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Study of Applicability of the Bifunctional System "Ethidium Bromide + Hoechst-33258" for DNA Analysis

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Abstract—Changes in absorbance and fluorescence excitation and emission spectra in the ultraviolet and visible regions of the system containing ethidium bromide (EtBr) and Hoechst-33258 (Ht) were investigated depending on various DNA quantities and the composition of the medium. It is noted that spectral properties of this system are determined by interactions of EtBr and Ht both with nucleic acid and with one another (for example, joint sorption of EtBr and Ht on DNA may involve fluorescent resonance energy transfer between the dye molecules). Thus, different modes of EtBr and Ht specificity to substrate and assay conditions suggest that combined use of these dyes provides some additional effects that may be interesting in terms of structure-functional study of nucleic acid. Some of these effects are considered in this paper.

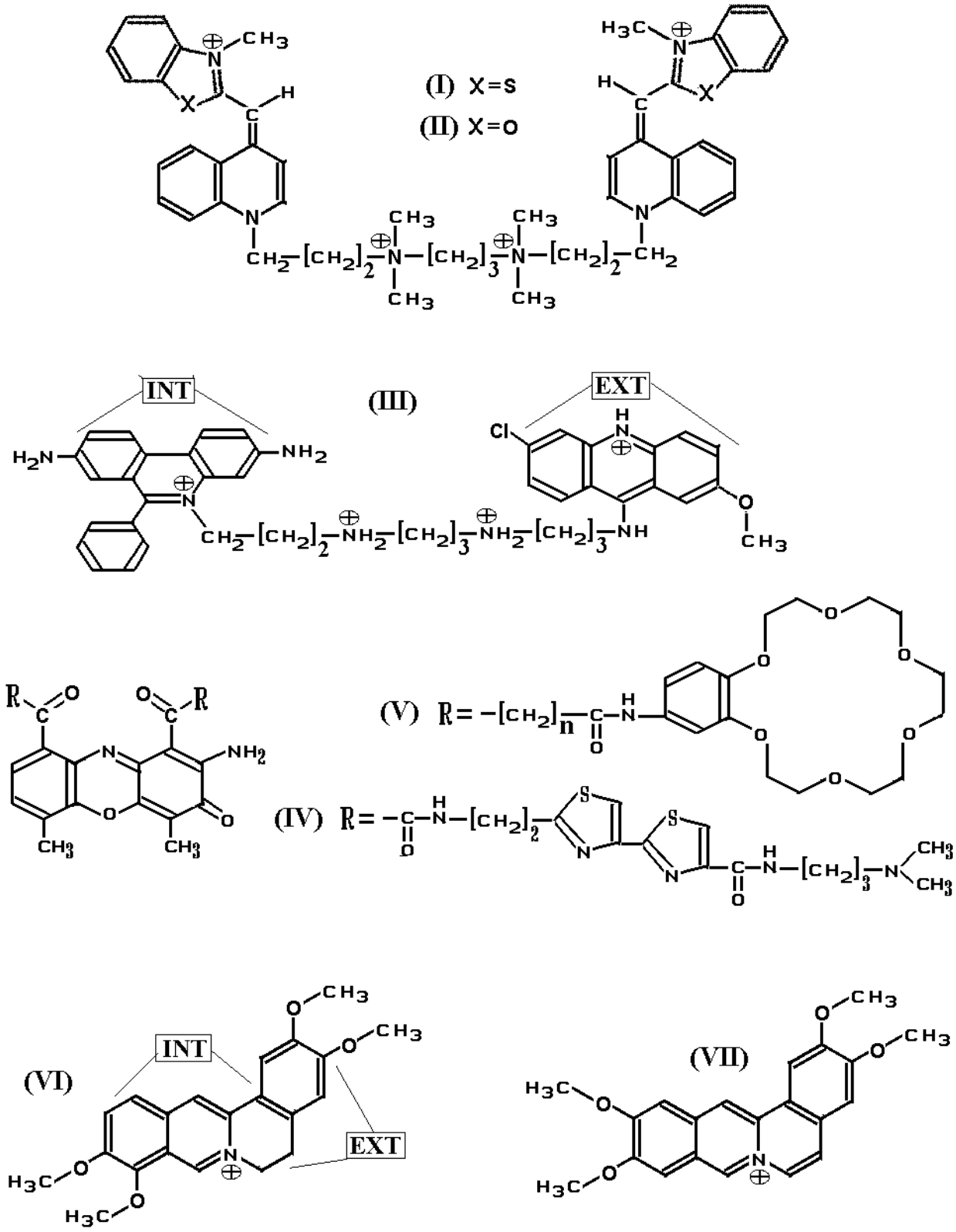
Key words: DNA, fluorophores, spectral properties, energy transfer

Artificially synthesized compounds that can bind to certain nucleotide sequences in a genome [1-6] (and especially those of these compounds that on such interaction, for example, can sharply alter intensity of their emission [7-12]) are becoming increasingly popular. All presently known compounds that reversibly (noncovalently) bind with polynucleotides are subdivided into two large classes by type of interaction with substrate: "polynucleotide external binding compounds" (non-intercalators) and intercalators. Non-intercalators bind to external parts of a polynucleotide molecule and do not alter integrity of the substrate structure; and there are mainly two primary and secondary orders of nucleic acid structure [9, 13-16]. Intercalators are inserted between complementary base pairs of the polynucleotide double helix; and they are mainly specific to secondary structure of nucleic acids and degree of their supercoiling [7, 17-20].

Many nucleotide-binding fluorophores are now widely used [7, 9, 16-18]. However, the development of the theoretical basis for design of new compounds (possessing higher sensitivity and specificity to substrate than earlier used compounds) is still an important task. The latter is also complicated by the fact that usually "potent" selective binding to substrate requires certain structural features of the ligand molecule, whereas detection of its binding requires other structural features of the ligand molecule.

One of the ways to solve this problem is to construct complex heterofunctional compounds consisting of several subunits, each of which is individually responsible for specific binding to substrate or for detection, respectively. If such heterofunctional compounds also consist of several subunits that can potentially bind to the substrate, it is reasonable to expect increased affinity and specificity (compared with monomeric constituents). Similarly, if such a complex compound contains several subunits that increase fluorescence during sorption onto substrate, we expect increased sensitivity of registration of such substrate compared with that of monomeric constituents. For example, compounds I and II (Scheme 1) are polynucleotide "bis-intercalators" exhibiting increased affinity to nucleic acids and higher fluorescence during interaction with them than any "mono-intercalators" known to date [21, 22]. However, construction of such complex heterofunctional compounds does not always result in development of end products with predictable properties. For example:

– in the case of compound III (Scheme 1) and related structures [23, 24] (which share properties of "classic bis-intercalators"), intercalation of both "functional" (ethidium and acridine) fragments into polynucleotide can take place only if the length of the linker joining these fragments together exceeds some critical value; otherwise, intercalation of only one (ethidium) fragment into nucleic acid would be observed and the second (acri-



"INT" and "EXT" show fragments of "heterofunctional" compounds intercalating or externally bound to nucleic acids

Scheme 1

dine) fragment binds to the external part of the polynucleotide;

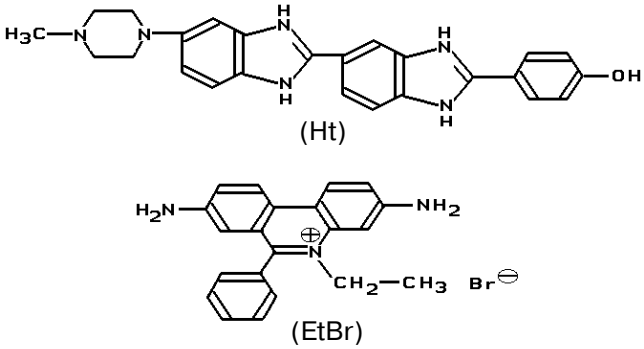
– at $r < 0.07$ (r is the amount of bound ligand per unit of substrate concentration) compound IV (Scheme 1) interacts by intercalation due to the presence of its bithiazole fragments, whereas at $r > 0.07$ it binds nonspecifically to polynucleotide due to the presence of the phenoxazine moiety, which itself can exhibit intercalating properties under other conditions [25];

– at $n = 1$ (i.e., when one CH_2-group links the phenoxazine moiety with the benzcrown fragment of the ligand molecule) and $r < 0.1$ compounds V (Scheme 1) interact with nucleic acids by intercalation due to the phenoxazine moiety; at $n = 0$ or 2, or at $n = 1$ with $r > 0.1$ they bind to nucleic acids via their benzcrown fragments, whereas at $n > 2$ they poorly interact with nucleic acids [26];

– compound VI (Scheme 1) demonstrates properties of both intercalator and non-intercalator (compound externally bound to nucleic acids), whereas its analog (VII) dehydrogenated in one of its aromatic cycles interacts with DNA as intercalator only [27].

So, use of systems including several nucleotide-specific fluorophores, which are indirectly linked together via the substrate but share concerted complex-forming and spectral properties, is more promising for analysis of nucleic acids.

One system might include two separately used fluorescent dyes, e.g., Hoechst-33258 (Ht) (2-[2-(4-hydroxyphenyl)-5(6)-benzimidazoyl-]-5(6)-(1-methyl-4-piperazinyl)benzimidazole) and ethidium bromide (EtBr) (2,7-diamino-10-ethyl-9-phenylphenanthridinum bromide) (Scheme 2).



Scheme 2

EtBr is an intercalator that lies between two base pairs of the polynucleotide double helix; it exhibits moderate preference to GC-rich sites. In the nucleic acid-binding state of EtBr is characterized by maxima of fluorescence excitation and emission localized in the visible part of spectrum at 520 and 605 nm, respectively [13, 28]. Ht is an outer nucleic acid binder specific for sites of polynucleotide double helix containing three sequentially positioned AT and only GC base pairs; in the DNA-binding state this dye is characterized by maxima of fluorescence excitation and emission in the visible part of spectrum at 350 and 455 nm, respectively [7, 29]. Thus, combined use of Ht and EtBr for nucleic acid analysis may give additional information not only about polynucleotide content in a sample [7, 30], but also about nucleic acid primary and secondary structure [31] and degree of supercoiling [11, 32-34].

Joint sorption of Ht and EtBr on a polynucleotide can also be accompanied by fluorescent non-radiative energy transfer. This phenomenon takes place when a wavelength region of fluorescent emission of one dye (energy donor; in our case this is Ht) is localized within the wavelength region of fluorescence excitation of the other dye (energy acceptor; in our case this is EtBr). In such case, when the primary excited molecule (energy donor) enters weak (dipole–dipole) interaction with another molecule (energy acceptor) positioned at a distance shorter than the emission wavelength of the first molecule, additional probability appears that the donor molecule will transit into an electron-oscillation state with lower energy, whereas the acceptor molecule may transit into a state with higher energy. Effectiveness of such resonant energy transfer (E is determined by the ratio of number of energy quanta transferred from donor to acceptor to total number of quanta emitted by donor during its transition from excited state to ground state under the same conditions but without acceptor) depends on the distance between the chromophore groups of excited donor and acceptor (f):

$$E = f_0^l / (f_0^l + f^l), \quad (1)$$

where f_0 is Forster critical radius (the f value at which probability of donor transition from excited state to ground state due to energy transfer to acceptor represents the sum of probabilities of all other processes leading to donor transition from excited state to the ground state under the same conditions but without acceptor); and $l = 2$, 4, or 6 (depending on dipole–dipole interactions between donor and acceptor molecules) [35-37].

It should be noted that phenomenon when fluorescence involves not only energy donor but also acceptor can be observed at distances between donor and acceptor molecules exceeding the wavelength of emission of the donor molecule. However, in this case the mechanism of energy transfer is different (the acceptor molecule reabsorbs quanta emitted by the donor) and the dependence E versus f also differs from Eq. (1). Phenomenon of fluorescent non-radiative resonance energy transfer can also be observed between various fragments of a "multifunctional" compound. It, for example, takes place in case of dye III (Scheme 1) [23, 24]. Also, considering systems containing nucleic acid and several nucleic-acid-specific lig-

ands, one should take into account the possibility of competition between these ligands for binding sites on their common high molecular weight substrate (nucleic acid) and some other things that have been analyzed in the literature.

MATERIALS AND METHODS

All measurements were carried out at constant concentrations of the dyes and variable DNA concentrations at 20-25°C. Absorbance spectra of all solutions studied were registered using a Beckman model 35 spectrophotometer (Austria). Fluorescence was analyzed using a Hitachi 850 spectrofluorimeter (Japan). Fluorescent spectra were recorded with monochromator slits for excitation and emission of 3 nm, using scan rate of 120 nm/min, response time of 2 sec, and normal photoelectric multiplication. All measurements were carried out in standard 1 cm cuvettes. Fluorescent spectra were corrected following the spectrofluorimeter manual and using a quantum counter based on standard alcohol solution of rhodamine B.

Substances were analyzed in the following buffers: 0.01 M NaCl + 0.01 M Na_2EDTA + 0.01 M Tris (pH 7.4) (1) and 19 parts (v/v) buffer 1 + 1 part of the lysing mixture (used for isolation of intact DNA from cells [38]) containing 2 M NaCl + 0.1 M Na_2EDTA + 0.01 M Tris (pH 8.0) + 0.5% (v/v) Triton X-100 (2).

Commercially available Ht, EtBr, Na_2EDTA (ethylenediamine tetraacetate, disodium salt), Tris (2-amino-2-hydroxymethyl-1,3-propane diol), and Triton X-100 (4-octyl-[2,4,6,8,10-decapentol]hydroxybenzene) were obtained from Serva (Germany). Calf thymus DNA obtained from Serva was used as the standard substrate; it had 58% AT pairs, average molecular mass (per one nucleotide) of 326 daltons, and molar absorbance coefficient ε_{260} = 6600 $M^{-1}\cdot cm^{-1}$. For homogenization and reduction of light scattering standard DNA was sonicated for 15 sec using a UZDN-2 (Russia) disintegrator at 0.3 A and 22 kHz. Other chemicals were of "chemically pure" grade. All concentrations (except specially stated) are given as molar concentrations.

RESULTS AND DISCUSSION

Figure 1 shows the individual spectral behavior of EtBr in the presence of various quantities of DNA in aqueous buffer 1 with low ionic strength. It is similar to our earlier observed individual spectral behavior of Ht under similar conditions [28]. However, wavelengths of fluorescent excitation and emission and ratios $\Delta A/\Delta C_{DNA}$ and $\Delta I/\Delta C_{DNA}$ differed (A, I, and C_{DNA} are optical density, fluorescence intensity, and DNA concentration in the system, respectively). Ratios of peak intensities for excitation and absorbance located in the ultraviolet (UV, 200-300 nm) and visible (VIS, 300-700 nm) parts of the spectrum were also different for Ht and EtBr (see table).

In contrast to the individual components, the Ht + EtBr system exhibited different behavior. For example, in the presence of DNA its absorbance spectra represented sum of individual spectra of Ht and EtBr taken separately under identical conditions (this argues against marked competition between these dyes for DNA binding sites). However, fluorescent spectra of this system were much more complex. For example, Fig. 2 shows changes of fluorescence spectra of the Ht + EtBr system at molar concentration ratio of the dyes C_{EtBr}/C_{Ht} = 8 in buffer 1 with low ionic strength in the presence of various quantities of

Spectral properties of dyes studied independently in buffer 1 in the absence and in the presence of DNA

Characteristics	Dye	
	(Ht)	(EtBr)
$\lambda_{ab,U}$	267	285
$\lambda^{D}_{ab,U}$	282	276
$\lambda_{ab,V}$	345	480
$\lambda^{D}_{ab,V}$	353	520
λ_{em}	497	595
λ^{D}_{em}	455	605
λ^{1}_{i}	350	510
λ^{D}_{i}	383	580
$\varepsilon_V \cdot 10^{-3}$	27.3	5.6
$\varepsilon_U/\varepsilon_V$	0.3	1.4
$\mu_V \cdot 10^{-6}$	11.7	0.3
μ_U/μ_V	0.3	1.7
$\varphi^{D}_{V}/\varphi_V$	41.6	9.9
$\eta^{1}_{V} \cdot 10^{-5}$	49.0	4.8
$\eta^{10}_{V} \cdot 10^{-5}$	40.7	2.9
$\eta^{D}_{V} \cdot 10^{-5}$	16.9	0.0

Note: λ_{ab} and λ_{em} (nm) are wavelengths of maximum absorbance (they coincided with maxima in excitation spectra) and fluorescent emission of dyes; λ_i (nm) is wavelength of isobestic points present in absorbance spectra during changes of the ratio DNA/dye; ε ($M^{-1}\cdot cm^{-1}$) and μ ($M^{-1}\cdot cm^{-1}$) are coefficients reflecting maximal value of optical density and fluorescence intensity of dye within the indicated wavelength range at its concentration 1 mol/liter; φ is quantum output of dye fluorescence (determined as described in [39]); η ($M^{-1}\cdot cm^{-1}$) is a coefficient reflecting the augmentation of fluorescence intensity of dye during increase in DNA concentration by 1 mol/liter (determined as described in [39]); indices "$_U$" and "$_V$" correspond to ultraviolet (λ = 200-300 nm) and visible (λ = 300-700 nm) regions of spectra; indices "^{1}", "^{10}", and "^{D}" mark values changed at ratios of molar concentration DNA/dye of 1, 10, and 100, respectively (other spectral characteristics were measured in the absence of DNA).

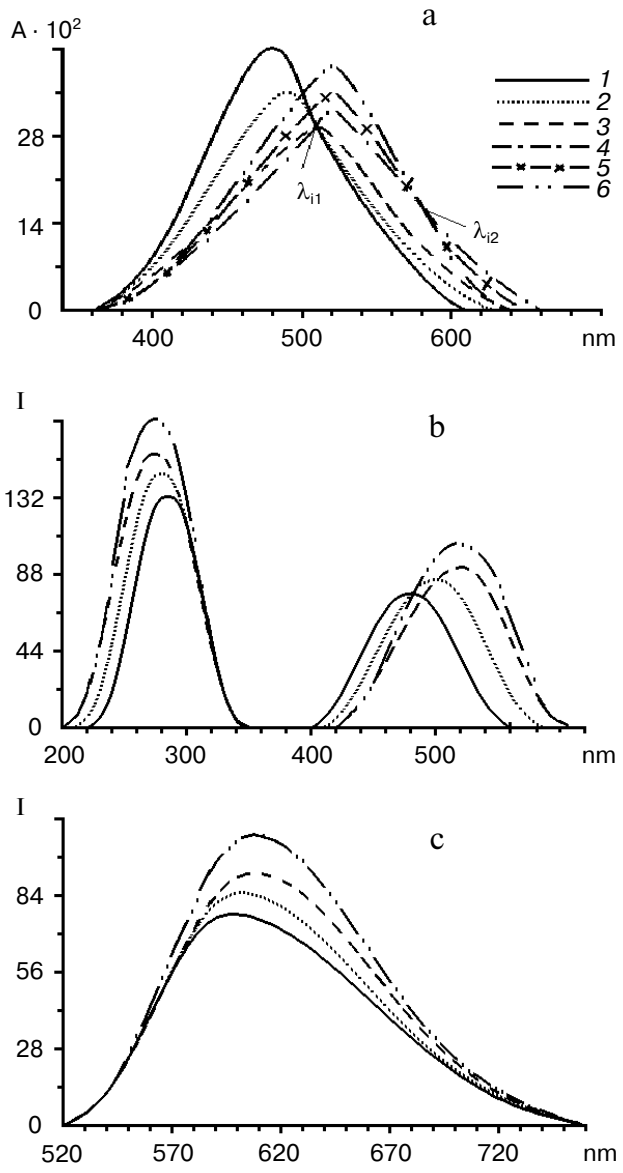


Fig. 1. Absorption (a), excitation (b), and emission (c) spectra of EtBr in the presence of various quantities of DNA in buffer 1. Curves *1-6* correspond to ratios of molar concentrations C_{DNA}/C_{EtBr} = 0, 5, 10, 15, 20, and 25; λ_{i1} and λ_{i2} designate isobestic points. Excitation spectra were recorded at the wavelength corresponding to maximum emission, whereas emission spectra were recorded at the wavelength corresponding to maximum of peak excitation of EtBr in visible part of the spectrum at each ratio of C_{DNA}/C_{EtBr} in the system. Optical density (*A*) was determined at $C_{EtBr} = 8 \cdot 10^{-5}$ M (a), whereas fluorescence intensity (*I*) was determined at $C_{EtBr} = 8 \cdot 10^{-6}$ M (b, c).

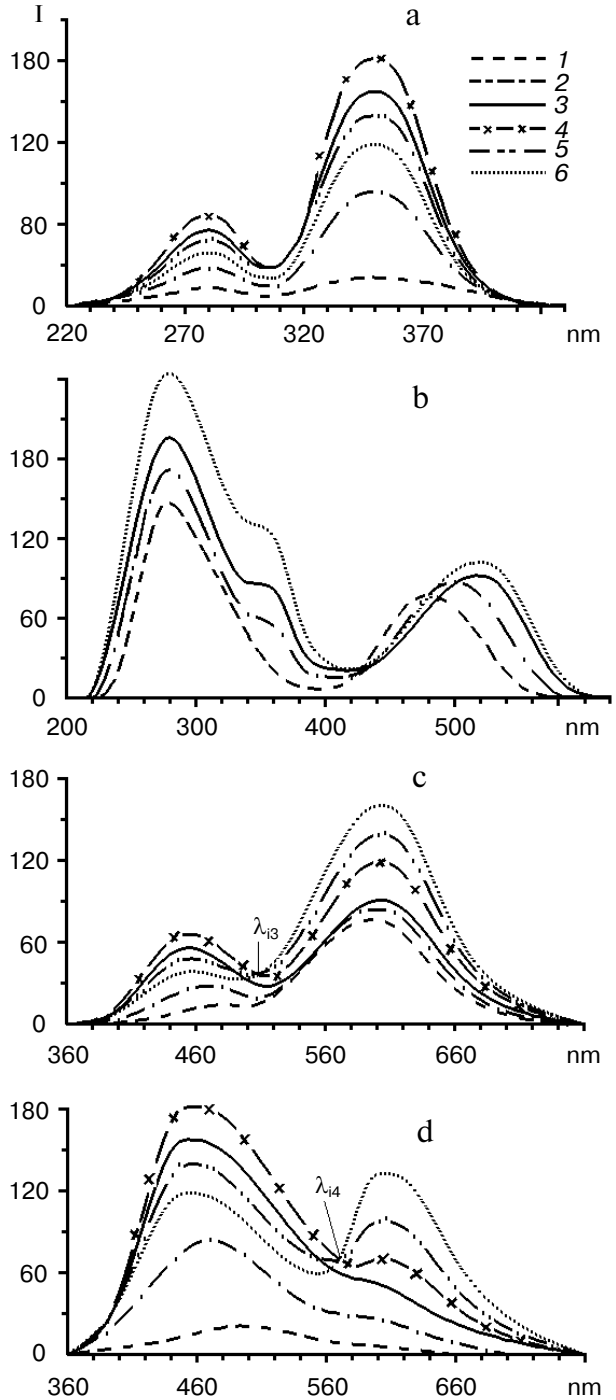


Fig. 2. Fluorescence spectra of the Ht + EtBr system (C_{EtBr}/C_{Ht} = 8) in the presence of various quantities of DNA in buffer 1 registered at fixed emission wavelengths λ_{em} = 455 (a) and 605 nm (b) and excitation wavelengths λ_{ex} = 280 (c) and 350 nm (d). Curves *1-6* correspond to ratios of molar concentrations C_{DNA}/C_{Ht} = 0, 5, 10, 15, 20, and 25 (at $C_{Ht} = 1 \cdot 10^{-6}$ M); λ_{i3} and λ_{i4} designate isobestic points.

calf thymus DNA in the range of molar concentrations C_{DNA}/C_{Ht} from 0 to 25 (ratio C_{DNA}/C_{EtBr} varied from 0 to 3).

Figure 2 shows that excitation spectra of the Ht + EtBr system, which are registered at the emission wavelength λ = 455 nm (corresponding to fluorescence emission maximum of Ht in the presence of DNA and absence of EtBr; see table), contain only two peaks with maxima at λ ~ 280 and 350 nm (which are also registered in the case of the binary Ht + DNA system and correspond to transition of DNA-bound Ht molecules from ground

state into excited state). However, in the tertiary system Ht + EtBr + DNA these peaks behaved similarly to the binary system Ht + DNA only up to the ratio C_{DNA}/C_{Ht} = 15; and the subsequent increase C_{DNA}/C_{Ht} (and C_{DNA}/C_{EtBr}, respectively) was accompanied already by reduction in the intensity of these peaks. Within the tertiary system Ht + EtBr + DNA this phenomenon can be attributed to resonant energy transfer from primary excited Ht molecules (maximum at λ = 353 nm) to EtBr molecules, which bind to DNA at a distance shorter than the wavelength of Ht emission (maximum at λ = 455 nm); these EtBr molecules can be excited within this region of wavelengths (maximum at λ = 520 nm, see table).

Also in fluorescence excitation spectra of the tertiary system Ht + EtBr + DNA registered at the emission wavelength λ = 605 nm (corresponding to maximum of fluorescence emission of EtBr in the binary system EtBr + DNA) besides two peaks with maxima at λ = 276-285 and 480-520 nm there was additional peak with maximum at λ ~ 350 nm (which was also registered in the case of the binary system EtBr + DNA).

In accordance with this fact, the fluorescence emission spectrum of the tertiary system Ht + EtBr + DNA excited at λ = 350 nm (corresponding to absorbance maximum in VIS region of the Ht spectrum) was characterized by two peaks (with maxima at λ = 455 and 605 nm); the first (with maximum at 455 nm) corresponded to the transition of primary excited Ht molecules into the ground state, and the second (with maximum at 605 nm) corresponded to transition of secondary excited EtBr molecules into the ground state. The peak with maximum at 605 nm increased in the whole range of concentration ratios C_{DNA}/C_{Ht} studied (from 0 to 25), whereas the peak with maximum at λ = 455 nm increased only up to the ratio C_{DNA}/C_{Ht} = 15 and after that it decreased. Within the range of concentration ratios C_{DNA}/C_{Ht} from 15 to 25 this resulted in appearance of an isobestic point at λ_{i3} = 570 nm in the fluorescence emission spectrum of the tertiary system Ht + EtBr + DNA exited at λ = 350 nm.

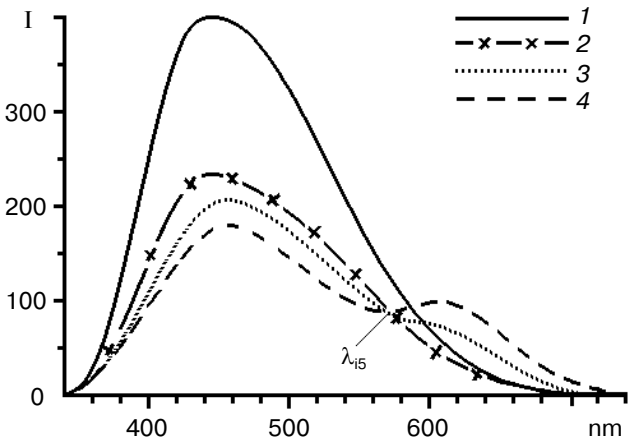


Fig. 3. Fluorescence spectra of Ht (curve *1*) and Ht + EtBr (C_{EtBr}/C_{Ht} = 8, curves *2-4*) in the presence of various quantities of DNA in buffer 2, registered at fixed excitation wavelength λ_{ex} = 350 nm. Curves *1* and *2* show spectra recorded in the absence of DNA. Curves *3* and *4* correspond to ratios of molar concentrations C_{DNA}/C_{Ht} = 10 and 25; λ_{i5} designates an isobestic point. $C_{Ht} = 1 \cdot 10^{-6}$ M.

On excitation of the tertiary system Ht + EtBr + DNA by light with λ = 280 nm (corresponding to UV absorbance maxima during Ht and EtBr binding to DNA, see table) behavior of fluorescence emission spectrum was nearly the same as above (with excitation of this system at λ = 350 nm, cf. Figs. 2c and 2d). However, peak intensity with maximum at λ = 605 nm was significantly higher than in the peak with maximum at λ = 455 nm. Within the range of ratios C_{DNA}/C_{Ht} from 15 to 25 an isobestic point was also observed at λ_{i4} = 505 nm but not at λ_{i3} = 570 nm as in the previous case. These data can be explained by the fact that EtBr fluorescence was determined not only by its secondary excitation due to neighboring Ht molecules, but also by its own primary UV excitation of EtBr molecules.

On excitation with light of λ = 520 nm (corresponding to the absorption maximum of EtBr in the VIS region during its binding to DNA) the tertiary system Ht + EtBr + DNA behaved as the typical binary system EtBr + DNA (see Fig. 1c).

In the case of altered ratio C_{EtBr}/C_{Ht}, the presence of additional substances selectively influencing spectral properties of one of these dyes, etc., the behavior of fluorescence spectra of the tertiary system Ht + EtBr + DNA may be significantly altered. For example, Fig. 3 shows fluorescence spectra of Ht and Ht + EtBr (C_{EtBr}/C_{Ht} = 8) in the presence of various DNA quantities in buffer 2. Earlier we found [39] that the presence of nonionic detergent Triton X-100 increased quantum output of Ht fluorescence in the VIS region similar to DNA, whereas spectral properties of EtBr were influenced to a lesser extent. This figure shows that in contrast to buffer 1 the tertiary system Ht + EtBr + DNA in buffer 2 demonstrated monotonous reduction in peak intensity of fluorescence emission with maximum at λ = 455 nm registered at excitation wavelength of 350 nm (corresponding to the absorbance maximum of Ht in the VIS region during its binding to DNA). This occurred with the increase in DNA concentration over all range of C_{DNA}/C_{Ht} ratios (from 0 to 25).

It should be noted that also in the absence of DNA in buffer 2 the intensity of the above mentioned peak of fluorescence emission of the system Ht + EtBr was markedly lower than that of the Ht peak used in the same concentration but without EtBr. This was obviously also the consequence of energy transfer from Ht to EtBr. However, in contrast to the non-radiative type of energy transfer (which was considered by us earlier and takes

place during binding of Ht and EtBr molecules to polynucleotide at a distance shorter than 450 nm), this is radiative type of energy transfer, which occurs at distances between donor and acceptor molecules exceeding the emission wavelength of the former (under these conditions a light quantum is initially emitted by an Ht molecule during its transition from excited into ground state, and then this quantum is absorbed by an EtBr molecule, which in turn transits into its excited state).

The most common case of altered intensity of fluorescence emission peaks with maxima at λ ~ 455 and 605 nm during excitation of the tertiary system Ht + EtBr + DNA by light with λ = 350 or 520 nm is shown in Fig. 4.

Below I consider a practical example of one possible protocol of analysis of genetic material using the system Ht + EtBr.

1) Prepare DNA for analysis. Mix 0.1 ml sample (e.g., whole blood) with 1 ml of the lysing mixture (see "Materials and Methods" section) and incubate resultant mixture for 5 min. Then, take 0.05 ml aliquot and mix it with 1 ml of buffer 1. This is solution A*. Set slits of excitation and emission monochromators of a spectrofluorimeter at 10 and 20 nm, respectively. Measure fluorescence intensity of solution A* and at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 350 and 470 nm, respectively, and detect I_F value for this sample.

2) Determine DNA concentration in the sample. Add 0.05 ml of standard Ht solution in buffer 1 (C_{Ht} = 10 μg/ml) to 1.05 ml of solution A*. This is solution B*. Measure fluorescence intensity of solution B* at λ_{ex} = 350 nm and λ_{em} = 470 nm and determine its I_H value. Add 0.02 ml of standard calf thymus DNA solution in buffer 1 (C_{DNA} = 60 μg/ml). This is solution C*. Measure fluorescence intensity of this solution at the same wavelengths as above and determine I_{DH}. Use the following equation for calculation:

$$C_{DH} = q \cdot C_{SD} \cdot (I_H - I_F)/(I_{DH} - I_H), \quad (2)$$

where q = 246 is DNA dilution in solution C* compared with the whole blood; and $C_{SD} = 3.3 \cdot 10^{-6}$ M is concentration of standard calf thymus DNA in solution C*.

3) Evaluate DNA supercoiling in the sample. Follow all steps of paragraph 2 using standard EtBr solution in buffer 1 (C_{EtBr} = 200 μg/ml) and determine C_{DE} value and calculate coefficient:

$$S = C_{DE}/C_{DH}. \quad (3)$$

In accordance with different specificity of EtBr and Ht to polynucleotide (see introductory part and literature data [11, 32-34]), increase in this coefficient should reflect reduction of DNA supercoiling in the sample.

4) Telomere sequence is a lengthy repeated oligonucleotide fragment representing the physical end of DNA molecules constituting the genome of multicellular living organisms. All vertebrates appear to have the same simple sequence repeat in telomeres: TTAGGG [40, 41]. Each cell division is accompanied by shortening of telomeric DNA (due to end-replication problem) on a certain number above telomere sequences. Thus, the length of telomeric site of DNA determines the maximal limit of the age of the organism because after total reduction of telomeric site of DNA subsequent cell divisions will be accompanied by reduction of the informative part of the genome. Telomeres also play some structural role in localization of chromosomes inside the cell nucleus, and telomere shortening is not a "simple biological clock" for cycles of cell divisions; shortening causes impairment of the whole functioning of the organism [42, 43]. Several cell types (including sex and stem cells, cells of lymphatic system, skin, uterus body, and cancer cells [40]) may restore the length of telomeric site of DNA at each cell division due to telomerase activity or an alternative

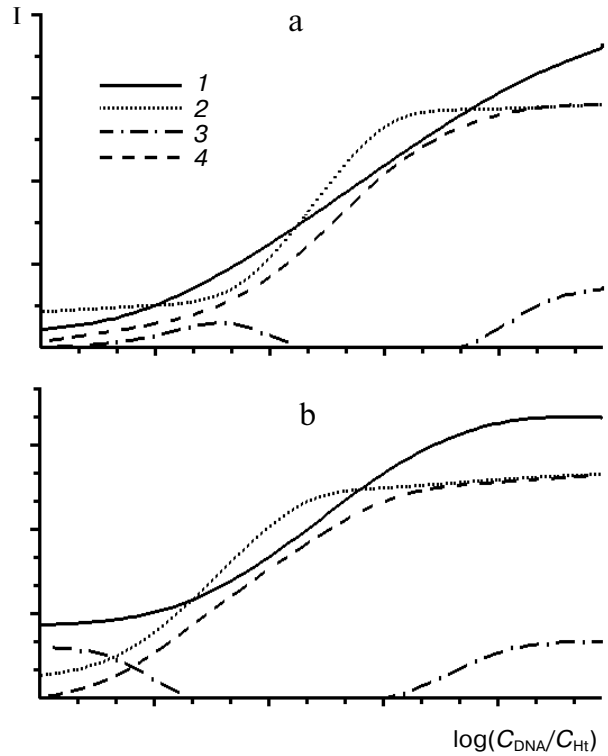


Fig. 4. Dependence of fluorescence intensity (I) on concentration ratios C_{DNA}/C_{Ht} (at constant C_{EtBr}/C_{Ht}) in buffers 1 (a) and 2 (b). Curves: *1*) change in I registered at excitation and emission wavelengths of λ_{ex} = 350 nm and λ_{em} = 455 nm for Ht (in the absence of EtBr); *2*) change in I registered at excitation and emission wavelengths of λ_{ex} = 520 and λ_{em} = 605 nm for EtBr irrespectively to the presence of Ht; *3*) change in I registered at excitation and emission wavelengths of λ_{ex} = 350 nm and λ_{em} = 455 nm for Ht in the presence of EtBr; *4*) change of I registered at excitation and emission wavelengths of λ_{ex} = 350 nm and λ_{em} = 605 nm for EtBr in the presence of Ht.

mechanism [44]. Thus, study of telomeres and end-replication problem may be important for elucidation of common mechanisms of aging of multicellular organisms and treatment of malignant tumors.

So, express analysis of telomeric DNA length can employ the Ht + EtBr system. For this purpose at first, we saturate DNA with Ht. Then we add EtBr, which exhibits lower specificity and lower affinity to polynucleotide substrate than Ht (this follows from literature data [7, 13, 28, 29] and our calculations based on the McGhee–van Hippel model [16, 45] implying independent interaction of Ht and EtBr with DNA), that is why can take on polynucleotide only not connected before Ht sites. Then we evaluate effectiveness of fluorescence resonance energy transfer from Ht to EtBr. It should correlate with number of sites on the DNA molecule located between AT- and GC-rich regions (where DNA-bound Ht and EtBr molecules are mutually located at a distance shorter than f_0). And a significant proportion of these sites should include $(TTAGGG)_n$ telomere sequence.

In the context of the above considered protocol of DNA analysis this should include measurement of fluorescence intensity of solution C* (see paragraph 2) at λ_{ex} = 350 nm and λ_{em} = 605 nm followed by determination of I_{FH}. Subsequent addition of 0.02 ml of standard EtBr solution in buffer 1 to 1.12 ml of solution C* yields solution D*. Measurement of fluorescence intensity of this solution at λ_{ex} = 350 nm and λ_{em} = 605 nm allows determining the I_{HE} value. The coefficient of energy transfer effectiveness can be determined using the following formula:

$$K = (I_{HE} - I_{FH})/C_{DH}. \quad (4)$$

If our consideration is correct, decrease in this coefficient would reflect shortening of telomeric sites of DNA length in the vertebrate (including human) genome.

This protocol of DNA analysis was tested on two groups of rats (in each group n = 20). The first and the second groups included 2-month-old rats weighing 140 g and 1.5-year-old rats weighing 350 g, respectively. We found statistically significant ($p < 0.01$) differences between means of S and K parameters for animals of first and second groups. This was expected because age-related changes of cellular genetic material (aging processes in rats occur about 20 times faster than in human) should be accompanied by reduction of DNA supercoiling and length of telomeric DNA. These results cannot be considered as strict evidence for correlation between S value and polynucleotide supercoiling and correlation between K value and telomeric DNA length. We did not consider that as a goal of this study. We have considered here a protocol for determination of S and K parameters as one possible example for use of the Ht + EtBr system for analysis of genetic material.

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