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BIOCHEMICAL ADAPTATION

Cadmium-Induced Oxidative Stress in the Bivalve Mollusk *Modiolus modiolus*

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Abstract—Cadmium-induced oxidative stress in the bivalve *Modiolus modiolus* is studied from the standpoint of the universality of the mechanism of free-radical oxidation. The kinetics of cadmium accumulation by the bivalve was revealed in a laboratory experiment. The gills accumulated higher Cd levels than the digestive gland. In the process of cadmium accumulation, there was an increase in lipid peroxidation products (malondialdehyde and lipofuscin) and a reduction in the total oxiradical scavenging capacity (TOSC). Cadmium induces oxidative stress in molluscan tissues through damage to the antioxidation system. Thus, TOSC can provide a useful biochemical indicator of early pathological changes in the cell or the organism, as well as of the environmental effects of heavy metal pollution.

Key words: oxidative stress, accumulation of cadmium, total oxiradical scavenging capacity, oxiradicals, lipid peroxidation.

The unique ability of bivalves to take out highly toxic cadmium from seawater and concentrate it in the body and tissues has attracted particular interest among researchers. The accumulation of cadmium may cause disturbances of different metabolic processes, both in the accumulating organism and in organisms of higher trophic levels compounding the food chain. Cadmium is a typical polytropic chemical agent capable of interacting with numerous cell structures and causing a spectrum of negative biochemical shifts: from inhibition of activity in individual enzymes and enzyme assemblages up to damage to the membrane structures [2, 4, 24, 28].

Of particular interest from the ecotoxicological point of view is the ability of heavy metals to induce oxidative stress [13, 21, 23], which is considered as a major pathogenic mechanism of cell metabolism disturbances [20, 22]. According to that concept, the intervention of an alien chemical agent, irrespective of its concrete mechanism of action, into oxidative metabolism is correlated, directly or indirectly, with increasing generation of highly reactive oxygen radicals (oxiradicals). The misbalance that arises between the pro- and antioxidative systems results in accumulation of products of oxidation of the basic classes of macromolecules, including lipids, proteins, and nucleic acids, which can entail pathological (destructive) processes in cells, called "oxidative stress" in the literature [20, 22]. However, there is almost no direct experimental evidence confirming the ability of cadmium to break the balance between pro- and antioxidative systems and to cause oxidative stress in tissues of marine invertebrates, in particular mollusks. On the other hand, variations in the content of low-molecular enzymes or in the activity of antioxidative enzymes in mollusks were recorded during field and laboratory studies [2, 11, 15]. This approach, although it is usually applied to reveal specific interrelations between different stressors and individual antioxidants, does not allow us to estimate unambiguously the degree of oxidative stress development in a biological system [17].

A new analytical method, named the method of determination of the total oxiradical scavenging capacity (TOSC) [14, 16] of biological samples by the authors, provides more reliable information on the condition of the protective potential of a biological system. Taking into account the importance of the oxidative stress parameters for estimation and forecast of ecological consequences of the effect of cadmium on marine ecosystems, we investigated the influence of the accumulation of this metal on the total oxiradical scavenging capacity of the antiradical system and the formation of products of membrane lipid oxidation in the tissues of the marine bivalve *Modiolus modiolus.*

MATERIAL AND METHODS

Adult individuals of the northern horse mussel, *Modiolus modiolus*, sampled in Peter the Great Bay, in the vicinity of Reineke Island, Sea of Japan, in July 2003 were used in the study. The mollusks, selected by size (7–10 cm), were kept in 140-liter aquaria for no less than 7 days prior to the experiment. Then the experimental mollusks were divided into 2 groups and kept

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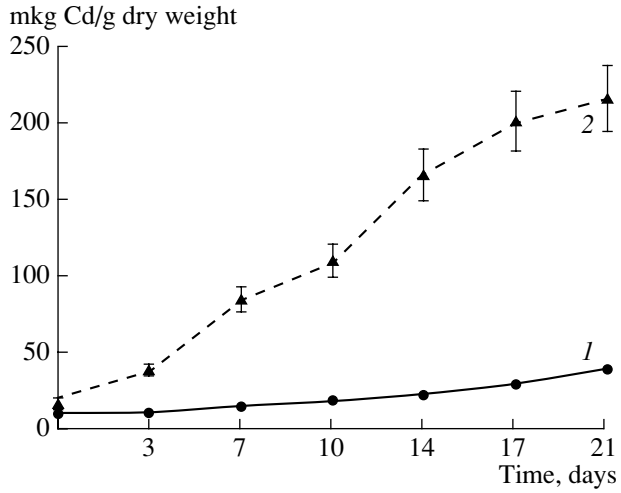

Fig. 1. Kinetics of cadmium accumulation by the tissues of *Modiolus modiolus. 1*—digestive gland, *2*—gills.

for 3 weeks in 100-liter aquaria with seawater and continuous aeration at a temperature of 18–20°C. A $CdCl_2$ solution was added into the aquaria with the group of tested animals up to a concentration of 100 μg/l. The water in the aquaria was changed daily. The control group of mollusks was kept in the same conditions as the test one, but no metal salt was added. In total, four series of tests were carried out.

On days 3, 7, 10, 14, 17, and 21, 5 individuals from the test group were sampled for determination of the contents of cadmium; the products of lipid peroxidation, namely malondialdehyde (MDA), and Schiff bases; and the total oxiradical scavenging capacity in the gills and digestive gland.

A technique based on the adsorption of bromphenol blue was applied for the determination of the protein content in the tissue homogenates [8]. Calibration curves were built using solutions of bull serum albumin, the concentration of which was estimated from the coefficient of molar extinction. The MDA content in the tissue homogenates was determined by color reaction with 2-thiobarbituric acid [3]. To prevent lipid peroxidation during MDA determination, an alcohol solution of ionol was added to the assay up to the final concentration of 5 mM. The MDA content was estimated taking the coefficient of molar extinction as 1.56×10^5 cm/M. The level of fluorescing products (such as Schiff bases) was determined with a Hitachi MPF-4 spectrofluorimeter at excitation wave lengths of 360 νm and fluorescence of 430 νm after extraction with a mixture of solvents (ether–ethanol, 1 : 3 volume ratio) [19]. The relative content of those compounds was converted into conditional units for a milligram of the protein (relative to fluorescence of a solution of 1 μm/ml quinine sulfate in 0.1 N H_2SO_4).

The determination of the total oxiradical scavenging capacity in tissues was based on the approach suggested by Winston *et al.* [27], with some original modifications, which amounted to the techniques of the registration of hydroxyl radicals (·OH). Instead of an unstable α-keto-γ-methiolbutyric acid, we used 2-oxybenzoic acid (salicylic acid) as a hydroxyl radical acceptor, and the product of its oxidation, the 2,4-dihydroxybenzoic acid (2,4-DHBA), was determined using high performance liquid chromatography (HPLC).

Hydroxyl radicals were produced in a Haber–Weiss reaction in the system (1.0 ml) containing 2 μM $FeCl_3$, 4 μM EDTA, and 200 μM ascorbic acid in 0.1 M phosphate buffer, pH = 7.5, in the presence of 0.7 mM salicylic acid. The reaction was initiated by the addition of Fe^{3+}, the obtained mixture was incubated for 60 min at 35°C. The produced hydroxyl radicals oxidized salicylic acid up to 2,4-DHBA (the control assay). Upon the addition of tissue homogenates of the north horse mussel containing low-molecular antioxidants ("quenchers" of hydroxyl radicals) to the incubatory mixture, the level of 2,4–DHBA reduced proportionally to the quantity of the added homogenate (10–60 μg of protein) (the test assay). The total oxiradical scavenging capacity was estimated with the following equation:

$$TOSC = (TA/CA \times 100) - 100,$$

where TA and CA are the quantities of the salicylic acid oxidation product and 2,4- DHBA, in the test and control assays, respectively. All TOSC values were normalized for a milligram of protein of the biological preparation.

The quantity of 2,4-dihydroxybenzoic acid in the incubatory mix was determined with HPLC (Knauer, Germany) with a UV detector (λ 310 μm) in an inverted phase system: C_{18} column (50 × 4 mm); the mobile phase contained a mixture of citric and acetic acids (0.03 I) titrated with sodium acetate up to pH of 3.6 [18]. The flow rate of the mobile phase was 0.5 ml/min.

The cadmium content was determined after mineralization of the molluscan tissues with a mixture of nitric and chloric acids (3 : 1) by the method of atomic absorptive spectroscopy in a torch flame, accurate to 1.0×10^{-5}%. The concentration of metal was evaluated for the dry weight of the tissues, which were dried up to a stable weight at a temperature of 85°C.

All numerical data are value averaged for four series of tests ± standard deviation.

RESULTS

The accumulation of cadmium by tissues of the *Modiolus modiolus* occurred linearly, but did not reach "saturation" for 3 weeks (Fig. 1). The respective rate of cadmium accumulation was 10–12 μg/day/g in the gill tissue and 1.5 μg/day/g in the digestive gland tissue. The accumulation of malonic dialdehyde and lipofuscin in the tissues of the north horse mussel exposed to cadmium was distinctly pronounced 7–10 days after the beginning of the experiment. By the end of the

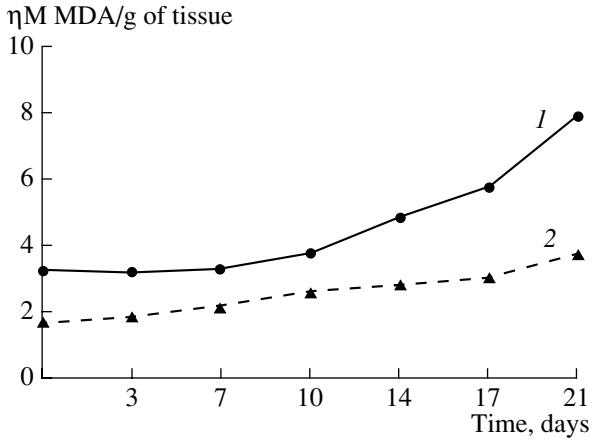

Fig. 2. The malondialdehyde (MDA) content in the tissues of *Modiolus modiolus* in the process of cadmium accumulation. *1*—digestive gland, *2*—gills.

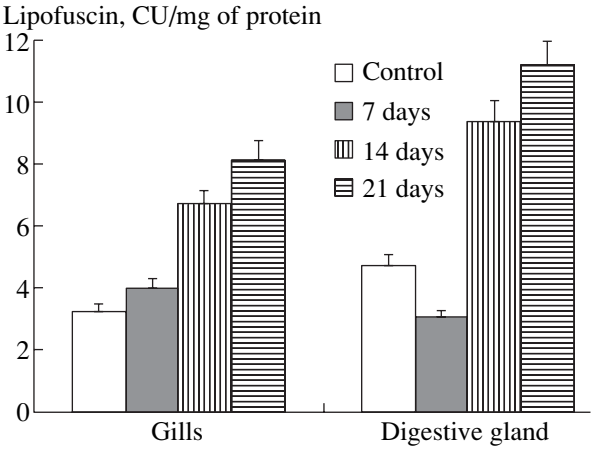

Fig. 3. Effect of cadmium accumulation on the generation of fluorescent products (lipofuscin) in the tissues of *Modiolus modiolus.*

experiment, the concentrations of MDA and lipofuscin in the digestive gland and gills had increased in more than 2 and 2.5 times, respectively (Figs. 2, 3).

The level of 2,4-DHBA decreased after addition of the digestive gland homogenate to the incubatory medium (test assay) (Fig. 4). The total oxiradical scavenging capacity of the digestive gland homogenates of the north horse mussel was almost 2 times higher than that of the gill homogenates and equaled 534 ± 22.1 and 270 ± 13.4 units/mg of protein, respectively (Fig. 5).

Concurrently with the accumulation of cadmium by mollusk tissues, TOSC variation was also recorded, being more pronounced in the gills. From the first days of the experiment, the TOSC rate decreased and, by the end of the experiment, did not exceed 50% of the initial level. Another pattern was observed in the digestive gland: a small increase of TOSC was recorded at the beginning of the experiment (1–5 days), but on the next day a tendency toward its gradual decrease became apparent (Fig. 5).

These data provide evidence of the inhibition of antioxidative activity in mollusk tissues under the effect of cadmium intoxication. However, the rate of this process in the gills and digestive gland was different.

DISCUSSION

The problem of the mechanism of the misbalance between the pro- and antioxidative systems still remains open. Cadmium, in contrast to copper and iron, was unable to participate directly in the production of active oxygen forms through oxidation–reduction reactions (the cycle of Haber–Weiss reactions). Numerous papers have come out with the assumption that cadmium can stimulate oxidative stress by depression of the antioxidative system. It was shown that cadmium is an inhibitor of enzymes that are included in the complex antioxidative system. However, that data, though providing evidence of the vulnerability of individual elements of antioxidant protection, cannot reflect completely the level of total oxidative damage of the entire cell system [22]. This is due to the fact that living organisms are complexly organized systems maintain-

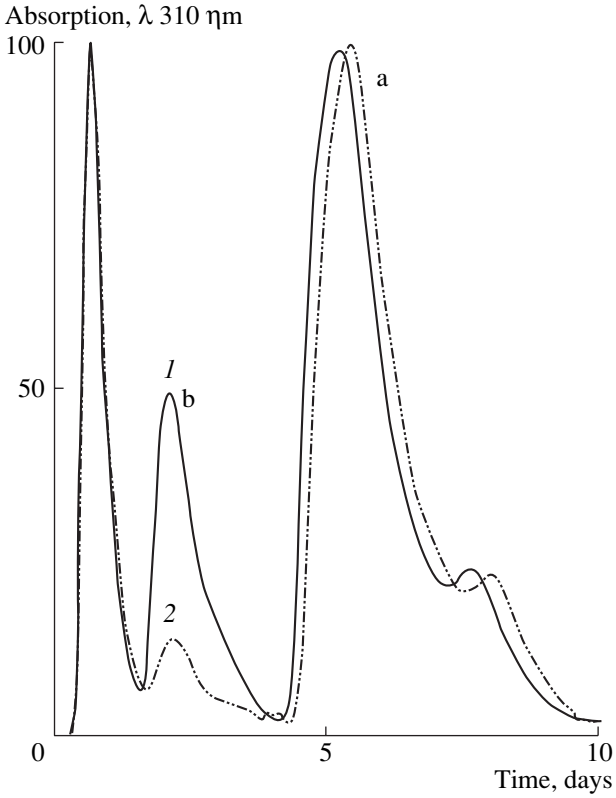

Fig. 4. Chromatogram of the separation of (a) salicylic acid and (b) a product of its oxidation, 2,4-hydrobenzoic acid, by inverted–phase HPLC in the digestive gland homogenate of *Modiolus modiolus. 1*—control assay, *2*—test assay.

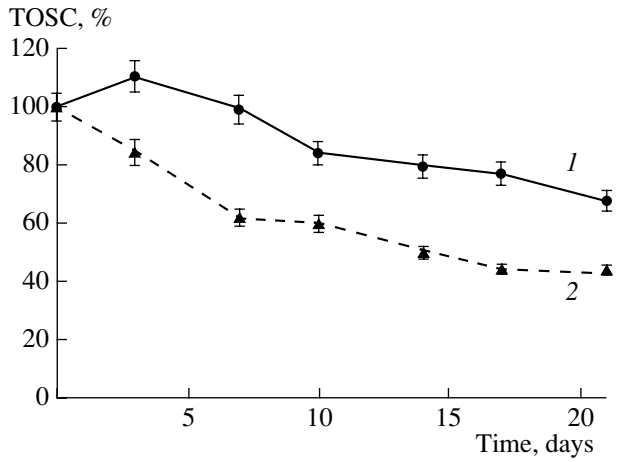

Fig. 5. Cadmium impact on the total oxiradical scavenging capacity (TOSC) in tissues of the mussel *Modiolus modiolus. 1*—digestive gland, *2*—gills.

ing and regulating the optimum level of functionally different endogenic antioxidants, enzymes, and bioantioxidants (low-molecular antioxidants). In such a system, a change in the level of one of the components of cell antioxidative protection may be compensated by induction of another protective component (or components). Moreover, it is believed that antioxidants can function cooperatively, providing the cell with a protective potential of greater force than would result from a simple sum of the individual contribution of each antioxidant [26].

In this study, to reveal the reason for cadmium-induced stress, we applied an approach that allowed us to reveal the ability of a biological system to inactivate highly toxic oxiradicals and to estimate the condition of the entire antioxidative system (integrated AO potential) [15–17]. The TOSC determination has shown that a gradual decrease in antiradical activity occurred during accumulation of cadmium in the tissues of the north horse mussel, though to different extents. It may be considered that the decrease in the capacity of the antioxidative system to inactivate oxiradicals observed in the experimental mollusks is the reason for the development of oxidative stress and the accumulation of lipid oxidative destruction products in the tissues of *Modiolus modiolus.*

The growth of antioxidative activity in the cells of the digestive gland in the initial stages of cadmium accumulation is apparently connected to intense synthesis of specific metal-bind proteins, metallothioneins (MT), in that period. Experimental data revealed that MT manifested antioxidative properties, being an effective "trap" for oxiradicals [9, 25]. However, as follows from the results of our tests, the contribution of those proteins to the general mechanism of antiradical protection under the condition of intense cadmium accumulation was almost not pronounced in the gills, and was of short-term character in the digestive gland. Moreover, it is necessary to emphasize, concerning the antiradical properties of MT, that molecules of these proteins are easily oxidized interacting with oxiradicals and lose their ability for metal binding [10]. In this connection, these are all reasons to believe that the ability of MT to intercept ("to quench") oxiradicals can provoke a degradation of the main system of metal detoxication in a cell.

Among the probable causes of the depression of antioxidative potential and the development of cadmium-induced oxidative stress, it is necessary to distinguish the ability of cadmium to enter into competitive interrelations and to disturb the metabolism of essential trace elements in mollusks, in particular, metals with variable valence, such as copper and iron [5]. Changes in the biological availability of these metals in a cell can facilitate their interactions with molecular oxygen and the formation of oxiradicals, e.g., hydroxyl radical ($\cdot$OH) [21], the most active prooxidant, whose hypergeneration results in fast "exhaustion" of the antioxidative system.

Whatever the concrete mechanism of cadmium ion intervention into oxygen metabolism was, the depression of the antioxidative potential of a cell is connected to the oxidative destruction of lipids, proteins, and nucleic acids. It entails disturbances in the mechanisms of the functioning of biochemical systems, an increase of destructive processes, and their consecutive manifestation at higher integrated levels [6]. The results of experiments on mollusks add new details to the general picture of the vulnerability of the biological system to the impact of cadmium ions, and allow us to join recommendations to apply TOSC for biomonitoring. The condition of the antioxidative system reflects, on the one hand, the adaptation potential of marine organisms and, on the other, the degree of the impact of adverse conditions in the environment. This parameter was successfully applied to ecotoxicological studies as a biomarker of oxidative stress in *Crenomytilus grayanus*, inhabiting Far Eastern seas, and in the Mediterranean mussel *Mytilus galloprovincialis* inhabiting polluted bays [1] and strongly eutrophic lagoons [7], and also in mussels transferred from clean water to polluted areas [12]. As a whole, the potential of this approach is very wide, but of special interest is the fact that, besides the diagnostic purposes, it can also be used for the purposes of prognosis, as far as it allows assessing the degree of susceptibility of an organism to oxidative stress [14].

Based on the results obtained, it may be concluded that cadmium induces oxidative stress in the tissues of *M. modiolus* via disorganization of the antioxidative system. The decrease in the capacity of the antioxidative system to inactivate free radicals may be considered a possible cause for oxidative stress and the accumulation of lipid peroxidation products in the tissues of *M. modiolus.*

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