Bulged Guanine is Uniquely Sensitive to Damage Caused by Visible-Light Irradiation of Ethidium Bound to DNA: A Possible Role in Mutagenesis

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Dedicated to my good friend and colleague Professor *André M. Braun* on the happy occasion of his 60th birthday

The interaction of ethidium bromide (= 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EB) with a series of duplex DNA oligomers having single-base bulges and single-base mis-pairs was investigated (*Fig. 1*). Physical and spectroscopic analysis reveals no definitive evidence for selective binding of EB at the bulge or mis-pair. However, irradiation of the bound EB with VIS light leads to lesions in the DNA selectively in the sequence having a bulged guanine. This reaction is attributed to the formation of an exciplex between the lowest excited singlet state of the EB and the bulged guanine. The exciplex is trapped by H_2O, which initiates a sequence of reactions that lead to piperidine-requiring strand cleavage at this site. Significantly, the damaged bulged guanine is not recognized by *E. coli* formamidopyrimidine glycosylase (Fpg), which is part of a base-excision repair system for oxidative damage to DNA. Thus, DNA containing a bulged guanine and having a bound intercalator may be irreparably damaged by exposure to VIS light, even though normal duplex DNA is relatively inert under these conditions.

1. Introduction. – Damaged or imperfect DNA structures initiate a cellular repair response that is indispensable to the maintenance of genome integrity [1]. Bulged (unpaired) bases in double-strand DNA are imperfections that can arise from recombination between sequences that are not fully homologous or from errors in replication. Bulged bases resulting from replicative errors are considered to play an important role in frame-shift mutagenesis [2], and they are often found to be sites of especially high reactivity [3]. Oxidative reactions are frequently implicated in mechanisms that lead to damage of DNA [4][5]. Many oxidative reactions are initiated by exposure to light, which is of significance because of the demonstrated link between light exposure and carcinogenesis [6]. Consequently, we have undertaken an investigation of light-induced oxidative reactions and their repair in DNA containing bulged and mis-paired structures.

Base bulges cause kinking of the DNA helical axis, which has been observed by electrophoretic retardation of the bulged DNA compared with their corresponding full duplexes [7–9]. Kinking in DNA duplexes by base bulges has been confirmed by NMR spectroscopy, fluorescence energy-transfer experiments, and electron microscopy [10–13]. A crystal structure has been reported for an oligonucleotide containing a single bulged adenosine [14]. The location of bulged nucleotides within the double helix has been examined by NMR spectroscopy. The extra base is thought to be stacked intra-helically in most cases [12][15]. However, pyrimidine bulges flanked by A·T rich sequences are found to be extra-helical [16][17]. In one case, a bulged cytosine was found to form both intra- and extra-helical structures in a temperature-dependent manner [18]. In another case, each guanine in a $(G)_3$ sequence that lacks one

complementary cytosine adopts a transient single-strand form, which is revealed by reaction with diethyl pyrocarbonate [19].

The insertion of a single-base bulge into one strand of duplex DNA destabilizes the molecule by approximately 4 kcal/mol at 37° [10][16][20][21]. The destabilization depends upon interactions with the base pairs directly neighboring the bulge and also with more distal base pairs [22]. Nucleic-base bulges are stabilized by intercalating drugs, a feature that has been suggested as a basis for their mutagenic properties [23].

Ethidium bromide (= 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EB) is one of the most thoroughly studied of the common DNA intercalators [24–26]. Consequently, it serves as a model for examination of many drug-DNA interactions. EB binds more strongly to DNA with a bulged base than to a perfect double helix [27], as do other dyes [28]. These findings are consistent both with enhanced binding on either side of the bulge or with strong EB binding to the bulged base on the outside of the helix. Other intercalators behave similarly [29].

It has been reported that irradiation of EB with VIS light yields spontaneous DNA-strand cleavage, which was attributed to H-abstraction from a deoxyribose [30][31]. However, *Kochevar* [32] found that irradiation of intercalated EB leads to efficient single-strand breaks only when it is carried out in the presence of an externally bound methyl viologen cosensitizer. Further, *Barton* and co-workers [33] described experiments showing that irradiation of EB with VIS light does not cause strand cleavage, but that UV exposure leads to both direct strand cleavage at the 5′-G of GG steps and to piperidine-sensitive lesions at guanines. More recently, *Barton* and co-workers reported long-distance electron transfer to excited ethidium only in oligonucleotides specially modified to contain easily oxidized bases [34][35]. Clearly, the nature and mechanism of damage to DNA caused by irradiation of bound EB is not well-established.

We report here a study of light-induced cleavage by EB of duplex DNA containing single-base bulges and base mis-pairs. A series of oligonucleotides was constructed that are identical except for the composition of a bulge or mis-pair region. In the bulge series **1**–**5** shown in *Fig. 1* (see below), DNA 20-mers contain an unpaired G, C, T, or A in the center of a $(T)_2(N)(T)_2$ sequence. In the mis-pair series **6**–**8**, a guanine is opposite either G, A, or T in the center of a $(T)_2(G)(T)_2$ sequence. We find that irradiation of EB either with VIS or UV light causes efficient piperidine-requiring DNA strand cleavage only in the structure having a bulged G. Irradiation of EB in the presence of the other bulged bases or mis-paired oligonucleotides gives much less-efficient strand cleavage, which occurs primarily at guanines. The bulged, damaged guanine is not excised by *E. coli* formamidopyrimidine glycosylase (Fpg), which is part of a base-excision repair system for oxidative damage to DNA [36][37]. These findings may be of relevance to light-induced carcinogenesis [38–40][41][42].

2. Materials and Method. – 2.1. *General.* Oligonucleotides were synthesized by standard, solid-phase 2-cyanoethyl phosphoramidite methods and purified by HPLC. The concentrations of the purified oligomers were determined by UV spectrophotometry at 260 nm. The *Klenow* fragment of DNA polymerase I and T4 polynucleotide kinase were purchased from *New England Biolabs* and used as received. [γ-^{32}P]ATP was from *Amersham* and used as received. Piperidine, ethidium bromide, methylene blue, and buffer ingredients were purchased from *Aldrich* and used without further purification. 'Histograms' from the autoradiograms were obtained by means of UTHSCSA Image Tool Version 1.27 in conjunction with a *HP Scanjet-IIcx* scanner. β-

Detection of polyacrylamide gel electrophoresis (PAGE) products was carried out with a *Scanalytics AMBIS* radiolytic imaging system β-emission detector. Irradiant counts were quantified over the area of each cleavage band.

2.2. *Radiolabeled DNA.* The 5′-end-labeling reactions were performed with T4 polynucleotide kinase and [γ-^{32}P]dATP. DNA Sample strands of 250–500 pmol were incubated with 2.0–5.0 µl [γ-^{32}P]ATP (5000–6000 Ci/mmol) and 1.0 µl (8 units) of T4 polynucleotide kinase in a total volume of 20–30 µl at 37° for 45 min. After incubation, the labeled DNA was suspended in denaturing loading buffer and purified on a 20% denaturing polyacrylamide gel. Labeled product was located within the gel by autoradiography. Bands corresponding to the DNA product were excised from the gel and eluted in 450 µl of elution buffer (0.5M NH_4OAc, 10 mM $Mg(OAc)_2$, 1.0 mM EDTA (ethylenediaminetetraacetic acid), and 0.1% SDS (dodecyl sodium sulfate)) at 37° for 4 h. The samples were centrifuged at 12000 *g* for 1–2 min pulses. Precipitation proceeded by the addition of 1.0 µl of glycogen and 700 µl of cold abs. EtOH to the samples. The reaction mixtures were vortexed, placed on dry ice for 30–45 min to further precipitate, and spun for 30 min at 12000 *g* in a *Savant*-µ*SpeedFuge* centrifuge. The supernatant was discarded. The resulting DNA pellets were washed twice at r.t. with 80% EtOH/H_2O and spin-dried with a *Savant Speed Vac Plus* for 30 min. The dried pellets were reconstituted, hybridized on a thermocycler at 90° for 5 min, and slowly cooled to r.t. within 4 h.

2.3. *Photocleavage Experiments.* Irradiation of 20-µl samples was accomplished by incubating labeled (5000 c.p.m.) and 5 µM of unlabeled strand with complementary strand in 10 mM sodium phosphate buffer solution containing the sensitizer and other components as indicated. Deuterated samples were combined with the appropriate aqueous, buffered reagents and lyophilized to dryness before dissolution in D_2O. Samples were irradiated in 1.5-ml micro-centrifuge tubes with a *Rayonet* photoreactor equipped with 8 (λ 350 nm) lamps. Irradiation with VIS light ($\lambda > 400$ nm, cutoff filter) was performed with an *Oriel-1000-W*-Hg/Xe lamp focused *ca.* 15 cm from a 40-µl sample soln. contained in a 120-µl ultra-micro-centrifuge tube.

Post irradiation, samples were apportioned and reserved for nonpiperidine and piperidine treatments. Portions for nonpiperidine treatment were precipitated as described above and vacuum spun-dried. The other portions were subjected to piperidine treatment that consisted of adding 1M (100 µl) of piperidine per sample. Each treated soln. was vortexed for 15 s. The sample mixtures were heated at 90° for 30 min. After heating, the samples were pulsed for 5–10 s with a *Savant Speed Vac Plus* centrifuge. With opened caps, the samples were dried in the *Savant* for 1 h at medium heat. To ensure that all piperidine was removed, 20 µl of H_2O was added to each sample and the drying process was repeated. This water-wash procedure was performed twice. Dried samples were dissolved in 5.0 µl of denaturing formamide loading buffer. The photocleavage products were separated electrophoretically on a 20% polyacrylamide sequencing gel and detected by autoradiography.

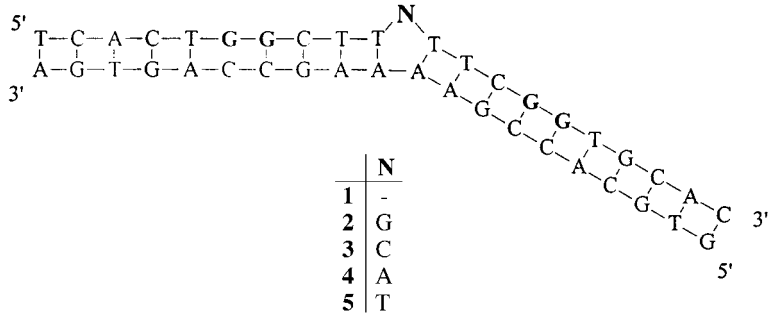
2.4. *Formamidopyrimidine Glycosylase* (Fpg) *Digestion.* The standard reaction mixture (10 µl of 50 mM *Tris*·HCl (pH 7.5), 2 mM EDTA, 70 mM NaCl, and 10 µg of Fpg) was incubated with 5 µM of DNA at 37° for 5 min. Reactions were terminated by heating the sample solns. to 70° followed by precipitation with EtOH at −20°. The reaction was analyzed by 20% polyacrylamide sequencing gel containing 7M urea.

2.5. *Melting Temperature* (T_m) *Determinations.* Thermal denaturation studies were performed in 10 mM sodium phosphate buffer soln. at pH 7. Sample solns. were placed in 1-cm-path-length quartz cells and monitored spectrophotometrically at 260 nm. A heating and cooling rate of 0.5°/min was maintained throughout the analysis. The T_m was obtained from first derivative maxima.

3. Results. – 3.1. *Studied Oligonucleotides. Fig. 1* shows the structures of the DNA oligonucleotides used in this work. The compounds in *Series I* and *Series II* contain 20 complementary base pairs. DNA **1** is a 'full-duplex', in which every base is paired with its complement. It serves as a control to reveal the affects of a base bulge or mis-pair. The oligonucleotides in *Series I*, DNA **2**–**5**, contain single-base bulges, G, C, A, and T, respectively, at the central position in the $(T)_2N(T)_2$ sequence. The strand containing the extra base is the one that will be 5′-labeled with ^{32}P. The oligonculeotides in *Series II* contain a single guanine in the center of the sequence on the labeled strand, $(T)_2G(T)_2$, which is opposite either a G, A, or T in DNA **6**–**8**, respectively.

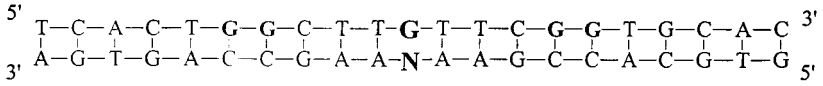
3.2. *Association of EB with DNA* **1** *and* **2**: *Melting Data. Fig. 2* shows the melting behavior (T_m) of 2.0 µM buffer solutions of DNA **1** and **2** determined by UV/VIS spectroscopy. In the absence of EB, these oligomers melt reversibly at 54.9 and 46.3°,

Series I:

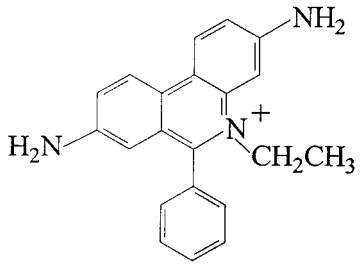


	N
1	-
2	G
3	C
4	A
5	T

Series II:



	N
6	G
7	A
8	T



EB

Fig. 1. *Hybridized oligonucleotides DNA* **1**–**8** *and ethidium bromide* (EB)

respectively. The bulged guanine in DNA **2** reduces the T_m by 8.6°, which is consistent with the previously reported destabilization caused by introduction of a single bulged base [10][16][20][21].

Ethidium bromide binds to duplex DNA with an equilibrium constant K_b of approximately 10^4 M^{-1} [27]. Addition of 1–4 equiv. of EB to solutions containing DNA **1** shows the expected increase in observed T_m (*Fig. 2*,*a*), but the effect is complex, which indicates multiple binding sites [27]. The addition of 1 equiv. of EB results in the appearance of at least two apparent transitions. The first T_m is at *ca.* 56° and the second at *ca.* 64°. The lower-temperature transition disappears as more EB is added to the

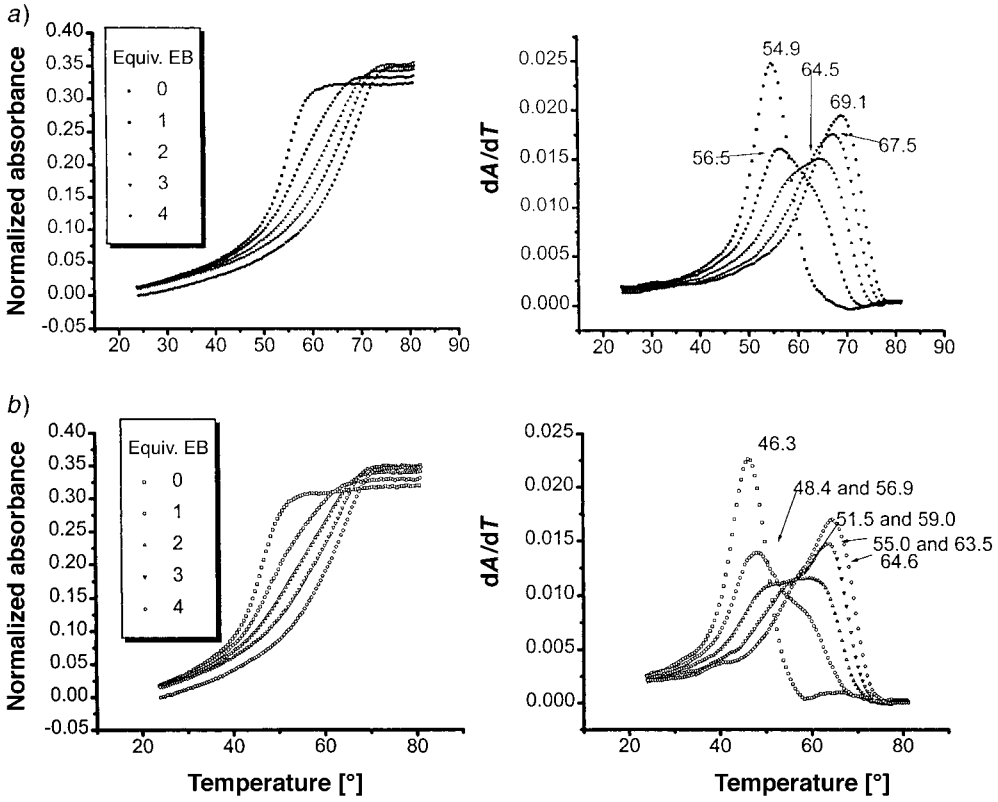


Fig. 2. *Melting temperature curves for* a) *duplex DNA* **1** *and* b) *duplex DNA* **2** *containing 0 to 4 equiv. of EB* (as indicated in the text box). Conditions: 5 μM **1** or **2** in sodium phosphate buffer solution at pH 7.0; melting behavior monitored by absorption spectroscopy at 260 nm; right-hand plots: first derivatives of the absorption data, with the corresponding T_m [°] values.

solution. When the ratio of EB to DNA **1** is 4 : 1, only a single transition having T_m at *ca.* 69° is observed. These findings indicate that there are at least two different modes for binding of EB to DNA **1**. The addition of EB to DNA **2** also causes stabilization of this compound, and the melting behavior is again complex (*Fig. 2,b*). Although the pattern of melting is the same, the extent of stabilization for DNA **2** is higher than for DNA **1**. The melting experiments performed on C-, A-, and T-bulged DNA **3**–**5** show the same complex melting behavior, and T_m values essentially the same as that for DNA **2** are observed.

The observed increase in the T_m of bulged DNA **2** by addition of EB is greater than that for full duplex DNA **1**. *Williams* and co-workers reported that methidiumpropyl EDTA binds to a C-bulged DNA duplex at two relatively strong sites having K_b of approximately $2.2 \cdot 10^5$ M^{-1}, which are presumed to be located at or near the bulge site [43]. However, there is no evidence for binding selectively at the bulge of DNA **2**, since the melting patterns of full duplex and bulged DNA compounds are essentially indistinguishable.

3.3. *Photocleavage of DNA by Irradiation of EB.* We examined the reactions of DNA **1**–**8** initiated by irradiation of EB with UV or VIS light to probe the effect of DNA-defect structures on light-induced oxidative cleavage. The absorption spectra of EB in buffer solution and bound to DNA are shown in *Fig. 3*. The spectra show two main transitions: a strong UV band with a maximum below 300 nm; and a weaker VIS band with a maximum above 470 nm. Both bands are shifted by the electronic interaction between EB and the DNA helix [44]. However, addition of either DNA **1** or DNA **2** to a solution of EB causes essentially identical shifts of the spectrum. Thus, this experiment reveals no distinct evidence for selective binding of the EB at the bulged guanine.

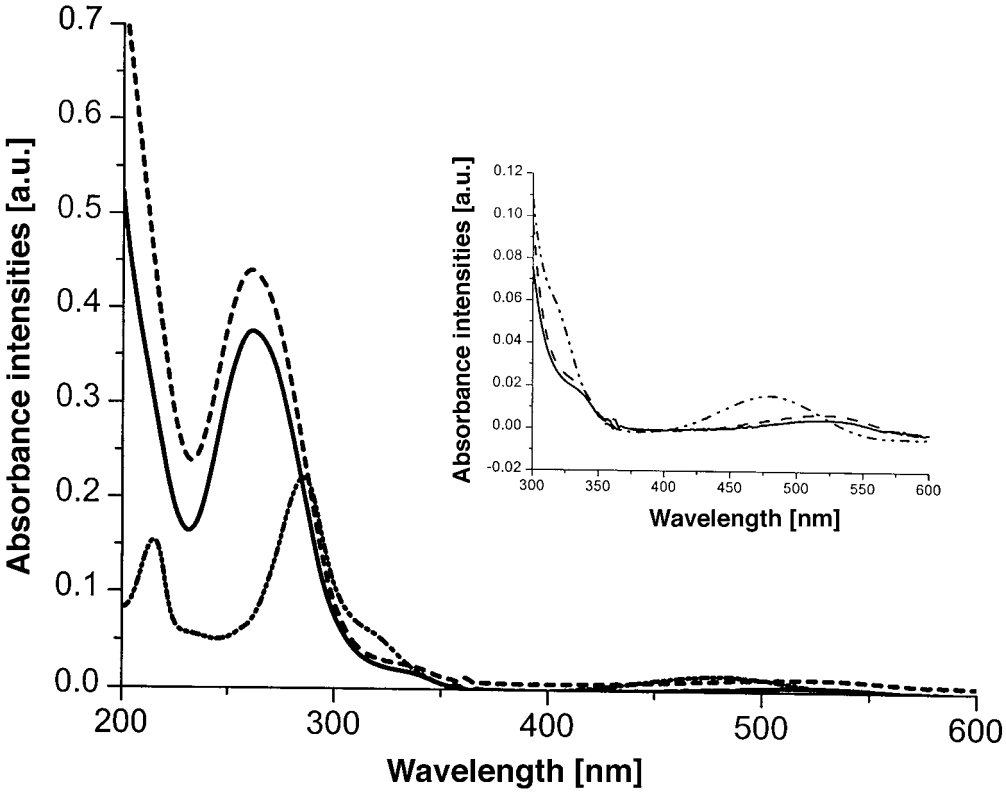


Fig. 3. *UV/VIS Absorbance spectra: EB* (2.0 μM) *in 10 mM sodium phosphate buffer solution* (···), *EB and 2.0* μM *of DNA* **1** (---), *and EB and 2.0* μM *DNA* **2** (——). Inset: expanded scale for the VIS-region absorption of EB.

3.4. *Irradiation with Visible Light.* Irradiation ($\lambda > 400$ nm) of a phosphate-buffered solution (pH 7.0) of ^{32}P-labeled DNA **1** containing from 1 to 8 equiv. of EB for up to 5 h does not cause a significant amount of strand cleavage even after treatment with piperidine. DNA **1** contains two GG steps on the labeled strand, and preferential strand cleavage at the 5′-G of this sequence has been shown to be indicative of one-electron oxidation of DNA [45–47]. This finding confirms the similar observation reported by

Barton and co-workers [33]. Clearly, reactions from EB*[1] (the lowest excited singlet state that is formed from VIS-light irradiation) with DNA do not yield a guanine radical cation (the product of one-electron oxidation). This is an expected result, since EB*[1] is not quenched rapidly by deoxyguanosine triphosphate in solution because the energetics of the electron-transfer reaction appear to be unfavorable [34][48].

In contrast, VIS-light irradiation of EB bound to 5′-^{32}P-labeled DNA **2** gives relatively efficient strand cleavage selectively at the bulged guanine. These data are shown in *Fig. 4*. There is no measurable strand cleavage when the samples are not irradiated or when irradiated samples do not contain EB. Strand cleavage is not detected unless the samples are treated with piperidine. The extraordinary sensitivity for detection of DNA-strand cleavage in radiolabeled samples requires careful control experiments. In particular, it could be that the small amount of UV light transmitted by the optical filter causes all of the observed reaction. This possibility was eliminated when doubling the number of filters was shown to have essentially no effect on the efficiency of cleavage at the bulged guanine. Additional control experiments were carried out on samples irradiated with UV light.

3.5. *Irradiation with UV Light*. Irradiation of phosphate-buffered solutions of ^{32}P-labeled DNA **1** containing from 1 to 8 equiv. of EB in a *Rayonet* photoreactor equipped with 350-nm lamps for 2 h causes a very small amount of strand cleavage after treatment with piperidine. Consistent with the earlier report [33], very long exposure to UV light does yield more-readily detectable cleavage at the GG steps of DNA **1**. In

Fig. 4. *PAGE Analysis of the VIS-light irradiation* ($\lambda > 400$ nm, 5 h) *of 5′-^{32}P-labeled G-bulged duplex* **2**) *in 10 mM sodium phosphate buffer solution. Lanes 2*, *3*, and *4* show the results of solutions of DNA **2** (5.0 μM) containing 1.00, 2.5, and 8.0 equiv. of EB. *Lane 1* is light control with no EB. The sample was subsequently treated with piperidine (30 min, 90°). The bulged G is indicated.

contrast, UV irradiation of DNA **2** for 2 h gives easily detected strand cleavage primarily at the bulged guanine after piperidine treatment, along with significantly less-efficient cleavage at the GG steps, see *Fig. 5*. In this regard, the primary difference between reactions of DNA **2** caused by irradiation of bound EB with VIS or UV light is emergence of the inefficient reaction at GG steps under the latter conditions. *Fig. 5* also shows the results of UV irradiation of EB bound to DNA **3**–**5**. No significant strand cleavage is detected at the bulged C, T, or A, even after piperidine treatment. Very inefficient cleavage at the GG steps is barely detectable in some samples.

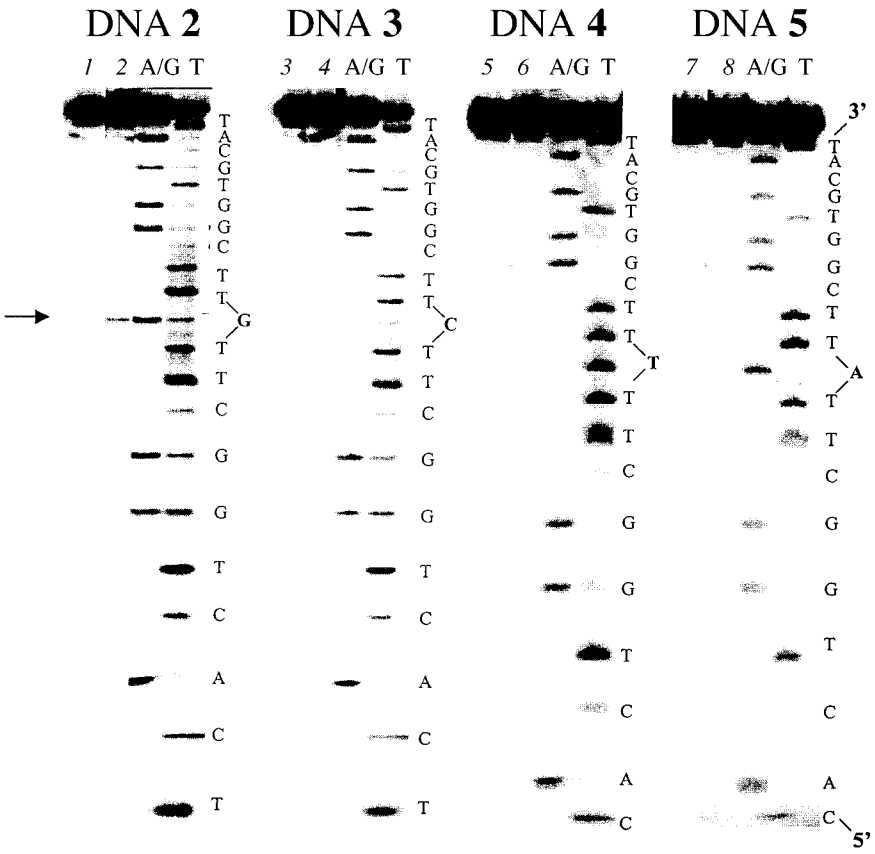


Fig. 5. *Autoradiograms showing cleavage products from the UV irradiation* (350 nm, 2 h) *of EB* (40 μM) *in 10 mM sodium phosphate buffer solutions containing DNA* **2**–**5** (each sample is 5.0 μM in duplex DNA). All samples were treated with piperidine (30 min, 90°) before analysis. *Lanes 1*, *3*, *5*, and *7* are dark controls (no light exposure). *Lanes 2*, *4*, *6*, and *8* are after the irradiation. The lanes marked A/G and T are *Maxim-Gilbert* sequencing lanes.

The reaction of electronically excited EB with mis-paired DNA was also examined. *Fig. 6* shows the results of UV irradiation of EB bound to DNA **6**–**8**. Compared with the results for the G-bulged DNA **2** (*Lane 2* in *Fig. 6*), GG, GA, and GT mis-pairs are very inefficiently damaged – the extent of cleavage at the mis-pair sites is approximately the same as at the GG step. These findings indicate that EB, excited

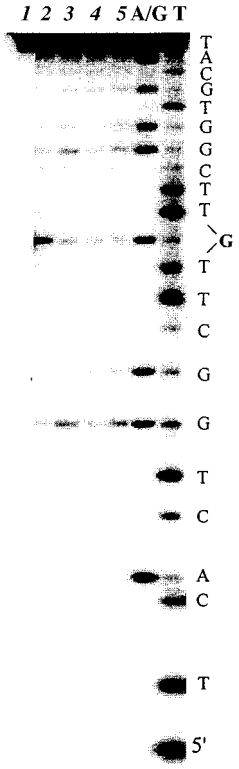


Fig. 6. *Autoradiogram showing cleavage products from the UV irradiation* (350 nm, 2 h) *of EB* (40 μM) *in 10 mM sodium phosphate buffer solution containing the various DNA duplexes.* All samples were treated with piperidine (30 min, 90°) after irradiation but before analysis. *Lane 1* is a dark control. *Lane 2* shows the results of the irradiation of a solution containing 5.0 μM DNA **2**. *Lanes 3–5* are matching experiments that show the results of irrradiation of DNA **6**–**8**, respectively. The lanes labeled A/G and T are *Maxim-Gilbert* sequencing experiments.

with either UV or VIS light, causes selective, piperidine-requiring cleavage at a G-bulge with relatively high efficiency. A series of experiments was conducted to elucidate the mechanism of this unexpected reaction.

3.6. *Mechanistic Investigation of G-Bulge Selective Cleavage.* Studies of light-induced reactions of DNA have identified singlet oxygen ($^1O_2$) as capable of causing damage selectively at guanines [49][50]. Singlet oxygen cleaves single-stranded or bulged guanines with greater efficiency than guanines confined to the helix [51]. We carried out a series of experiments to assess the role that $^1O_2$ plays in the EB-induced cleavage of DNA **2**.

The lifetime of $^1O_2$ increases approximately tenfold when the reaction solvent is changed from H_2O to D_2O [52]. This effect has been used to verify the participation of $^1O_2$ in reactions with DNA, since the increase in lifetime is manifested as a more efficient reaction [53]. We compared the efficiency of strand cleavage in DNA **2** from irradiation of EB in H_2O and D_2O solutions. The results shown in *Fig. 7* reveal that the cleavage efficiency at the bulged guanine increases slightly in D_2O, but there is no meaningful increase in strand cleavage at the GG steps. This finding poses a dilemma: if $^1O_2$ is the active reagent, an efficiency increase should be seen at all guanines, not selectively at the bulged G. To define the role of $^1O_2$ further, it was generated independently by irradiation of methylene blue (MB).

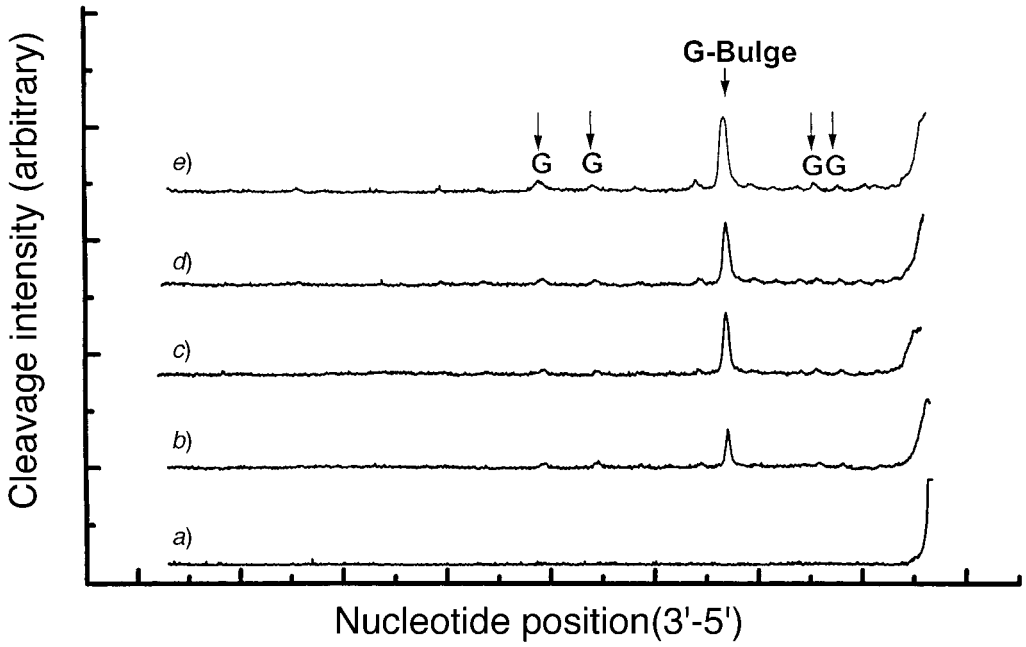


Fig. 7. *' Histograms' illustrating cleavage at the G-bulge of DNA* **2** *in H_2O or D_2O* (5.0 μM) *after irradiation of EB* (40 μM) *at 350 nm*. The samples were treated with piperidine before analysis. *a*) No irradiation, *b*) irradiation (1 h) in H_2O, *c*) same as *b*) but in D_2O, *d*) irradiation (2 h) in H_2O, and *e*) same as *d*) but in D_2O.

When irradiated with VIS light, MB sensitizes the formation of $^1O_2$ and causes DNA damage [49]. A solution of DNA **2** containing MB was irradiated, and strand cleavage was determined by PAGE and autoradiography. The results shown in *Fig. 8* reveal modest selectivity for cleavage at the bulged guanine by $^1O_2$ when compared with the reaction of EB, and there is significantly increased cleavage at the GG steps (trace *b*). In addition, the MB-sensitized cleavage is much more efficient in D_2O (trace *c*)) than it is in H_2O solution. These findings indicate clearly that $^1O_2$ does not play a significant role in the EB-initiated cleavage of DNA **2**.

The measurable solvent isotope effect in the EB-initiated cleavage of DNA **2** is traced to the effect of D_2O on the lifetime of EB^{*1}. Proton donation by water quenches the excited state of EB, and the lifetime of EB^{*1} is known to increase approximately 3-fold in D_2O [54]. These data suggest that the enhanced cleavage efficiency at the G-bulge in D_2O is a consequence of a direct reaction of EB^{*1} with guanine. We measured the lifetime of EB^{*1} bound to DNA **1** and to DNA **2** to search for evidence of selective binding at the bulged guanine.

Fluorescence lifetime measurements of EB are useful indicators of its local environment. When EB is intercalated in duplex DNA, it is protected from quenching by H_2O, and its lifetime increases from a value of *ca.* 2 ns to *ca.* 24 ns [54]. *Kallenbach* and co-workers [55] reported that EB exhibits a 12–16 ns component when it is bound to DNA containing a bulge or mis-pair. *Fig. 9* shows the fluorescence decay for EB complexed to DNA **1** or DNA **2**. Both decays fit very well to a double exponential having a very

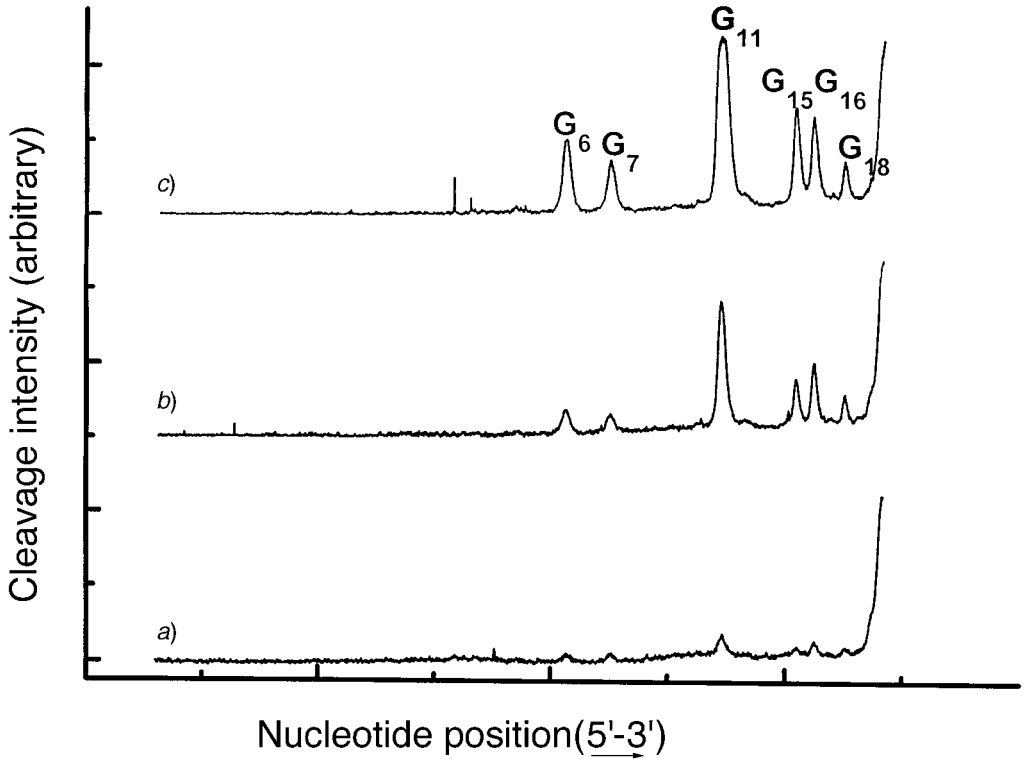


Fig. 8. *'Histogram' illustrating MB-sensitized* (VIS light, 150-W lamp, 4 min) *G-cleavage of DNA* **2** *irradiated in 10 mM sodium phosphate buffered H_2O or D_2O.* The samples were treated with piperidine before analysis. *a*) No irradiation, *b*) in H_2O, and *c*) in D_2O.

minor 1.6-ns component and a dominant 24-ns lifetime. We attribute the 1.6-ns component to unbound EB, and the 24-ns component is associated with the intercalated compound. These findings indicate that there is either no selective binding at the bulge of DNA **2**, or binding of EB at the bulge does not affect its lifetime measurably.

3.7. *Repair of Damaged Guanine by Fpg.* Oxidative reactions in duplex DNA at G generally lead to the formation of a 7,8-dihydro-8-oxoguanine (8-oxoG) moiety [56] which has an oxidation potential approximately 0.4 V lower than guanine [57]. Consequently, in the oxidizing environment that forms 8-oxoG, it is often subject to further oxidation [56]. *E. coli* formamidopyrimidine DNA glycosylase (Fpg) is an enzyme that cleaves at the 3′ and 5′ positions of the damaged guanine in duplex DNA, thereby removing the 8-oxoG, or a further oxidation product, which results in strand cleavage [36][58].

EB as irradiated in the presence of G-bulge-containing ^{32}P-labeled DNA **2**, and the reaction mixture was subsequently incubated with Fpg. Analysis of the reaction mixture by PAGE and autoradiography indicates that no Fpg-mediated strand cleavage occurs. As a control experiment, the one-base-short complementary strand (which causes the bulge) was displaced with an excess of fully complementary DNA after the irradiation but before treatment with Fpg. This displacement results in a duplex in

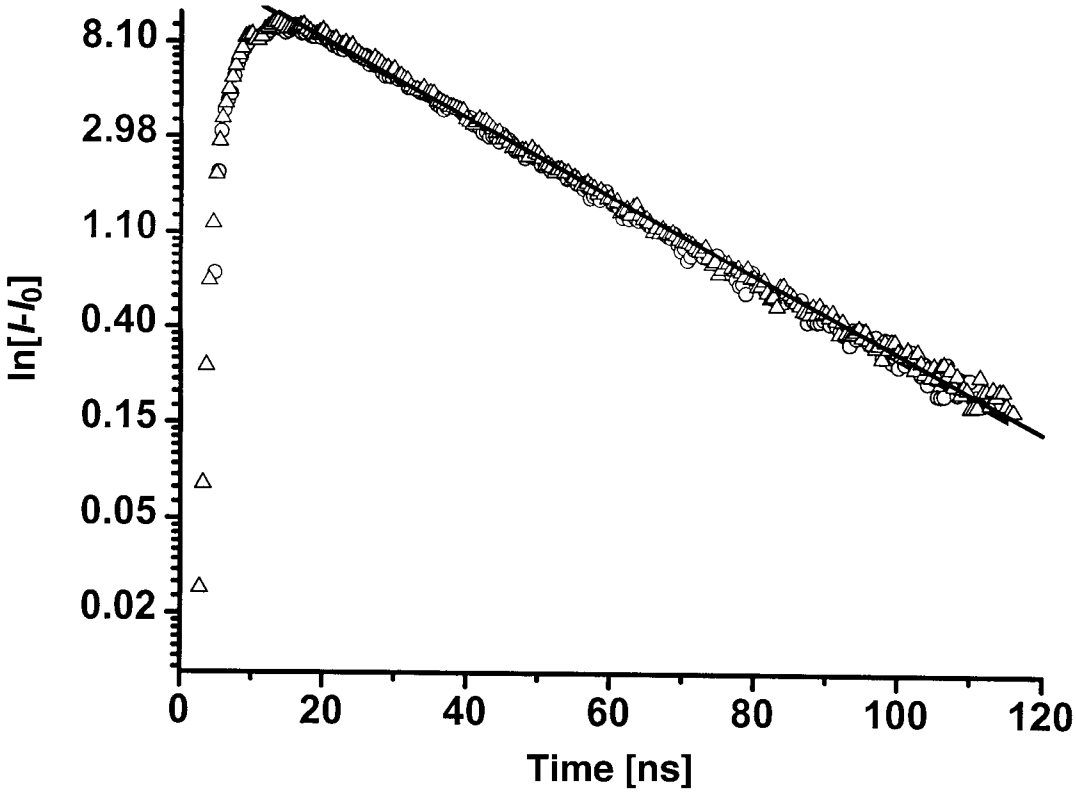


Fig. 9. *Fluorescence decay of 1.0 μM EB bound to 2.0 μM DNA* **1** (○) *or 2.0 μM DNA* **2** (△) *in phosphate-buffered solution*

which the formerly bulged G is paired with a C in a fully complementary duplex. This sample shows the same cleavage pattern and selectivity as irradiated samples of DNA (**2**) that are treated with piperidine (see *Fig. 10*). These results clearly indicate that the guanine oxidation products that are recognized by Fpg in the full duplex DNA are not excised when the damaged guanine is bulged.

4. Discussion. – 4.1. *Binding of EB to DNA* **1** *and DNA* **2**. We searched using melting behavior, absorption spectroscopy, time-resolved fluorescence measurements, and reactivity for direct evidence that EB binds specifically or selectively at or near the bulged guanine in DNA **2**. None of these experiments produced a result that requires preferential binding. However, irradiation of EB bound to DNA **2** with VIS or UV light does cause a selective reaction at the bulged guanine. In principle, this may be a consequence of migration of a charge (radical cation) that is introduced at a remotely bound EB to the bulged guanine where it is trapped by reaction with H_2O, or it could be due to a unique reaction from that fraction of the excited EB that is bound at or near the bulged guanine. The former explanation cannot account for reaction at the bulged guanine caused by VIS-light irradiation because the lowest-energy excited state of EB

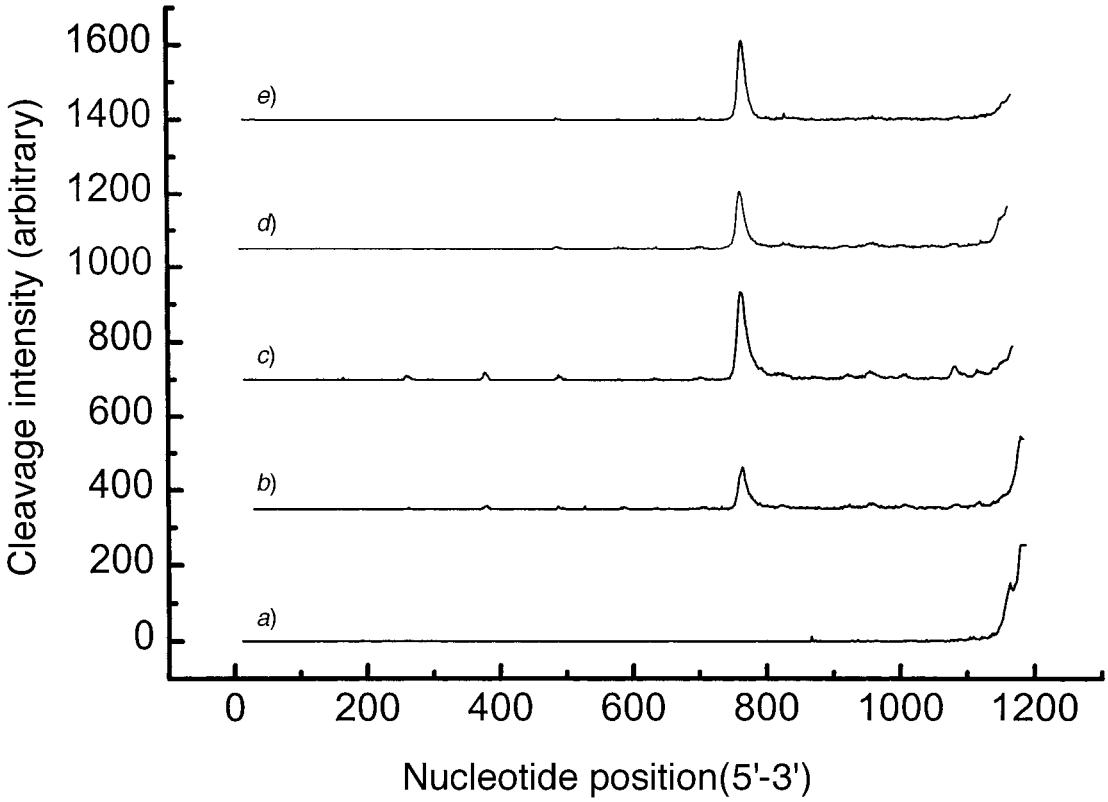


Fig. 10. *'Histogram' illustrating EB-sensitized* (VIS light, 4 h) *G-cleavage of DNA* **2** *in 10 mM sodium-phosphate buffered H_2O or D_2O.* The samples were treated with an excess of DNA fully complementary to the bulge-containing strand before treatment with piperidine or Fpg. *a*) No irradiation, *b*) in H_2O, piperidine treatment, *c*) in D_2O, piperidine treatment, *d*) in H_2O, Fpg-treated, and *e*) in D_2O, Fpg-treated.

lacks the oxidizing power required to convert a base to its radical cation [34][48]. It is possible energetically that an upper excited state of EB formed by UV irradiation introduces a radical cation into DNA, and this could be the cause of the inefficient cleavage at the GG steps observed uniquely under these conditions. While the experiments that compare the properties of EB bound to DNA **1** or DNA **2** do not provide evidence of strong, selective association at the guanine bulge, they do not exclude the possibility that some of the EB is bound at or near the bulged guanine. In consideration of these observations, we suggest that reaction at the bulged guanine resulting from VIS-light irradiation is initiated by EB bound at or near this site.

4.2. *DNA-Strand Cleavage Caused by Irradiation of EB.* It has been noted previously that irradiation of EB with UV light under a variety of conditions causes DNA-strand cleavage that appears to occur selectively at guanines [30–35]. The mechanism for this process is not clearly established in all instances; even so, this reaction is not a focus of this discussion. The primary new observations of this work are that VIS-light irradiation of EB causes a reaction uniquely at a bulged guanine, and that

UV irradiation gives highly selective reaction at that site. These are unexpected findings, which may have important implications for DNA damage, repair, and for the origin of mutations.

The control experiments reported above show plainly that strand cleavage initiated by VIS light is a result of the direct interaction between DNA and an excited state of EB. In particular, the reaction is not mediated by $^1O_2$. Furthermore, this reaction occurs selectively at a bulged guanine. VIS-Light irradiation of EB bound to DNA having bulged A, C, or T does not result in strand cleavage; and irradiation of EB bound to DNA having a G mis-paired with a G, T, or A does not result in strand cleavage. Clearly, there is a unique feature of the bulged guanine that enables reaction with the lowest-energy excited singlet state of EB.

Guanine has the lowest oxidation potential E_{ox} of the DNA bases [59][60], and, for this reason, it is often the primary target of oxidative damage [61][62]. However, this fact alone cannot explain why a bulged G is cleaved preferentially in comparison with G in a perfect duplex region or a G involved in a mis-pair. First, guanine in a G·C pair as part of duplex DNA is expected to have a lower E_{ox} than a bulged guanine [63][64]. Second, as previously noted, the lowest singlet state of EB lacks the oxidizing power to convert guanine to its radical cation in an exothermic process. Thus, there must be some special structural feature associated with the bulged guanine that enables its reaction with EB*[1].

Exciplex formation is commonly observed when an electronically excited electron acceptor interacts with an electron donor [65]. This process has been observed for excited intercalators with DNA [66]. An exciplex is an electronically excited species formed by a reaction between a localized excited state and a ground-state reagent. One characteristic of exciplexes is partial charge transfer from donor to acceptor. The degree of charge transfer is controlled primarily by energetic considerations, which are related to the E_{ox} of the donor, the reduction potential (E_{red}) of the excited acceptor, and to electrostatic factors that depend on the solvent dielectric constant and the distance between donor and acceptor. Since they are energetic, polarized intermediates, exciplexes often undergo chemical reactions.

We suggest that the selective reaction of the bulged guanine with bound EB*[1] is a consequence of exciplex formation. There is evidence that EB*[1] forms an exciplex in solution with guanine. *Barton*, *Zewail*, and co-workers report that the lifetime of EB*[1] increases when it is in the presence of 5 mM guanosine triphosphate (GTP) [48]. Consequently, there must be some interaction between GTP and EB*[1]. Although the nature of the complex formed between GTP and EB*[1] was not investigated, it is reasonable to assign it as an exciplex.

Since guanine has the lowest E_{ox} of the DNA bases, the exciplex (EB·G)*[1] formed between EB*[1] and G will have a greater degree of charge transfer than do the exciplexes formed from the other bases. This increase in charge transfer is consistent with a uniquely high reactivity for (EB·G)*[1], since the enabling process that leads to strand cleavage is reaction with H_2O [56]. The rate of this reaction will likely increase as the base component of the exciplex becomes more electron-deficient and electrophilic.

Exciplex formation generally requires electronic interaction between the two partners [65]. The strength of this interaction can be very strongly dependent upon the

relative orientation of the donor and acceptor. There is little restriction on possible orientations when the two partners are in solution. However, incorporation of the donor (guanine) in an ordered DNA structure will restrict the possible orientation geometries it can explore with EB*[1]. This fact provides a rationale for the observation that the cleavage reaction occurs efficiently uniquely for bulged guanines. Evidently, guanines involved in *Watson-Crick* pairs or in mis-pairs cannot form exciplexes with EB*[1] having sufficient charge transfer to permit their rapid reaction with H_2O. Furthermore, it has been shown that intercalators bind to bulged bases and increase the proportion of the looped-out structure [14]. Thus, EB bound at the bulged guanine site may stabilize an extrahelical conformation providing increased access to H_2O and an accelerated rate of reaction for the exciplex.

Finally, comparison of the results of UV- and VIS-light irradiation of EB suggest that, apart from reaction at a bulged guanine, DNA is relatively inert to reaction with EB*[1]. However, the upper excited states of EB (EB**[1]) formed by irradiation with UV light do cause more general DNA damage, albeit with significantly lower efficiency. This latter process is probably a result of one-electron oxidation by EB**[1] to form a base radical cation and its subsequent migration and reaction at a GG step [47].

4.3. *Unrepairable Damage at the Bulged G.* It has been proposed that proflavin, a DNA intercalator, induces frameshift mutations by stacking on looped-out bases [67]. Our findings suggest that intercalators may play an additional role in the sequence of events leading to mutations.

The primary product formed from oxidative reaction at guanine in duplex DNA is 8-oxoG. Further oxidation of 8-oxoG leads to imidazole ring-opened products and to an apurinic site [56]. The 8-oxoG is resistant to cleavage with piperidine [68], but it is an excellent substrate for Fpg – as are many products of further oxidation [58]. Our results show that the EB*[1]-initiated reaction of bulged guanine gives a product that results in strand cleavage when treated with piperidine. This finding suggests oxidation beyond 8-oxoG, which is reasonable because EB*[1] is a sufficiently strong oxidant to convert 8-oxoG to its radical cation in an exothermic process [57]. However, the damaged, bulged guanine is not cleaved when it is treated with Fpg, and, thus, the major repair mechanism for oxidative damage to DNA is inoperative [37]. Consequently, intercalators that normally do not damage DNA when exposed to VIS light may cause irreparable damage when bound to DNA having a bulged guanine. This finding may have meaningful implications for VIS-light-induced DNA mutations.

5. Conclusions. – The experimentally observable properties, such as T_m, spectral shifts, and fluorescent lifetime, of EB bound to fully complementary duplex DNA and to DNA having bulges and mis-pairs are indistinguishable. But irradiation of EB bound to DNA with VIS light causes lesions uniquely in DNA having a bulged guanine. UV Irradiation of the EB also results in especially selective reaction at the bulged G, but damage at other guanines, particularly GG steps, is also observed. The selective reaction of EB initiated with VIS light is attributed to reaction of the lowest excited singlet state of EB specifically with the bulged guanine. Evidence suggests that EB*[1], which is incapable energetically of oxidizing guanine to its radical cation, forms an exciplex with the bulged guanine that is attacked by water. This process initiates a cascade of reactions resulting, eventually, in the formation of a product(s) that causes

strand cleavage when the damaged DNA is treated with piperidine. Significantly, this product is not recognized by Fpg, an enzyme that plays a central part in base-excision repair of oxidatively damaged DNA. Thus, DNA is normally not damaged with high efficiency when bound EB is irradiated with by VIS light, but DNA containing a bulged guanine is damaged and that damage may not be easily repaired. As a result, the simultaneous occurrence of a bulged guanine, an intercalator, and light (even VIS light) may play some role in mutagenesis.

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