The study of complexes of groove binding ligand Hoechst 33258 with Calf Thymus DNA has been carried out. The data obtained revealed that the melting curves of the complexes of H33258 with DNA are monophasic at low ligand concentrations ($0 < r \leq 0.2$) and become biphasic at relatively high concentrations ($0.2 < r \leq 0.33$). This effect was revealed to depend on the ionic strength of the solution, and can also occur at high concentrations of the ligand. Comparison of the obtained data with the results for poly(rA)-poly(rU) and poly(dA)-poly(dT) shows a coincidence in the case of DNA and poly(rA)-poly(rU), while in the case of poly(dA)-poly(dT) the melting curves become biphasic at low ligand concentrations and actually do not depend on the ionic strength of the solution.

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**Keywords:** DNA, Hoechst 33258, multimodality, monophasic and biphasic melting curves.

**Introduction.** Understanding of the molecular mechanisms of action of biological activity of low-molecular weight compounds (ligands) underlies the design and synthesis of new, more effective preparations of practical importance [1]. Studies in this direction are important, since they allow to reveal questions concerning both conformation transformations of nucleic acids (NA) upon the interaction with ligands and the mechanisms of ligand binding with them. Particularly, ligands, specific to B-form of NA, can interact with A-form as well, but the specificity of several ligands is not limited only by affinity to A-NA (for instance, to RNA) [1]. There exists a group of ligands that preferably bind in minor groove of DNA in B-conformational state (netropsin, DAPI, various derivatives of Hoechst). This group of ligands preferably binds to A-form of DNA in the major groove (for instance, aminoglycosides) [1–5]. However, there are still no data on such ligands that demonstrate a clear preference for AT-rich regions of the DNA minor groove. The affinity of Hoechst derivatives for DNA is sufficiently high ($K_d \sim 10 \text{ nM}$). Compounds of the Hoechst family possess low cytotoxicity and penetrate well through cellular membranes [6–8]. These properties of Hoechst open up new possibilities for the application of its derivatives as modulators of cellular activity, as well as can become a basis for the design and synthesis of new drug preparations [6–8].

This work is aimed at studying the structural transitions of NA complexes with Hoechst 33258 (H33258) depending on the ligand concentration.

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Materials and Methods. Ultrapure Calf Thymus DNA (ctDNA), H33258 (“Sigma”, USA), EDTA (ethylenediaminetetraacetate), NaCl, trisodium citrate (chemically pure) were used in this work. All preparations were used without additional purification. The concentrations of the preparations used were determined spectrophotometrically using the following values of the extinction coefficients: \( \varepsilon_{260} = 6600 \, M^{-1} \cdot cm^{-1} \) for ctDNA, \( \varepsilon_{343} = 42000 \, M^{-1} \cdot cm^{-1} \) for H33258. The studies were carried out at the ionic strengths of the solution \( \mu = 0.002, 0.004 \) and \( 0.02 \, M \). All solutions contained monovalent Na\(^+\) cations and mixtures of bivalent cations form chelates with EDTA.

UV-melting of H33258 complexes with DNA was carried out on a UV/VIS PYE Unicam-SP-8-100 spectrophotometer (England). The thermostating cells were heated using SP 876 Series 2 program device. UV-melting was performed as described in [9]. Melting of DNA and its complexes with H33258 was carried out at a wavelength of 260 nm. The concentration ratio \( r = \text{ligand/DNA} \) varied in the range \( 0 \leq r \leq 0.33 \) (per nucleotide). When melting, the heating rate was 0.5°C/min, registration was carried out automatically through every 60 s. The data were displayed on a PC monitor in program software LabVIEW. Spectroscopic measurements were carried out in quartz cuvettes with a volume of 3 mL, an optical path length of 1 cm. The data were accumulated and analyzed using Excel software; the melting curves of the samples were also plotted in Excel. The melting curves were obtained as described in [9].

Results and Discussion. The A-form of nucleic acids is usually more dehydrated, and the water skeleton is absent in their minor groove, although it is present in the B-form of NA. This leads to a more unstable, double-stranded structure of the A-form of NA, since a decrease in the water component in the NA molecule leads to strengthening of repulsion between the electronegatively charged phosphate groups of opposite chains. That is why the A-form of NA is established at relatively high ionic strengths of the solution, at which the existing cations neutralize the negative charge of the opposite chains of NA. These peculiarities result in wider and shallower minor grooves as well as narrower and deeper major grooves of A-NA. In the case of B-DNA, the grooves have an opposite structure – shallower and wider major grooves and narrower and deeper minor grooves. Obviously, these structural peculiarities should certainly affect the affinity of groove binding ligands to NA [1].

Compounds from the Hoechst family are typical representatives of groove binding ligands (Fig. 1). When H33258 binds to DNA, it blocks DNA replication during cell division. Consequently, at low concentrations H33258 can actually be considered as an antibacterial or antitumor compound, while at high concentrations H33258 results in the destruction of the DNA structure. This ligand is a non-intercalator, since it preferably binds to AT-rich regions in the DNA minor groove. H33258 is also used as a DNA dye in both living and fixed cells, as well as in immunohistochecmical analyses. In the minor groove of B-DNA, H33258 binds to DNA by either high affinity (\( K_d \sim 1 \cdot 10^{-9} \, M \)) or low affinity (\( K_d \sim 1 \cdot 10^{-3} \, M \)), which corresponds to non-specific interaction of this ligand with sugar-phosphate skeleton of DNA [10]. Nonetheless, despite the abundance of experimental data, the mechanisms of H33258 binding to DNA cannot be considered definitively established, since this ligand can exhibit multimodality when interacting with NA.
Moreover, this ligand can initiate structural transitions in NA molecules, which can be detected by various methods, including UV-melting, which is one of the simple and highly informative methods. Based on this, we applied this method to study the interaction of H33258 with DNA depending on the ionic strength of the solution.

The melting curves of H33258 complexes with DNA at solution ionic strengths of 0.002, 0.004 and 0.02 M are presented in Fig. 2, a–c. It is obvious from Fig. 2 that at low concentrations of the ligand the melting curves are monophasic and shifted towards high temperatures. Another peculiarity of the melting curves is the decrease in the slope of these curves at low ionic strengths of the solution, which indicates the widening of the melting range of the complexes in relation to DNA. This fact cannot be interpreted from the point of preferable (specific) binding of H33258 to AT-pairs of DNA, since the melting temperature of AT-sequences – $T_{AT}$ should increase in this case; this conclusion follows from the shift of the melting curves towards high-temperature region. Due to this fact the width of the melting range, lying between $T_{AT}$ and $T_{GC}$, should decrease, which, in turn should result in enhancing of the slope of the melting curves. This is observed with an increase in the solution ionic strength. Particularly, at the ionic strengths of 0.004 M and especially 0.02 M, the slope of the melting curves increases, but at 0.02 M the width of the melting range of the complexes is less than that of DNA. Obviously, the specific (preferable) interaction of H33258 with AT-sequences is realized at the ionic strengths of the solution $\mu >0.004$ M; therefore, under conditions of low salt concentrations, the mechanism of H33258 binding to DNA differs from AT-specific interaction. It is also important to note that the melting curves of H33258 complexes with DNA at high ligand concentrations ($r >0.1$) (Fig. 2, a and b, curves 4 and 5) are biphasic. Analogous results were obtained for complexes of H33258 with poly(dA)-poly(dT) (B-form of NA) and poly(rA)-poly(rU) (A-form of NA) at different ionic strengths of the solution (Fig. 3, a and b) [11]. Particularly, it was revealed that at low concentrations of the ligand the melting curves of H33258 complexes with polyribonucleotide are monophasic, which indicates the transition of this polynucleotide and its complexes with the ligand from double-stranded (ds-) to single-stranded (ss-) state. Nevertheless, in the case of poly(dA)-poly(dT), a radically another scenario was observed, namely, the melting curves of the complexes were biphasic at low ligand concentrations (Fig. 3, a). Considering that the melting curve of poly(dA)-poly(dT) is smooth, it is obvious that this cannot results from the ds–ss transition of synthetic deoxyribonucleotide in a complex with a ligand. Based on this, we assume that H33258 induces structural transitions, which is reflected in the melting curves of the complexes. The opposition between the results obtained for DNA or nucleotide analogues poly(rA)-poly(rU) and poly(dA)-
poly(dT) appears to result from geometric differences in the NA grooves. However, this effect manifests itself independently of the change in the solution ionic strength for poly(dA)-poly(dT), despite the fact that at $\mu = 0.1 \, M$ it is less pronounced [11]. Preceding from this, we assume that the mentioned effect is a consequence of the structural peculiarities of poly(dA)-poly(dT), which were discussed in details in [12–17], as well as the influence of H33258 on the structure of this polynucleotide.

![Fig. 2. Melting curves of DNA (1) and its complexes with H33258 (2–5, a and b; 2–4, c) at the ionic strengths of the solution 0.002 $M$ (a), 0.004 $M$ (b) and 0.02 $M$ (c). Concentration of DNA was $\sim 6.5 \cdot 10^{-5} \, mol/L$; concentration ratio H33258 / DNA changed in interval $0 \leq r \leq 0.33$.](image)

It is known that various derivatives of H33258 possess a wide spectrum of biological activity, particularly, these compounds inhibit transcription and translation, they are toxic to human topoisomerase I and several lines of tumors, including mouse leukemia L1210 [18–22]. Despite the fact that H33258 binds preferentially to AT-sequences in the DNA minor groove, this may not underlie its toxicity to human topoisomerase I, since an analogous effect was not revealed in the case of other ligands binding in DNA minor groove. The toxicity of H33258 to topoisomerase I can be explained by the intercalation mode of binding in GC-rich regions of DNA [18–22]. Obviously, H33258 shows multimodality when interacting with DNA, which is confirmed by the results of our studies [4, 14, 23]. Taking this fact into account, we assume that the biphasic melting of H33258 complexes with poly(dA)-poly(dT) is due to duplex–triplex transitions, as a result of which the melting occurs in two phases; the biphasic transitions are observed when the absorption spectrum of the DNA triplex is recorded as a function of temperature. This manifests the dissociation of the DNA triplex into its counterpart duplex at low temperatures and further dissociation of the resulting DNA duplex into ss-structures at high temperatures [12, 13, 16]. The data obtained indicate that the ds-structure of DNA and poly(dA)-poly(dT) is more stable in the conditions of physiological solution or at relatively low ionic strengths, while the ds-form of poly(rA)-poly(rU) is metastable at low ionic strengths of the solution and in the presence of monovalent cations [11]. H33258, which is in a cationic form in the solution, apparently induces a transition of synthetic polynucleotides from ds- to triple- and ss-state.
However, minor groove-binding compounds such as H33258 stabilize DNA duplex much better than DNA triplex, assuming that relevant transformations take place in the minor groove at the ligand binding to the duplex major groove [16].

**Conclusion.** Thus, the data we obtained indicate that H33258 can exhibit multimodality when interacting with NA, though this ligand binds not only by different mechanisms, depending on the solution ionic strength [23], but also provokes structural reconstructions in molecules of NA. The analysis of received results indicates that H33258 initiates a transition from a double-stranded to a three-stranded state not only for DNA and poly(dA)-poly(dT), but also for poly(rA)-poly(rU), although for RNA analogue this effect is less expressed [11]. These properties of H33258 underlie the biological effect of this ligand on a cell and can have an important role from the practical point of view, considering that both the nucleotide sequence of NA and secondary or tertiary structures of them are prominent for the regulation of gene expression. Much attention is paid to intermolecular triplexes, which is explained by their potential therapeutic capacity to inhibit the gene expression, which in turn is very important for the treatment of cancer and other human diseases. These triplexes are able to target and inactivate the disease-causing genes, to stimulate DNA repair as well as recombination pathways, to provoke site-specific mutations and to interfere with DNA replication. From this point of view, the usage of small molecules is very perspective for the regulation of triplex-DNA conformations, as well as for specific interaction with these DNAs. These studies are valuable for designing more specific and efficient triplex-related gene targeting agents [24].

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