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RESEARCH ARTICLE

ANTIBACTERIAL PROPERTIES OF NEW CHITOSAN-BASED SCHIFF BASES.

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Abstract

Chitosan, a derivative of chitin, one of the most widespread natural polysaccharides, is of great interest in terms of practical application. The importance of this polymer and its derivatives cannot be overestimated since it is currently used in such spheres of human activity as medicine, agriculture, food and textile industries, nuclear medicine, pharmaceutics and cosmetology, ecology and others. Its antibacterial properties are of particular interest.

In the A.I. Alikhanyan National Science Laboratory Foundation (Yerevan Physics Institute) the works are being conducted on obtaining/synthesizing new samples of chitosan Schiff bases. The present work is devoted to the study of the antibacterial activity of newly synthesized Schiff bases derivatives of chitosan. As test cultures in studying their antibacterial properties, several aerobic gram-negative and one gram-positive bacteria were used.

It has been reliably shown that at least a two of the five samples of chitosan and its derivatives studied by us had pronounced antibacterial properties. The effectiveness of the preparations impact differed from culture to culture. Of all the cultures tested, the wild type of the bacterial culture of Bacillus cereus proved to be the most sensitive. The term “effective ratio” (ER) is proposed for the shift of selected ratios one way or another, which resulted either in a complete stop of cell proliferation or cessation of the chitosan derivative to affect the ability of cells to reproduce.

Introduction:-

Chitosans are deacetylated derivatives of chitin, a natural polysaccharide, a widely distributed in nature polymer performing protective and supporting function in many insects, marine arthropods, fungi, etc. The raw material for chitin obtaining, mainly, is the waste of seafood production. At present, chitin/chitosan systems and their various derivatives have found wide application in practice. Just a brief enumeration of the fields of chitosan and its derivatives application indicates the importance of this compound (Ahmed and Ikram, 2017). Chitosan and some of its derivatives are biocompatible, have low toxicity, have bactericidal, anticoagulant, chelating (complex-forming) properties (Wang et al., 2009; Liu 2012, **Subhapradha et al.,** 2013; Gritscha et al., 2018), characterized by high radiation stability (Chmielewski, 2010), demonstrate anti-tumor properties (Wang et al., 2009; Gupta and Karar, 2012). Taking into account chelate-forming abilities of chitin/chitosan systems, efficient sorbents for bacterial cells and many heavy metals and a number of their compounds have been synthesized on their basis and studied (Guibal,

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2004; Varma et al., 2004; Tariq et al.,2010; Jiang et al., 2011; Kocak et al., 2012; Farag and Mohamed, 2013). The possibilities of their medical application are of interest (Kumirska et al., 2011). Owing to the radiation stability and the ability to form chelates also with radionuclides (Alirezazadeh and Garshashbi, 2003; Oshita et al., 2009; Suc and Ly, 2011), in particular, with ^{157}Gd, ^{166}Ho, (Chmielewski, 2010), ^{153}Sm (Samani et al., 2010), they are actively used in practical nuclear medicine. Chitosan compounds after preliminary radiation treatment are used as plant growth stimulators (Hegazy et al., 2009).

There are also works testifying to the increase of the antibacterial properties of chitin/chitosan systems after their radiation treatment (Morhsed et al., 2011).

Of particular interest are the antibacterial properties of chitosan and its derivatives. Quite often, the publications devoted to chitosan and synthesis of its derivatives, contain data on their antibacterial properties. This paper is devoted to the investigation of the antibacterial properties of the synthesized new Schiff bases derivatives of chitosan described in our co-author Gavalyan's paper (2016).

Materials And Methods:-

Chemicals and Media

Antibacterial properties of chitosan (one preparation) and some of its derivatives (four preparations) synthesized in A.I. Alikhanyan National Science Laboratory Foundation (Yerevan Physics Institute) (AANL-YerPhI) by our co-author Gavalyan (Gavalyan, 2016), namely: Cs-pure (a), CsSBRTCl (b), CsSB80Cl (c), CsSBRTBr (d), CsSB80Br (e) were studied in the work.

All other used chemicals were of analytical grade or higher without further purification except for technical agar-agar (Japan) that was carefully washed with large amount of distilled water. To grow test bacteria, standard media – meat-peptone agar (MPA) and meat-peptone broth (MPB) were used.

Used Strains. Cultures of Microorganisms:-

As cultures for testing the antibacterial properties of synthesized chitosan derivatives, classical bacterial species were selected, such as: *(a)* a strain of *Escherichia coli* K-12 AB-1157; *(b)* a wild strain of opportunistic pathogen culture of *Ps. aeruginosa*; *(c)* an unidentified culture of *Pseudomonas sp.* A-27, isolated by us from the soil. This culture has high tolerance to the presence of thiamethoxam, the neonicotinoid, active constituent of the pesticide "Actara". In the literature there is no unequivocal answer to the question to what extent this insecticide is harmless for soil microorganisms. A special study conducted by us has shown that it presents a sufficient danger for bacteria actively participating in improving the fertility of the soil (KhachatryanandMkrtchyan, 2016). We found it reasonable to include it into the list of test cultures.

All three cultures are gram-negative. As a gram-positive culture a wild strain of spore-forming bacterium *Bac. cereus*, known as an opportunistic microorganism, capable of causing sporadic food poisoning in humans was selected.
The temperature at which all cultures were grown in all experiments was 30^{o}C.

Antibacteral Properties Check Methodology:-

There is a number of publications where various techniques are described evaluating the antimicrobial properties of various preparations, including chitin-chitosans (Ortega-Ortiz, 2010; Liu, 2012; Pundir et al., 2013; Mohamed et al., 2013; MohyEldin et al., 2015), however, all of them did not fully satisfy us. For a clearer understanding of the research results, we found it more expedient to combine a number of methods, so that we could more or less clearly evaluate the survival of bacteria of different species at a given antimicrobial preparation concentration by direct counting of surviving cells. For this purpose, we developed a generalized methodology based on the modification of the methods proposed in the works (Jayavanth et al., 2011; Morhsed et al., 2011; Ortega-Ortiz et al., 2010; Han et al., 2012). It is this technique that was used in our further studies.

Below, on the example of *Bacillus cereus*, a detailed description of the steps undertaken by us is presented. We have chosen *Bacillus cereus* as a case requiring more detailed analysis, since bacilli are spore-forming cultures. When working with this culture, the studies were performed: (*i*) with fresh culture – with the predominant content of vegetative cells in the logarithmic growth phase; (*ii*) with a suspension of endospores obtained by pasteurizing the culture in a stationary growth phase; (*iii*) with an "old" culture (containing both vegetative cells and endospores).

For all other test cultures, the operations performed were similar to those performed with the culture of vegetative cells of bacilli.

To obtain the culture in a logarithmic growth phase, a tube with 5 mL of MPB was inoculated with the material taken from an 18-hour colony grown on MPA. The tube was left overnight in a thermostat. In the morning, 1 mL of the overnight culture was transferred to 4 mL of sterile broth, and refreshed for 2 hours on a reciprocal shaker (120 rev/min). An aliquot of the suspension of the refreshed culture was run through a series of dilutions using sterile tap water to achieve a cell concentration suitable for evaluating the effect of chitosan derivatives by plating on MPA (for counting colony forming units: CFU). The number of transfers providing a convenient for count the number of colonies on the surface of agar plate was determined experimentally.

After determining the number of necessary transfers, the suspension in all three cases was transferred to the MPB in the penultimate stage, both in the experimental and the control series. And only after that, in order to reach the required final cell concentration, a suspension of cells in the broth (0.2 mL each) in the test series was transferred to a final tube with the testing chitosan derivative (volume 1.8 mL), and in the control series – to the same volume of sterile tap water .

It was assumed that despite a ten-fold dilution, the resulting broth would be sufficient to ensure the proliferation of the culture in both cases, and experiments confirmed this assumption.

An aliquot of 0.1 mL of the resultant final volumes of the mixture, after its incubation for 1 hour in a thermostat at 30°C, was plated on MPA, the residue was incubated for another 23 hours and the plating was repeated on MPA to check residual proliferation. Here it should be noted that the same order of actions was followed when working with *Ps. aeruginosa*, distinguished by greater resistance to antimicrobial agents showing significantly more intensive proliferation compared to other tested Gram-negative cultures. When working with *E. coli* K-12 and *Pseudomonas sp.* A-27 plating was carried out only after the 24-hour incubation.

Further, to test the reaction of endospores on chitosan derivatives, plating on MPA was performed from a pasteurized suspension. For this purpose, a suspension for the experiment with the "old" culture was prepared by transferring part of the colony of the 48-hour culture from the MPA plate into a test tube with sterile tap water. The resulting volume of the suspension was divided into two parts. One part (*ii*) was treated exactly according to the scheme described above. The other (*iii*) was heat-treated (pasteurized) by incubating it for 20 minutes at 80°C in a water ultrathermostat and plated on MPA to determine the number of surviving spores. This made it possible to comprehend the necessary number of dilutions and further manipulations with spores completely identical with the experiment described above.

The aftereffects were evaluated by comparing the number of CFU on MPA plates grown after plating the experimental (with the chitosan derivative solution) and control (sterile tap water) cell suspensions.

In all cases, the concentration of chitosan derivatives was used at the limit of their solubility (about 500 μg/ml), except for the case with *Ps. aeroginosa*, when it became necessary to reduce the concentration of the chitosan derivative (about 200 μg/ml). The data presented in the Tables are the average of the five replicates of each experiment and the dispersion over them. As a rule, the data fluctuated within 5-10% of the average value, therefore the specific scatter figures in the reduction tables are only in a few, from our point of view, principle cases.

Results:-

Of the five samples investigated in the present study, namely, Cs-pure (a), CsSBRTCl (b), CsSB80Cl (c), CsSBRTBr (d), CsSB80Br (e), at this stage of the study, samples (b) and (c) proved to be the most interesting from the standpoint of demonstrating antibacterial abilities.

As indicated in the Materials and Methods section, the techniques used to evaluate the effectiveness of the chitosan derivatives impact were, in our view, too formal. We considered it necessary to select such experimental conditions that will enable a direct numerical estimate of the ratio of microorganism cells in the aqueous suspension to the used concentration of the chitosan (Cs) derivative (CCsD-Concentration of Chitosan Derivative), at which residual proliferation does not occur at all or is insignificant. To ensure the validity of the evaluation of the effect of the antimicrobial preparation, we tried to introduce into the experimental medium practically identical numbers of cells

of the test cultures. Obviously, if the chitosan derivative has antibacterial properties and there is its excess in the experimental medium, the cell proliferation will cease completely. If the ratio is shifted in favor of bacterial cells, the amount of chitosan will not be sufficient to prevent proliferation. The ratio at which the desired effect is observed, we called an "Effective Ratio" (ER).

It is clear that the problem posed this way could be solved by varying the ratios of cell concentration of the test cultures and the chitosan derivative. And since it was impossible to increase the concentration of the antimicrobial preparation, we had, if necessary, to decrease the concentration of the test cells. And in fact, only in one case, in testing the culture of *Ps. aeruginosa* it was necessary to reduce the concentration of chitosan. The following Tables show the data obtained as a result of using this approach.

Table 1:- Evaluation of survival of test cultures under the influence of chitosan derivatives (data for sample (b) are given).

Culture	QCFU*	CFU in exp. tubes in 24 h	CFU in control tubes in 24 h	% of exp. to control in 24 h
(a)E.coli K-12	$1.5*10^2$	0	$8.5*10^8$ +/- $0.3*10^8$	0
(b) Ps. sp. A-27	$2.0*10^2$	$2.0*10^2$	$8.1*10^8$ +/- $0.6*10^8$	$2.5*10^{-5}$
(a) Ps. aeruginosa	$1.6*10^2$	$8.8*10^5$	$9.0*10^8$ +/- $0.4*10^8$	$0.97*10^{-2}$

*Designations: QCFU - quantity of CFU, inserted to the experimental and control tubes.

Based on the Table 1, it is easy to evaluate the order of inhibition of cell proliferation of test cultures comparing control and experimental data. It can be seen that the survival of *Ps.aeruginosa* turned out to be the most significant among the tested cultures. Therefore, it was decided to conduct an additional cycle of experiments, trying to ensure a higher ratio of chitosan to cell suspension. With this purpose, we reduced the concentration of cells inserted into experimental tubes, while slightly reducing chitosan concentration in the hope to catch ER. The results are shown in Table 2.

Table 2:-Evaluation of survival of P. aeruginosa culture in the presence of a sample b.

Drug concn.	QCFU (CFU/ml)	CFU/ml in exp. (in 1h)	CFU/ml in control (in 1h)	CFU/ml in exp. (in 24h)	CFU/ml in control (in 24h)
Variant I (200 mg/ml)	$6.5*10^1$	$2.5*10^1$	$6.5*10^1$	$1.4*10^3$	$1.8*10^8$
Variant II (500 mg/ml)	$6.4*10^1$	0	$6.4*10^1$	0	$1.6*10^8$

Obviously, we managed to come very close to what we called an "effective ratio".
Table 3 summarizes the reaction of the gram-positive spore-forming bacterial culture of *Bacillus cereus*, known as an aerobic sporulating opportunistic microorganism, causing sporadic human food poisoning.

Table 3*:*-Evaluation of the survival of B. cereus culture in the case of the action of CsD

Culture B. cereus	QCFU (CFU/ml)	CFU/ml in exp. (in 1h)	CFU/ml in control (in 1h)	CFU/ml in exp. (in 24h)	CFU/ml in control (in 24h)
Old culture, I	$1.0*10^3$	0	$6.2*10^3$	0	$0.8*10^7$
Old culture, II	$1.0*10^4$	$1.5*10^1$	$6.2*10^4$	0	$1.8*10^7$
Spores	$1.7*10^4$	$1.7*10^4$	$1.7*10^4$	$6*10^3$	$4.3*10^7$
Vegetative cells	$3.0*10^4$	$2.2*10^2$	$2.8*10^4$	$7*10^1$	$2.7*10^7$

Discussion:-

The data presented in the above Tables, regarding the maximum CsD/cell suspension ratio factor (recall that the concentration of CsD in all cases was a little bit lower than 500 μg/ml – almost the solubility limit in water), can be interpreted as follows: for (*a*) the chitosan derivative inhibits proliferation of *E. coli* cells, and the available concentration of sample (b) was sufficient to kill all cells of this culture in suspension in this ratio; for the strain *Ps.sp.* A-27 (*b*), this ratio allowed to actually inhibit the proliferation of cells of this culture; for (*c*) the proliferation of the cells of *Ps.aeroginosa*, being somewhat inhibited by the presence of sample (b), continued, and as can be seen from Table 1, by the 24^{th} h the cell concentration increased significantly, though amounting to only 0.097% of

control. That is why the additional cell/concentration ratios of the CCsD were selected to find the ER. Table 2 shows that it was possible to select cell/CsD ratios, in which the culture in the experiment was at the survival limit. Even in the case of Variant I (CCsD is equal to 200 μg/ml), where a certain percentage of survivors was observed, their number, compared to the number of cells proliferated under control conditions, was less than 0.001%.

In the literature there is information on the effect of the derivatives of chitosans on bacilli (Morhsed, 2011), but in these studies we did not find data that take into account the peculiarity of the bacilli to form endospores. As detailed in the Materials and Methods section, it seemed to us necessary to conduct a more detailed study of this culture.

As can be seen from the Table, the selected cell/CsD ratios allow one to register greater efficacy of the antimicrobial preparation impact on this culture. Moreover, as in the case of *Ps. aeruginisa*culture, in this case, we also evaluated the time factor. As it turned out, just one hour after the incubation, 98.8% of all *B. cereus* vegetative cells in the presence of sample (b) were killed. A more impressive picture was observed after 24 hours. It is also seen from Table 3 that endospores proved to be much more resistant to the action of the test chitosan derivatives, however, their number also significantly decreased in comparison with those proliferated under control conditions.

Conclusion:-

The data obtained by us on the effect of chitosan derivatives on the cells of all the tested Gram-negative cultures make it possible to assume that two of the Schiff base samples studied, namely, samples (b) (CsSBRTCl) and (c) (CsSB80Cl) did have the ability to suppress the proliferation of cells of the tested cultures. The antibacterial ability of both derivatives has turned out to be almost the same, so in this paper only the data obtained as a result of testing sample (b) are given.

The effectiveness of the preparations differed from culture to culture. Of the three gram-negative cultures tested, the culture of *Escherichia coli* K-12 AB-1157 proved to be the most sensitive, *Pseudomonas aeruginosa* - the least. However, even in the case of *Ps. aeruginosa*, the effect of the chitosan derivative proved to be very significant, since its presence suppressed the proliferation of culture cells by 4 orders of magnitude compared to the control. The shift of the selected ratios one way or another from the ER led either to a complete stop of the cell proliferation, or cessation of the chitosan derivative to affect the ability of cells to reproduce.

We believe that further development of the proposed approach can provide an effective tool for a real numerical evaluation of this technique.

Gram-positive culture of *B. cereus* appeared to be more sensitive, so, the concentration of 500 μg/ml completely suppressed proliferation of the cells introduced into the experiment in the amount of CFU $1.0*10^3$ after an hour, whereas at CFU $1.0*10^4$ the growth was suppressed only after 24 hours. Endospores were somewhat more stable.

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