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PHYSIOLOGICAL AND BIOCHEMICAL MECHANISMS OF CONTACT INTERACTION OF NANOPARTICLES OF GOLD WITH *BACILLUS ANTHRACIS* VACCINE STRAIN *STERNE* 34F2 CELLS

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Summary. The article presents the results of experimental studies of mechanisms of Aurum nanoparticles contact interaction on *Bacillus anthracis* vaccine strain.

This research was aimed to study the physiological and biochemical mechanisms of influence of Aurum nanoparticles (AuNP) on *B. anthracis* cells (strain *Sterne* 34F2 — productive vaccine strain).

The cell cultures biomass of *B. anthracis* (strain *Sterne* 34F2) was used as a model for our experiments. The sterile aqueous dispersion of Aurum nanoparticles with average size 19.0±0.9 nm and initial concentration of 19.3 μg/cm^3 for the metal was used for accumulation of *B. anthracis* (strain *Sterne* 34F2) biomass and with average size of 30.0±0.6 nm in initial concentration of 38.6 μg/cm^3 and 77.2 μg/cm^3 by the metal — in experiments on the study of physiological and biochemical mechanisms of interaction of cells. The effectiveness of the interaction of *B. anthracis* cells (strain *Sterne* 34F2) with AuNP was evaluated by a membrane filter method with a followed by determination of optical density changes of mixture on a spectrophotometer. The value of H^+-ATPase activity (KF 3.6.3.6) in total membrane fraction (TMF) bacterial cells was recorded by the accumulation of inorganic phosphorus (P_i). Respiratory activity (RA) of *B. anthracis* was measured by oxygen of electrode Clarks type. Measured parameter was the maximum speed of reducing the concentration of oxygen in the environment measurement, reduced to a unit of bacterial biomass.

According to results of the impact of nanoparticles of Aurum (gold, AuNP) for basic the physiological and biochemical indicators of *B. anthracis* cells (strain *Sterne* 34F2) appeared promising metal nanoparticles in a concentration range 2.90–8.69 μg/cm^3 for metal, as indicated by the presence of ATPase and respiratory activity stimulation and is consistent with the highest accumulation of cells research AuNP along with the intensification of proliferative activity of *B. anthracis* cells.

The mechanisms of gold accumulation in *B. anthracis* cells have the metabolism-dependent nature which is characteristic only active metabolized cells, and desplayed within our experiment involving certain assets metabolism (determining role of the transmembrane potential and its generators — ATPase, respiratory activity (RA)) in the overall regulation of the metabolic system and functional organization (proliferation activity) of the bacterial cells.

Keywords: *Bacillus anthracis*, bacterial cell, Aurum (gold) nanoparticles, contact interaction, proliferative activity of H^+-ATPase activity, respiratory activity

Introduction. The problem of epidemic and epizootic wealth regarding Anthrax in Ukraine belongs to the list of national priorities in order to provide state measures for of animal and people health protection from the especially dangerous diseases.

Anthrax is the multispecies zoonotic disease of the agricultural, domestic and wild animals and humans. Once it has been emerged in a particular area it can take root, keeping the threat of outbreaks in decades (Ipatenko, 1996).

The causative agent of Anthrax — *Bacillus anthracis* — is one of the aerobic spore-forming bacteria. Under favorable conditions (ambient temperature not below 12°C) pathogen can form spores. In spore form it may survive in the soil, remaining viable and maintaining pathogenicity long time. The soil that contaminated by Anthrax spores, could be the possible source of infection for susceptible animals or humans for 70 years and more.

The main source of Anthrax agent are the sick animals that spread the pathogen to the environment with urine, faeces, milk, bloody discharge even before the clinical signs of the disease.

There are thousands of unsatisfactory points for Anthrax where over the years there were outbreaks of the disease today in Ukraine.

Current scientific views on the issue of anthrax changed its vector – the causative agent of Anthrax

ranks the first in the list of agents applied to bioterrorism (Barth et al., 2004; Turnbull, 2008).

The leading role in the prevention of Anthrax occupies vaccination, that should be applied in all susceptible animals. Only in Ukraine, according to current statistics, the annual amount of using vaccines is 30–40 million doses. Currently, national manufacturers of veterinary immunological means not fully satisfy the existing effectively level of vaccine against Anthrax in animals, that encourages to the search for ways to improve existing preparations, particularly through the using of nanoparticles of metals in vaccine production biotechnology.

Among the variety of the existing nanoparticles of metals a special attention attracted nanoparticles of Aurum (gold) (Chen, Mwakwari and Oyelere, 2008). It causes a great interest perspectives for usage of the metal nanoparticles in technology of design of highly efficient diagnostic tools and targeted to prevention and treatment means and measures (Fu et al., 2005; Hainfeld et al., 2006; Cai et al., 2007), the attention of researchers mainly focused on the study of the biological effects of exposure nanogold at the cellular level (Feng, Tianjun and Li, 2008; Zhang et al., 2009).

Study of the impact of Aurum nanoparticles on proliferative activity and immunogenicity of bacterial cells of different taxonomic classes, including *B. anthracis*, made earlier (Dibkova et al., 2009; Ushkalov et al., 2011a, 2011b; Roman'ko, 2012; Roman'ko, Machusskiy and Ushkalov, 2013) indicates the possibility of controlling and managing the intensity of physiological and biochemical responses of bacterial cells as a result of metal nanoparticles of a certain size and concentration, their safety for microbial cells that introduction vaccine preparation of nanoparticles gold can contribute significantly increase its immunogenicity and economic efficiency of the production process.

However, the identified high biological activity of Aurum nanoparticles demonstrates the need for a detailed study of the mechanisms of their effects on *B. anthracis* cells from the *Sterne* 34F2 strain. The study of the interaction of bacterial cells with colloidal particles of gold demonstrates the significant role of them in colloid-chemical aspects of this process (De Roe, Courtoy and Baudhuin, 1987; Olsen and Bernstein, 1989). It has been proved the dependence of cytotoxic, immunotoxic, genotoxic, mutagenic and other negative effects of Aurum nanoparticles from the form, concentration and particle size. The a correlation between the established nature of the listed effects and biological compatibility, reduction of toxic membrane-alternating products of lipid and protein oxidation in biomembranes of prokaryotic cells (lipid peroxidation products and nitrites derivatives OMP), lack of inhibition of inflammatory cytokines TNF-α and IL1-β where described. They convincingly indicate the possibility of nanosized colloidal gold using to create the therapeutic and immunological means (Paciotti et al., 2004; Shukla et al., 2005; Dybkova et al., 2009; Dybkova, 2010; Roman'ko et al., 2010; Roman'ko, 2010). The consensus on the mechanisms of action of biocompatibility and bioavailability of nanoparticles of gold on the microbial cell was not formed today. So the safety or toxicity of nanometals for certain groups of organisms, and a prerequisite for the development and implementation of any biotechnological processes were not determined.

The purpose of this work was to study the physiological and biochemical mechanisms of influence of Aurum nanoparticles on *B. anthracis* cells (strain *Sterne* 34F2 – productive vaccine strain).

Materials and methods. The research were carried out on the model experiments using cell cultures biomass of *B. anthracis* (strain *Sterne* 34F2) from the collection of the National Center of Microorganism Strains in State Scientific Control Institute of Biotechnology and Strains of Microorganisms (Kyiv, Ukraine).

The sterile aqueous dispersion of gold nanoparticles weve synthesized by chemical condensation in an water solution, using the original protocol, developed at the F. D. Ovcharenko Institute of Biocolloidal Chemistry of NAS of Ukraine (Kyiv, Ukraine). Notation conventions of Aurum nanoparticles used in the work is AuNP.

Nanoparticles were synthesized by restoring the Aurum hydrochloric acid ($HAuCl_4$) by sodium citrate in the presence of potassium carbonate. Used nanoparticles of gold had spherical geometry.

Study of the size and form of gold nanoparticles were performed by laser-correlation spectrometry (LCS) and transmission electron microscopy (TEM). Measurements were performed on laser correlation spectrometer 'Zetasizer-3' (Malvern Instruments Ltd., UK). Visualization of prototypes nanoparticle metal was performed by transmission electron microscopy with an electron microscope 'JEOL JEM-1230' (Tokyo Boeki Ltd., Japan). Monodisperse LCS method allows to determine accurately the constant rate of particle diffusion and calculate their hydrodynamic diameter, based on the assumption of spherical geometry of the particles (Rawle, 1994).

Were used (accumulation of biomass of *B. anthracis* (strain *Sterne* 34F2)) AuNP water dispersion medium size 19.0±0.9 nm with initial concentration of 19.3 μg/cm^3 for the metal. In experiments on the study of physiological and biochemical mechanisms of interaction of cells strain *Sterne* 34F2 *B. anthracis* – were used AuNP with average size of 30.0±0.6 nm in initial concentration of 38.6 μg/cm^3 and 77.2 μg/cm^3 by the metal, respectively (Fig. 1, 2A, 2B).

For culturing of the cells and *B. anthracis* biomass accumulation standard commercial environment hydrolyzate medium of fish meal and Hottynheris medium (pH 7.2–7.6) was used (as a source of nitrogen). Amino nitrogen 100–120 mg%, with added by aseptic conditions gold dispersion of nanoparticles average size of ~19 nm in the ratio 1:2, 1:4, 1:6, 1:8, 1:10, 1:12 and 1:20 respectively has been used. The sterilization of prepared culture medium was performed by autoclaving at 0.7 atm. Later it was maintained at a temperature 37±1°C for 48 hours for the purpose of controlling sterility.

Prepared medium groming capacity was studied by adding to 1 cm^3 of medium the culture of *B. anthracis* with a known concentration. It was cultivated at a temperature 37±1°C for 20–24 hours.

As a 'control' standard environment-commercial hydrolyzate medium and fish meal Hottynhes's medium (pH 7.2–7.6) and amino nitrogen (100–120 mg%) without adding AuNP were used.

The effectiveness of the interaction of bacterial *B. anthracis* cells (strain *Sterne* 34F2) with AuNP average size of ~30 nm membrane filter method was evaluated by determining of optical density changes of mixture, containing metal nanoparticles and bacterial cells filtrates, received using membrane filters with pore diameter 0.15–0.17 mm. Optical density values were recorded for λ 520–550 nm (absorption maximum AuNP different sizes) spectrophotometer ('SHIMADZU UV-1800', Japan).

Defining features of the interaction was conducted under conditions of constant final concentration of bacterial cells in a range of dispersion AuNP dilution in the culture medium — 1.1, 2.8, 5.5, 8.3, 11.0, 16.5, 22.0 μg/cm^3 for the metal, respectively.

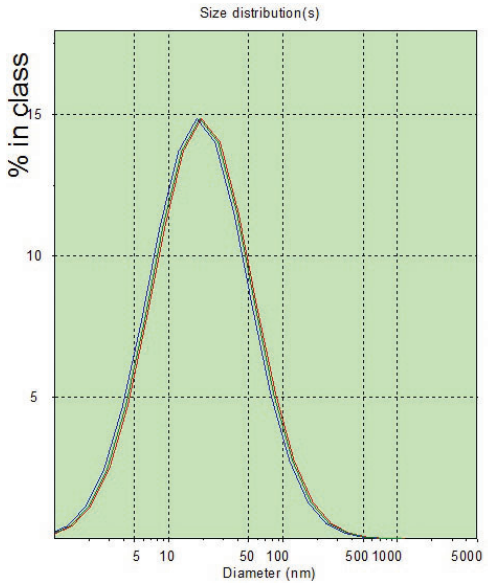
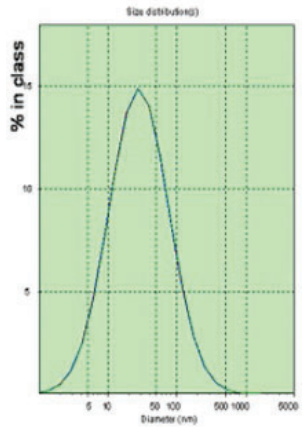


Figure 1. The average size AuNP, (19.0±0.9 nm) (according LCS)



Run	Angle	KCps.	ZAve	Poly	Fit	Time
1	90.0	219.0	30.1	1.000	0.000928	16:29:28
2	90.0	218.9	29.2	1.000	0.000754	16:33:45
3	90.0	220.5	30.3	1.000	0.000826	16:38:01
Average		219.5	**29.9**	1.000		
+/-		0.9	**0.6**	0.000		

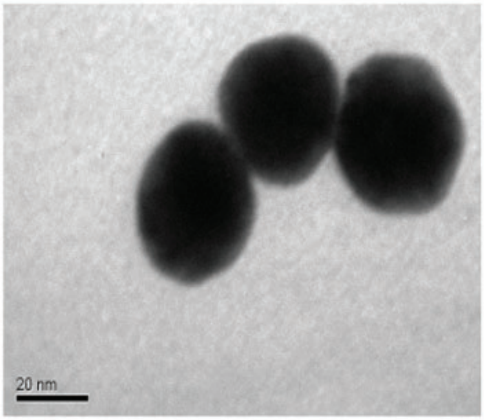


Figure 2. AuNP, (30.0±0.6 nm) properties (form and size): A — according to LCS, B — according to TEM

The quantyty of linked gold (%) was calculated using the formula $(1–D/D_0)\times100\%$, where D — optical density of AuNP filtrate after contact with bacterial cells, D_0 — density filtrate 'control' preparations AuNP. Cultivation of 'control' preparations AuNP was performed with distilled water, the amount of dispersion AuNP, which added to the contact mixture remained unchanged. Contact interaction time was 5 min.

The value of H^+-ATPase activity (KF 3.6.3.6) total membrane fraction (TMF) bacterial cells was recorded by the accumulation of inorganic phosphorus (P_i) concentrations in the environment is determined by Fiske-Subbarou method (Fiske and Subbarow, 1925). Testing has been performed by the incubation in medium with the following composition: 10 mM Tris-HCl, 3 mM $MgCl_2$, 3 mM ATP (pH 7.5). The duration of incubation was 10 min. Quantity of the membrane protein — 60–70 μg/cm^3. The reaction is initiated by the introduction of conditions for the incubation environment TMF aliquots of cells and stopped by adding 1 cm^3 10% solution TCAA.

AuNP dispersion was made in TMF medium and incubated for 3 min. The range of concentrations in the contact mixture AuNP — 1.10–23.16 μg/cm^3. The 'control' sample AuNP instead of adding 10 mM Tris-HCl buffer.

In all experiments, 'control' for non-enzymatic hydrolysis of ATP served incubation environment without it TMF. As the 'control' on endogenous phosphorus in the environment was used TMF, which included only the TMF in aqueous solution.

Respiratory activity (RA) of *B. anthracis* was measured by oxygen of electrode Clarks type (MO128, Mettler Toledo, Switzerland). Measured parameter was the maximum speed of reducing the concentration of oxygen in the environment measurement, reduced to a unit of biomass used bacterial strains (specific RA).

The speed of reducing the concentration of oxygen in the environment measurement AuNP dispersion have been tested in with incubating bacterial cells. The range of concentrations in the contact mixture AuNP — 0.39–7.70 μg/cm^3 for the metal. The 'control' sample was added instead AuNP 5 mM Tris-HCl buffer.

ATP-hydroxymethyl aminometan has been used in the study manufactured by 'Gibco RBL', Scotland, other reagents — by domestic production qualification 'analytical grade'.

The calculation the average values has been done in at least 3 repeats in a series of 5 independent experiments. Statistical analysis of the results was carried out in accordance with common requirements (Lakin, 1990) using Student's test ($P<0.05$).

Results and discussion. Previous research found, that the adding of dispersions AuNP (average size ~19 nm) to cultivation nutrient medium for *B. anthracis* (strain *Sterne* 34F2) increased the cells proliferative activity and the level of biomass accumulation of germs.

Thus, when cultured in the commercial standard environment ('control') increment of biomass *B. anthracis* experimental strain accounted for no more than 10^9 colony forming units (CFU/cm^3), whereas for terms of adding to the environment AuNP level biomass accumulation anthrax increased and reached by value in 10^{15} CFU/cm^3, respectively.

It was established that the maximum increasing of the level of biomass growth of bacterial cells has been observed in the culture medium containing AuNP in dilution of 1:8 (concentration gold $1{,}90\times10^{-3}$ g/l) and 1:10 (concentration gold $2{,}38\times10^{-3}$ g/l), recorded to a dilution of culture *B. anthracis* 10^{-15} inclusive. Increasing of biomass of anthrax meaningfully CFU at 2 and 6 logarithms exceeded in a 'control'.

When cultured microbial mass ranging from 10^{-10} dilution in the medium containing AuNP range of concentrations (1:2–1:6), 1:12, and 1:20, as well as in the standard nutrient medium ('control') recorded growth retardation of *B. anthracis* by studied strain.

The research results have been protected by a declarative patent of Ukraine for a utility model (Holovko et al., 2011).

The Figure 3 shows the concentration curves of AuNP medium size ~30 nm in intact (Fig. 3, 1) and thermo inactivated (Fig. 3, 2) cells of *B. anthracis* (strain *Sterne* 34F2).

The data show that in the range of low concentrations AuNP (1.1–5.5 μg/cm^3 for the metal) weak accumulation of metal nanoparticles bacterial cells: the number of linked gold does not average exceed 50% observed (Fig. 3, 1).

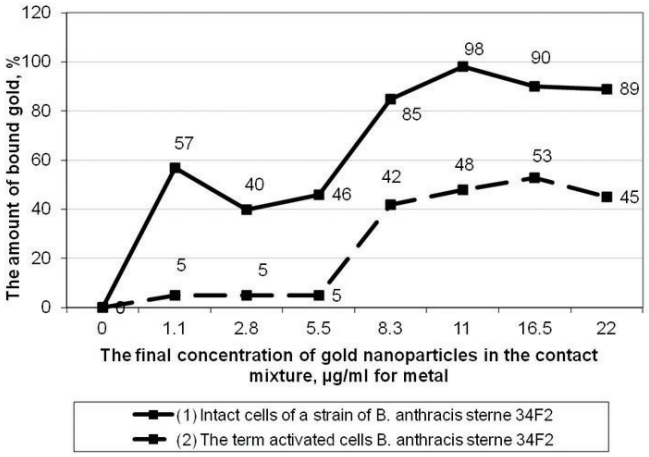


Figure 3. The concentration of binding curves AuNP (average size of ~30 nm) intact (Fig. 3, 1) and thermoinactivated (Fig. 3, 2) cells *B. anthracis* (strain of *Sterne* 34F2)

Efficiency of the AuNP interactions with *B. anthracis* cells significantly increased in concentrations of nanoparticles range (8.3–22.0 μg/cm^3 for the metal). Thus, the quantity number of Aurum bound by of Anthrax cells in the concentration range was averaged 90%.

Maximum accumulation of the AuNP by bacterial cells (98%) has been observed in the final concentration of nanoparticles 11.0 μg/cm^3 (initial concentration dispersions AuNP — 38.6 μg/cm^3 of the metal).

Inactivation of bacterial cells *B. anthracis* (strain *Sterne* 34F2) led to a drop in the efficiency of AuNP binding on average 40–50% (Fig. 3, 2). Thus, accumulation AuNP thermoinactivated cells in the range of low concentrations (1.1–5.5 μg/cm^3 for the metal) was virtually absent. The level of binding of AuNP by anthrax cells in a concentration range 8.3–22.0 μg/cm^3 for the metal was not exceed 50%.

Consequently, studies indicate that the mechanisms of contact interaction AuNP with average size ~30 nm

with of bacterial cells of *B. anthracis* strain research can be defined as passive localization by electrostatic, coordination and other types of connection (especially the structural organization of the cell surface), so and metabolism-dependent accumulation that is characteristic only active metabolized cells (determining role transmembrane potential and its generators).

The hypothesis about way do determine the basic mechanisms of the interaction of Aurum nanoparticles of *B. anthracis*, agreement with the literature (Ipatenko, 1996). So, at prompting the typical properties of *B. anthracis* and *B. cereus*, that was studied by the methods of electron microscopy indicated that *B. anthracis* cells had a thick cell membrane and more advanced membrane structure, due to increased activity of oxidation-reduction enzymes.

The presence of the folded structure of the membrane, increasing of the membrane enzymes, including H^+-ATPase, and the earlier data demonstrated stimulation of proliferative activity of *B. anthracis* (*Sterne* 34F2 strain) under the AuNP influence, suggest that the predominant mechanism of interaction with nanoparticles of tested strain is metabolism-dependent accumulation. It plays a decisive role for functioning transmembrane potential bacterial cell.

H^+-ATPase is the main generator of transmembrane potential bacterial cells. The H^+-ATPase activity of TMF cells and respiratory activity of intact of *B. anthracis* (*Sterne* 34F2 strain) cells under the influence AuNP were studied.

Figure 4 demonstrates the graph changes H^+-ATPase activity in TMF cells under the influence of AuNP average size ~30 nm to *B. anthracis* cells in the range of concentrations, compared with control values.

The most pronounced effect on the H^+-ATPase activity (by increasing its activity to 30%) TMF-growed bacterial cells comparing to control, was observed under the influence of AuNP in concentration 8.69 μg/cm^3 for the metal.

The gold nanoparticles the range of concentrations 2.90–23.16 μg/cm^3 in contact interaction with TMF-growed cells of strain *B. anthracis* has been commitment inhibition with of ATPase. Its values in overall significantly increased relative to the 'control', which indicated no deceleration rate of ATP hydrolysis in the membranes of bacterial cells.

In addition, high level of bacterial cells energy of has been the regulatory factor in their ability for active accumulation of metals nanoparticles (Danilovich et al., 2007), and also consistent with the hypothesis put forward by us regarding the mechanisms of metabolism-dependent accumulation of nanoparticles gold in *B. anthracis* cells.

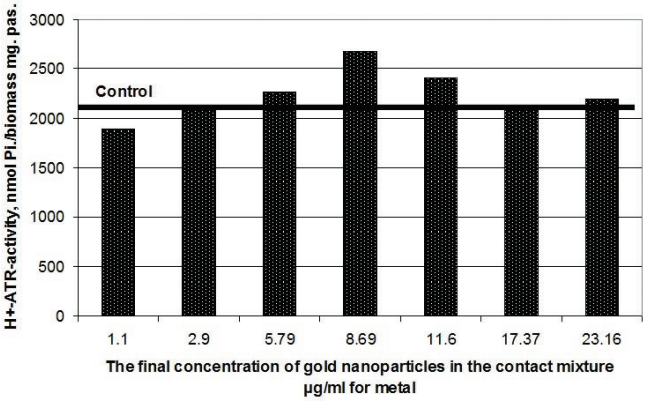


Figure 4. Changes in the level of H^+-ATPase activity TMF of bacterial cells *B. anthracis* (strain of *Sterne* 34F2) under the influence of AuNP (average size of ~ 30 nm) in the range of concentrations

Since respiratory activity is the major physiological reaction of microorganisms (Basnak'yan, Borovkova and Kuz'min, 1981), it could be used as an integral criteria for assessing the stability of the biological potential of the bacterial cell.

However, AuNP addition to the incubation medium led to a significant stimulation of the respiratory activity of the *B. anthracis* cells (Fig. 5).

It was proved that as a result of adding AuNP (average size ~30 nm) in the concentration range 0.39–7.70 μg/cm^3 for the metal to the incubation medium bacterial cells of *B. anthracis* (strain *Sterne* 34F2) led the increasion of RA on average by 74% compared with 'control'.

This concentration dependence for AuNP impact on the value of RA is not installed.

As a result of the RA study during incubation of concentration range of *B. anthracis* cells with AuNP demonstrated the safety of nanoparticles for the physiological status of Anthrax bacterial cells.

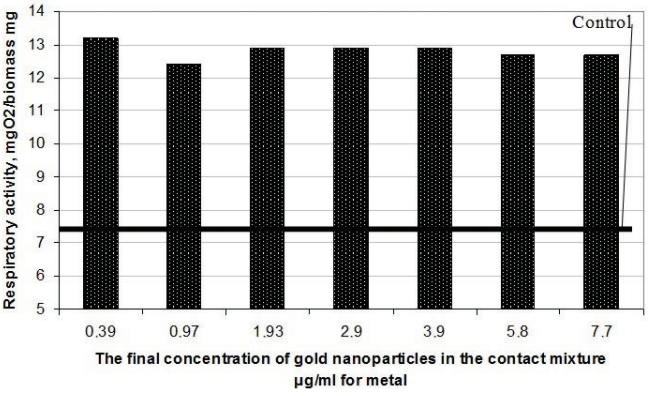


Figure 5. Changes in the level of respiratory activity of *B. anthracis* cells (strain of *Sterne* 34F2) under the influence of AuNP (average size of ~30 nm) in the range of concentrations

Conclusion. According to results of the study impact of sold nanoparticles of basic physiological and biochemical indicators of *B. anthracis* cells (strain of *Sterne* 34F2) it was appeared promising metal nanoparticles in a concentration range 2.90–8.69 μg/cm^3 for metal, as indicated by the presence of ATPase stimulation and respiratory with activity. This is also consistent with the highest accumulation of cells research AuNP along with the intensification of cell proliferative activity of agent cells.

Thus, the results indicate AuNP strong biological activity, their biocompatibility and bioavailability of the conditions of contact interaction of bacterial cells of *B. anthracis* (strain *Sterne* 34F2). On the other hand — along with the possible presence of a passive localization, mechanisms of gold accumulation in *B. anthracis* cells with metabolism-dependent nature has been deserited. It demonstrates peculiar only active metabolized cells, and reflectes within our experiment involving certain assets metabolism (determining role transmembrane potential and its generators — ATPase, RA) in the overall regulation of metabolic and functional organization systems (proliferation activity) of bacterial cells.

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