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Determination of Silver Ions Toxicity in Short-Term and Long-Term Experiments Using a Luminescent Recombinant Strain of *E. coli*

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Abstract. The effects of silver ions on the luminescent recombinant strain of *Escherichia coli* carrying *luxCDABE* operon of *Vibrio fischeri* were investigated. The toxicity of silver ions was determined in 30 minutes and in chronic 24 hours experiments. Changes in the luminescence intensity and in the growth rate of bacteria were considered as a measure of silver ions toxicity within the range of concentrations applied. The effect of silver ions was demonstrated to be strongly dependent on the concentration of bacteria and on the medium composition. EC_{50} values were 0.018 mg/l after 30 min exposure and 0.014 mg/l after 10 hours of bacterial growth. Comparison of two modifications of the experiment showed that silver ions have a strong non-specific toxicity, as well as a specific effect on bacterial cells.

Keywords: silver ions; bacterial luminescence test; *E. Coli*; toxicity; bacterial growth.

Introduction.

It is well known that silver ions effectively inhibit growth of bacteria, including *Escherichia coli*. This quality is widely used for water disinfection and in many biomedical applications. However, the toxicity of silver ions for bacteria has not been sufficiently studied in respect to exposure duration or to the influence of the concentration of bacterial cells. This information may be valuable for understanding the features of various materials that contain silver-based nanoparticles.

Previously, the effect of silver ions on the bacteria *E. coli* has been evaluated mainly by changes in the bacterial growth rates. It was found that different strains of *E. coli* have different tolerance for silver ions, which was attributed to differences in their genomes [1]. However, it has also been reported that in long-term experiments, the minimal concentrations of silver ions that affect bacteria can be considerably different for the same strains, ranging from 0.075 mg/l to 0.3 mg/l [2, 3].

One of the most advanced methods to determine biological activity of various compounds is marine luminescent bacteria test. Recently, it was applied to evaluate the effects of silver ions on the bacteria. This fast (up to 30 minutes) standard method is widely used to test the toxicity of chemical agents and their mixtures, and allows to monitor the process in real time [4].

Decrease in bacterial luminescence is proportionally dependent on the toxic effect of an agent tested. Any change in the cellular metabolism can lead to a rapid change in the intensity of bioluminescence, since luciferase, the enzyme responsible for bioluminescence, directly or indirectly responds to various external stimuli [5]. One of the test modifications deals with bioluminescent phenotypes of non-marine bacteria, such as *E. coli*. In this case, together with all advantages of the test, it is possible to avoid obligatory addition of NaCl that significantly interferes with detection of silver ions.

The bioluminescence-based test on *E. coli* allows for fast determination of non-specific toxicity of a sample. In addition, another modification of the method was recently developed, whereby prolonged chronic experiments during active bacterial growth are carried out. In this case, if a substance can influence protein biosynthesis of genetic stability, the toxicity does not decrease over time, but rather can considerably increase. It was shown that toxicity of some specifically acting chemical agents was underestimated [6]. The toxicity of substances affecting biosynthetic pathway of growth and reproduction can only be evaluated if tested at a certain period of the cell cycle. Thirty minutes of incubation is too short to cover reproduction cycle of bacteria.

The tests of silver toxicity on luminescent bacteria, mainly using the commercial kit Microtox (based on the marine bacterium *Vibrio fischeri*) have revealed quite controversial results. During exposer times of 15 to 30 minutes, EC_{50} (which is the main test parameter and designates the concentration that causes 50% reduction in sample luminescence comparing to the control) varied in a wide range of values, such as 9.5 mg/l [7], 2.39 mg/l [8], 0.59 mg/l [9], 0.46 mg/l [10] and 0.18 mg/l [11]. Moreover, an extremely different result was reported by Deheyn *et al*, 2004 [12]. 108 mg/l of silver nitrate solution only slightly inhibited bacterial luminescence during 30 minutes of exposure. In addition, on non-marine luminescent bacteria (*E. coli* modified with genes *luxCDABE* from *Photorhabdus luminescence*) the minimal affecting $AgNO_3$ concentration was found to be 0.34 mg/l and the toxicity threshold was 2.6 mg/l [13]. Besides that, in a chronic 22 hours modification of the experiment assessing growth rate of the luminescent bacteria *V. fischeri* it was found that EC_{50} of silver ions was 7.9 mg/l [14].

The objective of our study was to investigate the effect of cell concentration and of the presence of lyophilization medium on the sensitivity of bacteria to different concentrations of silver ions in 30-minutes experiments, using a recombinant bioluminescent strain of *E. coli* that carries the *luxCDABE* operon from *V. fischeri*. In addition, we compared the effects of short-term (30 minutes) and long-term (chronic, 24 hours) exposure to silver ions, which revealed both non-specific and specific effects of Ag^+ on the bacteria.

Materials and Methods. A luminescent *E. coli* strain was constructed using standard techniques. The bacterium *E. coli* K12 TG1 was used as a recipient of hybrid plasmids with the insertion of luxCDABE of Photobacterium leiognathi 54D10 (from the collection of M. V. Lomonosov Moscow State University). The luxCDABE genes were isolated by the use of the vector plasmid pUC19 [15]. Freeze-dried cells were rehydrated in distilled water for 30 minutes. Then, "working suspension" of bacteria was prepared by diluting the stock solution to the necessary concentration. For extraction from the lyophilization medium, the bacteria were centrifuged at 3,000 g for 10 min, followed by re-suspension of the pellet in distilled water to appropriate concentration. Measurements of luminescence were carried out for 5 and 30 minutes. Samples contained 100 μl of working suspension and either 900 μl of distilled water (control) or 900 μl of silver nitrate water solution of different concentrations (test) [16].

For long-term experiments, the luminescent *E. coli* were first grown at 25°C with shaking at 200 rpm up to the mid-logarithmic phase of growth in LB medium (5g/l NaCl, 10g/l tryptone, 5g/l yeast extract). Then the cells were diluted in LB medium to 10^4 cells/ml and mixed with different concentrations of $AgNO_3$, followed by incubation of total volumes of 1.5ml in 12 ml vials in stationary conditions at 25°C for 24 hours. Afterwards, one part of the sample was used for growth rate analysis and the other part, for the measurement of bioluminescence. Measurements were done in at least 3 replicates. Data represent mean ± SD from three measurements. One of three

independent experiments, all with similar results, is shown. The error in all the experiments did not exceed 10%.

Bacterial bioluminescence intensity was measured using Luminometer 1251 BioOrbit (Finland) and Biotox-10 (Russia) instruments, during 30 minutes for short-term experiments and every 2 hours in chronic 24-hours experiments. The number of bacteria was determined on photoelectrocolorimeter KF77 (Poland) at 590 nm wave length, based on a previously generated standard curve.

The intensity of the luminescence was measured in imp/sec. The toxicity index was calculated according to the formula

$$T = 100 \cdot (I_0 - I_t)/I_0, \quad (1)$$

where I_0 and I_t are bioluminescence intensity of the control and of test samples, respectively. Determination of the toxicity index was carried out in at least 3 replicates for both, control and test samples. EC_{50} was calculated with gamma-function according to the formula [4]

$$\Gamma = (I_0 - I_t)/I_t, \quad (2).$$

For media preparations, Difko reagents were used, as well as domestic brands of chemically pure and analytical grade compounds. Silver nitrate was purchased from Sigma.

Results and Discussion.

First, we determined the dependence of the toxicity index on the concentration of silver ions in a standard test with 30 min. exposure. The response of bioluminescent bacteria to Ag^+ addition was very rapid, and the higher was the salt concentration the faster saturation effect was achieved. The greatest change in luminescence intensity took place in the first 5 minutes and then remained almost constant up to 30 minutes of experiment duration, which was observed for all the concentrations tested. Notably, the two curves that describe a 5 minutes and a 30 minutes experiments are almost identical (Fig. 1).

The similarity of the two curves suggests that within 5 minutes, almost all active sites that can interact with Ag^+ at certain concentration are occupied. Moreover, the number of active sites increased with increasing Ag^+ concentration, which may indicate that there are sites with different affinity to silver.

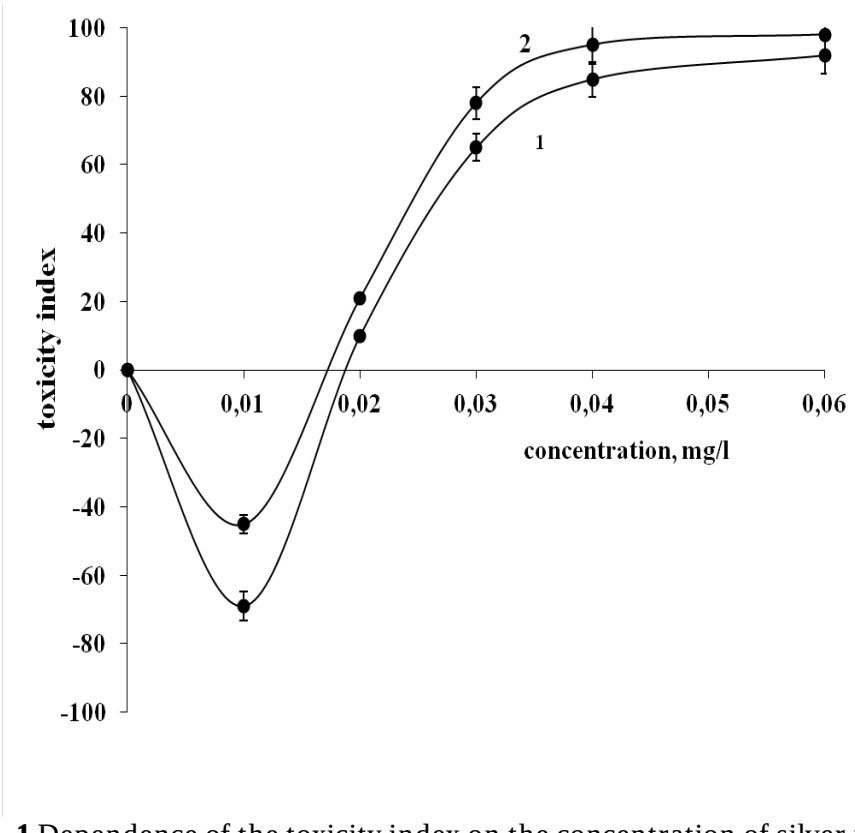

Fig. 1 Dependence of the toxicity index on the concentration of silver ions.

Measurements of luminescence were done as described in Materials and Methods during 5 minutes (curve 1) and 30 minutes (curve 2). Samples contained 100 µl of working suspension and either 900 µl of distilled water (control) or 900 µl of silver nitrate water solutions at different concentrations, as indicated (test). The concentration of cells was 1.7×10^7 cells/ml. Data represent mean ± SD from four measurements. One of three independent experiments, all with similar results, is shown.

We then tested if the sensitivity of the method depends on the number of cells in a sample and/or on the content of the media, e.g. freeze-dry (lyophilization) medium or cultivation medium in the sample. We found that the cells that were not washed from the lyophilization medium and tested at the concentration of 2×10^8 cells/ml (Fig. 2) were an order of magnitude less sensitive to silver ions than pre-washed cells at about the same concentration (Fig. 3), with EC_{50}=0.27 mg/l and EC_{50}=0.025 mg/l, respectively. Notably, a ten-time dilution of unwashed cells with water (down to 2×10^7 cells/ml) produces a similar effect, EC_{50}=0.022 mg/l (Fig. 1).

The most likely explanation of this phenomenon is the ability of Ag^+ to form non-toxic complexes with components of the medium, so that unwashed *E. coli* cells become less sensitive to silver ions due to their decreased effective concentration. Diluting or washing the cells decreases medium concentration and thus, increases bacterial sensitivity.

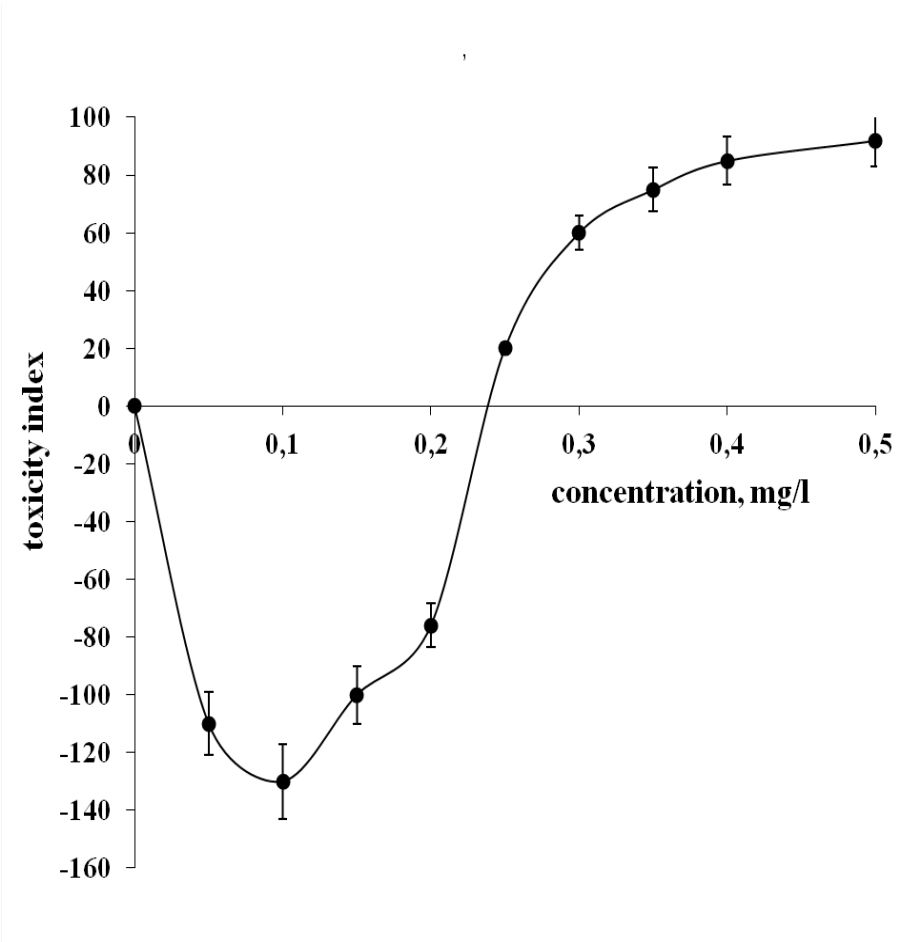

Fig. 2 Dependence of the toxicity index of unwashed cells on the concentration of silver ions at 30 min exposure.

Unwashed cells at 2.0×10^8 cells/ml concentration were analyzed. 100 µl cell suspension was mixed with 900 µl silver nitrate solutions at different concentrations, as indicated. Data represent mean ± SD from four measurements. One of three independent experiments, all with similar results, is shown.

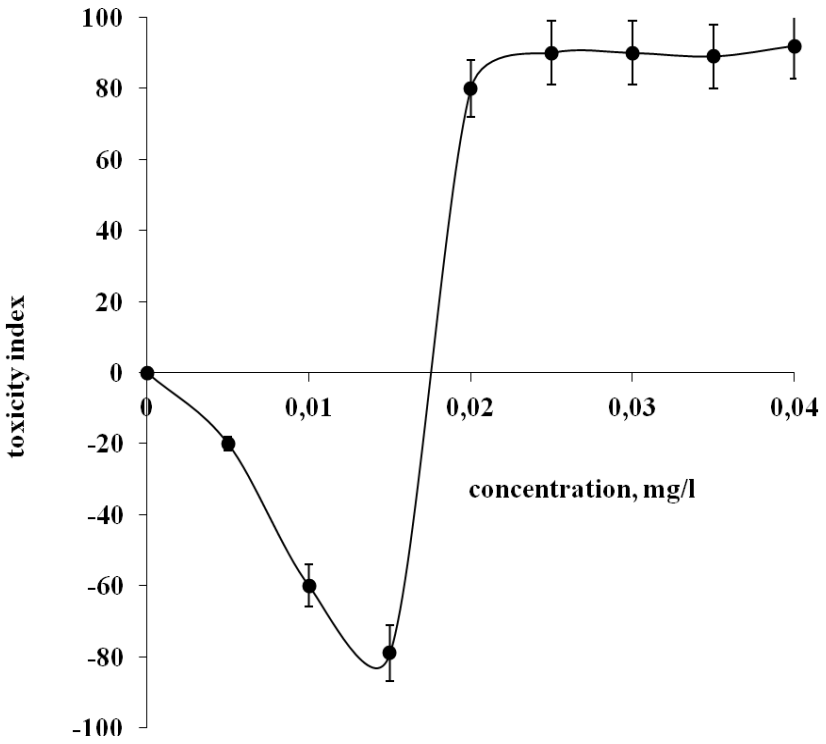

Fig. 3 Dependence of the toxicity index of pre-washed cells on the concentration of silver ions at 30 min exposure.

Cells were pre-washed to get rid of lyophilization medium as described in Materials and Methods and analyzed as described in Figure 2 legend at 1.5×10^8 cells/ml concentration. Data represent mean ± SD from four measurements. One of three independent experiments, all with similar results, is shown.

Interestingly, at certain low concentrations of silver ions negative values of the toxicity index relatively to the control samples were observed (Figs. 1-3). One possibility is that at low concentrations, silver ions primarily react with and competitively inhibit the respiratory chain of electron transfer. Due to a competition between respiratory and luminescence systems [3], the flux of reduced equivalents is increased in the luminescence chain and, consequently, luminescence is increased in test samples relatively to the control, resulting in negative values of the toxicity index. Indeed, the ability of Ag^+ to inhibit respiratory chain of *E. coli TG1* at several sites has been previously reported [17]. Furthermore, at higher silver ions concentrations, other targets are probably affected, including the luminescence chain itself. Thus, within a narrow range of concentrations, the toxicity index reaches the maximum value of 100.

Finally, to determine the specificity of the effect of silver ions on the bacteria, we performed so called chronic experiments, whereby bacteria were grown in the presence of silver ions for up to 24 hours. We found that all Ag^+ concentrations applied (from 0.015 mg/l to 0.12 mg/l) were toxic, at least from 2 to 18 hours of incubation with silver ions (Fig. 4). The most evident changes of the toxicity index occurred within 10 hours of cell growth and a clear dependence on silver concentration was detected. The minimal reliably determinable silver toxic level calculated from Γ-function (EC_{10}) was 0.0072 mg/l, the concentration when the sample was considered toxic (EC_{20}) was 0.0091 mg/l and EC_{50} was 0.013 mg/l. The apparent decrease of toxicity observed in the final stages of cell cultivation could be related to a deceleration in utilization of metabolites necessary for functioning of the fluorescence system due to the presence of Ag^+, as compared to control cells, or due to cell adaptation, but is presently unclear and needs further investigation.

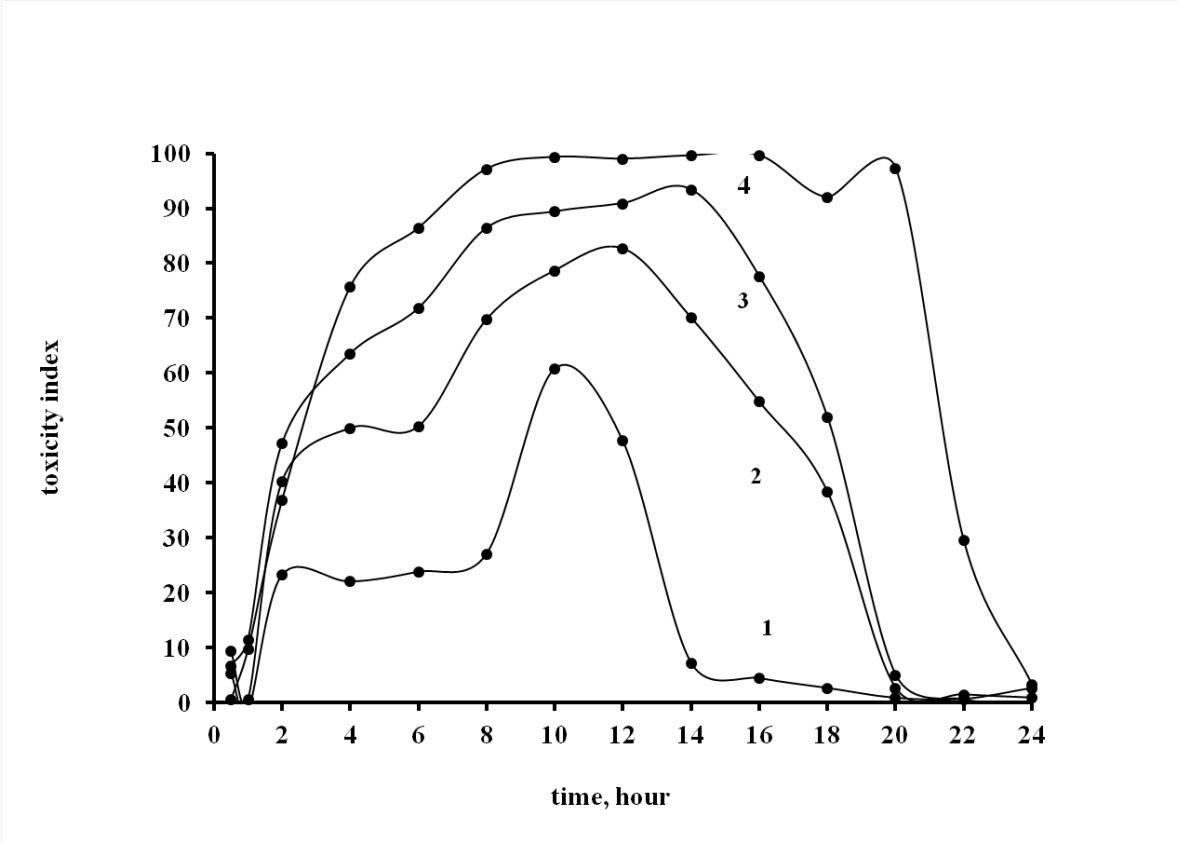

Fig. 4 Time-dependent toxicity of silver ions in the long-term toxicity assay. Cells *E. coli* were grown until mid-logarithmic phase in LB medium, followed by incubation at 10^4 cells/ml at 25°C for 24 hours with the following concentrations of silver ions: 0.015 mg/l (curve 1), 0.03 mg/l (curve 2), 0.06 mg/l (curve 3), 0.12 mg/l (curve 4). The measurements were done in at least the replicates, mean is shown. All errors did not exceed 10%.

In parallel to measuring the luminescence intensity, growth of bacteria was also measured in chronic experiments, beginning at 14 hours of cultivation when changes in bacterial growth were most evident (Fig. 5).

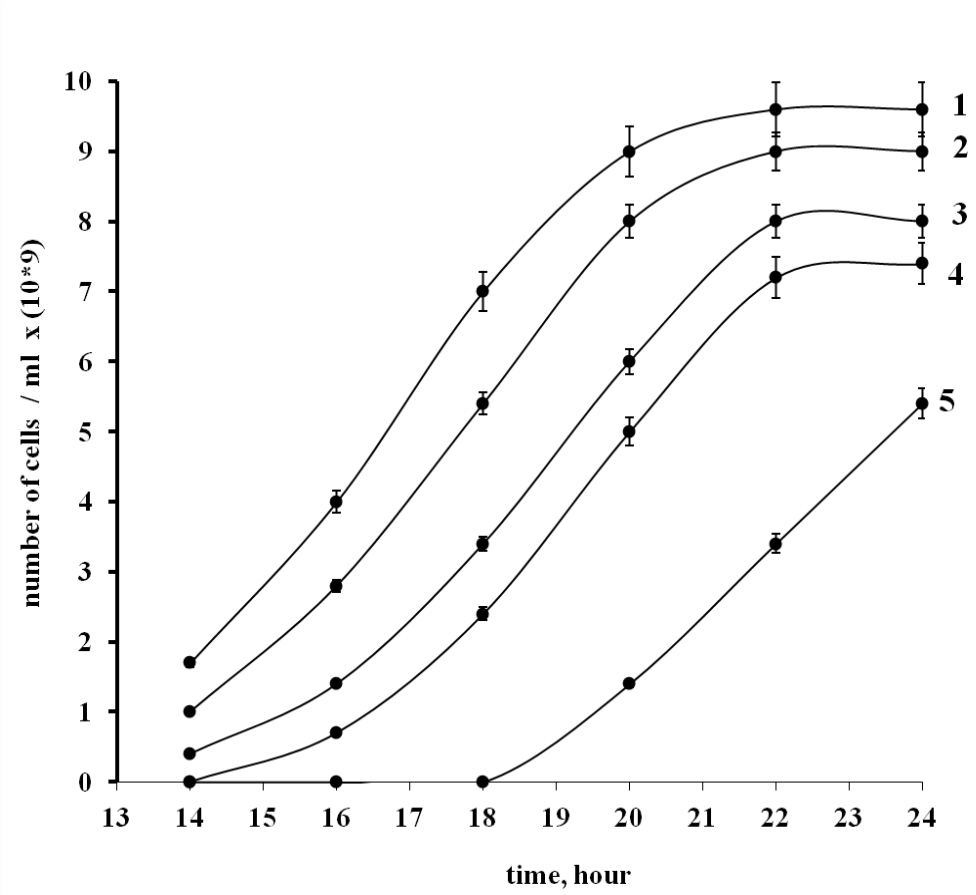

Fig. 5 The effects of different concentrations of silver ions on the growth of *E. coli* at 14-24 hours of culture in LB medium.

Cells *E. coli* were prepared as described in Figure 4 legend, using the following concentrations of $AgNO_3$: no $AgNO_3$ (curve 1), 0.015 mg/l (curve 2), 0.03 mg/l (curve 3), 0.06 mg/l (curve 4), 0.12 mg/l (curve 5). Cell number was measured from 14 to 24 hours every two hours as described in Materials and Methods. Data represent the means of four independent measurements. One of three independent experiments, all with similar results, is shown.

Cell growth was found to be sensitive to all silver ion concentrations tested. Thus, at the lowest silver concentration (0.015 mg/l) cell number comprised 93.7% of the control, whereas at the highest silver concentration (0.12 mg/l) it was 56.2% of the control. Of note, the maximum suppression of luminescence and of growth required different amount of time. Thus, the maximum inhibition of growth was achieved after 22-24 hrs, whereas the maximum effect on the toxicity index required only 10 hours. Nonetheless, growth inhibition and the toxicity index correlated in respect to Ag^+ concentration, so that the greater the inhibition of growth, the higher the toxicity index. This suggests that silver ions have specific effects on the bacteria, and these effects can be multifarious.

The bacterial luminescence tests on many chemicals, including antibiotics, have revealed their toxicity in chronic experiments. Moreover, their sensitivity threshold may be up to a thousand times low than in 30 min experiments, which is explained by apparent specific activity of antibiotics revealed during active bacterial growth [6, 18]. In case of silver ions, this difference in Ag^+ concentration is about 20 times: EC_{50} = 0.27 mg/l for unwashed cells in 30 min experiments, EC_{50} = 0.014 mg/l in a chronic experiment in the presence of 5 g/l NaCl in LB medium, when the same 10^8 cells/ml concentration of cells was used. Remarkably, in chronic experiments, the EC_{50} for luminescence was much less than the silver ions concentration required for 50% inhibition of cell growth (about 0.12 mg/l).

Our experiments demonstrate that silver ions rapidly penetrate into cells at very low concentrations (0.002 mg/l for bacteria washed from medium), and within 30 minutes complete

extinction of luminescence occurs, which indicates very high level of toxicity. This short time is only sufficient to reveal so-called non-specific activity [6], which is very high in case of silver ions.

In the course of chronic experiment, even in the presence of 5 g/l NaCl in the medium, silver ions likely strike additional targets associated with specific activity, such as protein biosynthesis and functioning of the genetic apparatus, leading to a prolonged increase in the toxicity index and a decrease in the growth rate of bacteria. Indeed, it is known that silver ions react with SH-groups of vital proteins and enzymes, with DNA, distort ionic balance, inhibit the absorption of phosphate, block the respiratory system and electron transfer, and strike many other targets [3, 17, 18, 19].

In **conclusion**, our study has demonstrated that chronic bacterial bioluminescent test is a promising method for fast determination of relative toxicity of metals and other pollutants. Moreover, this rapid screening method is a relatively inexpensive alternative to *in vivo* bioassays on higher organisms and can be used for risk assessment of various chemical agents and their mixtures. In combination with the standard 30 min bioluminescence assay, the growth inhibition assay provides a more reliable toxicity test that can be used to evaluate substances with delayed toxicity.

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Исследование токсичности ионов серебра в кратковременном и хроническом опытах на биолюминесцентном рекомбинантном штамме E. Coli

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Аннотация. Исследовали действие ионов серебра на бактерии рекомбинантного люминесцентного штамма Escherichia coli с опероном luxCDABE из Vibrio fischeri. Эксперименты проводили, определяя как токсичность в 30-минутном опыте, так и в хроническом 24-часовом варианте. По изменению интенсивности биолюминесценции и роста клеток в присутствие ионов серебра судили об их токсичности в исследуемом интервале концентраций. Показано, что действие ионов серебра сильно зависит от концентрации бактерий и среды инкубации. Величины EC50 составляли 0,018 мг/л при 30 минутной экспозиции и 0,014 мг/л на 10 часов роста бактерий. Сопоставление данных двух опытов указывают на то, что ионы серебра обладают как сильной неспецифической токсичностью, так и специфическим действием на бактериальные клетки.

Ключевые слова: Ионы серебра; бактериальных тест люминесценции; E. Coli; токсичности; рост бактерий.