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APPLIED PROBLEMS OF ARID LAND DEVELOPMENT

Influence of Temperature on the Functional Characteristics of the Key Enyzme of Energy Metabolism in the Skeletal Muscles of Small Ground Squirrels (*Sphermophilu spygmaeus* Pall.) of Steppe Ecosystems

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Abstract—The significant seasonal temperature fluctuations in the arid zones of northwestern Dagestan lead to a broad population of heterothermic mammals that have the unique ability to fall into a state of hibernation at low ambient temperatures. Their significant fluctuations in physiological and biochemical processes during and after hibernation indicate that heterotherms have flexible mechanisms to change the functional characteristics of key metabolic enzymes. In this paper, the temperature dependence of the lactate dehydrogenase (LDH) activity in the skeletal muscles of the small ground squirrel *Spermophilus pygmaeus* Pall. was studied during the period of summer wakefulness, deep hibernation, and at various stages of awakening, accompanied by warming of the animal bodies to temperatures of 10, 20, 30, and 37°C. It was found that the LDH activity substantially decreases in hibernating ground squirrels, regardless of its incubation temperature in vitro. In the dynamics of warming, there is an increase in LDH activity, the progressive nature of which is most pronounced in the range of animal body temperatures of 1.6–20°C. The temperature dependence of LDH activity in Arrhenius coordinates was approximated with nonlinear graphs; their inflection position during hibernation shifted to the low temperature region, returning to the control values after total warming. Hibernation contributed to a significant increase in the energy and enthalpy of LDH activation. In the warming dynamics, the levels of these parameters are disproportionally reduced and reach the control values of summer animals. The results supported the assumption that the mechanisms of LDH catalysis are substantially modified during hibernation and at various stages of awakening in the skeletal muscles of ground squirrels and had an adaptive character.

Keywords: hibernation, awakening, ground squirrels, muscles, lactate dehydrogenase, temperature dependence, activation energy, activation enthalpy

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INTRODUCTION

The steppe ecosystems of northwestern Dagestan, which are located in the Pre-Caspian arid zones, are exposed to significant seasonal temperature fluctuations, from –24°C in winter to 40.4°C in summer (Gasanov et al., 2013). This results in a broad population of small mammals characterized by a unique strategy of temperature adaptation called heterothermy. One feature of heterothermy is that heterothermic animals go into a torpid state, often called hibernation (winter sleep), at low ambient temperatures and conditions of food deficiency.

Hibernation is characterized by significant deceleration of the metabolic rate and body temperature (up to 1–1.5°C, suppression of a wide range of physiological functions, such as cardiac output, respiratory rate, heart contractions, and peripheral vasoconstriction (Geiser, 2004; Jørgensen et al., 2014). Moreover, the biochemical processes and organ and system functioning in hibernating animals reorganize such that a certain homeostasis is maintained, even at extremely low temperatures (Storey, K.B. and Storey, J.M., 2004).

Hibernation lasts for several months and is accompanied by periodic awakenings, during which the animals to warm up for 1.5–2.5 h before normothermy, and the biochemical and physiological organism parameters soon return to the euthermic level (Carey et al., 2003). Such major changes in an animal's physiological state require flexible adaptive mechanisms for the temperature of glycolytic enzymes, with L–lactate NAD-oxydereductase as the main enzyme (1.1.1.27, lactate dehydrogenase, LDH). Changes in LDH activity in the muscles are particularly interesting, since body cooling primarily results in their heat loss. Muscles play an important role in

heat production when a heterothermic animal exits the torpid state (Carey et al., 2003). To summarize, successful hibernation requires adaptive changes of individual cellular functions in skeletal muscles.

In our work we studied functional characteristics, i.e., activity, activation enthalpy, and activation energy, of LDH in skeletal muscles of the ground squirrel at different body temperatures corresponding to the following physiological conditions: summer wakefulness, deep hibernation (the middle of the bout), and induced warming. The results may become an important step in the discovery of the molecular mechanisms of compensatory and adaptational reactions of animal enzymes with a resistant strategy of adaptation at low body temperatures.

EXPERIMENTAL

Study objects. The studies involved the small ground squirrels *Spermophilus pygmaeus Pall.* weighing 250–300 g. They were caught in the Buynaksk region of Dagestan (42°55′ N, 47°20′ E, and 320 m A.S.L. BS) and kept in standard vivarium conditions.

Modeling of winter sleep and awakening. The animals were divided into six groups. The first group consisted of animals kept awake in summer (control group), the second included animals in deep winter hibernation, while the third to sixth groups were comprised of animals at different stages of induced wakefulness. To provoke winter sleep at the end of October, the ground squirrels were transferred into a dark room with a temperature of 2–5°C. After several days the animals went into hibernation with a decrease in body temperature (t_b) of approximately 1.6 ± 0.4°C. The medium duration of bout was 14 days after 1 month. For experimental purposes the animals were taken in the torpid state in the middle of a bout. To promote awakening the animals were moved to a room with a temperature of 20°C. The animals were involved in the experiments after reaching a t_b of 10, 20, 30, and 37°C.

Preparation of mitochondrium-free cytosol. The animals were decapitated, and the gastrocnemius muscles were then isolated. Weighed portions of tissue were homogenized in 0.1 M phosphate buffer (pH 7.4). Homogenate was centrifuged at 600 *g* for 10 min. The obtained supernatant was again centrifuged at 15000 *g* for 10 min.

Measurement of LDH activity. The LDH activity was determined with a spectrophotometer according to the decrease in the $NADH_2$ level ($\lambda = 340$ nm) in the reaction medium (Halilov et al., 2018). The LDH activity was assessed in an incubation temperature range of 5–37°C at a pyruvate concentration of 0.3 mM in the incubation medium. The enzymatic activity was calculated based on the results, and linear anamorphoses were constructed in the Arrhenius coordinates used to determine energy activation.

Statistical analysis. Statistical analysis of the results was performed with the help of one-way analysis of variance (ANOVA) in the Statistica 8.0 software. The significance of the results was determined with the Fisher's criterion at a p level of 0.05. Each curve on the plots of the temperature dependence of LDH activity was constructed based on the mean values of eight independent experiments. The data in the Tables are presented as mean ± standard error.

RESULTS AND DISCUSSION

The LDH activity in the skeletal muscles was studied in ground squirrels during the periods of summer wakefulness, deep hibernation, and different stages of awakening accompanied by body warming to temperatures of 10, 20, 30, and 37°C. Figure 1 shows that there is a significant reduction of LDH activity in ground squirrels in the torpid state; it was 75% of the control.

The warming of animals to 10°C resulted in a substantial (71.4%) increase in LDH activity with respect to hibernation. As compared to hibernation, the rate of LDH catalysis was 3.1 times higher at a t_b of 20°C, 3.8 times higher at 30°C, and 4.1 times higher at 37°C. It should be noted that the LDH activity in vitro[1] was determined at incubation temperatures that nearly corresponded to the body temperatures of ground squirrels. Thus, Fig. 1 fully represents the actual functional activity of the enzyme in vivo.

A question then arises as to whether the observed effects of hibernation and warming on LDH activity are associated with the direct influence of temperature on the enzyme or adjustable changes in the physicochemical properties in hibernation cycle.

To detect the etiology of changes in the enzymatic activity of hibernators, it seems to be necessary to study the dependence of LDH activity on the animal body temperature in vitro at different incubation temperatures of the enzyme. Figure 2 demonstrates a significant decrease in the LDH activity of hibernating animals at almost all LDH incubation temperatures and its progressive elevation in the dynamics of animal warming to the euthermic state. This implies that the perturbation of LDH activity in hibernation and awakening were related to certain adaptive changes in the enzymatic level that make it possible to regulate its activity depending on the physiological condition of animal and its body temperature rather than the direct influence of temperature on LDH, which is accompanied by changes in balance of van der Waals forces in the enzyme molecule.

Interestingly, the dependence diagrams of LDH activity from animal body temperature are nonlinear,

[1] Editor's note: In vitro (from Latin, in glass) is the term for an experimental technology in which the studies are conducted in test-tubes outside the living organism. In general this term is opposed to in vivo, or an experiment on a living organism that is performed on humans or a live model.

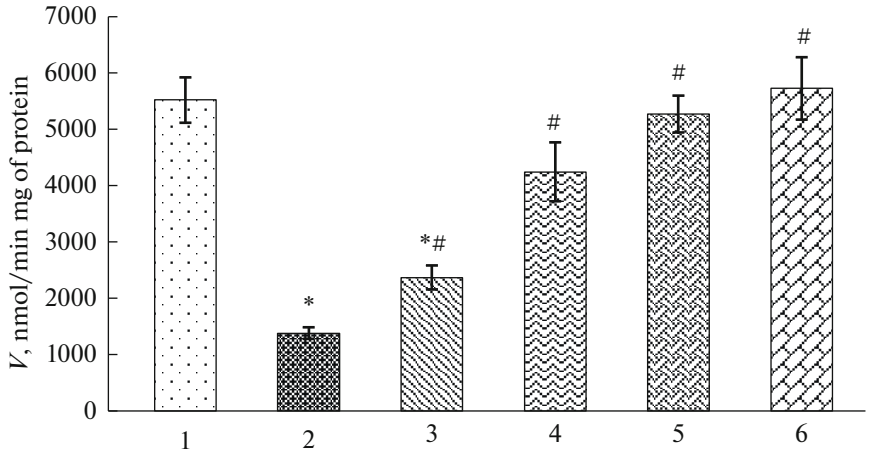

Fig. 1. LDH activity in the skeletal muscles of ground squirrels during the period of summer awakening (1), deep hibernation (2), and warming to a t_b of 10 (3), 20 (4), 30 (5), and 37°C (6); significant differences are marked with * in comparison with control and # in comparison with hibernation.

which is related to inadequate increases in the efficiency of enzyme catalysis within a t_b range of 1.6–20°C (the sharper and more prominent increase) and 20–37°C (a slow and insignificant increase). Therefore, abrupt changes occur in the mechanisms of LDH functioning when t_b reaches 20°C, which helps stabilize the LDH activity on a certain level close to normothermy.

To study the mechanisms of changes in LDH activity during hibernation in further detail, we studied thermodynamic parameters of the enzyme as calculated via the analysis of linear anamorphoses of the temperature dependence in Arrhenius coordinates (Fig. 3). In almost all of the studied conditions, the temperature dependence of the enzyme was approximated with nonlinear graphs, in particular, two lines crossing at specific temperatures. The presence of curve breaks on graphs of the temperature dependence of activity of the cytosolic enzyme may be associated with the fact an extremely narrow temperature range leads to one of two expected events: either the enzyme conformation trigger changes or a there is a phase transition in the substrate fixating it. It is known that glycolytic enzymes in muscles produce a supramolecular complex with F–actin as a binding anchoring subunit (Menard et al., 2014). This integration of glycolytic enzymes allows not only fast tunneling of the substrate but also regulation of the glycolytic flow (Puchulu-Campanella et al., 2013). It is interesting that early studies of the LDH temperature dependence in homoiothermic animals have demonstrated its linearity, unlike that in heterothermic animals (Halilov et al., 2018). The LDH structure, as well as the structure of many components of the glycolytic metabolon, is quite conservative in an evolutionary sense; therefore, the feature of the LDH temperature dependence discovered in our trial in heterotherms is unlikely to be related to profound changes in the structural organization of the enzyme or its anchor substrate. All that is left to assume the existence of some kind chemical modification of LDH that enables an acute change of its temperature sensitivity.

It is clear that the break point transitions towards a lower temperature on the graph in the torpid state. Thus, for example, the point of thermal transition in the control was around 20°C, while it was 10°C in torpidity. The warming of animals did not significantly affect the position of the break point in the Arrhenius plot. However, the complete warming of ground squirrels to a t_b of 37°C resulted in a transition of the break point to that in the control animals. To summarize, the changes in the enzymatic activity and break-point transition in hibernation and the warming dynamics

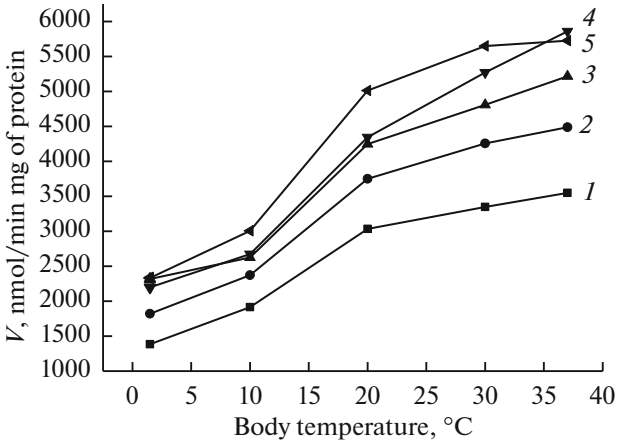

Fig. 2. Dependence of LDH activity of skeletal muscles on the t_b of ground squirrels at different temperatures of enzyme incubation in vitro. (*1*) 5; (*2*) 10; (*3*) 20; (*4*) 30; (*5*) 37°C.

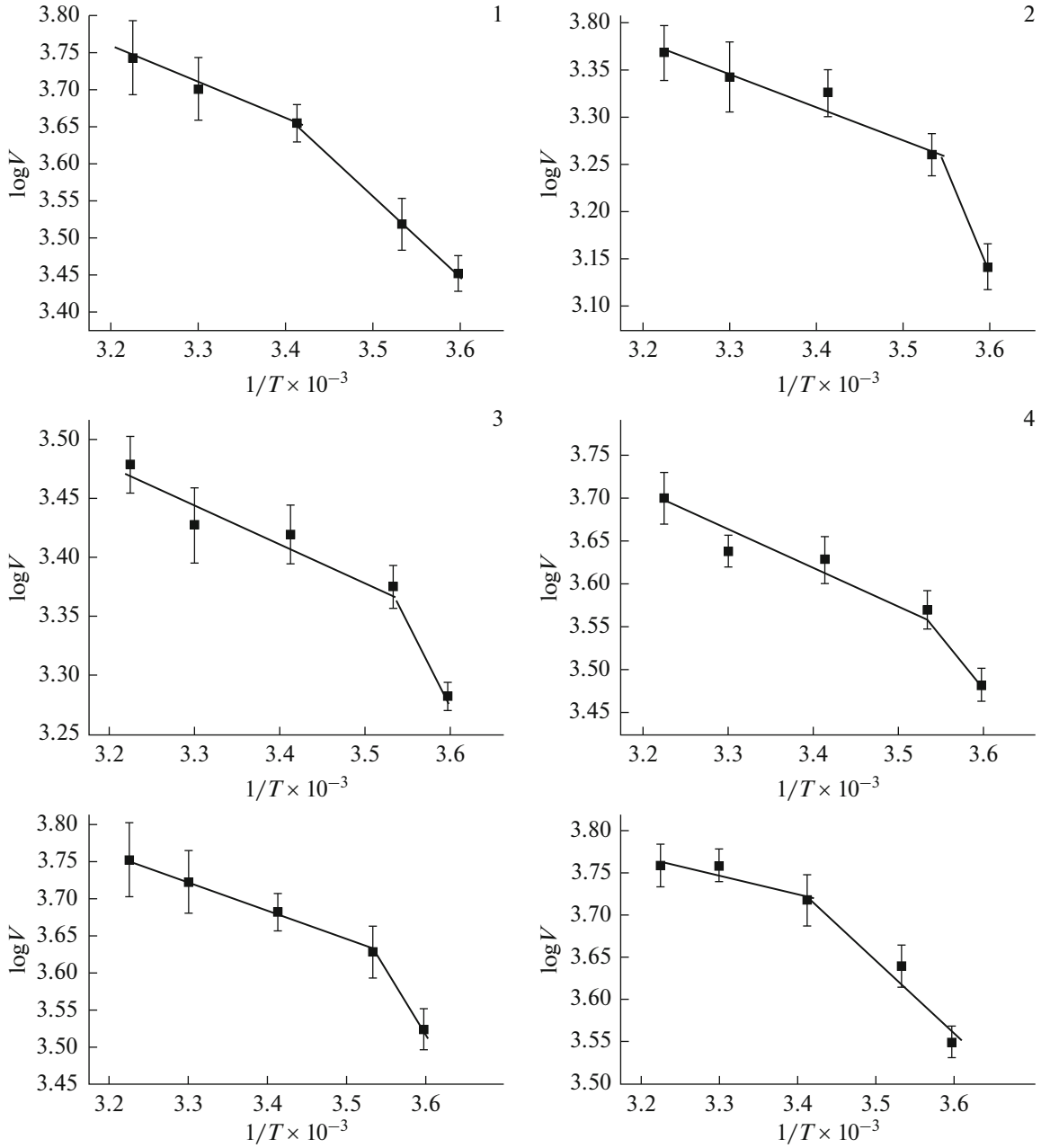

Fig. 3. Dependence of LDH activity of skeletal muscles on t_b in ground squirrels in hibernation and in the dynamics of induced warming. (1) Control; (2) hibernation; (3) to 10°C; (4) to 20°C; (5) to 30°C; and (6) to 37°C.

are shown on the graphs. Moreover, there was a change in the slopes of the curves used to calculate in which the corresponding effective energies and activation enthalpies based on their angle tangents.

The data presented in Table 1 demonstrate that the activation below the break point (Ea_2) in deep torpor substantially increases (by 59.6%) as compared to the control. The warming of animals to 10°C was accompanied by an insignificant (21%) decrease in Ea_2 in comparison to sleeping animals. An increase in the body temperatures of torpid animals to 20°C significantly decreased Ea_2 in LDH to the level of ground squirrels awake in summer after complete warming. As for the activation energy above the break point (Ea_1), it also increased in hibernation, whereas it reduced to the control level after the complete warming of heterothermic animals. Nevertheless, the amplitude of Ea_1 fluctuations was less expressed compared to Ea_2.

Effective activation enthalpies were calculated from the activation energies according to the formula $\Delta H = \Delta E_a - RT$. Therefore, the changes in this ther-

Table 1. Thermodynamic parameters of LDH in the skeletal muscles of ground squirrels in winter sleep and in the dynamics of induced warming

Animal condition	Ea_1, kJ/mol K	Ea_2, kJ/mol K	ΔH_1, kJ	ΔH_2, kJ
Control	5.14 ± 0.47	22.52 ± 1.52	2.67 ± 0.27	20.01 ± 1.35
Deep hibernation	6.88 ± 0.52*	35.93 ± 2.63*	4.41 ± 0.33*	33.65 ± 2.46*
Warming, t_b 10°C	6.30 ± 0.72	28.66 ± 2.90	3.83 ± 0.43	26.25 ± 2.25
Warming, t_b 20°C	7.07 ± 0.69*	24.32 ± 1.73#	4.60 ± 0.44*	21.89 ± 1.55#
Warming, t_b 30°C	6.37 ± 0.38	22.58 ± 1.94#	3.90 ± 0.23	20.07 ± 1.72#
Warming, t_b 37°C	4.58 ± 0.36#	22.93 ± 2.00#	2.11 ± 0.16#	20.35 ± 7.77#

Statistical differences are marked with * as compared to control and # in comparison with hibernation.

modynamic parameter during hibernation cycle had the same nature as the activation energy.

Thus, the incubation temperature range of 10–20°C was associated with an abrupt change in many thermodynamic LDH parameters. Notably, the critical temperature was 20°C in normothermic animals, while warming it was 10°C in sleeping animals and those in process of. This indicates that the chemical modification of the enzyme that dramatically changes its thermodynamic parameters is reversible.

Do the changes in activity and the LDH temperature dependence found in our experiment in hibernation and awakening have a biological significance, and what could be the reasons for them? Winter sleep is an evolutionarily developed adaptation that allows minimization of all of the energy expenses of an organism. Numerous studies suggest that there is an extremely low level of oxidative metabolism in hibernator tissues residing in the torpid state (Ruf et al., 2012). Hibernation is characterized by an adjustable suppression of energy-consumptive processes, i.e., transcription, translation, and active transport, and reorganization of the fuel metabolism from carbohydrate to lipid (Wang and Lee, 2011). The study of Vermillion et al. (2015) showed that the levels of expression glycolytic enzymes decreased in the skeletal muscles of heterotherms during winter sleep. These results correlated well with the data of proteomic analysis, which demonstrated low levels of glycolytic proteins in the muscles of hibernating, thirteen-lined ground squirrels (Anderson et al., 2016). The contribution of proteosomic degradation to the decrease in the LDH level is miniscule in hibernation, since its adjustable suppression occurs in the torpor period, which makes it possible to limit the loss of muscle mass during torpidity (Cotton, 2016).

Therefore, the discovered suppression of LDH activity in hibernation may primarily be associated with the decrease in its cellular concentration, which leads to a decrease in the number of glycolysis cycles and thus efficiently exhausts the muscles' glycogen supplies. However, our data showed that LDH in sleeping animals was characterized by higher energy values and enthalpy activation. These thermodynamic parameters of the enzyme depended not on the expression level but on the specific flow of the catalytic process itself, which was defined by the spatial configuration of the enzyme and its conformation lability. For instance, the changes in those structural-functional properties of the enzyme may result from the expression of various molecular forms of LDH in they hibernation cycle, and each of them is different in terms of the thermodynamic parameters. However, the high energy consumption related to the formation of new proteins cast doubt on the differential expression of genes of various LDH subunits. Therefore, the differences in the activity and temperature dependence between the LDH of awake and sleeping animals are probably associated with a reversible, post-translational protein modification.

Phosphorylation is the most promising mechanism of LDH chemical modification. It was found that the differential phosphorylation of LDH in different poikilothermic animal species entering the hypometabolic state leads to the depression of enzymatic activity (Xiong and Storey, 2012; Katzenback et al., 2014). Analysis via Western blot with specific antibodies against phosphoserine and phosphotreonin discovered that the LDH of torpid heterothermic animals was substantially more phosphorylated on serine and threonine as compared to animals awake in summer (Ruberto, 2015). AMP-dependent protein kinase (AMPK) is a potential candidate for the role of the enzyme responsible for LDH phosphorylation. AMPK is a kinase sensitive to the ratio of [AMP] to [ATP][2] and is described as an energetic energy sensor. It was found that the AMPK activity is enhanced in hibernating chipmucks (Yamada et al., 2019), which is probably related to the decrease in the ATP cellular level during the torpid period.

The metabolism is activated in the period of interbout awakenings of ground squirrels and is accompa-

[2] Editor's note: Adenosine triphosphate or adenosinetriphosphoric acid (ATP) is a nucleoside triphosphate that is important in the metabolism of energy and substances in organisms. ATP is a universal source of energy for all biochemical processes occurring in the living organisms.

nied by an increase in body temperature. Numerous physiological processes intensify, too, such as the heartbeat, respiration, circulation, etc. The elevation of t_b in the dynamics of awakening primarily happens due to the force of nonshivering thermogenesis in brown fat and then by the shivering of skeletal muscles, which begins after the t_b reaches ~20°C. High thermogenesis rates continue until the animal reaches normothermy, with many substrates mobilizing for energy production (Carey et al., 2003), and the lipid metabolism switches to carbohydrate (Karpovich et al., 2009). Intense skeletal muscle contraction, which is characterized by an incomplete recovery of the circulation rate, can contribute to the development of hypoxia in these tissues. Hence, a high LDH activity in the skeletal muscles favors an increase in number of anaerobic glycolysis cycles and thus plays the key role in the energy supply of their contractile activity. Interestingly, the temperature of intense muscle shivering in heterotherms corresponds to the temperature of the break point in the plots of the LDH activity dependence on the body temperature of heterotherms (Fig. 2). This indicates that the LDH plays the leading role in the regulation of shivering thermogenesis, the intensity of which influences the rate of biochemical and physiological processes.

The warming of animals to normothermy develops rapidly for 1.5–2.5 h. the significant increase in LDH activity that we detected in the early stages of warming (t_b 10°C), when the rates of energy-consuming processes, i.e., transcription and translation, still do not reach the levels of euthermic animals, was unlikely related to the increased expression of this enzyme. Moreover, the discovered decrease in the values of the enzyme's thermodynamic parameters suggests that the transformations are structural-functional. The increased catalysis efficiency of the enzyme is most probably associated with a process that opposes phosphorylation, in particular, dephosphorylation, which involves specific protein phosphatases, on the initial stages of awakening.

Thus, our experimental data regarding the activity and temperature dependence of LDH activity in sleep and the dynamics of awakening support the assumption that there is an adjustable reprogramming of the metabolism in the hibernation cycle in the skeletal muscles of ground squirrels that is probably based on posttranslational modifications of this crucial enzyme of the glycolytic pathway of carbohydrate catabolism. The thermodynamic LDH parameters are probably the point of application of the regulatory signals that switch the metabolism from anaerobic in the case of insufficient muscle perfusion to aerobic, from carbohydrate to lipid metabolism, and vice versa.

CONCLUSIONS

(1) The LDH activity in skeletal muscles during the hibernation of ground squirrels substantially decreases. The dynamics of warming is associated with a progressive elevation of LDH activity, which is more prominent in the animal body temperature range of 1.6–20°C.

(2) The temperature dependence of LDH activity was approximated in nonlinear plots in Arrhenius coordinates with a break point that transitions in hibernation to the area of low temperatures in the skeletal muscles of ground squirrels, while complete warming is characterized by the values found in ground squirrels that are awake in the summer.

(3) Hibernation favors a substantial increase in the energy and enthalpy of LDH activation. The levels of these parameters disproportionally decrease in the warming dynamics, reaching the values of ground squirrels awake in the summer.

(4) The activity and catalytic mechanisms of LDH substantially change in the skeletal muscles of ground squirrels during the hibernation period and at different stages of awakening and have an adaptive nature.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on animal welfare. All applicable guidelines for experiments with laboratory animals (Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes).

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