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Acid Phosphatase Complex from the Freshwater Snail *Viviparus viviparus* L. under Standard Conditions and Intoxication by Cadmium Ions

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Abstract—Acid phosphatases differing in both subcellular localization and substrate specificity were isolated for the first time from the liver of the freshwater snail *Viviparus viviparus* L. by preparative isoelectrofocusing. One of five characterized phosphatases is highly specific to ADP and the others can hydrolyze (at variable rate) a series of natural substrates. A scheme is proposed for the involvement of the studied phosphatases in carbohydrate metabolism. We have also studied some peculiarities of the effect of Cd^{2+} *in vitro* and *in vivo* on the activities of individual components of the acid phosphatase complex and corresponding changes in metabolism of the freshwater snail as a new test-object allowing the estimation of toxicity in water.

Key words: multiple enzyme forms, acid phosphatase, mollusks, freshwater snail, toxic effect of cadmium, biochemical adaptation

Acid phosphatase (AP) is one of most common and metabolically important hydrolytic enzymes. In cells of the majority of organisms AP is present in multiple forms that differ in molecular weight, p*I*, and pH optimum of activity and may express differing activities towards a variety of natural substrates [1-3]. Due to these features, AP complex possesses broad catalytic capabilities and in a great number of cases can substitute for or duplicate many specific phosphatases [4]. In particular, there is evidence that AP expresses phosphoprotein phosphatase [5, 6], glycerophosphocholine phosphatase [7], nucleotidase, and pyrophosphatase [8-11] activities and therefore is involved in anabolic, regulatory, and destructive processes associated with reparation or death of an organism [12, 13].

It is worth noting that multiple AP forms detected by a conventional enzyme-electrophoretic method with model substrates (preferably α-naphthyl phosphate) might represent not only acid phosphatase (EC 3.1.3.2). In some cases, AP forms "conceal" specific phosphatases, such as glucose-1-phosphatase (EC 3.1.3.10) and fructose-1,6-diphosphatase (EC 3.1.3.11), and each may be represented by its own molecular forms.

The "classic" acid phosphatase (EC 3.1.3.2) is often a glycoprotein containing sialic acids (predominantly N-acetylneuraminic acid), which are present as oligomers or monomer residues tautomerized into cycles [14]. The saturation of protein core of the enzyme molecule with sialic acid residues occurs already in the course of its synthesis and transport along the endoplasmic reticulum, causing acidic shifts in both p*I* and pH optimum for AP enzymatic activity [15]. Significant variation in number of N-acetylneuraminic acid residues bound to the AP polypeptide is mainly responsible for AP divergence to form a complex of multiple molecular forms [16]. Post-translational modifications and alternative splicing of the transcripts are other causes [17]. Besides, there is evidence for gene heterogeneity of AP molecular forms (isoenzymes), the number of which is relatively stable for a distinct tissue, organ, and whole organism, providing a specificity for their metabolism and individuality [17, 18].

AP synthesized *de novo* is transported to the dictyosomes of the Golgi apparatus, in which the final maturation of the enzyme occurs via the inclusion of a large bulk of sialic acid residues and packing into primary lysosomes [19, 20]. Along with lysosomal forms, other AP forms exist that are localized only in cytosol and are characterized by individual values of electrophoretic mobility and p*I* [7, 21].

Catalytic activity of AP depends on inorganic ions. In particular, inhibitory effects of Hg^{2+} [22, 23], Zn^{2+} [6,

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24], Al^{3+} [25, 26], and Mg^{2+} [27] were reported for AP *in vitro*. However, AP isolated from *Pseudomonas aeruginosa* [25] is activated in the presence of bivalent cations of copper, zinc, and especially magnesium. Manganese is thought to activate acid phosphatases at least with respect to some substrates. There are suppositions and, in some cases, exact indications of the incorporation of Mn^{2+} into the active center of AP [28, 29]. However, to all appearances Mn^{2+} is not a solitary cofactor for AP. The AP found in batatas tubers contains trivalent manganese [30], and the prosthetic group of AP from *Branchiostoma belcheri* contains iron [31]. Earlier, it had been already reported about iron-containing APs, and a possible role of iron in the binding of phosphate had been discussed [32, 33]. However, most reports on the structural organization of AP contain no data on the presence of any metal in the molecule [17, 34-36]. There is evidence for indifference of AP *in vitro* to manganese and some other ions [37, 38].

The problem of the influence of metal ions on AP activity *in vivo* is interesting as well. Previously, we detected distinct alterations in the AP activity in freshwater mollusks under intoxication caused by Cd^{2+} [39]. However, both the functional role of molecular heterogeneity of AP in the snail under the present study and the role of distinct members of AP complex in the molecular adaptation of the organism to toxic agents remained unknown. The study presented here gives data on the physical and chemical properties and the functional role of AP forms isolated from the freshwater snail under normal conditions and under intoxication by cadmium ions.

MATERIALS AND METHODS

Materials. Freshwater snails (*Viviparus viviparus* L., family Viviparidae, order Architaenioglossa, class Gastropoda, phylum Mollusca) were trapped in the shore of the river Vyaz' near the village Tishkovo, Moscow Region. The acclimation of the collected animals under laboratory conditions was conducted in a microcosm of 60 liters water containing higher plants, as well as benthos and plankton, common for habitats of the freshwater snail. The acclimation was conducted for 2-3 months at 16-17°C under illumination by sunlight, regular water replacement, and constant aeration.

Toxicological experiment. Both the experimental and control groups of animals (five or six individuals each) were placed into glass containers filled with filtered aquarium water (about 1 liter) in the absence of foreign flora or fauna. The aqueous solution of chemically pure cadmium chloride with the concentration of Cd^{2+} of 0.01 mg/liter was used for intoxication of experimental groups. The exposure was for 72 h. The environmental conditions (temperature, aeration regime, and illumination) remained unchanged. At the end of the exposure time, the animals were taken from the water and immediately prepared.

Protein extraction and subcellular fractionation. The organs and tissues isolated from the snails and sometimes the whole shell-free bodies were rinsed twice with 10 volumes of 0.15 M NaCl solution, scissored, and homogenized by grinding in a mortar with quartz debris in extracting solution (0.5% Triton X-100 and 1 mM EDTA, disodium salt), 8 ml per gram of biomass. Proteins were extracted for 3-5 min at 4°C followed by centrifugation at 10,000*g* for 20 min at 0°C. The supernatant was decanted and dialyzed overnight against 1000 volumes of bidistilled water.

Particular subcellular fractions from the hepatic cells (hepatopancreas) of snails were isolated by differential centrifugation according to the method of Sawant et al. [40] with slight modifications. Centrifugation in a sucrose density gradient was used to purify "light" mitochondrial fractions and lysosomes. With this aim the liver isolated from five to six snails (overall tissue weight 2-3 g) was scissored, rinsed twice in 0.15 M NaCl solution, and homogenized on ice (0°C) in eight volumes of 0.25 M sucrose solution with 1 mM EDTA (disodium salt) using a Potter–Elvehjem tissue homogenizer. The obtained homogenate was filtered through a kapron sieve and centrifuged at 300*g* for 10 min at 0°C to separate undivided whole cells and large cell debris. The sediment was discarded and the supernatant was repeatedly centrifuged at 750*g* for 10 min at 0°C to precipitate nuclei. The nuclei were washed by resuspension of the pellet three times in 0.25 M sucrose solution and re-harvesting each time by centrifugation at 750*g* for 10 min at 0°C. The waste water was discarded, and the fraction of "heavy" mitochondria was prepared by centrifugation at 3500*g* for 10 min at 0°C from the supernatant obtained after the first harvesting (sedimentation) of the nuclei. The fraction of "heavy" mitochondria was washed twice by re-sedimentation from 0.25 M sucrose solution by centrifugation under the same conditions. The supernatant obtained after the first sedimentation of "heavy" mitochondria was centrifuged at 16,500*g* for 20 min at 0°C. The supernatant was used further as the cytosolic fraction, and the pellet was re-suspended in 0.5 M sucrose solution and applied on a step sucrose density gradient: lower layer, 0.7 M; middle layer, 0.6 M; and upper layer, the sample (0.5 M). The centrifugation was carried out at 6000*g* for 30 min at 0°C (VAC 601 ultracentrifuge, Germany; a rotor with swinging tubes, 3 × 5). On completion of the centrifugation, the orange pellet of "light" mitochondria was accumulated on the bottom of centrifuge tubes, and a lysosomal fraction was formed as an opalescent suspension within the thickness of the gradient (on the border of 0.7 and 0.6 M layers). The lysosomal suspension was picked up using a syringe with blunt needle and centrifuged at 17,000*g* for 20 min

at 0°C. The pellets of "light" mitochondria and lysosomes were washed once and repeatedly centrifuged at 17,000*g* for 20 min at 0°C from 0.7 and 0.5 M sucrose solutions, respectively.

All obtained subcellular fractions were resuspended in minimum volume (200-600 μl) of extracting solution, freeze–thawed three times, and homogenized on ice (0°C) using a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 20,000*g* for 30 min at 0°C, and the supernatants were decanted and dialyzed overnight against bidistilled water. The purity of subcellular fractions was monitored enzymatically by specific activities of acid phosphatase, acid ribonuclease, and cytochrome oxidase [40].

The total protein in extracts was determined by the Bradford method [41] using aqueous ovalbumin solutions (100-1000 μg/ml) as standards.

Electrophoresis and isoelectrofocusing of proteins. Electrophoresis of proteins was conducted in polyacrylamide gel slabs 1 mm thickness with the step buffer system by Davis [42] (as recommended by the protocol of Hoefer, USA) using a vertical SE 600 electrophoresis device (Hoefer) and 337 IE power supply (LKB, Sweden). The concentration of the separating gel was 5.7%, the ratio of N,N′-methylene-*bis*-acrylamide concentration to the sum of monomers was 2.6%; these values were 3.9, and 20% for concentrating gel, respectively. The protein extracts before electrophoresis were diluted with bidistilled water and mixed with tenfold sample solution (625 mM Tris-HCl buffer, 40% glycerol, and 0.1% bromophenol blue, pH 6.8) to the final protein concentration 150 μg/ml. The electrophoresis was conducted at 4°C. The constant voltage of 150-200 V was applied during the entrance of proteins into the concentrating gel and the formation of start zone, then the voltage was increased to 300 V, the electrophoresis duration under these conditions being 4-5 h.

The protein extracts obtained from subcellular fractions (lysosomes and cytosol) were isoelectrofocused. A Multiphor universal device for horizontal electrophoresis (LKB) and the 337 IE power supply (LKB) were used in this study. The isoelectrofocusing of proteins was conducted in Ultrodex granulated gel (LKB) with ampholytes of pH working ranges 3.5-10, 6-8 (LKB), and 3-7 (Serva, Germany). The anode electrode solution was 1 M H_3PO_4, and a cathode electrode solution was 1 M NaOH. A protein extract (3 ml) with the concentration of 5-7 mg/ml (diluted with bidistilled water, if necessary) was applied into the gel. The temperature during all the isoelectrofocusing process was set at 5°C. At the first moment the current of 24 mA and the voltage about 900 V were applied to the electrodes. After 20, 40, 60, and 90 min of the separation the decrease in current was compensated by the increasing up to its initial value or to the maximum permitted value, while the voltage increased up to 1200 V. The current stabilized at the level of 4-5 mA after 14-15 h, whereupon the process was stopped.

The proteins were eluted from the gel with 0.15 M NaCl at 4°C overnight. After pH was determined in the eluates, they were dialyzed against bidistilled water.

Determination of acid phosphatase activity. Enzymatic activity of AP was determined spectrophotometrically from the rate of hydrolysis of model substrates. The enzyme amount providing the increase in the content of the enzymatic reaction product by 1 μmole per 1 min was taken as 1 unit of the enzyme activity (U). The specific activity was expressed in units per 1 mg protein (U/mg).

In most cases, the AP activity was determined by the method of Bessey et al. [43] using sodium *p*-nitrophenyl phosphate (Sigma, USA) as a substrate.

The substrate specificity of AP was characterized by the rate of phosphate production (the method of Fiske–Subbarow modified by Heinonen and Lahti [44]). The following substrates were used: sodium *p*-nitrophenyl phosphate (Sigma), sodium α-naphthyl phosphate (Lachema, Czech Republic), disodium α-glycerophosphate (Serva), disodium β-glycerophosphate (Serva), disodium glucose 1-phosphate (Reanal, Hungary), disodium glucose 6-phosphate (Serva), disodium fructose 6-phosphate (Serva), trisodium fructose 1,6-diphosphate (Serva), disodium ribose 5-phosphate (Serva), disodium adenosine 5′-triphosphate (Reanal), disodium adenosine 5′-diphosphate (Reanal), and disodium adenosine 5′-monophosphate (Reanal).

The AP activity in polyacrylamide gel was detected by the hydrolysis of sodium α-naphthyl phosphate and subsequent diazo (Griess) reaction of free naphthol with fast blue B dye by the method of Lojda et al. [45] modified in our study for the treatment of gel slabs. After electrophoresis, the gel slab was removed from the device and incubated for 20 min without substrates at 37°C in 100 ml of 0.2 M acetate buffer (CH_3COOH/CH_3COONa), pH 4.1. Then the gel was placed in a fresh portion of 0.2 M acetate buffer containing 10 mM α-naphthyl phosphate and incubated for 5-20 min (depending on the enzyme purity) at 37°C. Zones containing AP activity were visualized via 10-15 min incubation at room temperature with aqueous solution of fast blue B dye (Lachema) added into the incubation medium to the final concentration of 0.5 mg/ml. The stained polyacrylamide plates were fixed in 7% acetic acid. The obtained zymogram was photographed using a Gel Doc 1000 gel-documenting video system (Bio-Rad, USA), and the images were processed using an accompanying application for the determination of electrophoretic mobility to anode (R_f) and relative enzymatic activity of each AP form. Absolute values of activity were derived from the relative portions using the data on the total AP activity with *p*-nitrophenyl phosphate.

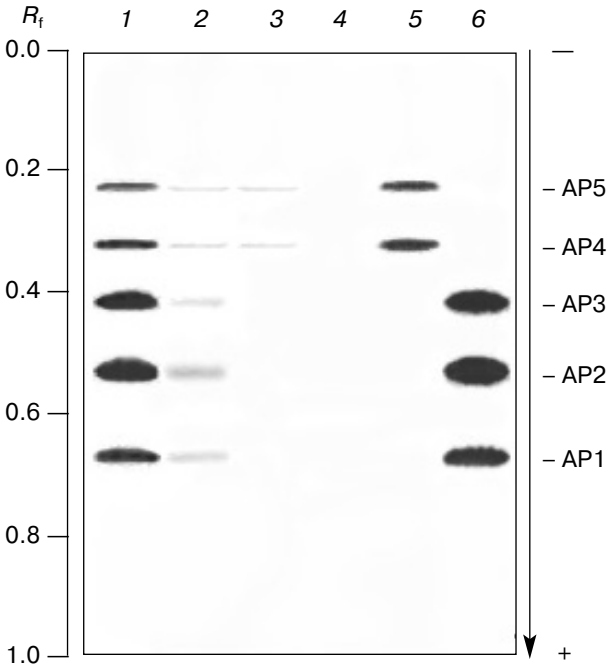

Fig. 1. Zymograms of acid phosphatase complex from various subcellular fractions of freshwater snail: *1*) cell homogenate; *2*) nucleus; *3*) "heavy" mitochondria; *4*) "light" mitochondria; *5*) lysosomes; *6*) cytosol. The arrow indicates the electrophoresis direction from cathode to anode.

RESULTS AND DISCUSSION

The study on multiple molecular forms of AP in various organs (muscle, liver or hepatopancreas, intestine, and gonads (male and female separately)) of the freshwater snail by the method of enzyme electrophoresis has shown that five AP forms are active in this mollusk under normal conditions, which we have designated AP1 to AP5 in accordance with their anode mobility. The AP complex is represented completely only in liver, hence we used this organ as a material most suitable for the study of multiple functional AP forms in the freshwater snail.

The analysis of subcellular localization of AP revealed that a significant enzymatic activity is present only in lysosomal extract and cytosolic fraction (Fig. 1). Some AP activity in both nuclear fraction and "heavy" mitochondria is probably an artifact [46, 47].

The AP complex in cytosolic fraction is represented by AP1, AP2, and AP3, unique forms of the enzyme that differ from lysosomal ones in substantially higher electrophoretic mobility and enzymatic activity (see Fig. 1). These AP forms are obviously differentiated in their genesis, and their activity cannot be associated with the activity of lysosomal apparatus of the cell.

We further purified all of the AP forms revealed in lysosomal and cytosolic fractions by two or three sequential isoelectrofocusing steps, first in broad and then in narrow pH ranges (3.5-10; 6-8 and 3-7, respectively). As a result, we succeeded in separation and isolation of individual AP forms, each characterized by a single zone of activity on the electrophoregram with constant R_f value; hence, they were well-suited for the study on their functional characteristics (Table 1). We cannot exclude the presence of other proteins possessing no activity with α-naphthyl phosphate in these isolated samples.

The final step of the study on multiple AP forms was the determination of their substrate specificity in respect to various synthetic and natural phosphoric acid esters (Table 2). The substrate specificity of AP3 localized in cytosol is of particular interest. This enzyme expresses hydrolytic activity to the sole natural substrate adenosine diphosphate. The hydrolysis rate for ADP is 3.6-fold higher than that for *p*-nitrophenyl phosphate, whereas ATP and AMP are not hydrolyzed at all.

Table 1. Characteristics of acid phosphatase forms isolated from the liver of the freshwater snail

Enzyme form	Subcellular localization	R_f in 5.7% polyacrylamide gel	p*I*	Activity, U/mg	
				initial homogenate	after purification
AP1	cytosol	0.67	4.9	0.205	22.40
AP2	cytosol	0.53	5.8	0.269	22.30
AP3	cytosol	0.41	7.1	0.105	8.48
AP4	lysosomes	0.32	6.3	0.053	4.98
AP5	lysosomes	0.23	6.6	0.050	5.08

Table 2. Substrate specificity of acid phosphatases from the liver of the freshwater snail

Substrate	Activity, %*				
	AP1	AP2	AP3	AP4	AP5
p-Nitrophenyl phosphate	100	100	100	100	100
α-Naphthyl phosphate	75.4	—	—	63.8	48.6
α-Glycerophosphate	55.7	16.7	0.0	32.8	18.6
β-Glycerophosphate	73.8	16.7	0.0	14.8	13.2
Glucose 1-phosphate	19.8	—	0.0	—	0.0
Glucose 6-phosphate	73.8	—	0.0	—	0.0
Fructose 6-phosphate	57.4	27.8	0.0	53.4	—
Fructose 1,6-diphosphate	103	55.6	0.0	0.0	0.0
Ribose 5-phosphate	31.1	—	0.0	—	—
ATP	52.5	—	0.0	23.8	14.3
ADP	10.0	15.2	360	15.6	0.0
AMP	0.0	12.3	0.0	12.6	—

* Symbol "—", no data available.

The presented data indicate that AP3 is a specific and thereby rather unusual enzyme. The properties and functions of hydrolases hydrolyzing nucleoside diphosphates are often considered in the literature; however, only two of them are well known yet. Nucleoside diphosphatase (EC 3.6.1.6) isolated from liver and kidney of vertebrates hydrolyzes not only ADP, but also inosine diphosphate, guanosine diphosphate, uridine diphosphate, and D-ribose-5-phosphate [48]; another enzyme, so-called apyrase (EC 3.6.1.5), which was first identified in plants but is common in mammals, hydrolyzes ATP and ADP [49, 50]. Upon the data of the same authors, both enzymes use bivalent ions of alkaline earth metals (Mg^{2+} and Ca^{2+}, respectively) as cofactors. However, because AP3 is characterized by a total absence of hydrolytic activity to D-ribose 5-phosphate and ATP (see Table 2) and also is independent of the presence of Mg^{2+} or Ca^{2+} in the reaction medium (treatment with 1 mM EDTA, disodium salt), which is characteristic of most acid phosphatases, we cannot consider this enzyme as nucleoside diphosphatase or apyrase. The acid ADPase from the liver of the freshwater snail is apparently a novel newly discovered nucleoside diphosphate phosphohydrolase.

Another important characteristic feature of AP complex from the liver of the freshwater snail is the ability of two forms (cytosolic AP1 and AP2) to hydrolyze the ortho-phosphoric acid diester—fructose 1,6-diphosphate (see Table 2), whereas the "classic" AP is a hydrolase specific to monoesters [51]. Moreover, our data indicate that AP1 and AP2 more rapidly cleave fructose 1,6-diphosphate than other natural substrates. There are no indications in the literature on facts like these, but a contamination of AP1 and AP2 fractions with fructose-1,6-diphosphatases (if their isoelectric points are overlapping) that is not possible to determine by electrophoresis on zymograms with α-naphthyl phosphate due to absolute specificity to the substrate immanent for fructose-1,6-diphosphatases is not ruled out.

Lysosomal AP4 and AP5 forms are apparently nonspecific, but do not hydrolyze some natural orthophosphates. In particular, AP4 is not active toward fructose 1,6-diphosphate and AP5 toward glucose 1-phosphate, glucose 6-phosphate, and fructose 1,6-diphosphate. AP4 and AP5 hydrolyze substantially slower other natural substrates than *p*-nitrophenyl phosphate (Table 2).

Divalent cadmium ion present *in vitro* in the incubation medium for zymograms at the concentration of 1 mg/liter inhibits virtually all phosphatases studied here (Table 3). Interestingly, the inhibition effect of cadmium monotonously decreases to statistically insignificant values ($p > 0.99$) as the electrophoretic mobility of AP forms increases and the isoelectric point value decreases. In particular, AP5, the form that is the least mobile to the

Table 3. Effect of cadmium chloride on the activities of acid phosphatases from the liver of the freshwater snail

Enzyme form	Activity, U/mg		
	in the absence of Cd^{2+} (control)	Cd^{2+} in the reaction mixture for the activity determination	Cd^{2+} in the living medium for mollusks
AP1	0.164	0.161*	1.151
AP2	0.222	0.217*	0.961
AP3	0.112	0.069	0.0
AP4	0.094	0.038	0.426
AP5	0.091	0.027	0.407
AP6	0.0	0.0	0.309

* Difference with control is statistically insignificant by Student's *t*-test ($p > 0.99$).

anode (p*I* 6.6), is inhibited by 70%, whereas the most mobile forms AP1 (p*I* 4.9) and AP2 (p*I* 5.8) are virtually not inhibited.

The actual concentration of Cd^{2+} *in vivo* was proved significantly lower than *in vitro*. Significant alterations in activity of AP complex occur already at Cd^{2+} concentration of 0.01 mg/liter ($CdCl_2$ was present in the medium where the snails were kept for 72 h); these changes are commonly opposite to those under the influence of Cd^{2+} *in vitro* and, hence, are induced at the level of metabolism of cells, organs, or the whole body. These alterations are summarized as a significant elevation of activity of virtually all the multiple AP forms found under standard conditions (with the exception of AP3 which, in contrast, is completely inactivated) and also as the induction of activity of a novel form, AP6 (Table 3, Fig. 2).

The substrate specificity of the form AP6 induced *in vivo* by Cd^{2+}, which was revealed by enzyme-electrophoresis (Fig. 2) and then isolated from lysosomal fraction of the mollusk liver using isoelectrofocusing, was characterized. AP6 (R_f 0.21, p*I* 7.1) is specific to fructose 6-phosphate (71% as compared with *p*-nitrophenyl phosphate), slowly hydrolyzes α-glycerol phosphate (19%), and does not hydrolyze any natural substrate.

The activity of AP6 directed to the decrease in fructose 6-phosphate level must apparently slow down the glycolytic cleavage of carbohydrates and accumulation of free fructose on the condition that the enzyme goes out of lysosomes into the intracellular medium, which is very possible under intoxication of the organism [52, 53] (Fig. 2). Moreover, under the action of Cd^{2+} *in vivo* a drastic increase occurs in activities of cytosolic AP1 and AP2, the forms which are most active to fructose 1,6-diphosphate, and lysosomal AP4 hydrolyzing preferentially fructose 6-phosphate and α-glycerol phosphate. In connection with this, it is interesting to note that another specific phosphatase (AP3), in our opinion, activating the hydrolysis through the action of glycogen phosphorylase, is completely inactivated under the intoxication of the mollusk by cadmium.

As a result of all these events, an inhibition of catabolic processes and accumulation of free carbohydrates and glycerol must occur in the hepatic cells of the mollusk under the intoxication, in accordance with the scheme proposed here (Fig. 3). In nature, such "expectant" strategy may take place, for instance, during the preparation of mollusks for the winter rest [54]. A gradual slow-down of carbohydrate decay and, hence, stopping of all energy processes, economy of glycogen and lipid stores, glycerol and sorbitol accumulation as cryoprotectors, etc. are characteristic of the mollusk under the stress conditions due to environmental temperature decrease. It is interest-

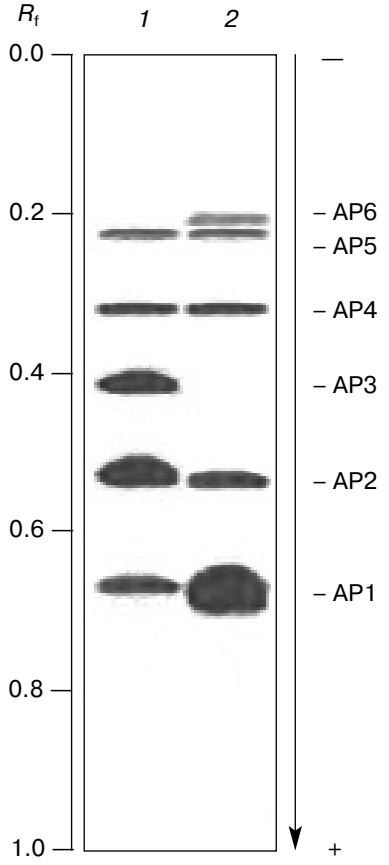

Fig. 2. Zymograms of acid phosphatase complex after the action of Cd^{2+} at the concentration of 0.01 mg/liter *in vivo*: *1*) control (standard conditions); *2*) after 72 h exposure. Arrow indicates the direction of electrophoresis from cathode to anode.

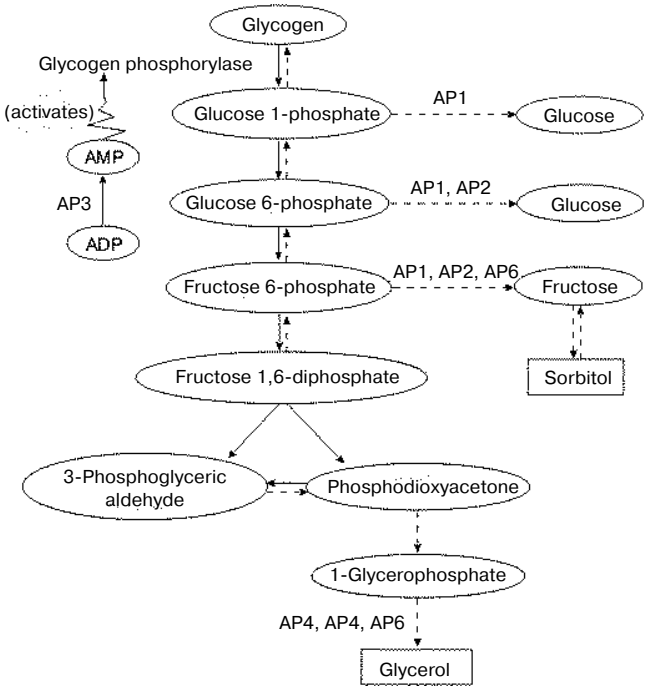

Fig. 3. Possible role of acid phosphatase complex in regulation of carbohydrate metabolism in the freshwater snail (explanation see in text). Designations: in ovals, metabolic intermediates; in rectangles, metabolic end products.

ing to note that in a number of laboratory experiments inducing other stresses in animals, for instance, hyperthermia or starvation, the analogous events happened in these animals, which are now known as the nonspecific adaptation syndrome [55]. The effect of Cd^{2+} at the concentration of 0.01 mg/liter on a snail during 72 h apparently resulted in similar conditions for the mollusk and led to the respective adaptation changes in the action of AP complex.

In conclusion, we would like to note an obvious applied aspect of the present study. The total activity of AP complex and the spectrum of its forms in liver of the freshwater snail represent safety criteria of the state of the mollusk's organism and can be used as markers in biotesting of water pollution with toxic compounds.

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