

# Spectrophotometrical study of mechanisms of cytidin analogues and ethidium bromide binding with DNA

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*To study the mechanisms of cytidine and its biologically active analogues binding to DNA we analyzed the binding of these ligands to DNA in the presence of well-known intercalator ethidium bromide (EtBr). Thereto, we have carried out the detailed spectrophotometric research of EtBr-DNA mixtures absorption in the presence of cytidine and its analogues in the wide range of wavelengths and DNA concentrations. Cytidine derivatives containing aza group in the cytosine ring (6AZC, AZAfur and AZAxyl) compete with EtBr for the DNA binding sites. The binding constants and binding site sizes of ligand-DNA complexes were calculated via absorption spectra optimization programs COMP and DALSMOD. Unmodified in the cytosine ring ligands (cytidine and Ara-C) do not compete with EtBr for the DNA binding sites, however, they contribute to the change in concentration dependencies of titration curves in the region of low DNA concentrations. This phenomenon can be explained by the cytidine and Ara-C influence on the DNA conformation in the presence of EtBr at low  $P/D_{EtBr}$  values, where  $P/D_{EtBr}$  is the phosphate/EtBr ratio.*

*Key words: cytidine analogues, DNA, models of binding, spectrophotometry, ethidium bromide.*

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**Introduction.** Nucleosides represent the class of biologically active agents, interacting with DNA and influencing its function in cells. Many representatives of this group are the pharmaceutical medications of gene-directed action, widely used in the antitumour therapy of leukemia and other diseases [1-5]. Interaction of therapeutically active nucleosides with DNA in cell causes inhibition of nucleic acid synthesis and apoptosis [6]. For example, one of the most effective

drugs used in the treatment of leukemia, namely, cytosine arabinoside (Ara-C), is phosphorylated inside cells by their enzymes to cytotoxic form of cytosine arabinoside triphosphate and is incorporated into DNA by DNA polymerase [7, 8]. After Ara-C incorporation into DNA, further DNA synthesis is inhibited, what leads to the cell death. Another cytidine (Cyd) analogue 6-azacytidine has antitumour and antiviral activity [9-12]. Unlike Ara-C, there are no exact data on 6AZC biological action. In literature there is only description of indirect influence of 6AZC on virus reproduction inhibition which results in decrease of virus DNA and polypeptide synthesis [9, 10]. Inhibition of

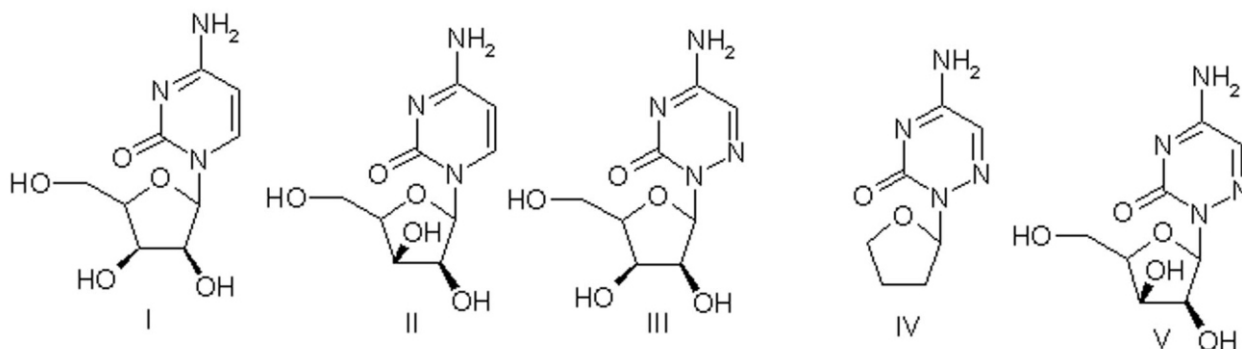


Fig.1 Chemical structures of cytosine (Cyt) (1), cytosine arabinoside (Ara-C) (2), 6-azacytosine ribofuranoside (6AZC) (3), 6-azacytosine tetrahydrofuran (AZAfur) (4), 6-azacytosine xylofuranoside (AZAxyl) (5)

tumor growth is also assumed to be associated with decrease of macromolecules synthesis [11, 12].

Thus, the mechanism of 6AZC influence on DNA biosynthesis and 6AZC interaction with DNA remains unknown. In addition to incorporation of nucleosides into DNA molecule with cell enzymes it is also possible that they intercalate into DNA or have the groove binding. Nevertheless, the molecular mechanisms of their interaction with DNA still remain unclear. In this respect, it is interesting to study possible mechanisms of interaction of cytidine analogues with DNA using new theoretical (modeling of the processes) and experimental (competitive binding) approaches.

One of the informative methods of study on the ligand-DNA interactions is spectrophotometry which allows not only detecting the ligand-DNA complex formation and but also revealing the characteristics of possible mechanisms of such interaction by appropriate modelling approaches [13, 14].

As absorption spectra of nucleosides are very similar to the nucleic acids absorption spectra, we have used the method of study of their competitive binding to DNA in the presence of colored label. We used ethidium bromide (EtBr) as label-ligand, absorbing in the VIS spectra region.

**Materials and Methods.** Commercial calf thymus DNA has been purchased from *Serva*. Ethidium bromide has been purchased from *Fluka* (Switzerland). Cytidine and its analogues (cytosine arabinoside (Ara-C), 6-azacytosine ribofuranoside (6AZC), 6-azacytosine tetrahydrofuran (AZAfur), 6-azacytosine xylofuranoside (AZAxyl)) have been synthesized in the

Institute of Molecular Biology and Genetics NAS of Ukraine. Concentrations of nucleosides have been determined by the weight method.

Chemical structures of the nucleosides studied are shown in Fig.1.

Concentrations of DNA ( $C_p$ , moles of phosphates) and EtBr ( $C_D$ ) have been determined by absorption spectroscopy using molar extinction coefficients at absorption maximum:  $\epsilon_{260} = 6.4 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  for calf thymus DNA and  $\epsilon = 5.85 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  for EtBr [15]. All absorption measurements have been carried out on Specord M 40 (Germany) spectrophotometer in thermostatic quartz cells, having light paths of 10 and 20 mm. Complexation studies have been carried out in phosphate buffer ( $2.5 \cdot 10^{-2} \text{ M KH}_2\text{PO}_4$ ,  $2.5 \cdot 10^{-2} \text{ M Na}_2\text{HPO}_4$ , pH 6.86). P/D is phosphate/dye ratio. All preparations have been used without further purification.

As there was a possibility that cytidine and its analogues could make heteroassociates with EtBr, we have checked their influence on the EtBr spectrum in the absence of DNA. We have found that these nucleosides do not influence EtBr spectrum and therefore there is no heteroassociation between these ligands.

Calculation of binding parameters of nucleoside-DNA complexes (binding constants and binding site sizes) has been performed according to two different models of binding.

In *Model 1* only one type of nucleoside-DNA and EtBr-DNA complexes is presumed. Values of ligands binding sites  $n_1$  and  $n_2$  are allowed to vary in a wide region of values. Equilibrium concentrations of free and bound ligands for every mixture with total concentra-

tions of ligands and DNA  $C_{Di}$ ,  $C_{pi}$ , respectively, are calculated according to equations (1)-(4):

$$\frac{R_1}{m_1} K_1 \left[ \frac{1}{1 + \frac{R_1 n_1}{R_2 n_2} + R} \right]^{n_1} \quad (1) \quad (R_1 n_1 + R_2 n_2 + R); \quad (1)$$

$$\frac{R_2}{m_2} K_2 \left[ \frac{1}{1 + \frac{R_1 n_1}{R_2 n_2} + R} \right]^{n_2} \quad (1) \quad (R_1 n_1 + R_2 n_2 + R); \quad (2)$$

$$C_{D1} = m_1 R_1 C_p; \quad (3)$$

$$C_{D2} = m_2 R_2 C_p. \quad (4)$$

Equations (1) and (2) describe the competitive binding of two ligands to DNA [17]. Equations (3) and (4) represent the law of mass balance for ligands.

The following designations have been used in equations (1) – (4):  $m_1$  and  $m_2$  are the equilibrium concentrations of free ligands, where index 1 is used for nucleosides and index 2 for EtBr,  $R_1$  и  $R_2$  – are the shares of bound ligands determined as quotient from the division of corresponding complexes concentrations to  $C_p$ ,  $K_1$ ,  $K_2$  – are the binding constants for ligands 1 and 2 on binding sites  $n_1$  and  $n_2$  DNA bases per ligand,  $R$  is the sum ( $R_1 + R_2$ ).

In *Model 2* binding of nucleoside to DNA is described with the assumption on the fixed value of binding site size  $n_1=1$  (one nucleoside per one DNA phosphate), when EtBr binds to DNA in a cooperative manner, *i.e.* value is varying ( $\alpha$  is cooperative parameter which characterizes the probability of ligand binding to the adjacent binding sites). Such model allows taking into account changes in the spectrum of the bound EtBr when it does not have neighbours (high  $P/D_{EtBr}$  values) or when it may have neighbours (low  $P/D_{EtBr}$  values). To calculate the equilibrium concentrations of EtBr-DNA-nucleoside mixture in this case we use the equation system, analogous to the one described above but instead of equation (2) the McGhee and von Hippel equation for cooperative binding [18] is introduced. Such binding model was described by us in [19]. The  $R_1 C_p$  value is the sum of concentrations of

monomerically bound ligands (without neighbours) and ligands having neighbours. The concentration of the monomerically bound ligand is calculated using the following relation  $C_{m.b.} = (R_1 - C_D) / R_1 C_D$  [17]. Such separation of concentrations is necessary because we assume that monomerically bound ligands and ligands having neighbours can have different absorption spectra.

Calculation of the equilibrium composition of mixtures and binding constant values according to Models 1 and 2 was carried out via COMP [20] and DALSMOD [21, 22] optimization programs, respectively. COMP and DALSMOD optimization programs have been developed as new versions of the original DAL program [23] by changing the procedure of equilibrium concentration calculation. In COMP optimization program the equations (1) – (4) are used in order to calculate the equilibrium concentrations in the study on complexation of ligands (or drugs) with polymeric matrices [19]. Detailed description of DALSMOD optimization program is given in [20, 21]. In these programs optimal values of molar extinction coefficients, binding constants and binding site sizes for each type of complex are calculated through minimization of the sum of squares of deviations of experimental absorptions  $A_{ij}^0$  from calculated ones  $A_{ij}$ , in wide wavelength and concentration ranges.

The values of absorption  $A_{ij}$  are calculated according to the Beer's law:

$$A_{ij} = \sum_k \epsilon_{jk} l C_{ki},$$

where  $\epsilon_{jk}$  is the molar extinction coefficient of  $k^{\text{th}}$  component in  $j^{\text{th}}$  wavelength, and  $c_{ki}$  is the equilibrium concentration of corresponding component in every  $i^{\text{th}}$  mixture,  $l$  is the optical path length.

The optimization procedure is terminated when further iterations of optimized parameters ( $K_i$  and  $\alpha_{ij}$ ) do not improve the value of optimized function for each model tested. At the end of optimization process the values of both  $Q$  and  $Q_{\text{lim}}$  Hamiltonian factors [23] are calculated:

$$Q = \left\{ \sum_{ij} (A_{ij}^0 - A_{ij})^2 / \sum_{ij} (A_{ij}^0)^2 \right\}^{1/2}; \quad (5)$$

$$Q_{\text{lim}} = \left\{ \sum_{ij} (e^{-\alpha_{ij}} - A_{ij}^0)^2 / \sum_{ij} (A_{ij}^0)^2 \right\}^{1/2}, \quad (6)$$

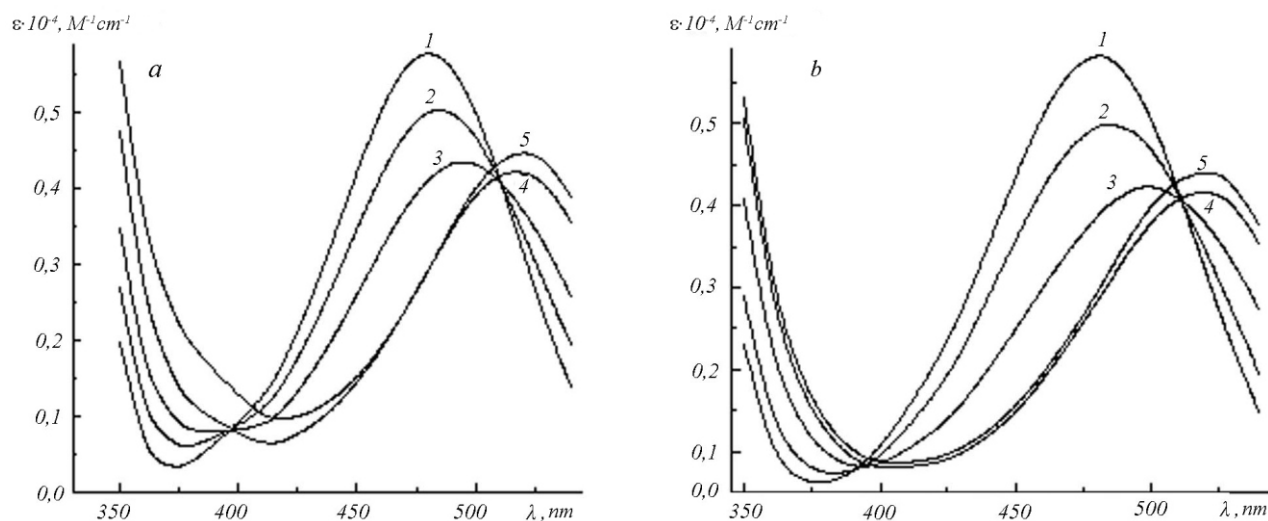


Fig.2 Absorption spectra of EtBr-DNA mixtures at  $C_{\text{EtBr}} = 1.145 \cdot 10^{-4}$ ,  $P/D = 0$  (1), 1.0 (2), 2.0 (3), 5.0 (4), 21.7 (5) (a), EtBr-DNA-6AZC mixtures at  $C_{\text{EtBr}} = 9.865 \cdot 10^{-5}$  M,  $C_{6\text{AZC}} = 1.124 \cdot 10^{-4}$  M,  $P/D = 0$  (1), 1.1 (2), 2.7 (3), 7.3 (4), 13.4 (5) (b).

where  $e_{ij}$  is the deviation of absorbance in the  $i^{\text{th}}$  mixture corrected to the 1% error in the total component concentrations and the 0.005 optical unit error in the measurement of absorbance.  $Q$  and  $Q_{\text{lim}}$  characterize the correspondence of the binding model to the experimental data. The selected model (and corresponding binding parameters  $n_1$ ,  $n_2$  and  $\dots$ ) satisfy the experimental absorption data if  $Q < Q_{\text{lim}}$ .

**Results and Discussion.** Fig.2, *a* shows the absorption spectra of EtBr-DNA mixtures in wide DNA concentration range ( $(0 \text{--} 2.5) \cdot 10^{-3}$  M). It can be seen that not all the spectra of the studied  $P/D_{\text{EtBr}}$  region go through the isobestic points ( $\lambda = 390$  nm and  $\lambda = 510$  nm). This is the evidence of formation of more than one EtBr-DNA complex. Formation of several types of EtBr-DNA complexes is confirmed by other authors [24-26]. At the same time, many authors insist that at low  $P/D_{\text{EtBr}}$  values when EtBr molecules bind closely to each other, the  $B > A$  conformational transition of DNA molecule can be observed [27-31]. As many authors say that EtBr intercalates into DNA, we can assume that there is intercalation both in the adjacent base pairs and intercalation according to the law of exclusion of the nearest neighbour. Absorption spectra of these complexes are different. This results in appearance of the pseudoisobestic points on the EtBr-DNA absorption

spectra. Also titration curves of EtBr-DNA mixtures could have stages because of different molar extinction coefficients of complexes (Fig.3, *a*, curves 1 and 3).

Let us consider the way cytidine and its analogues exert influence on EtBr binding to DNA. The EtBr-DNA absorption spectra change weakly in the presence of nucleosides (Fig.2, *b*) but these changes can be clearly seen in the titration curves of EtBr-DNA mixtures in the absence and in the presence of these competing ligands (Fig. 3-5).

All figures show titration curves at  $\lambda = 340$  nm. Spectral changes at this wavelength, belonging to the second absorption band with the absorption maximum at  $\lambda = 270$  nm, are more significant than in another absorption maximum at  $\lambda = 480$  nm. One can see from the figures that different nucleosides cause different changes of EtBr-DNA mixtures titration curves. Thus, in the presence of unmodified cytosine analogues (cytidine and AraC) (Fig.3), the stage on the EtBr-DNA-nucleoside titration curve becomes more evident. But further increase of  $P/D_{\text{EtBr}}$  value differences in titration curves in the absence and in the presence of these nucleotides is lower. One can assume that these derivatives are not direct competitors of EtBr during its binding to DNA but instead they change DNA hydration environment and maintain DNA molecule in

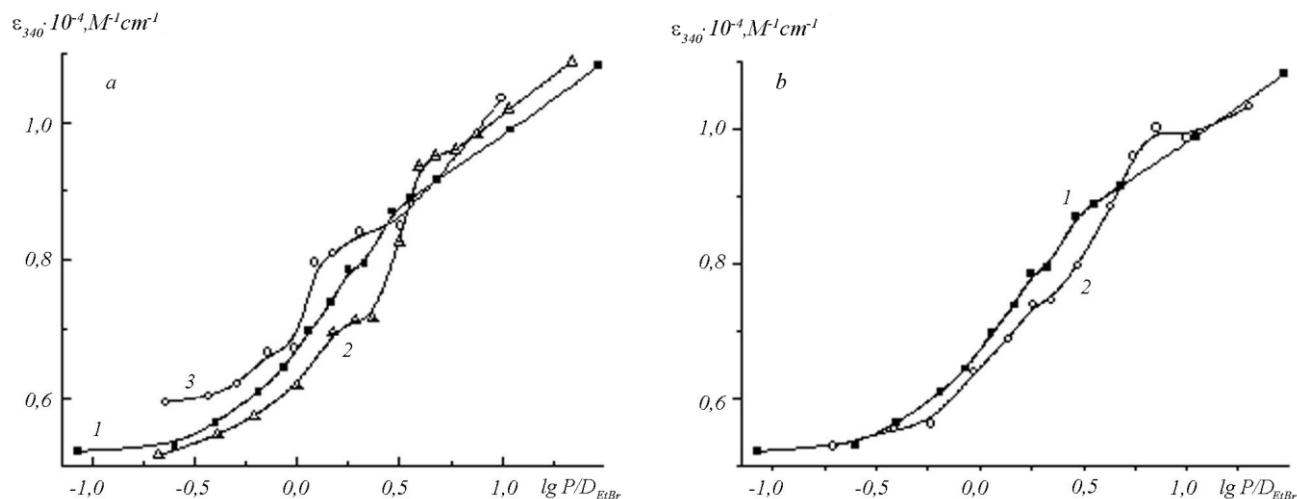


Fig.3 Concentration dependencies of the absorption of EtBr-DNA mixtures ( $C_{\text{EtBr}} = 1.145 \cdot 10^{-4}$ ) (1, a, b) and EtBr-DNA-AraC ( $C_{\text{EtBr}} = 4.7 \cdot 10^{-5}$ ,  $C_{\text{AraC}} = 4.48 \cdot 10^{-4}$ ) (2) (a) and EtBr-DNA-Cyt mixtures ( $C_{\text{EtBr}} = 4.9 \cdot 10^{-5}$ ,  $C_{\text{Cyt}} = 1.3 \cdot 10^{-4}$ ) (2) (b) at  $\lambda = 340$  nm of  $P/D_{\text{EtBr}}$

A-like conformation. As none of the models described reveals conformational changes of DNA in the binding process, we can not calculate the binding parameters of these derivatives with DNA. Nevertheless, we can assume that cytidine and AraC influence EtBr binding to DNA in a different way, for example, they can interact with DNA functional groups which are not involved in binding with EtBr but thus produce sterical hindrance for EtBr binding to DNA molecule.

As seen from Fig. 4 and 5, modified cytidine analogues (6AZC, AZAfur and AZAxyl), having aza group in cytosine ring, compete with EtBr for DNA binding sites. This is evident from the increase of the mixture absorption caused by DNA concentration rise. Also stages on EtBr-DNA titration curves in the presence of these ligands decrease, *i.e.* 6AZC, AZAfur, and AZAxyl bind to DNA in B-conformation. Binding parameters of complexes of these ligands with DNA in the presence of EtBr is calculated using the models of binding described above.

We have calculated binding parameters of 6AZC, AZAfur and AZAxyl complexes with DNA using the *Model 1* and COMP optimization program. As molar extinction coefficients of the two EtBr complexes with DNA are similar and in the presence of

competing nucleosides the amount of EtBr aggregates on DNA is decreased, in this model we assume that EtBr forms only one type of complexes with DNA. Values of binding constants and binding site sizes are shown in Table 1.

It can be seen from Table 1 that *Model 1* is sufficient to describe spectral changes which take place in nucleotide-DNA-EtBr systems, as  $Q < Q_{\text{lim}}$  in all cases. Also it is evident that binding site sizes for all nucleosides are approximately one DNA base per one nucleoside molecule.

As EtBr forms two complexes with DNA with two different molar extinction coefficients we have used *Model 2* which takes this fact into account, assigning  $n_1 = 1$ . Values of binding parameters for nucleosides calculated via *Model 2* using DALSMOD optimization program are shown in Table 2.

*Model 2* is also sufficient to describe the nucleotide-DNA-EtBr system, as in all cases  $Q < Q_{\text{lim}}$ . It can be seen that binding constants of 6AZC-DNA and AZAxyl-DNA complexes, calculated via two different binding models, coincide. This fact evidences that 6AZC and AZAxyl are not sensitive to differences of EtBr-DNA complexes molar extinction coefficients as they are competitors of only second type of complexes.

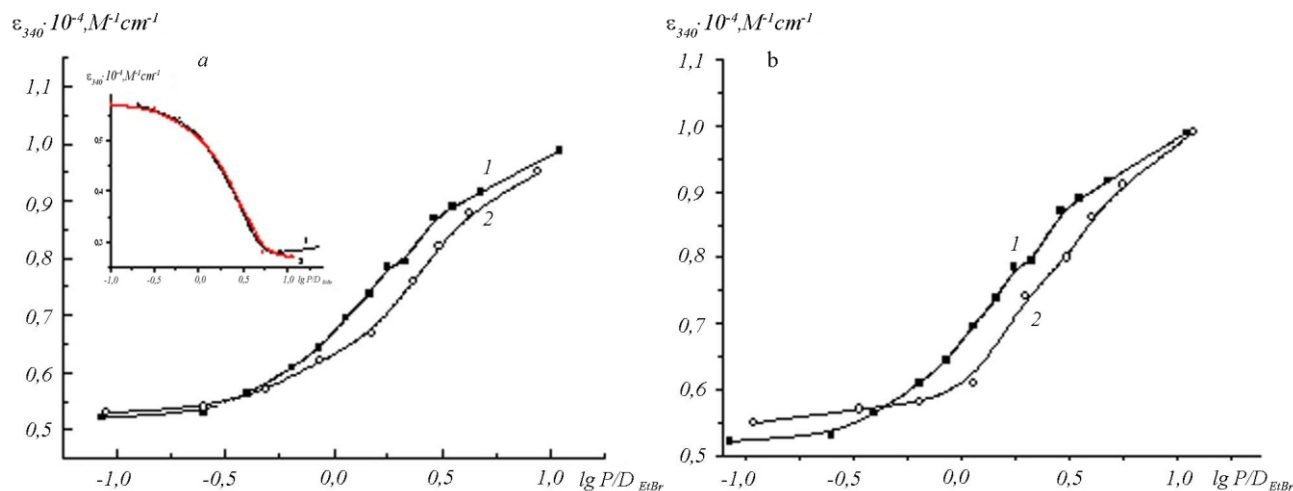


Fig.4 Concentration dependencies of the absorption of EtBr-DNA mixtures ( $C_{\text{EtBr}} = 1.145 \cdot 10^{-4}$ ) (1 *a, b*) and EtBr-DNA-AZAfur mixtures ( $C_{\text{EtBr}} = 7.77 \cdot 10^{-5}$ ,  $C_{\text{AZAfur}} = 3.01 \cdot 10^{-5}$ ) (2) (*a*) and EtBr-DNA-6AZC ( $C_{\text{EtBr}} = 7.54 \cdot 10^{-5}$ ,  $C_{\text{6AZC}} = 6.92 \cdot 10^{-4}$ ) (2) (*b*) at  $\lambda = 340$  nm of  $P/D_{\text{EtBr}}$ . Inset on Fig.4, *a*: Concentration dependencies of the absorption of EtBr-DNA mixtures ( $C_{\text{EtBr}} = 1.145 \cdot 10^{-4}$ ) (1) and EtBr-DNA-AZAfur mix-

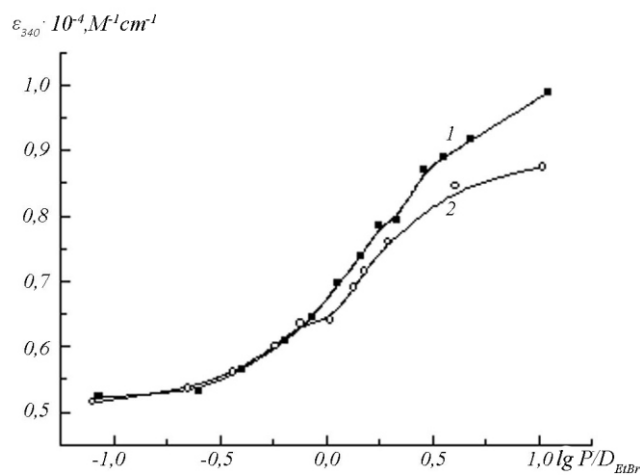


Fig.5 Concentration dependencies of the absorption of EtBr-DNA mixtures ( $C_{\text{EtBr}} = 1.145 \cdot 10^{-4}$ ) (1) and EtBr-DNA-AZAxyl mixtures ( $C_{\text{EtBr}} = 1.28 \cdot 10^{-4}$ ,  $C_{\text{AZAxyl}} = 3.51 \cdot 10^{-4}$ ) (2) at  $\lambda = 340$  nm of  $P/D_{\text{EtBr}}$ .

Differences in binding constants of AZAfur-DNA complex are probably related to the fact that in *Model 1* formation of two EtBr-DNA complexes with different molar extinction coefficients is not taken into account, but AZAfur is a competitor of both EtBr-DNA complexes. Also it is significant that binding constants of ligands with different OH-groups position in furanose ring vary

considerably, *i.e.* both structure and conformation of glycoside fragments are essential for binding of these ligands to DNA.

As binding site sizes for 6AZC, AZAfur and AZAxyl complexes with DNA are approximately one DNA base per ligand molecule, these nucleosides do not show AT- or GC- binding specificity and do not intercalate into DNA because about 4-5 DNA bases per ligand are needed for the intercalative complex. Therefore, one can assume that 6AZC, AZAfur, and AZAxyl can interact with DNA by their azo groups (N-6 atom of threazine bases) and DNA aminogroups. EtBr also interacts with the DNA aminogroups while intercalating into DNA [32].

**Conclusions.** Detailed spectrophotometric study of cytidine and its analogues binding to DNA has shown that EtBr can be used as colored label in spectrophotometric absorption studies in VIS region of spectra. Analysis of nucleoside-DNA-EtBr titration curves has shown that unmodified cytidine analogues (cytidine and AraC) do not compete with EtBr for DNA binding sites. On the other hand, these nucleosides influence EtBr binding to DNA at low  $P/D_{\text{EtBr}}$  values. Modified cytidine analogues (6AZC, AZAfur and AZAxyl) compete with EtBr for DNA binding sites at high  $P/D_{\text{EtBr}}$  values.

Binding parameters of nucleoside complexes with DNA calculated via COMP optimization program using Model 1 and via DALSMOD optimization program using Model 2

Parameter	6AZC	AZAfur	AZAxyl
<i>Model 1</i>			
$K_1, M^{-1}$	(1,4 0,5) $10^4$	(2,5 0,3) $10^4$	(9,5 0,5) $10^{24}$
$n_1$	1,1 0,1	1,2 0,1	1,1 0,1
$n_2$	2,9 0,1	3,0 0,4	2,5 0,1
$Q$	0,027	0,024	0,018
$Q_{lim}$	0,031	0,035	0,030
<i>Model 2</i>			
$K_1, M^{-1}$	(1,3 0,3) $10^4$	(8,3 1,3) $10^3$	(8,7 1,5) $10^2$
$n_1$	1,0	1,0	1,0
$n_{21}$	2,8 0,1	2,9 0,1	2,9 0,1
$Q$	0,027	0,023	0,013
$Q_{lim}$	0,036	0,031	0,033

We can assume that both azagroups of these nucleosides and their glycoside fragment interact with DNA. It is shown that proposed system of equations (1) – (4) as well as the model of competitive binding proposed by Nechipurenko [17] and COMP optimization program [20] can be used for calculation of binding parameters of ligands, competing for DNA binding sites. Binding constants of 6AZC and AZAxyl complexes with DNA calculated via two different models of binding coincide very well. Binding constants of complex of AZAfur which does not have OH-groups in furanose ring with DNA calculated via two different models of binding do not coincide. This could be related to the fact that EtBr forms two complexes with DNA and this is not taken into account in *Model 1*. Binding constants of 6AZC and AZAfur complexes with DNA have much higher values than for those for AZAxyl, hence nucleoside sugar structure and conformation influence greatly their ability to bind to DNA.

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Спектрофотометрическое исследование механизмов связывания аналогов цитидина и бромистого этидия с ДНК

## Резюме

Для выяснения механизмов связывания цитидина и его биологически активных производных с ДНК исследовано их взаимодействие с ДНК в присутствии интеркалятора бромистого этидия (ЭБ). Проведен спектрофотометрический анализ электронных спектров поглощения смесей ЭБ–ДНК в присутствии цитидина и его аналогов в широкой области длин волн и концентраций ДНК. Аналоги цитидина, содержащие дополнительный атом азота в гетероцикле (6AZC, AZAfur и AZAxyl), конкурируют с ЭБ за места связывания на ДНК. Константы ассоциации и параметры мест связывания образующихся комплексов для этих производных рассчитаны с помощью программ оптимизации спектров поглощения смесей ЭБ–ДНК–нуклеозид COMP и DALSMOD. Немодифицированные по цитозинового кольцу нуклеозиды (цитидин и Ага-С) не являются конкурентами ЭБ за места связывания на ДНК, однако в их присутствии изменяются концентрационные зависимости кривых титрования ЭБ в области низких концентраций ДНК. Это можно объяснить влиянием упомянутых нуклеозидов на структурные или конформационные изменения матрицы ДНК в присутствии ЭБ в области низких значений  $P/D_{ЭБ}$ , где  $P/D_{ЭБ}$  – отношение общих концентраций ДНК и ЭБ.

Ключевые слова: аналоги цитидина, ДНК, модели связывания, спектрофотометрия, бромистый этидий.

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