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PHOSPHATE TRANSPORT IN *MICROCOCCUS LYSODEIKTICUS*

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Summary

Phosphate accumulates in *Micrococcus lysodeikticus* cells against a concentration gradient, by an energy-dependent process. The phosphate transport is derepressed during phosphate deprivation. The derepression process is inhibited by chloramphenicol. The apparent K_m of phosphate transport is 4.3 μM. The activation energy of the transport is 21 kcal per mol in the temperature range of 0—29°C, and 4.9 kcal per mol between 29 and 40°C. The rate of the transport increases in presence of K^+ and Mg^{2+}. Arsenate is a competitive inhibitor of phosphate transport, having an apparent K_i of 6.0 μM. Sulfhydryl reagents, respiratory inhibitors and uncouplers of oxidative phosphorylation inhibit phosphate transport.

Introduction

The nature and the properties of phosphate (P_i) transport systems have been investigated in various bacterial strains, such as *Escherichia coli* [1], *Bacillus cereus* [2], *streptococcus faecalis* [3,4] and *Micrococcus pyogenes* [5,6]. Phosphate is an integral constituent of essential cell components and metabolites, and its transport into the cell, as well as its fate following entry, varies widely.

In a previous study we have shown that *Micrococcus lysodeikticus* accumulates considerable amounts of inorganic polyphosphate, concentrated in unique-shape subcellular structures [7,8]. The rate and the level of polyphosphate accumulation increased markedly after phosphate deprivation. (Esh'har, Z., Avigad, G. and Friedberg, I., unpublished). The intensive accumulation of polyphosphate suggested that phosphate-deprived cells of *M. lysodeikticus* might exhibit an intensive uptake system for P_i.

Abbreviations: TKM buffer, 50 mM triethanolamine · HCl buffer, pH 7.8, containing 100 mM KCl and 2 mM $MgSO_4$; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

In this communication, the presence of an active P_i transport system in *M. lysodeikticus* has been demonstrated, and its regulatory properties are described.

Materials and Methods

Organism and culture media. M. lysodeikticus ATCC 4698 (Fleming) was grown on a defined medium (based on the media described by Grula [9] and McDonald [10], composed of 5 g L-glutamic acid, 1 g L-arginine, 0.5 g L-tryosine, 0.4 g L-phenylalanine, 20 mg DL-methionine, 5 g NaCl, 1.36 g KH_2PO_4 and 20 mg $MgSO_4 \cdot 7H_2O$ per l. The pH was adjusted to pH 7.8 with KOH. A solution containing 0.5 g $FeSO_4 \cdot 7H_2O$ per l, and a solution composed of 6.0 g inosine, 0.2 g thiamine · HCl and 0.01 g biotion per l were sterilized by filtration, and 10 ml of each solution were added to 1 l medium. Growth was carried out in a shaker, at 30° C.

Starvation for phosphate. Cells were grown on the defined medium until they reached the late logarithmic phase of growth, harvested by centrifugation, washed twice and resuspended in phosphate-less medium, to a final turbidity of about 100 units when read in the Klett-Summerson colorimeter, adapted with a No. 54 filter. The phosphate-less medium had the same composition as the defined medium, except that KH_2PO_4 was replaced by 50 mM triethanolamine · HCl buffer, pH 7.8. Growth was carried out in a shaker, at 30° C, until the growth stopped, usually after 8—10 h, when the turbidity of the cell suspension was 250—300 Klett units.

P_i and arsenate uptake assay. Cells were harvested by centrifugation, washed twice with 50 mM triethanolamine buffer, pH 7.8, containing 100 mM KCl and 2 mM $MgSO_4$ (TKM buffer) and resuspended 1 : 50 (1 g wet wt. per 50 ml) in the same buffer. For the standard uptake experiment, cells were suspended in TKM buffer (200 µl of 1 : 50 cell suspension per 1 ml), in flat-bottom flasks, to ensure adequate aeration. Measurements of P_i or arsenate uptake were initiated by addition of either $^{32}P_i$, or [^{74}As]arsenate to 0.1 mM. 200-µl samples were withdrawn after 15, 30, 60 and 120 s, filtered through a membrane filter of 0.45 µm pore size (Sartorius), and immediately washed with 5 ml of TKM buffer. The filters were dried, and the radioactivity of the material on the filters was measured with a Packard 3330 scintillation spectrometer. The amount of radioactivity was plotted vs. time and the uptake rate was calculated from the slope, which was linear at least for 2 min.

Analytical procedures. Soluble phosphates were extracted from the cells by incubation in cold, 5% trichloroacetic acid for 15 min. P_i in the cells was assayed according to Avron [11]: Ammonium molybdate was added to the soluble phosphate fraction, and the phosphomolybdate complex formed was further extracted into the organic phase of isobutanol/benzene. The radioactivity of samples of the cold trichloroacetic acid extract, the organic phase and the water phase, was measured to determine the total soluble phosphates, P_i, and other soluble phosphates in the cells, respectively. The osmotic space of the bacteria was determined according to Rottenberg et al. [12], using ^3H_2O and [^{14}C]mannitol. Alkaline phosphatase activity was measured as described [13]. Protein was determined by the biuret method [14]. Amino acids, biotin,

inosine, thiamine, CCCP, and valinomycin were purchased from Sigma Chemical Co., St. Louis, Mo. $^{32}P_i$ was purchased from the Israel Atomic Energy Commission, Israel, and [^{74}As]arsenate from The Radiochemical Centre, Amersham, England. All chemicals employed were analytical grade materials.

Results

The effect of P_i deprivation on P_i uptake

As shown in Fig. 1, the rate of P_i uptake by *M. lysodeikticus* cells, grown in the presence of P_i, was relatively low, 1—3 nmol P_i per min per mg cell protein. The rate of P_i uptake was gradually increased, after transferring the cells into a P_i-less medium. Maximal rate of uptake (40—100 nmol P_i per min per mg cell protein) was reached at the end of the growth period (about two generations) and from then on the rate of uptake began to decrease. Chloramphenicol, when added during P_i starvation, stopped the increase of the uptake rate, and its removal resulted in a further increase (Fig. 1). Chloramphenicol, when added to the assay system, did not inhibit the transport itself. This effect indicates that the increase in the rate of P_i uptake is correlated with de novo protein synthesis.

It is apparent from the data presented in Fig. 2 that addition of P_i to growth medium during P_i starvation resulted in an immediate decrease of the rate of P_i uptake. On the other hand, addition of organic phosphate, such as β-glycero-

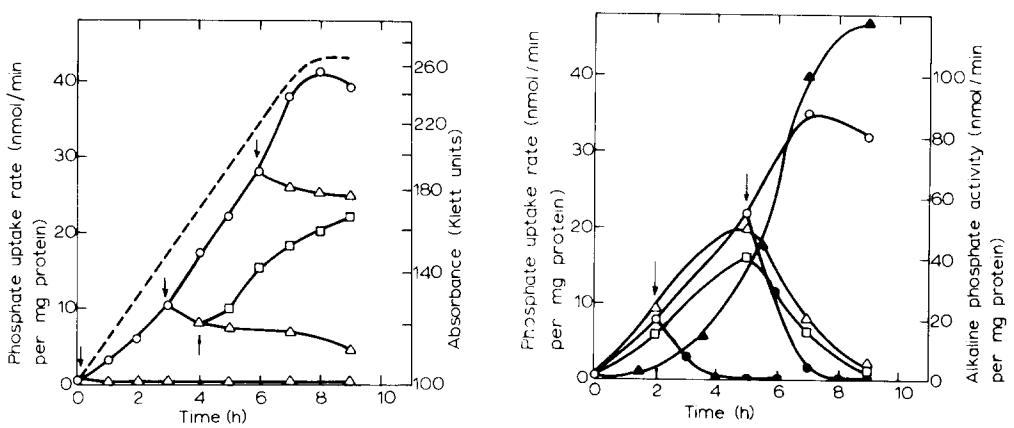


Fig. 1. The effect of P_i deprivation and chloramphenicol on P_i transport in *M. lysodeikticus*. Cells were grown on a P_i-containing medium, harvested, washed and resuspended in P_i-free medium. The growth was followed turbidometrically (- - - - - -). Samples were withdrawn at indicated times, and the initial rate of P_i uptake was measured as follows: ○———○, no additions; △———△, 100 μg/ml chloramphenicol was added at indicated times (arrows pointing down); □———□, chloramphenicol was subsequently washed out (arrow pointing up). Details of the procedures as described in Materials and Methods.

Fig. 2. The effect of P_i and β-glycerophosphate on the rate of P_i transport during P_i-starvation of *M. lysodeikticus*. Cells were grown in a P_i-containing medium, washed and resuspended in a P_i-free medium. The initial rate of P_i transport was measured in the following systems: (1) ○———○, no additions. (2) △———△, 1 mM β-glycerophosphate was added at zero time. (3) □———□, 10 mM β-glycerophosphate were added at zero time. (4) ●———●, 10 mM P_i were added at indicated times (arrows pointing down). ▲— —▲, alkaline phosphatase activity was measured in system 1. Procedures as described in Materials and Methods.

phosphate, did not affect the increase of the uptake rate during the first 5 h of growth on P_i-less medium. After that time period, the rate of P_i uptake gradually decreased, apparently as a consequence of the release of P_i from β-glycerophosphate, by alkaline phosphatase. As was further shown, the activity of alkaline phosphatase increased during P_i starvation (Fig. 2).

Kinetics of P_i uptake

The rate of P_i uptake by P_i-starved cells was linear for about 30 min, until a level of 1000 nmol of total phosphate per mg cell protein was achieved. This increase of the phosphate level was followed by a decrease and stabilization at a concentration of 600 nmol per mg cell protein (Fig. 3).

As shown in Fig. 4, a considerable amount of the P_i that had been taken up by the cells was subsequently found in the trichloroacetic acid-insoluble fraction. This fraction was shown to be composed mainly of polyphosphate [7,8]. The level of free P_i within the cell increased during the first minute of uptake up to 30 nmol per mg protein, then decreased during the following 2 min, and from then on was stabilized at a steady-state level of 10 nmol P_i per mg protein.

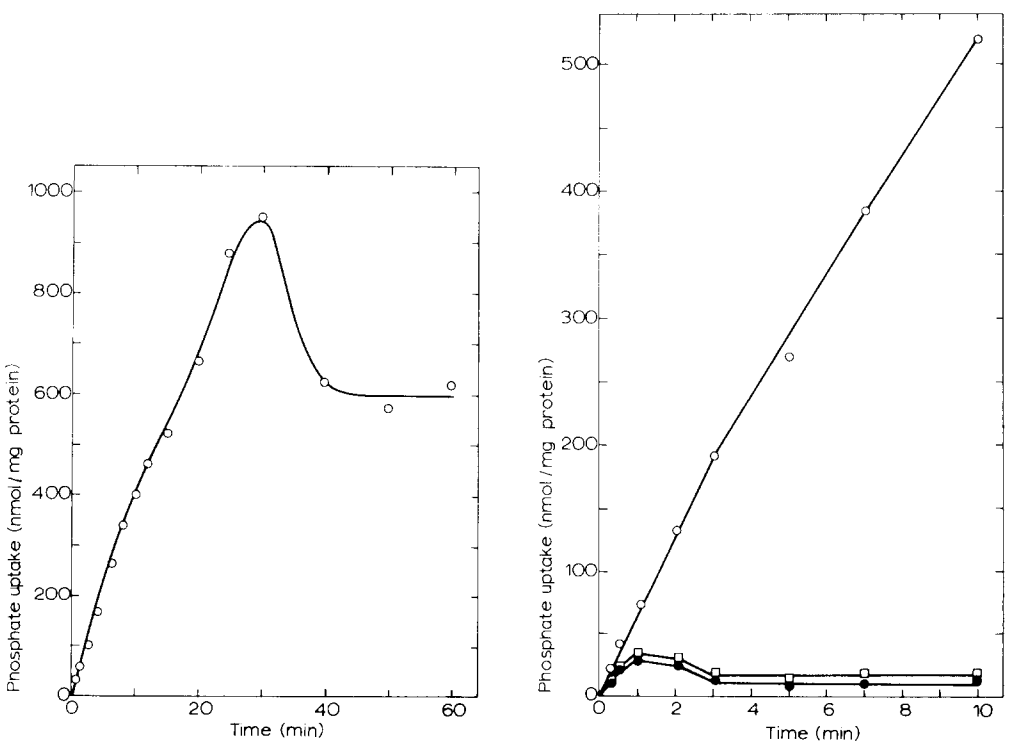


Fig. 3. Accumulation of phosphate by P_i-starvated *M. lysodeikticus* cells. Starvation of P_i and assay of phosphate level in the cells were carried out as described in Materials and Methods.

Fig. 4. The distribution of phosphate taken up by *M. lysodeikticus* cells. The procedures for P_i starvation, P_i uptake and fractionation of phosphate compounds were carried out as described in Materials and Methods. ○———○, total phosphate; □———□, trichloroacetic acid-soluble phosphate; ●———●, orthophosphate (P_i).

According to the measurements of the osmotic space, the inner concentrations of free P_i were found to be 6.3 mM at 1 min after P_i addition, and 2.1 mM during the steady-state stage. Thus, although most of the P_i taken up by the cells is subsequently polymerized, a maximal concentration gradient of 1 : 63 was obtained after 1 min and a gradient of 1 : 21 was established during the steady-state stage. When the direct measurements of free P_i within the cells were repeated with lower external P_i concentrations, $1 \cdot 10^{-5}$ and $1 \cdot 10^{-6}$ M, the maximal internal concentrations were found to be 7.5 and 2.26 mM, respectively. Thus, the respective concentration gradients were 1 : 750 and 1 : 2260.

The apparent K_m for P_i uptake was found to be 4.3 μM, using outer concentrations of P_i in the range $1 \cdot 10^{-6}$—$5 \cdot 10^{-4}$ M. (Fig. 7). The apparent value of V was found to be 133 nmol P_i per min per mg cell protein, although this value will depend on the degree of derepression of the uptake system.

Effect of ions

The data presented in Table 1 show that P_i transport was slightly but consistently stimulated by K^+ and Mg^{2+}. Optimal concentrations were found to be 100 mM and 2 mM of K^+ and Mg^{2+}, respectively. Mg^{2+} alone had only a slight effect on the rate of P_i transport, but when it was added in presence of K^+, a further stimulation was observed. Na^+ had a slight inhibitory effect on the rate of P_i transport. Ca^{2+} alone stimulated the transport rate, but when Ca^{2+} was added in the presence of Mg^{2+}, the rate decreased by 15% (data not shown).

The concentration of H^+ had only a mild effect on the rate of P_i transport, in the pH range of 5—9 (Fig. 5). Maximal rate of transport was at pH 8, and a second peak of transport activity was found at pH 6.5.

Effect of temperature

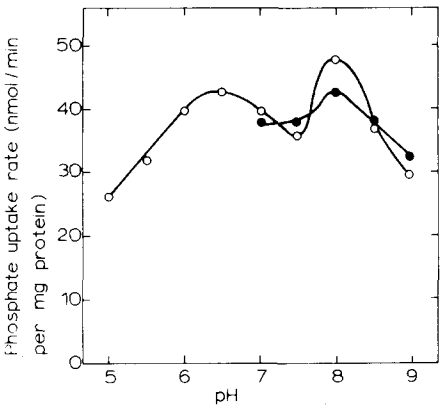
The rate of P_i transport increased with temperature elevation, in the range of 0—40° C, and decreases at higher temperatures. When the data were plotted according to Arrhenius, a biphasic curve was obtained (Fig. 6). The activation energy for P_i transport was calculated to be 21 kcal per mol P_i between 0 and 29° C, and 4.9 kcal per mol P_i between 29 and 40° C.

TABLE I

EFFECTS OF K^+, Na^+ and Mg^{2+} ON P_i UPTAKE

Rate of P_i uptake was measured as described in Materials and Methods. Relative rate of 1.00 was equal to 20 nmol of P_i/min per mg protein.

K^+ (mM)	Mg^{2+} (mM)	P_i uptake relative rate	K^+ (mM)	Mg^{2+} (mM)	P_i uptake relative rate	Na^+ (mM)	Mg^{2+} (mM)	P_i uptake relative rate
—	—	1.00	—	—	1.00	—	—	1.00
5	—	1.29	5	1	1.36	0.1	—	0.84
20	—	1.64	20	2	1.70	100	—	0.89
100	—	1.71	100	2	2.00	0.1	2	0.89
200	—	1.57	200	2	2.00	1	2	0.86
—	1	1.07	20	5	1.43	10	2	0.89
—	10	1.07	100	5	2.00	100	2	1.00



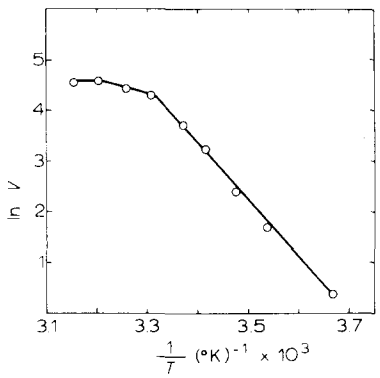


Fig. 5. The effect of H^+ concentration on the rate of P_i transport in *M. lysodeikticus*. The initial rate of P_i transport was measured as described in Materials and Methods, using either triethanolamine/maleate buffer ○———○; or triethanolamine—HCl buffer ●———●.

Fig. 6. Activation energies of P_i transport in *M. lysodeikticus*. The initial rate of P_i transport was measured as described in Materials and Methods, at different temperatures. The data were plotted according to Arrhenius, and the activation energies were calculated.

Effect of Inhibitors

As shown in Table II, sulfhydryl reagents inhibited P_i transport. The inhibition caused by *p*-hydroxymercurybenzoate was partially reversed by the subsequent addition of dithiothreitol. The reversion was not complete, since dithiothreitol itself mildly inhibited the transport. Table III shows that respiratory inhibitors and uncouplers of oxidate phosphorylation inhibited the

TABLE II

EFFECT OF SULFHYDRYL REAGENTS ON P_i AND ARSENATE UPTAKE

The rate of P_i and arsenate uptake was measured as described in Materials and Methods. Preincubation of the cells with *N*-ethylmaleimide and *p*-hydroxymercurybenzoate was carried out at room temperature for 5 min. When dithiothreitol was added to cells preincubated with *p*-hydroxymercurybenzoate, the preincubation proceeded for additional 5 min. Relative rate of 1.00 equals to 47 nmol of P_i, and 16 nmol of arsenate per min per mg protein.

Addition	Concentration (mM)	P_i uptake relative rate	Arsenate uptake relative rate
None	—	1.00	1.00
N-Ethylmaleimide	1	0.51	0.41
	10	0.08	0.13
p-Hydroxymercurybenzoate	0.01	0.71	0.63
	0.10	0.32	0.40
p-Hydroxymercurbbenzoate	0.10		
+ dithiothreitol	5	0.66	0.72
Dithiothreitol	5	0.76	0.78

TABLE III

EFFECT OF RESPIRATION INHIBITORS AND UNCOUPLERS ON P_i AND ARSENATE UPTAKE

The rate of P_i and arsenate uptake was measured as described in Materials and Methods. Preincubation with the inhibitors was carried out at room temperature for 5 min. Relative rate of 1.00 equals to 50–100 nmol of P_i or 15–20 nmol of arsenate per min per mg protein, in different experiments, KCl concentration was 100 mM, unless other concentrations are indicated. When lower concentrations of KCl were applied, the relative rate was compared to respective controls without inhibitor.

Additions	Concentration (mM)	P_i uptake relative rate	Arsenate uptake relative rate
None	—	1.00	1.00
KCN	1	0.33	0.34
	10	0.03	0.11
2,4-Dinitrophenol	1	0.91	0.78
	10	0.17	0.11
CCCP	0.001	0.56	0.51
	0.005	0.33	0.35
	0.010	0.30	0.28
Valinomycin	0.002	0.08	0.07
	0.004	0.02	0.05
Valinomycin	0.002		
+ 10 mM KCl		0.90	1.05
+ 1 mM KCl		0.98	1.03
+ 0.1 mM KCl		1.10	1.12

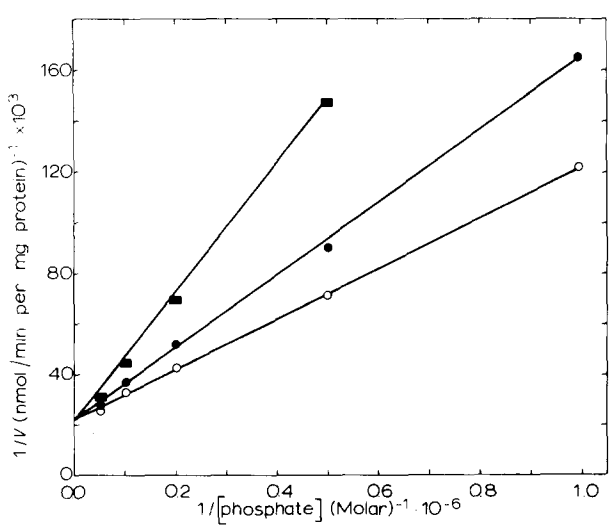


Fig. 7. Inhibition of P_i transport in *M. lysodeikticus* by arsenate. The initial rate of P_i transport was measured as described in Materials and Methods without arsenate ○———○ and in presence of 2.5 μM ●———● or 10 μM ■———■ arsenate. The data were plotted according to Lineweaver and Burk.

transport of P_i. KCN, a respiratory inhibitor, exerted almost complete inhibition. Proton ionophores, 2,4-dinitrophenol and CCCP inhibited the transport markedly, but not completely. The inhibition by valinomycin, a K^+ ionophore, was more effective, and almost complete inhibition was exerted in presence of 100 mM KCl. When lower concentrations of KCl, up to 10 mM, were applied, valinomycin did not inhibit P_i transport. Arsenate, a non-metabolized analogue of P_i, was found to be a competitive inhibitor for P_i transport, with an apparent K_i of 6.0 μM. (Fig. 7). The uptake of arsenate was inhibited by sulfhydryl reagents, respiratory inhibitors and uncouplers of oxidative phosphorylation to a similar extent as P_i uptake (Table II and III).

Discussion

The data presented show that the regulation of P_i transport by *M. lysodeikticus* cells is consistent with the mechanism of repression-derepression. During P_i deprivation, the rate of P_i transport is gradually increased. This process is arrested upon addition of chloramphenicol, a protein synthesis inhibitor, indicating that de novo protein synthesis is essential for the increase of P_i transport by P_i-starved cells. P_i by itself, or one of its products, might be involved in the regulation of the transport mechanism, since growth in the presence of P_i results in a slow rate of P_i transport, and addition of P_i to the medium during P_i starvation brings about a gradual decrease in the transport rate. This effect is specific for inorganic phosphate, since organic esters of phosphate do not affect the derepression process. The decrease in the rate of P_i transport by addition of P_i to the medium during P_i starvation is more pronounced than that caused by chloramphenicol. It might be that P_i not only represses the synthesis of components of the transport system, but also inhibits their function. A similar inactivation of the P_i uptake system in the eucaryotic microorganism, *Chlorella* was suggested to result from turnover of the uptake system components [15]. The effect of P_i deprivation on *M. lysodeikticus* cells is more pronounced than in other bacterial strains. In *B. cereus* [2] and *E. coli* [1] the rate of P_i transport is increased twice and 20 times, respectively, during P_i starvation, whereas in *M. lysodeikticus* the rate is increased by a factor of 40—100.

The kinetics of P_i uptake in *M. lysodeikticus* are different from the kinetics shown in other bacterial strains. In *M. lysodeikticus* the P_i uptake is linear until the maximal level is reached, whereas in *E. coli* [1] and *B. cereus* [2], the kinetics of P_i uptake are biphasic: An initial rapid rate is followed by a slower one. This biphasic kinetics was explained by the presence of two transport systems, of which the rapid one is subjected to inhibition when a "primary pool" of P_i within the cell is filled up [1]. Since in *M. lysodeikticus* the P_i taken up by the cells is subsequently polymerized, it might be postulated that the pool of free P_i is only partially loaded; therefore, it does not exert inhibition on P_i uptake, and monophasic kinetics are maintained.

The rapid and extensive P_i uptake by cells previously subjected to P_i starvation, is followed by a decrease of the total phosphate level within the cells, after a maximal level has been achieved. This phenomenon of "overplus accumulation" [16] is known in other microorganisms, and was explained as a

result of regulatory mechanisms of polyphosphate metabolism [17].

The marked change in the activation energy of P_i transport at 29°C from 21 to 4.9 kcal per mol, might have resulted from a phase transition in the membrane, as was shown for other membranal enzymes and active transport systems [18,19]. The apparent K_m for P_i transport, 4.3 μM, is similar to the K_m values described for other bacteria: 0.7 and 9.2 μM for *E. coli* [1] and 10 μM for *S. faecalis* [3]. The stimulation of P_i transport by K^+ and Mg^{2+} was also described in other bacteria [1—3,20]. Although the stimulation by K^+ and Mg^{2+} in *M. lysodeikticus* is less pronounced than in other P_i transport systems, the effect seems to be specific, since Na^+ and Ca^{2+} have a slight inhibitory effect.

Cations were found to be involved in the transport of various substrates, by cation-substrate symport [21—23]. In transport of anions, such as P_i, the cation has a role of a counter ion as well. There is much evidence, that proton is the counter ion for accumulation of P_i by mitochondria [24,25]. In bacteria, however, further work is required to search for cotransport of cation with P_i [22], although it was suggested that any permeant cation might serve to compensate for the anionic charge [4].

The external pH has a mild effect on the initial rate of P_i uptake in a wide range of pH 5 to pH 9. However, two peaks of uptake activity, at pH 8 and pH 6.5 were observed. The presence of two pH optima might be explained by the effect of the external pH on the proton gradient (ΔpH) and the membrane potential ($\Delta\psi$) [26], the two parameters of the proton motive force which was suggested to be the driving force for active transport [21]. The relative amount of the ion species, $H_2PO_4^-$ and HPO_4^{2-}, which affects P_i uptake [27], is also changed with the expernal pH.

Arsenate is a competitive inhibitor for P_i transport, as was described for other bacterial strains [1—4,28,29]. Sulfhydryl reagents inhibit the transport of P_i in *M. lysodeikticus*, indicating that -SH groups are involved in the mechanism of the active transport [1,30,31].

Although most of the P_i taken up by the cells is subsequently polymerized [7,8], its transport is performed against a concentration gradient, and a gradient higher than 1 : 2000 is established when the external concentration is low. The transport of P_i is an energy-dependent process, since it is inhibited by respiratory inhibitors and uncouplers of oxidative phosphorylation. The transport of arsenate, a non-metabolized analogue of P_i, is affected by the inhibitors and the uncouplers similarly to P_i transport. This implies that the main effect of the inhibitors is on the P_i uptake process, when measured by its initial rate, and not on its subsequent metabolism.

The effect of the ionophores, such as CCCP and valinomycin, is in accordance with the chemiosmotic hypothesis, suggesting that a proton motive force is the driving force for the active transport process [21—23]. Under the experimental conditions used, it seems that P_i transport in *M. lysodeikticus* is primarily driven by membrane potential, since the inhibition by valinomycin, a K^+ ionophore, is almost complete, whereas only partial inhibition was exerted by CCCP, an ionophore of H^+. A more detailed study on the effect of ionophores on P_i transport is a subject of a further investigation.

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