
| $preg^{c-2}$ | normal | 0 034±0 038 | 2 38±0 93 | 0 16 $10^{-9}$±0 2 $10^{-2}$ | 1 53±1 02 |
| | P-starved | 0 034±0 038 | 0 36±1 36 | 0 16 $10^{-9}$±0 2 $10^{-2}$ | 5 11±1 23 |

Although the fit has improved, the differences between the new parameters listed here and the ones in Table II are probably not significant

Vogel's minimal medium (normal cells) and for cells grown in this medium and then transferred to P-free medium plus 2 % sucrose for 3.5 h (P-starved cells). In general, satisfactory results were obtained by fitting the data from all strains jointly, holding $K_{\frac{1}{2}(I)}$ and $K_{\frac{1}{2}(II)}$ in common and allowing $J_{max\,(I)}$ and $J_{max\,(II)}$ to vary for each strain and condition. Only in the case of *preg*$^{c-2}$ did the joint fit deviate conspicuously from the data, so that a separate fit of *preg*$^{c-2}$ (normal and P-starved) was also done. The computer results are presented in Table II and in Figs 3–5.

The $K_{\frac{1}{2}}$ values from the joint fit of wild-type strain 74-OR8-1a and the five mutant strains were essentially identical to those previously reported for wild-type strain R421a. 0.174 (0.166) mM for system I and 0.00263 (0.00216) mM for system II. The amounts of systems I and II in wild-type strain 74-OR8-1a were also quite similar to those in strain RL21a: system I had a $J_{max}$ of 1.77 (compared with 1.52) mmol/l cell water per min in normal (repressed) cells and decreased somewhat during phosphorus starvation, while system II had a very low $J_{max}$ (0 02 compared with 0.16 mmol/l cell water per min) in normal cells and increased markedly (to 4 38 compared with 5 19 mmol/l cell water per min) during phosphorus starvation

Significant differences were seen in the mutants. As expected, the greatest changes were in system II: *nuc*-1 and *nuc*-2 had no detectable system II when grown in normal Vogel's medium (and *nuc*-2, even when phosphorus starved). By contrast, *pcon*$^{c-6}$, (T)*pcon*$^{c-2}$ and *preg*$^{c-2}$ all formed substantial amounts of system II during growth in Vogel's medium: the $J_{max}$ values were 1.36, 2.07 and 2.66 (or 1 53, see legend to Fig. 5) mmol/l cell water per min, respectively, corresponding to 31, 47 and 61 % (or 35 %) of the derepressed values for the wild-type strain When P-starved, *preg*$^{c-2}$ showed a further increase in the $J_{max}$ of system II to 5 84 (or 5.11) mmol/l cell water per min, slightly above the derepressed value for the wild type. The other two constitutive mutants were not examined in detail during phosphorus starvation, but it is likely from the results in Fig. 2 and from other similar data (not shown) that system II also increases in *pcon*$^{c-6}$ and (T)*pcon*$^{c-2}$ under these conditions.

There were also smaller changes in system I in some of the mutants (Table II). When grown in Vogel's medium, *nuc*-2 had an abnormally low $J_{max}$ for system I (0.283 compared with 1.77 mmol/l cell water per min), and the $J_{max}$ increased (to 1.54 mmol/l cell water per min), rather than decreasing as in the wild-type strain, upon phosphorus starvation. In addition, two of the constitutive mutants (*pcon*$^{c-6}$ and (T)*pcon*$^{c-2}$) had slightly elevated levels of system I in Vogel's minimal medium. These differences are discussed below.

## DISCUSSION

From the results originally described by Lehman et al. [3] and extended in the present paper, it is clear that phosphate transport system II is under the control of three regulatory genes: *pcon-nuc*-2, *preg* and *nuc*-1. Other enzymes which are needed for the scavenging of phosphorus from the environment are known to be regulated in parallel; they include an acid phosphatase [5], an alkaline phosphatase [3, 6], one or more nucleases [7] and *O*-phosphorylethanolamine permease (Metzenberg, R. L., personal communication). From studies of dominance and epistasis, and from the properties of revertants of the various regulatory mutants, Metzenberg and his co-workers [3, 6, 8, 9] have concluded that both positive and negative control elements

act in a definite sequence to regulate the production of this group of enzymes

The relationship of phosphate transport system I to system II and to the various regulatory genes is less clear. On the basis of the present data, we cannot rule out the possibility of a minor stimulatory effect of *pcon*, and a minor inhibitory effect of *nuc*-2, on the synthesis of system I. We think it more likely, however, that system I is constitutive, and that the relatively small changes in its activity among the mutant strains and during phosphorus starvation reflect variations in the intracellular concentration of some regulatory molecule (possibly inorganic phosphate itself).

Overall, phosphate transport in *Neurospora* follows a now-familiar microbial pattern. dual transport systems, with one being derepressed (along with related enzymes) when there is a particular metabolic need for its substrate. Further understanding of systems I and II will be helped, as in these other cases, by the isolation of structural-gene mutants and by the purification of components of the transport systems.

Finally, now that the kinetic parameters for phosphate transport have been determined in a variety of strains, it is of interest to examine the relationship between transport and growth. In order for logarithmic-phase cells to maintain a steady-state concentration of cell phosphorus, net phosphate influx must be $J = 0.693\ P_c/t_D$, where $J$ is the influx in mmol/l cell water per min, $P_c$ is the cell phosphorus concentration in mmol/l cell water, and $t_D$ is the doubling time (in min) for cell mass [1] This equation, applied to 74-OR8-1a, *pcon*$^{c-6}$ and *nuc*-1 (data from Table I), gives required transport rates of 1 09, 1.46 and 0 93 mmol/l cell water per min, comfortably smaller than the transport rates that are possible (1 78, 4 27 and 1.40 mmol/l cell water per min, respectively) on the basis of the measured kinetic parameters of systems I and II (Table II) and a phosphate concentration of 37 mM (Vogel's minimal medium) For *nuc*-2, however, there is something of a paradox. The required transport rate, given the doubling time and cell phosphorus concentration in Table I, is 1.07 mmol/l cell water per min, but the rate calculated from the Michaelis parameters in Table II is only 0 28 mmol/l cell water per min (although it would rise under conditions of phosphorus starvation to 1 53 mmol/l cell water per min). Thus, even during growth at high external phosphate concentrations, *nuc*-2 appears somehow to balance between the unstarved and starved states, and is an interesting strain to examine further.

ACKNOWLEDGEMENTS

We are grateful to Dr Robert Metzenberg for supplying the mutant strains and for helpful discussions during the course of the work, and to Mr Anthony Mongillo for expert technical assistance. The work was supported by U. S Public Health Service Research Grant GM 15761 and by Research Career Development Award GM 20163 to C W. Slayman.

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