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EXPERIMENTAL ARTICLES

Inorganic Polyphosphates and Phosphohydrolases in *Halobacterium salinarium*

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Abstract—*Halobacterium salinarium* grown in a liquid medium consumed up to 75% of phosphates originally present in the growth medium and accumulated up to 100 μmol P_i/g wet biomass by the time it entered the growth retardation phase. The content of acid-soluble oligophosphates in the biomass was maximum at the early stage of active growth and drastically decreased when cells reached the growth-retardation phase. The total content of alkali-soluble and acid-insoluble polyphosphates changed very little throughout the cultivation period (five days). The polyphosphate content of *H. salinarium* cells was close to that of yeasts and eubacteria. The pyrophosphatase, polyphosphatase, and nonspecific phosphatase activities of *H. salinarium* cells were several times lower than those of the majority of eubacteria. The specific activity of pyrophosphatase, the most active hydrolase of *H. salinarium*, gradually increased during cultivation, reaching 540 mU/mg protein by the end of the cultivation period. Half of the total pyrophosphatase activity of this halobacterium was localized in the cytosol. The molecular weight of pyrophosphatase, evaluated by gel filtration, was 86 kDa. The effective K_m of this enzyme with respect to pyrophosphate was 115 μM.

Key words: inorganic polyphosphates, phosphohydrolases, *Halobacterium salinarium.*

Inorganic polyphosphates, the linear polymers of orthophosphoric acid, are key metabolites of the phosphorus metabolism of bacteria. These biopolymers are widespread in microorganisms [1], where their role is manifold [2]. Together with phosphohydrolases, polyphosphates promote bacterial survival under unfavorable conditions. For instance, *Escherichia coli* cells with an enhanced content of cellular polyphosphate showed better survival under osmotic and salt stress conditions [2]. In this connection, it would be of interest to study polyphosphates and the enzymes of phosphorus metabolism in microorganisms living in extreme habitats. The elucidation of the role of polyphosphates in archaebacteria must be important not only to researchers dealing with the physiology of this group of bacteria but also to general evolutionary biochemists. The content and metabolism of polyphosphates in archaebacteria are still poorly studied. The choice of the archaebacterium *Halobacterium salinarium* for investigating the metabolism of polyphosphates was due to its well-studied bioenergetic and membrane transport systems [3, 4], whose role in this metabolism is now recognized [2]. Earlier, halobacterial alkaline phosphatases were studied in *Halobacterium halobium* [5], *Halobacterium cutirubrum* [6], and *Halobacterium marismortui* [7].

The aim of this work was to study the dynamics of polyphosphates and some phosphohydrolases during the growth of *H. salinarium*

MATERIALS AND METHODS

Strain and cultivation conditions. *Halobacterium salinarium* ET1001 used in this study was kindly donated by T.S. Kalebina (Moscow State University). The strain was grown at 37°C in shaken flasks with 200 ml of liquid medium containing (g/l) NaCl, 250; KCl, 2; sodium citrate, 3; $MgSO_4$, 20; peptone (Framenia, United States), 7; and K_2HPO_4, 0.4.

Culture growth was monitored by weighing the wet biomass, which was collected by centrifugation at 5000 *g* for 40 min and washed twice with the growth medium without phosphate and peptone. The use of the wet biomass for the evaluation of the cellular content of polyphosphates and protein was dictated by the possibility of misestimating the dry weight of the biomass due to the disruption of cells in the process of their washing.

Preparation of the cell homogenate. A weighed portion of the biomass was mixed with distilled water to cause cell lysis and then with Tris–HCl buffer (pH 7.2) at a final concentration of 25 mM. Cells were homogenized using a glass homogenizer with a teflon

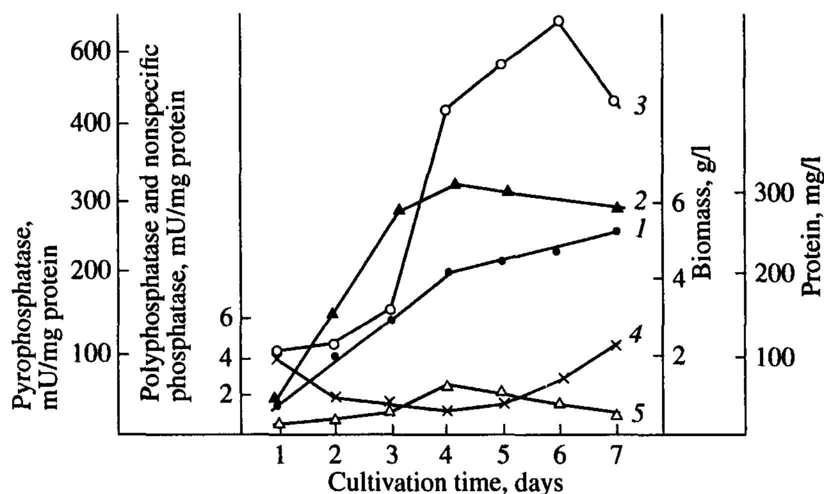

Fig. 1. Phosphohydrolase activities in the homogenate of *H. salinarium* cells grown in the phosphate-containing medium: *1*, biomass; *2*, biomass protein; *3*, pyrophosphatase activity; *4*, polyphosphatase activity; and *5*, nonspecific phosphatase activity.

pestle. This and all other procedures were carried out at 0–4°C.

Fractionation of the cell homogenate. The cell homogenate was centrifuged at 5000 *g* for 10 min. The resultant precipitate 1 contained mainly fragments of the cell envelope. The supernatant was fractionated by centrifugation at 14000 *g* for 40 min into precipitate 2 (the membrane fraction) and the transparent supernatant liquid (the cytosol fraction). The supernatant and precipitates suspended in 25 mM Tris–HCl buffer (pH 7.2) were assayed for various phosphohydrolase activities.

Enzyme assays. All enzymes were assayed at 30°C. The nonspecific phosphatase activity was determined from the hydrolysis rate of *p*-nitrophenyl phosphate measured at pH 8.4 in the presence of 10 mM $MgSO_4$ [8]. Other phosphohydrolase activities were determined from the rate of formation of orthophosphate (P_i) in a 1-ml reaction mixture [8]. The reaction mixture for the assay of exopolyphosphatase activity contained 0.08 mM polyphosphate with an average chain length of 25 P, 1 mM $CoSO_4$, and 200 mM KCl in 50 mM Tris–HCl buffer (pH 7.2). The reaction mixture for the pyrophosphatase contained 1 mM pyrophosphate, 10 mM $MgSO_4$, and 200 mM KCl in 50 mM Tris–HCl buffer (pH 8.4). All of the modifications of the enzyme assay procedures are specified below.

The effect of various reagents on enzymes was studied by preincubating them at 20°C for 5 min in the respective assay mixture. The reaction was started by adding the substrate of the particular enzyme.

One unit of enzyme activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of the substrate (or produces 1 μmol of P_i from the substrate) in 1 min.

Polyphosphates with average chain lengths of 9, 15, 188 (Monsanto, United States), and 25 P (Sigma) were purified of ortho- and pyrophosphate by gel filtration on Sephadex G-10 as described earlier [8].

Triphosphate and pyrophosphate used in this work were purchased from Sigma (United States) and Koch-Light (United Kingdom), respectively.

The protein concentration was determined by the method of Lowry *et al.* [9] using bovine serum albumin as the standard.

The molecular weight of the pyrophosphatase was determined by gel filtration on a Sephacryl S-300 column (1.6 × 80 cm) with an eluant representing 20 mM Tris–HCl buffer (pH 7.2) with 4 mM $MgSO_4$, 100 mM NaCl, and 1 mM dithiothreitol. The elution volume of pyrophosphatase was determined by mea-

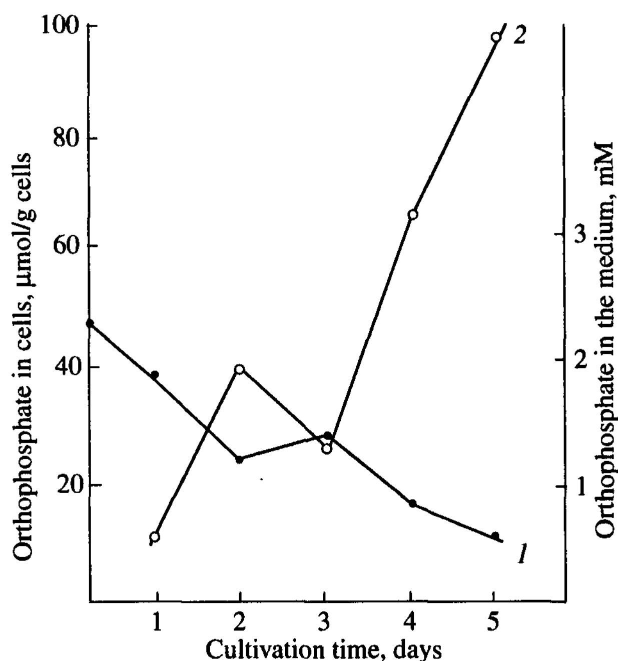

Fig. 2. Concentration of orthophosphate in (*1*) the cultivation medium and (2) cells of *H. salinarium.*

suring its activity in chromatographic fractions. The column was calibrated using ferritin (440 kDa), catalase (232 kDa), β-amylase (200 kDa), aldolase (158 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), ribonuclease (13.7 kDa), and blue dextran (~2000 kDa).

Extraction of phosphates from cells was carried out as described by Kulaev [1] and Vagabov *et al.* [10]. Inorganic phosphate, pyrophosphate, and oligophosphates were extracted from cells by incubating them at 0°C for 30 min in 0.5 N $HClO_4$ under continuous stirring. The acid-extracted biomass was removed by centrifugation at 10000 *g* for 20 min, and the nucleotide phosphates present in the supernatant were removed by 30-min sorption on Norit A activated charcoal. The resultant preparation was referred to as the fraction of acid-soluble phosphates. Alkali-soluble polyphosphates were prepared similarly, by extracting the biomass with 0.05 N NaOH (pH 12).

The amount of polyphosphates in these fractions was estimated from the difference in the orthophosphate contents of fractions before and after their hydrolysis in 1 N HCl at 100°C for 10 min [1, 10].

Polyphosphates remaining in the biomass after extraction with cold $HClO_4$ (the so-called acid-insoluble polyphosphates [1, 10]) were estimated by hydrolyzing them into orthophosphate with 0.5 N $HClO_4$ in a boiling water bath for 30 min. The supernatant obtained after the removal of the residue biomass by centrifugation at 10000 *g* for 20 min was analyzed for the amount of orthophosphate.

Orthophosphate and pyrophosphate were evaluated as described earlier [8].

RESULTS AND DISCUSSION

Dynamics of polyphosphates during the growth of *H. salinarium*. The growth of *H. salinarium* in liquid medium with phosphate persisted for at least 7 days, with the most active period of growth between the first and fourth days (Fig. 1). The protein content of cells increased within the first four days and then slightly decreased (Fig. 1).

By the fifth day of growth, *H. salinarium* cells consumed up to 75% of phosphates from the medium. The orthophosphate content of cells gradually increased in the course of their cultivation with a parallel decrease in the concentration of orthophosphate in the medium, except for day 3 of growth, when the situation was quite the opposite (Fig. 2). The content of orthophosphate in 5-day-old cells comprised no less than one-third of its initial content in the medium (100 μmol P_i/g wet biomass).

The phosphates of *H. salinarium* cells could be separated into three fractions, known as acid-soluble, alkali-soluble, and acid-insoluble fractions [1, 10]. The acid-soluble fraction contained oligophosphates and pyrophosphate (the latter was detected only after

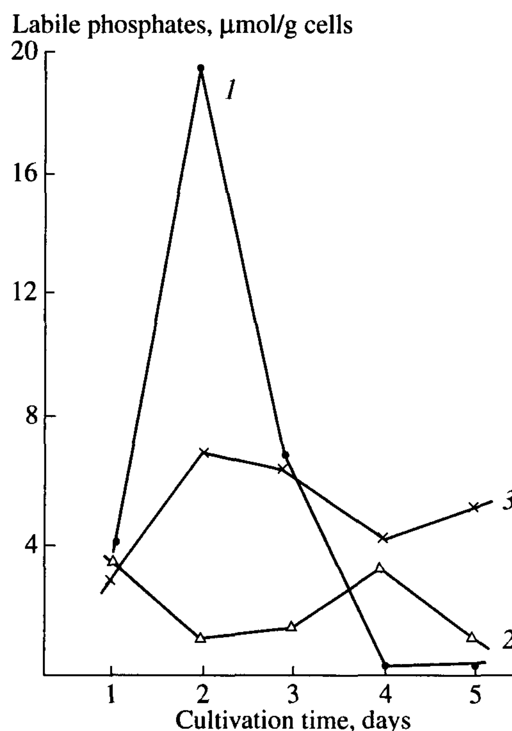

Fig. 3. Dynamics of different fractions of polyphosphates in *H. salinarium* cells in the process of their cultivation: *1*, acid-soluble polyphosphates; *2*, alkali-soluble polyphosphates; and *3*, acid-insoluble polyphosphates.

4–5 days of cultivation in amounts not exceeding 10% of the total content of labile phosphates of this fraction). Two other fractions contained high-molecular polyphosphates [1, 10].

Acid-soluble phosphates underwent considerable changes during the cultivation of *H. salinarium* (Fig. 3): their content was maximum (19.5 μmol P_i/g

Table 1. Effect of phosphate in the incubation medium of *H. salinarium* on phosphohydrolase activities

Parameter	Incubation time, h	Incubation medium	
		+P	−P
Wet biomass, g/l	16	3.4	3.8
	40	5.7	5.1
	64	6.8	5.8
Nonspecific phosphatase, mU/mg protein	16	1.4	4.9
	40	2.2	18.3
	64	2.2	20.6
Polyphosphatase, mU/mg protein	16	0.7	1.1
	40	1.1	1.8
	64	1.1	1.5
Pyrophosphatase, mU/mg protein	16	100	136
	40	408	194
	64	495	113

Table 2. Pyrophosphatase activity in different subcellular fractions of *H. salinarium* prepared from 1.065 g of wet biomass

Fraction	Protein, mg	Pyrophosphatase activity	
		U/mg protein	Total, U
Homogenate	48.0	0.43	20.6
Cell envelope (precipitate 1 after centrifugation at 5000 *g* for 10 min)	2.0	3.0	6.0
Membranes (precipitate 2 after centrifugation at 14000 *g* for 40 min)	5.8	0.24	1.4
Cytosol (supernatant after centrifugation at 14000 *g* for 40 min)	35.0	0.29	10.2

wet biomass) on the second day of cultivation and then considerably decreased. Changes in the fractions of alkali-soluble and acid-insoluble polyphosphates were much less pronounced: the total content of labile phosphates in these fractions ranged from 6.3 to 8.4 μmol P_i/g wet biomass throughout the cultivation period.

The cellular content of polyphosphates in the archaebacterium *H. salinarium* is close to that in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, which accumulate from 11 to 20.5 μmol P_i/g wet biomass [11], and in some bacteria, such as *Corynebacterium xerosis* and *Staphylococcus albus* [1].

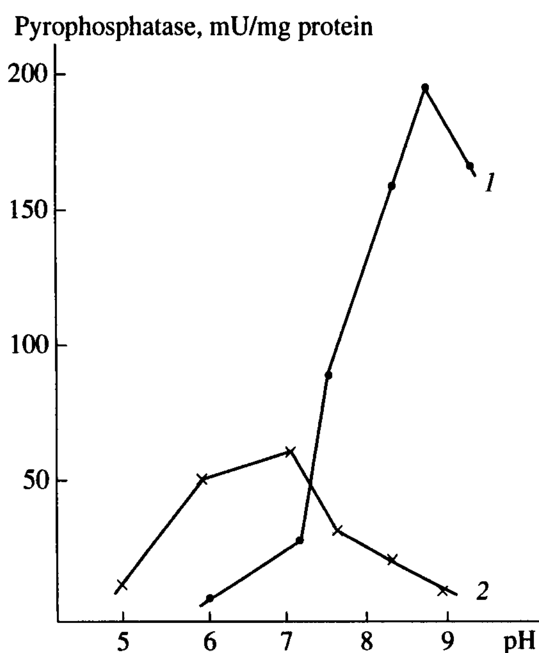

Fig. 4. Dependence of the cytosolic pyrophosphatase activity of *H. salinarium* on pH in the presence of (*1*) 10 mM Mg^{2+} and (2) 1 mM Co^{2+}.

However, the typical content of polyphosphates in eubacteria is 1–2 orders lower [1].

H. salinarium cells accumulated phosphates in the form of both ortho- and polyphosphates. On the fifth day of cultivation, the cellular concentration of orthophosphates was more than ten times higher than that of polyphosphates. The role of polyphosphates in archaebacteria is far from being well understood. Some authors believe that archaebacterial polyphosphates may be involved in the phosphorylation of proteins and in the regulation of glycogen metabolism [12, 13].

Phosphohydrolase activities during the growth of *H. salinarium*. The polyphosphatase and nonspecific phosphatase activities of *H. salinarium* cells changed very little throughout the cultivation period and did not exceed 4.8 and 2.6 mU/mg protein, respectively. At the same time, pyrophosphatase activity began to increase on the fourth day and peaked on the sixth day of cultivation, reaching 540 mU/mg protein (Fig. 1). On the fourth day, pyrophosphatase, polyphosphatase, and nonspecific phosphatase activities could be detected in the culture liquid. On the seventh day of cultivation, these activities were equally distributed between the culture liquid and cells.

Polyphosphatase activity in cells of *H. salinarium* was found to be lower than that in cells of *E. coli* [14], *Aerobacter aerogenes* (18 mU/mg protein) [15], *Acinetobacter johnsonii* (27 mU/mg protein) [16], and *S. cerevisiae* (48 mU/mg protein) [8]. The pyrophosphatase activity of *H. salinarium* cells was also lower than the pyrophosphatase activity of yeasts [8] and *E. coli* cells (4 U/mg protein) [17].

Effect of orthophosphate in the growth medium on phosphohydrolase activities. It is known that some phosphohydrolases are induced in cells grown in phosphate-free media [1]. In our experiments, *H. salinarium* cells grown for 3 days in a phosphate-containing medium (+P) were washed twice with a phosphate-free medium (–P), placed in media either containing phosphate (+P) or not (–P), and incubated at 37°C on a shaker. In both media, the increase in the biomass was almost the same (Table 1); however, the behavior of phosphohydrolases in these media was different. Polyphosphatase activity in the two media studied did not considerably change. At the same time, pyrophosphatase activity increased almost fivefold in the course of incubation in the (+P) medium and somewhat decreased during incubation in the (–P) medium. Nonspecific phosphatase activity was higher in the (–P) medium than in the (+P) medium by a factor of 3.5 after 16 h of incubation and by a factor of 9.4 after 64 h of incubation.

The different behaviors of phosphohydrolase activities during the growth of *H. salinarium* and its incubation in the phosphate-deficient medium indicate that several enzymes are responsible for these activities.

The alkaline phosphatases of some halobacteria have been isolated and characterized [5–7], but poly-

phosphatases and pyrophosphatases in these bacteria remain to be studied.

Some properties of the polyphosphatase activity of *H. salinarium*. The activity of yeast and eubacterial polyphosphatases strongly depends on bivalent cations and pH [8, 14]. The polyphosphatase of *E. coli*, which additionally depends on monovalent K^+ ions, hydrolyzes high-molecular-weight polyphosphates more readily than low-molecular-weight substrates [14]. Conversely, the polyphosphatase activity of *H. salinarium* depended only slightly on pH within a range of 6.0 to 8.4, exhibiting a small maximum at pH 7.2. Monovalent K^+, Na^+, and NH_4^+ ions, as well as bivalent Co^{2+}, Mg^{2+}, and Zn^{2+} ions, virtually did not stimulate this activity. Polyphosphates with average chain lengths of 9, 15, 25, 45, and 188 P were hydrolyzed at about equal rates. The high hydrolysis rate of triphosphate (18.5 mU/mg protein) can be explained in part by its nonspecific hydrolysis by pyrophosphatase [8].

Some properties of the pyrophosphatase activity of *H. salinarium*. Pyrophosphatase activity was the highest phosphohydrolase activity of *H. salinarium*. Table 2 illustrates the distribution of this activity among different fractions of 5-day-old *H. salinarium* cells. Precipitate 1, representing mainly cell-wall fragments, contained 29% of the total pyrophosphatase activity of the cell homogenate. The specific pyrophosphatase activity of this fraction was sevenfold higher than that of the homogenate. These data suggest that the pyrophosphatase of *H. salinarium* is localized in the cell envelope or on the cell surface. This suggestion is confirmed by the data showing that about 20% of the total pyrophosphatase activity of cells could be washed off from them with the growth medium without phosphate and peptone and that, after four days of cultivation, pyrophosphatase activity began to accumulate in the culture liquid. Pyrophosphatase activity could be easily extracted from precipitate 1 by washing it with 50 mM Tris–HCl buffer (pH 7.2) supplemented with 1 M KCl, 50 mM $MgSO_4$, and 10 mM dithiothreitol.

The pyrophosphatase activity of precipitate 2, which contained membrane fragments and probably bacteriorhodopsin, made up less than 7% of the total pyrophosphatase activity of the cell homogenate. At the same time, the cytosol fraction contained about 50% of the total pyrophosphatase activity of the cell homogenate. In this connection, further experiments were carried out using this fraction.

The optimum pH of cytosolic pyrophosphatase depended on the bivalent ion present in the incubation medium: the optimum pH was about 9.0 in the presence of Mg^{2+} and 7.2 in the presence of Co^{2+} (Fig. 4).

Table 3 summarizes data on the effect of different concentrations of Mg^{2+} and Co^{2+} on the pyrophosphatase activity. The optimum concentration of Mg^{2+} at pH 9.0 was 50 mM, and that of Co^{2+} at pH 7.2 was 1 mM. In the absence of these ions, the pyrophosphatase activity comprised no more than 2% of the maximum value.

Table 3. Effect of Mg^{2+} at pH 9.0 and Co^{2+} at pH 7.2 on the cytosolic pyrophosphatase of *H. salinarium*

Cation	Concentration, mM	Specific activity, mU/mg protein
Mg^{2+}	0	3.8
	0.5	7.3
	2.5	86.5
	10.0	171.0
	20.0	183.0
	50.0	195.0
	100.0	173.0
Co^{2+}	0	7.6
	0.1	9.2
	0.5	26.0
	1.0	69.0
	2.5	46.0

Table 4. Effect of monovalent cations on the pyrophosphatase activity of *H. salinarium* at different concentrations of Mg^{2+} (pH 9.0)

Salt added	Specific activity, mU/mg protein		
	Mg^{2+} concentration, mM		
	0	10	50
None	5.9	60	93
KCl, 200 mM	13.0	81	101
NaCl, 200 mM	11.0	85	101
NH_4Cl, 200 mM	16.0	–	125
Ammonium acetate, 200 mM	19.0	79	122

In the absence of Mg^{2+}, monovalent cations increased this activity by 2–3 times. The presence of Mg^{2+} ions in the incubation medium considerably diminished the effect of monovalent cations (Table 4).

Further experiments with the cytosolic pyrophosphatase of *H. salinarium* were performed by measuring its activity in a medium with pH 9.0 containing 200 mM KCl and pyrophosphate and Mg^{2+} ions in a proportion of 1 : 50. In this medium, the dependence of the reaction rate on the pyrophosphate concentration was described by the Michaelis–Menten equation. The effective K_m of pyrophosphatase with respect to pyrophosphate calculated by the Lineweaver–Burk method was found to be 115 μM.

Sodium fluoride, an efficient inhibitor of pyrophosphatases, suppressed the pyrophosphatase activity of *H. salinarium* by 44% at a concentration of 1 mM and almost completely at a concentration of 10 mM. Other inhibitors of phosphohydrolases (orthovanadate, sodium azide, and heparin), as well as iodoacetamide

and *N*-ethylmaleimide (modification reagents for –SH groups in proteins), failed to inhibit pyrophosphatase. In the presence of 50 mM Mg^{2+}, EDTA at concentrations of 1 and 10 mM stimulated pyrophosphatase activity by 40 and 60%, respectively. As shown earlier for the polyphosphatase of the yeast envelope [8], such an effect of EDTA can be due to the removal of heavy metal ions, which are inhibitory to many enzymes, from the incubation medium.

The molecular weight of the cytosolic pyrophosphatase of *H. salinarium* determined by gel filtration on Sephacryl S-300 was found to be 86 kDa. For comparison, the pyrophosphatase of *Sulfolobus acidocaldarius*, the only well-studied pyrophosphatase of archaebacteria, is a homohexamer with a molecular weight of subunits of about 20 kDa [18]. Eubacterial pyrophosphatases are also homohexamers with about the same molecular weights of subunits [19].

Yeast pyrophosphatases differ from prokaryotic pyrophosphatases in cellular location, molecular weight, and structure [20]. Generally, microbial pyrophosphatases are highly diverse and need further investigation.

Thus, pyrophosphatase is the most active phosphohydrolase of *H. salinarium*. The activity of this enzyme greatly varies in the course of cultivation and depends on the concentration of phosphate in the medium. *H. salinarium* cells consume phosphorus compounds from the medium and accumulate them in the form of ortho- and polyphosphates in amounts dependent on the growth phase. *H. salinarium* is a suitable object for studying the role of polyphosphates and respective enzymes in evolutionary ancient prokaryotes.

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