ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 8, pp. 887-900. © Pleiades Publishing, Ltd., 2007.
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Fluorescent DNA Probes: Study of Mechanisms of Changes in Spectral Properties and Features of Practical Application

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Received November 30, 2006
Revision received April 19, 2007

Abstract—Spectral data and nucleic acid complex formation properties for more than 30 both newly synthesized and widely used fluorescent nucleotide-specific compounds of various classes have been analyzed. These include phenylbenzene, bisbenzimidazole, psoralen, angelicin, tetrahydrocarbazole, oxophenoxazine, and others. The main rules of a generalized model adequately explaining changes in fluorescent properties of synthetic, low molecular weight nucleotide-specific dyes depending on their chemical structure, mode of interaction with substrate, properties of assay medium, etc. are proposed. Fluorescent nucleotide-specific dyes have been originally used in newly developed methods for: express evaluation of "generalized microbial dissemination" of liquid media; evaluation of possible genotoxic effects of various foodstuffs, pharmaceutical drugs, hazardous environmental factors (including their combined effects on living organisms), etc.

DOI: 10.1134/S0006297907080111

Key words: DNA analysis, DNA fluorophores, spectral analysis, biotesting

Compounds exhibiting specific binding to certain nucleotide sequences in genomes attract increasing attention. For example, such compounds can used for medical treatment of various oncological [1-5], infectious [6-8], and genetically determined diseases, as well as radioprotectors [9], antiseptic preparations (e.g. in systems of blood disinfection [10]), and research tools (e.g. for site-directed modification of nucleic acids [11] and blockade of their various sites), etc.

All these compounds are subdivided into two large classes depending on type of specific reversible interaction with the substrate: intercalators (which are inserted between complementary base pairs of polynucleotide double helix, and which are mainly specific to the second and higher orders of structure organization of nucleic acids) [12-16]; compounds exhibiting polynucleotide external binding are mainly specific to the first and second order structural organizations of nucleic acids [17-21] (Fig. 1). Stabilization of these nucleotide ligand complexes usually involves hydrogen bonds, which are formed between electron excessive nitrogen or oxygen atoms of the ligand molecule and corresponding heteroatoms of nitrogenous bases of the polynucleotide substrate. In some cases, stabilization may also occur via other electrostatic interactions and stacking interactions between ligand and substrate [17, 22].

The range of applications of such compounds is determined not only by their affinity and selectivity to particular sites of nucleic acids (this emphasizes biological activity of such ligands); properties convenient for registration such as fluorescence and sharp changes of emission in response to interaction with polynucleotide extend areas of potential use of such compounds. These include diagnostic systems for qualitative and quantitative analysis of nucleic acids in various cells under different conditions of the body [23, 24], research studies [13, 17, 25, 26], ecological monitoring [27], and many other applications.

However, in spite of such diversity of potential applications of fluorophores, specific to particular sites of nucleic acids, the range of real practical use of such compounds is not as wide as it could be. The development of methods based on already known nucleotide-specific fluorophores is not a very promising approach. There is no theoretical basis for prediction of features and behavior of putative nucleotide-specific fluorophores, and therefore for corresponding synthesis of new ligands (rather than only empirical search for new compounds with desired properties).

Thus, the present study represents an attempt to propose common rules for a general theory linking together optical properties of such ligands with their chemical

Abbreviations: EB) ethidium bromide; Ht) Hoechst-33258.

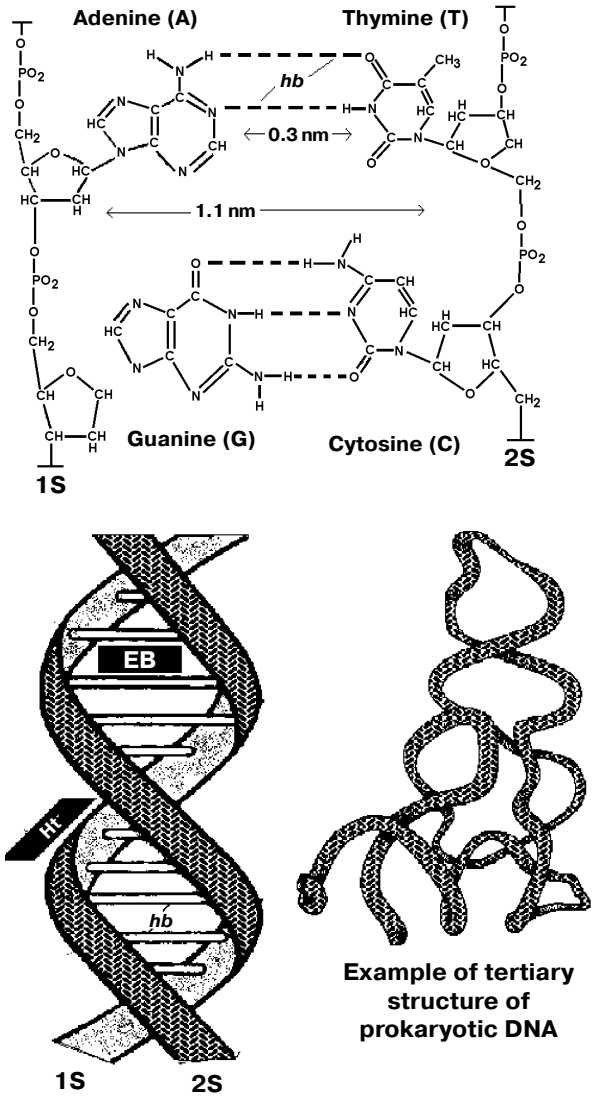

Fig. 1. Schemes of DNA structure and interaction of intercalators (EB) and non-intercalators (Ht) with it. Abbreviation "hb" designates hydrogen bonds between complementary bases of strands 1 and 2 of DNA (1S and 2S, respectively).

structure, characteristic features of their interaction with a substrate, type of microenvironment, composition of an assay medium, etc. using the investigation of the properties of more than 30 synthetic low molecular weight fluorescent DNA-specific compounds belonging to phenylindole, phenylbenzene, bisbenzimidazole, psoralen, angelicin, tetrahydrocarbazole, oxophenoxazine, and other series. This paper also considers new opportunities for practical application of these compounds.

MATERIALS AND METHODS

Figure 2 shows structural formulas of the fluorescent nucleotide-specific dyes used in this study. Compounds I-IX have been synthesized at the Department of Organic Chemistry, Russian Chemical-Technological University, as described [28]. Compounds X, XI, XX, XXII, and XXXI were purchased from Serva (Germany), and compounds XII-XIV, XXI, and XXIII-XXX have been synthesized at the Departments of Organic Chemistry and Molecular Biotechnology, St. Petersburg State Technological Institute as described in [29-33]. Mode of interaction of compounds X-XXXI with DNA was validated by viscosimetric data and optical anisotropy of free and fluorophore-bound DNA as described in [34].

Titration was carried out at constant dye concentration and various DNA concentrations at 20-25°C using standard buffer (St-buffer) containing 0.01 M NaCl, 0.01 M Na_2EDTA, and 0.01 M Tris (pH 7.4 ± 0.1). In experiments with whole cells rather than isolated and purified DNA, lysing mixture was used to destroy cell walls and plasma and nuclear membranes to provide access of a dye from an "extracellular" solution to the intracellular DNA; this mixture contained 2 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris, and 0.5% Triton X-100 (v/v) (pH 8.0 ± 0.1) [35]. Hydrogen or ionic bond formation between nucleic acid and a dye was inhibited by means of aqueous solutions containing St-buffer and 4 M urea (pH 7.6 ± 0.1) or St-buffer and 2 M NaCl (pH 8.0 ± 0.1), respectively [22]. High viscosity or low dielectric permeability was achieved by means of the aqueous solution containing St-buffer and 50% (w/w) sucrose or 2-propanol, respectively [36], etc. Calf thymus DNA (58% AT base pairs; mean molecular mass of 326 daltons per nucleotide, molar absorbance coefficient ε_{260} = 6600 $M^{-1}\cdot cm^{-1}$) was used as standard (St) substrate. The dry DNA preparation obtained from Serva was dissolved in distilled water and sonicated using an UZDN-2 instrument (Russia) for 15 sec at 0.3 A and 22 kHz. This results in formation of DNA fragments of 35 kD. Na_2EDTA, Tris, and Triton X-100 (4-octyl-(2,4,6,8,10-decapentol)oxybenzene) were also obtained from Serva. Other chemicals were of chemically pure grade.

Registration of absorbance and fluorescence spectra and calculation of fluorescence quantum yields (φ) and also parameters of complex formation between dyes and DNA by means of Scatchard's model [37-39] were carried out as described in [22]. Values of adsorption constant *K* (inverse of the concentration of free ligand in the system under conditions when it occupies half of potential binding sites on the substrate [37-39]) and *n* (maximal possible number of ligand molecules that can bind to one DNA molecule divided by total number of nucleotide pairs in it [37, 38]) are given only for cases when relative error of *K* and *n* values calculated by experimental data of interaction of particular compounds with DNA did not exceed 9%. Scatchard's model has been selected for calculation of the parameters of complex formation in the studied systems for the following reasons: i) among widely used models (which we have described in detail previ-

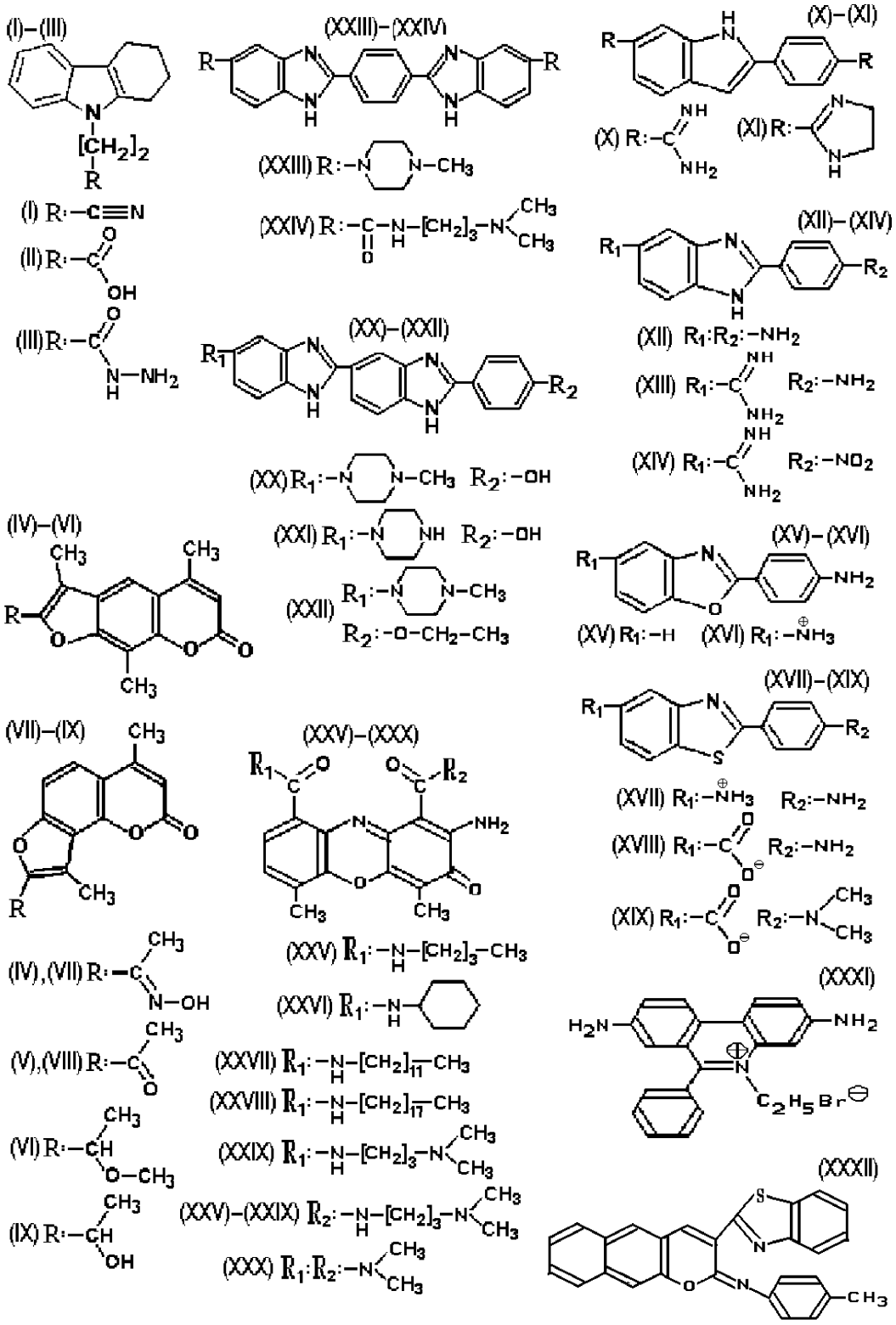

Fig. 2. Structural formulas of dyes of 1,2,3,4-tetrahydrocarbazole (I-III), 4,8,4′-trimethylpsoralen (IV and V), 4,4′-dimethyl angelicin (VII-IX), 2-phenylindole (X and XI), 2-phenylbenzazole (XII-XIX), bis-benzimidazole (XX-XXIV), 2-amino-4,6-dimethyl-3H-3-oxophenoxazine (XXV-XXX), and 2,7-diamino-9-phenylphenanthridinium (XXXI) series.

ously [39]) it is the simplest one (in contrast to theory, use of more complex models for practical calculations often reduces reliability of results compared with those obtained using this model for analysis of experimental results); ii) to exclude nonspecific substrate–ligand interactions, data of fluorometric titration of isolated and purified DNA were used instead of photometric titration data; iii) to exclude other effects influencing substrate–ligand interaction (e.g. statistical, cooperative, and other effects, which are taken into consideration in the models by McGhee–van Hippel, Crothers, Zasedatelev–Gurski, and others [39]) only titration data obtained at ratios of molar concentrations of DNA and ligand of $C_S/C_L >> 1$ have been used.

RESULTS AND DISCUSSION

Carbazoles I-III. Compounds of this series exhibit significant antimicrobial and antitumor activity [2, 4, 40]. In aqueous media, absorption spectra of 1,2,3,4-tetrahydrocarbazoles (I-III) are characterized by the presence of two peaks in the visible and UV regions: 240 and 290 nm (free dyes) or 255 and 310 nm (DNA-bound state). Fluorescence spectra have excitation peaks at similar wavelengths and a single emission peak at 380 nm (free dye) or 360 nm (DNA-bound dye).

Reversible interaction of compounds I-III with the polynucleotide was characterized by intercalation of the dye molecule between two DNA strands. Besides stacking interactions, this was also determined by formation of both hydrogen bonds and other types of electrostatic interactions between the ligand molecule and nitrogenous bases of the nucleotide substrate. This was accompanied by the increase in intensity of both absorbance and fluorescence of the DNA-bound dye compared with the free dye. Data of Table 1 show that the values of fluorescence quantum yield (φ) of these compounds in 2-propanol and in aqueous medium were closer in the DNA-bound state than the values corresponding to free (polynucleotide unbound) state. The values characterizing the unbound state increased with the decrease in electron acceptor properties of a modified external ("exocyclic") substituent versus the polycyclic tetrahydrocarbazole chromophore system of these dyes. This suggests that the chromophore system of compounds I-III may exhibit rather active fluorescence. However, in polar solvent this fluorescent activity is inhibited due to attraction of some electron density of the dye chromophore system to an external substituent. In the case of specific interaction with polynucleotide or in medium with low dielectric permeability (e.g. in 2-propanol, where spectral characteristics of compounds I-III were similar to that for the dye–DNA complex), these effects of the external substituent on the chromophore system of a ligand are significantly attenuated. This results in approaching of total fluorescence quantum yield of the dye to the φ value of its chromophore system in the absence of any substituents.

Table 1. Properties of 1,2,3,4-tetrahydrocarbazoles

Compound	I	II	III
$\varepsilon^{W}_{290} \cdot 10^{-4}$	1.3	1.4	1.3
φ_W	0.33	0.38	0.41
φ_P	0.48	0.49	0.49
φ_P/φ_W	1.5	1.3	1.2
φ_D	0.45	0.46	0.47
φ_D/φ_W	1.4	1.2	1.1
$K \cdot 10^{-6}$	2.4	26	7.4
$n \cdot 10^{3}$	25	7.4	13

Note: ε ($M^{-1} \cdot cm^{-1}$) is molar absorption coefficient (determined at the wavelength corresponding to maximal peak in the absorption spectrum); φ is fluorescence quantum yield; K (M^{-1}) and n are adsorption constant and "specificity coefficient" calculated by Scatchard's model for the complex of dye–standard DNA (see "Materials and Methods") in standard buffer; indices designate: 2-propanol (P) and also standard buffer in the absence of standard DNA (W) and in its presence at concentrations saturating dye (D).

Psoralens IV-VI and angelicins VII-IX. Compounds of these series are rather actively used in phototherapy (e.g. for psoriasis), blood disinfection, site-directed modification of RNA, and other purposes [6, 8, 10, 11]. Table 2 shows the main spectral and complex forming properties of compounds IV-IX (and also described in our publication [22]). The most interesting features of these compounds (from the viewpoint of goals and tasks of this study) consist in the following: first, changes in quantum yields of fluorescence (φ) of compounds IV-IX is similar to those described for carbazoles I-III. However, trimethylpsoralens IV-VI had a bit higher φ values, than similar dimethyl angelicins. In part, these quantitative differences can be attributed to additional electron donor properties of additional methyl groups in the corresponding molecule and in part due to different configuration of the polycyclic chromophore system. Second, each of compounds IV-IX was characterized by at least three (rather than one) "fine" types of reversible intercalation, specific interaction with the polynucleotide; they predominate at various ratios of DNA and dye concentrations. Affinity of trimethylpsoralens (IV-VI) to DNA (evaluated by K_i values, Table 2) was two times lower, whereas specificity (evaluated here as the values inverse to n_i of Table 2) two times higher than corresponding parameters of dimethyl angelicins. Third, according to literature data [6, 8, 10, 11] prolonged irradiation (for 1 h, wavelengths 250-370 nm) may initiate for-

mation of tighter, covalent bond formation between psoralen or angelicin compounds and nucleic acids.

Phenylindoles X and XI and phenylbenzazoles XII-XIX. Compounds X and XI are now widely used as commercially available dyes interacting with DNA in a non-intercalation manner (in contrast to the above-considered compounds I-IX). Tables 3 and 4 summarize the main spectral and complex forming properties of compounds X-XIX (properties of X-XIV have been described in our previous study [41]).

These dyes exhibit the following most interesting features (within the framework of goals and tasks of the present study). First, free compound X has lower value of fluorescence quantum yield in standard buffer in DNA-free state (φ_W) than compound XI. This difference is due to amidine terminal substituents of compound X, exhibiting stronger acceptor properties of electron density than imidazoline groups of compound XI. However, in standard buffer these dyes bound to DNA demonstrate similar values of fluorescence quantum yield (φ_D), which are

Table 2. Properties of 4,8,4′-trimethylpsoralens IV-VI and 4,4′-dimethyl angelicins VII-IX

Compound	IV	V	VI	VII	VIII	IX
$\varepsilon_{330}^{W} \cdot 10^{-4}$	0.83	0.91	0.75	0.93	0.98	0.85
φ_W	0.096	0.13	0.19	0.069	0.10	0.13
φ_P	0.29	0.29	0.30	0.25	0.25	0.26
φ_P/φ_W	3.0	2.6	1.5	4.1	3.1	2.2
φ_D	0.24	0.24	0.25	0.21	0.21	0.22
φ_D/φ_W	2.4	2.1	1.3	3.0	2.4	1.7
$K_1 \cdot 10^{-6}$	0.035	0.031	0.017	0.067	0.064	0.044
$n_1 \cdot 10^{3}$	120	110	99	210	240	260
$K_2 \cdot 10^{-6}$	0.0045	0.0039	0.0021	0.0088	0.0081	0.0055
$n_2 \cdot 10^{3}$	39	37	35	75	83	90
$K_3 \cdot 10^{-6}$	0.019	0.018	0.0093	0.037	0.035	0.024
$n_3 \cdot 10^{3}$	28	27	24	53	58	63

Notes: Indices designate parameters calculated by the Scatchard model for prevailing types of binding between standard DNA and ligands of the considered compounds in standard buffer at the ratios of molar DNA and dye concentrations C_S/C_L): 3-20 (1), 20-65 (2), and 65-100 (3), respectively. Other designations are as in Table 1. In absorption spectra of compounds IV-IX in the visible (VB) and ultraviolet (UV) regions there were two peaks with maximums at 255 nm (disappeared in 2-propanol) and 330 nm; in fluorescence spectra there were corresponding excitation peaks and a single emission peak with maximum at 455 nm.

Table 3. Properties of 2-phenylindoles X and XI and 2-phenylbenzimidazoles XII-XIV

Compound	X	XI	XII	XIII	XIV
λ_{AB}^{W}	340	355	330	330	330
λ_{AB}^{D}	350	365	330	340	340
λ_{EM}^{W}	455	465	450	455	455
λ_{EM}^{D}	455	450	450	455	455
$\varepsilon_W \cdot 10^{-4}$	2.0	1.7	3.1	1.6	0.52
φ_W	0.0063	0.0078	0.23	0.0047	0.0038
φ_D	0.20	0.22	0.13	0.10	0.09
φ_D/φ_W	24	32	0.55	24	23
$K \cdot 10^{-6}$	4.9	8.4	14	7.8	7.9
$n \cdot 10^{3}$	26	6.9	4.1	6.5	6.6

Notes: λ_{AB} and λ_{EM} (nm) designate wavelength maximums in absorption and fluorescence emission spectra, respectively. Other designations are the same as in Table 1. Besides the listed peaks, these compounds have smaller absorption (and also fluorescence emission) peaks with maximums at 265-275 nm observed in aqueous media.

Table 4. Properties of 2-phenylbenzoxazoles XV and XVI and 2-phenylbenzothiazoles XVII-XIX

Compound	XV	XVI	XVII	XVIII	XIX
λ_{AB}	325	340	350	340	370
λ_{EM}	390	510	520	445	465
$\varepsilon_W \cdot 10^{-4}$	1.5	0.60	1.7	1.3	2.6
φ_W	0.87	0.13	0.07	0.55	0.66
φ_D	0.074	0.072	0.053	0.055	0.056
φ_D/φ_W	0.085	0.55	0.76	0.10	0.085

Notes: All designations are the same as in Table 3. Compound XVI has an additional peak at λ_{EM} = 400 nm, binding with DNA causes a shift of λ_{AB} to 345 nm. Compound XVII has additional peaks at λ_{AB} = 315 nm and λ_{EM} = 430 nm and binding to DNA causes a shift of λ_{AB} = 350 nm to 355 nm. In aqueous media, these compounds have smaller absorption (and also fluorescence emission) peaks with maximums at 260-280 nm.

two times higher than for compounds XII-XIV; evidently, the π-excessive indole fragment of the chromophore system of compounds X and XI exhibits stronger and more stable fluorescent properties than similar benzimidazole fragment of compounds XII-XIV, which exhibits π-amphoteric properties.

Second, among 2-phenylbenzimidazole compounds considered here, compound XII has the highest value φ_W = 0.23; binding to DNA decreases its intensity and fluorescence quantum yield. This may be attributed to the presence of two external amino groups, exhibiting electron donor properties with respect to the phenylbenzimidazole chromophore system. However, compound XIII differing from compound XII only by the presence of the amidine substituent (demonstrating electron acceptor properties with respect to the polycyclic chromophore system of this compound) at its benzimidazole end has significantly lower value φ_W = 0.0047 in standard buffer. During specific interaction of compound XIII with a polynucleotide, the fluorescence yield in aqueous medium nearly reaches the value (φ_D = 0.1) observed in the case of compound XII bound to DNA. Compound XIV in which the second terminal amino group (at the phenyl end of the molecule) has been substituted for a nitro group (demonstrating electron acceptor properties with respect to the chromophore system of this dye) exhibits similar behavior. This compound is characterized by the lowest φ_W = 0.0038 among all 2-phenylbenzimidazole derivatives considered in this study.

Third, comparison of properties of benzoxazoles XV and XVI and benzothiazoles XVII-XIX shows that these compounds are characterized by both decreased intensity and quantum yield of fluorescence during specific interaction with the polynucleotide in the aqueous medium: the value of φ_D was higher in benzoxazoles XV and XVI than in benzothiazoles XVII-XIX, but lower than in benzimidazoles XII-XIV; the value φ_W = 0.13 for benzoxazole XVI was significantly lower than the corresponding value for benzoxazole XV (φ_W = 0.87). This may be explained by preferential protonation $H_3N^{(+)}$ of the modified amino group of compound XVI (exhibiting electron acceptor properties with respect to the phenylbenoxazole fragment), whereas the second (unmodified) external amino group of benzoxazoles XV and XVI exists in solution as preferentially nonionized form, exhibiting electron donor properties with respect to the chromophore system of these compounds. Similarly, substitution of amino group (compound XVII) for carboxyl group (compound XVIII) causes significant increase in φ_W (from 0.07 to 0.55), which my be explained by the electron donor influence of the deprotonated form of carboxyl group $^{(-)}$OOC of this dye on the chromophore system (in the nonionized form carboxyl group would demonstrate electron acceptor properties with respect to the phenylbenzothiazole fragment). Finally, in the case of compounds XVIII (φ_W = 0.55) and XIX (φ_W = 0.66) the ionized form external dimethylamino group (compound XIX) is a weaker acceptor in ionized form and the non-ionized form is more potent donor of electron density for the phenylbenzothiazole moiety than (non)ionized forms of amino groups of compound XVIII.

Thus, we can conclude that the mechanism responsible for changes in fluorescence of 2-phenylindoles and 2-phenylbenzazoles (interacting with DNA in non-intercalation manner) is similar to the mechanism underlying fluorescence changes in tetrahydrocarbazoles, and also in trimethylpsoralens and dimethyl angelicins (intercalating into polynucleotide). However, one should take into consideration: i) in spite of similar effect of external substituents, the increase in fluorescence properties occurs in the order benzothiazole > benzoxazole > benzimidazole > indole; ii) the considered compounds can exist in solution in several equilibrium forms differing by ionization of their external substituents.

Bis-benzimidazoles XX-XXIV. Two compounds of this series (XX and XXII) are now widely used as commercially available fluorescent dyes interacting with DNA in a non-intercalation manner (similarly to compounds

X-XIX). Table 5 summarizes the main spectral and complex-forming properties of compounds XX-XXIV (they have also been described in our previous studies [36, 39]). These dyes exhibit some interesting features relevant to the goals and tasks of the present study. In contrast to all previously considered compounds, bisbenzimidazoles have a slightly different mechanism of fluorescence. This takes place because mutual donor–acceptor effects of various fragments on electron density can be efficient only in the case of their mutual orientation within one plane. In the first four compounds of this series (XX-XXIII) phenyl and both benzimidazole fragments capable of active fluorescence can undergo mutual rotation. In spite of evident electron donor properties of terminal groups with respect to the phenylbisbenzimidazole chromophore system, these compounds are characterized by quite low values of fluorescence quantum yield (φ_W) measured in free (DNA unbound) dyes using standard buffer. Specific binding of these compounds to DNA increases (φ_W) up to values significantly increasing those obtained for phenylmonobenzimidazoles XII-XIV. Similar increase was observed after addition of sucrose, increasing the viscosity of the medium. The latter stabilizes the planar structure of the phenylbisbenzimidazole chromophore system due to interaction of long, branched hydrophilic terminal dimethylaminopropyl carbamoyl groups with water molecules. This gives a reasonable explanation why fluorescence quantum yield (φ_W) of free (DNA unbound) compound XXIV is about five times higher than in other bis-benzimidazoles studied, but one order of magnitude less than in compound XXIV (φ_D) bound to DNA. These terminal groups exhibit electron acceptor properties with respect to the phenylbisbenzimidazole chromophore system. This conclusion is also supported by the ratio of fluorescence quantum yield for free (DNA-unbound) compound XXIV measured in aqueous buffer in the absence and in the presence of added sucrose (φ_{SUC}/φ_W). It was significantly lower than for other bisbenzimidazoles studied.

Oxophenoxazines XXV-XXX. The oxophenoxazine moiety is the chromophore of actinomycin D, a known antibiotic intercalating into DNA. Table 6 summarizes the main spectral and complex forming properties of 2-amino-3H-3-oxophenoxazine compounds XXV-XXX (they have also been described in detail in our previous studies [42]). In the standard buffer and in free (DNA-unbound) state all compounds of this series (except the last one, in which terminal dimethylamino groups cannot form hydrogen bonds) demonstrated the following pH behavior; increase in pH value from 6.0 to 9.0 causes (i) increased fluorescence intensity (at constant value of quantum yield), and (ii) significant changes in absorption spectrum (by both amplitudes and wavelengths of peak maximums accompanied by visible changes in color of the solution). This can be attributed to the simultaneous presence of several equilibrium forms of most oxophenoxazines in aqueous medium. Some forms (S1) prevail at alkaline pH values and absorb light within the range of wavelengths 250-350 nm and are relatively fluorescent. Others (S2 with many intramolecular hydrogen bonds leading to the decrease of electron saturation of phenoxazine moiety) predominate at acidic pH values, absorb light in the range 400-500 nm, and they do not fluoresce (Fig. 3).

Table 5. Properties of bis-benzimidazoles

Compound	XX	XXI	XXII	XXIII	XXIV
λ_{AB}^{W}	345	350	345	360	330
λ_{AB}^{D}	355	355	350	370	350
λ_{EM}^{W}	500	500	495	555	400
λ_{EM}^{D}	455	455	455	500	400
$\varepsilon_W \cdot 10^{-4}$	2.7	3.6	2.4	1.7	1.3
φ_W	0.011	0.0091	0.013	0.0086	0.051
φ_D	0.49	0.48	0.50	0.40	0.42
φ_D/φ_W	44	53	40	46	10
φ_{SUC}/φ_W	7.0	6.8	7.1	10	2.0
$K \cdot 10^{-6}$	4.6	4.6	4.6	4.2	7.2
$n \cdot 10^{3}$	8.9	9.0	11	16	8.4

Notes: The index (SUC) designates parameters determined in 50% (w/w) solution of sucrose in standard buffer. Other designations are the same as in Table 3. In aqueous media, the compounds listed in this table have additional smaller absorption (and also fluorescence emission) peaks of lower intensity with maximums in the range of wavelengths 260-280 nm. Fluorescence emission spectra of compound XXIV also have additional maximums at 380, 420, and 450 nm; at $C_S/C_L > 160$ there is additional type of substrate binding accompanied by a decrease in intensity and fluorescence quantum yield during subsequent increase in C_S/C_L in the system.

Table 6. Properties of 2-amino-3H-3-oxophenoxazines

Compound	XXV	XXVI	XXVII	XXVIII	XXIX	XXX
$\varepsilon_W \cdot 10^{-4}$	0.68	0.59	0.71	0.76	0.69	0.72
φ_W	0.0093	0.0081	0.0067	0.013	0.0085	0.041
φ_D	0.11	0.11	0.10	0.12	0.11	0.13
φ_D/φ_W	12	14	15	9.2	13	3.3
φ_P/φ_W	1.2	0.32	0.10	0.13	0.83	0.21

Notes: Indices designate 2-propanol (P) and standard buffer, pH 8.0, in the absence of DNA (W) and in the presence of DNA concentrations saturating a dye (D). Other designations are the same as in Table 3. In aqueous media, the compounds listed in this table have additional absorption peaks with maximums at 270 and 330 nm (315 nm in compound XXVI) and 440 nm (450 nm in compounds XXIX and XXX; 430 and 450 nm in compound XXVI). The peak at the longest wavelength is not duplicated in the fluorescence excitation spectra (however, ε_W values are shown for this peak). Others correspond to the peaks at fluorescence emission spectra: 400 nm (absent in compounds XXVII and XXVIII) and 470 nm (450 nm in compounds XXVII and XXVIII).

In addition it should be mentioned that besides one of several "potent and specific" types of binding with nucleic acids (their parameters are shown in corresponding tables), compounds I-XXXI also exhibited weaker and less specific type of binding seen in standard buffer at low ratios of concentrations DNA/dye. This type was mainly determined by formation of van der Waals and ionic interactions between dye molecules and negatively charged polynucleotide phosphate backbone (such binding was accompanied by a decrease in intensity of both absorbance and fluorescence of the DNA-bound dye.)

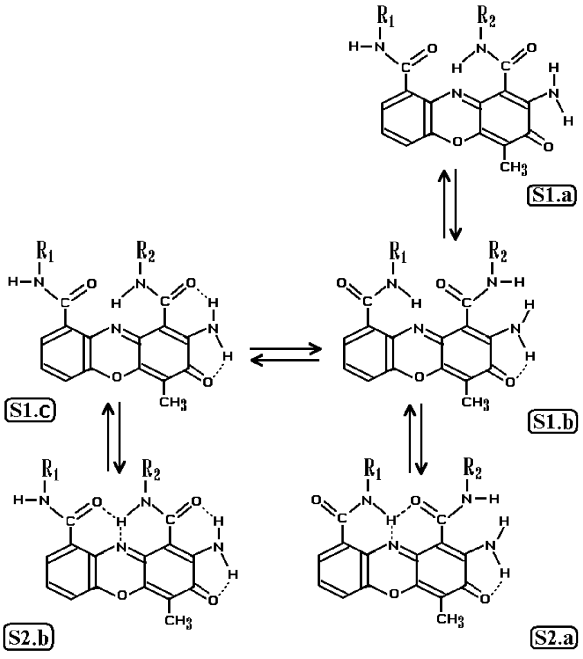

Fig. 3. Probable structures of equilibrium forms, in which actinomycins XXV-XXIX exist in aqueous medium at neutral pH and the scheme illustrating mutual transitions of these forms (broken lines show possible hydrogen bonds).

Second, in 2-propanol spectra of fluorescence emission of compounds I-XXX contained peaks at wavelengths corresponding to similar maximums in aqueous medium in the presence of saturating concentrations of DNA (λ^{D}_{EM}); in absorption spectra (and spectra of fluorescence excitation, respectively) only one peak at the maximum wavelength corresponding to similar maximum in the aqueous medium in the presence of saturating concentration of DNA (λ^{D}_{AB}) was observed in the visible region of the spectrum. Third, addition of nonionic detergent, Triton X-100, to the aqueous medium caused the same changes in absorbance and fluorescence spectra of compounds I-XXX as addition of 2-propanol.

A general model describing changes in fluorescence properties of nucleotide-specific dyes. Taking into consideration the above issues, a common mechanism responsible for changes in fluorescence properties of low molecular weight dyes specific to nucleotide consists in the following.

1. Only structures containing developed coupled electron system including both π-electrons of double or triple bonds and also unshared *n*-electrons of heteroatoms have an opportunity for active fluorescence.

2. Such coupling is usually achieved when the electron system has a rather rigid coplanar system (i.e. when all its fragments are positioned in one plane). If this precondition is not realized (as in the case of bisbenzimidazoles analyzed in this study), fluorescence requires the presence of additional factors involved in stabilization of planar structure of a dye molecule. This decreases a radiationless loss of energy absorbed by a dye molecule due to mutual rotation of fragments of the dye molecule and increases the probability of radiation transitions of electron from excited to ground state. These additional factors include: specific interaction between a dye and a substrate; significant viscosity of the medium for dye molecules (e.g. viscosity achieved in 50% aqueous sucrose solution); the existence of certain external substituents in the dye molecule (e.g. carbamoyl groups), etc.

3. In polar solvent the fluorescence quantum yield (φ) of free (unbound) molecule of the dye, containing chromophore system with rather developed and rigid coupled electron structure, can be influenced by introduction of external substituents into the dye molecule. Substituents exhibiting electron acceptor properties with respect to the chromophore will decrease the fluorescence quantum yield, whereas substituents exhibiting electron donor properties with respect to this chromophore will increase this parameter. These effects of substituents on the chromophore system of the dye ligand is significantly attenuated during specific interaction with polynucleotides (or in media with low dielectric permeability such as 2-propanol) and the resultant total fluorescence quantum yield becomes closer to φ value exhibited by its chromophore system in the absence of any substituents.

4. In certain cases (including some phenylmonobenzazole and oxophenoxazine derivatives considered here), the same compound can exist in solution as several equilibrium forms differing by ionization degree of the chromophore system and its substituents, by amount of intra- or intermolecular hydrogen bonds, etc. The concentration ratio of these forms may change depending on pH value, type of solvent, etc.

Thus, construction of an effective DNA-specific fluorophore requires initial selection of structure of potential fluorescently active chromophore system, which should contain one or several fragments, possessing a developed system of coupled bonds and including one or several 5- or 6-atom ring aromatic cycles (condensed in the case of intercalator construction) and at least one heteroatom (endocyclic nitrogen or oxygen); the latter will represent a center of specific binding between the constructed dye and a polynucleotide.

Such chromophore systems are always present in all known DNA-specific chromophores. However, the molecule of a potential dye should also contain one or several external substituents of optimal structure, which should be located at particular positions. This represents a rather complex task because these substituents should:

– not create steric problems for interaction the constructed ligand and substrate (as in the case of tert-butyl or similar structures);

– exhibit hydrophilic properties (otherwise the constructed fluorophore will have poor solubility in aqueous media);

– include functional groups containing nitrogen or oxygen as heteroatoms (which may serve as additional centers of specific binding between the constructed fluorophore and a polynucleotide);

– exhibit electron acceptor properties with respect to the chromophore system of the constructed dye under conditions of its potential binding with DNA; these properties determine some decrease in dye fluorescence in aqueous media (this decrease should not be very pronounced or it may be possible that relative increase in fluorescence during dye binding to the substrate would be rather high, but absolute values required for reliable registration in practice will be too low).

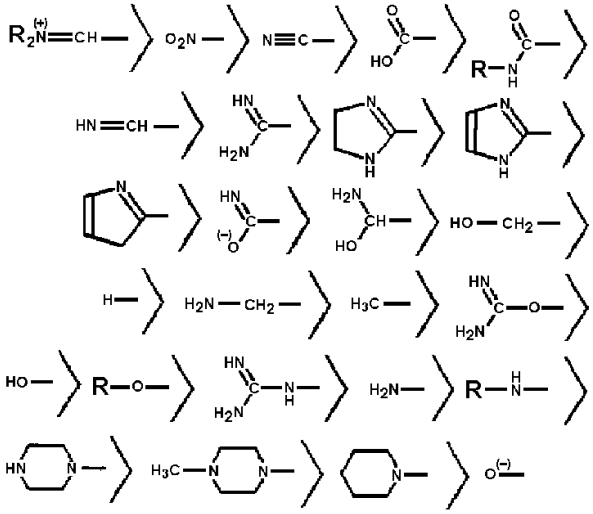

Fig. 4. External substituents arranged in the decreasing order of their electron acceptor (and increasing order of electron donor) activity with respect to the chromophore system of a dye.

For convenient selection, such substituents can be arranged in some order by their electron acceptor activity as shown in Fig. 4. Selection of optimal structure of a fluorophore specific to nucleic acids should take into consideration sensitivity of spectral and complex-forming properties to changes in assay procedure (temperature, pH, viscosity, etc.) and to the presence of various contaminants (changing dielectric permeability of the medium, competing with the main ligand for substrate binding, or alternatively competing with substrate for ligand binding, etc.); it is also important to take into consideration whether this compound is commercially available or technology for its synthesis is reasonable and the compound itself is stable in solution (and it does not exhibit aggregate formation of adsorption on vial walls), etc. Finally, our own experience indicates that for practical application of compounds as nucleic acid-specific fluorophores the coefficient of fluorescent sensitivity η (reflecting the value of augmentation of fluorescence intensity of the ligand during the increase in substrate concentration by 1 mole/liter at given ratio of substrate and ligand concentrations) is more important than the maximal value of the ratio of fluorescence quantum yield of the considered dye in the presence and in the absence of DNA (φ_D/φ_W). The value of coefficient η mainly depends on the ratio of substrate and ligand concentrations (C_S/C_L) in the system, the constant of dye adsorption on DNA (K) rather than φ_D/φ_W (Fig. 5).

Rapid method for evaluation of microbial dissemination of liquid media. We have also developed a new method

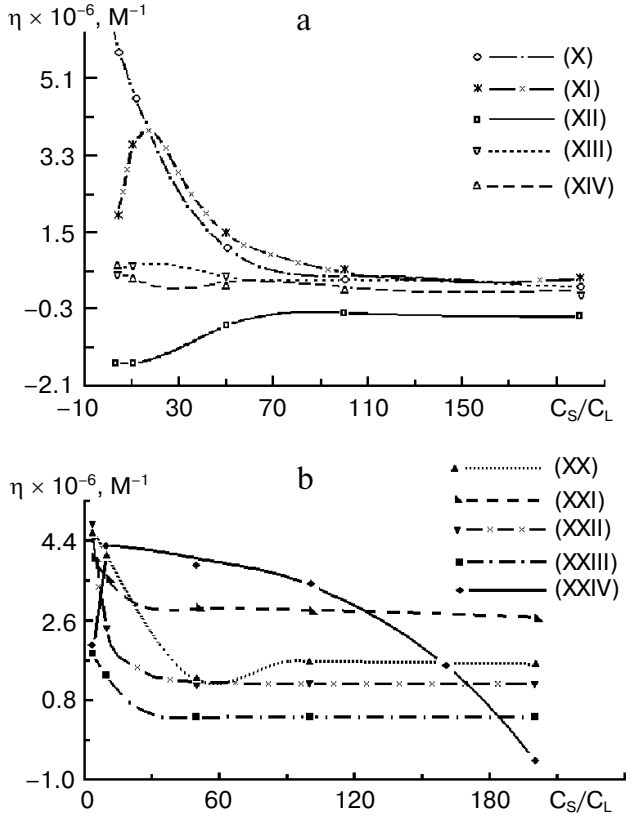

Fig. 5. Dependence of the value of coefficient of fluorescent sensitivity η (reflecting augmentation of fluorescence intensity of the dye during the increase in substrate concentration by 1 M) on the ratio of molar concentrations substrate/ligand (C_S/C_L) in standard buffer for compounds X-XIV (a) and XX-XXIV (b) demonstrating the highest relative change in fluorescence quantum yield during polynucleotide binding among all dyes investigated in this study.

for practical application of fluorescent nucleotide-specific compounds. The first one was developed for rapid evaluation of microbial dissemination of liquid media, which is one of the important parameters used in evaluation of sanitary hygienic state of reservoirs, monitoring of water purification process, and also biotechnological industry, etc.

Standard methods used for these purposes usually include seeding of an analyzed material onto cultivation medium followed by incubation at constant temperature under sterile conditions for one or more days and calculation of microbial colonies formed. These methods clearly require specialized stationary conditions and are time consuming. Our method can analyze samples in real time mode and can be used under "field-work" conditions (i.e. right after sample aspiration). It includes the following steps.

One milliliter of a sample added to a fluorimetric cell is mixed with 0.05 ml of a lysing mixture, and after incubation for a few minutes at $t° = 25 \pm 3°C$ (until equilibration of fluorescence parameters), background fluorescence intensity I_{B1} is determined. A commercial preparation of the DNA-specific fluorophore DAPI (compound X; 10 μg/ml in standard buffer) is added in the volume 0.1 ml to the analyzed sample (the background fluorescence intensity of this solution I_{B2} has to be determined earlier). The sample is mixed again and incubated at $t° = 25 \pm 3°C$ until equilibration of fluorescence parameters, and then the fluorescence intensity of the sample I_D is determined. After addition of internal standard (0.02 ml of standard DNA solution, 60 μg/ml in standard buffer), new fluorescence intensity I_{SD} is determined. The total level of microorganisms in this sample is calculated as:

$$C_M = a_0 + a_1(I_D - I_{B1} - I_{B2})/(I_{SD} - I_D),$$

where the empiric coefficients a_0 and a_1 are determined using a calibration curve, which has been plotted using standard solutions with known content of microorganisms.

Using cordless TKO-100 microfluorimeter (Serva), this method can determine total number of microorganisms in the sample from 20 cells/ml, with error not more that 30%, and one assay takes about 10 min. And the hypothesis that data obtained using this method belong to the same generation as data obtained using the standard method [43] (Fig. 6) has been confirmed with 95% probability. This method was patented [44], and its subsequent development was supported by grant PD03-1.3-40 from the St. Petersburg government.

If the lysing mixture used for treatment of cells is substituted by an antibiotics forming a channel in the external wall and cell membrane, the DNA-specific fluorophore will stain only prokaryotic cells (their presence is definitely unwanted in the brewing and other process). Subtracting these data from the total number of microorganisms in the sample determined by means of the DNA-specific fluorophore and the lysing mixture as described, it

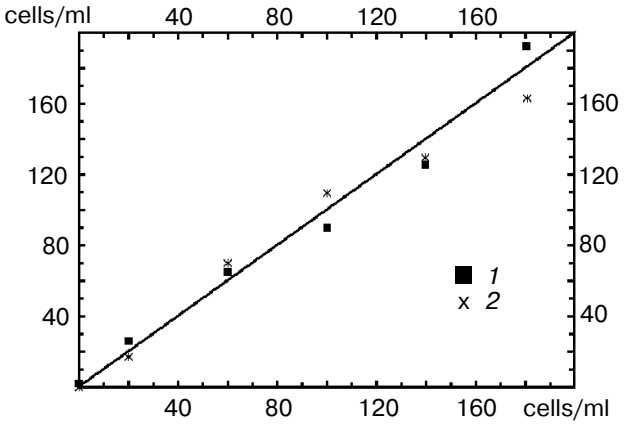

Fig. 6. Comparison of data obtained by the measurement of *E. coli* cell content in standard samples (abscissa) using the method approved by GOST 18963-73 [43] (*1*) and by the method proposed in this study (*2*) (ordinate).

is possible to evaluate number of eukaryotic cells in the analyzed sample (their presence is unwanted in production of fermented milk and other products). In stationary systems (for example such as the water purification industry for population and technical needs) the DNA staining with fluorophore can be registered by a flow cytofluorimeter rather than compact portable instruments. In this case, evaluation of microbial dissemination can be carried out in real time mode and use of stationary equipment will increase sensitivity of such evaluation. However, in each case (when microbiological purity is determined not only in water but in a rather complex multicomponent mixture) it is important to be sure whether the influence of the above-mentioned components on fluorescence of a fluorophore in the absence of microorganisms may be left out of consideration (because the influence of "contaminations" on the mode of fluorophore interaction with DNA is already included into consideration by the "internal standard"). In the case of negative result, it is better to select the other fluorophore (many of them have been considered here), which is more convenient for use under certain assay conditions, or change the assay procedure.

DNA analysis using a combined system of two dyes exhibiting coordinated properties. For evaluation of the suggestion that combined use of several fluorescent nucleotide-specific dyes with coordinated properties may significantly increase sensitivity and informativeness of analysis compared with independent use of these compounds, we have chosen the system of two widely used commercially available fluorescent nucleotide specific dyes ethidium bromide (EB) and Hoechst-33258 (Ht) (compounds XXXI and XX, respectively). The first dye (EB) is a known intercalator that lies between two base pairs of the double helix; it exhibits some preference to GC-rich sites, but generally is mainly specific to the second and higher orders of structural organization of DNA molecules. The second dye (Ht) is a non-intercalator, exhibiting external binding to a DNA molecule; it preferentially binds to specific sites of the double helix containing three sequentially positioned AT pairs and one GC base pair. In DNA binding state, these dyes are characterized by the following maximums of fluorescence excitation and emission: 350 and 455 nm (Ht) and 520 and 605 nm (EB). Consequently, joint binding of these dyes to the polynucleotide substrate can induce fluorescence resonance energy transfer, when excitation of fluorescence of an energy donor (in our case Ht) will cause fluorescence not only of these molecules but also molecules of an energy acceptor (in our case EB). This phenomenon occurs due to overlapping of fluorescence wavelength of the energy acceptor and the region of wavelengths of fluorescence emission of the donor (Fig. 7). Effectiveness of such energy transfer will strongly depend on the distance between molecules of donor and acceptor (see [45] for details).

Thus, certain evidence exists that combined use of Ht and EB can evaluate not only total DNA in a sample

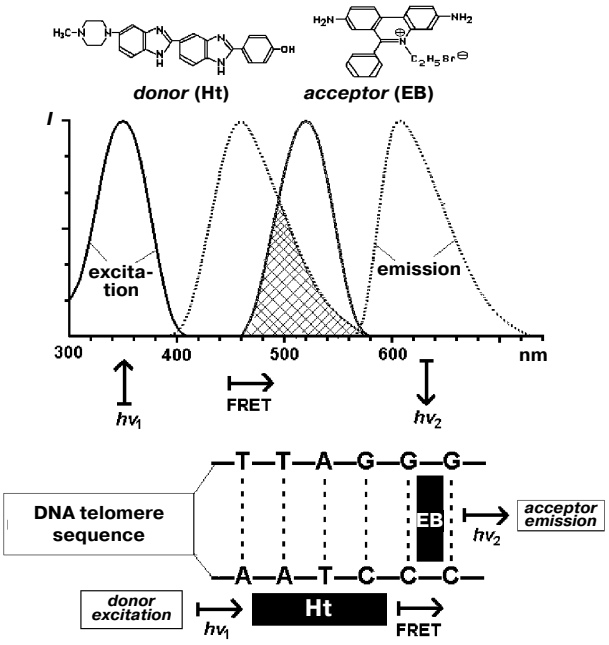

Fig. 7. Scheme of fluorescence resonance energy transfer (FRET) between molecules of Hoechst-33258 (XX; Ht) and ethidium bromide (XXXI; EB). Designations: I, fluorescence intensity; $h\nu_1$ and $h\nu_2$, light quanta absorbed by Ht and emitted by EB, respectively.

but also changes in its structure. As a practical example of one possible scheme of analysis of genetic material by means of the system "Ht + EB" we may propose the following protocol.

Add 1 ml of the lysing mixture to 0.1 ml of a sample (e.g. whole blood), mix, and leave for 5 min. After this period mix 0.05 ml of the resultant solution with 1 ml of standard buffer. This yields solution 1, which is used for determination of background fluorescence intensity $I_{B_{Ht}}$ (at the wavelengths of excitation and emission of 350 and 455 nm) and $I_{B_{EB}}$ (at the wavelengths of excitation and emission of 520 and 605 nm).

Adding 0.05 ml of a solution containing 10 μg/ml of Ht in standard buffer to 1.05 ml of solution 1 yields solution 2, which is also used for determination of the fluorescence intensity I_{Ht} as described above for $I_{B_{Ht}}$. Subsequent addition of 0.02 ml of a solution containing 60 μg/ml of standard DNA in standard buffer to 1.1 ml of solution 2 yields solution 3, which is also used for determination of the fluorescence intensity $I_{D/Ht}$ as described above for $I_{B_{Ht}}$. DNA concentration in the sample is determined using the following formula:

$$C_{D/Ht} = gC_{SD}\,(I_{Ht} - I_{B_{Ht}})/(I_{D/Ht} - I_{Ht}), \qquad (1)$$

where $g = 246$ is a dilution factor for leukocyte DNA in solution 3 compared with the whole blood and $C_{SD} = 3.3 \cdot 10^{-6}$ M is concentration of standard DNA in solution 3.

Adding 0.05 ml of a solution containing 200 μg/ml EB in standard buffer to 1.05 ml of solution 1 yields solution 4, which is used for determination of fluorescence intensity I_{EB} (at the wavelengths of excitation and emission of 520 and 605 nm, respectively). Subsequent addition of 0.02 ml of a solution containing 60 μg/ml of standard DNA in standard buffer to 1.1 ml of solution 4 yields solution 5, which is also used for determination of the fluorescence intensity $I_{D/EB}$ as described above for I_{EB}. Then the "structuring coefficient" K_S is calculated using the following formula:

$$K_S = C_{D/EB}/C_{D/Ht}, \quad (2)$$

where $C_{D/EB} = gC_{SD}[I_{EB} - I_{B_{EB}}]/[I_{D/EB} - I_{EB}]$. From our viewpoint, change in coefficient K_S reflects certain changes in tertiary and higher orders of structural organization of a genome in the sample (because EB exhibits higher specificity to higher orders of genetic material organization than Ht).

Determine $I_{B_{Ht/EB}}$ (fluorescence intensity of solution 2) at the wavelengths of excitation and emission of 350 and 605 nm, respectively. Adding 0.02 ml of the solution containing 200 μg/ml of EB in standard buffer to 1.12 ml of solution 2 yields solution 6, which is used for determination of fluorescence intensity $I_{Ht/EB}$ as described above for $I_{B_{Ht/EB}}$. Then calculate the coefficient of energy transfer in the sample K_E using the formula:

$$K_E = (I_{Ht/EB} - I_{B_{Ht/EB}})/C_{D/Ht}. \quad (3)$$

In our opinion, the decrease in K_E (as shown in Fig. 7) may reflect (to some extent) reduction of telomere length. In vertebrates (including man) telomeres represent multiple repeats of TTAGGG nucleotide sequence at DNA ends; the number of such repeats is reduced after each cell division due to incomplete DNA replication, and therefore it limits maximal life span of living organisms. Telomeres also play regulatory and some other functions in living organisms [46-50].

This protocol of DNA analysis was tested using two groups of rats (in each group $n = 30$) of 2 months and 1.5 years old. Results of this study revealed statistically significant (with probability of 99%) difference in K_S and K_E parameters. This is consistent with our initial suggestion that age related processes (aging of rats occurs 20 times faster than of man) should be accompanied by impairments of genetic material in eukaryotic cells particularly due to reduction in telomere length (using the classical method described in [53], this decrease was about 6300 bp per chromosome end for the groups of animals studied) and other changes in the structure of nucleoprotein complex.

This protocol of DNA analysis has also been used for identification of genotoxic effects of various foodstuffs, pharmaceutical preparations, and unfavorable environmental factors on living organisms and also for evaluation of individual radiosensitivity of animal and human blood cells. Their adequateness is considered below.

Other methods employing the DNA-specific fluorophores. We have also developed a method for identification of genotoxic effects of various foodstuffs and pharmacological preparations on living organisms including combined effects on these organisms of other products, preparations, and environmental factors specific for particular regions. This is especially important because of intensive development of pharmacology and wide use of various genetically modified products and other biotechnological products. We also developed a method for identification of genotoxic effects of low doses of radiation and other environmental factors during their combined action on living organisms. This method can be used for complex evaluation of impairments in the ecological situation of certain regions even in cases when "standard" methods do not register increases in maximally acceptable concentrations of such factors but their combinations may cause significant damaging effects on man and other living organisms.

These methods are based on long-term treatment of animals with an analyzed product or drug or exposure of such group of animals to the action of certain unfavorable factors in combination (if necessary) with other products, preparations, or environmental factors. At certain time intervals, blood samples are taken from these animals and treated with the lysing mixture. Using a special dye (or a system of a few dyes – for example, ethidium bromide and Hoechst-33258 considered above) and a fluorimeter, total content and structure of genetic material in the samples are analyzed, and normalized versus number of leukocytes (normal mature erythrocytes circulating in blood of mammals lack DNA, mitochondria, ribosomes, intracellular membranes) and compared with similar parameters obtained for animals of a control group (as well as the parameters obtained for animals of the experimental group before the beginning of the treatment). Based on this comparison one can conclude the existence or lack of any genotoxic effect in the analyzed group of animals.

These methods have been tested for evaluation of therapeutic and prophylactic effect of cycloferon (an interferon inducer) during radiotherapy of fibrosarcoma [52], for evaluation of the effect of products of the Pollustrovo factory on living organisms of St. Petersburg and Leningrad Region (by contract with this factory), and also for evaluation of the ecological situation in some natural reservoirs of the Leningrad Region. Data obtained correlated with subsequently observed decrease in life span of experimental animals and also with results obtained during analysis of the same samples by means of scoring of unstable chromosome aberration in cultured lymphocytes and other cytological methods.

We have also developed a method of pilot evaluation of individual radiosensitivity of human (and animal)

blood cells. This method is based on the following. A blood sample (1 ml is enough) taken from a patient before the beginning of radiotherapy is diluted with standard buffer and separated into two parts; one is subjected to radiation, the other one served as control. Each of these parts is incubated at 37°C for 3 h (for repair of primary damages) and treated with the lysing mixture. Using a special dye (or a system of a few dyes) and a fluorimeter, total content and structure of genetic material in the samples are analyzed and normalized versus number of leukocytes. Comparison of the values can indicate degree of damaging effect of radiotherapy (using particular dose) on a particular patient. The described method has been successfully employed in more than 50 patients of the Central Research Institute for Roentgenology and Radiology. This method demonstrated significant 95% correlation with results obtained in the same patients by means of scoring of unstable chromosome aberration in cultured lymphocytes, one of the most common methods for early identification of irradiation in man. However, the latter method requires more material and is more labor and time consuming than our method (7 days versus 4 h).

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