ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 11, pp. 1260-1265. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © I. L. Tsvetkov, A. S. Konichev, 2009, published in Biokhimiya, 2009, Vol. 74, No. 11, pp. 1548-1554.

Stress-Induced Changes in Accumulation of Sorbitol and in Activities of Concomitant Enzymes in Digestive Gland of Freshwater Snail

I. L. Tsvetkov* and A. S. Konichev

Moscow State Regional University, ul. Radio 10a, 107005 Moscow, Russia; fax: (495) 261-2228; E-mail: i_tsvetkoff@mail.ru

Received February 16, 2009
Revision received March 25, 2009

Abstract—Sorbitol content was determined in the digestive gland of freshwater snail (*Viviparus viviparus* L.) in different seasons and in a short-term experiment on the water temperature decrease and on intoxication with cadmium chloride. In the model experiments, changes in activities of enzymes involved in sorbitol metabolism (acid phosphatases, sorbitol dehydrogenase, and aldose reductase) were also studied. Sorbitol was accumulated by the snail in response to the temperature decrease (as a cryoprotectant) and under conditions of acute intoxication (as a probable metabolic regulator or a nonspecific protective factor). However, the mechanisms of this accumulation are different: on cold adaptation sorbitol is produced as a result of reduction of glucose under the influence of aldose reductase, and on intoxication sorbitol is mainly produced from fructose under the influence of sorbitol dehydrogenase. Pathways of the sorbitol accumulation and its re-involvement into metabolism are not always the same, and this might be a mechanism for regulation of carbohydrate metabolism (at the initial stage of glycolysis) on adaptation to unfavorable factors of the environment.

DOI: 10.1134/S0006297909110121

Key words: mollusks, cold adaptation, intoxication, cryoprotectants, sorbitol metabolism

Sorbitol, glycerol, and some other polyatomic alcohols are important reserve metabolites in animals that regulate the density and viscosity of biological fluids such as blood, lymph, and intercellular fluid [1]. These substances are metabolic byproducts not directly involved in carbohydrate metabolism; therefore, their accumulation in the organism can vary in very broad limits and lead to changes in some physical parameters of the organism's liquid phase, in particular, decrease the freezing temperature. Therefore, polyols and other glucose derivatives are reasonably called natural antifreezes or cryoprotectants, and this feature is especially important for poikilothermic animals that are usually living under conditions of low temperature or need to adapt to strong fluctuations in temperature (life under conditions of sharp continental climate and associated periods of deep rest, freezing into ice in winter, and also reproduction- or food-associated migrations) [2, 3].

The accumulation in plasma of glucose metabolism byproducts (mainly glycerol) is supposed to be an adaptation mechanism to decreased environmental temperature also in homoeothermic animals. In particular, from the general biological viewpoint insulin-dependent diabetes mellitus (type I) can be considered as a rudimentary form of increased cold resistance [4].

However, similarly to the majority of other metabolites, sorbitol and glycerol serve not only as cryoprotectants, but participate in some other biochemical functions. Most obviously they store energy side by side with glycogen, especially in evolutionarily primitive animals. In particular, glycerol accumulated in plasma of *Salmonidae* at decreased temperature is immediately involved in carbohydrate metabolism when the environmental temperature increases. Thus, this energy store can be used significantly earlier than stored glycogen [2]. Another cooperating function of metabolic byproducts is associated with an increase in osmotic pressure that provides a decrease in the fluidity of plasma membranes by glucose derivatives and, as a consequence, an increase in resistance to oxidative stress and some toxic agents [5]. But the role of the carbohydrate metabolism byproducts can be even more complicated. In particular, sorbitol is shown to induce apoptosis in tumor cells of human bone marrow via decrease in the membrane potential of mito-

* To whom correspondence should be addressed.

chondria, accumulation of cytochrome *c*, and activation of caspase 3 [6]. Thus, studies on changes in the contents of sorbitol, glycerol, and similar compounds can reveal unexpected interrelationships in the regulatory mechanisms of metabolism, including mechanisms associated with adaptation to stress, which also can induce apoptosis [7, 8].

Moreover, changes in the contents of metabolic byproducts depend on activities of the corresponding enzymes, especially dehydrogenases (α-glycerophosphate dehydrogenase, sorbitol dehydrogenase, aldose reductase) and phosphatases (α-glycerophosphatase, glucose-1-phosphatase, glucose-6-phosphatase, fructose-6-phosphatase, and probably nonspecific phosphatases with broad substrate specificity) [3, 9-11]. An increase in the activity of these enzymes (or in their mRNAs) is often identified with accumulation of glycerol and sorbitol because these parameters are etiologically interrelated. We have studied this interrelationship: we have determined the sorbitol contents and activities of acid phosphatase (EC 3.1.3.2), sorbitol dehydrogenase (EC 1.1.1.14), and aldose reductase (EC 1.1.1.21) in the digestive gland of a gastropod, the freshwater snail *Viviparus viviparus* L., in its natural environment in different seasons and in response to acute exposure to cold or a toxic agent.

MATERIALS AND METHODS

The animals were collected on the Vyaz' river (Tishkovo village, Moscow Region) during different seasons: in autumn (beginning of November before becoming icebound), in winter (middle of January from under the ice), in spring (middle of April after the thaw), and in summer (June). The animals were brought to the laboratory within 1.5-2 h in vessels with fresh water. To study the season-associated changes in biochemical parameters, the animals were killed immediately; for experiments, they were acclimated to the laboratory conditions (a microcosm) for a week at room temperature (~18°C) with 24-h aeration and illumination with natural light. Water (~80 liters) and higher plant fragments as food were brought from the animal collection site. For experiments, 15-20 animals were placed into separate vessels (~5 liters) where they were exposed to the presence of cadmium chloride (0.005 mg Cd^{2+}/liter) or low temperature (4-6°C) virtually without illumination for 1, 4, and 11 days. After exposure, 5-6 animals were taken from each group including the control (microcosm), and their digestive glands were extirpated, washed in 0.15 M NaCl, slightly dried on a filter paper, weighed on an analytical balance, and homogenized in cold in fivefold greater volume of 0.15 M NaCl. The homogenate was centrifuged for 10 min at 4°C and 10,000*g*. The supernatant was taken and, if necessary, stored frozen (for determination of sorbitol and acid phosphatase) or at −18°C mixed with 50% glycerol (for determination of dehydrogenases).

Total protein was determined in the resulting extracts by the Lowry method [12]. Acid phosphatase was determined by the rate of accumulation of *p*-nitrophenol, the product of *p*-nitrophenyl phosphate hydrolysis (disodium salt from Fluka (Germany)) [13], sorbitol dehydrogenase and aldose reductase were determined by the reduction rate of NAD^+ (dihydrate from Fluka) and of $NADP^+$ (disodium salt from Fluka), respectively, in the reaction with D-sorbitol (Fluka) [14]. Activities of the enzymes were expressed in standard activity units per mg protein (U/mg).

The qualitative composition of polyols and approximate contents of sorbitol were determined by high performance liquid chromatography (HPLC) in an ion-exclusion variant with detection by refractometry. This was performed using an 8 × 250-mm column with Reprogel-H^+ polystyrene divinylbenzene sulfite (with H^+ replaced by Na^+) sorbent and with water as the mobile phase. Chromatography was performed at 80°C with elution rate 0.5 ml/min for ~20 min.

Before the chromatography, the extract of the snail's digestive gland was purified by double treatment with water-saturated phenol and then by triple extraction of phenol with water-saturated chloroform. The aqueous phase was separated by centrifugation for 5 min at 10,000*g*. The purified extract was diluted 50-fold in deionized water. The chromatogram was considered to be terminated when the peak of the residual phenol eluted. A new chromatogram was started after the column had been washed for 20 min and the value of the refractive index had stabilized between 0.0002 and 0.0005.

More accurately sorbitol contents were determined in the initial extract by the rate of its oxidation with sorbitol dehydrogenase from sheep liver (purified preparation of ~6 U/mg from Fluka) in the presence of NAD^+ and D-sorbitol as a standard for the calibration curve [15]. The sorbitol contents were expressed in μmol per gram tissue.

Significance of the results was evaluated by the Student test at the probability of 0.95 [16].

RESULTS

Sorbitol contents in the snail's digestive gland strongly depended on the season and could vary more than 100-fold (table). The sorbitol accumulation was the highest in winter, the lowest in summer, and in autumn and spring its contents were about twofold higher than in summer. These values stayed virtually the same on the mollusks' acclimation in the laboratory and throughout the experiment, up to 11 days of exposure.

On short-term experimental temperature decrease, the sorbitol level in the digestive gland of the animals changed similarly in all seasons: it increased monoto-

nously, which was especially pronounced in summer when it increased by more than 400% versus the control. The difference with respect to the control was the least in winter. No accumulation of sorbitol seemed to occur in winter, possibly because of the presence of its level sufficient for supporting viability in cold water.

Intoxication with cadmium is an unnatural external influence on the mollusks. Nevertheless, it induced a similar response — sorbitol was accumulated in the digestive gland, and its amount became twofold higher than in the control already after one-day exposure. On the fourth day the sorbitol contents additionally increased 1.5-fold, and then it decreased but remained higher than in the control. Such changes were observed in all seasons, including winter when the sorbitol contents reached 220 μmol/g tissue, which was the highest value recorded by us.

Thus, decreasing water temperature and intoxication similarly influenced the freshwater snail, inducing sorbitol accumulation in the digestive gland. The rates of this accumulation and absolute values of sorbitol contents were slightly different, but sorbitol clearly serves as a protective factor not only on decrease in temperature (this is known from the literature) but also upon intoxication. The changes observed in the experiments displayed season-related features, but they did not significantly contribute to the established regularity.

Elucidation of changes in activities of enzymes that determine pathways of sorbitol transformations allowed us to better understand the mechanism of adaptation of the freshwater snail to temperature decrease and to cadmium toxicity. In particular, some forms of acid phosphatase characterized by us as AP1, AP2, AP4, and AP6 (the latter being induced in the snail on intoxication by cadmium) were active towards glucose-1-phosphate, glucose- and fructose-6-phosphates, and also towards fructose-1,6-diphosphate. Hydrolysis of these metabolites inhibits decomposition of carbohydrates in the digestive gland and leads to accumulation of free carbohydrates, glucose, and fructose [9]. Their further reduction under the influence of dehydrogenases—sorbitol dehydrogenase (L-iditol:NAD^+ 5-oxidoreductase, EC 1.1.1.14) and aldose reductase (alditol:$NADP^+$ 1-oxidoreductase, EC 1.1.1.21)—seems to promote the accumulation of sorbitol. Another byproduct of metabolism, glycerol, seems to be similarly produced from α-glycerophosphate. However, we failed to detect by HPLC glycerol and other polyols in the extract from the snail's digestive gland. It seems that sorbitol in this mollusk is the only polyol accumulated in the organism as a metabolic byproduct (Fig. 1).

Activities of dehydrogenases and acid phosphatases measured in summer on temperature decrease and on

Changes in sorbitol contents in the digestive gland of freshwater snail on decreasing water temperature and on intoxication with cadmium chloride in different seasons

Duration of experiment, days	Sorbitol content, μmol/g tissue			
	autumn	winter	spring	summer
	Initial values (before experiment)			
0	1.31 ± 0.08	156 ± 4	1.37 ± 0.07	0.68 ± 0.06
	Control			
1	1.18 ± 0.04	161 ± 4	1.21 ± 0.05	0.80 ± 0.05
4	1.03 ± 0.04	134 ± 2	1.33 ± 0.03	0.71 ± 0.03
11	n.d.	165 ± 2	n.d.	0.88 ± 0.03
	Decreased temperature			
1	1.88 ± 0.05 (+)	178 ± 3 (+)	1.99 ± 0.04 (+)	1.38 ± 0.03 (+)
4	1.66 ± 0.07 (+)	138 ± 2 (+)	2.24 ± 0.04 (+)	2.15 ± 0.02 (+)
11	n.d.	163 ± 3 (–)	n.d.	4.47 ± 0.02 (+)
	Intoxication			
1	n.d.	220 ± 5 (+)	2.55 ± 0.07 (+)	1.71 ± 0.02 (+)
4	n.d.	211 ± 3 (+)	3.50 ± 0.07 (+)	2.01 ± 0.03 (+)
11	n.d.	194 ± 3 (+)	n.d.	1.14 ± 0.02 (+)

Note: n.d., no data; (+), significant difference compared to control with $p = 0.95$ by Student's test; (–), insignificant difference.

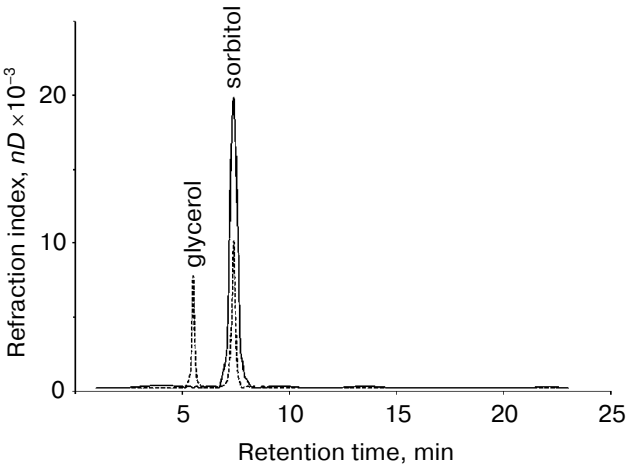


Fig. 1. Chromatogram (HPLC) of polyols from the digestive gland of mollusks. The dashed line shows standard solutions of glycerol and sorbitol, and the solid line represents the extract from the digestive gland of the freshwater snail. Time of sorbitol retention was 7.4 ± 0.2 min.

exposure to $CdCl_2$ depended on the experimental conditions. Thus, after exposure for one day, the activity of acid phosphatase decreased by 28% in response to cold and by 14% in response to the intoxication. On the fourth day, the intoxication caused a strong increase in the enzyme activity (nearly by 270%), whereas in response to decreased temperature the enzyme activity slightly increased but did not overcome the control value. On the 11th day the activity of acid phosphatase sharply decreased to 50% above the control in the presence of $CdCl_2$ and virtually to zero upon the decrease in temperature (Fig. 2).

Upon the decrease in temperature for one day, activities of dehydrogenases increased synchronously by nearly 100% (Fig. 2). During the subsequent 4 and 11 days of the experiment sorbitol dehydrogenase was virtually inactivated, whereas the activity of aldose reductase gradually increased by approximately 170%.

Similar changes were observed under the influence of cadmium chloride, but the activity of sorbitol dehydrogenase was prevalent except on the 11-day exposure when the aldose reductase activity increased by nearly 190% and overcame the sorbitol dehydrogenase activity.

DISCUSSION

Phosphatases and dehydrogenases are major effectors in the adaptive accumulation or the decrease in contents of metabolic byproducts in animal tissues [3, 10, 11]. In our experiments, this is confirmed by data on the stress-induced changes in sorbitol and accompanying enzymes in the digestive gland of the freshwater snail. A scheme of conversions is proposed that is a development of an earlier hypothesis about the functional role of individual phosphatases [9]. According to this scheme, sorbitol can accumulate in the digestive gland by two pathways: reduction from glucose (on decrease in temperature) and from fructose (mainly on intoxication). These pathways are used depending on the stress-inducing influence (Scheme).

On the decrease in environmental temperature, the mollusks demonstrated the increase in activities of dehydrogenases already after one day and, correspondingly, contents of sorbitol produced from similar amounts of glucose and fructose also increased in the digestive gland. But then the activity of sorbitol dehydrogenase sharply decreased, and the further accumulation of sorbitol could occur only through reduction of glucose.

Acid phosphatases are virtually inactive on the decrease in temperature, but glucose and fructose can be accumulated in the organism also as free substances. Thus, contents of free carbohydrates in body fluids (plasma, coelomic fluid, muscle cell cytoplasm) of the pond snail (*Lymnaea stagnalis* L.) were maximal in summer, then slightly decreased but remained high until October, when the water temperature could decrease to 0°C [17]. Therefore, it was supposed that free carbohydrates, especially glucose and fructose, could serve as cryoprotectants; however, the same authors found that

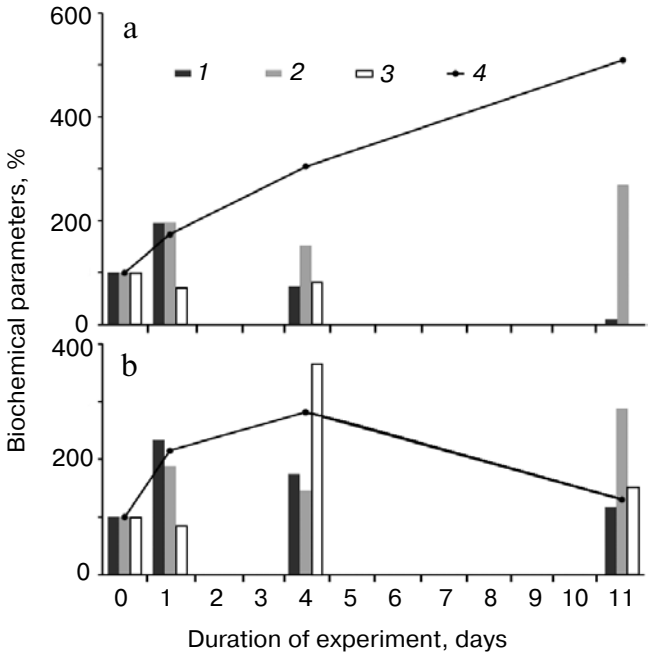
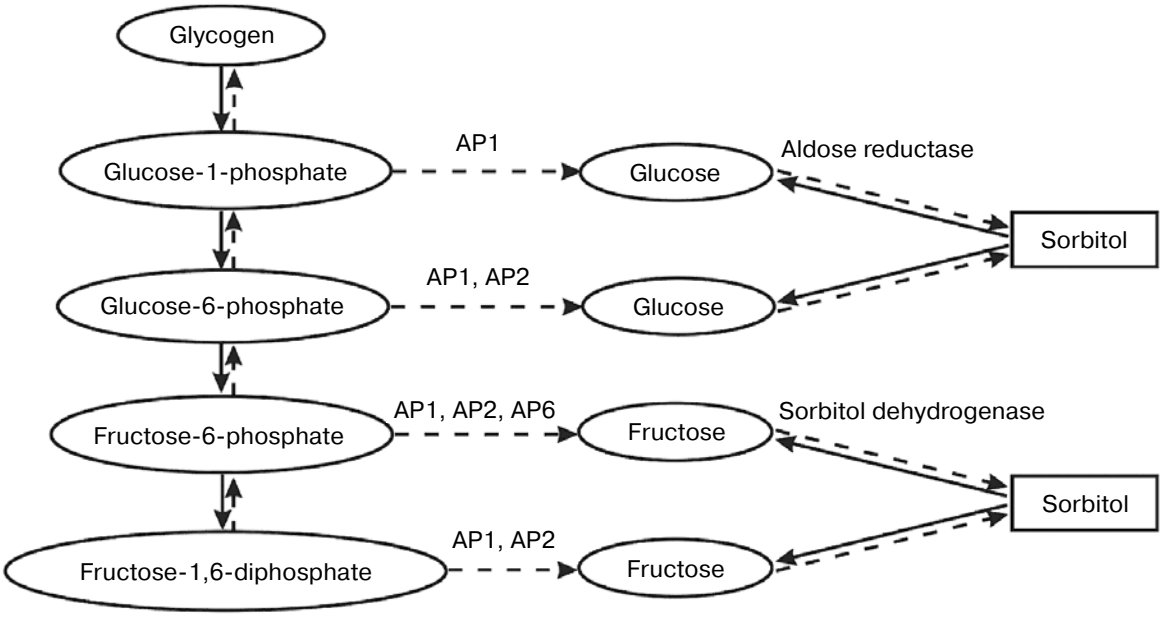


Fig. 2. Changes in sorbitol contents (*4*) and activities of concomitant enzymes (*1-3*) from the digestive gland of freshwater snail on decrease in temperature (a) and on cadmium intoxication (b). Enzyme activities (mU/mg) taken as 100% were as follows: sorbitol dehydrogenase (*1*), 3.4 ± 0.2; aldose reductase (*2*), 2.5 ± 0.2; acid phosphatase (*3*), 26 ± 1.



Scheme of carbohydrate transformations and production of sorbitol in the digestive gland of freshwater snail. Intermediary products of metabolism are in ellipses, the end products of metabolism are in rectangles; solid arrows show reactions of carbohydrate decomposition, and dashed arrows show reactions inhibiting carbohydrate decomposition and promoting accumulation of metabolic byproducts; AP, acid phosphatase

under real conditions of decreasing water temperature in winter the contents of free carbohydrates became minimal. The comparison of these data with our findings indicates that in freshwater gastropods sorbitol seems to be the only cryoprotectant and also a possible metabolic regulator on intoxication, whereas glucose and fructose are its precursors; therefore, their amounts in the organism are high (especially in the warm season) and can be rapidly resupplied if necessary by hydrolysis of food or stock polysaccharides. The re-involvement of free carbohydrates into glycolysis can be regulated by corresponding kinases.

Upon intoxication, sorbitol was mainly produced from fructose. This was promoted by the more pronounced increase in the activity of sorbitol dehydrogenase as compared to the activity of aldose reductase and especially in the activities of acid phosphatases converting fructose-6-phosphate into fructose upon intoxication [9]. However, sorbitol was accumulated in the snail's digestive gland only in the beginning of the experiment. Upon 11-day exposure its contents sharply decreased mainly due to its conversion into glucose, because the activity of sorbitol dehydrogenase approached the control value and the aldose reductase activity increased nearly twofold (Fig. 2).

Most likely, sorbitol in mollusks acts not only as a cryoprotectant but also as a protective factor of broader spectrum capable of reducing the deleterious effect of different influences including intoxication. In this respect, sorbitol is similar to free carbohydrates (glucose, maltose), which act in yeast as nonspecific protective metabolites by reducing the fluidity of cell membranes [5]. The decrease in the sorbitol contents in the mollusks under study 11 days after the introduction of the toxic agent might be explained by a real decrease in the toxic load due to the partial absorption of cadmium by the living organisms and glass walls of the vessel and also due to specific adaptation of the mollusks to cadmium through its binding by metallothioneins.

Thus, adaptive responses of the freshwater snail exposed to cold and a toxic agent are fundamentally the same in the result represented by sorbitol accumulation (at least during 4 days of exposure), but their mechanisms are different: by participation of phosphatases (the involvement of phosphatase is significant on intoxication and negligibly small on adaptation to cold); by carbohydrates-precursors of sorbitol (fructose on intoxication, glucose and fructose on adaptation to cold); and by products of sorbitol oxidation (fructose upon intoxication, glucose on temperature decrease).

REFERENCES

1. Driedzic, W. R., and Ewart, K. V. (2004) *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.*, **139**, 347-357.
2. Lewis, J. M., Ewart, K. V., and Driedzic, W. R. (2004) *Physiol. Biochem. Zool.*, **77**, 415-422.
3. Driedzic, W. R., Clow, K. A., Short, C. E., and Ewart, K. V. (2006) *J. Exp. Biol.*, **209**, 1016-1023.
4. Moalem, S., Storey, K. B., Percy, M. E., Peros, M. C., and Perl, D. P. (2005) *Med. Hypotheses*, **65**, 8-16.

5. Alves-Araujo, C., Pacheco, A., Almeida, M. J., Spencer-Martins, I., Leao, C., and Sousa, M. J. (2007) *Microbiology*, **153**, 898-904.
6. Marfe, G., Morgante, E., Di Stefano, C., Di Renzo, L., De Martino, L., Iovane, G., Russo, M. A., and Sinibaldi-Salimei, P. (2008) *Arch. Toxicol.*, **82**, 371-377.
7. Tsvetkov, I. L., and Konichev, A. S. (2006) *Ecological Biochemistry of Hydrobionts* [in Russian], Moscow State Regional University Publishers, Moscow.
8. Popov, A. P., Tsvetkov, I. L., and Konichev, A. S. (2008) *Biochemistry (Moscow)*, **73**, 937-942.
9. Tsvetkov, I. L., Popov, A. P., and Konichev, A. S. (2003) *Biochemistry (Moscow)*, **68**, 1327-1334.
10. Frenette, G., Thabet, M., and Sullivan, R. (2006) *J. Androl.*, **27**, 233-239.
11. Liebscher, R. S., Richards, R. C., Lewis, J. M., Short, C. E., Muise, D. M., Driedzic, W. R., and Ewart, K. V. (2006) *Physiol. Biochem. Zool.*, **79**, 411-423.
12. Lowry, O. H., Rosenbrought, N. J., Farr, A. L., and Randal, R. L. (1951) *J. Biol. Chem.*, **193**, 265-275.
13. Bessey, O. A., Lowry, O. H., and Broock, M. J. (1946) *J. Biol. Chem.*, **164**, 321-329.
14. O'Brien, M. M., Schofield, P. J., and Edwards, M. R. (1983) *Biochem. J.*, **211**, 81-90.
15. Bergmeyer, H. U. (1970) *Methoden der Enzymatischen Analyse*, Bd. 2, Academie-Verlag, Berlin, pp. 1292-1299.
16. Plokhinsky, N. A. (1961) *Biometry* [in Russian], Siberian Division, Academy of Sciences of USSR Publishers, Novosibirsk.
17. Karanova, M. V., and Gakhova, E. N. (2007) *Sravnit. Ontogenet. Biokhim.*, **43**, 258-264.