



**INVESTIGATION OF DNA ISOLATED FROM YEASTS CANDIDA  
GUILLIERMONDII NP-4 AND FROM BACTERIA  
ESCHERICHIA COLI BY FLUORESCENCE ANALYSIS**

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**ABSTRACT**

*The aim of present study was the comparative investigation of fluorescence peculiarities (wavelength and intensity) of complexes of ethidium bromide with DNA: isolated from low eukaryotic organisms - yeasts Candida guilliermondii NP-4 after their X-irradiation and post-radiation incubation of cells, and from prokaryotic organisms - E.coli of wild type and from Crohn's patients gut microflora. Ethidium bromide (3.8-diamino-5-ethyl-6-phenylphenanthridium bromide) was used as a fluorescence probe, which intercalate into plane between base pairs of DNA. As a result, the fluorescence intensity of Ethidium bromide increases. The investigation of interaction of DNA with Ethidium bromide was realized by gradually increase of dyestuff concentration in DNA solution, or by titration of DNA with ethidium bromide.*

*The obtained data suggest that DNA isolated from prokaryotic cells is saturated with ethidium bromide faster than DNA isolated from eukaryotic cells. After X-irradiation and post-radiation incubation (repair) of yeast cells the increase of saturation speed of DNA with Ethidium bromide performs that X-irradiation causes structural changes in DNA, such as single-stranded breaks, a part of which turns into double-stranded breaks of DNA during the post-radiation repair. So the deepening of DNA damages takes place during the repair process.*

*In case of Crohn's disease development the changes in DNA structure also take place. In particular, the methylation level of DNA increases, which brings to increase of saturation speed of DNA with Ethidium bromide.*

**KEYWORDS:** fluorescence, DNA, eukaryotic cells, prokaryotic cells, ethidium bromide, yeasts Candida guilliermondii, X-irradiation, Crohn's disease.

**INTRODUCTION**

In field of biology and medicine the efficiency of investigations in molecular level is greatly depended on use of different modern physical and chemical methods, such as high-performance liquid chromatography, affinity and ion exchange chromatography, spectroscopy, including fluorescent spectroscopy. Side by side with many methods of receiving physical information about biological structures and biochemical processes the investigations of fluorescence spectrums are largely used in biochemistry. These investigations

allow realizing the quantitative and qualitative analysis of different components of biological systems, investigate their statement, and interaction with other components. But not every macromolecules are endowed with fluorescent properties. Thus, for fluorescence of nuclei acids are responsible the nitrogen bases which have a combined system of chemical bindings, but are endowed with fluorescence of very low intensity. In early investigations it has been shown that from all nitrogen bases of DNA only thymine is endowed with fluorescent properties, but his intensity is too low and is not useful for detection of thymine in biological systems [Udenfriend S et al., 1969]. So the investigation of own DNA fluorescent parameters is not informative and gives no essential information about DNA properties, functional and structural

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statements. Therefore, in investigations of nucleic acids, especially of DNA, it is accepted to use the specific fluorescence probes – dyestuffs, which intercalate to DNA double strand sectors and interact with its nucleotides. As a result of such interaction the fluorescence wavelength and intensity of dyestuffs are sharply changed. The formed complexes are used for study of structural damages of DNA and for getting the information about structural features of investigated system, as well as secondary structure and morphology of DNA [Frayfelder D, 1980]. In our investigations the ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridine bromide, EB) was used as a fluorescence probe (Fig. 1).

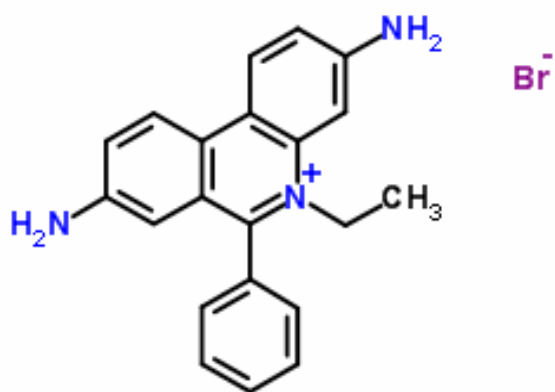


FIGURE 1. The structure of ethidium bromide [Sambrook J, Russel D, 2012].

Ethidium bromide is refers to classical intercalators. A series of investigations in recent years have shown that EB, depending on the concentration and environmental conditions, interacts with DNA in several ways - intercalation and external binding [Baranovsky S.F., et al, 2009], and the value of the binding constant varies in the interval  $10^4 \text{ M}^{-1} \div 10^7 \text{ M}^{-1}$  [Vardevanyan P.O. et al, 2003; Hayashi M., et al, 2007].

This ligand is a convenient marker (probe) to investigate triple systems by different methods, as well as widely accepted dyestuff for staining of DNA, nucleus and chromosomes in luminescence microscopy, flow cytometry and fluorescence investigations [Zhang G., et al, 2012; Dehkordi M.N., et. al, 2012; Alonso A., et al., 2006; Iermak Ie., et.al, 2011]. EB at low concentrations intercalates into plane between pairs of DNA bases, resulting in an additional stacking interaction, which leads to stabilization of the double-stranded struc-

ture of DNA [Vardevanyan P.O., et al, 2008]. By intercalation onto double stranded molecule of DNA the plane chromophore fragments of EB are built in (intercalate) between adjacent base pairs of double helix of DNA. Wherein, they provide additional stabilization of the complex DNA-EB due to the formation of hydrogen bindings between the proton-donor and proton-acceptor groups of the dyestuff and nitrogen bases, as well as electrostatic interactions between the positively charged atoms with negatively charged phosphate groups of DNA. The base pairs, moving apart, free the place for the dyestuff molecule and remain perpendicular to the axis of the DNA helix. Wherein, the molecule is in the van der Waals contact with the base pairs and, accordingly, is parallel to them. EB is characterized by absence of specificity to DNA-sequences. One molecule of EB intercalate approximately into 2.5 base pairs, as a result of which the fluorescent intensity of EB increases up to 20 times [Sambrook J, Russel D, 2012]. It has been shown that in B-form DNA, which is the basic conformation *in vivo* statement of double stranded DNA, by intercalation of EB takes place the axis shift by 8 degrees, which brings to DNA transition to supercoiling state [Vardevanyan P et al., 2008]. This ligand is antibacterial, as well as a mutagenic substance (i.e., has a certain cytotoxicity) [Vardevanyan P et al., 2008], it is also considered a carcinogen and teratogen [Saeidnia, S, Abdollahi M, 2013].

Thereby fluorescence investigation of DNA-EB complexes is one of the sensitive methods of spectroscopy and can give important information about the structure and conformation of DNA. This method has especially high importance in studies of DNA carrying various types of structural damages or conformational changes, which lead to a disruption of the binding of ethidium bromide to DNA.

The aim of this study was the investigation of fluorescence wavelength and intensity of complexes of ethidium bromide with DNA isolated from low eukaryotic organisms-yeasts *Candida guilliermondii* NP-4 after X-irradiation and post-radiation incubation of cells, and from prokaryotic organisms - *E.coli* of wild type and from Crohn's patients gut microflora.

It is known that X-irradiation causes various structural damages in DNA: single- and double-strand breaks, intermolecular cross-links and oth-

ers [Hall E, Giaccia A, 2012]. By this point of view the comparative investigation of fluorescence parameters of complexes of EB with DNA isolated from non-irradiated, X-irradiated and repaired yeasts is very interesting.

Crohn's disease is a chronic autoimmune inflammatory disease of the gastrointestinal tract, characterized by the onset of a granulomatous inflammatory process. The development of the disease contributes to various factors, such as immunological, infectious, lifestyle, as well as genetic [Panés J et al., 2007]. The latter indicates that during Crohn's disease development, the changes in the structure or conformation of DNA are possible.

Our early studies have shown the methylation level of DNA is changed in case of Crohn's disease. Based on this result, we have also investigated the fluorescence of DNA isolated from *E.coli* wild type and from gut microflora of Crohn's disease patients.

#### MATERIAL AND METHODS

The subjects of this investigation were DNA isolated from chicken blood erythrocytes (Reanal), from yeasts *Candida guilliermondii* NP-4, from *E.coli* wild type bacteria and from *E.coli* bacteria of gut microflora of Crohn's patients.

Dry DNA from chicken blood erythrocytes from Reanal was used as a high-polymer and highly purified standard. DNA was solved in standard saline solution (0.1xSSC) by concentration 0.5mg/ml immediately before use. The DNA solution for fluorescence analysis was diluted until concentration of  $8 \times 10^{-5} M$ .

The bacterial biomasses were received from Laboratory of Molecular Biology and Biotechnology of Armenian National Agrarian University and were investigated immediately after receiving. DNA from bacteria cells was isolated by J. Marmur (1961). Bacterial biomasses were incubated in buffer of 0.15M NaCl+0.1M EDTA+0.015M sodium citrate+ 0.1M tris + 0.1% Triton X-100 (pH=8.2), at 60°C, during 40min, then the lysates were incubated in presence of 2.5% sodium dodecyl sulfate, at 55°C, during 40 min., after which the lysates were treated with NaCl and with mixture of chloroform-isoamyl alcohol (24:1). Then the mixture was centrifuged and the DNA recovered from the supernatant by a double volume of ethanol. DNA purification from RNA fragments

was performed with enzyme ribonuclease, and final DNA purification was carried out with solutions of sodium dodecyl sulphate and NaCl. DNA isolated by this method corresponds to the criteria of purity, nativity and polymerity, accepted in the literature, which were calculated from the spectral parameters of the DNA solution:  $A_{260}/A_{230} \geq 2$ ,  $A_{260}/A_{280} \geq 1.8$  [Britten R et al, 1974].

*Cultural medium and yeast biomass getting:* Yeast cells were incubated in liquid medium on a shaker, under the temperature of 30°C, with 4000Lux of light, during 24 hours. The yeast biomass was isolated from cultural liquid by centrifugation by 5000rpm, during 10 min. Then the yeast cells were washed by distilled water [Davtian M et al., 2010].

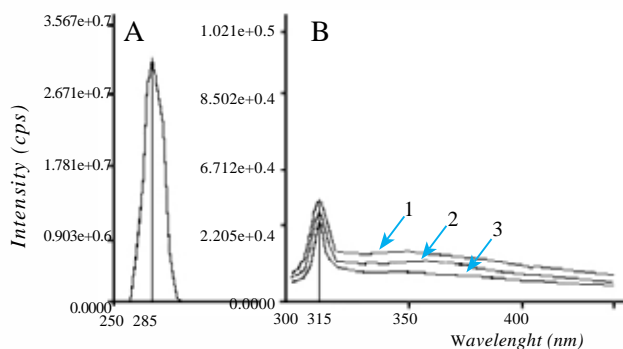
*Yeast cells X-irradiation:* Yeast cells irradiation was realized in stationary phase of growth, on X-ray machine Dron-3 (Russia), with Cu-X-ray tube, by wavelength of  $\lambda = 1.54 \times 10^{-10} m$ . The dose rate was 1080Gray/min, and the total absorbed dose was 540 Gray.

Isolation and purification of DNA from yeasts was realized according to J. Marmur's method (1961) modified by us. Yeast cells are characterized by the presence of a strong cell wall, so at first we carried out chemical, thermal and enzymatic treatment of the cell wall, in order to obtain protoplasts of yeast cells. The yeast biomass was treated with a 4-fold volume of 0.1M tris + 0.1M EDTA + 0.5%  $\beta$ -mercaptoethanol (pH = 8) solution at 30°C, for 30 min. Then, the treatment was continued with solution of 0.05M cis-HCl + 0.1M  $K_2HPO_4$  (pH=7.8) with addition of an enzyme-containing medium (1g of cytohelicase per 10 g of yeast biomass in 1M  $KH_2PO_4$  + 0.1M  $MgSO_4$  solution) at 32°C, for 30 min. As a result of this treatment, the yeast cells are transformed into spherical protoplasts. The optimal conditions for each stage of thermal and enzymatic treatment of yeast cells were experimentally selected by us. The formation of spherical protoplasts was observed under microscope (Biolar, Poland) [Marutyán S et al., 2016]. As described above further processing of the obtained protoplasts and isolation of DNA was carried out for bacterial DNA.

Investigation of fluorescence wavelength and intensity was realized by fluorescent spectrometer FluoroMax™ (SPEX, France). Data treatment has been done with hardware DN3000F. The excitation

wavelength for DNA was  $\lambda=285\text{nm}$  with intensity  $I=3.1 \times 10^7\text{cps}$ , and the fluorescence wavelength -  $\lambda=315\text{nm}$  with intensity  $I = 4.4 \times 10^4\text{cps}$  (Fig. 2). Thus, the fluorescence intensity of yeast DNA is  $10^3$  times lower than the excitation intensity, so the own DNA fluorescence is not used for investigations of its structure or conformation. Therefore for DNA fluorescence investigations the EB was used as fluorescence probe. For EB solution the excitation wavelength was  $\lambda=480\text{ nm}$ , and the fluorescence wavelength -  $\lambda=584\text{ nm}$ . In case of complex forming of EB with DNA the excitation wavelength shifts to a longer wavelength region and is  $\lambda=510\text{nm}$ , and the fluorescence wavelength -  $\lambda=584\text{ nm}$  (Fig. 3). The investigation of interaction of DNA with EB was realized by gradually increase of dyestuff concentration in DNA solution, or by titration of DNA with EB. A solution of EB with a concentration of  $0.15\text{ mM}$  was taken as the basic solution. DNA solution of  $2.5\text{ ml}$  with a concentration of  $8 \times 10^{-5}\text{M}$  was collected in a quartz cuvette and  $10\ \mu\text{l}$  of EB solution was added in each step for DNA titration. Together with the EB concentration increase in DNA solution, the concentration of DNA was decreased, which was taken in account during data calculating. At the same time, we checked the fluorescence of solutions of EB and DNA at the excitation wavelength of  $\lambda = 510\text{ nm}$ . The obtained data showed that when excited at a given wavelength, fluorescence with very low intensity is observed for both EB and DNA (Fig. 3)

**Reagents and data processing:** All the reagents of analytical grade were used throughout. Each experiment was repeated at least three times; average



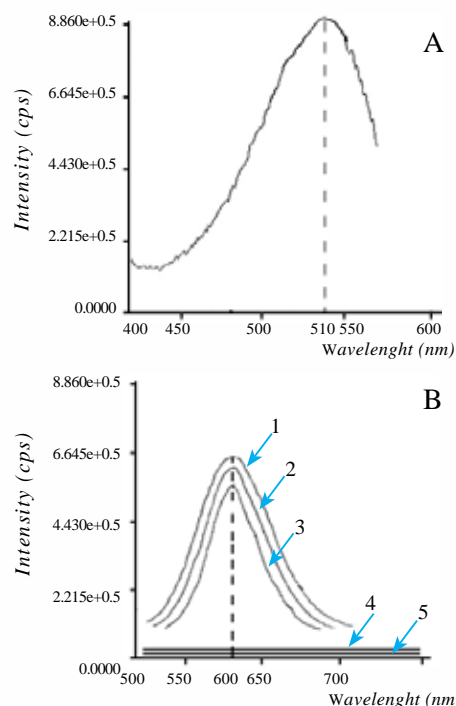
**FIGURE 2.** Fluorescence spectrums of DNA: A- excitation spectrum, B – Fluorescence spectrum. 1 – DNA from chicken blood erythrocytes, 2- DNA from yeasts *Candida guilliermondii* NP-4, 3 – DNA from bacteria *E.coli* wild type.

statistical errors are given in tables 1, 2. The changes were validated by calculation of Student's validity criteria ( $p$ ); the differences between experiments were valid if  $p < 0.05$ .

## RESULTS

The results of investigation of DNA fluorescence parameters isolated from different objects have shown that by gradually increase of EB concentration in DNA solution the increase of fluorescence intensity of complexes DNA-EB takes place until the certain concentration of dyestuff, after which there is no increase of fluorescence intensity in case of increasing of EB concentration. Thus, DNA saturation with EB occurs. The corresponding data are given in table 1.

In order to make fluorescence data of DNA from different objects comparable, the fluorescence spectrums were reached the same scale (i.e. were scaled), so instead of absolute values of fluorescence intensity their relative values  $I_x/I_{x_{\max}}$  were discussed (Fig. 4). For discussing of obtained data we choose the  $1/2$  value of  $I_x/I_{x_{\max}}$ , as it is accepted when working with the saturation curves [Nelson D, Cox M, 2010]. As the obtained data have shown,



**FIGURE 3.** Fluorescence spectrums of complexes DNA-EB: A- excitation spectrum, B- fluorescence spectrums, 1 – DNA from chicken blood erythrocytes, 2 – DNA from yeasts *Candida guilliermondii* NP-4, 3 – DNA from bacteria *E.coli* wild type, 4 – EB solution, 5 – DNA solution.

TABLE 1

Fluorescence intensity of complexes EB with DNA from different objects (n=5, p&lt;0.05)

$C_{EB} \times 10^{-6} M$	$C_{DNA} \times 10^{-5} M$	$C_{EB}/C_{DNA}$	Chicken blood erythrocytes	Fluorescence intensity, $\times 10^5$ cps				
				Yeast DNA from <i>Candida guilliermondii</i> NP-4			DNA from bacteria <i>E.coli</i>	
				Non-irradiated	X-irradiated	Repaired	Wild type	Crohn's disease patients
0.6	7.78	1:125	0.28±0.02	0.4±0.03	0.5±0.04	0.5±0.04	0.09±0.006	0.132±0.01
1.19	7.75	1:66.6	0.5±0.05	0.8±0.06	1.05±0.08	0.9±0.08	0.13±0.01	0.21±0.018
1.77	7.72	1:43.5	0.8±0.07	1.2±0.1	1.5±0.12	1.3±0.12	0.16±0.01	0.24±0.021
2.36	7.69	1:38.5	1.1±0.09	1.64±0.15	2.02±0.15	1.7±0.14	0.18±0.017	0.26±0.02
2.94	7.66	1:25.6	1.3±0.08	2.1±0.2	2.2±0.21	2.05±.2	0.21±0.02	0.27±0.023
3.52	7.63	1:21.7	1.5±0.11	2.5±0.21	2.5±0.23	2.4±0.21	0.23±0.021	0.28±0.027
4.08	7.60	1:18.5	1.7±0.13	2.9±0.27	2.9±0.26	2.7±0.24	0.25±0.024	0.29±.023
4.65	7.57	1:16.6	1.9±0.17	3.2±0.3	3.5±0.32	2.8±.26	0.26±0.02	0.3±0.03
5.21	7.54	1:14.5	2.1±0.2	3.6±0.32	3.8±0.31	3.02±0.3	0.27±0.021	0.31±.003
5.76	7.51	1:12.9	2.3±0.21	3.9±0.34	4.1±0.38	3.15±0.3	0.28±0.026	0.315±0.029
6.32	7.48	1:11.9	2.56±0.23	4.3±0.39	4.4±0.4	3.3±0.29	0.29±0.02	0.318±0.024
6.87	7.45	1:10.3	2.8±0.19	4.5±0.41	4.6±0.42	3.4±0.28	0.3±0.028	0.32±0.03

NOTES:  $C_{EB}$  – concentration of ethidium bromide in DNA solution,  $C_{DNA}$  – concentration of DNA,  
 $C_{EB}:C_{DNA}$  – ratio of concentration of ethidium bromide to DNA.

the concentration of EB that describes the state of semi saturation with dyestuff of DNA isolated from chicken blood erythrocytes is  $6.32 \times 10^{-6} M$  (table 1), and the ratio  $C_{EB}:C_{DNA}=1:12$ , in the other words, when to 12 pairs of nucleotides of DNA corresponds 1 molecule of EB in solution.

A similar picture is also observed when studying the fluorescence of DNA isolated from yeast cells: with a gradual increase in the concentration of EB, an increase in the intensity of fluorescence to a saturation state is observed, and besides the

concentration of EB in the state of semi saturation of DNA by the dyestuff is  $4.23 \times 10^{-6} M$  (Table 2), and the ratio of  $C_{EB}:C_{DNA}=1:18$ , in other words, when 18 pairs of DNA nucleotides corresponds to 1 molecule of EB in solution. In the case of DNA isolated from the bacteria of *E. coli* wild type, the concentration of EB in the semi saturated state of the DNA with the dyestuff was  $1.83 \times 10^{-6} M$  and  $C_{EB}:C_{DNA}=1:43$  (Table 2).

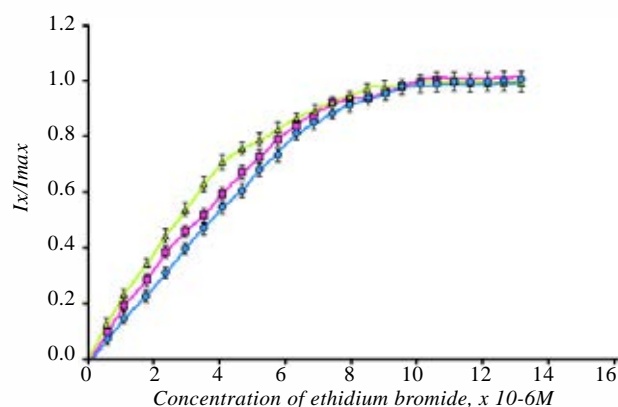
*Investigation of DNA from X-irradiated and repaired yeasts:* The fluorescence of DNA changes

TABLE 2

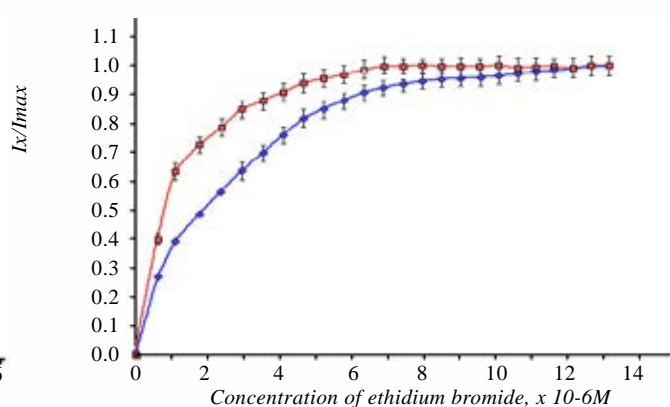
Concentrations of DNA and EB and their ratio in state of semi saturation of DNA with dyestuff (n=5, p&lt;0.05)

	$C_{EB}, \times 10^{-6} M$	$C_{DNA}, \times 10^{-5} M$	$C_{EB}:C_{DNA}$	
DNA from chicken blood erythrocytes	6.32±0.5	7.49±0.6	1:12	
DNA from yeasts <i>C.guilliermondii</i> NP-4	Non-irradiated	4.23±0.2	7.61±0.5	1:18
	X-irradiated	3.56±0.3	7.63±0.6	1:22
	Repaired	3.28±0.3	7.65±0.4	1:24
DNA from bacteria <i>E.coli</i>	Wild type	1.83±0.1	7.72±0.6	1:43
	Crohn's disease	0.86±0.07	7.76±0.7	1:100

NOTES:  $C_{EB}$  – concentration of ethidium bromide in DNA solution,  $C_{DNA}$  – concentration of DNA,  
 $C_{EB}:C_{DNA}$  – ratio of concentration of ethidium bromide to DNA.



**FIGURE 4.** Dependence of fluorescence intensity of complexes EB-DNA from yeasts *Candida guilliermondii* NP-4 from ethidium bromide concentration (scaled curves) 1- non-irradiated yeasts (upper line), 2 – X-irradiated yeasts (middle line), 3 – repaired yeasts (lower line)



**FIGURE 5.** Dependence of fluorescence intensity of complexes EB-DNA from bacteria *E.coli* from ethidium bromide concentration (scaled curves). 1- *E.coli* bacteria wild type (upper line), 2 – *E.coli* bacteria from Crohn's disease patients gut microflora (lower line).

under the influence of various extreme factors on cells, such as X-irradiation. We have realized a comparative investigation of the fluorescence of DNA-EB complexes after X-irradiation of yeast cells, and also after 24-hour post-radiation repair (Fig. 4). After the yeast cells were exposed to the X-irradiation, the DNA fluorescent parameters were changed: the semi saturation of DNA with EB for X-irradiated yeast cells is confirmed by concentration of dyestuff  $C_{EB}=3.56 \times 10^{-6}M$  and by ratio  $C_{EB}:C_{DNA}=1:22$  (table 2), and for 24-hour repaired yeast DNA by concentration of dyestuff  $C_{EB}=3.28 \times 10^{-6}M$  and by ratio  $C_{EB}:C_{DNA}=1:24$ .

The obtained data have shown that the irradiated DNA was saturated with EB faster, than native DNA, and for repaired DNA the semi saturation rate is the highest. Since the difference in the fluorescence intensity of non-irradiated and X-irradiated DNA in conditions of semi saturation with EB is insignificant, and the difference is observed only in the saturation rate, it can be assumed that in the X-irradiated DNA there are damages of secondary structure caused by X-irradiation, such as single-strand breaks. Considering the fact that EB intercalates into a double-stranded DNA molecule, damages of secondary structure of DNA lead to a disruption of intercalation of the dyestuff, consequently a smaller amount of dyestuff binds to DNA. In case of repaired DNA the saturation rate is highest, and it can be assumed that in this case DNA carries a larger number of damages than X-irradiated DNA, including unrepaired single-

strand breaks, intermolecular DNA-DNA and DNA-protein cross-links, and double-strand breaks that may occur in DNA molecule in post-irradiation repair process, because of the so-called “wrong repair” [Glazunov A, Lobachevsky P, 1988]. The presence of double-stranded breaks in the repaired yeast DNA was demonstrated by our early electrophoretic studies [Marutyán S et al., 2016]. Due to the higher number of damages in secondary structure of DNA, the intercalation of EB into DNA is further complicated, and a smaller amount of dyestuff binds to DNA, which affects the rate of DNA saturation with EB.

*Investigation of DNA of bacteria E.coli at Crohn's disease.* For the development of Crohn's disease there is a hereditary predisposition, and one of the causes of the disease is genetic. Proceeding from this, we assumed that during the development of disease there are shifts in the structure of DNA, for example, point mutations. Our preliminary DNA studies have shown that in Crohn's disease, the degree of DNA methylation increases [Pepoyan A 2015], as a result of which electrostatic and hydrophobic interactions between nitrogen bases change, and this leads to changes in the conformation of the DNA molecule. This fact gave grounds to believe that in Crohn's disease, the fluorescence of DNA of *E. coli* from the gut microflora of Crohn's patients can also be changed. Therefore, in next stage of our work we have investigated the fluorescence of complexes EB-DNA isolated from *E.coli* bac-

teria from gut microflora of Crohn's patient (Fig. 5). The obtained data have shown, that in this case the EB concentration in state of semi saturation of DNA with dyestuff was  $0.86 \times 10^{-6} M$ , and  $C_{EB}:C_{DNA}=1:100$ . Thus, the saturation rate of DNA with EB increases during the development of Crohn's disease.

#### CONCLUSION

The obtained data suggest that DNA isolated from prokaryotic cells is saturated with ethidium bromide faster than DNA isolated from eukaryotic cells. It is possible that these differences are related to the features of prokaryotic DNA: eukaryotic DNA differs from bacterial by redundancy of genetic information, i.e. in the eukaryotic cell there is a large number of repeating sequences [Ugar-

kovich D, 2005; Alberts B et al., 2014]. It is possible that this circumstance leads to the fact that the saturation of eukaryotic DNA with EB occurs at its higher concentrations than for prokaryotic DNA. In case of X-irradiation and post-radiation repair of yeasts, the change in DNA saturation rate with EB shows that X-irradiation leads to structural damages in DNA, including single-strand breaks, some of which turn into double-strand breaks in the process of post-radiation repair, so there is deepening of yeast DNA damages during repair process.

In case of development of Crohn's disease, there is also a change in the structure of DNA, in particular - the degree of methylation of DNA increases, which in its turn leads to an increase in the rate of DNA saturation with EB.

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