Development of industries, particularly chemical industries, has led to increase of content of heavy metal ions (HMI) and other chemical compounds in the environment to such concentrations, under which they produce toxic effect. This leads to a necessity to study mechanisms of toxic actions, particularly of HMI, as basic pollutants, and to realization of global character of the problem of chemical pollution of environment.

One of the main events of the end of XX-th century was the UN General Assembly on ecology problems, which was held in Rio de Janeiro (June 1st-14th, 1992). Two international conventions were held at that session- (a) Convention on Biological Diversity; and (b) Framework Convention on Climate Change. According to a number of experts, division of world into developed and developing countries is anachronism, since geopolitical state of the planet has changed to such extent during recent decades that usage of these two categories makes no sense. *Per se* forum in Rio de Janeiro set new ideology, new outlook of the modern civilization, this could be defined as environmentalism. Ideology of environmentalism sets forth a new view on the world, on interrelation of mankind and biosphere. Most important in it – acknowledgment of dependence of each human being and humanity in general on the state of biosphere. Level of society development is determined not only by social and economic conditions, but also by the state of planet environment in general and in each country. Due to this, control of quantity of toxic compounds in environment and mechanism of their action on living organisms of various levels of organization is one of elements of environmental thinking, since increase of level of chemical pollution in one point of the planet would inevitably lead to global changes in biodiversity, biosystem productivity and changing state of people's health on the whole planet.

As it has already been said, HMI are widely spread toxic pollutants of the aquatic environment of the planet. In some cases, content of HMI has reached such a high level in surface and subsoil waters, that they produce direct toxic action on human body. Thus, according to World Health Organization (WHO), level of arsenic in subsoil waters in some regions of Eastern Bengal, India significantly exceeds allowed concentrations, which endanger not less than 50 million people, which is just characterized by authors as nature catastrophe. For another Indian region (Sukinda, Orissa, India), where about 2.6 million people reside, high concentration of chrome compound pollutants in subsoil waters represent high risk. Millions of people in many other regions of the planet are directly exposed to toxic action of ions of copper, lead, cadmium and other metals.

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*Correspondent author- Telephone: +38- 057-707-53-40
E-mail: padalko@univer.kharkov.ua*
It has to be noted, that HMI have the ability to get accumulated in organisms at a higher concentration and manifest toxic effects. Besides, various plants and living organisms are able to selectively accumulate various HMI. Consumption of such plants and organisms as food, raw material for production of medicines or other remedies may lead to toxic effects. Therefore, HMI can endanger human health not only through polluted potable water, but also through food products, household chemicals and also through direct contact in the process of industrial production.

Considering negative influence of HMI on human organism, it is necessary to allocate their direct influence on organism functions and indirect influence. Thus, relatively high concentrations of HMI in an organism induce development of cancer diseases, embryo-toxicity, pathology of liver, respiratory apparatus, etc. In cases of indirect influence, which may be manifested under low concentrations of HMI in an organism, depression of functions of immune system, secretory system and detoxication system can be observed. Their bioaccumulation and chronic effect can result in alteration of functional states of organisms and development of various metabolic diseases. It is evident that only fundamental research of molecular mechanisms of toxic action of HMI on biological systems can be a basis for ecological toxicology and means of development of systems of protection of human organism from their negative influence.

Modern toxicology has moved from the field of medical study of poison properties and development of means of prevention and cure to the category of medico-biological disciplines. Moreover, we can speak today about formation of ecological toxicology, the one of the main tasks of which can be described as-(1) study of toxicity mechanisms with consideration of factors that influence sensitivity (resistance) of various objects to toxicants; and (2) study of mechanisms of adaptation and imprinting of various species, since their different mechanisms of adaptation could result in changing population’s structure and biocenosis in general.

Study of mechanisms of biological action of HMI has one more important aspect. Majority of HMI are essential elements and their deficiency (lower than physiological level for various metals and types of organism) in organism also results in development of various pathologies.

Thus, essence of the problem of biological action of HMI is in their bifacial nature, which manifests itself in their complex dose-dependent effect, which may depend on various reasons and factors. Some of which are- concentration of metal ions in organism; intake (ingress) of HMI into organism; metabolic activities of a biological system that are exposed to HMI (age, gender, functional and food load, etc.); temporal nature of changes of kinetic characteristics of HMI in organism; and interrelation of HMI on the nature of mineral structure in biological systems, which is characterized by specific features of chemical properties of metal ions and state of mineral homeostasis, etc.

It is clear that a uniform concept of biological action of HMI with consideration of species and age aspects can be established only on the basis of exhaustive knowledge of influence of the above mentioned factors on formation of biological responses of organism. The present review deals with some of these issues, namely, characteristic of dose dependency of HMI action, feasible mechanism of formation of organism resistance in cases of chronic action of HMI and time-based characteristics of preservation of HMI action for resistance of organism.

**Mechanisms of toxic action of heavy metal ions**

Manifestation of HMI toxicity depends on various factors, the most important of which are chemical properties of an element, its role in metabolism of a particular organism (essential or not) and their concentration in biological systems. As a rule, relatively high doses of HMI produce acute toxicity, which manifests itself in lethal outcome or fast development of pathological states. In cases of acute toxicity, HMI directly influence metabolic processes in organism. Several points of HMI action application can be marked out when they manifest acute toxicity.

First of all, this is their ability to interrelate with proteins, and most of all, SH groups of various proteins. Besides, enzymes with SH groups in their structure get selectively inactivate, whereas ferments that do not contain SH groups can preserve their activity in HMI presence or even activate, which results in cell enzyme profile pattern changing.

Thus, it is shown that lithium is highly effective in the treatment of bipolar disorders and has multiple effects on embryonic development, glycogen synthesis, hematopoiesis and other biological processes. A variety of enzymes have been proposed as potential targets of lithium action, including inositol monophosphatase, a family of second
messeger, and the protein kinase glycogen synthase kinase-3 (Ref. 22-24).

Effect of CdCl₂ (0-50 mM) on enzymes of leaves in growing pea (Pisum sativum L.) has been studied 25. Alterations in the activated oxygen metabolism of pea plants have been observed as evidenced by an increase in lipid peroxidation and carbonyl-groups content, as well as a decrease in catalase, superoxide dismutase and glutathione peroxidase levels has been noted in the study group compared with the control group 26.

Inhibiting activity of enzymes is manifested by lead, zinc, nickel, copper and other HMI, and this inhibition of some ferments results in changing blood cell functions, in particular, erythrocytes, cells of liver, brain and other tissues 27-30. These manifestations result in changing structural and functional properties of cell membranes and other cell organelles at various biological objects 31.

Such selectivity in HMI action will result in formation of new metabolic pattern and not only in inhibition of enzymes activity. They can also produce increase of activity of enzymes. Thus, intraperitoneal introduction of cadmium, zinc, copper, cobalt and other metals produce increase of activity of heme oxygenase 32.

Such an increase of ferment activity under increase of HMI content in a cell can have various mechanisms: both specific and non-specific. Specific mechanisms of activating action of metals are those cases when a metal ion enters active center of enzymes (as in Cu, Zn-dependent superoxide dismutase). Non-specific – compensatory increase of activity of some enzymes with the result of decrease of activity or inactivation of alternative metabolic pathways. This is important in a sense that enzyme systems should be considered as dynamic systems having various alternative metabolic variants. Hence, HMI in toxic concentrations, inhibiting activity of some enzymes, increasing activity of other ferments and influencing a diversity of proteins, thereby change cell functions, forming “disadaptive” pattern of enzymic activity and changing the very system of cell communications in organism.

One more molecular effect of HMI action is induction of production of free radicals and, as a result, develops oxidative stress 21,28,29. As it is known, oxidative stress launches a cascade of metabolic changes, in particular, peroxidation of lipids, oxidation of proteins and nucleic acids. The complex of these metabolic changes results in changing respective signaling pathways (as calcium homeostasis), which is accompanied with changes in content of intracellular calcium, which, in its turn, influences a big number of metabolic processes and, in particular, promotes reduction of membrane potential, release of cytochrome C, launch of mitochondrial-dependent apoptosis, and changing enzymes activity 29,33,34.

Various HMI can “use” various pathways and their combinations in realization of toxic effect. However, independent from ways of molecular realization on cell and organism level there is a relatively single-type response of organism–development of pathology and lethal outcome. However, initial “state” of biological system significantly influences manifestation of metal toxicity. Thus, copper (1.25, 2.5 and 5.0 mM) and cobalt (20, 40 and 80 mM) have slowed down growth, inhibited photosynthesis and respiration in Chlorella pyrenoidosa, however, their toxic effect can change significantly depending on conditions of cultivation 35.

It is known that HMI are capable of damaging erythrocytes membrane, however, this effect depends not only on metal ions and their doses, but also on the state of erythrocytes 36. Cadmium suppressed induced proliferation of lymphocytes, but at the same time, this effect depended on the stage of cell stimulation, i.e. on functional state of cells 37.

As it is known, metals (in particular, cadmium) contribute to development of osteoporosis, anemia and lung, prostate, pancreas and kidneys cancers. It is attributed by the International Agency for Research on Cancer in US to the first category of carcinogens 38. There are indications that cadmium produces dose-dependent increase of cases of cancer of interstitial cells of testicles. Besides, frequency of development of adenoma increases in case of deficiency of Zn in food 39. Still many results testify to interrelation of HMI, which may manifest increase or decrease of effects of HMI on each other.

Malignant transformation of cells was also induced by chromium ions and this has been correlated with increase of frequency of chromosomal aberrations 40. Similarly, cytotoxicity and transformation activity has been shown for arsenic, nickel and other metals 14,41,42.

It can be assumed that mechanism of carcinogenic action of HMI is realized through direct influence of
metal ions on genome. It is confirmed with experimental data on conformation changes of DNA, DNA rupture and its depurinization\(^{43}\). Genotoxic effects of HMI are shown in micro-algae culture\(^{44}\). At the same time, there are opinions on indirect action of metal ions on genome, which can be mediated by their action on enzymic systems of replication and transcription\(^{35}\), or their action is mediated through oxidative stress\(^{46,47}\).

The quoted results of own research and available literature on molecular mechanisms of toxic action of HMI allow drawing several conclusions- (i) action of HMI is heavily polyfunctional and represented both as direct influence on proteins, nucleic acids and the very mineral composition of cells and tissues, and as indirect influence through oxidative stress and induction of alternative metabolic pathways; and (ii) toxic action of HMI depends on their chemical properties, concentration of HMI in biological liquids and functional properties of biological objects.

**Biological effects of HMI depending on concentration**

In case of HMI dose, better to say, their concentration in biological systems, being lower than the toxic one, two strategies of organism reaction can be realized- (i) adaptive strategy, which manifests itself in formation of resistance to toxic action of HMI; and (ii) disadaptive strategy, which manifests itself in development of chronic pathologies. Disadaptive strategy is conditioned by bio-accumulation of HMI in organism and formation of alternative metabolic pathways. Complexity of the problem lies in the fact that these two strategies could be realized simultaneously and such a division is mostly a methodological approach to experimental study of resistance phenomenon. In some cases, depending on the rate of HMI accession into organism, one or the other strategy gets apparent. Another serious methodological problem in establishing general biological pattern of HMI action is selection of test doses for different objects. First, sensitivity to HMI action is different for different objects and, consequently, different doses are required to obtain similar biological effect. Second, components introduced into system *in vitro*, required for creation of optimal conditions of enzymes functioning, can exert influence upon HMI action, which also produces difficulties for benchmark research\(^{48}\).

With the purpose of proper conduct of benchmark research of HMI action on various biological objects and systems *in vitro*, we proceeded-(a) not from HMI dose, but from assessment of biological response, produced by a wide range of doses or concentration of metal ions; (b) from controlling several indicators in one object simultaneously (DNA synthesis, RNA synthesis, RNA transport, etc.), i.e. used system level in our research; and (c) in the *in vitro* experiments, to account for possible influence of reagents on HMI action on cell nucleus or other organelles, we educe organelles from organs with various functional activity (for example, intact and regenerating liver), which allows taking into account influence of functional properties of organs and organism in general on HMI action\(^{48}\).

Thus, in case of assessment of primary effect of action of copper ions on functional activity of genome of *Chlorella vulgaris* cells, the range of doses of copper sulphate is selected on the basis of the dose, which produces 50% depression of growth of culture for 1-3 days of cultivation. It has been found that 0.4 µmol/l dose of copper sulphate increases specific radioactivity of DNA by 53% compared to reference variant\(^{48}\). Effect of stimulation of DNA synthesis rate decreases with the increase of dose up to 1 µmol/l, although it is still higher than the reference variant. When the dose was 2 µmol/l, specific radioactivity of DNA in *Chlorella vulgaris* cells coincide with the reference level and decreases by 21-31% compared with the reference level, when the dose has been increased to 4 and 6 µmol/l (Fig. 1A).

We cite these results since they clearly show pronounced dose dependency, which consist of three stages: stimulation stage, latent stage (quantitative correspondence with the reference level) and stage of function depression. In order to determine the character of dose-dependent responses, other indicators from other model systems are used\(^{49,50}\).

We will give examples of changing protein synthesis rate and intensity of proliferation of *Chlorella vulgaris* cells. It has been found that the same three-phase response, depending on the dose of copper sulphate in culture medium, exists both for protein synthesis rate and DNA synthesis (Fig. 1A). Phase of stimulation, latent phase and phase of inhibition are expressed under concentrations 0.4, 2, 4 and 6 µmol/l and by rate of accumulation of *Chlorella vulgaris* cells in culture (Fig. 1B).

Such a three-phase responses of cells in micro-algae have also been observed with introduction of other metals into media\(^{44}\).
In order to identify manifestation of three-phase response to primary action of HMI, we determine dose dependency of activity of RNA-polymerases of nuclei of cells of rat liver in the \textit{in vitro} system\textsuperscript{48}. It has been found that presence of 8.5 mM of copper sulphate in medium results in increase of activity of enzymes by 3.9 times compared with reference level (Fig. 2). It is necessary to note that effect of increase of activity of RNA-polymerases pronounces. If copper ions concentration in the medium increases by 10 times, this is accompanied with inhibition of enzymes activity by 75\%. Further increase of metal concentration in medium up to 850 mM results in 80\% inhibition of activity of RNA-polymerases (Fig. 2). It is necessary to note, that the three-phase answer of RNA-polymerases activity has been shown and in case of change of functional activity of liver cells (induced by partial hepatectomy), though quantitatively this effect differs from control variant (Fig. 2).

Study of influence of copper ions in the system of microsomes in a wide range of concentrations shows that activity of marker enzymes of microsomal fraction-glucose-6-phosphatase increases by 40\% with concentration at 0.005 mM and increase of metal concentration is accompanied by linear decrease of activity of this ferment\textsuperscript{48}. Drop of activity of glucose-6-phosphatase in the range of concentrations 0.005-5 mM is accompanied by linear increase of MDA content in medium\textsuperscript{48}. Consequently, three-phase dose-dependent character of copper ions action also manifests itself in the system of microsomes.

Numerous studies have shown that HMI induce free radical processes\textsuperscript{21}. Thus, it has been established that lipid peroxidation is involved in cadmium-related toxicity\textsuperscript{51}. After the administration of lead alone, the activity of superoxide dismutase (SOD) decreases in liver. Cadmium exposure and combined exposure to lead and cadmium lead to decrease in GSH content and increase in thiobarbituric acid (TBARS) levels\textsuperscript{52}.

However, other authors have shown that cadmium does not produce lipid peroxidation and does not potentiate lipid peroxidation and hepatotoxicity of \textit{CCl}_4, while iron or vanadate which produces lipid peroxidation alone potentiates lipid peroxidation and hepatotoxicity of \textit{CCl}_4\textsuperscript{53}. This indicates that other mechanisms, different to oxidative stress, may be involved in hepatic damage\textsuperscript{54}. Contradictions in the above examples from literature also reveal complex dose-dependent effect of HMI action. It may be assumed that mechanisms of dose-dependent three-phase response of biological systems are realized through the character of distribution of metal ions in a cell or system \textit{in vitro}. If so, it is expected that depending on HMI concentration in a cell, their distribution may be different. Identification of the pattern of distribution of copper ions in cells shows that it depends on dose and scheme of introduction (time interval between introductions)\textsuperscript{55,56}. Character of
intracellular distribution of nickel ions in liver is also influenced by functional alteration of its activity, which was induced by partial hepatectomy.

On the basis of numerous data, it may be assumed that a mechanism of consecutive binding of HMI with cell components functions in biological systems, which determines their dose-dependent action. This indicates that there are several dose-dependent stages of binding metal ions (copper in particular) with biological system components (Fig. 3).

First stage of binding manifests itself under metal ions concentrations only slightly higher than physiological requirements of the cell (we can speak about relative concentrations, since physiological requirements are different for different organisms). These concentrations in the cell provide specific binding of metal ions with molecules of metallothionein type and other proteins that provide specific binding with metals. Such a specific structural alteration of molecules provides induction of metabolic processes, directed at increase in synthesis of RNA and a number of proteins, and enzymes activity. This is a compensatory-adaptive reaction.

The second stage of binding manifests itself with further increase of metal ions concentration in the system. Besides, metal ions bind not only with specific proteins, but also non-specifically with various cell components. The shown data in respect of stimulation of activity of RNA-polymerases and glucose-6-phosphatase in in vitro system can be an example of such non-specific stimulation. It may be assumed that stimulation effect of metal ions is formed by stages of specific and non-specific stimulation, which are conditioned by relevant stages of binding (Fig. 3).

Third stage of binding manifests under further increase of concentration of metal ions, which results in additional non-specific binding with molecules, which already form complexes with metal, and are accompanied by enzymes inactivation (RNA-polymerases, glucose-6-phosphatase, etc.), decrease of synthesis of DNA and proteins in cells. Moreover, activation of lipid peroxidation, accompanied with structural and functional changes of the whole metabolism of the cell is possible under high concentrations of copper ions. This binding stage corresponds with the effect of inhibition of metabolic processes and toxicity in general (Fig. 3).

It has to be noted that so-called latent phase of metal ions action can be overlapping of processes of stimulation and inhibition, leading to quantitative compliance with reference level. However, metabolic processes differ from the reference level at this stage qualitatively. These differences are exposed in response reaction of the biological system to exogenous impacts. Thus, it has been shown that Tetrahymena pyriformis cells responded to action of hormones at this stage differently from the reference variant. Time of biological system “being in” so-called latent phase has big importance for the process of adaptation of this system to action of HMI.

Consequently, depending on HMI concentration in biological systems, they can manifest various effects from stimulation to inhibition and toxicity. However, these effects can also modify the functional state of the biological system.

Study of mechanisms of organism adaptation to HMI action is an extremely complex task. First of all,
because adaptation is a highly dynamic process and there are no appropriate methods of study of time processes in biological systems yet. Most often, we study several static points in such situations, after which we build a possible dynamic series, which does not always allow understanding of truly temporal character of functioning of biological processes. At the same time, it is not possible to understand mechanisms of HMI toxic action without knowledge of mechanisms of organism adaptation to long-term influences.

**Mechanism of formation of bio-resistance to HMI action**

Study of mechanisms of adaptation and formation of bio-resistance to HMI toxic action has been started with the discovery of phenomenon of stress-protein induction. It has been shown that under certain concentrations, HMI induce synthesis of specific proteins metallothioneins (MT).

Literature is available MT studies. Besides, the major part of these studies is devoted to structural and functional organization of MT genes and structure of stress-proteins. Metallothioneins are found in cells of animals, plants, unicellular eukaryotic organisms and even prokaryotic organisms. This is a group of low-molecular metalloproteins with high content of cysteine. MT molecule has a “nucleus” consisting of two metallothionein clusters. Cluster A contains 11 cysteine residua and capable of binding 5-6 copper atoms. Cluster B contains 9 cysteine residua and binds 4 atoms of zinc, cadmium and 6 atoms of copper. Polypeptide chain forms two big spiral turns around this “nucleus”.

Metallothionein genes contain numerous regulatory elements, which provide interaction with specific sensor proteins. According to some authors, HMI bind with specific nuclear regulatory proteins, which correlate with promotive areas of MT genes. Thus, well-studied sensor protein *Saccharomyces cerevisiae* ACET binds with Cu$^{2+}$ or Ag$^{+}$, and this results in alteration of its conformation, after which it becomes possible to bind it with MT gene promotor, and induction of synthesis of mRNA metallothioneins. A number of sequences that participate in induction of expression with participation of ions of cadmium and zinc have been discovered for MT-I gene of mouse. These sequences are called MRE. They regulate expression of MT gene and are located remotely from it, i.e. they possess enhancer properties. MRE also form a complex with specific proteins after their binding with metal ions.

Results of some studies allow to draw conclusion that MT expression is regulated at the level of transcription with participation of metal ions. Since HMI presence in organism is accompanied by MT induction in cells of various organisms (mammals, plants, bacteria), it indicates that this phenomenon has general-biological character. Probably, there are age specific features of MT synthesis induction. Thus, high level of MT content in liver has been observed in 2 and 4 day old normal new-born chickens and then their content sharply reduces compared to other liver proteins. Generally the young animals are less sensitive to metals and some authors connect this with a higher level of MT, which has been shown to alter the hepatic subcellular distribution of cadmium such that less binds to subcellular organelles and more binds to metallothionein located in the cytosol. At the same time, content of MT in kidneys, liver and lungs does not change in 3, 12 and 24 months old rats. As regards the level of cadmium MT induction, it was higher in 12 and 24 months old animals than in 3 months old rats.

Consequently, background level of MT in postnatal ontogenesis of mammals changes a little (at least, in the range adult–ld), and MT induction ability has age-dependent character. However, these issues need further studies.

Synthesis of metallothionein increases in presence of metal ions, but also increases in presence of other compounds. For example induction of MT expression has been observed in liver after administration of CCl$_4$ and ethanol.

Most probable, MT induction is a non-specific cell response to action of various factors: radiation, high and low temperatures, hypoxia, water deficit and salinity, and various chemical compounds. It can be assumed that MT induction provides general resistance to cells and organism against various negative factors. Mechanisms of such stability are not clear enough, as well as specific role of MT in formation of such a stability.

It is possible to believe that MT are polyfunctional proteins, which participate in metabolism regulation under standard physiological conditions, and their role is not reduced to formation of resistance to HMI action only.

It is shown that MT is engaged in regulation of intracellular homeostasis of zinc, cadmium and copper, in particular, depositing, transport and their
redistribution in the cell. Metallothioneins take part in regulation of metal-containing factors of transcription, regulate expression of genes of some proteins, and transcription of genes of enzymes. Thus, taking part in regulation of expression of such a big range of regulatory proteins, MT provides such processes at the cellular level as apoptosis, proliferation, differentiation and regeneration of tissues.

It is shown that along with physiological processes, in which MT take part, they determine, under high level of MT expression, apoptosis stability of transformed cells and, thereby, rate of growth of aggressive tumour. They can perform the function of scavenger of free radicals and control cellular ox-red-homeostasis.

Thus, the shown data allow in drawing a conclusion that MT is an evolution-conservative group of proteins with polyfunctional properties. MT synthesis in the cell can be induced by various physiological and chemical compounds. However, the levels of MT induction can be different depending on the nature of inductor. The nature of MT induction has tissue-specific and age-dependent character. Along with various other physiological functions, MT takes part in formation of organism resistance to HMI action. We do not know yet about a specific mechanism of formation of organism resistance to HMI action, and, may be due to no common mechanism.

It can be deduced that HMI perform polyfunctional action on biological systems. Direction of their action depends on various conditions and factors like, HMI dose, ways of their introduction into organism, and functional state of the system (age and other differences).

We put forward a hypothesis, according to which induction of MT and other stress-proteins launch cooperative alterations in the whole metabolic system of the cell and organism in general, that result in formation of new epigenotypes as a result of increased resistance of the organism to HMI action and possibly other stress-factors. Or, in other words, action of MT and other stress-proteins has non-specific, polyfunctional character.

In order to test this hypothesis, experiments were conducted on micro-algae Dunaliella viridis Teod. Earlier, we obtained in our laboratory the culture of D. viridis resistant to copper ions at lethal concentrations for this species.

It has been found that content of total proteins in D. viridis cells, resistant to copper ions (CuR D. viridis), decreases compared to cultures sensitive to copper ions (CuS D. viridis) by 23% and this decrease is correlated to decrease of rate of protein synthesis in them. Cells of CuR D. viridis do not contain water-soluble 70 kDa proteins and show a new 35 kDa protein fraction (Fig. 4). Thus, additional heat treatment of cells does not influence on a spectrum of water-soluble proteins (Fig. 4).

Consequently, adaptation of D. viridis cells to high (toxic) concentrations of copper ions in the medium is accompanied by decrease of the rate of synthesis of total proteins in cells, reduction of their quantity in the cell and alteration of composition of proteins, which are extracted from cells by 25 mM of tris-HCl buffer (pH 7.6).

Even more deep changes take place during adaptation of cells to copper ions in genome expression of these cells. Thus, cells of CuR D. viridis contain 1.6 times less of total RNA, which is conditioned by decrease of the number of ribosomes in them compared to CuS cultures. However, these changes in metabolism of proteins and RNA do not influence intensity of growth of CuR and CuS cultures of D. viridis. These cultures also has slight differences in cell morphology.

Formation of D. viridis cell resistance to copper ions accompanies by increase of a number of lipids.

![Fig. 4](image-url)---Polyacrylamide gel electrophoresis of water-soluble proteins of cells of CuS- and CuR-cultures D. viridis (age of cultures of 14 days). [Lane 1- CuS-culture in standard conditions of cultivation; Lane 2- CuS-culture after heat treatment at 45°C; 1.5 min; Lane 3- CuR-culture in standard conditions of cultivation; and Lane 4 - CuR-culture after heat treatment at 45°C; 1.5 min; and kit – protein with known molecular weight]
and, first of all, of neutral lipids in cells. Thus, content of steroids increases by 3.2 times, non-esterified fatty acids by 8.2 times and triacylglycerols by 10.6 times, compared to cells of CuS cultures of \textit{D. viridis}. Content of phospholipids fractions, in particular phosphatidyl-choline and phosphatidylethanol-amine increase by 3.7 times and phosphatidyl-inositol by 6.4 times in cells adapted to copper ions, compared to the cells of CuS cultures of \textit{D. viridis} that do not adapt to copper ions \cite{86}. Consequently, formation of \textit{D. viridis} cell resistance to toxic concentrations of copper ions accompanies by general over haul of all structures of the cell, which result in development of epigenotype different from that of CuS cultures of \textit{D. viridis} (Fig. 5). It should be noted that CuR \textit{D. viridis} shows quantitative changes in metabolism and also qualitative changes in genome expression, which become apparent in changing spectrum of synthesizing proteins. These changes have a co-operative character and form epigenotype against the background of high concentrations of copper ions and can provide manifestation of some new properties of these cells. In order to test this assumption, stability of CuR culture of \textit{D. viridis} to high temperatures has been determined \cite{82}. It has been found that if culture cells, which are not adapted to high concentrations of copper ions (CuS \textit{D. viridis}), stay for 1.0 and 1.5 min at 45°C, however, 50\% of cells died after 1 h of exposure. If this culture stays at 45°C for 2 min 90\% of cells die 1 h later. Consequently, cells of the reference strain of CuS \textit{D. viridis} are rather sensitive to rise of temperature. At the same time, if cells, resistant to toxic concentrations of copper ions – CuR \textit{D. viridis} – are exposed to thermal shock, all cells remain alive after 1 min of heat shock at 45°C, and when time of heat shock is increased to 2 min, rate of cell death is 3 times lesser than in case of CuS \textit{D. viridis}. It accompanies also by formation of new epigenotypes as CuR and CuS \textit{D. viridis} (Fig. 5).

Consequently, formation of resistance to copper ions in \textit{D. viridis} culture accompanies by formation of increased tolerance to high temperature too. This indicates that formation of resistance to toxic concentrations of copper ions is a non-specific response of cells, which bears a co-operative character. Such changes result in formation of a new epigenotype of these cells, which is also characterized by higher tolerance to high temperature.

If we assume that formation of resistance of organism to HMI action is explained by co-operative change of metabolism with formation of new epigenotypical variants, this allows explaining differences in response reactions for HMI action in animals of different ages.

\textbf{Age specific features of formation of resistance for HMI action}

It has been found that in the result of numerous successive introductions of copper sulphate in rats at the dose that constitutes 30\% of the lethal dose for these animals, they form resistance even to subsequent introduction of lethal dose of this toxicant \cite{56}. It is well known about ability of rats and other mammals to adapt to long-term exposure to copper ions \cite{83-85,88}. However, the mechanisms of this phenomenon are not clear.

Studying the mechanism of formation of adaptation to long-term HMI action, it is necessary to differentiate between resistance effect and hormesis effect. Hormesis effect is the effect of tolerance to lethal doses of toxicant after preliminary exposure to lower doses of the same toxicant. Some authors consider hormesis effect as a result of overcompensation response of homeostatic compensatory systems under exposure to low doses of stressor \cite{86-88}.

Hormesis effect is found not in animals only; it is, probably, a common biological phenomenon and has been described for various toxicants. Thus, it is shown in Dunaliella viridis \cite{89}, Chlorella \cite{90}, amphibia \cite{87}, and even macrophage culture \cite{88}.

Hormesis effect has been studied in recent years. Majority of researchers consider that it is related with MT synthesis induction or with specific metal-binding proteins. Thus, Luza and Speisiu \cite{91} have shown that binding copper ions with MT is a form of “safe storage” of metal in cytoplasm. Growth of MT pool in
cytoplasm provides binding of free metal ions and, thereby, it prevents generation of hydroxyl radicals.

It has been observed that after introduction of toxic doses of metal such an organism is capable of accumulating a big number of metal ions, since it contains more specifically HMI binding proteins. Experimental results, which show that hormesis effect results in 10-15 times increase of MT in liver, testify in favor of these data. Zinc ions induce MT synthesis and this provides further decrease of copper level in organism and, consequently, its toxicity. Perhaps, MT provides depositing of metal ions and also their washout from organism. Decrease of HMI toxicity can be correlated rather with elimination of toxic action of metal ions than with MT induction itself. Influence of preliminary introduced ethanol on decrease of lethal outcome and hepatic toxicity of cadmium, which can be resulted to lower interaction between metal ions and molecular targets, testifies in favour of this. Rate of death from high doses of metal can be reduced not only by means of preliminary treatment with lower doses of xenobiotics, but also by means of a stressor of different nature, for example, cold stress. This points at nonspecific action of MT in protection of cells from HMI.

Besides, quite convincing and logical are data that show that MT induction is not the only one or not the main mechanism of manifestation of hormesis effect. Thus, young animals are more sensitive to action of copper ions if compared to adults, however, MT content in reference and experimental grown-ups is the same. Perhaps, this phenomenon is connected with the fact that only a small part of copper ions is bound with MT, besides, synthesis induction does not occur even at copper doses up to 500 µg/g of body wt.

Interesting experiments have been conducted with LEC (Long-Evans cinnamon) rats. These rats accumulate copper ions in liver fast and they have hepatitis when they are 4 months old. Besides, majority of copper ions in them are bound with MT, but the level of liver damage is much higher than that of rats with low content of MT. Consequently, MT does not provide full protection from toxic action of copper ions and available results do not allow unambiguously interpret role of MT in adaptation to HMI and in formation of hormesis effect.

Data is available about participation of oxidative stress in formation of hormesis. Mild or medium intensive exposure results in adaptation through Nrf2 (nuclear factor erythroid 2–related factor 2) a number of enzymes, first of all antioxidant ones, which allows keeping the level of free radical pro-oxidants at a relatively low level–such a stage of response is adaptive by nature and provides cell stability. A number of experiment testify in favor of activation of peroxide processes in presence of HMI, copper in particular. Thus, particularly, increase of MDA level is shown in liver tissue of LEC rats, which are characterized with spontaneously high level of copper ions content in liver. At the same time, there are studies that demonstrate that copper toxicity and possible hormesis effect has nothing to do with LPO activation.

Perhaps, manifestation of hormesis effect, as one of the most vivid mechanisms of induced resistance, is realized in different ways. This depends on various factors and, in particular, age of animals, scheme of toxicant introduction, dose, etc. However, the basis of formation of resistance and hormesis effect are generalized changes of many, if not all, metabolic links, which provide homeostatic functions of the cell.

In order to solve this important issue, the study of influence of age of experimental animals on formation of tolerance to multiple actions of copper ions in toxic concentrations has been conducted. It is shown that 3 mg dose of copper sulphate per 100 g of body weight are lethal for Wistar rats, besides, lethal outcome of experimental animals occurred in 1-2 h after introduction. In this case, if experimental animals get the dose of 1 mg/100 g body wt, i.e. about 33% of the lethal dose and 48 h later the lethal dose (3 mg/100 g) is introduced, death of a number of animals occurs significantly later and 10% of experimental animals survives (Fig. 6, Curve 2). Such an increase of resistance to lethal doses testifies manifestation of hormesis effect. Hormesis effect depends on a number of preliminary introductions of relatively low doses of copper sulphate. Thus, it increases after two and especially three successive introductions of copper sulphate in small doses (1 mg/100 g; Fig. 6, Curves 3 and 4).

It has to be noted that after 3 successive introductions of copper sulphate in doses of 1 mg/100 g body wt with 48 h interval and subsequent lethal dose, 70-75% of experimental animals survive and death of few animals occur in 15-24 h. This points at the fact that death of animals in these cases is provoked by reasons other than primary toxic reaction, produced by the lethal dose.

Increase of a number of preliminary introduction of small doses (1 mg/100 g body wt) to more than 3 times
with further introduction of lethal doses in the same animals do not increase, but, on the contrary, reduce survival effect compared with three successive introductions of 1 mg/100 g body wt (Fig. 6, Curve 5). Consequently, preliminary introductions of non-lethal doses of copper sulphate (1 mg/100 g body wt) provides manifestation of hormesis effect depending upon number of preliminary introductions of copper sulphate. This dependency has S-shape nature, i.e. it increases at 1 to 3 introductions and sharply reduces at 6 preliminary introductions in cases after 48 h of introductions of copper sulphate.

As it has been mentioned, the mechanism of hormesis effect is not quite clear. Due to this, study of various factors that influence this phenomenon is of interest. Of biggest interest in this respect are biological factors, such as age of animals, functional state of organism, etc. Unfortunately, there are few such studies, but it has been shown that animals of different age manifested different tolerance to action of HMI.

To assess manifestation of hormesis effect in respect of lethal doses of copper sulphate, 3 non-lethal doses (1 mg/100 g) are preliminary introduced to young (3 months old) and old (20 months old) animals, after which lethal doses are introduced (3 mg/100 g body wt). It turns out that, under this scheme, resistance of young rats increases by 10 times compared to a single lethal dose. Resistance to lethal dose, under this scheme of introduction of copper sulphate, also increases in old animals by 4 times. These results allow in drawing a conclusion that old animals are less tolerant to toxic action and their effect of induced stability is less pronounced than that of young animals.

It is known that liver plays the key role in performance of the function of xenobiotic detoxication. It has been reported that the function of detoxication, the integral indicator is the hexobarbital sleep time, may be depressed under multiple introductions of HMI. It has been reported that three successive introductions of copper sulphate (1 mg/100 g body wt) at 48-h interval do not influence the hexobarbital sleep time in young animals, but increase by 3 times in old rats compared to the relevant age reference level. Consequently, three successive introductions of copper sulphate (1 mg/100 g body wt) at 48-h interval do not change the function of detoxication of liver in young animals, but significantly depress it in old (20 months) animals.

Such pronounced differences in the function of liver detoxication against the background of multiple introductions of copper sulphate can be explained by the fact that 20 months old rats’ liver binds more copper ions that liver of 3 months old rats. In order to test this assumption, we have determined content of copper ions in various compartments of liver cells 12 h after three successive introductions of copper sulphate (1 mg/100 g of body wt) every 48 h. It has been observed that cell nuclei, microsome fractions and, especially, mitochondrial and MT fraction of old animals, contained more copper ions in comparison with young rats (Fig. 7). It is necessary to note that...
protein fraction, which contains MT, bound 2.6 times more copper ions compared to young animals. However, this does not provide necessary protection of mitochondria of old animals and they bind 2 times more copper ions than young ones (Fig. 7), which can be correlated to manifestation of lower tolerance to copper ions in old animals compared to young animals.

This character of intracellular distribution of copper ions and, in particular, maximum in mitochondria can result in induction of free radical processes and increase of peroxide processes of macromolecules in the cell. It has been found that LPO level in old animals, induced by copper ions and other HMI, is higher compared to relevant indicators of young animals. However, a higher level of LPO in old animals in this case, perhaps, is the result of maximum accumulation of copper ions in the liver of old rats compared to young ones. Maximum accumulation of copper ions in mitochondria of liver in 20-months old rats compared to 3-months old rats after introduction of copper sulphate (1 mg/100 g of body wt), results in depression of detoxication function in liver and LPO increase. This can be explained by the fact that relation of liver weight and body weight changes with age, and as consequence, on introducing HMI per body wt unit, “load” on liver of old rats may be higher than that of young rats.

In a separate series of experiments with Wistar rats of different age group, we have identified ontogenetic changes in relation between liver weight and body weight. It has been found that relation between liver and body weight has age-dependent character and especially significant change up to 4 months age, which lead to trustworthy reduction of relative liver weight in ontogenesis.

Maximum toxicity of copper sulphate for old animals in comparison with young ones can be correlated with the fact that, when calculating dose per unit of body weight of animal, old animals receive more copper ions per unit of liver weight. If dose of copper sulphate is calculated per 1 g of liver, and not per unit of body weight, the dose of 1mg/100g of body weight for young animals equals the dose of 0.57 mg/100 g for old animals in order to produce the same “load” for liver. The experiment establishes that introduction of 0.57mg/100g instead of 1mg/100g to old animals and introduction of 1mg/100g to young animals produces equal toxicant load on liver, and old ones shows the same tolerance to toxic action of copper ions as young ones.

As it has already been mentioned that copper ions, as well as other HMI, are able to bind with various macromolecules of cell, besides, depending on dose, stages of specific and non-specific binding can be marked. We have isolated protein from liver cytosol with molecular mass of 12 kDa and named copper-binding protein (CBP)\(^{56}\).

If copper sulphate (1 mg/100 g body wt) is introduced to young animals three times at an interval of 48 h, 5.3 \(\mu\)g of Cu\(^{2+}\) gets bound on 1 mg of this protein, with background level of its binding being 0.733 \(\mu\)g per 1 mg of protein. However, this produces significant increase of copper ions content in microsomes, mitochondria and proteins of liver cytosol. These results give ground to affirm that when introducing sufficiently higher concentration of copper ions, a new pattern of intracellular distribution of copper ions is formed. But for all that, CBP, although binding considerably more Cu\(^{2+}\) compared to other studied cell compartments, do not provide “protection” of these functionally important cell compartments from toxic action of copper ions.

Consequently, several successive introductions of copper sulphate into organism form a new, different from basic (reference) pattern of intracellular distribution of copper ions. Character of distribution pattern, on the one hand, depends on metabolic peculiarities of the biological system at the moment of HMI introduction, and, on the other hand, induces new variants of alternative metabolic cycles. In the case when these new metabolic variants are stable, i.e. they can provide formation of imprinting of adaptive pattern of distribution of copper ions, introduction of lethal doses of this toxicant ensures hormesis effect.

An experiment has been conducted to assess possible contribution of imprinting into formation of hormesis effect in old and young rats (Bozhkov A., unpublished data). For this, influence of time interval between copper sulphate introductions on the character of pattern of intracellular distribution of copper ions and, thus, on ability of metabolic system to “retain” former state (pattern character) has been identified. Young and old animals have received copper sulphate (1mg/100g body wt) three times and after 1 month of the last introduction, repeated introduction again according to the same scheme and determined the pattern of intracellular distribution of copper ions after 48 h of the last introduction. It turns out that one month after the last introduction of copper sulphate to experimental animals, copper ions...
completely washes out from all liver compartments and its contents remain absolutely similar to that of the reference (basic) level (Bozhkov A., unpublished data).

In the case when experimental animals are again given introduction of copper sulphate three times, after one month since the first scheme of introduction, even more pronounced “adaptive” pattern of intracellular distribution of copper ions in liver cells compared to the first series of introductions is formed in these animals. This effect manifests itself in increase of content of copper ions in CBP and corresponding decrease in microsomes, mitochondria and cytosol, i.e. protective effect or hormesis. Thus, CBP of 3-month old rats binds up to 15 µg of Cu²⁺ per 1 mg of protein, which is nearly 3 times more compared to the first series of copper sulphate introduction (Fig. 8).

Consequently, one month interval between introductions of copper sulphate provides formation of a new “adaptive” pattern of intracellular distribution of copper ions, which can be called a prolong increasing hormesis effect. These results allow affirming that hormesis effect has to do with formation of new metabolic variants in response to experimental impacts, in the basis of which is the metabolic process imprinting ability.

Identification of content of copper ions in old animal liver cell compartments under this time scheme of copper sulphate introduction show that they have maximum copper ions binding with CBP (30 µg of Cu²⁺ per 1 mg), which is 2 times more compared to young ones and 43 times more compared to the reference level. At the same time, content of copper ions in mitochondria and microsomes of old animals is more compared to young ones (3.7 and 2.4 times, respectively; Fig. 8). However, when comparing content of copper ions in these compartments after the second series of introduction with their content after the first series of copper sulphate introduction, content of Cu²⁺ decreases in cytosol and microsome proteins, and somewhat increases in mitochondria (Fig. 8). Consequently, if introducing copper sulphate per unit of body weight, a more number of copper ions “fell at” old rat liver compared to young ones. After the repeated scheme of copper sulphate introduction, the old animals form a new pattern of intracellular distribution of copper ions, which differs not only from that of the first series of introductions, but also from that of young animals. Such a specific “adaptive” pattern of intracellular distribution of copper ions in liver cells can result in hormesis effect. “Adaptive” pattern of intracellular distribution of copper ions is preserved one month at least, i.e. it is remembered (imprinting effect).

Results of research clearly point at the fact that traditional comparison of response reactions of young and old animals to action of heavy metal ions or other xenobiotics, introduced per animal body weight unit is not always correct.

Mechanisms of specific binding of metal ions with MT and other metal-proteins are still not properly studied, and this task is rather complex, since mammal cells contain at least 10 isoforms of metal-proteins. Binding of HMI with other cell proteins and cell components, which take part in the processes of transport, storage and expression of HMI, is studied even less. Taking into account the fact that various molecular forms produce a common dynamic system, it can be suggested that the cell is capable of forming various metabolic variants (patterns) and each of these variants will provide manifestation of effects of toxicity, adaptation, hormesis or imprinting. All these phenomena, which can be induced by heavy
metal ions, and can be presented as a number of metabolic variants in the organism.

**Conclusion**

Study of mechanisms of HMI action is of interest not only for toxicologists, biochemists and molecular biologists, but also for ecologists, epidemiologists and specialists in the field of environmentology, since HMI is one of the most powerful factors of environment that influence on development of biota and their evolution. One of the approaches in solution of this topical and extremely complex task is the search for common regularities of action of HMI on biological systems.

Heavy metal ions exert influence upon functioning of all biological systems independent of the level of their organization, function of enzymes, structure of chromatin and gene expression, structure of membranes, ion content of cells and other systems. These effects are different from stimulation of biological functions to inhibition, manifestation of acute and chronic toxicity and even lethal outcome of organism, i.e. they are polyfunctional. Biological effects, induced by heavy metal ions, can be divided, according to realization in time, into acute and chronic. Polynonspecificity in respect of HMI action means that the same metal can “use” different mechanisms of realization of its effects. For better understanding of these processes, they can be presented as mechanisms of direct action of HMI on macromolecules of the cell and indirect action through induction of alternative metabolic pathways and co-operative changes of the whole metabolic system of the cell. Small amount of HMI in organism could produce direct specific action on activity of enzymes and induction of metabolic changes, which manifests itself in formation of organism resistance to HMI and even other experimental factors of environment (temperature). High concentration of HMI in organism produces not only their specific but also non-specific binding with various molecules, launch of free radical processes, dysfunction of a wide range of cell functions and development of pathologies or manifestation of toxic effect. It is quite evident that the most important factor in identification of biological HMI action is the dose or concentration of HMI in biological systems. However, along with this, manifestation of biological effects can be modified or altered by functional properties of biological systems. Different tolerance potential to HMI action in young and old organisms, functionally active and not active biological systems, can be conditioned by these distinctive features.

When determining dose dependencies of HMI action, it is necessary to take into account the rate of changes of HMI concentration in organism or biological system, i.e. rate of its growth. Single-stage increase of HMI to high concentrations in organism may produce manifestation of acute toxicity. At the same time, if the same concentration of HMI in organism reaches gradually during a long period of time (during several days), alternative metabolic pathways may be induced in the organism and it may display resistance to HMI action. Rate of changes of HMI concentrations in the organism or biological system may determine the choice of strategy of realization of HMI effect and, consequently, the biological effect. Various HMI strategies can be named—MT induction and co-operative changes in metabolic system; formation of a new fermentative pattern of the cell; oxidative stress; and genome changes, etc. It is quite evident that none of the above strategies is realized just as that. As a rule, all of them are interrelated, however, in different cases the share of each of these strategies can vary. Which of the possible strategies will dominate, at the same time determining physiological effects, will depend on the rate of changes of concentration of HMI in biological systems, functional activity of organism at the moment of HMI action and on chemical properties of HMI, more precisely, their complex. In this respect we can describe about the concept of time-based alterations of concentration of HMI (TACMI) in biological systems. On the basis of TACMI concept formation of organism resistance to HMI action also can be explained. In the event of slow increase of concentration of HMI in organism, it produces induction of MT and other stress-proteins, co-operative changes in the whole metabolic system. This, first of all, results in formation of new specific epigenotypes, which provide higher resistance (hormesis effect) not only to HMI that induced this effect, but also to such stress-factors as high temperature (at least, for micro-algae cells). The S-shape character of hormesis effect can also testify in favor of TACMI concept. Slow increase of metal concentration as a result of three successive introductions of small doses of copper sulphate into organism with 48 h interval between introductions testifies to a dose-dependent effect of induction of
adaptive processes. Increase of a number of such introductions up to 6, on the contrary, sharply reduces hormesis effect, which could be connected with bioaccumulation of HMI and arrival at toxic concentrations of xenobiotics in the organism or a cell.

Relatively slow increase of concentration of HMI in organism was accompanied by generalized change of metabolism, which ensure formation of a new, different from the basic (reference) one, pattern of intracellular distribution of HMI. Character of the pattern of intracellular distribution, on the one hand, depends on metabolic specific features of biological systems at the moment of HMI action, and, on the other hand, it provides for induction of new alternative metabolic pathways. In the case when these newly induced metabolic variants will be saved protractedly in details, i.e. they will manifest phenomenon of imprinting of adaptive pattern of HMI distribution; and introduction of lethal doses of toxicants to these animals will provide for hormesis effect.

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