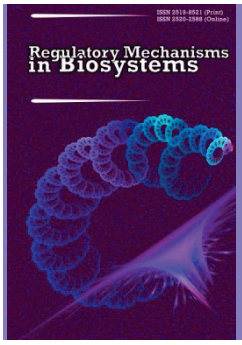

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Influence of potassium p-aminobenzene thiosulfate on the membrane potential and ATPase activity of the plasmatic membrane of the embryos of weatherfish (*Misgurnus fossilis*)

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We studied the effects of the newly synthesized biologically active compound potassium aminobenzene thiosulfonate on electrophysiological parameters of the embryos of weatherfish (*Misgurnus fossilis* L.), in particular the dynamics of transmembrane potential (TMP) of the plasmatic membranes of the weatherfish and the activity of the membrane enzyme Na^+/K^+–ATPase during synchronous cleavage of blastomeres in early embryogenesis. A slight impairment of electrogenesis of the cellular membranes under the action of potassium aminobenzene thiosulfonate indicates changes in the permeability of plasmatic membrane and transport of electrogenic ions. This was related to the inhibition of biosynthetic processes in the first hours of the development of embryos, which led to 60.6 ± 2.6% decrease in the activity of membrane pump when subject to high (10^{-3} M) concentration of potassium aminobenzene thiosulfonate. Its activity further recovered to the level of the control only in 10^{-8} M concentration. Also, we conducted a comparative analysis of the effects potassium aminobenzenethiosulfonate had on the activity of the membrane pump of embryos in *in vivo* and *in vitro* experiments. We determined that the action of the examined compound depends on the presence of a corresponding concentration in the embryo incubation medium. To characterize the variability of changes in the activity of membrane pump of the embryos in the conditions of action of potassium aminobenzene thiosulfonate, we determined constants of semi-inhibition (I_{50}) by linearization of the developed concentration-effect curves using Hill's plot. To determine which factors contribute to the changes in the activity of membrane pump the most, namely, various concentrations of potassium p-aminobenzene thiosulfonate, duration of the development of embryos or other factors that had not been taken into account, we performed a dispersion analysis of how these factors affect the variability of the studied parameter. We determined that the extent of variability of the activity of the membrane pump is also determined by the effect of different concentrations of potassium p-aminobenzene thiosulfonate, and the factor of time of embryo development. The electronic-microscopic study of weatherfish's blastomeres subject to potassium p-aminobenzene thiosulfonate revealed the changes in the ultrastructure of mitochondria, which led to inhibition of their matrix and electron transport chain, and therefore decrease the efficiency of ATP production and energy-dependent processes.

Keywords: potassium p-aminobenzene thiosulfonate; transmembrane potential; weatherfish embryos; ultrastructure.

Introduction

Studying synthesis of new biologically active compounds and subsequent search for new potentially medical drugs are relevant scientific-technological tasks. Special attention should be paid to the development of synthetic analogues of natural bioregulators (Filimonov, 1999; Steiner, 2001; Gafurov & Makhmutova, 2005; Crowley & Dando, 2022), which would be able to exert higher or lower biological action and thereby regulate the physiological activity of the cell, organ or the organisms in general. The development of chemistry of sulfur-containing organic compounds is of significant scientific and practical importance. Special attention should be paid to derivatives of thiosulfoacids of the general formula RSO_2SR'. Besides the practical importance, those compounds are of great theoretical interest as models for studying the interrelations between the structure, reaction ability and biological activity. Because of their high reaction ability and a broad spectrum of the biological action, thiosulfonates are proposed as drugs, pesticides, preservatives of fruits and vegetables, biocides for the protection of materials from biodamage. They display a vast range of the biological action and have notable medicinal properties while being low-toxic, and are more stable than their close analogue, a natural antibiotic allicin – an active compound of the Alliaceae family – garlic (*Allium sativum* L.), which is used not only as food, but also as a means of treating cardiovascular diseases and cancer (Rahman & Lowe, 2006; Roy et al., 2016; Fuchs et al., 2018).

Allicin is the commonest typical sulfur-containing compound in raw garlic. It is synthesized from alliin (Block, 1985). A study revealed that allicin has a broad spectrum of antimicrobial activity towards Gram-positive and Gram-negative bacteria, including antibiotic-resistant bacteria (Wallock Richards et al., 2014; Wu et al., 2015; Loi et al., 2019). Furthermore, allicin exerted antiviral, antifungal and antiparasitic activities (Getti & Poole, 2019). It was reported (Müller et al., 2016) that allicin displayed antimicrobial activity through S-allil-mercapto-modification of thiol-containing proteins in bacteria, thus causing their death, against the background of decrease in glutathione, induction of protein aggregation and inactivation of essential enzymes. Reiter et al. (2017) determined the antimicrobial action of allicin against pathogenic bacteria of *Pseudomonas*, *Streptococcus* and *Staphylococcus* genera, including multiresistant strains. Allicin prevented the formation of biofilm by inhibiting early bacterial adhesion (Lihua et al., 2013; Ranjbar et al., 2015). Also, by preventing the formation of reactive oxygen species through regulation of phase II detoxificating enzymes, allicin increased the level of cellular glutathione in the

cultivated endothelial cells of the vessels (Iciek, 2009). However, allicin is unstable, and was demonstrated to be ruined or metabolized in blood in a couple of seconds (Lawson & Wang, 1993; Freeman & Kodera, 1995). Therefore, the application of allimicin is limited to inhalations or external use due to its instability.

During the biotransformations, allicin transforms into other sulfur compounds, in particular, ajoene, diallyl sulphides, vinyl sulfur-containing derivatives, S-allilcystein and D-allilmercaptocystein. Ajoenes are also typical sulfur-containing compounds of garlic oil. Their fungibacteriocidal action has been confirmed (Ohta et al., 1999; Maluf et al., 2008). S-allicystein prevented thrombus formation by inhibiting the aggregation and adhesion of platelets, which significantly affects the course of cardiovascular pathologies (Rai, 2009). Diallyl sulfide (DAS) and diallyl disulfide (DADS) exert hypolipidemic effects, which – according to the authors (Song et al., 2009) – in *in vivo* experiments were associated with the fact that those compounds inhibited the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), an essential enzyme of cholesterol synthesis. Diallyl disulfide prevented *Pseudomonas* spp. from the formation of biofilm by suppressing the bactericidal adhesion through the inhibition of synthesis of the virulent factors (Høiby, 2017). It was demonstrated that DAS and DADS are able not only to reduce the cholesterol level but also prevent the formation of cancer cells (Hirsch et al., 2009). Despite the fact that the molecular cytotoxic mechanisms of DAS and DADS are not presented in those studies, the cellular cycle was observed to cease during G2/M phase because of the initiation of apoptosis and activation of tumour-suppressing proteins.

During the research on the biological activity of thiosulfates, the greatest attention was paid to their antimicrobial action and determining the mechanism of their action in biological objects (Iciek et al., 2009; Nakamoto et al., 2020; Borlinghaus et al., 2021). Because of the high index and broad range of the antimicrobial activity of thiosulfoesters, their stability and low toxicity, those compounds were proposed as medical agents (Field et al., 1964).

Currently, there is known a broad spectrum of sulfur-containing drugs: streptocyde, norsulfasol, sulfadimethoxine, phtalazol, disulformine and others. It has to be noted that the group of sulfonamides contains not only antibacterial sulfonyl amides – derivatives of sulfanyl (n-aminobenzen sulfon) acid. Such drugs as tolbutamide, chlorpropamide, cyclamide and chlorcyclamide exert hypoglycemic activity. Benztiadiazins are strong diuretics, in particular cyclomethiazide, which displays a direct action towards the reabsorption of sodium and chlorine ions in the kidney tubules. Etamide is used in medicine as a drug that regulates the purine metabolism during gout, polyarthritis and kidney stone diseases. Therefore, the newly synthesized thiosulfonates are interesting and promising compounds for the development of drugs.

Materials and methods

The studies of PAT were conducted on embryos of a freshwater species *Misgurnus fossilis* L. weatherfish. It belongs to the Cobitidae family, Cypriniformes order. Weatherfish can be kept in the laboratory conditions with no special requirements, obtaining its oocytes and spermia is relatively easy, it has short embyogenesis and some other specifics that make it convenient for experimental in vivo and in vitro studies. Therefore, weatherfish are broadly used in the studies of a number of problems in modern biology, including biophysical, biochemical, cytological and embryological (Hoida, 1993; Zinchenko et al., 2018).

The ovulation of weatherfish was stimulated by intramuscular injection of chorionic gonadropin to the females (500 units). The caviar had been obtained 36 h after the stimulation and was fertilized in Petri dishes using the classic method (Hoida, 1993). The testicles were obtained after decapitation and autopsy of the abdominal cavity of males. Five-ten minutes after the fertilization, the zygotes were rinsed and incubated in the Holtfreter's physiological solution at the temperature of 20–22 ºC. The development stages were visually controlled under a Sigetamb-401 binocular microscope.

To determine the changes in TMP during the first cleavages and blastulation in the conditions of minimal damage of morphological and functional density of embryos, we used a microelectronic device for electrophysiological studies (Hoida, 1993). To carry out the biochemical studies, the weatherfish embryos were incubated in a solution of potassium n-aminobenzene thiosulfonate during the first cleavage of blastomeres, namely 60, 150, 210, 270 and 330 min after conception of the ovicells during the stages that correspond to the first cleavage of zygote (2 blastomeres), forth (16 blastomeres), sixth (64 blastomeres), eighth (256 blastomeres) and tenth (1,024 blastomeres), respectively.

The microsome fraction of the membranes of weatherfish embryos was obtained using the method of differential centrifugation in the sacharose density gradient. At first, the embryos were homogenized in a buffer solution of the following composition (mmol/L): sacharose – 120.0, KCl – 130.0, $MgCl_2$ – 5.0, Tris-HCl – 10.0 (pH 7.4; 4 °C). Then, the residuals of embryo vitelline were settled by centrifugation (10 min., 1,600 g). The supernatant fluid that had been enriched by fragments of plasmatic and reticular membranes, obtained after 10 min centrifugation at 10,000 g, was kept at the temperature of 20 °C (Lutsyk et al., 1986).

The activity of Na^+/K^+-ATPase (EC 3.6.1.37) (in μmol of P_i/min per 1 mg of protein) of cells at different blastulation stages was evaluated according to the difference in the content of inorganic phosphate (P_i), formed in the incubation environment in the presence and absence of membrane fragments in it, and accounting for the content of endogenic P_i in the membrane preparation. The amount of the product of P_i reaction was tested using the modified Fiske-Subbarow method (Fiske & Subbarow, 1925), and the concentration of protein in the membrane preparation – using the method of O. H. Lowry (Lowry et al., 1951).

In the studies, we used CP reagents: EGTA, NaN_3 (Merk, Germany); ouabain (Fluka, Switzerland); PAT (Acros, Belgium); Tris, thapsigargin (Sigma, USA).

The differences between the values in the control and experimental groups were determined using ANOVA, where the differences were considered significant at $P < 0.05$ (with Bonferroni correction). The results were identified as mean ± standard error ($x \pm SE$) at $n = 5$ (Morgan et al., 2012).

Potassium n-aminobenzene thiosulfonate (PAT) was synthesized at the Department of Technology of Biologically Active Compounds, Pharmacy and Biotechnologies of the NU Lviv Polytechnic University according to the following scheme of transformations (Fig. 1) (Lubenets, 2003).

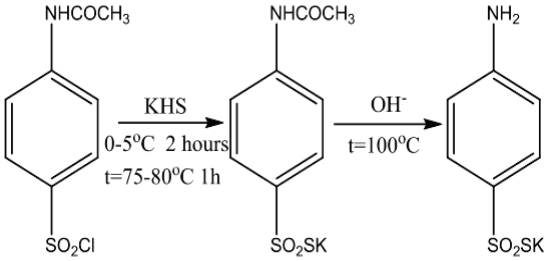

Fig. 1. Synthesis of potassium n-aminobenzene thiosulfonate

The structure and individuality of this compound was confirmed using the methods of thin-layer chromatography (TLC), the data of infrared spectroscopy (IR), proton nucleomagnetic resonance (H1NMR) and the data of element analysis.

Results

Right after we had made the protocols of plasmatic membrane of the weatherfish embryos in the Holtfreter's solution using a microelectrode, we recorded the transmembrane potential (TMP), the amplitude of which accounted for (–13.4 ± 2.3) mV (n = 5) (Fig. 2, curve 1) and slowly, over 15–20 min, it increased to the typical level for this stage – the first cleavage (2 blastomeres). The period of fluctuations of the membrane potential in the control throughout this cleavage of blastomeres was approximately the same, equaling 31 min, corresponding to the duration of the cell cycle.

As we see in the curve, the further changes in TMP are fluctuating, changing in phases: increases and decreases in each mitotic cycle, while the amplitude values gradually increase from –10 to –14 mV, peaking

(–58.3 ± 2.8) mV (n = 5) during the sixth cleavage (64 blastomeres) (on graph 2 of Figure 1, the range of increasing potential from –18.3 to –76.6 mV). The phase of reaching this TMP level was longer than its decline phase, which is characteristic for the stage of early development, when the cleavage grooves are meridional and always perpendicular to the previous. After the sixth cleavage, the pattern of the curve of TMP dynamics changed in the following cycles: the phases of increase and decline became even, and the level of maximal values decreased from 58.3 to 52.2 mV. This particular period was characterized by the establishment of latitudinal cleavage grooves (cleavage VI), formation of morulae, and complete desynchronization of cleavage (divisions IX and X). Hyperpolarization of the membrane (increase in TMP level) of weatherfish embryos occurred during the interphase of cellular cycle, depolarization (decline of TMP) during mytosis, which was the highest during the prometaphase.

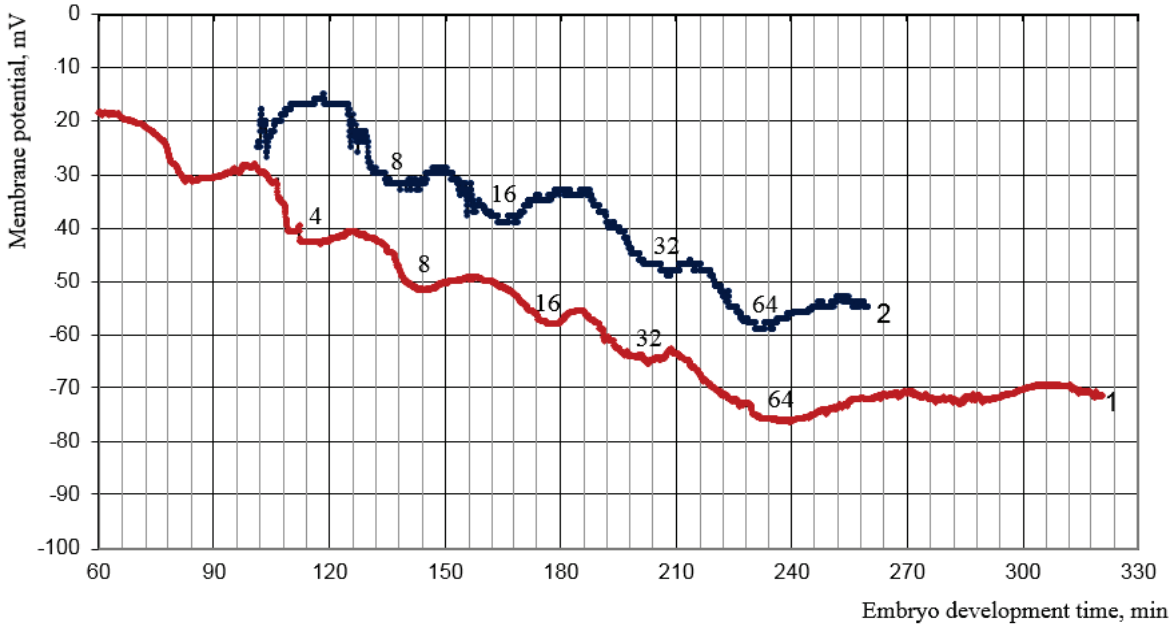

Fig. 2. Influence of potassium n-aminobenzene thiosulfonate in 10^{-3} M (*2*) concentration on the transmembrane potential during early development of the weatherfish embryos, compared with the control (*1*)

Dynamics of the TMP significantly changes as a result of the influence of external factors – physical and chemical, and is a sensitive indicator of cellular homeostasis, which made us consider some aspects of the influence of chemical factors, particularly, thiosulfoacid salts on the changes in TMP. The continuous monitoring of TMP of the embryos incubated in the environment supplemented with the studied compound revealed insignificant aperiodic changes in its level. As we see in TMP curve (Fig. 2, curve 2) and data in the table (Table 1), the fluctuation periods coincided with the mitotic cycles of synchronous cleavage of blastomeres, compared with the control.

Table 1
Values of the periodic changes (min.) in the membrane potential during the synchronous cleavages (2–64 blastomeres), when subject to potassium n-aminobenzene thiosulfate, compared with the control

Stage, blastomeres	2	4	8	16	32	64
PAT (n = 3)	–	–	25.2 ± 2.4	34.8 ± 2.2	31.9 ± 0.7	37.5 ± 3.5
Control (n = 5)	30.2 ± 1.2	28.2 ± 1.8	29.2 ± 2.1	30.5 ± 1.6	27.2 ± 2.3	51.8 ± 2.2

Note: the fluctuations of membrane potential were impossible to see at the stages of 2nd and 4th blastomeres.

If TMP fluctuations continued (Fig. 2, curve 2), the third (8 blastomeres) and sixth (64 blastomeres) cleavages were 25.2 ± 2.4 and 37.5 ± 3.5 min and were somewhat shorter than in the control (where the period of fluctuations was 29.2 ± 2.1 min in third cleavage and 51.8 ± 2.2 min in the sixth. By contrast, the fourth and fifth cellular divisions were characterized by an insignificant increase in the duration of TMP fluctuations, by 4.3 and 4.9 min, respectively. Moreover, when the maximal values of MP fluctuations were preserved, compared with the control, we determined significant 5.3 and 5.1 mV ($P < 0.05$) increases in the fluctuation of amplitude (Table 2) during the third cleavage at the stage of 32 blastomeres.

Such results indicate insignificant, nonetheless still an impairment in electrogenesis of the cellular membrane against the background of PAT influence and indicate changes in the permeability of plasmatic membranes and transport of electrogenic ions that can alter the activity of a number of enzymes, including Na^+/K^+-ATPase. This assumption has been confirmed by the results of our *in vivo* and *in vitro* studies of Na^+/K^+-ATPase activity of the weatherfish embryos subject to potassium salts of para-aminobenzene thiosulfoacid in high (10^{-3} M) and low (10^{-8} M) concentrations during the 6th h of development.

Table 2
Values of amplitude (mV) changes in the membrane potential during the synchronous cleavages (2–64 blastomeres), when subject to the action of potassium n-aminobenzene thiosulfate, compared with the control

Stage, blastomeres	2	4	8	14	32	64
PAT (n = 3)	–	–	16.1 ± 1.3	9.1 ± 1.4	14.2 ± 1.5	13.0 ± 2.2
Control (n = 5)	13.1 ± 0.9	14.2 ± 1.5	10.8 ± 1.6	10.1 ± 1.4	9.1 ± 1.3	14.2 ± 1.1

Note: fluctuations in the membrane potential were impossible to observe at the stages of 2nd and 4th blastomeres.

Throughout the *in vitro* studies of Na^+/K^+-ATPase activity, we determined that PAT action (in 10^{-3}–10^{-8} M concentrations of) during 6th h of the development of embryos led to notable changes in the activity of Na^+/K^+-ATPase of the embryos, compared with the control. We determined that the action of high concentrations of the compound caused 60.6 ± 2.6% decrease in the activity of the enzyme of embryos (Fig. 3) and was characterized by the inhibiting effect. Gradual decrease in the concentration of thiosulfonate in the incubation environment caused gradual increase in the activity of enzyme, compared with the control, and in some cases, there was insignificant decrease (2 and 16 blastomeres), and in some cases even increase in the activity of the enzyme (insignificant during the 6th and 10th cleavages and significant during the 8th cleavage).

Therefore, in the *in vitro* studies, we determined that the action of thiosulfonate caused statistically significant dose-dependent changes in the activity of membrane-bound enzyme of the embryos. The greatest inhibiting effect was seen if 10^{-3}–10^{-7} M concentrations of the biologically active compounds were present in the incubation environment, whereas 10^{-8} M concentrations increased Na^+/K^+-ATPase activity in the embryos, compared with the control.

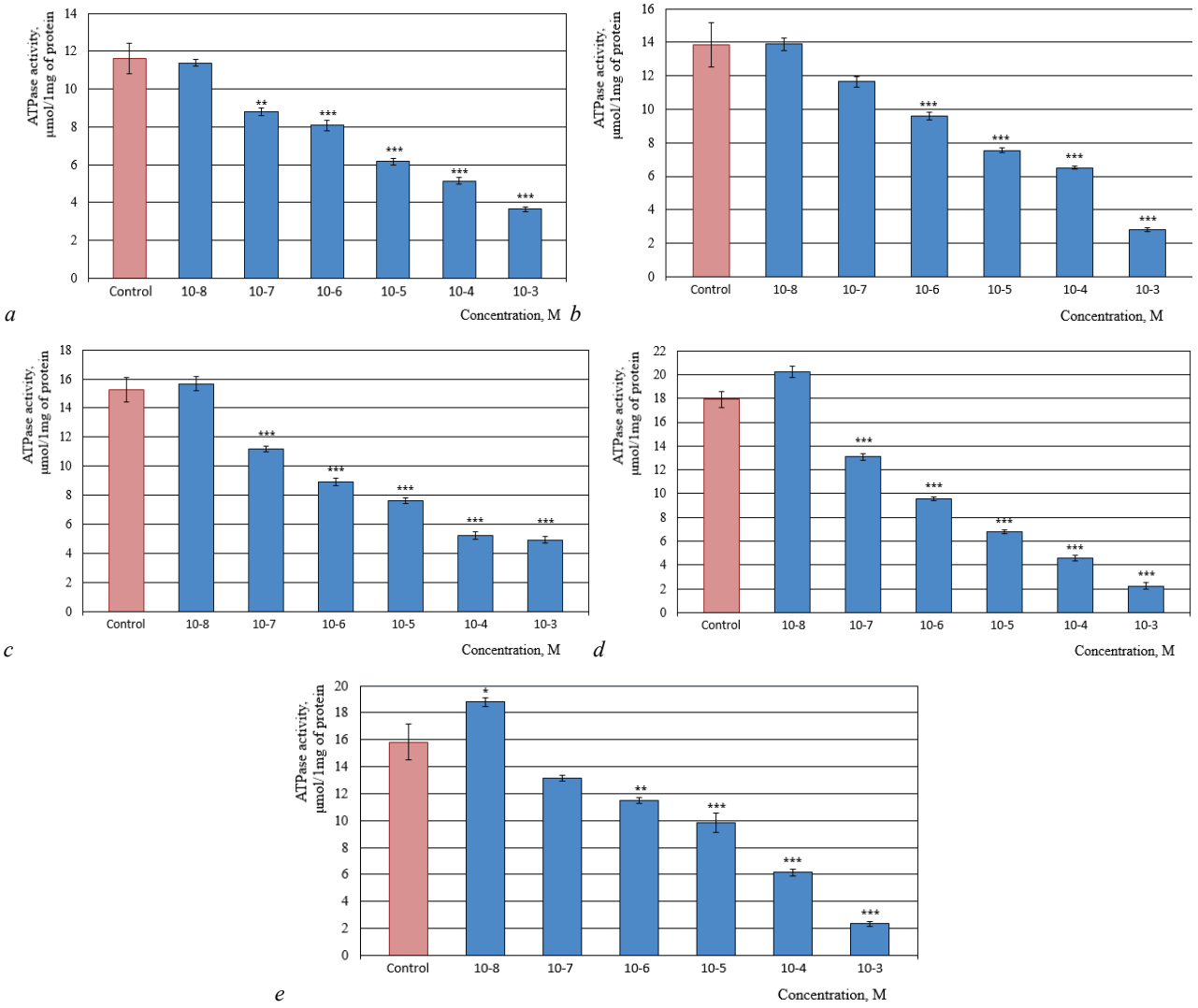

Fig. 3. Changes in *in vitro* activity of Na^+/K^+-ATPase of the embryos subject to potassium п-aminobenzene thiosulfonate during the synchronous cleavages of blastomeres: *a* – 2 blastomeres, *b* – 16 blastomeres, *c* – 64 blastomeres, *d* – 8 division, *e* – 10 division; compared with the control; x ± SE, n = 10; * – P < 0.05 – the difference is statistically significant between control and experimental groups; ** – P < 0.01; *** – P < 0.001

For a detailed understanding of the action mechanism of the newly synthesized compound, we carried out a series of in vivo studies of PAT effect in 10^{-3} and 10^{-8} M concentrations on the activity of Na^+/K^+-ATPase of the embryos during all the stages of synchronous division of blastomeres (Fig. 4). As revealed by the studies, the examined compound led to decrease, significant in most cases, in the activity of Na^+/K^+-ATPase of the embryos, compared with the control. Subject to high PAT concentration, in the first hours of the development, the embryos (10^{-3} M) were observed to have significant 78.4 ± 10.4% decrease in the activity of membrane enzyme. In the following hours of the development cleavage VI (210 min) and cleavage VIII (270 min), there occurred gradual increase in the activity of Na^+/K^+-ATPasa of the embryos subjects to both the concentrations of the studied compounds, i.e. recovery of the enzyme activity, compared with the control. There we observed decrease in Na^+/K^+-pump, on average by 46–48%, compared with the control.

At the last stage of synchronous cleavage of blastomeres cleavage X (330 min), after the action of high and low concentrations, we saw continuation of rapid increase in the activity of membrane enzyme, unlike the control, where the activity of Na^+/K^+-ATPase decreased almost down to the previous values. The action of low concentration of the compound restored the activity of enzyme almost to the control value, while the high concentrations of PAT decreased the activity of ATPase by 22.2 ± 0.5%, compared with the control. Thus, we determined that in vivo action of thionsulfonate led to significant dose-dependent changes in the activity of the membrane-bound enzyme of the embryos: the greatest inhibiting impact was seen when 10^{-3} M concentration of the biologically active compounds was present in the incubation medium, during the first hours of development, whereas adding thiosulfoacids in low concentration (10^{-8} M) to the incubation medium caused recovery of the activity of Na^+/K^+-ATPase of embryos up to the control level.

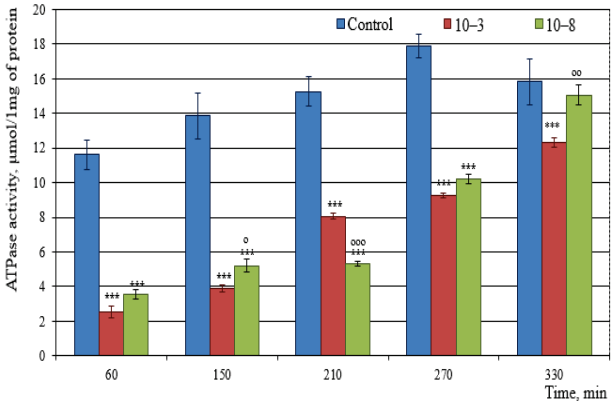

Fig. 4. Changes in *in vivo* activity of Na^+/K^+-ATPase of the embryos subject to potassium p-aminobenzene thiosulfonate during the synchronous cleavages of blastomeres, compared with the control (x ± SE, n = 10): * – the difference between the control and experimental groups P < 0.05; ** – P < 0.01; *** – P < 0.001; ^{o} – the difference between different concentrations (10^{-3} and 10^{-8} M) P < 0.05; ^{oo} – P < 0.01; ^{ooo} – P < 0.001

Derivatives of thiosulfoacids obviously have an indirect effect on the activity of Na^+/ K^+-pump. By being involved in the chain of metabolic transformations in the cell, they can likely affect other hydrolyses as well, for example Ca^{2+}-ATPase and phosphodiesters.

For the further understanding of the inhibiting effect of derivatives of thiosulfoacids, we performed a comparative analysis of PAT effect in 10^{-3} and 10^{-8} M concentrations (Fig. 5) on the activity of Na^+/K^+-ATPase of the embryos in *in vivo* and *in vitro* experiments during the early embryogenesis. We determined that the action of the examined compound differs by its effect in *in vivo* and *in vitro* experiments, and also depends on presence of a certain concentration of the biologically active compound in the embryo incubation medium.

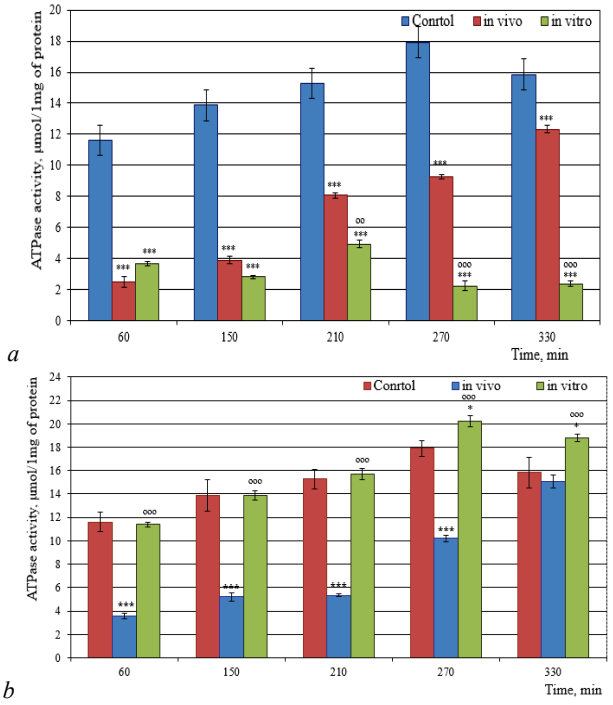

Fig. 5. Changes in *in vivo* and *in vitro* activity of Na^+/K^+-ATPase of the embryos subject to the PAT in 10^{-3} (*a*) and 10^{-8} M (*b*) concentrations during the early embryogenesis, compared with the control; x ± SE, n = 10; see Figure 4

Therefore, in vitro influence of the PAT in 10^{-3} M concentration significantly inhibited the activity of Na^+/K^+-ATPase of the embryos at all the stages of their development, and the values of activity of the enzyme remained within low ranges (3.9 to 5.3 µmol P_i/h per 1 mg of protein). The *in vivo* action of thionsulfonate had a slightly different effect, particularly recovery of the activity up to the level of control, i.e. significant increase in the activity of Na^+/K^+-ATPase of the embryos during all the examined development stages, compared with *in vitro* action of thionsulfonate. Perhaps, in *in vivo* conditions, only a fraction of molecules of derivative thionsulfoacids binds with enzyme molecules, as indicated by decrease in the enzymatic activity of Na^+/K^+-ATPase, compared with the control. Most PAT molecules or their primary metabolites can presumably modulate other links of metabolic transformations in embryo cells, which in turn enhances the metabolic processes in embryos that are intensively developing and growing.

Against the background of the action of low PAT concentration (10^{-8} M), we determined a similar pattern of its activity in *in vivo* and *in vitro* conditions, where the enzymatic activity grew in both cases. When the high thionsulfonate concentration (10^{-3} M) was used, we saw an opposite effect of its action in *in vivo* and *in vitro* conditions.

In *in vivo* conditions, this compound inhibited the activity of the membrane enzyme to a higher degree than in *in vitro* conditions. Therefore, the pattern of changes caused by 10^{-8} mol/L PAT concentration was statistically significant and similar, and such caused by 10^{-3} M concentration was significant and opposite.

It has to be noted that *in vivo* and *in vitro* actions of 10^{-8} M PAT concentration, at the stage of the 10th division of blastomeres, there were seen 95.2% and 18.8% recoveries of the Na^+/K^+-ATPase activity, compared with the control. In our opinion, PAT in low concentrations is a modulator of metabolic processes in embryos, intensifying the metabolic processes.

Therefore, inhibition of Na^+/K^+-ATPase of the weatherfish embryos at early stages of embryogenesis by thionsulfonates can be explained by their ability to disrupt the integrity of membrane, ruining proteins at the same time, thereby decreasing the activity of the examined membrane enzyme, and this in turn confirms its embryotoxicity. However, those biologically active compounds easily metabolize in low concentrations and intensify the metabolic processes in embryos that in any case are intensely developing and growing, and this may explain the partial recovery of Na^+/K^+-ATPase activity in our studies.

To characterize the variability of changes in Na^+/K^+-ATPase activity of the weatherfish embryos in the conditions of PAT action, we identified the semi-inhibition constants (I_{50}) by linearization of the dose-effect curves in logarithmic coordinates (Fig. 6) (Dotsenko & Taradina, 2017). Values of I_{50} for the studied factors at different stages of the development were determined at the cross point of the developed straight lines and abscissa axis. The linearized graphs of the development were quantified using the smallest squares (r = 0.90–0.99).

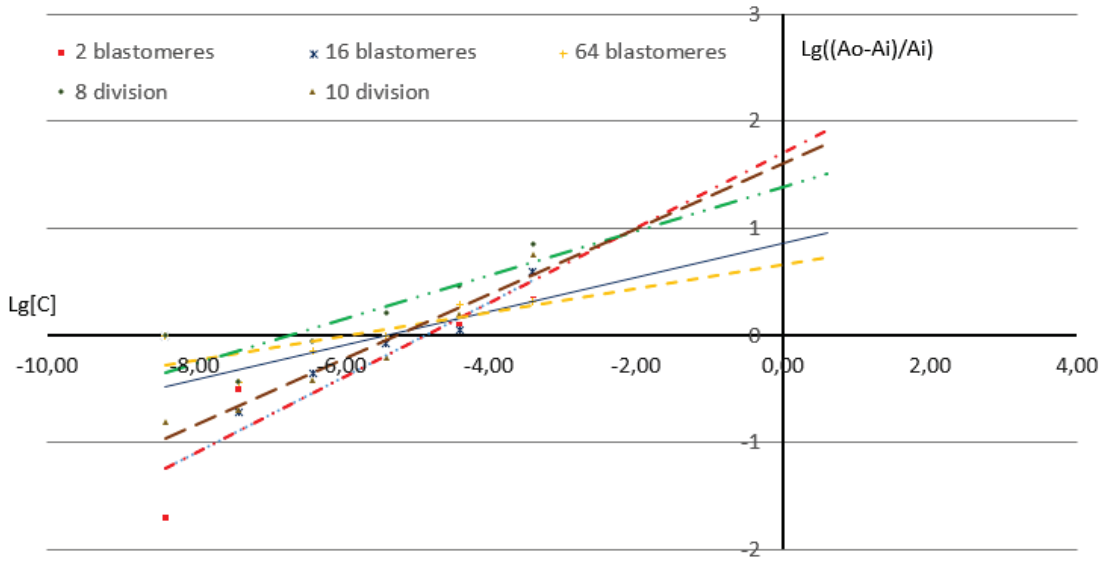

Fig. 6. Linearization of the concentration dependency of Na^+/K^+-ATPase inhibition of the weatherfish embryos at the stages of 2, 16, and 64 blastomeres, 8th and 10th cleavages of blastomeres by potassium para aminobenzene thiosulfonate in the Hill's coordinate system

It has to be noted that at different stages of cleavage of blastomeres, the sensitivity (affinity) of Na^+/K^+-ATPase to the action of biologically active compounds significantly changed.

The highest values of I_{50} semi-inhibitions constants, i.e. the lowest extent of inhibition of the activity of the membrane enzyme by the biologically active compound, were observed at the stages of the 2nd, 16th and 10th cleavages of blastomeres (Table 3). This in turn indicates the stability of embryos to the influence of the biologically active compound.

Table 3

Values of Na^+/K^+-ATPase I_{50} semi-inhibition constants (mol/L) of the weatherfish embryos by derivates of thiosulfoacids at different development stages

Derivative of thiosulfoacid	Development stages of wheaterfish embryos				
	2 blastomeres	16 blastomeres	64 blastomeres	8th cleavage	10th cleavage
PAT	$1.36 \cdot 10^{-5}$	$4.15 \cdot 10^{-6}$	$1.17 \cdot 10^{-6}$	$1.82 \cdot 10^{-7}$	$5.55 \cdot 10^{-6}$

Thiosulfoacids likely owe their stability to the ability to form solid complexes with molecules of other compounds, thus preventing them from going through the cellular membrane.

The greatest inhibiting effect of derivatives of thiosulfoacids, i.e. lowest I_{50} values, was found at the stages of the 6th and 8th cleavages of blastomeres. This correlates with the biosynthetic processes that are developing at this development stage in the embryo cells, which need redistribution of pools of high-energy bonds. Those stages of embryo development are considered the most sensitive to the action of any external factors, i.e. there is needed a small introduction of the active compound into the incubation environment in order to decrease the activity of Na^+/K^+-ATPase of the plasmatic membranes of embryos by 50%.

To identify which of the factors contributes to changes in Na^+/K^+-ATPase activity the most – namely, various concentrations of biologically active compounds, the duration of the development of embryos or other factors that had not been taken into account – we performed a dispersion analysis of the influence of those factors (according to relative fractions) on variability of a studied parameter. This statistical method allows for adequate expertise of variable experimental material.

By using the dispersion analysis, which is sufficient to produce significant results with small sampling, (Bakhrushyn, 2011), we determined relative fractions of PAT influence on the variability of Na^+/K^+-ATPase activity of the weatherfish embryos.

The single-factor dispersion analysis revealed the effects of PAT concentrations (10^{-3}–10^{-8} M) on the variability of Na^+/K^+-ATPase activity at various development stages of the weatherfish (Fig. 7). The lowest effect of the compound was observed at the stage of 16 blastomeres (150 min), relative fractions of which were 85.0% on average.The effect was the strongest at stage 8 of the blastomere cleavage (270 min), relative fractions – on average 96.7% (P = 0.99). At the stage of 2 blastomeres (60 min), the share of PAT effect accounted for 87.3%. Thiosulfoacids decreased their tendency towards variability of the enzyme on 6th h of the development (10th division – 330 min).

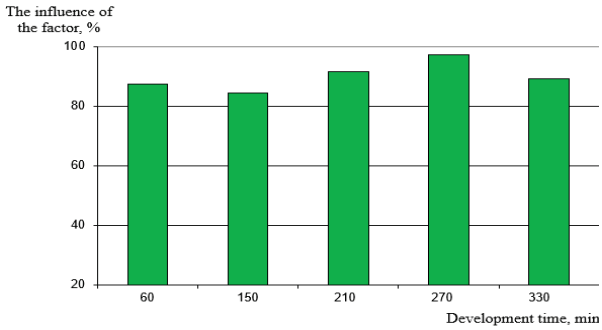

Fig. 7. Evaluation of influence of various concentrations (10^{-3}–10^{-8} M) of potassium p-aminobenzene thiosulfonate on the activity of Na^+/K^+-ATPase of the weatherfish embryos at different development stages

Therefore, Na^+/K^+-ATPase of the membranes of the embryos were most sensitive to the changes in all PAT concentrations between 210 to 270 min of the development, and least sensitive on the stage of 16 blastomeres (150 min). In order to compare the influence of various concentrations of derivative of thiosulfoacids (10^{-3}–10^{-8} M) on the activity of Na^+/K^+-ATPase of the membranes of blastomeres of the weatherfish embryos during different periods of their development, we performed 12 series of two-factor dispersion analysis, the results of which are presented in Fig. 8 (n = 5).

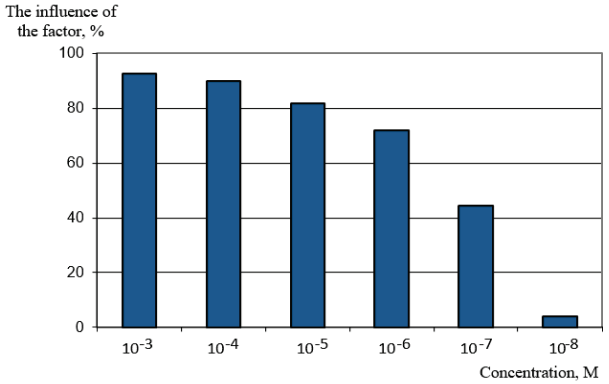

Fig. 8. Evaluation of the influence of various concentrations (10^{-3}–10^{-8} M) of potassium p-aminobenzene thiosulfonate on the activity of Na^+/K^+-ATPase of the weatherfish embryos at different development stages

We determined the contributions of different concentrations of the examined compound and time factor against the background of other factors that had not been taken into account in our experiment (Fig. 9), and also evaluated the significance of effects of the studied factors. The significance level for the results of the influence of concentration factor (10^{-3}–10^{-7} M) of derivatives of thiosulfoacids equals P < 0.01, except the effect of PAT in 10^{-8} M (P = 0.83) concentration.

Therefore, we found that the changes in activity of Na^+/K^+-ATPase of embryos are determined by the action of various concentrations of the examined biologically active compound. This compound had the greatest effect (89.9–92.7%) in 10^{-3}–10^{-4} M concentrations, further having lower effect on the changes of the enzyme activity (with decrease in thiosulfonate concentrations), and minimal effect was observed after using the low concentration of 10^{-8} M, which for PAT was 3.9%.

The two-factor dispersion analysis revealed that the contribution of the time factor to the general variability of enzymatic activity of potassium/sodium ATPase subject to the action of biologically active compound was insignificant when high 10^{-3}–10^{-6} M concentrations were used, being within 2.5–20.2%. For 10^{-7} and 10^{-8} M concentrations, the contribution of the time factor to the change of Na^+/K^+-ATPase activity was significant, measuring 51.8–90.6% (P = 0.99, see Fig. 9).

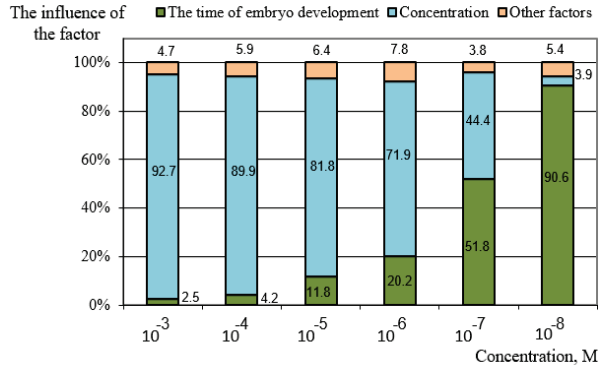

Fig. 9. Two-factor dispersion analysis of effects of various PAT concentrations and duration of the embryos' development on the activity of Na^+/K^+-ATPase of the weatherfish embryos at different stages of their development, where "other factors" were random factors, contributions of which the dispersion analysis subtracts; "concentration" is the influence of thionsulfonates in a given concentration indicated on x axis; "time of development" is duration of the embryos' development (since the studies were carried out during different development stages)

Those results suggest that the influence of the indicated concentrations of biologically active compounds changed during the development of the weatherfish embryos. Therefore, the variability of the activity of Na^+/K^+-ATPase of membranes is determined by the effects of various concentrations of potassium salts of thiosulfoacids, and the time factor of the development of embryos. The dispersion analysis revealed a quite insignificant contribution of other, unaccounted, factors on the changes of activity of examined membrane enzyme for the PAT– 3.8–6.4%.

Because during early embryonic development, the embryos are an adequate test system to study the toxicity of compounds, it is practical to analyze the ultrastructural variations of intracellular structures and organelles that accompany the metabolic changes against the background of the action of biologically active compounds. To study the ultrastructural changes of blastomeres of the Misgurnus fossilis embryos subject to derivatives of thiosulfoacids, we chose 10^{-8} and 10^{-3} M concentrations. The study was carried out during the tenth stage of development.

The least expressed changes in the ultrastructure of all organelles of blastomeres of the weatherfish embryos – as compared with the control – were observed when 10^{-8} M concentration of derivatives of thiosulfoacids were present in the incubation environment (Fig. 10). We saw that –similarly to the control – after the embryo cells had been incubated in the solution of potassium p-aminobenzene thiosulfonate in low concentration, cytoplasm of blastomeres was characterized by homogenous cytosol and contained channels of a-granular (aER) and cisterns of granulated endoplasmatic reticulum (gER), had accumulation of polysomes (PS), average-sized rounded mitochondria (MC), single lysosomes (L) (Fig. 10b), and multivesicular bodies (MB).

In the sixth hour of development (10th stage), the studied samples were observed to have extension of gER cisterns and partial disorganization of their membranes (Fig. 10b). Channels of the aER were also larger than in the control. There appeared many large phagosomes and lysosomes, mitochondrial matrix with the content of low electronic density. If high 10^{-3} M concentration was present in the medium where those thiosulfonates were incubated, there were significant changes in the ultrastructural organization, compared with the control. Changes in the ultrastructural organization of embryo cells had signs of necrotic changes (Fig. 10d).

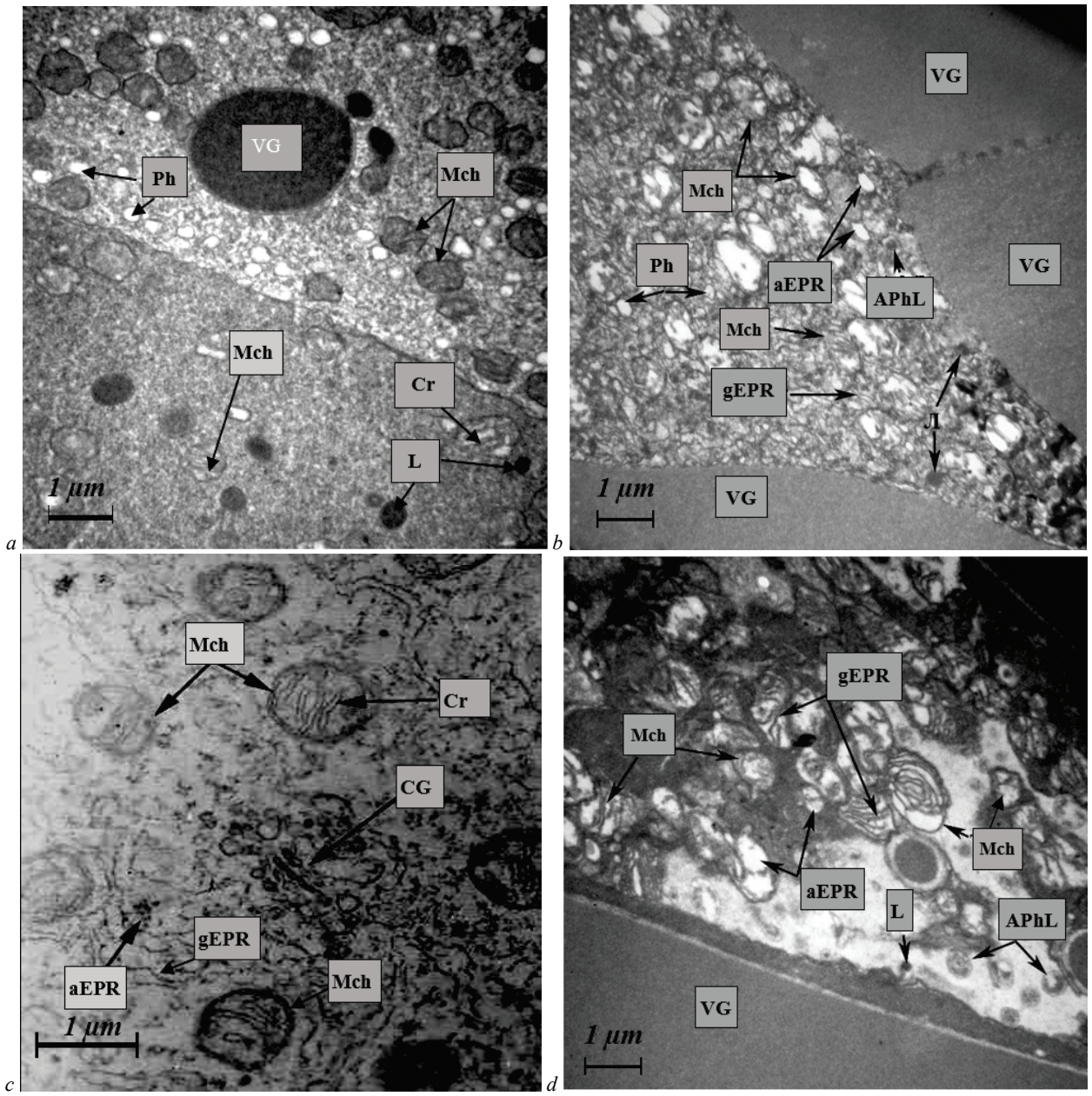

Fig. 10. Ultrastructure of the weatherfish embryos at the 10th cleavage stage in the normal conditions (*a*, *c*) and subject to PAT (*b*, *d*) in 10^{-8} M (*b*) and 10^{-3} M (*d*) concentrations: hereinafter: aER – a-granular ER; gER – granular ER; YG – yolk granules vitelline granule; MC – mitochondrial cristae; L – lysosomes; APhL – autophagolysosomes; M – multivesicular bodies; MC – mytochondria; PS – polysomes; PS – phagosomes; GC – Golgicomplex; SL – secondary lysosomes

In the regions with initial necrosis, cytoplasm of blastomeres was characterized by non-homogenic cytosol, which could be a consequence of coagulation of cytoskeleton proteins, destruction and ruination of cytoplasmic enzymatic complexes. The cytosol was seen to have hypertrophic aER channels, filled with low-electronic-density complexes. In deep layers of cytoplasm, there was significant ruination of cER membranes, which resulted in breakdown of polysomes into monosomes and their disorganization, indicating inhibition of protein biosynthesis. Also, we observed changes in mitochondria. Those organelles were characterized by irregular shape with ruined cristae and content of matrix of low electronic density (Fig. 10d).

Against the background of the action of this concentration of compound, there were regions of notable colliquative necrosis. Such regions were characterized by presence of large amount of increased phagosomes, multivesicular bodies, hypertrophic aER channels and disorganized gER cisterns, which had already lost ribosomes. Plasmatic membranes of vitelline granules were indistinct and loose, though retained their integrity. Membranes of phagosomes were indistinct as well. Cytosol was almost absent, because the entire field of view was occupied by large phagosomes, suggesting the process of necrotic ruination of cells, produced by high concentration of derivatives of thiosulfoacids.

Therefore, PAT influence on the ultrastructural organization of blastomeres at early stages of the development of wheatherfish embryos was concentration-dependent. Presence of the low concentration of derivative of thiosulfoacids in the incubation environment caused no significant impairments, whereas the high concentration led to notable paranecrotic or necrobiotic processes that are related to impairments of viscosity of cytoplasm and disorganization of the enzymatic cell system.

The electronic-microscopic study of the weatherfish blastomeres of embryos incubated in presence of the PAT revealed that the cytoplasm peeled off, which was associated with – first of all – easy penetration of the compound into the cell, also suggesting rarefaction of cytoplasm.

As is known, an important role in cell pathology belongs to inactivation of ion-transporting enzymes, the active center of which contains thiol groups, first of all ATP-hydrolases. Inactivation of such enzymes slows the "pumping" of calcium or sodium ions from cells and increases the intracellular concentrations of those ions. It also damages the organelles as a result of increase in osmotic pressure. Under the action of difference of electronic potentials on the membranes, a larger number of sodium ions enter the cell, and potassium ions enter mitochondria. As a result, the osmotic pressure inside blastomeres increases, leading to edema, causing even greater damage to membranes and ultimately the development of necrotic processes.

Discussion

The values of transmembrane potential are known to be the indicators characterizing the physical-chemical and physiological properties of cells, and also the level of their structural-functional organization. In particular, membrane-bound fluctuations take part in the regulation of cellular proliferation and are an important indicator of homeostatic condition of the cells subject to both chemical and physiological factors (Galyk et al., 2021). Increase in TMP potential during the early development of embryos of many animals coincide with changes in the activity of Na^+/K^+-ATPase and their homegenates (Ohara et al., 1993; Kadir et al., 2018). Those experiments revealed that Na^+/K^+-ATPase makes a certain contribution to hyperpolarization of embryo membranes while its blastomeres are dividing. The changes that we observed in the electronic characteristics of TMP of cells of the embryos of weatherfish subject to derivatives of thiosulfuacids suggest that they modulate permeability of the plasmatic membrane and ion transport, accompanied by changes in the activity of a number of enzymes, namely – Na^+/K^+-ATPase. Therefore, high PAT concentrations (10^{-5}–10^{-3} M) inhibited the activity of Na^+/K^+-ATPase at all the development stages on average by 48.5–77.8%.

The results of comparative analysis of effects of derivatives of thiosulfoacids on the changes in the Na^+/K^+-ATPase activity of wheatherfish embryos indicated that *in vivo* and *in vitro* PAT actions differ depending on concentration. Therefore, *in vitro* influence of 10^{-3} M concentration of this compound notably inhibited the activity of Na^+/K^+-ATPase of the embryos at all the stages of their development. At the same time, thiosulfate in *in vivo* conditions had an opposite effect, causing recovery of Na^+/K^+-ATPase activity up to the level of the control at all the examined stages of weatherfish development.

Perhaps, the changes caused by in vitro action of high PAT concentration were caused by a direct interaction between molecules of derivatives of thiosulfoacids and Na^+/K^+-ATPase. In *in vivo* conditions, only a fraction of molecules bound with Na^+/K^+-ATPase, and most thiosulfonates molecules or their primary metabolites are able to modulate other links of metabolic transformations of embryos.

Mampuys et al. (2019) revealed that high sulfunulating ability of thionsulfates blocks the nucleophilic fragments of nucleic acids and proteins, thus leading to impairment of the enzymatic activity, cellular respiration and cell division of esthers. A probable mechanism of those reactions is given in Figure 11.

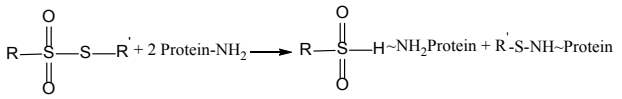

Fig. 11. Blocking of nucleophilic fragments of nucleic acids and proteins

The influence of thiosulfonates in low concentrations (10^{-8} M) was the same in both *in vivo* and *in vitro* conditions, because in both conditions, the activity of the enzyme significantly increased during the development of embryos. It has to be noted that at stage 10 of cleavage of balstomeres subject to *in vivo* action, we determined recovery of Na^+/K^+-ATPase activity by 95.2%, compared with the control, and it exceeded its activity in the control during the in vitro action by 18.8%.

In our opinion, potassium p-aminobenzene thiosulfonate in low concentrations easily metabolizes and increases the intensity of metabolic processes in embryos that are actively developing and growing during that period. This may explain the recovery of Na^+/K^+-ATPase activity in our studies.

As is known, decrease in the TMP can take place because of both increase in the ion permeability of the membrane and equilibration of the concentrations of electrogenic ions as a result of the activity of cellular pump in the conditions of a direct action of the compounds towards Na^+/K^+-ATPase, or during decrease in the intercellular level of ATP as a result of impairment of biogenetic processes in mitochondria. This is evidenced by the results of our study of the ultrastructure of embryo cells subject to action of high concentrations of all the derivatives of thiosulfoacids. We observed ruination of cristae and decrease in the electronic density of content of mitochondrium matrix, most likely resulting from osmotic imbalance and edema of this organelle.

Experimental studies of the starfish embryos (Mita & Obata, 1984) revealed that depolarization of the plasmatic membrane leads to intense release of Na^+ ions to the cell. Such an increase in cytoplasmatic sodium induces opening of potential-managed potassium and potassium channels, and also release of Ca^{2+} ions from cellular depots (Ummarino, 2017) and increases the intracellular concentrations of those ions as a result of activation of Na^+/Ca^{2+}-exchanger, which is why mitochondria become damaged as a result of impaired chemiosmotic balance, and therefore their edema. Thus, decrease in Ca^{2+} in mitochondria reduces the efficiency of oxidative phosphorilation, which significantly decreases ATP synthesis, and thus energetic provision of Na^+/K^+-ATPase. Moreover, decrease in intracellular potassium during early embryogenesis stops the cell division and inhibits the synthesis of RNA and protein (Urrego et al., 2014). This is coherent with the results of our studies of the ultrathin organization of protein-synthesizing organelles – gER and ribosomes. Also, presence of a large amount of multivesicular bodies that play the key role in sorting of the products of intracellular metabolism indicates intensive sequestration of membrane proteins and cytoplasmic molecules in their vesicles, which further can translocate into lysosomes for catabolism (Luzio et al., 2010) or release in exosomes into extracellular environment (Hanson & Cashikar, 2012).

A likely cause of the changes in the ultrastructure of mitochondria that we found is inhibition of enzymes of their matrix and electron transport chain, decreasing the efficiency of ATP production and energy-dependent processes (Totha et al., 2020). In such conditions, membranes of orga-

nelles with impaired structures have no ability to support the needed level of membrane potential, leading to loss of control of ion flows and mediator systems. As a result, necrotic processes in the cells and tissues may develop.

Furthermore, a reason for the impairment of the structure of membranes of mitochondria can be opening of pores of non-specific permeability (Permeability Transition Pore), decreasing the membrane potential ($\Delta\Psi m$) and edema of mitochondria (Briston et al., 2017; Wacquier et al., 2020). Opening of the pores stimulates the formation of active oxygen species, impairment of oxidative phosphorilation, depletion of mitochondrium pool of ATP and inactivation of ion-transporting enzymes. Furthermore, release of cytochrome c and apoptogenic proteins, specifically AIF (apoptosis inducing factor – inducing apoptosis factor), from the intermembrane space of mitochondria causes programmed death of cells.

Thus, the derivatives of thiosulfoacids affect the cells of weatherfish embryos during the early embryogenesis at the membrane level, namely as a result of impairment of electrogenesis of the plasmatic membrane of weatherfish embryos.

Conclusions

The action of thiosulfonates led to significant dose-dependent changes in the activity of membrane-bound enzyme of the embryos. If the high concentration (10^{-3} M) of the biologically active compound was present in the incubation environment, we saw the greatest inhibiting effect, whereas introduction of the low concentration (10^{-8} M) of potassium paraaminobenzene thiosulfonate caused recovery of the activity of Na^+/K^+-ATPase of the embryos, compared with the control. Obviously, low concentrations of thiosulfonates or their primary metabolites are able to become involved in the metabolic processes of embryos and increase the intensity of metabolism in embryos that are intensively developing and growing.

The study of TMP of embryo cells subject to PAT action revealed insignificant aperiodical changes in its level – the periods of third (8 blastomeres) and sixth (64 blastomeres) cleavages lasted 25.2 ± 2.4 and 37.5 ± 3.5 min and were shorter than in the control (where the fluctuation period of the third cleavage equaled 29.2 ± 2.1 min and sixth respectively 51.8 ± 2.2 min). By contrast, the fourth and fifth cellular divisions were characterized by insignificant prolongation of the period of TMP fluctuations, by 4.3 and 4.9 min, respectively. Moreover, if the maximal MP fluctuations continued to increase, compared with the control, we saw significant ($P < 0.05$) increase in the amplitude of fluctuations during the third cleavage, equaling 5.3 and 5.1 mV at the stage of 32 blastomeres. Such results indicate insignificant, but nonetheless real impairment in the electrogenesis of cellular membranes against the background of PAT influence, and indicate changes in the permeability of membrane and transport of electrogenetic ions that can alter the activity of a number of enzymes, including Na^+/K^+-ATPase.

Given the aforesaid, we can state that thionsulfonates are interesting and promising compounds for the development of drugs and search for antimycotic and antiproliferative drugs and could be broadly used in chemiotherapy of oncological diseases and treatment of autoimmune pathologies.

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