Оригинальные исследования **Original researches**

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VIRUCIDAL AND BACTERICIDAL EFFECTS OF ELECTROCHEMICALLY ACTIVATED ANOLYTE AND CATHOLYTE TYPES OF WATER ON CLASSICAL SWINE FEVER VIRUS AND BACTERIUM *E. COLI DH5*

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Introduction

The phenomenon of electrochemical activation of water (EAW) is a set of electrochemical and electrical processes occur in water in the electric double layer (EDL) type of electrodes (anode and cathode) with non-equilibrium electric charge transfer through EDL by electrons under the intensive dispersion in water the gaseous products of electrochemical reactions [1]. In 1985 EAW was officially recognized as a new class of physical and chemical phenomena.

As a result of the treatment of water by a constant electric current at electric potentials equal to or greater than the decomposition potential of water (+1,25 V), water goes into a metastable state, accompanied by electrochemical processes and characterized by the abnormal activity levels of electrons, the redox potential, and other physical-chemical parameters (pH, E_h, ORP) [2].

The main stage of electrochemical treatment of water is the electrolysis of water or aqueous solutions with low mineralization as aqueous solutions of 0,5–1,0 % sodium chloride (NaCl) [3], which occurs in the electrolysis cell, consisting of the cathode and the anode separated by a special semipermeable membrane (diaphragm) which separates water to alkaline fraction – the catholyte and acidic fraction – the anolyte (Fig. 1). When the passing of electric current through water, the flow of electrons from cathode as well as the removal of electrons from water at the anode, is accompanied by series of redox reactions on the surface of the cathode and anode [4]. As the result, new elements are being formed, the system of intermolecular interactions, as well as the composition of water and the water structure are changed [5, 6].

The products of electrode reactions are the neutralized aqueous admixtures, gaseous hydrogen and oxygen generated during the electrolytic destruction of H_2O molecules, metal cations (Al^{3+}, Fe^{2+}, Fe^{3+}) in the case of metal anodes made of aluminum and steel, the molecular chlorine. Wherein at the cathode is generated the gaseous hydrogen, and at the anode – oxygen. Water also containes a certain amount of hydronium ions (H_3O^+) depolarizing at the cathode with formation of the atomic hydrogen:

$$H_3O^+ + e^- \rightarrow H + H_2O, \quad (1)$$

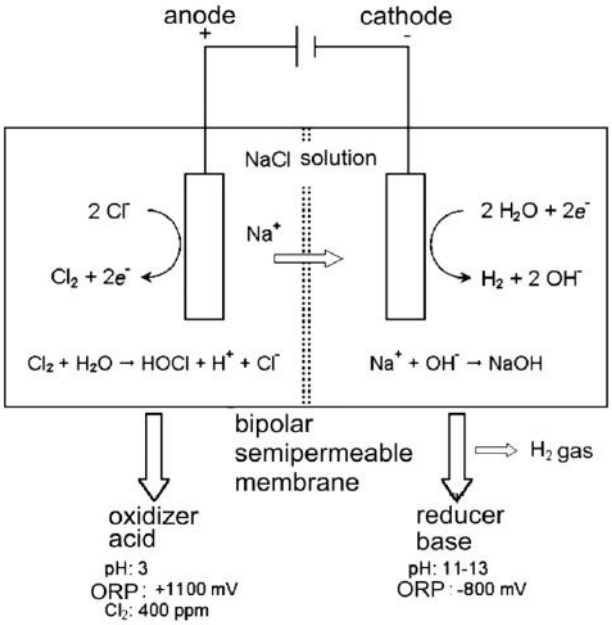

Fig. 1. The diaphragm electrolysis method for the preparation of acid (anolyte) and alkali (catholyte) solutions via the electrochemical activation of sodium chloride

In an alkaline environment there occurs the disruption of H_2O molecules, accompanied by formation of the atomic hydrogen and hydroxide ion (OH^-):

$$H_2O + e^- \rightarrow H + OH^-, \quad (2)$$

The reactive hydrogen atoms are adsorbed on the surfaces of the cathode, and after recombination are formed the molecular hydrogen H_2, released in the gaseous form:

$$H + H \rightarrow H_2, \quad (3)$$

At the same time at the anode is released the atomic oxygen. In an acidic environment, this process is accompanied by the destruction of H_2O molecules:

$$2H_2O - 4e^- \rightarrow O_2 + 4H^+, \quad (4)$$

In an alkaline environment, the source of oxygen source is OH^- ions, moving under the electrophoresis from the cathode to the anode:

$$4OH^- \rightarrow O_2 + 2H_2O + 4e^-, \quad (5)$$

The normal redox potentials of these reactions compiles +1,23 V and +0,403 V, respectively, but the process takes place in certain conditions of electric overload.

The cathodes are made of metals that require high electrical voltage (lead, cadmium), allow to generate the reactive free radicals as Cl*, O*, OH*, HO_2*, which react chemically with other radicals and ions.

In bulk oxidative processes a special role plays products of electrolysis of water – oxygen (O_2), hydrogen peroxide (H_2O) and hydrochlorine acid (HClO). During the electrolysis, an extremely reactive compound formed – H_2O_2, the formation of which occurs due to the hydroxyl radicals (OH*), which are the products of the discharge of hydroxyl ions (OH^-) at the anode:

$$2OH^- \rightarrow 2OH^* \rightarrow H_2O_2 + 2e^-, \quad (6)$$

where OH^* – the hydroxyl radical.

The chlorine-anion is transformed to Cl_2:

$$2Cl^- \rightarrow Cl_2 + 2e^-, \quad (7)$$

Gaseous Cl_2 forms highly active oxidants: Cl_2O; ClO_2; ClO^-; HClO; Cl*; HO_2*. The parameters of pH, the redox potential, ORP and the electrical conductivity of the anolyte/catholyte depend on different factors including the ratio of water volumes in the two electric chambers, the material of electrodes, NaCl concentration, the temperature, electric voltage and processing time [7,8].

The electrolysis cell can be regarded as a generator of the above mentioned products, some of them, entering into the chemical interaction with each other and water impurities in the interelectrode space, providing additional chemical treatment of water (electrophoresis, electroflotation, electrocoagulation) [9]. These secondary processes do not occur on the electrode surface, but in the bulk water. Therefore, in contrast to the electrode processes they are indicated as the volume processes. They generally are initiated with increasing the temperature of water during the electrolysis process and with increasing the pH value.

As a result of the cathode (catholyte) treatment water becomes alkaline: its ORP decreases, the surface tension is reduced, decreasing the amount of dissolved oxygen in water, increases the concentration of hydrogen, hydroxyl ions (OH^-), decreases the conductivity of water, changes the structure of hydration shells of ions [10]. By external characteristics the catholyte – is a soft, light, with an alkaline taste liquid, sometimes with white sediment; its pH = 10–11, ORP = -200…-800 mV.

On physical and chemical parameters the catholyte has the significantly enhanced electron-donating properties, and getting into the physiological fluids of an organism can enhance the electron-background for a few tens of millivolts [11]. The catholyte reportedly has antioxidant, immunostimulating, detoxifying properties, normalizing ORP, metabolic processes (increases the ATP synthesis, modification of enzyme activity), stimulates the regeneration of tissues, increases the DNA synthesis and stimulates the growth and division of cells by increasing the mass transfer of ions and molecules across the cell membrane, improves trophic processes in tissues and blood circulation [12]. It was also reported that catholyte with the redox potential at -700…-100 mV favorizes the development of anaerobs, whereas the anolyte with the redox potential at +200…+750 mV supports the growth of aerobs [13]. The antibacterial effect of the catholite is differentiated: the bactericidal effect is appeared relative to Enterobacteriaceae, resistant to it are enterococci and the group of streptococci B, and against Gram-negative microorganisms – only the bacteriostatic effect [14].

The electrochemically activated solutions of the catholite, depending on the strength of the transmitted electric current may be of several types:

C – alkaline catholyte (pH > 9,0; ORP = -700…-820 mV), the active components – NaOH, O_2, HO_2^-, HO_2*, OH^-, OH*, HO_2^-, O_2;

CN – neutral catholyte (pH = 9,o; ORP = -300…-500 mV), the active components – O_2, HO_2^-, HO_2*, H_2O_2, H^+, OH^-.

As a result of the anode (anolyte) treatment water becomes acid reaction, the ORP increases slightly, the surface tension is slightly reduced, the conductivity increases, the amount of the dissolved oxygen and chlorine in water also increases, whereas the amount of hydrogen decreases [15]. The anolyte is a brownish, acid, with a characteristic odor and taste the liquid with a pH = 4–5 and ORP = +500…+1100 mV. The specific anolyte toxicity when being administered in the stomach and applying to the skin refers to the class 4 of harmful substances according to the Russian Standard GOST 12.1.007-76, with the minimal toxicity within this class. When being inhaled the anolyte with oxidants content of 0,02 % and total mineralization 0,25–0,35 % does not irritate the respiratory system and mucous membranes of the eyes. When introduced into the organism, the anolyte has no immunotoxic action and increased chromosomal aberrations in the bone marrow cells and other tissues, and it has no cytogenetic activity. When being heated to 50 ^{0}C the bactericidal activity of the anolyte is increased by 30–100 % [16].

The electrochemically activated solutions of the anolyte are divided into four main types:

A – acidic anolyte (pH < 5,0; ORP = +800…+1200 mV), the active components – HClO, Cl_2, HCl, HO_2*;

AN – neutral anolyte (pH = 6,0; ORP = +600…+900 mV), the active components – HClO, O_3, HO^-, HO_2*;

ANK – neutral anolyte (pH = 7,7; ORP = +250…+800 mV), the active components – HClO, ClO^-, HO_2^-, H_2O_2, O_2, Cl^-, HO*;

ANKD – neutral anolyte (pH = 7,3; ORP = +700…+1100 mV), the active components – HClO, $HClO_2$, ClO^-, ClO_2*, HO_2*, H_2O_2, O_2, O_3, Cl^-, HO^-, O*.

The anolyte has antibacterial, antiviral, antifungal, antiallergic, anti-inflammatory, antiedematous and antipruritic effect, may be cytotoxic and antimetabolite action without harming the human tissue cells [17]. The biocide elements in the anolyte are not toxic to somatic cells, as represented by oxidants, such as those ones produced by the cells of higher organisms.

Studies on the virucidal effect of the anolyte are rare and insufficient, basically on the possibilities of applying the anolyte in the implementation of effective control of viral diseases in humans and animals and especially on particularly dangerous viral infections, as staphylococcal Enterotoxin-A [18]. One of them is the classical swine fever (CSF), prevalent

in different regions of the world and inflicting heavy economic losses. It is caused by enveloped viruses belonging to the genus *Pestivirus* of the family *Flaviviridae* [19, 20]. The resistance and inactivation of the virus of CSF virus is a subject of extensive research. Although it is less resistant to external stresses other than non-enveloped viruses, it retains its virulence for a long period of time: in frozen meat and organs – from a few months up to one year; in salted meat – up to three years; in dried body fluids and excreta – from 7 to 20 days. In rotting organs it dies for a few days and in urine and faeces – for approx. 1–2 days. In liquid fertilizer it can withstand 2 weeks at +20 ^{0}C, and over 6 weeks at +4 ^{0}C. Its thermal resistance may vary depending on the strain type, but the inactivation is dependent mostly on the medium containing the virus. Although the CSF virus loses its infectivity in cell cultures at +60 ^{0}C for 10 min, it is able to withstand at least 30 min at t = +68 ^{0}C in defibrinated blood. It is relatively stable at pH = 5–10, and the dynamic of the inactivating process below pH = 5 depends on the temperature.

According to J.A. Sands [21] and U.S. Springthorpe [22], the effective disinfection of viruses whose infectivity is associated with the elements of the casing is achieved by disinfectants dissolving fats, surfactants, disinfectants or fatty acids, organic solvents (ether and chloroform), detergents, proteases, and common disinfectants. It is believed that 2 % solution of sodium hydroxide is most suitable for the disinfection of spaces contaminated with them. It is thought that to achieve the effective electrochemical disinfection it is necessary to irreversibly damage the RNA [23].

Investigations conducted by other authors [24] were carried out with *E. coli*, using as a desinfectant the anolyte with ORP equal or greater than +1100 mV and pH = 5,5, obtained via electrolysis of diluted NaCl solution on planktonic cells of a strain of *E. coli JM109*. It was demonstrated that within 5 min of influence all cells were inflated and burst. Also, it was occurred a full destruction of proteins, DNA and RNA. Supposedly the anolyte enters the cells provoking structural and functional damages on the cell‘s membrane and cell‘s wall.

Similar research was performed by S.V. Kumar et al. [25]. They evaluated the inactivation efficacy of anolyte of pH = 2,7 and ORP = + 1100 mV on *Escherihia coli O157:H7*, *Salmonela enteritidis* and *Lusteria monocytogenes*. As it was demonstrated on five strains of *E. coli E06* (milk), E08 (meat), E10 (meat), E16 (meat) and E22 (calf feces), all patogens were significantly reduced (7,0 logCFU/ml) or fully destroied (8,0 logCFU/ml) after 2 to 10 min inactivation by the anolyte in the temperature range from +4 ^{0}C to +23 ^{0}C. Supposedly, the low pH value of the anolyte makes sensitive the outer cell‘s membrane, thus facilitating HClO to enter the cell and further destroy it.

However, it should be noted that the pharmacological studies of electrochemically activated solutions of water and their virucidal effects and toxicity have not yet been completely evaluated. Therefore, the purpose of this research was to study the antiviral virucidal effect: 1) of the anolyte in different dilutions on classical swine fever virus in cell culture and organ suspensions; 2) of the anolyte/catholyte on a strain of *E. coli DH5a*, and 3) to determine how the virocidal effect relates to local maximums in NES-spectra of the anolyte and catholyte[1].

Experimental Part

Material and Methods

The studies of the antiviral activity of the anolyte were performed at the National Reference Laboratory of Classical and African Swine Fever, section “Exotic and Especially Dangerous Infections” of the National Diagnostic and Research Veterinary Medical Institute (Sofia, Bulgaria). Experiments were conducted with the anolyte obtained by the electrolysis apparatus “Wasserionisierer Hybrid PWI 2100” equipped with four titanium electrodes coated with platinum. 0,3 % solution of chemically pure sodium chloride (NaCl) in distilled water was used for the electrolysis. The obtained anolyte had pH = 3,2 and ORP = +1070 mV. The interaction of the anolyte with the virus suspension was carried out at a temperature +22 ^{0}C.

A cell culture of porcine origin sensitive to the CSF virus was used: a continuous cell line was PK-15. Contamination of cell cultures was carried out with the standard cell culture test virus 2,3 (Bulgaria) with a cell titre 107,25 $TCID_{50}$/ml and organ suspension of internal organs (spleen, kidney, lymph node) of wild boar originating from the last outbreak of CSF in Bulgaria in 2009. The titer of the established virus in the suspension was $10^{4,75}$ $TCID_{50}$ ml.

To establish the virucidal activity of the anolyte, the inocula prepared for contamination of cell culture (cell culture virus) were treated with the following dilutions of the anolyte in sterile distilled water: 1:1 (50 %), 1:2 (33,33 %), 1:3 (25 %), 1:4 (20 %). These dilutions were mixed with inocula in proportion 1:1 (100 µl of the CSF virus suspension and 100 µl of the appropriate anolyte concentration). The time of action was conformed to the period, at which it was methodologically necessary to “capture” any viral presence in the cell culture. Upon the infection of a cell monolayer, the mixture was removed after the end of the exposure period of 1 h. Upon the infection of a cell suspension, the mixture, otherwise, was not removed.

To establish the virucidal activity of the anolyte on the CSF virus in the suspension, a different dilution was used: the inoculum was mixed directly with the concentrated anolyte in anolyte-inoculum ratios 1:1; 3:1; 7:1 and 15:1 respectively. Since it is known that the growth of the virus does not cause a cytopathic effect, therefore, for demonstration of its presence, immunoperoxidase plates dyeing were used. The cells were fixed and the viral antigen was detected after binding to a specific antibody labeled with peroxidase. The organs exude 1 cm^3 of tissue, which was homogenized in a mortar with 9 ml of the cell culture medium containing antibiotics, in order to obtain 10 % of organ suspension. Sterile sand was added to improve the homogenization. The samples were kept at room temperature for 1 h, after that they were

[1] Such a dependence was established between the local maximum (-0,1387 eV; 8,95 µm) in the NES-spectrum of the catholyte that suppresses the development of tumor cells (Ignatov & Mosin, 2014).

centrifuged for 15 min at 2500 g. The supernatant was used to infect the cells. In case of cytotoxic effect, the parallel dilutions of the homogenates were prepared in proportions 1:10 and 1:100. From the suspensions into multi well (24-well) plates were added 200 µl of the inoculums with coverage of 50–80 %. Cell cultures were incubated at t = 37 ^{0}C for 1 h in order to "capture" an eventual virus if presented, then they were rinsed once with PBS and fresh media were added. Alternatively, the plate was filled directly (cell suspension), since the preliminary studies had found that the anolyte did not induce a cytotoxic effect.

The cell cultures were incubated for 72–96 h at t = +37 ^{0}C in a CO_2 incubator. The procedure with preparation of the positive and negative control samples was similar. The positive control sample was a reference strain of the CSF virus. The immunoperoxidase technique with using a horseradish peroxidase was used for the enzymatic detection of antigen-antibody complexes in cell cultures. The fixation of the plates was carried out thermally for ~3 h at t = +80 ^{0}C in a desiccator. In the processing was used a primary monoclonal antibody C 16, diluted in proportion 1:50, and secondary antibody RAMPO, diluted in proportion 1:50. For the immunoperoxidase staining was used 3 % H_2O_2 and AEC (dimethylformamide and 3-amino-9-ethylcarbazole) in acetate buffer. The antibody-antigen complex was visualized by the peroxidase reaction with the substrate.

A polymerase chain reaction (PCR) to amplify the segments of the RNA was carried out in real time scale. The cell culture and organ suspensions were examined for the presence of the CSF viral genome by the PCR in real time (real-time RT-PCR, one step, TagMan), one-step according to Protocol of the Reference Laboratory for CSF of EU. For RNA extraction was used the test QIAamp Vital RNA Mini Kit, Qiagen Hilden (Germany). The initial volume of the biological material was 140 µl, and the elution volume – 60 µl.

For amplification of PCR was used the test Qiagen OneStep RT-PCR Kit in a total volume of 25 µl, and template volume of 5 µl. In the PCR were used primers A 11 and A14, and probe TaqMan Probe–FAM–Tamra.

PCR studies were carried out with a thermo cycler machine "Applied Biosystems 7300 Real Time PCR System" with the temperature control for reverse transcription at t = +50 ^{0}C – 30:00 min, inactivation of reverse transcriptase and activation of Taq at t = 95 ^{0}C – 15:00 min, denaturation at t = +95 ^{0}C – 00:10 min, extension at t = +60 ^{0}C – 00:30 min for 40 cycles.

The second study on the antimicrobial activity of the anolyte/catholyte was performed at the Institute of Molecular Biology of the Bulgarian Academy of Sciences (BAS). The two electrochemical solutions were prepared with using the Activator-I, developed at the Institute of Information and Communication Technologies at BAS. For this, drinking water without aditional quantity of NaCl was used. This led to pH = 3,0 and ORP = +480 mV for the anolyte, and pH = 9,8 and ORP = -180 mV for the catholyte.

Bacterial strain used in these experiments was *E. coli* DH5α with genotype: *fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi1 hsdR17.*

The Colony Forming Units (CFU) technique was used in this study to assess cellular viability. The conditions for the bacterial cultures growth were as described in our previous paper [26]. The bacterial cells were cultivated on the LB-medium (pH = 7,5) with 1 % bactotryptone; 0,5 % yeast extract; 1,0 % NaCl at t = +37 ^{0}C. After overnight cultivation of bacteria 100 µl samples of culture liquids were taken, centrifuged for 1 min at 10000 g and the pellet of bacterial cells was resuspended in 100 µl of the anolyte or the catholyte. As control samples were used the bacterial samples, re-suspended in non-electroactivated water. Different dilutions of cells were spread on LB-agar Petri plates. After the overnight incubation at t = +7 ^{0}C the appeared bacterial colonies were counted. The viable cells were calculated as a percentage from the CFU. The CFU obtained from culture liguids treated with non-electrochemically activated water were accepted as 100 %.

The NES method was used for the estimation of energy of hydrogen bonds of the anolyte, catholyte and deionized water in order to make a supposition about the spectrum characteristics. The device measures the angle of evaporation of water drops from 72 0 to 0 0. As the main estimation criterion was used the average energy (ΔEH...O) of hydrogen O...H-bonds between individual H2O molecules in water's samples. The NES-spectrum of water was measured in the range of energy of hydrogen bonds 0,08–0,387 eV or λ = 8,9–13,8 µm with using a specially designed computer program.

Results and Discussion

Research into the effects of electro-activated aqueous NaCl (anolyte)on the CSF virus

As shown in Fig. 2 the cytoplasm of cells infected by the CSF virus was stained in the dark reddish brown color (positive reaction), whereas in the uninfected cells it was colorless. That indicates on the presence of viral antigen in the samples.

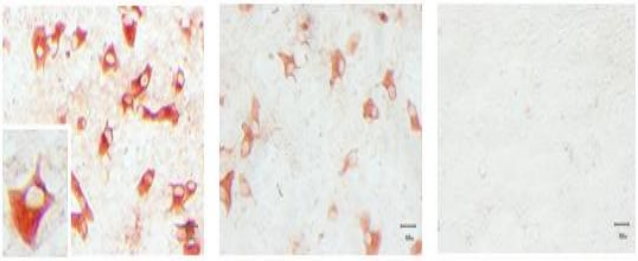

Fig. 2. The established presence of viral antigen in cell cultures (left) and a negative control (right)

Table 1 summarizes the results of different experiments of the virucidal action of the anolyte on the cell culture suspension of the CSF virus upon infecting cell monolayer PK-15. As is shown in Table 1, upon treatment of the viral inoculum with the anolyte in a 1:1 dilution, there was no viral growth in the four infected wells of the plate, upon 1:2 dilution there was no growth in two of the wells, the other two were reported

as positive. Upon treatment with the anolyte at dilutions 1:3 and 1:4, the result was identical: no growth in one of the contaminated wells of the plate, and poor growth – in the other three. The results obtained by infection of CSF virus a cell monolayer and cell suspension were identical.

Table 2 summarizes the results of studies aimed at the evaluation of the virucidal effect of the anolyte on organ suspension containing CSF virus upon infecting a cell monolayer PK-15 with the virus. According to the data, upon treatment of the CSF viral inoculum (organ suspension) with the anolyte in all dilutions, there was no viral growth in the four infected wells of the plate.

Evidently, the anolyte has a destructive influence on the envelope of the CSF virus, wherein the main antigens (proteins) are localized. Studies of the viral inocula used in the tests by means of polymerase chain reaction (PCR) in real time demonstrated the presence of a genome (RNA) in them, also after the treatment with the anolyte. Some shortening of the time was proved (the decreased number of amplification cycles), required for the formation of a fluorescent signal, respectively, a positive reaction for a genome, closely correlated with the exposure under the treatment of the viral inocula. The longer the exposure of processing with the anolyte, the sooner the presence of the viral RNA in the PCR was detected. According to one of our co-authors (Stoil Karadzhov), this may serve as an indirect indication that anolyte destroys the CSF virus envelope, which, in its turn, facilitates the extraction of viral RNA and its more rapid reading by the fluorescent signal. However, there is still no sufficient convincing evidence on the impact of different concentrations of the anolyte on CSF viral particles. The analogous experiments carried out by Russian and German researchers were carried out mainly with the concentrated anolyte. The maximum virucidal effect detected in those experiments confirmed a strong virucidal action of the electrochemically activated aqueous solution of NaCl on the CSF virus. The difference in the results evidently is due to the use of lower concentrations of NaCl in our experiments. We attributed essential significance to the fact that we determined the concentration limit (25 %) of the well demonstrated by the virucidal activity. In this aspect the further studies on reducing the time of the virucidal action, and the conducting of experiments in the presence of biofilms which protect viruses would be promising.

Research into the antibacterial effects of the anolyte and catholyte on a strain of E. coli DH5a

In order to assess the effect, if any, of the electrochemically activated water solutions (catholyte/anolyte) on bacterial cells we treated the cultures of a strain of *E. coli DH5a* by the catholyte. After the treatment of bacterial cells the colonies appearing on the plates with 2 % agar were obtained, produced by survived cells, which were further counted by the CFU method. Therefore, the number of colonies was presented on Figure 3 as a percentage of viable cells. It can be seen from Figure 3 that bacterial cells of *E. coli DH5a* treated with the catholyte hardly survived the treatment with only approximately 15 % of the cells being survived. This clearly shows that the electrochemically activated water produced from the cathode possesses a strong bacteriocidal activity on the strain of *E. coli DH5a*.

Notably, the anolyte also showed slight antibacterial effect. Thus, approximately, 73 % of the bacterial cells of *E. coli DH5a* survived the electrochemical treatment with the anolyte. In summary, it is assumed that both types of the electrochemically activated water solutions (catholite/anolyte) possess antibacterial effect on the strain of *E. coli DH5a*, however it is obvious that the catholyte has a stronger bacteriocide effect than the anolyte.

Table 1

The virucidal action of the anolyte on cell culture suspensions of the CSF virus upon infecting cell monolayer PK-15

Contamination of CC with:	Dilutions of anolyte (100 µl)	Total volume of the inoculum (µl)	Concentration of anolyte in %	Number of wells	Result: positive/ negative
Virus 200 µl	–	200	–	4	4/0
Virus 100 µl	1:1	200	25	4	0/4
Virus 100 µl	1:2	200	16,51	4	2/2
Virus 100 µl	1:3	200	12.5	4	3/1
Virus 100 µl	1:4	200	10	4	3/1

Table 2

The virucidal action of the anolyte on organ suspensions containing CSF virus upon infecting cell monolayer PK-15

Contamination of CC with:	Dilutions of anolyte (100 µl)	Total volume of the inoculum (µl)	Concentration of anolyte in %	Number of wells	Result: positive/ negative
Virus 200 µl	–	200	–	4	4/0
Virus 100 µl	1:1	200	50	4	0/4
Virus 50 µl	3:1	200	75	4	0/4
Virus 25 µl	7:1	200	87	4	0/4
Virus 12,5 µl	15:1	200	94	4	0/4

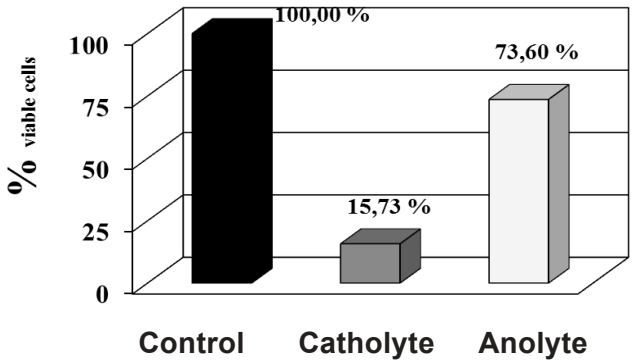

Fig. 3. Percentage of viable cells of *E. coli DH5a* after the electrochemical treatment with the catholyte and anolyte relative to the non-electrochemically activated water

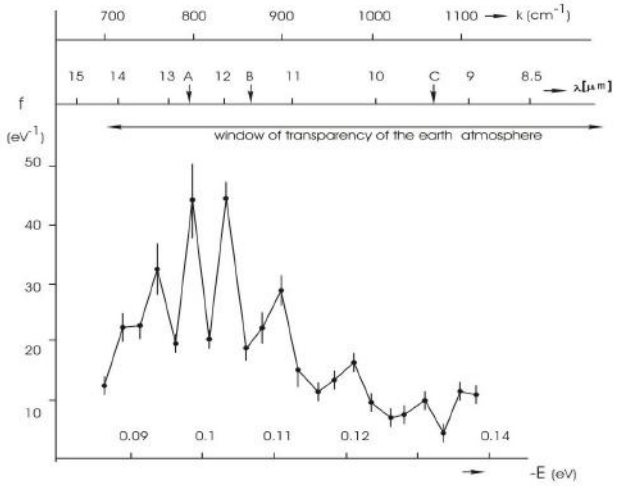

Fig. 4. NES-spectrum of deionized water (chemical purity – 99,99 %; pH – 6,5–7,5; total mineralization – 200 mg/l; electric conductivity – 10 μS/cm). The horizontal axis shows the energy of the H...O hydrogen bonds in the associates – E (eV). The vertical axis – energy distribution function – f (eV^{-1}). *k* – the vibration frequency of the H–O–H atoms (cm^{-1}); λ – wavelength (λm).

NES and DNES methods in spectral analysis of the anolyte and catholyte

Other method for obtaining useful information about the structural changes in water and the average energy of hydrogen bonds is the measuring of the energy spectrum of the water state. It was established experimentally that at evaporation of water droplet the contact angle θ decreases discretely to zero, whereas the diameter of the droplet changes insignificantly [27]. By measuring this angle within a regular time intervals a functional dependence f(θ) can be determined, which is designated as "the spectrum of the water state" (SWS) [28]. For practical purposes by registering the SWS it is possible to obtain information about the averaged energy of hydrogen bonds in an aqueous sample. For this purpose the model of W. Luck was used, which consider water as an associated liquid, consisted of O–H…O–H groups [29]. The major part of these groups is designated by the energy of hydrogen bonds (-E), while the others are free (E = 0). The energy distribution function f(E) is measured in electronvolts (eV^{-1}) and may be varied under the influence of various external factors on water as temperature and pressure.

For calculation of the function f(E) experimental dependence between the water surface tension measured by the wetting angle (θ) and the energy of hydrogen bonds (E) is established:

$$f(E) = bf(\theta) / [1 - (1 + bE)^2]^{1/2}, \quad (8)$$

where $b = 14{,}33\ eV^{-1}$; $\theta = \arccos(1 - bE)$

The energy of hydrogen bonds (E) measured in electronvolts (eV) is designated by the spectrum of energy distribution. This spectrum is characterized by non-equilibrium process of water droplets evaporation, thus the term "non-equilibrium energy spectrum of water" (NES) is applied.

The difference $\Delta f(E) = f$ *(samples of water) – f (control sample of water)* – is designated as the "differential non-equilibrium energy spectrum of water" (DNES) [30].

The DNES-spectrum measured in milielectron volts (0,001 eV) is a measure of changes in the structure of water as a result of external factors. Figure 4 shows the characteristic NES-spectrum of deionized water made from 25 independence measurements performed in a period of one year.

The average energy ($\Delta E_{H...O}$) of hydrogen H…O-bonds among individual molecules H_2O was calculated for the catholyte and the analyte by NES- and DNES-methods. We studied the distribution of local maximums in catholyte and anolyte solutions. The local maximum for the catholyte in the NES-spectrum was detected at -0,1285 eV, for the anolyte – at -0,1227 eV, and for the control sample of deionized water – at -0,1245 eV. The calculations of $\Delta E_{H...O}$ for the catholyte with using the DNES method compiles (-0,004±0,0011 eV) and for the anolyte (+1,8±0,0011 eV). These results suggest the restructuring of $\Delta E_{H...O}$ values among individual H_2O molecules with a statistically reliable increase of local maximums in DNES-spectra of the catholyte and anolyte (Table 3).

For the catholyte the biggest local maximum was detected at -0,1387 eV, or at 8,95 μm. In 1992 A. Antonov performed experiments with the impact of different types of water on tumor mice cells. It was detected a decrease in the NES-spectrum compared with the control sample of cells from healthy mice. There was also a decrease of the local maximum at -0,1387 eV, or 8,95 μm in DNES-spectra. Notably, the local maximum at 8,95 μm was detected with the negative value. It should be noted that for the catholyte the local maximum in the DNES-spectrum was detected with the positive value at +133,3 eV^{-1}.

For the catholyte the biggest local maximum in the DNES-spectrum was detected at -0,1312 eV, or 9,45 μm. It should be noted that for the treatment of influenza in medical drugs is included $Al(OH)_3$ [31]. The local maximum in this case was measured at -0,1326 eV, or at 9,35 μm.

The evaluation of the possible number of hydrogen bonds as percent of H_2O molecules with different values of distribution of energies is presented in Table 4. These

Table 3

Local maximums of catholite and anolyte solutions in NES- and DNES-spectra

-E(eV) x-axis	Catholyte	Anolyte y-axis (eV^{-1})	Control sample y-axis (eV^{-1})	DNES Catholyte	DNES Anolyte	-E(eV) x-axis	Catholyte y-axis (eV^{-1})	Anolyte y-axis (eV^{-1})	Control sample y-axis (eV^{-1})	DNES Catholyte y-axis (eV^{-1})
0,0937	0	0	0	0	0	0,1187	0	66,7	66,7	-66,7
0,0962	0	0	0	0	0	0,1212	66,7	0	0	66,7
0,0987	0	0	0	0	0	0,1237	0	0	0	0
0,1012	66,7	66,7	33,3	33,4	33,4	0,1262	0	0	66,7	-66,7
0,1037	0	0	33,3	-33,3	-33,3	0,1287	0	0	66,7	-66,7
0,1062	0	0	0	0	0	0,1312	33,3	100	33,3	0
0,1087	0	0	0	0	0	0,1337	33,3	33,3	33,3	0
0,1112	0	0	0	0	0	0,1362	0	0	0	0
0,1137	0	66,7	66,7	-66,7	0	0,1387	200	66,7	66,7	133,3
0,1162	0	0	0	0	0	–	–	–	–	–

Table 4

Energy distribution of catholyte and anolyt e solutions in electrochemical activation of sodium chloride

-E(eV) x-axis	Catholyte y-axis, % $(-E_{value})/(-E_{total\ value})$	Anolyte y-axis, % $(-E_{value})/(-E_{total\ value})$	-E(eV) x-axis, % $(-E_{value})/(-E_{total\ value})$	Catholyte y-axis, % $(-E_{value})/(-E_{total\ value})$	Anolyte y-axis, % $(-E_{value})/(-E_{total\ value})$
0,0937	0	0	0,1187	0	16,7
0,0962	0	0	0,1212	16,7	0
0,0987	0	0	0,1237	0	0
0,1012	16,7	16,7	0,1262	0	0
0,1037	0	0	0,1287	0	0
0,1062	0	0	0,1312	8,4	24,8
0,1087	0	0	0,1337	8,4	8,4
0,1112	0	0	0,1362	0	0
0,1137	0	16,7	0,1387	49,8	16,7
0,1162	0	0	–	–	–

distributions are basically connected with the restructuring of H_2O molecules with the same energies. This serves as the base for evaluating the mathematical model explaining the behavior of the anolyte and catholyte regarding the distribution of H_2O molecules to the energies of hydrogen bonds [32].

Conclusions

The experimental results prove the strong influence of different types of electrochemically activated water solutions (catholyte/anolyte) on various microbes and viruses. They are in accordance with the results obtained by other researchers, and demonstrate the strong biocidal effect of the anolyte toward the CSF virus. Also, the interesting results on the antibacterial effect were obtained when a strain of *E. coli DH5a* was treated with the catholyte and anolyte, respectively. Unexpectidely, the catholyte with ORP ≈ -180 mV and pH = 9,8 demonstrated the better biocidal effect than the anolyte with ORP ≈ +500 and pH = 3,9. We tried to relate the antimicrobial and antiviral action of electrochemically activated water with the characteristics of the NES-spectrum. There is an indication about such a connection but more thorough research is needed to prove it. For example, the inverse biocidal effect between the catholyte and anolyte in case of a strain of *E. coli DH5a* requires a clear explanation.

The results of the research are formulated as follows.

1. The anolyte did not affect the growth of the cell culture PK-15;
2. The anolyte administered at a concentration of 25 %, exerts a strong virucidal effect on a cell culture virus, and a weaker antiviral activity at concentrations of 16,51 %, 12,5 % and 10 %;
3. The anolyte exerted a strong virucidal effect at concentrations of 50 %, 75 %, 87 % and 94 % over the CSF virus in cell culture suspensions;
4. The catholyte supresses the growth of *E. coli* up to 85 % while anolyte is at least three times less effective;
5. The local maximum in the DNES-spectrum of the catholyte was detected at 9,85 μm; there was a decrease of this local maximum in water with mice tumor cells;
6. The local maximum in the DNES-spectrum of the anolyte was detected at 9,45 μm; at 9,35 μm occurred the effect of inflammation from virus of influenza;
7. The mathematical model of the catholyte and anolyte regarding the distribution of H_2O molecules to the energies of hydrogen bonds was evaluated.

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VIRUCIDAL AND BACTERICIDAL EFFECTS OF ELECTROCHEMICALLY ACTIVATED ANOLYTE AND CATHOLYTE TYPES OF WATER ON CLASSICAL SWINE FEVER VIRUS AND BACTERIUM *E. COLI DH5*

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This article outlines the results on the antimicrobial action of electrochemically activated water solutions (anolyte/catholyte), produced in the anode and cathode chamber of the electrolitic cell. Under laboratory conditions the cell culture and suspensions of classical swine fever (CSF) virus were treated with the anolyte. After inoculating them with cell cultures, the viral presence (the presence of viral antigen) was measured using the immunoperoxidase technique. It was found that anolyte did not affect the growth of the cell culture PK-15; viral growth during the infection of a cell monolayer with a cell culture virus was affected in the greatest degree by the anolyte in 1:1 dilution and less in other dilutions; whereas the viral growth at the infection of a cell suspension with the CSF virus was affected by the anolyte in dilution 1:1 in the greatest degree, and less by other dilutions; viral growth at the infection with a virus in suspension of the cell monolayer was affected by the anolyte in all dilutions. Unexpectedly, the stronger biocidal effect of the catholyte was observed when a strain of *E. coli DH5* was treated by the anolyte and catholyte, respectively. In order to provide additional data about the antiviral activity of the electrochemically activated water and the distribution of H_2O molecules according to the energies of hydrogen bonds, the non-equilibrium energy spectrum (NES) and differential non-equilibrium energy spectrum (DNES) of the anolyte and catholyte were measured.

Key words: anolyte, catholyte, *E. coli DH5*, CSF virus, NES, DNES

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ВИРУЛИЦИДНЫЙ И БАКТЕРИЦИДНЫЙ ЭФФЕКТЫ ЭЛЕКТРОХИМИЧЕСКИ АКТИВИРОВАННЫХ РАСТВОРОВ КАТОЛИТА И АНОЛИТА НА КЛАССИЧЕСКИЙ ВИРУС СВИНОГО ГРИППА И БАКТЕРИЮ E. COLI DH5

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В статье описываются результаты антимикробного действия электрохимически активированных водных растворов (анолит/католит), полученных в анодной и катодной камере электролитической ячейки. В лабораторных условиях культура клеток суспензии вируса свиного гриппа была обработана анолитом. После прививки их с культурами клеток присутствие вируса (наличие вирусного антигена) было измерено с использованием иммунопероксидазного метода. Было обнаружено, что анолит не влияет на рост культуры клеток РК-15; вирусной рост при заражении клеточного монослоя замедлялся вирусом в наибольшей степени при разведении анолита в пропорции 1:1 и менее в других разведениях; в то время как вирусный рост при инфекции клеточной суспензии с вирусом замедлялся анолитом в наибольшей степени в разведении 1:1, и менее в других разведениях; вирусный рост при инфекции вирусом суспензии клеток монослоя зависел от присутствия анолита во всех разведениях. Неожиданно сильный биоцидный эффект католита наблюдался при обработке штамма *E. coli DH5* анолитом и католитом соответственно. Для получения дополнительных данных о противовирусной активности электроактивированных растворов воды, а также о структурных изменениях, были измерены неравновесный энергетический спектр (НЭС) и дифференциальный неравновесный энергетический спектр (ДНЭС) анолита и католита.

Ключевые слова: анолит, католит, *E. coli DH5*, вирус свиного гриппа, НЭС, ДНЭС

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ВІРУЛІЦИДНИЙ І БАКТЕРИЦИДНИЙ ЕФЕКТИ ЕЛЕКТРОХІМІЧНИХ АКТИВОВАНИХ РОЗЧИНІВ КАТОЛІТУ І АНОЛІТУ НА КЛАСИЧНИЙ ВІРУС СВИНЯЧОГО ГРИПУ І БАКТЕРІЮ *E. COLI DH5*

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У статті описуються результати антимікробної дії електрохімічно активованих водних розчинів (аноліт/католіт), отриманих в анодній і катодній камері електролітичної комірки. У лабораторних умовах культура клітин суспензії вірусу свинячого грипу була оброблена анолітом. Після щеплення їх з культурами клітин присутність вірусу (наявність вірусного антигену) було виміряно з використанням імунопероксидазного методу. Було виявлено, що аноліт не впливає на ріст культури клітин РК-15; вірусне зростання при зараженні клітинного моношару сповільнювалося вірусом найбільшою мірою при розведенні аноліта в пропорції 1:1 і менше в інших розведеннях; в той час як вірусне зростання при інфекції клітинної суспензії з вірусом сповільнювалося анолітом найбільшою мірою в розведенні 1:1, і менше в інших розведеннях; вірусне зростання при інфекції вірусом суспензії клітин моношару залежало від присутності аноліту у всіх розведеннях. Несподівано сильний біоцидний ефект католіту спостерігався при обробці штаму *E. coli DH5* анолітом і католітом відповідно. Для отримання додаткових даних щодо противірусної активності електроактивованих розчинів води, а також про структурні зміни, були виміряні нерівноважний енергетичний спектр (НЕС) і диференційний нерівноважний енергетичний спектр (ДНЕС) аноліту і католіту.

Ключові слова: аноліт, католіт, *E. coli DH5*, вірус свинячого грипу, НЕС, ДНЕС.

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STUDYING THE BIOLOGICAL INFLUENCE OF HEAVY WATER (^2H_2O) ON PROCARYOTIC AND EUCARYOTIC CELLS

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Introduction

The most interesting biological phenomenon is the ability of some microorganisms to grow on heavy water (^2H_2O) media in which all hydrogen atoms are replaced with deuterium [1, 2]. ^2H_2O has high environmental potential in biomedical studies due to the absence of radioactivity and poccebility of detecting the deuterium label in the molecule by high-resolution methods as NMR, IR, and mass spectrometry that facilitates its use as a tracer in biochemical and biomedical research [3].

The average ratio of $^1H/^2H$ in nature makes up approximately 1:5700 [4]. In natural waters, the deuterium is distributed irregularly: from 0,02–0,03 mol.% for river water and sea water, to 0,015 mol.% for water of Antarctic ice – the most purified from deuterium natural water containing in 1,5 times less deuterium than that of seawater. According to the international SMOW standard isotopic shifts for ^2H and ^{18}O in sea water: $^2H/^1H = (155,76\pm0,05).10^{-6}$ (155,76 ppm) and $^{18}O/^{16}O = (2005,20\pm0,45).10^{-6}$ (2005 ppm). For SLAP standard isotopic shifts for ^2H and ^{18}O in seawater make up $^2H/^1H = 89.10^{-6}$ (89 ppm) and for a pair of $^{18}O/^{16}O = 1894.10^{-6}$ (1894 ppm). In surface waters, the ratio $^2H/^1H = \sim (1,32–1,51)\cdot10^{-4}$, while in the coastal seawater – $\sim(1,55–1,56)\cdot10^{-4}$. The natural waters of CIS countries are characterized by negative deviations from SMOW standard to $(1,0–1,5)\cdot10^{-5}$, in some places up to $(6,0–6,7)\cdot10^{-5}$, but however there are also observed positive deviations at $2,0\cdot10^{-5}$.

The chemical structure of ^2H_2O molecule is analogous to that one for H_2O, with small differences in the length of the covalent H–O-bonds and the angles between them. The molecular mass of ^2H_2O exceeds on 10% that one for H_2O. The difference in nuclear masses stipulates the isotopic effects, which may be sufficiently essential for the $^1H/^2H$ pair [5]. As a result, physical-chemical properties of ^2H_2O differ from H_2O: ^2H_2O boils at +101,44 ^{0}C, freezes at +3,82 ^{0}C, has maximal density at +11,2 ^{0}C (1,106 g/cm^3) [6]. In mixtures of ^2H_2O with H_2O the isotopic exchange occurs with high speed with the formation of semi-heavy water ($^1H^2HO$): $^2H_2O + H_2O = {}^1H^2HO$. For this reason deuterium presents in smaller content in aqueous solutions in form of $^1H^2HO$, while in the higher content – in form of ^2H_2O. The chemical reactions in ^2H_2O are somehow slower compared to H_2O. ^2H_2O is less ionized, the dissociation constant of ^2H_2O is smaller, and the