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EFFECTS OF HIGH-INTENSITY UV RADIATION ON ISOLATED AND DNA-INTERCALATED ETHIDIUM BROMIDE

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High-intensity picosecond laser flash photolysis has been used for investigating ethidium bromide (EtBr). The quantum yield of primary photoproducts as a function of the laser intensity has been obtained. DNA intercalated EtBr and water solutions of EtBr have been investigated. The results have been compared. They show that two-photon processes are predominantly responsible for generation of hydrated electron (e^-_{aq}). The primary processes of DNA photosensitization have also been discussed.

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1. Introduction

Photoprocesses play an important role in the investigations on DNA structure, dynamics and damages. The photosensitization strategy exploits the ability of several organic molecules to intercalate or bind to DNA in sequence- and structure-specific manner and to introduce lesions by long wavelength ultraviolet light (UVA) irradiation. The ethidium bromide (EtBr) has a high affinity for DNA (binding amounts to $2.5 \cdot 10^6$ M^{-1}) [1] and, as a consequence of intercalation, dramatic changes in the photophysical properties of the dye molecule are observed. For example, the fluorescence lifetime of EtBr increases from 1.8 ns in aqueous solution to 23 ns when intercalated in DNA [2–4]. The intercalation of EtBr between the strands of DNA has been the subject of many investigations [1,3,4]. A recently proposed laser version claimed to introduce a high yield with DNA base damage and strand scission at intercalation sites [5]. The effect has been tentatively explained by a resonance-inductive energy transfer from the biphotonicaly excited chromofore to DNA. However, in our previous flash-photolysis and EPR studies [6–8], we have shown that ionization and generation of highly reactive hydroxyl radicals are also important processes in high intensity laser photolysis of DNA photosensitizers, including EtBr. Thus, at present, it is not clear whether high intensity laser photosensitization of DNA occurs through the intermediary of dye cation radicals or involves an energy migration mechanism. It is well established that light absorption by aromatic compounds in aqueous solution may result in photoejection of electrons, which subsequently (in a time of about 10^{-13} s) hydrate to give e^-_{aq} at photon energies lying well below their gas phase ionization potential [9,10]. In the conditions of pulse irradiation, the possibility of electron ejection via two-photon absorption must always be considered [11,12].

In this article, we describe a picosecond "pump-probing" technique in a comparative study of hydrated electron generation in EtBr, isolated and in intercalation complex with DNA, using high-intensity laser radiation at 355 nm. We determine the possible primary processes from excited states of EtBr by measuring the quantity of irreversibly decomposed molecules under UV laser photolysis.

2. Materials and experimental technique

EtBr (2,7-diamino -10-ethyl-9-phenylphenanthridinium bromide) (Fig. 1) was purchased from the firm Sigma and used without further purification. Calf thymus DNA (Sigma) was purified from proteins by phenol extraction.

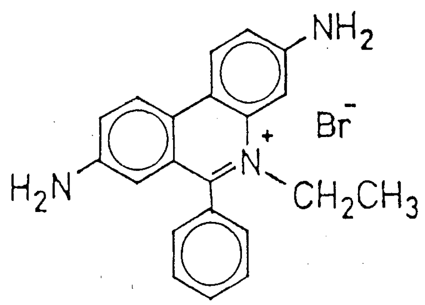

*Fig. 1. Ethidium bromide molecule (*EtBr*).*

Experiments have been carried out in 10 mM phosphate buffer, 5 mM NaCl pH 7.6 at EtBr concentration $c_e = (1 - 60) \cdot 10^{-5}$ M, and DNA concentration was $c_d = (1.0 - 1.5) \cdot 10^{-3}$ M. The absorption was $A = 0.033$ and $A = 0.09$ for EtBr $c_e = 3 \cdot 10^{-4}$ M, and EtBr-DNA complex $c_d = 1.5 \cdot 10^{-3}$ M, respectively, at 355 nm in a 0.1 cm long cell. Simplified scheme of the picosecond flash photolysis setup is shown in Fig. 2. Laser excitation was provided by pulses of 30 ps duration from a mode-locked Nd:YAG laser system. The average pulse energy of the 355 nm radiation at the exit of the third harmonic was between 3 and 8 mJ. The exciting beam was formed by a cylindrical lens of a focal length $f = 40$ cm. The dimensions of the exciting beam

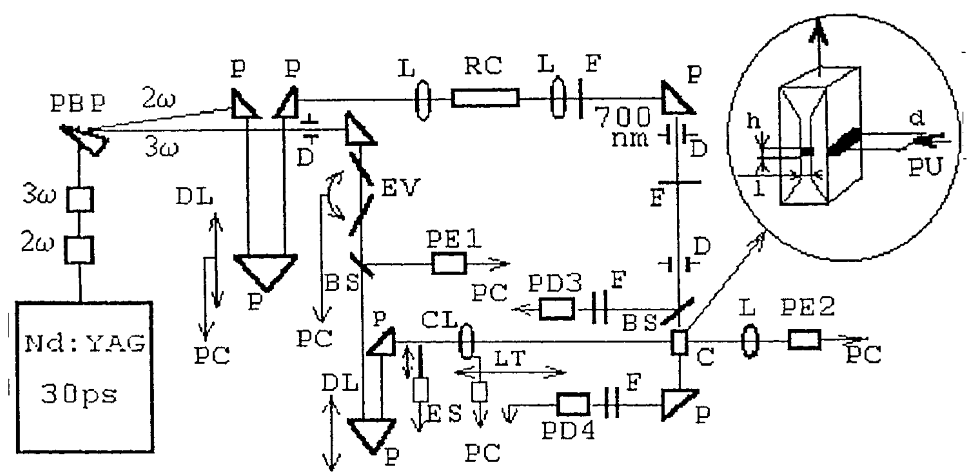

Fig. 2. Simplified scheme of the photolysis setup. The notations are: BS – beam splitter, C – cell ($h = 0.03$ cm, $d = 0.55$ cm, $l = 0.1$ cm*), CL – cylindrical lens, D – diaphragm, DL – delay line, ES – electromagnetic shutter, EV – energy variation, F - - filter, L – lens, LT – linear translation, P – prism, PBP – Pelin-Broca prism, PC – connection to personal computer, PD3 and PD4 – photodiodes, PE1 and PE2 – pyroelectrical energy meters, PU – pump and RC – Raman cell. The* Nd:YAG *laser generates a beam with* $\lambda = 1064.1$ nm*,* 2ω *is the second harmonic (*$\lambda_2 = 532$ nm*) and* 3ω *is the third harmonic (*$\lambda_3 = 355$ nm*).*

at the position of the 0.1 cm cell was adjusted to $h = 0.03$ cm and $d = 0.6$ cm. The solution flowed through a quartz cell at a rate adjusted to provide a fresh solution for every laser shot. The exciting beam was perpendicular to the probe beam, and it entirely overlapped the probe beam in the cell. The excitation energy was set by rotating two antiparallel quartz plates (EV in Fig. 2) while the keeping beam geometry in the cell unchanged. The variation of the time interval between the pumping (355 nm) and the probing (700 nm) beams in the cell was achieved by an optical delay line. Laser-pulse-induced formation of e^-_{aq} is conveniently studied by measuring transient absorbance at 700 nm, near the maximum of the e^-_{aq} absorption band, the extinction coefficient being 18000 M^{-1} cm^{-1} [13]. For simplicity, the probing beam ($\lambda = 700$ nm) was obtained by stimulated Raman scattering (2^{nd} Stokes) in CH_3CN solution and subsequent cut-off filtering. The experimental error of D_{700} measurement at 700 nm was less than $5 \cdot 10^{-3}$ (i.e ± 0.1 %) when averaging over 80 shots. The quantum yield (QY) of hydrated electrons was defined as the ratio between the number of generated electrons and of photons absorbed by ground-state molecules [14], and it was calculated using the experimental D_{700} values as described in Refs. 7 and 8. Linear absorption approximation was used because the direct determination of the real

QY of a photoprocess occurring upon nonlinear excitation was not possible. All data were calculated in real time in a personal computer.

3. Results and discussion

The dose response of the quantum yield (QY) of hydrated electron generation is shown in Fig. 3a. The solid line represents a fit using the formula from Ref. 17 for optical thin layer

$$QY = \frac{1}{\sigma_1 E}\left(1 - \frac{\exp(-\sigma_1 E)}{1-\sigma_1/(\varphi\sigma_2)} - \frac{\exp(-\varphi\sigma_2 E)}{1-\varphi\sigma_2/\sigma_1}\right)$$

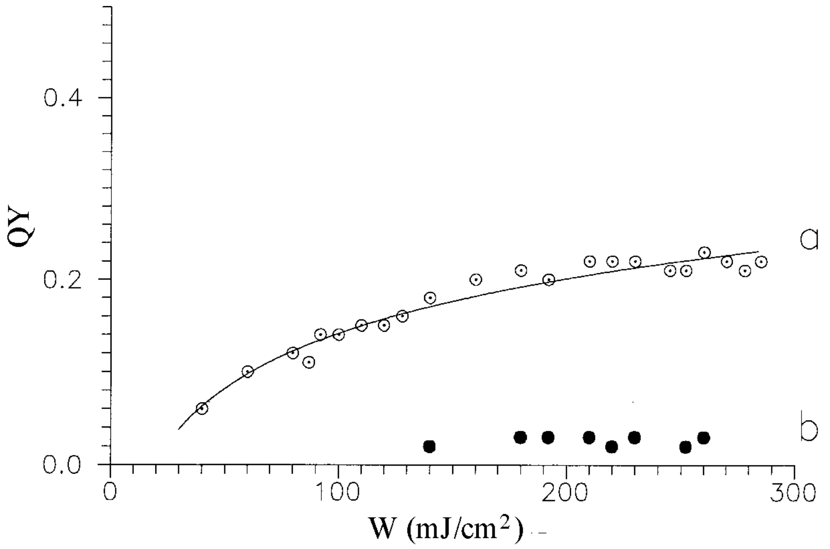

Fig. 3. Quantum yield of hydrated electron generation for different laser doses (delay time is 1.7 ns*) and the theoretical fit (the curve) for a)* EtBr, *concentration* $c_e = 3 \cdot 10^{-4}$ M, *b) DNA intercalated* EtBr, *concentrations:* EtBr $c_e = 3{\cdot}10^{-4}$ M *and DNA bases* $c_d = 3{\cdot}10^{-3}$ M.

where E is the exciting laser dose (number of photons per pulse); σ_1 and σ_2 are the absorption cross-sections of the $S_0 \to S_1$ and $S_{1,2} \to S_n$ transitions, respectively. S_0 is the ground state and S_i are the excited states of EtBr molecule, and φ is the quantum yield of ionization from the S_1 and S_2 states. In order to interpret the influence of excitation energy on the results, we turn to the scheme in Fig. 4. There are two pathways for second photon absorption from the excited EtBr molecule. The energy of the photon ($\lambda = 355$ nm) corresponds to the excited S_2 state. The second photon may be absorbed from S_2, but S_2 has a short lifetime. During the pumping pulse, nonirradiated relaxation to the longer living S_1 state and absorption from S_1 to S_n are possible, too. Both pathways may generate free electron (e^-) and EtBr cation-radical. The fluorescence proves the population of the S_1 state. The lifetime of the S_1 state is 1.8 ns for free EtBr, and 23 ns for intercalated in DNA EtBr. We ignore transition to triplet states in the cases of 30 ps pump pulse. On the other hand, the energy of S_n corresponds to the energy of excited water molecule. After resonance energy transfer from highly excited EtBr

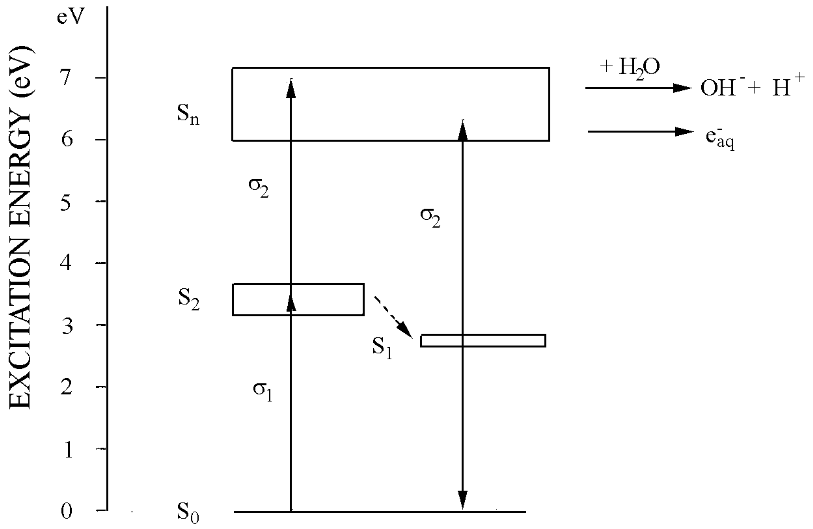

Fig. 4 Scheme of the electronic states of EtBr. *S_0 is the ground state, S_1 and S_2 are the excited states and S_n is the highly excited state. σ_1 is the absorption cross-section for the absorption transition $S_0 \rightarrow S_2$ and σ_2 is the absorption cross-section for the absorption $S_2 \rightarrow S_n$ and $S_1 \rightarrow S_n$ transitions. The transition $S_1 \rightarrow S_0$ shows the the fluorescence.*

to water, OH^-, H^+ and e^-_{aq} are generated [8]. For EtBr, the absorption cross-section is $\sigma_1 = 7 \cdot 10^{-18}$ cm^2 . The best fit shown in Fig. 3a corresponds to $\varphi\sigma_2 = 2.3 \cdot 10^{-18}$ cm^2. Since no appreciable change of the absorption at 355 nm was observed in the whole intensity range, we assume that $\sigma_1 \approx \sigma_2$ and $\varphi \approx 0.20$. The results for σ_2 and φ cannot be determined separately in this procedure.

When the double strand DNA with the base concentration $c_d = 3 \cdot 10^{-3}$ M was added to EtBr with $c_e = 3 \cdot 10^{-4}$ M, we obtained about 100% of intercalated complexes, without free EtBr. Then the maximum of the absorption moved from 480 nm to 500 nm. Figure 3b shows that the quantum yield of hydrated electron from intercalated complexes decreased by a factor of about five, not depending on the delay. At the moment, we have no unambiguous explanation of that finding. No change of D_{700} at a time delay of up to 2 ns after the flash was observed (the time resolution was 30 ps), indicating the absence of electron recombination in this time interval.

When pulses of 40 ns duration were used, the transient absorption at 700 nm was very low at fluencies up to several tenths of J/cm^2 (not shown), corresponding to hydrated electron QY of less than $(1 - 2) \cdot 10^{-2}$. Similar findings have already been reported for hematoporfirine [7].

DNA strand breakage has been observed to occur upon UV lamp irradiation ($\lambda = 350$ nm) [15] and N_2 laser irradiation ($\lambda = 337$ nm) [5] of EtBr DNA complexes. The corresponding QYs were $5 \cdot 10^{-8}$ and $1.9 \cdot 10^{-5}$, respectively, at saturation intensity. At low intensity lamp irradiation, DNA cleavage was explained by electron transfer from the excited EtBr to DNA bases, the low quantum yield being

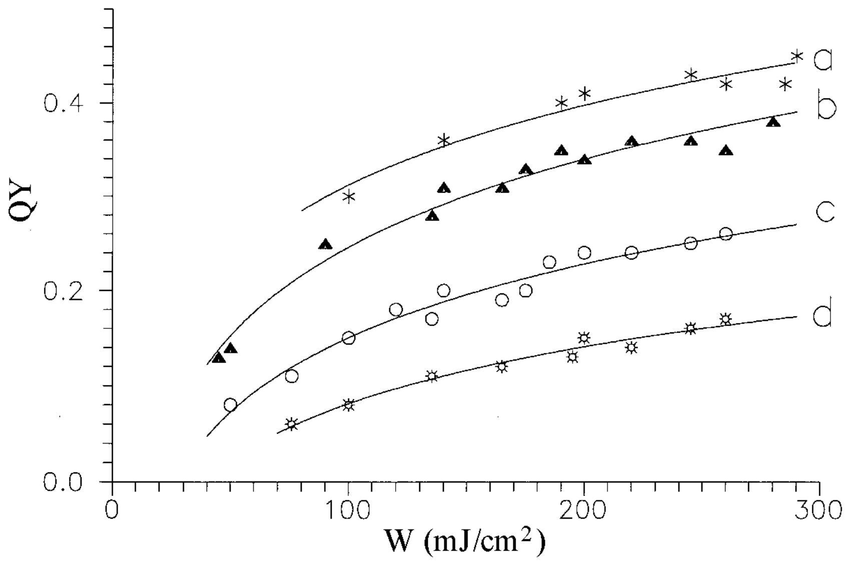

Fig. 5. Quantum yield of hydrated electron generation for different laser doses without delay, for different EtBr *concentrations: a)* $c_e = 1.87 \cdot 10^{-5}$ M, *b)* $c_e = 7.5 \cdot 10^{-5}$ M, *c)* $c_e = 3 \cdot 10^{-4}$ M *and d)* $c_e = 6 \cdot 10^{-4}$ M.

due to a fast back-transfer of electron. When methyl viologen (MV) was added to EtBr – DNA complexes, the QY of DNA strand scission increased by a factor of about 10. The MV was supposed to prevent back-transfer of electron, thus making possible the oxidation of the adjacent base by oxidized EtBr [16]. The use of two DNA binding substances instead of one leads to a decrease of the quantum yield of strand scission.

When high intensity laser pulses were used, the quantum yield of DNA cleavage increased by more than 3 orders of magnitude due to the biphotonic excitation and presumably also by the fast energy transfer [5]. However, no experimental evidence in support of the involvement of energy transfer has been presented. Our electron generation studies showed that biphotonic generation of hydrated electrons is a predominant process at high laser intensity. Figures 3, 5 and 6 show nonlinear dependence of hydrated electron quantum yield on laser dose for a number of EtBr concentrations in water solution. The generation of hydrated electron after two-quantum EtBr excitation may occur by two processes:

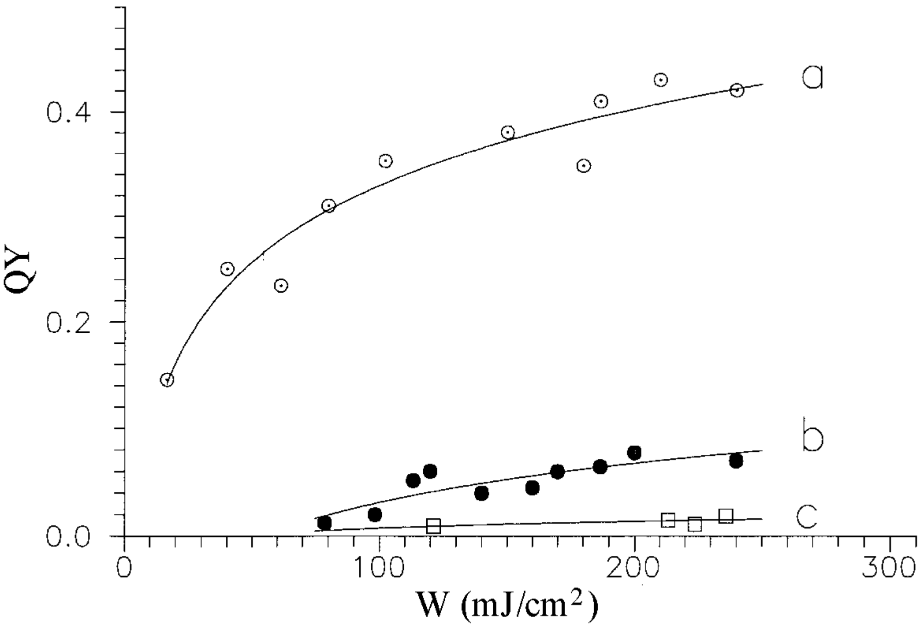

Fig. 6. Quantum yield of hydrated electron generation without delay for different laser doses of: a) isolated EtBr, *concentration:* $c_e = 1.5 \cdot 10^{-4}$ M, *b) DNA intercalated* EtBr - EtBr, *concentrations:* $c_e = 1.5 \cdot 10^{-4}$ M *and the base concentration:* $c_d = 3 \cdot 10^{-3}$ M, *and c) DNA intercalated* EtBr *with added methylviologen, the concentrations:* $c_e = 1.5 \cdot 10^{-4}$ M, $c_d = 3 \cdot 10^{-3}$ M *and* $c_m = 1.5 \cdot 10^{-3}$ M, *for* EtBr, *DNA bases and methylviologen molecules, respectively.*

1) the EtBr ionization (Fig. 7a), and

2) the water ionization (Fig. 7b).

In each of the processes, two absorbed photons lead the EtBr molecule to a highly excited state S_n (EtBr**). In the first process, direct ionization is possible to cation radical (EtBr$^+$). The ejected electron hydrates in the bulk. In the second process, the resonance energy transfer from a highly excited state (EtBr**) to H_2O molecule may lead to the excited water molecule (H_2O^*). At least two processes are possible if the water molecule is in the excited state: generation of H^+ and OH^-, and e^-_{aq}. This corresponds to the results shown above on registered hydrated electrons as well as to our previous results on registered OH^- radicals [8]. Figure 6 shows laser dose responsible for the quantum yield of hydrated electron generation without delay in the cases of isolated EtBr (Fig. 6 a), DNA intercalated EtBr alone (Fig. 6 b) and with added methylviologen (Fig. 6 c). The control experiments with 34 ns delay show generation of e^-_{aq}, excluding absorption at 700 nm from S_1. The DNA intercalation of EtBr leads to a competition of other possible processes, Fig. 8).

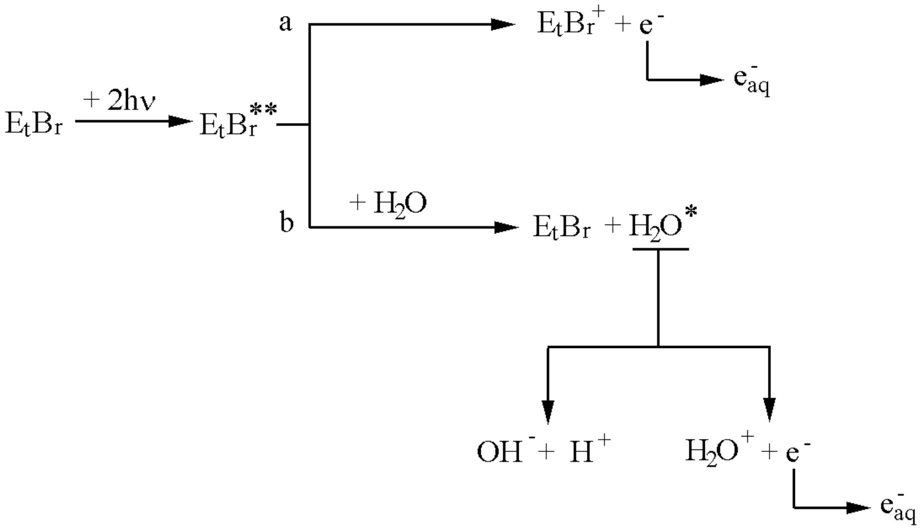

Fig. 7. Scheme of possible primary processes after two-photon EtBr *excitation. a)* EtBr *ionization and b) resonance energy transfer to water. The notations are:* $EtBr^+$ *– cation radical* EtBr, $EtBr^{**}$ *– highly excited* EtBr, H_2O^* *– excited water molecule,* e^-_{aq} *– hydrated electron,* H_2O^+ *– ionized water molecule,* e^- *– free electron.*

Once the electrons have been ejected and hydrated via the pathway shown in Fig. 8a , the resulting EtBr cation radicals ($EtBr^+$) have enough time to react with the neighbouring bases, leaving presumably base cation radicals and other reactive nucleoside intermediates BOH (OH - aducts) and B-H (H abstractions), which are known to lead to direct strand scission or to its labilization. On the other hand, energy transfer to a neighbouring base may lead to the base ionization (Fig. 8b) and modification or strand energy migration. It is difficult to differentiate between the processes (a) and (b), because products are similar. We don't know about the possibility of resonant energy transfer to water, but keeping in mind the known characteristics of DNA-EtBr complexes, it doesn't seem to be very important. Not only the S_1 state lifetime is changed when EtBr intercalate, but the maximum of absorption band moves from 485 nm to 500 nm. This shows strong complex influence on EtBr excited states. But if the solution contains free EtBr, the generation of hydroxil radicals trough water dissociation must be considered. The decreasing of hydrated electron quantum yield, when adding of methylviologen (Fig. 6), shows undoubtedly the participation of the ionization in the previous processes. Briefly, our results are in

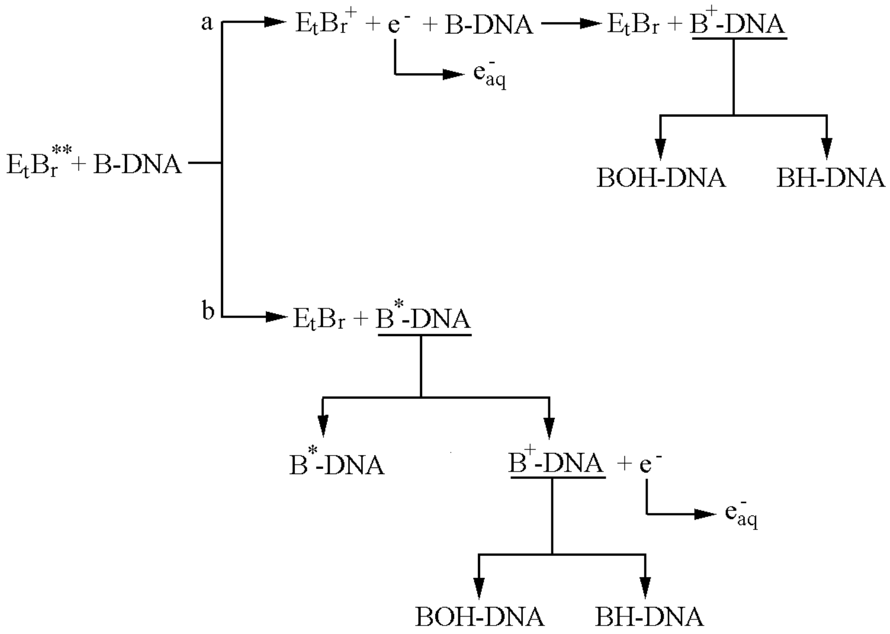

Fig. 8. Scheme of possible primary processes after two-photon excitation of DNA-intercalated EtBr *a)* EtBr *ionization and b) resonance energy transfer from highly excited* EtBr *to DNA base. The notations are:* $EtBr^+$ *– cation radical* EtBr, $EtBr^{**}$ *– highly excited* EtBr, H_2O^* *– excited water molecule,* e^- *– free electron,* e^-_{aq} *– hydrated electron, B-DNA – connected to DNA base,* B^**-DNA – excited connected to DNA base,* B'^**-DNA – another excited connected to DNA base,* B^+*-DNA – ionized connected to DNA base, BOH-DNA and BH-DNA – OH and H products.*

a good agreement with a dye-cation-radical-mediated DNA damage rather than an energy transfer mechanism. EtBr works as a "Type I" (free radical) photosensitizer. Further studies including chemical and electrophoretical analysis of laser-induced sensitized lesions in DNA are in progress. The most important advantage over the known methods, including classical photochemistry, is the rapidity of photolesions formation - within a single picosecond laser pulse, thus enabling conformational dynamic studies to be carried out in-situ or even in-vivo.

4. Conclusions

The highly excited free EtBr in aqueous solution generates free electrons by direct ionization and/or OH^-, H^+ and e^-_{aq} by energy transfer to water. EtBr is an effective photosensitizer. We believe that dye-cation-radical-mediated DNA damage is more probable than the energy transfer mechanism.

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UČINCI UV ZRAČENJA VISOKOG INTENZITETA NA IZDVOJEN I S DNA INTERKALIRAN ETIDIUM BROMID

Istraživali smo etidium bromid (EtBr) pomoću pikosekundne bljeskovne fotolize velikog intenziteta. Odredeni su kvantni prinosi fotoprodukata u ovisnosti o intenzitetu laserskog snopa. Istraživao se je EtBr u vodenoj otopini i interkalirani s DNA. Rezultati se usporeduju i oni pokazuju da pretežno dvofotonski procesi tvore hidrirane elektrone e^-_{aq}. Raspravljaju se takoder primarni procesi fotosenzitiranja DNA.