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EXPERIMENTAL ARTICLES

Dependence of the Intracellular Concentrations of Univalent Ions and Hydrogenase Activity on the Salt Composition and pH of the Medium in the Haloalkaliphilic Sulfate-Reducing Bacterium *Desulfonatronum thiodismutans*

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Abstract—It has been shown that the intracellular concentrations of Na^+, K^+, and Cl^- ions in *Desulfonatronum thiodismutans* depend on the extracellular concentration of Na^+ ions. An increase in the extracellular concentration of Na^+ results in the accumulation of K^+ ions in cells, which points to the possibility that these ions perform an osmoprotective function. When the concentration of the NaCl added to the medium was increased to 4%, the concentration gradient of Cl^- ions changed insignificantly. It was found that *D. thiodismutans* contains two forms of hydrogenase—periplasmic and cytoplasmic. Both enzymes are capable of functioning in solutions with high ionic force; however they exhibit different sensitivities to Na^+, K^+, and Li^+ salts and pH. The enzymes were found to be resistant to high concentrations of Na^+ and K^+ chlorides and Na^+ bicarbonate. The cytoplasmic hydrogenase differed significantly from the periplasmic one in having much higher salt tolerance and lower pH optimum. The activity of these enzymes depended on the nature of both the cationic and anionic components of the salts. For instance, the inhibitory effect of NaCl was less pronounced than that of LiCl, whereas Na^+ and Li^+ sulfates inhibited the activity of both hydrogenase types to an equal degree. The highest activity of these enzymes was observed at low Na^+ concentrations, close to those typical of cells growing at optimal salt concentrations.

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Haloalkaliphilic bacteria are exposed to high salt concentrations and pH. It is common knowledge that halophilic microorganisms accumulate K^+ ions or low-molecular-weight organic compounds (osmolytes) at high extracellular salt concentrations [1]. These compounds normalize cell turgor pressure by decreasing the osmotic gradient between the cell and the environment. At millimolar extracellular concentrations of K^+, its intracellular concentration may exceed the intracellular concentration of Na^+ by many times [2].

Most cytoplasmic enzymes of alkaliphiles remain active at neutral pH [3]; therefore, it is essential for these organisms to maintain intracellular pH near these values. Alkaliphilic organisms utilize the electrochemical H^+ (or Na^+) gradient for energy conservation. Alkaliphiles maintain such a ΔpH that the proton concentration in the cytoplasm is higher than in the medium. In these organisms, pH homeostasis is maintained due to the operation of electrogenic Na^+/H^+ antiporters, which provide for the Na^+ efflux and proton uptake [4].

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It has previously been reported that the involvement of molecular hydrogen in the generation of the proton gradient in some species of neutrophilic sulfate-reducing bacteria requires the presence of several hydrogenases with different cellular localizations [5]. Protons are reduced by cytoplasmic hydrogenase, and the produced molecular hydrogen diffuses through the cytoplasmic membrane to the periplasm, where one or several hydrogenases oxidize hydrogen to protons, accepting its electrons. The protons are used for ATP synthesis via a reversible ATPase. The electrons are passed through the cytoplasmic membrane to reduce endogenous or exogenous electron acceptors. A unique aspect of these processes is the transition of protons through the cytoplasmic membrane without the direct involvement of membrane-bound reductases or proton pumps.

The alkaliphilic and moderate halophilic sulfate-reducing bacterium *Desulfonatronum thiodismutans*, isolated from soda Mono Lake (United States), grows at NaCl concentrations of 1–7% (the optimum is 3%) and pH 8.0–10.0 (the optimum is 9.5) in carbonate–

bicarbonate media supplemented with formate or ethanol [6]. This organism is capable of chemolithotrophic growth with hydrogen according to the following reaction:

$$SO_4^{2-} + H^+ + 4H_2 \longrightarrow HS^- + 4H_2O \ (\Delta G^0 = -152 \text{ kJ/mol}).$$

The hydrogenase of sulfate-reducing hydrogen-utilizing bacteria is the main catabolic enzyme which catalyses the reversible reaction of hydrogen oxidation and reduction. The biochemical and bioenergetic functions of hydrogenases differ depending on the physiological properties of these bacteria. In haloalkaliphilic sulfate reducers, the enzyme, especially the periplasmic one, operates under conditions which are very close to those of the environment, where the Na^+ concentration is high and the proton concentration is extremely low.

We have previously demonstrated that the hydrogenases of haloalkaliphilic sulfate reducers from soda lakes are fairly tolerant to high Na^+ concentrations and extremely resistant to high pH values [7].

The goal of the present work was to investigate the concentrations of univalent ions in the *D. thiodismutans* cells as dependent on the extracellular Na^+ concentrations and pH values, as well as to study the localization of hydrogenases and their dependence on salt concentrations and pH.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The study was carried out using the type strain of *Desulfonatronum thiodismutans* MLF1^T (= ATCC BAA-395^T = DSM 14708^T) obtained from E.V. Pikuta. *D. thiodismutans* was cultivated under strictly anaerobic conditions at 37°C in a carbonate–bicarbonate medium (pH 9.5) containing (g/l) Na_2SO_4, 3.0; NaCl, 30.0; Na_2CO_3, 2.76; $NaHCO_3$, 24.0; KCl, 0.2; KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; NH_4Cl, 1.0; $Na_2S \cdot 9H_2O$, 0.4; 0.04% resazurin, 2 ml; yeast extract, 0.2; sodium formate, 5.0; and Whitman trace element solution [8], 1 ml. Oxygen was removed from the medium by boiling it and then bubbling with nitrogen for 15 min. $NaHCO_3$, yeast extract, and reducing agent were added to the medium after cooling. The reduced medium was dispensed into 500 ml flasks under a flow of nitrogen. The flasks were autoclaved at 121°C for 30 min.

The medium was inoculated with mid-logarithmic cultures in a proportion of 1–3 vol %.

Cell fractionation. To obtain the periplasmic and cytoplasmic hydrogenases, cells from 700 ml of a culture grown to the exponential phase were harvested by centrifugation at 16000 *g* at 4°C for 15 min in a Sigma 6K15 centrifuge. The cell pellet was then suspended in 5 ml of *Tris*–HCl anaerobic buffer (pH 7.8) containing 0.5 M sucrose, 5 mM EDTA, and 5 mM $MgCl_2$. The suspension was then supplemented with lysozyme (3.5 mg per 1 ml of the culture). Spheroplasts were formed after 4-h incubation at 37°C. Cell lysis was monitored microscopically. The resulting spheroplasts were separated from the periplasmic fraction by centrifugation at 9000 *g* for 20 min in a Jouan C3i centrifuge. The precipitated spheroplasts were resuspended in 5 ml of 0.05 M anaerobic Tris–HCl buffer (pH 9.5) containing 5mM $MgCl_2$ and 2 μg/ml DNase. The suspension was disrupted by ultrasound in a UZDN-1 0.063/22 disintegrator in four 3-min bursts at 22 kHz. After removal of cell debris by centrifugation at 9000 g, the cytoplasmic fraction was obtained [9]. Protein was quantified by the method of Lowry et al. [10].

Hydrogenase activity was determined according to the reduction rate of benzyl viologen with hydrogen measured at 37°C in thermostated anaerobic cuvettes at 600 nm with a Specord spectrophotometer (Germany). The reaction mixture (1.2 ml), which contained 1 ml of 0.05 anaerobic M Tris–HCl buffer, pH 9.2 (or another value when the effect of pH on hydrogenase activity was studied), 0.05 ml of 10 mM benzyl viologen, and 0.05 ml of 20 mM dithionite, was bubbled with oxygen-free hydrogen for 2 min and incubated for 10 min at 37°C, after which the reaction was started by the addition of 0.05 ml of the enzyme preparation.

To study the effect of salts on the hydrogenase activity, a series of 0.05 M anaerobic Tris–HCl buffer solutions (pH 9.5) containing different salt concentrations was used.

The effect of pH on the hydrogenase activity was studied using Tris–HCl buffers in which the pH was adjusted to the desired values by titration of the initial anaerobic buffer (pH 9.5) with oxygen-free 1 N HCl or 1 N NaOH.

Determination of the intracellular concentrations of Na^+, K^+, and Cl^- ions. To determine the intracellular concentrations of these ions, bacteria were grown in modified media which did not contain NaCl or were supplemented with 4% NaCl. To obtain a medium with pH 8.0, Na_2CO_3 was replaced with an equimolar amount of $NaHCO_3$; the medium was dispensed into vials and sterilized, and its pH was adjusted to 8.0 by the addition of sterile 1 N HCl.

An exponential-phase culture was centrifuged at 9000 *g* for 25 min in a Jouan C3i centrifuge. The supernatant was discarded, and the tube was dried with a piece of filter paper. The precipitated cells were resuspended in 10 ml of deionized water and subjected to ultrasonic disruption in a UZDN1-0.063/22 disintegrator in three 10-min bursts. Cell debris was removed by centrifugation at 9000 *g*, and the supernatant was used to determine the ion concentrations.

The concentration of inorganic ions was determined by high-performance liquid chromatography on a Staier chromatograph (Akvilon, Russia) with a conductometric detector. The determination of chloride ions was carried out in 3 mM carbonate buffer in an A 1.2 separation column (4.6 mm × 100 mm). The elution rate was 1.5 ml/min; the volume of the introduced sample was 20 μl. The determination of Na^+ and K^+ ions was carried out in a 2 mM HNO_3 solution in a C1P separa-

Ratios between the intracellular and extracellular concentrations of Na^+, K^+, and Cl^- in *D. thiodismutans*

Cultivation conditions of *D. thiodismutans*	Na^+_{extra} (M)	Na^+_{intra} (M)	$Na^+_{intra}/Na^+_{extra}$	K^+_{extra} (M)	K^+_{intra} (M)	K^+_{intra}/K^+_{extra}	Cl^-_{extra} (M)	Cl^-_{intra} (M)	$Cl^-_{intra}/Cl^-_{extra}$
NaCl, pH 9.5 0.46 M $[Na^+]_{total}$*	0.46	0.28	0.61	0.006	0.025	4.17	0.03	0.016	0.53
NaCl, pH 8.0 0.48 M $[Na^+]_{total}$*	0.44	0.23	0.52	0.007	0.03	4.29	0.04	0.009	0.23
NaCl, (4%) pH 9.5 1.15 M $[Na^+]_{total}$*	1.14	1.16	0.98	0.005	0.19	38	1.13	0.76	0.67

Note: Values averaged over no less than three experiments are presented.

*Theoretical concentrations calculated on the basis of the total content of sodium salts.

tion column (4 mm × 150 mm). The elution rate was 1.5 ml/min; the volume of the introduced sample was 20 μl.

The concentration of ions was determined using the formula: $C = (V_1C_1 - V_2C_2)/V_3$, where C is the concentration of the ion in cells, V_1 is the sample volume, C_1 is the concentration of the ion in the sample, V_2 is the volume of intercellular water, C_2 is the concentration of the ion in intercellular water, and V_3 is the volume of intracellular water.

The volume of intercellular water was determined with the use of blue dextran 2000 T, which does not penetrate cells. The cell suspension was precipitated by centrifugation at 5000 *g* and quantitatively transferred to centrifuge tubes dried to a constant weight. The total volume of intercellular and intracellular water was determined according to the difference in weight between wet cell mass and cell mass dried to a constant weight. To determine the intercellular volume, the precipitates were resuspended in 3 ml of a blue dextran solution (50 mg/ml). The resultant suspension was centrifuged at 5000 *g* for 20 min. The cell pellet was resuspended in 3 ml of distilled water and recentrifuged at the same settings. The dextran concentration in the supernatant was determined according to the calibration curve depicting the dependence of the optical density at 630 nm on the concentration of this polysaccharide. The intracellular volume was calculated as the difference between the total and intercellular water volumes [11].

The statistical analysis and graphical visualization of data were performed using the Adjacent average and B-Spline methods implemented in the Origin 7.5 software package.

RESULTS AND DISCUSSION

The intracellular concentrations of Na^+, K^+, and Cl^- ions in *D. thiodismutans* were determined (Table). The cells were grown in a medium that did not contain NaCl or contained 4% NaCl; the extracellular ion concentrations were compared with the intracellular ones.

It can be seen from the table that at a Na^+ concentration in the medium of 0.462 M, the intracellular Na^+ concentration was 0.28 M. With an increase in the Na^+ concentration in the medium to 1.15 M, the intercellular Na^+ concentration increased four times (to 1.16 M); i.e., the Na^+ gradient was eliminated due to equilibration of the intracellular Na^+ concentration with the extracellular one.

The intracellular K^+ concentration exceeded its extracellular concentration and increased by many times with an increase in the Na^+ content in the medium; the K^+ gradient increased by an order of magnitude.

At a Na^+ concentration in the medium of 0.462 M, the content of Cl^- in cells was relatively low (0.016 M). With an increase in the NaCl content in the medium, the intracellular Cl^- concentration was maintained at a level proportional to its content in the medium (Table). These results differ from those which we obtained earlier when studying haloalkaliphilic acetogens and denitrifiers [12, 13]. For instance, denitrifiers of the genus *Halomonas* do not accumulate Na^+, K^+ and Cl^-. In these organisms, organic osmolytes perform an osmoprotective function. In the acetogens *Tindallia magadiensis* and *Natroniella acetigena*, an increase in the NaCl content in the medium by 1.5–2 fold is accompanied by the accumulation of Na^+ ions; the intracellular and extracellular concentrations of Cl^- concentrations become equal, and the intracellular concentration of K^+ does not depend on the extracellular NaCl concentration.

An increase in the pH of the growth medium from 8.0 to 9.5 (the upper boundary for the growth of the bacterium under study) had practically no effect on the intercellular concentrations of Na^+, K^+, and Cl^- (table).

When considering the activity of the intracellular enzymes of halophilic eubacteria, especially their salt tolerance, it is necessary to emphasize that some of

them exhibit maximal activity in the absence of salts; in others, the peak activity is observed at moderate NaCl concentrations. However, many enzymes are not only capable of functioning at high salt concentrations but even require the presence of salts to maintain their stable state and activity [14–16].

We have previously demonstrated the presence of two types of hydrogenase—periplasmic and cytoplasmic—in *D. lacustre*. These hydrogenases differ in their activity and sensitivity to specific inhibitors [9]. The results on the fractionation of *D. thiodismutans* cells obtained in the present work show that *D. thiodismutans* also have hydrogenase activities in the periplasm (the maximum value was 16.5 μmol/(min mg of protein)) and in the cytoplasm (the maximum value was 3.5 μmol/(min mg of protein)).

The study of hydrogenase activity in the periplasm and cytoplasm of *D. thiodismutans* cells (pH 9.5) at various concentrations of NaCl, KCl, LiCl, $NaHCO_3$, Na_2SO_4, and Li_2SO_4 showed higher sensitivity of the periplasmic hydrogenase to these salts (see Figs. 1 and 2, where maximum activities of the enzymatic preparations were taken as 100%).

The maximum activity of the periplasmic hydrogenase occurred at 0.25 M NaCl and KCl. After that, it decreased by 60–70% and remained constant as the concentrations were raised from 1 to 4.27 M and 3.36 M (for NaCl and KCl, respectively) (Figs. 1a, 1b). LiCl had a profound inhibitory effect on the periplasmic hydrogenase as its concentration was raised from 0 to 2.5 M (Fig. 1c).

The nature of both the anion and cation influenced the hydrogenase activity. In the presence of the Li and Na sulfates, the maximum activity of the periplasmic hydrogenase was observed at low salt concentrations. After that, it decreased by 80% (Figs. 2b, 2c).

$NaHCO_3$ is the main mineral component of the growth medium. In this connection, we studied the effect of this salt on the enzymatic activity; its concentration was varied from 0 to 1.2 M. Both the periplasmic and cytoplasmic hydrogenases were resistant to bicarbonate; their peak activity was observed at 0.1–0.2 M of $NaHCO_3$ (Fig. 2a). We have previously obtained similar data on the two hydrogenase forms of *D. lacustre* [7].

The effect of chloride on the cytoplasmic hydrogenase activity was somewhat different. For instance, NaCl and KCl had a slight inhibitory effect on the enzyme as the concentrations of these salts increased (Figs. 1a, 1b). LiCl had a stimulatory effect on the cytoplasmic hydrogenase at concentrations from 0 to 0.5 M; in the presence of 2.5 M LiCl, the activity of the cytoplasmic hydrogenase comprised 30% of its maximum activity (Fig. 1c).

The effect of both anions and cations on the activity of hydrogenases is illustrated by the example of Li^+ and Na^+ sulfates. In the presence of Li^+ and Na^+ sulfates, the maximum activity of the periplasmic hydrogenase

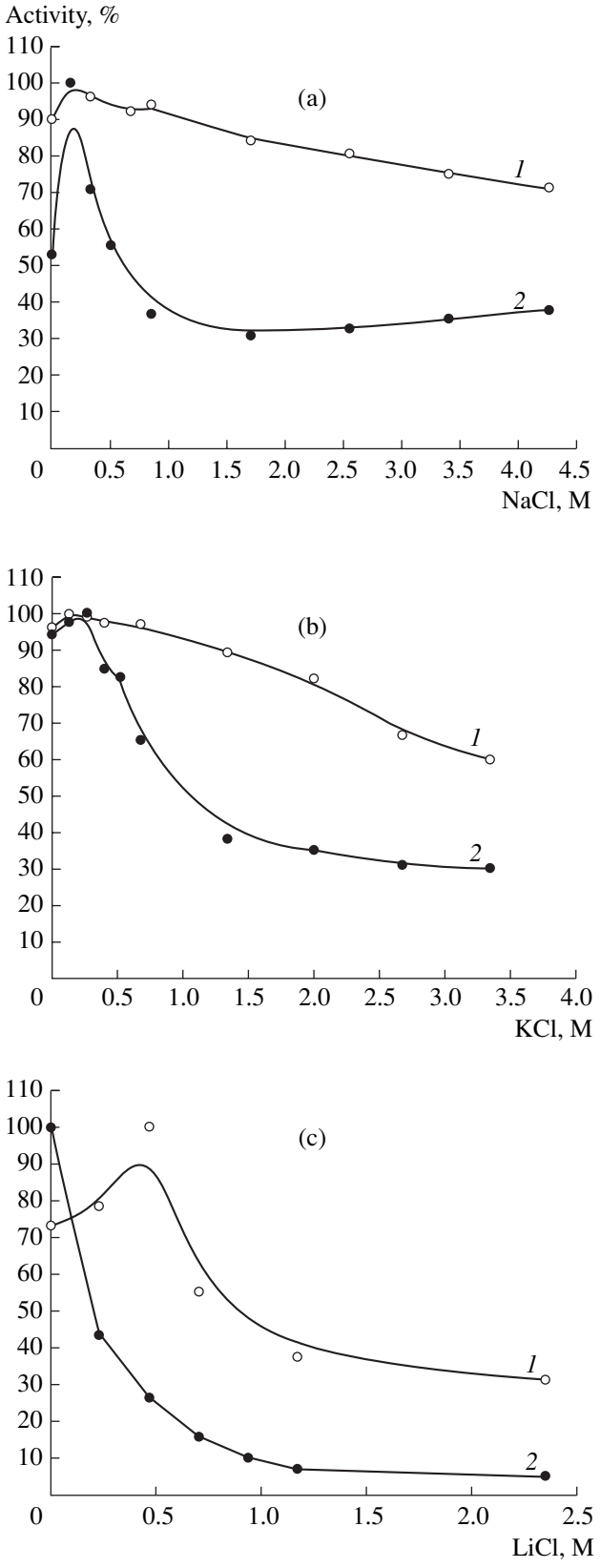

Fig. 1. Effect of (a) Na^+, (b) K^+, and (c) Li^+ chlorides on the activity of the (*1*) cytoplasmic and (*2*) periplasmic hydrogenases of *D. thiodismutans*.

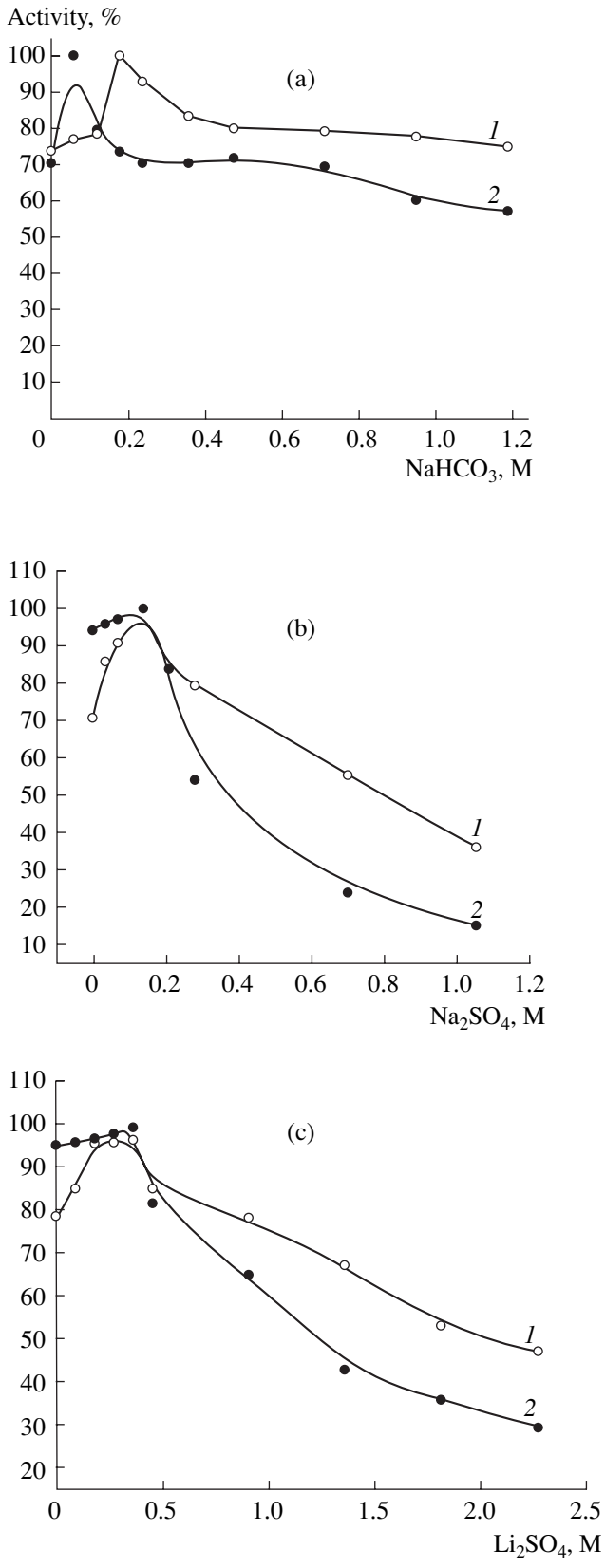

Fig. 2. Effect of (a) $NaHCO_3$, (b) Na_2SO_4, and (c) Li_2SO_4 on the activity of the (*1*) cytoplasmic and (*2*) periplasmic hydrogenases of *D. thiodismutans*.

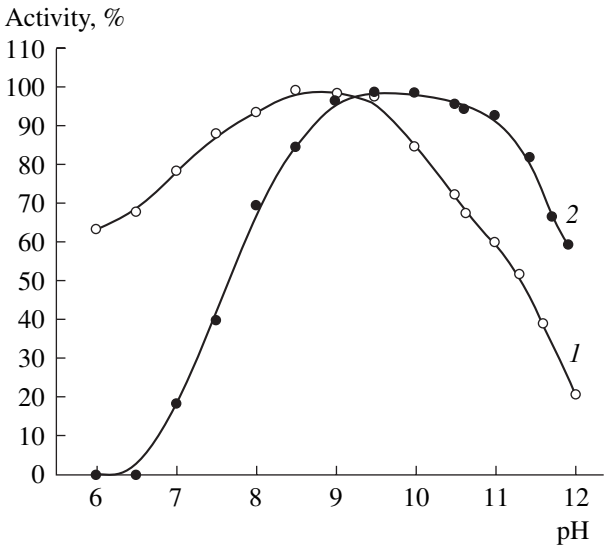

Fig. 3. Effect of pH on the activity of the (*1*) cytoplasmic and (*2*) periplasmic hydrogenases of *D. thiodismutans*.

was observed at low salt concentrations (Figs. 2a, 2b). However, these salts had a stimulatory effect on the cytoplasmic hydrogenase at Na_2SO_4 and Li_2SO_4 concentrations of 0–0.2 and 0–0.5 M, respectively. The activities of the periplasmic and cytoplasmic hydrogenases decreased at the maximum salt concentrations by 80 and 65%, respectively (Figs. 2b, 2c).

The study of the effect of pH on the activity of periplasmic and cytoplasmic hydrogenases showed that these enzymes are resistant to a wide range of pH values. The periplasmic hydrogenase remained active within the pH range of 7.0 to 12.0 and reached a maximum of its activity at pH 9.0–9.5, which roughly coincides with the pH growth optimum (Fig. 3).

The maximum activity of the cytoplasmic hydrogenase was observed at pH 8.0. The enzyme was active within a pH range of 6.0 to 12.0. The lower pH optimum of the cytoplasmic hydrogenase indirectly indicates that the intracellular pH in alkaliphiles is two–three points lower than the extracellular pH, as has been shown for alkaliphilic aerobes [4].

Thus, the intracellular concentrations of Na^+, K^+ and Cl^- ions in *D. thiodismutans* depend on extracellular NaCl concentrations. The multiple increase in the K^+ concentration that occurs as the extracellular Na^+ concentration increases points to a probable osmoprotective function of K^+ [17]. The apparent lack of equivalence between anion and cation concentrations in the cell most likely results from the binding of a portion of the cations with macromolecules and other constituents of the cell. Despite the relatively small content of intracellular ions, the periplasmic and cytoplasmic hydrogenases were stable in solutions with molar salt concentrations. The periplasmic hydrogenase was more sensitive to high concentrations of K^+, Na^+, and Li^+ salts. The activity of hydrogenases was dependent on the

nature of the salts. For example, Na^+ and Li^+ sulfates had a similar inhibitory effect on the activity of both types of hydrogenase. The maximum activity of hydrogenases was observed at low concentrations of the studied salts, which were close to the detected intracellular concentrations of Na^+, K^+, and Cl^- ions. The pH optimal for the cytoplasmic hydrogenase was 1.0–1.5 points lower than the pH optimal for the periplasmic hydrogenase.

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