Russian Journal of Plant Physiology, Vol. 47, No. 6, 2000, pp. 761–771. Translated from Fiziologiya Rastenii, Vol. 47, No. 6, 2000, pp. 865–876.

Functional Properties of Lactate Dehydrogenase from *Dunaliella salina* and Its Role in Glycerol Synthesis

E. A. Saburova*, N. V. Avseenko*, N. B. Simonova*, L. I. Elfimova, N. A. Pronina***, and V. E. Semenenko†, *****

** Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow oblast, 142292 Russia; e-mail: saburova@mail.ru*

*** Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow oblast, 142292 Russia*

**** Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya ul. 35, Moscow, 127276 Russia*

Received August 4, 1999

Abstract—The dependence of the catalytic properties of lactate dehydrogenase (LDH, EC 1.1.1.27) from a halophilic alga *Dunaliella salina*, a glycophilic alga *Chlamydomonas reinhardtii*, and from porcine muscle on glycerol concentration, medium pH, and temperature was investigated. Several chemical properties of the enzyme from *D. salina* differentiated it from the LDH preparation obtained from *C. reinhardtii* and any homologous enzymes of plant, animal, and bacterial origin. (1) V_{max} of pyruvate reduction manifested low sensitivity to the major intracellular osmolyte, glycerol. (2) The affinity of LDH for its coenzyme NADH dropped in the physiological pH region of 6–8. Above pH 8, NADH virtually did not bind to LDH, while the enzyme affinity for pyruvate did not change considerably. (3) The enzyme thermostability was extremely low: LDH was completely inactivated at room temperature within 30 min. The optimum temperature for pyruvate reduction (32°C) was considerably lower than with the enzyme preparations from *C. reinhardtii* (52°C) and porcine muscle (61°C). (4) NADH greatly stabilized LDH: the ratio of LDH inactivation constants in the absence of the coenzyme and after NADH addition at the optimum temperature in the preparation from *D. salina* exceeded the corresponding indices of LDH preparations from *C. reinhardtii* twelve times and from porcine muscle eight times. The authors believe that these LDH properties match the specific metabolism of *D. salina* which is set at rapid glycerol synthesis under hyperosmotic stress conditions. The increase of cytoplasmic pH value produced in *D. salina* by the hyperosmotic shock can switch off the terminal reaction of the glycolytic pathway and thus provide for the most efficient utilization of NADH in the cycle of glycerol synthesis. As LDH is destabilized in the absence of NADH, this reaction is also switched off. In the course of alga adaptation to the hyperosmotic shock, glycerol accumulation and the neutralization of intracellular pH stabilize LDH, thus creating the conditions for restoring the complete glycolytic cycle.

Key words: Dunaliella salina - lactate dehydrogenase - kinetics - glycerol synthesis

INTRODUCTION

As compared to other extremely halophilic organisms, in particular bacteria, the unicellular alga *Dunaliella salina* possesses a unique ability to survive a wide range of salt concentrations (0.1 to 5.5 M NaCl) in the external medium [1], while extremely halophilic bacteria perish at 1.5 M NaCl [2]. Such a unique capacity for adaptation is facilitated by the synthesis of glycerol: at high intracellular concentration, glycerol counterbalances the high osmotic pressure of the medium. Glycerol synthesis in *D. salina* starts by starch degradation to dihydroxyacetone phosphate, and the subsequent steps of glycerol synthesis and degradation are mediated by several enzymes [3, 4].

What is the trigger of rapid glycerol synthesis and dissimilation (glycerol is completely synthesized or degraded in about 30 min [1])? This question is among the basic issues of osmoregulation in *Dunaliella.* Due to the biochemical mechanisms of osmoregulation, when many enzyme activities are highly dependent on pH value, the changes in intracellular pH values regulate the direction of enzyme reactions engaged in the cycle of glycerol synthesis and degradation. Thus, in the illuminated cells of *D. parva*, *D. acidophila*, and *D. tertiolecta*, the cytoplasm is alkalized by 0.5 pH unit, whereas the shift of 0.2 pH units was produced by saline stress both in the light and darkness [5, 6]. Meanwhile many enzymes are known to respond to minute functional pH fluctuations (0.2 to 0.4 units) when nonspecific changes of protein solubility in the water–

† Deceased.

Abbreviations: DHAK—dihydroxyacetone kinase; GAPDH—glyceraldehyde phosphate dehydrogenase; GPase—glycerol phosphatase; GPDH—glycerol phosphate dehydrogenase; LDH—lactate dehydrogenase.

1021-4437/00/4706-0761$25.00 © 2000 MAIK "Nauka / Interperiodica"

organic solutions alter the equilibrium between the free soluble enzyme and the enzymes bound to membranes or compartmentalized in the enzyme complexes [7]. Moreover, pH values affect the steric configuration of the active enzyme center and thus determine its affinity for substrate [8]. To illustrate, let's take a well-known case of α-stat regulation by the substrate semisaturation characteristic of some enzymes, including LDH, regardless of the intracellular conditions [9]. This mechanism of LDH control was demonstrated in mammals and fish [9]. However, there are no data for the catalytic properties of LDH from *D. salina* cells. Also, we do not understand the role of glycolytic enzymes in glycerol synthesis, nor the glycerol effects on the catalytic properties of glycolytic enzymes.

The presence of osmolytes (polyols and sugars) in the cell helps maintain its osmotic status. These compounds are traditionally regarded as neutral osmolytes because they do not participate in intracellular chemical reactions [1, 9]. The previous reports [10–16] demonstrated that both polyols and sugars might regulate catalytic processes by creating the optimum conditions for enzyme functioning under conditions of osmotic stress.

Our goal is to elucidate the molecular mechanisms of algae adaptation to the extremely high saline concentrations in their habitats. We focused the present study on the catalytic properties of LDH from the halophilic alga *D. salina* as compared to LDH from a nonhalophilic alga *C. reinhardtii*, which belongs to the same class of algae. Two other tasks of this study were to clarify the role of glycerol as the major intracellular osmolyte in stabilizing the cytoplasmic enzymes of *D. salina* and to investigate whether the halophilic algae manifested the phenomenon of α-stat regulation.

MATERIALS AND METHODS

Plant material. We investigated two species of algae belonging the same class, Chlorophyceae: an extremely halophilic *Dunaliella salina* Teod. (strain IPPAS D-209 from the IPPAS collection of microalgae at the Institute of Plant Physiology, RAS), which produces glycerol as an intracellular osmolyte, and a nonhalophilic *Chlamydomonas reinhardtii* Dang (strain IPF $K^+(137^+)$ from the collection of microalgae at the Institute of the Fundamental Biological Problems, RAS [17]). For further comparison of algal to mammal cells, porcine muscle was used.

D. salina cells were grown and monitored as described in [17] in a medium containing 2.5 M NaCl with a pH of about 7.5 maintained throughout the course of culturing. *C. reinhardtii* cells were grown as described in [18] in a medium containing 0.1 M NaCl with the pH maintained at 7.5.

Cell-free extract preparation. Cells of *D. salina* washed preliminarily with 0.9 M NaCl were broken down at 0°C in the medium containing the 0.1 M Na–phosphate buffer, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.2 mM NADH, and the inhibitors of protease activities, 1 mM phenazine methosulfofluoride, 1 mM benzomidin, and 5 mM aminocaproic acid. The homogenate was centrifuged for 15 min at 25000 *g*, and the supernatant was used for assessing LDH activity. The enzyme preparation diluted with glycerol by 50% (w/v) maintained its activity for three weeks when kept at –9°C. The LDH preparation from *C. reinhardtii* cells was obtained following the same procedure, except that NADH was omitted from the homogenizing medium.

LDH from porcine skeletal muscle (isoenzyme M_4) was isolated as described in [19]. The specific activity of the electrophoretically homogeneous preparation was 320 μmol NADH/(min mg protein) at 25°C and pH 6.4.

Measurements of LDH activity. LDH activity was determined by the rate of pyruvate to lactate reduction. The changes in the optical density at 340 nm (NADH absorption range) were followed using a Specord M40 spectrophotometer (Germany) in a thermostatic cell against the control without pyruvate. Except in the experiments stipulated below, the enzymatic reaction was started with an aliquot of the cell-free extract. The activity is presented as the change in the optical density at 340 nm per min. The kinetic parameters, the maximum rate, and the Michaelis constant, were calculated from $1/V$ by $1/S$ dependence using the linear regression equations. The points on the curves and their bars correspond to the means and their standard errors from four or five experiments.

Measurements of LDH inactivation rate. To estimate the constant of LDH (apoenzyme) inactivation, we measured LDH activity in the buffer solution as related to the period of enzyme exposure to the assigned pH and temperature values. Fifty microliters of the cell-free extracts in the 0.1 M Na–phosphate buffer, pH 7.5, were added to 1.8 ml of the same buffer at the particular pH and exposed to the particular temperature for the assigned period. Next, the mixture was rapidly cooled to 20°C, NADH and pyruvate were added to the corresponding final concentrations of 0.2 and 1 mM, and LDH activity was measured. The exposure ranged from one to ten minutes, with a one-minute interval. The data were plotted as a dependence of activity on exposure. This dependence could be described as the one-step irreversible enzyme transition of the native enzyme N into the denatured form D. The constant of inactivation k_{in} was calculated from an exponential approximation of N to D transition. The rate of holoenzyme (E-NADH) inactivation was estimated in the same way, except that NADH was preliminarily added to the incubation medium to the final concentration of 0.2 mM. The inactivation of the ternary complex (E-NADH–pyruvate) was followed directly in the spectrophotometer cell in the course of measuring the activity of the enzymes. In this case, the cell-free extract was added to the reaction mixture, thus starting

the reaction of pyruvate reduction. Enzyme inactivation was followed by a decrease in the slope of the product–time curve (without inactivation, this slope did not change for several minutes). In this way, the time-related decline of the slope (decrease in the first derivative of the product–time curve) characterized enzyme inactivation. The selected concentrations of NADH and pyruvate were high enough to saturate the enzyme in the course of the whole inactivation period, and under these conditions, the change in the pyruvate reduction rate corresponds to the change in the catalytic stage of the enzyme reaction, that is, V_{max}.

Measurements of the reversibility of glycerol effects on the kinetic parameters of the LDH reaction. These measurements were performed by diluting the reaction mixture with a volume equal to the initial volume of the substrate solution (10 mM pyruvate and 0.02 mM NADH in 0.1 M phosphate buffer, pH 7.5) without glycerol and determining the enzyme activity. The measurements thus obtained were doubled to account for the diluted enzyme concentration. As a result, we found that dilution did not change the adjusted rates of pyruvate reduction by more than 5%. This evidence shows that glycerol effects on LDH preparations from two algae were completely reversible.

Viscosity of glycerol solutions of various concentrations was determined from the reference tables as described in [12].

Measurements of pH values were performed before and following the determinations of LDH activities using a PH-340 pH-meter (Russia) to the precision of 0.01 pH unit. The pyruvate and NADH solutions were preliminarily brought by alkali and acid to the particular pH level used for an inactivation experiment. Starting the enzymatic reaction with 30 to 50 µl of the cell-free extract, pH 7.5, slightly shifted the pH of the reaction mixture, and therefore the final pH value was determined after assessing the enzyme activity.

RESULTS

Glycerol Effects on LDH Activity

The study of glycerol effects on pyruvate reduction to lactate catalyzed by LDH from *D. salina* (Fig. 1) showed that glycerol affected both the maximum reaction rate (V_{max}) and pyruvate affinity of the enzyme ($1/K_M$). Similar curves were obtained to describe the pyruvate-reducing activity of LDH from *D. salina* as depending on NADH concentration and to relate LDH activity from *C. reinhardtii* to NADH and pyruvate concentrations. Substrate inhibition of LDH, characteristic of the porcine muscle enzyme [11] at pyruvate concentrations exceeding 3–4 mM, was not observed in algal LDH preparations at pyruvate concentrations as high as 20 mM, with and without glycerol. All curves fit well into the Michaelis–Menten kinetics, and therefore we could calculate K_M and V_{max} values for both LDH preparations. As glycerol concentration increased, the LDH affinity for pyruvate increased (K_M values decreased) in both preparations (Table 1). The value of V_{max} was most evidently affected by glycerol: at 63% glycerol concentration, the V_{max} value declined more than threefold in *D. salina* and fivefold in *C. rein-*

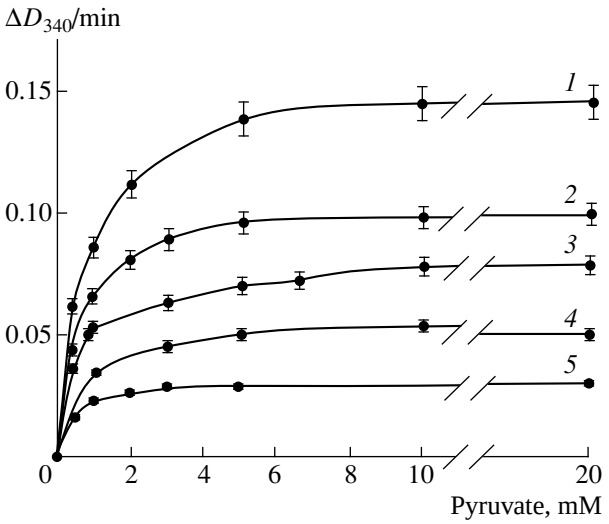

Fig. 1. The activity of the LDH preparation from *D. salina* as related to pyruvate and glycerol concentrations.
(*1*) Without glycerol; (*2*–*5*) respectively, 12.5, 25, 50, and 63% (w/v) glycerol in 0.1 M phosphate buffer, pH 7.5, containing 0.2 mM NADH.

Table 1. The kinetic constants of pyruvate and NADH binding to the LDH from *D. salina* and *C. reinhardtii* as related to the glycerol concentration in the reaction medium

Glycerol, % (w/v)	K_M, mM		V_{max}, %
	pyruvate	NADH	
D. salina			
0	0.70 ± 0.07	0.048 ± 0.007	100
12.5	0.55 ± 0.06	–	95
25	0.42 ± 0.04	–	75
33	0.36 ± 0.04	–	66
50	0.26 ± 0.03	–	48
63	0.18 ± 0.02	0.034 ± 0.005	32
C. reinhardtii			
0	0.65 ± 0.07	0.030 ± 0.005	100
12.5	0.53 ± 0.05	–	78
25	0.42 ± 0.04	–	58
33	0.30 ± 0.03	–	50
50	0.20 ± 0.03	0.039 ± 0.005	30
63	0.09 ± 0.02	–	18

Note: NADH concentration for measuring K_M for pyruvate was 0.2 mM; pyruvate concentration for measuring K_M for NADH, 10 mM; mean K_M values were calculated from four replicates; – means no data available.

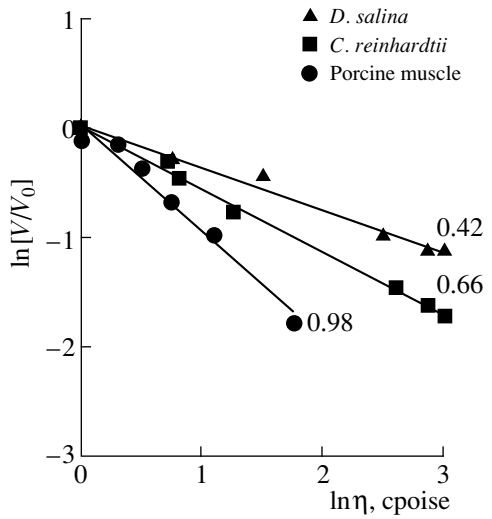

Fig. 2. The rate of pyruvate reduction catalyzed by the LDH preparations from various organisms as related to the viscosity of the medium.

(V_0 and V) The rates of reduction in the absence and presence of glycerol; (η) viscosity. The numbers at the curves are δ values.

hardtii LDH. The special experiments established that the decrease in V_{max} in both cases was completely reversible (see the Materials and Methods section).

Previously we investigated the catalytic properties of porcine muscle LDH in several organic solvents varying in viscosity and dielectric conductance and demonstrated that viscosity considerably affected the enzymatic activity limited by the stage of conformational changes accompanying the catalysis [12].

V_{max} as the Function of Viscosity

The analysis of our data (Fig. 2) showed that V is the function of viscosity of the reaction medium. This function is described with the Kremers equation

$$k = A\eta^{-\delta}e^{-E_a/RT},$$

where k is the rate constant and E_a is the activation energy of the pyruvate-reducing reaction, R is the universal gas constant, T is the Kelvin temperature, and A and δ are empirical constants. With V proportional to the rate constant of the catalytic step (when the reaction is saturated with substrates), the Kremers equation can be presented as

$$V(\eta) = A^*\eta^{-\delta}e^{-E_a/RT}$$

or, in the logarithmic form,

$$\ln V(\eta) = \ln A^* - \delta\ln\eta - E_a/RT.$$

At the constant temperature, the former equation is transformed into

$$\ln V(\eta) = \ln V_0 - \delta\ln\eta,$$

where $\ln V_0 = \ln A^* - E_a/RT$ is a constant coefficient independent of viscosity and equal to the logarithm of the reaction rate in the absence of glycerol at $\eta = 1$, whereas δ is the slope of the line describing the reaction rate vs. viscosity in dual logarithmic coordinates.

The slopes of the graphs in Fig. 2 were used to estimate the δ values (0.42 and 0.66 for the LDH preparations correspondingly from *D. salina* and *C. reinhardtii*, as compared to the index of 0.98 previously determined for the porcine muscle preparation [12]).

The effect of viscous solutions on the reaction rate was completely reversed when glycerol-containing media were diluted with the buffer. It follows that the multiple glycerol effects comprise of an enhanced affinity for pyruvate (K_M declined as the glycerol concentration increased) and of enzyme inhibition due to an almost twofold k_{cat} decrease (V_{max} dropped at 33% glycerol concentration in the reaction mixture, see Table 1). These data demonstrated that glycerol, as an osmotically active substance, is genuinely neutral, that is, it does not considerably affect the rates of enzyme-catalyzed reactions. However, such a neutral effect is derived from summing up two reciprocal trends (the increased substrate affinity and the decreased V_{max}). The comparison of the LDH preparations from two algal species differing in their tolerance to saline stress showed that the glycerol effect was the lowest in halotolerant *D. salina*, with the neutral effect of glycerol most evident both when LDH was saturated with the substrate and under the low physiological pyruvate concentrations.

pH Effects on LDH Activity

K_M values for pyruvate and NADH presented in Figs. 3a and 3b were calculated from the curves describing LDH saturation with the corresponding substrates under the steady-state kinetics and varying pH conditions. With two algal preparations of LDH, K_M values for pyruvate were not affected until the pH level reached 8.9 (Fig. 3a). In contrast, K_M values for NADH dramatically increased in *D. salina* LDH preparation when the pH value exceeded 8, whereas with *C. reinhardtii* preparation, K_M values were not affected by a pH as high as 10. The pH-dependencies of the K_M values for pyruvate and NADH in the preparation from *D. salina* were similar to those of the K_M value for pyruvate in the muscle LDH [11]. It is noteworthy that in the latter case, the K_M value for pyruvate depends on ionization of His195, which binds pyruvate in the enzyme active center at pK of about 7.5 to 7.7, and is a mechanism tuning mammalian cell metabolism [9]. The dramatic decrease in the affinity for NADH characteristic of *D. salina* LDH preparation at the neutral pH values is quite unique and was not observed with the mammalian preparations [20] or the enzyme from the nonhalophilic alga *Chlamydomonas.* We therefore presume that the pH-stat regulation of LDH activity in

D. salina depends on the modulated affinity for NADH rather than pyruvate as in the mammalian preparations.

The V_{max} values of LDH from *D. salina* did not change within pH 6–8 and decreased at higher pH values (see Fig. 5 below) due to complete and irreversible protein denaturation.

Effect of Temperature on LDH Activity

Using LDH preparations from *D. salina* and *C. reinhardtii*, we established the optimum conditions for enzyme functioning in a wide range of temperatures and investigated the effects of glycerol–water solutions on the temperature-dependent enzyme activities. The enzyme from *D. salina*, which is functional *in vivo* in the presence of 4–5 M glycerol, was the least thermostable, independent of the presence of glycerol. The optimum temperature for the *D. salina* preparation (31°C) was considerably lower than the corresponding index for *C. reinhardtii* (52°C). Glycerol decreased V_{max} and practically did not affect the value of T_{opt}. Such decrease in enzyme activity in the presence of glycerol resulted from the higher viscosity of the medium constraining the conformational changes in the functional enzyme molecule, rather than from enzyme degradation (Fig. 2). This conclusion was drawn from the parallel studies of the various stages of the reaction catalyzed by porcine muscle LDH when affected by organic solvents that changed the viscosity of the reaction medium [12]. Thus, V_{max} decrease in glycerol solutions does not mean reduced enzyme stability. The effect of glycerol stabilizing the protein structure against various denaturing agents is well known. Previously, we showed [13] that judging by the constant of the inactivation rate, glycerol did stabilize proteins; however, this effect was observed only at room temperature. The stabilizing effect of glycerol was the lowest in the *D. salina* preparation and the highest with porcine LDH. It is noteworthy that, at higher temperatures (over 50°C), glycerol no longer stabilized proteins; on the contrary, it denatured proteins. Apparently this phenomenon explains the fact that the optimum temperature indices of the enzymes under study were not affected by glycerol (Fig. 4).

The data illustrated in Fig. 4 showed that under the supraoptimum temperatures, both enzyme preparations were rapidly inactivated. The thermal inactivation at these temperatures was accompanied with the disruption of enzyme secondary structure, as shown in the porcine muscle LDH preparation investigated by circular dichroism at the optical band of peptide group absorption [13]. We believe that similar processes were characteristic of the two algal LDH preparations, as the enzyme inactivation at the temperature exceeding T_{opt} was irreversible in all three LDH preparations under study.

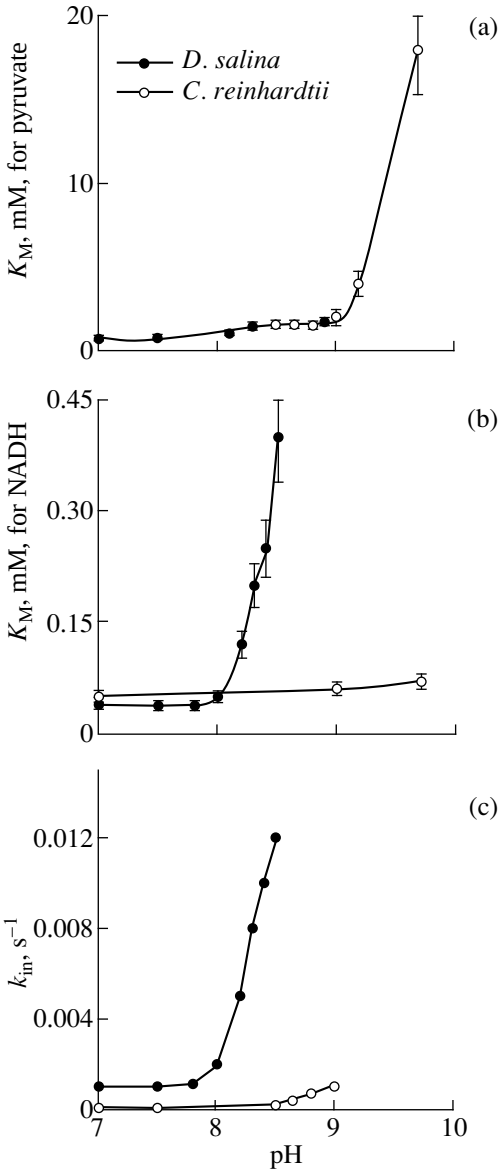

Fig. 3. The Michaelis constants of binding (a) pyruvate and (b) NADH to LDH as depending on pH, and also (c) the constants of inactivation rates for the LDH preparations from *D. salina* and *C. reinhardtii*.

The values of k_{in} were calculated from the data presented in Fig. 5.

The Effect of the Medium pH on the LDH Stability to Thermal Denaturation

The product–time exposure curves, at various pH values for the LDH preparation from *D. salina*, demonstrated that at pH 7.5, this dependence was linear within the whole period of the measurements (Fig. 5). This means that the reaction rate was steady for at least 2–3 min. At pH 8 and above, the linear dependence was broken, and the slope of the curves decreased in the experiments with the *D. salina* preparation, while, with the enzyme from *C. reinhardtii*, the reaction rates were not changed within the whole pH range for a 3-min period (the data

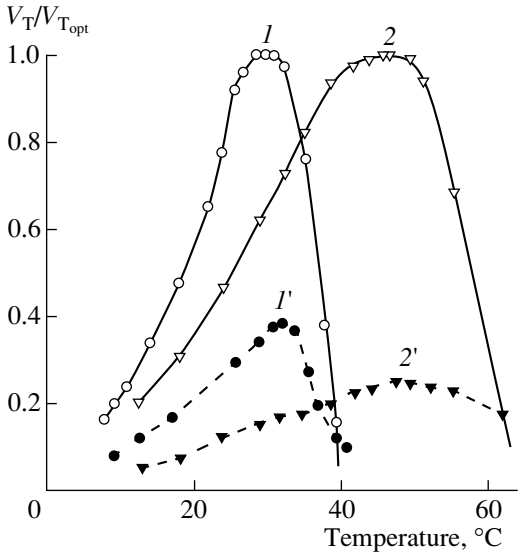

Fig. 4. The relative activities of the LDH preparations from (*1*, *1*') *D. salina* and (*2*, *2*') *C. reinhardtii* as related to the temperature (*1*, *2*) in the absence and (*1*', *2*') in the presence of glycerol (63%, w/v).

At temperatures exceeding T_{opt}, the reaction rate decreased during the measurement period, and therefore V_T values were determined within the initial 10-s period, $V_{T_{opt}}$, the reaction rate at the optimum temperature.

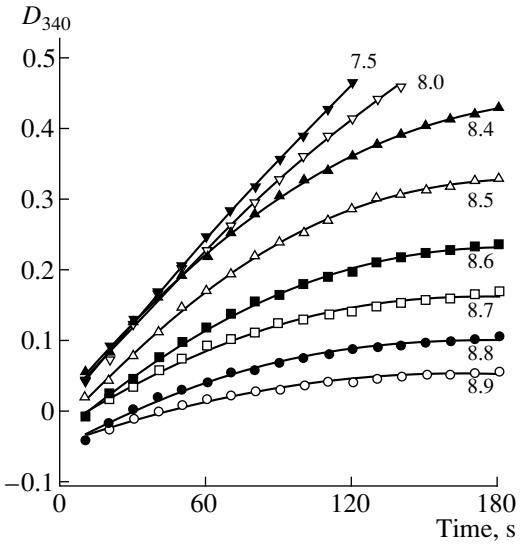

Fig. 5. Pyruvate–lactate transformation at 20°C catalyzed by the LDH preparation from *D. salina* depending on pH (pH values shown at the curves).

The constants of inactivation rates calculated by the exponent approximation of the first derivative of these curves are presented in Fig. 3c. The reaction medium included 0.1 M phosphate buffer, pH 7.5; 10 mM pyruvate; and 0.2 mM NADH.

are not given). The analysis of the data derived from these curves at each point of time vs. exposure showed that the reaction rate declined with time, that is, the enzyme was inactivated during the period of measurements. Using the exponential approximation of this decline vs. time, we estimated the exponents corresponding to the constants of the enzyme inactivation rate, k_{in}, as the pH function. These data for the LDH preparation from *D. salina* are presented in Fig. 3c. The value of k_{in} was low in the neutral pH region and dramatically increased above pH 8. This enzyme inactivation observed with the *D. salina* preparation was absent in the case of LDH from *C. reinhardtii* (Fig. 3b) and other plant cells [10]. Above pH 8, LDH from *D. salina* was completely inactivated following 30-s exposure at 21°C, even in the presence of the coenzyme.

We investigated the reversion of LDH inactivation when the value of pH was returned back from alkaline to neutral (so-called pH shock, see Materials and Methods). Such experiments were carried out at the very beginning of the enzyme reaction and following several time intervals. To do this, we suspended monitoring of the reaction and diluted the reaction mixture twofold to bring the pH down to 7.5. In all cases, that is, at the beginning and the end of the reaction, the reaction rate when calculated to account for protein content did not increase notably, that is, did not exceed the initial value at pH 7.5 by more than 10%. This shows that the alkaline denaturation was practically irreversible.

The question is what process would destabilize LDH above pH 8? Either ionization of amino acid residues into an electrostatic pattern unfavorable to the native enzyme structure, or the dissociation of the coenzyme (at pK 7.8) could make LDH unstable. The second hypothesis seems more probable as the pH dependency for K_M for NADH and the pH dependency for k_{in} coincide (cf. Figs. 3b and 3c). In addition, we will show below that NADH binding to the LDH apoenzyme from *D. salina* decreased the constant of the inactivation rate by almost an order of magnitude, that is, it considerably stabilized the enzyme. This evidence also supports the idea that the pH-dependent inactivation of the enzyme preparation from *D. salina* is determined mainly by the pH dependence of the enzyme affinity for NADH: a pH increase resulted in NADH dissociation from the enzyme, with the complete loss of the latter's activity.

The Effect of LDH Substrates on the Enzyme Stability to Thermal Denaturation

Inactivation curves were obtained for the LDH preparations from the two algae by following the enzyme activity after the enzyme preparations were incubated at the corresponding temperatures (Fig. 6). The inactivation rate constants, calculated by the exponent approximation of the first derivative of these curves, are presented in Table 2. NADH addition to the reaction mixture considerably stabilized the enzyme from *D. salina* by bringing down the inactivation rate constant

from 0.45 to 0.037 min^{-1} (Table 2, Fig. 6a, curves *1*, *2*). The subsequent pyruvate addition did not considerably change the stability of the enzyme–NADH complex (Fig. 6a, curve *3*). In contrast, when NADH was added to the LDH preparation from *C. reinhardtii*, it did not enhance the stability of the enzyme protein, but rather the stability slightly diminished (Fig. 6b, curves *1*, *2*), whereas when both NADH and pyruvate were added, LDH in this triple complex was stabilized (Fig. 6b, curve *3*), and k_{in} at 56°C dropped from 0.116 min^{-1} in the apoenzyme to 0.058 min^{-1} in the ternary complex (Table 2).

Within the whole range of temperatures under study, denaturation of the LDH preparations from the two algae is described by the same exponential equation. Therefore, we could calculate the inactivation rate constants as the slopes of ln activity against time curve. Using k_{in} values thus obtained, the values for activation energy for the free enzyme E_A^*, the holoenzyme E_A^*(NADH), and the ternary complex E_A^*(NADH + pyruvate) were calculated from the Arrhenius plots. The evidence presented in Table 3 and Fig. 6 shows that the formation of the dual protein–NADH complex plays the major role in stabilizing LDH from *D. salina*, whereas in the case of the LDH preparation from *C. reinhardtii*, only the ternary complex formation could partially stabilize the enzyme against thermal denaturation. Our data on LDH stabilization by NADH and on pH dependence of the enzyme affinity for NADH presume that, in the case of *D. salina*, the pH control of LDH activity is carried out by modulating the enzyme affinity for NADH and, as a result, by changing its activity, while the activity of enzyme preparations from *C. reinhardtii* and mammalian cells are attuned by an affinity for pyruvate.

The data presented in Tables 2 and 3 show that NADH binding enhanced the enzyme thermostability in the case of LDH from *D. salina*, while the stability of LDH preparations from *C. reinhardtii* and porcine muscle [10] was not affected. In the latter case, only pyruvate binding with the formation of the ternary enzyme–NADH–pyruvate complex could stabilize these enzyme preparations against the effects of temperature.

The stabilizing effect of NADH is a specific property of LDH from *D. salina* (Fig. 6). This conclusion does not exclude the possibility of the existence in the algal cell of other, as yet unidentified metabolites protecting the enzyme protein from thermal inactivation.

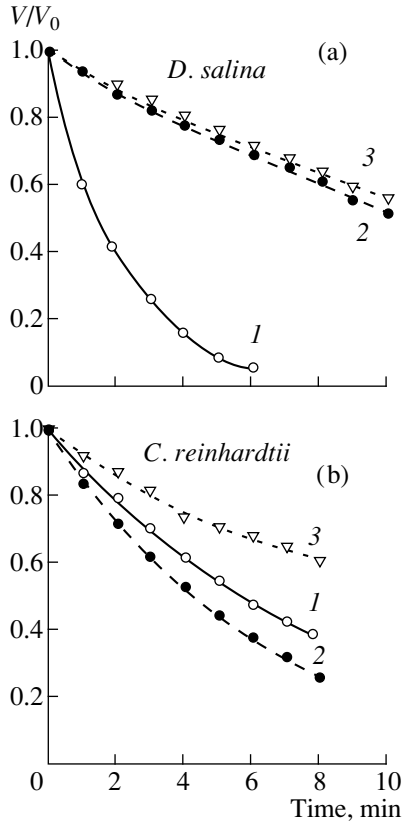

Fig. 6. Effect of substrates on the kinetics of thermal inactivation of the LDH preparations from (a) *D. salina* and (b) *C. reinhardtii* at 30 and 56°C, respectively.
(*1*) Apoenzyme; (*2*) enzyme + 0.025 mM NADH; (*3*) enzyme + 0.25 mM NADH + 10 mM pyruvate. The reaction mixture as in Fig. 2.

DISCUSSION

Dehydrogenases comprise the class of enzymes most conserved in their structure and functions across all three kingdoms of plants, animals, and microorganisms [14–16]. The conserved structure of the active center, the configuration of the polypeptide backbone, and the regulatory centers binding the allosteric effectors make these proteins especially valuable for comparative studies of their functions in the cells of organisms adapted to extreme habitats during the process of evolution. At present, the kinetic properties of LDH from the halotolerant alga *D. salina* are not known, due to the extreme instability of the enzyme. We found a way to enhance LDH stability in cell-free extracts to a level where one can assess some of its catalytic parameters, and evaluate the effects of glycerol, the major intracellular osmolyte of *D. salina*, on these parameters.

Cell viability depends on adaptational mechanisms that evolved to meet environmental changes. *D. salina* cells respond to hyperosmotic shock by rapid glycerol synthesis. Our data showed that such an increase in glycerol synthesis in *D. salina* cells was produced by shutting off one of the pathways of NADH expenditure, glycolysis, and in particular, the step of pyruvate to lactate transformation. Figure 7 illustrates schematically the pathways of glycerol metabolism as derived from the already published evidence [1, 22]. Glycerol is synthesized from polysaccharides along the glycolytic

Table 2. The constants of LDH inactivation rate, k_{in}, in various complexes

Temperature, °C	k_{in}, min^{-1}		
	E	E-NADH	E-NADH–pyruvate
	D. salina		
27	0.32 ± 0.04	0.028 ± 0.003	0.027 ± 0.003
30	0.45 ± 0.05	0.037 ± 0.005	0.031 ± 0.005
32	0.54 ± 0.06	0.046 ± 0.006	–
35	0.75 ± 0.08	0.072 ± 0.006	–
39	0.92 ± 0.12	0.084 ± 0.009	–
	C. reinhardtii		
52	0.10 ± 0.02	0.12 ± 0.01	0.080 ± 0.010
56	0.116 ± 0.02	0.175 ± 0.02	0.058 ± 0.007

Note: E, apoenzyme; E-NADH, enzyme in the presence of 0.2 mM NADH; E-NADH–pyruvate, the ternary complex. The value of k_{in} was averaged from three independent experiments; the standard error was estimated by approximating an experimental inactivation curve with the exponential function; – means no data available.

pathway until the latter branches at dihydroxyacetone phosphate, a common metabolite of glycolysis *per se* and the pathway of glycerol synthesis. The coenzyme NADH is essential for the further reduction of dihydroxyacetone phosphate to glycerol 3-phosphate. Thus,

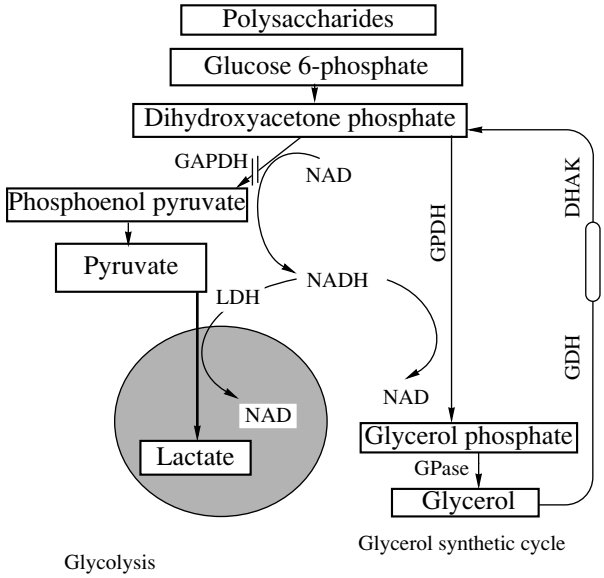

Fig. 7. The metabolic pathways of glycerol transformation in *D. salina* cells.

The shaded area represents the segment of the glycolytic pathway shut down by hyperosmotic shock to release NADH for glycerol synthesis.

DHAK—dihydroxyacetone kinase; GAPDH—glyceraldehydephosphate dehydrogenase; GDH—glycerol dehydrogenase; GPase—glycerolphosphatase; GPDH—glycerolphosphate dehydrogenase.

Table 3. The values of energy activation for LDH denaturation as (E) apoenzyme, holoenzyme (E–NADH), and the ternary complex (E–NADH–pyruvate) calculated from the temperature dependencies of the denaturation rate constants measured with the corresponding complexes

Species of algae	E_A^* of denaturation, kJ/mol		
	E	E–NADH	E-NADH–pyruvate
D. salina	67 ± 6	155 ± 20	168 ± 17
C. reinhardtii	218 ± 20	231 ± 25	243 ± 26

Note: The precision of E_A^* assessment depends on the precision of extrapolating the dependence $\ln k_{in}$ by reversed temperature by the linear function. The values of E_A^* are the means from four replications.

NADH is utilized simultaneously in two metabolic pathways, at the final stage of glycolysis which is catalyzed by LDH, and in the glycerol-3-phosphate dehydrogenase stage of glycerol synthesis. The two metabolic steps compete because of the similar values of NADH-binding constants characteristic of two dehydrogenases: K_M values for binding NADH at the standard conditions are 20 μM for LDH (our data) and 26 μM for glycerol-3-phosphate dehydrogenase [23]. Thus, the competition between the enzymes of the glycolytic and glycerol-synthesizing pathways is quite probable.

When affected by hyperosmotic shock, the algal cells mobilize their metabolic potential for rapid glycerol synthesis and therefore must run out of NADH. To make up for NADH deficiency, cells must lower their expenditure in the glycolytic pathway. The unique properties of *D. salina* LDH provide for such regulation: (a) the enzyme affinity for NADH declines as the pH is increased from 7.5 to 8.0 due to the increased alkalinity of the intracellular space caused by hyperosmotic shock [5, 6]; (b) NADH deficiency caused by glycerol synthesis decreases LDH stability. We conjecture that when the cytoplasm is alkalized by the hyperosmotic shock, the catalytic properties of LDH help shut down the final step of glycolysis (this segment of the glycolytic pathway is shaded in Fig. 7).

With osmotic shock alleviated, *D. salina* cells were shown to rapidly restore the initial metabolic indices. The fate of the accumulating metabolites, which would shut down the reduction of pyruvate to lactate, is an interesting problem, which is difficult to solve because the concentrations of these metabolites rapidly change. Apparently an enormous excess of carboxylases in *D. salina* cells (about 70% of the total protein content [24]) facilitates the rapid utilization of accumulated metabolites, including pyruvate.

Under a steady high state glycerol concentration, cells must still have a source of energy, like ATP, available for the complete glycolytic cycle in the presence of LDH. Glycerol does not considerably affect the affinity of LDH preparation from *D. salina* for NADH (Table 1); however, glycerol can greatly stabilize the enzyme [13], especially at low levels of physiological temperature.

Glycolysis is of primary importance for energy generation in all cells, including the unicellular green alga *D. salina*. The adaptation of halotolerant *D. salina* cells to high external salinity depends on the rapid synthesis of its basic osmolyte, glycerol, from starch, as the major source of carbon. Enzyme responses to the considerable changes in the intracellular glycerol concentration must redirect the metabolic routes under the osmotic stress conditions.

The present study demonstrated that endogenous glycerol affected various facets of the catalytic process: it increased the enzyme affinity for the substrate (K_M for pyruvate declined) and decreased V_{max}. It is noteworthy that although V_{max} declined in the presence of glycerol, the stability of the protein structure was not reduced, as in the case of denaturing agents; to the contrary it even increased. Other organic osmolytes, sorbitol, sucrose, etc., produce similar effects: all these compounds decrease the activities of NAD-dependent dehydrogenases and other enzymes with the catalytic activities restricted by conformational transitions. In such cases, the decline in enzyme activity results from the enhanced microviscosity of the medium [25, 26] brought about by these organic osmolytes. Meanwhile, though the polymers of higher molecular weight (over 1 kD) change the viscosity of the medium, they do not change the microviscosity and thus do not considerably affect catalysis [13].

Pick *et al.* [27] described an alternative mechanism of osmoregulation in *D. salina*: instead of enhanced glycerol synthesis, cells increase their inorganic phosphate concentration by reducing cell volume under the hyperosmotic conditions. These authors believe that an increase in phosphate concentration can directly or indirectly trigger the enzymes of glycerol synthesis. Glycerol synthesis in *D. salina* under hyperosmotic shock is accompanied by polymerization of inorganic orthophosphate to polyphosphate [28]. Such a process decreases the osmolarity of the cell contents and therefore cannot promote osmoregulation. However, one can interpret the combination of these processes by cell inclination to maintain the necessary amount of water and to accumulate more energy-rich compounds for glycerol synthesis (the free energy of the phosphoanhydride bond in polyphosphate is similar to that of ATP). Furthermore, under alkaline stress induced by the addition of ammonium chloride to the nutrient medium, polyphosphates are rapidly hydrolyzed with concomitant acidification and level off at the intracellular pH status [28]. The study by Pick *et al.* [22, 28] demonstrated the importance of the intracellular pH status as a trigger for glycerol synthesis and degradation and the concomitant processes of coupled biochemical reactions.

Pronina and Semenenko [4] believe that cells of *D. parva* and *D. salina* maintain a steady pH value around 7.1 to 7.2 whatever changes might occur in the environment, that is, CO_2 concentration, illumination, and salinity. However, the dramatic changes in the external condition can temporarily shift the intracellular pH, which later returns back to its initial value. Thus, in *D. tertiolecta* cells, illumination produced a short-term alkalization by 0.5 pH units, yet the saline stress alkalized the cytoplasm by 0.2 pH units both in the darkness and in the light [6]. It follows from our kinetic data (Fig. 3) that the alkalization in *D. salina* cells may decrease LDH activity by reducing the enzyme affinity for NADH. Detachment of the coenzyme results in the loss of enzyme stability, that is, it curbs the pathway for pyruvate transformation into lactate.

Numerous experiments by Alexandrov and his students (see [29]) established the thermoresistance of enzymes in thermophilic organisms. In the species under study, the enzyme-denaturing temperatures exceeded the temperatures characteristic for the habitats by 30–40°C and the temperatures invoking cell death by 15–20°C. Most enzymes maintain these differences, and Alexandrov relates it to the dynamic flexibility of polypeptide chains essential for catalysis under varying habitat temperatures.

The LDH preparation from *D. salina* is a rare exception to this trend. We demonstrated (Fig. 4) that, when the halophilic algal cells of *D. salina* and the ordinary algal cells of *C. reinhardtii* are grown at the same temperature, the enzymes produced in the two species differ in thermostability by 20°C. The question is why in halophilic *D. salina* such a thermolabile enzyme is necessary for the last stage of the glycolytic pathway, while other enzymes, e.g., carboanhydrase [30], carboxylase [31], glycerol-3-phoshate dehydrogenase [32], and also glycolytic enzymes are much less labile? We tend to explain such a characteristic trait of LDH in *D. salina* by its peculiar mechanism of adaptation to the saline stress using competition for NADH between LDH of the glycolytic pathway and GAPDH in the pathway of glycerol synthesis (Fig. 7). Let us add that the initial steps of glycolysis in *D. salina* cells are localized in the chloroplast, whereas its final step, that is, pyruvate reduction, is localized in the cytoplasm [27, 33]. It is noteworthy that, while in *C. reinhardtii* cells, the glycolytic events are also compartmentalized, such segregation takes place at the earlier step of dihydroxyacetone synthesis, with all subsequent processes of dihydroxyacetone reduction to glycerophosphate occurring in the cytoplasm [34]. In the higher plants, all these enzymes, including LDH, the terminal enzyme of glycolysis, are cytosolic. We believe that the separation of catalytic reactions between the two compartments provides bet-

ter control over the "hot" steps of metabolic processes under stress conditions induced by wider local changes in proton or inorganic phosphate concentrations, which can specifically regulate enzyme activities in the corresponding compartments. The key question is whether LDH is inactivated by strong alkalization *in vivo* to the same extent as *in vitro*, as we cannot exclude the possibility that the enzyme is additionally stabilized by some cell components.

Stabilization of enzymes, particularly of NAD-dependent dehydrogenases, with their coenzyme, has been demonstrated in most investigated organisms. Thus, the comparative study of the coenzyme-induced stabilization of NAD-dependent dehydrogenases from endo- and ectothermic animals demonstrated different stabilizing effects of the coenzyme [35]; the authors believe that these differences are essential for adaptation. Stabilization of the LDH preparation from *D. salina* with NADH (Tables 2, 3) exceeded by many times the effect of NADH on the enzymes of endothermic animals. Among other peculiar traits of the LDH preparation from *D. salina*, the dramatic stabilizing effect exerted by NADH is the most intriguing. In response to the changes in the external saline concentration, *D. salina* cells synthesize glycerol for about two hours [36]. As the active glycerol synthesis comes to its end, NADH concentration must increase as it is not any longer consumed in the osmolyte synthesis. In addition, pH value declines to its standard level of 7.2. The decrease in pH value results in the enhanced LDH affinity for NADH (Fig. 3b) and the lowered constant rate of enzyme inactivation (Fig. 3c). Higher NADH concentration and higher LDH affinity for NADH activate the enzyme and provide for the glycolytic pathway, running its full length.

Thus, by comparing the osmolyte effects on the successful stages of the reaction catalyzed by the LDH preparations from *D. salina* and *C. reinhardtii*, we come to the following conclusions:

(1) In the presence of glycerol, the affinity of both LDH preparations for pyruvate increased several times, while the affinity for NADH did not change. The glycerol-exerted changes in viscosity affected the maximum rate of pyruvate reduction by the *D. salina* preparation to a lesser extent than by *C. reinhardtii* LDH.

(2) The mechanisms for enzyme regulation by pH level differ in the two algal species. While the affinity for NADH is changed in *D. salina*, the affinity for pyruvate is instrumental in *C. reinhardtii* and other organisms.

(3) The kinetic study of thermal inactivation along the range of temperatures demonstrated an extremely low thermostability of the LDH preparation from *D. salina*; in contrast to the preparations from other organisms, the stability of the *D. salina* enzyme was considerably enhanced by NADH.

These kinetic data presume that *D. salina* cells maintain homeostasis in a wide range of intracellular glycerol concentrations by modifying the catalytic enzyme constants of substrate binding and by enzyme stabilization revealed at the stage of conformational transitions.

REFERENCES

1. Ginzburg, M., *Dunaliella*: A Green Alga Adapted to Salt, *Adv. Bot. Res.*, 1987, vol. 14, pp. 93–183.
2. Skulachev, V.P., *Energetika biologicheskikh membran* (Energy Metabolism of Biomembranes), Moscow: Nauka, 1989.
3. Goyal, A., Brown, A.D., and Gimmler, H., Regulation of Salt-Induced Starch Degradation in *Dunaliella tertiolecta*, *J. Plant Physiol.*, 1987, vol. 127, pp. 77–96.
4. Pronina, N.A. and Semenenko, V.E., Molecular and Cellular Organization of CO_2-Concentrating Mechanisms in the Cells of Photoautotrophic Microalgae, *Al'gologiya*, 1991, vol. 1, pp. 80–92.
5. Gimmler, H., Kugel, H., Leibfrits, D., and Mayer, A., Cytoplasmic pH of *Dunaliella parva* and *Dunaliella acidophila* as Monitored by *in vivo* ^{31}P-NMR Spectroscopy and DMO Method, *Physiol. Plant.*, 1988, vol. 74, pp. 521–530.
6. Goyal, A. and Gimmler, H., Osmoregulation in *Dunaliella tertiolecta*: Effects of Salt Stress and External pH on the Internal pH, *Arch. Microbiol.*, 1989, vol. 152, pp. 138–142.
7. Karasev, V.A., Stefanov, V.E., and Kurganov, B.I., *Nadmolekulyarnye struktury: organizatsiya, funktsionirovanie, proiskhozhdenie* (Supramolecular Structures: Organization, Functions, and Origin), Moscow: VINITI, 1989.
8. Abad-Zapatero, C., Griffith, J.P., Sussman, J.L., and Rossmann, M.G., Refined Crystal Structure of Dogfish M_4 Apo-Lactate Dehydrogenase, *J. Mol. Biol.*, 1987, vol. 198, pp. 445–467.
9. Hochachka, W. and Somero, G.N., *Biochemical Adaptation*, Princeton: Princeton Univ. Press, 1984. Translated under the title *Biokhimicheskaya adaptatsiya*, Moscow: Mir, 1988.
10. Saburova, E.A., Markovich, D.S., and Goldstein, B.N., Study of the pH-Dependence on Structure and Functional Properties of Lactate Dehydrogenase (M_4). The Indirect Cooperation in Lactate Dehydrogenase, *Mol. Biol.* (Moscow), 1987, vol. 11, pp. 332–344.
11. Saburova, E.A. and Markovich, D.S., Anion Effect on the Inhibition of Lactate Dehydrogenase by Pyruvate, *Mol. Biol.* (Moscow), 1978, vol. 12, pp. 737–746.
12. Demchenko, A.P., Ruskyn, O.I., and Saburova, E.A., Kinetics of the Lactate Dehydrogenase Reaction in High-Viscosity Media, *Biochim. Biophys. Acta*, 1989, vol. 998, pp. 196–203.
13. Simonova, N.B., Saburova, E.A., Shirshikova, G., Elfimova, L.I., Pronina, N.A., Fal'kovich, T.N., and Semenenko, V.E., Heat Stability of Lactate Dehydrogenases from Halophilic Alga *Dunaliella salina* and Nonhalophilic *Chlamydomonas reinhardtii* in the Aqueous Glycerol Solutions, *Dokl. Akad. Nauk*, 1995, vol. 345, pp. 549–554.
14. Eszes, C.M., Sessions, R.B., Clarke, A.R., Moreton, K.M., and Holbrook, J.J., Removal of Substrate Inhibition in a

Lactate Dehydrogenase from Human Muscle by a Single Residue Change, *FEBS Lett.*, 1996, vol. 399, pp. 193–197.

15. Dym, O., Mevarech, M., and Sussman, J.L., Structural Features that Stabilize Halophilic Malate Dehydrogenase from an Archaebacterium, *Science*, 1995, vol. 267, pp. 1344–1346.
16. Eisenberg, H., Life in Unusual Environments: Progress in Understanding the Structure and Function of Enzymes from Extreme Halophilic Bacteria, *Arch. Biochem. Biophys.*, 1995, vol. 318, pp. 1–5.
17. *Katalog kul'tur mikrovodoroslei v kollektsiyakh SSSR* (Catalogue of Microalgal Cultures in the Collections of the USSR), Semenenko, V.E., Ed., Pushchino: ONTI, 1991.
18. Semenenko, V.E. and Abdulaev, A.A., Parametric Direction of β-Carotene Biosynthesis in *Dunaliella salina* Cells in an Intensive Culture, *Fiziol. Rast.* (Moscow), 1980, vol. 27, pp. 31–41 (*Sov. Plant Physiol.*, Engl. Transl.).
19. Jecsai, G., Crystalline Lactic Dehydrogenase from Pig Skeletal Muscle, *Acta Physiol. Acad. Sci. Hung.*, 1961, vol. 20, pp. 339–346.
20. Enzymes and Metabolic Pathways Database (EMP), http://www.empproject.com.
21. Wynn, R.M., Bertsch, J., Bruce, B.D., and Malkin, R., Green Algal Cytochrome B_6-F Complexes: Isolation and Characterization from *Dunaliella salina, Chlamydomonas reinhardtii*, and *Scenedesmus obliquus*, *Biochim. Biophys. Acta*, 1988, vol. 935, pp. 2115–2122.
22. Bental, M., Pick, U., Avron, M., and Degani, H., Polyphosphate Metabolism in the Alga *Dunaliella tertiolecta: 1. D. salina* Studied by 31P-NMR, *Biochim. Biophys. Acta*, 1991, vol. 1092, pp. 21–28.
23. Haus, M. and Wegmann, K., Glycerol-3-Phosphate Dehydrogenase (EC 1.1.1.8) from *Dunaliella*: Purification and Kinetic Properties, *Physiol. Plant.*, 1984, vol. 60, pp. 283–288.
24. Podesta, F.E., Colombo, S.L., and Andreo, C.S., Purification and Characterisation of the Light and Dark Forms of Phosphoenolpyruvate Carboxylase from the Dicot Plant *Amaranthus viridis* L., *Plant Cell Physiol.*, 1995, vol. 36, pp. 1471–1476.
25. Saburova, E.A., Regulation of Dehydrogenases by Osmolites in Organisms Differing in Halotolerance, *Doctoral (Biol.) Dissertation*, Pushchino, 1997.
26. Khoshtariya, D.E. and Goguadze, N.G., The Activity of Myosin ATPase as Dependent on a Medium Viscosity, *Biofizika*, 1986, vol. 31, pp. 220–223.
27. Bental, M., Pick, U., Avron, M., and Degani, H., The Role of Intracellular Orthophosphate in Triggering Osmoregulation in the Alga *Dunaliella salina*, *Eur. J. Biochem.*, 1990, vol. 188, pp. 117–122.
28. Pick, U., Bental, M., Chitlaru, E., and Weiss, M., Polyphosphate-Hydrolysis—a Protective Mechanism against Alkaline Stress? *FEBS Lett.*, 1990, vol. 274, pp. 15–18.
29. Alexandrov, V.Ya., *Reaktivnost' kletok i belki* (Cell Reactivity and Proteins), Leningrad: Nauka, 1985.
30. Fisher, M., Gokhman, I., Pick, U., and Zamir, A., A Salt-Resistant Plasma Membrane Carbonic Anhydrase Is Induced by Salt in *Dunaliella salina*, *J. Biol. Chem.*, 1996, vol. 271, pp. 17718–17723.
31. Rabbani, S., Beyer, P., Lintig, J., Hugueney, P., and Kleinig, H., Induced Beta-Carotene Synthesis Driven by Triacylglycerol Deposition in the Unicellular Alga *Dunaliella bardawil*, *Plant Physiol.*, 1998, vol. 116, pp. 1239–1248.
32. Haus, M. and Wegmann, K., Glycerol-3-Phosphate Dehydrogenase (EC 1.1.1.8) from *Dunaliella tertiolecta*: 2. Influence of Phosphate Esters and Different Salts on the Enzymatic Activity with Respect to Osmoregulation, *Physiol. Plant.*, 1984, vol. 60, pp. 289–293.
33. Ginzburg, M. and Ginzburg, B.Z., Ion and Glycerol Concentration in 12 Isolates of *Dunaliella*, *J. Exp. Bot.*, 1985, vol. 36, pp. 1064–1074.
34. Klein, U., Compartmentation of Glycolysis and of the Oxidative Pentose-Phosphate Pathway in *Chlamydomonas reinhardtii*, *Planta*, 1986, vol. 167, pp. 81–86.
35. Leibman, D.Ya. and Elfimova, L.I., Coenzyme Effect on the Conformational Stability of Glyceraldehide-3-Phosphate Dehydrogenase in the Muscles of Ecto- and Endothermic Animals, *Biokhimiya*, 1977, vol. 42, pp. 1960–1964.
36. Balnokin, Yu.V., The Ion Homeostasis in Halotolerant Algae, *Doctoral (Biol.) Dissertation*, Moscow, Inst. Plant Physiol., Russ. Acad. Sci., 1995.