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PHOTONUCLEASE ACTIVITY OF TAYLOR'S BLUE

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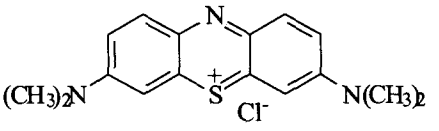
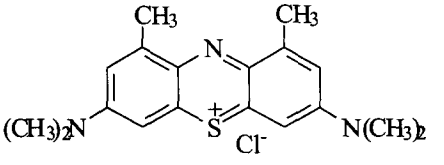
Abstract: Taylor's blue (1,9-dimethylmethylene blue, $DMMB^+$) associates with DNA, at least in part, through intercalation as is evidenced from the red shift in the absorption maximum, diminution of the fluorescence, and induced circular dichroism in the presence of nucleic acid. Irradiation of $DMMB^+$/covalently closed circular supercoiled ΦX174 phage DNA complex at $\lambda > 520$ nm leads to DNA nicking in a dose-dependent manner.

There is considerable recent interest in the use of photosensitizing agents to treat several types of tumors and inactivate viruses in blood products because of the selectivity of this methodology in terms of time and space. Among the various classes of compounds (e.g., psoralens,[1] metal complexes,[2] and red light-absorbing dyes[3]) currently under intense investigation, the latter are particularly attractive since these are activated with light of wavelengths > 600 nm. Such wavelengths are transparent to biological molecules, lie within the photochemotherapeutic window (600–900 nm), and have better tissue penetration.[4] Methylene blue (MB^+, 3,7-bis(dimethylamino)phenothiazin-5-ium chloride; see structure below) is a cationic phenothiazine dye that has been extensively studied for its photodynamic activity,[5] and is currently used for photosterilization of blood in Germany. It predominantly kills extracellular viruses via possibly targeting the nucleic acid.[6] MB^+ creates single-strand breaks in DNA, specifically at guanine sites.[7] Nicking is initiated by direct oxidation of the base and by an, as yet undefined, oxygen-dependent mechanism. Taylor's blue (3,7-bis(dimethylamino)-1,9-dimethylphenothiazin-5-ium chloride; 1,9-dimethylmethylene blue; $DMMB^+$; see structure below) is a structurally related dye that carries two additional methyl groups. This dye has recently been shown to be phototoxic to viruses in cellular blood components[6a,6b] and murine mammary tumor cell line, EMT-6.[8] In both studies, a higher photolethality for $DMMB^+$ relative to MB^+ was noted. This was attributed to a higher yield of photosensitized formation of singlet oxygen, and a greater level of association with DNA as a result of increased lipophilicity. Another added bonus of



MB^+



$DMMB^+$

$DMMB^+$ is that it kills both intra- and extracellular viruses, in contrast to MB^+ that is effective predominantly against extracellular viruses. Though nucleic acid has been suggested as a potential target for $DMMB^+$-photosensitized inactivation of viruses in blood,[6a] no direct evidence has yet appeared to support this proposal. This has prompted us to investigate the interaction of $DMMB^+$ with DNA and, in this communication, we

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demonstrate that the irradiation of $DMMB^+$ in the presence of covalently closed circular supercoiled ΦX174 phage RFI DNA leads to nicking of the nucleic acid in a dose-dependent manner. Control experiments confirm that DNA strand breaks are observed only when the dye and DNA are exposed to light. A possible role for singlet oxygen in the nicking mechanism is also discussed.

The absorption spectrum of $DMMB^+$ (Taylor's blue)[9] in 50 mM phosphate buffer (pH 7) displays a maximum at 650 nm and a shoulder at 602 nm, corresponding to the monomeric and metachromatic absorption for the dye, respectively.[10] Upon addition of native calf thymus DNA to a $DMMB^+$ solution at a low P/D ratio, the absorption spectrum exhibits small hypsochromic and large hypochromic effects in the metachromatic peak at ca. 600 nm (cf. Table 1). This is consistent with the metachromatic behavior of positively charged dyes, including $DMMB^+$, wherein they exhibit hypsochromic and hypochromic shifts in their absorption spectra due to

Table 1. Effect of P/D ratio on the absorption spectrum[a] of $DMMB^+$

P/D Ratio	Metachromatic peak, nm (% hypochromicity)	Monomer peak, nm (% hypochromicity)
0	602	650
1	600 (15.4)	651 (14.5)
2	599 (21.2)	651 (22.9)
5	600 (19.2)	651 (21.7)
10	604 (9.6)	656 (7.2)

[a] The dye solution (1 μM) in 50 mM phosphate buffer (pH 7) was treated with native calf thymus DNA at the indicated P/D ratios.

aggregations through cooperative binding via intercalation and surface binding.[10] A maximum of 21-23% hypochromicity is observed at a P/D = 2 (see Table 1). A progressive red shift in the absorption maximum of the dye is noted upon further titration of the $DMMB^+$ solution with larger concentrations of calf thymus DNA (see Figure 1), suggestive of intercalation as one of the binding modes of the dye to the nucleic acid. A maximum of 10 nm red shift is observed at the phosphate/dye ratio of > 40. No clear isosbestic point is observed in the isotherms, an indication that $DMMB^+$ associates with DNA in more than one binding mode (i.e., intercalative and electrostatic binding). This conclusion is supported by the fact that the presence of DNA leads to hyperchromicity instead of hypochromicity as the P/D ratio increases. The hyperchromicity is ascribed to deaggregation of the dye in the presence of DNA, as has been observed with other phenothiazine dyes[11] (including MB^+). The foregoing UV-Vis data are substantiated by fluorescence studies under aerobic conditions whereby $DMMB^+$ emission at 678 nm is found to be quenched by ca. 50% in the presence of DNA at P/D = 163 (data not shown).

The association of $DMMB^+$ with DNA through intercalation and surface binding was further confirmed by induced circular dichroism (ICD) data. Using a 25 μM solution of $DMMB^+$ and native calf thymus DNA (0.14 mg/ml) in 50 mM phosphate buffer, we observe a peak at ca. 580 nm and two troughs at ca. 645 and 685 nm (cf.

Figure 2). The trough at 645 nm is attributed to the intercalated $DMMB^+$, whereas the peak and trough at 580 and 685 nm, respectively, are the result of exciton splitting by the dye surface-bound to the DNA.[10] The intensities of these spectral signatures vary as a function of the dye concentration, using a fixed concentration of the nucleic acid.[10] As one would expect, the $DMMB^+$ is CD inactive in the absence of DNA (data not shown).

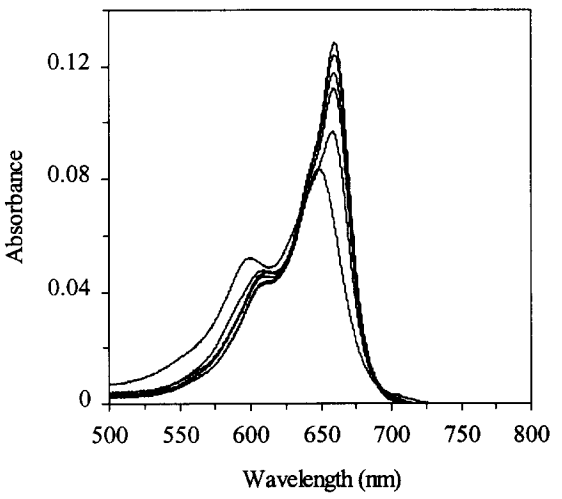

Figure 1. Variations in the absorption spectra of $DMMB^+$ induced by native calf thymus DNA in 50 mM phosphate buffer (pH 7). The dye concentration in each curve is 1 μM and the P/D ratios are 0, 20, 30, 40, 50, 100, 250, and 750 as the absorption maxima shift from left to right. For purposes of clarity, the titration curves for the P/D = 30, 250, and 750 have been omitted.

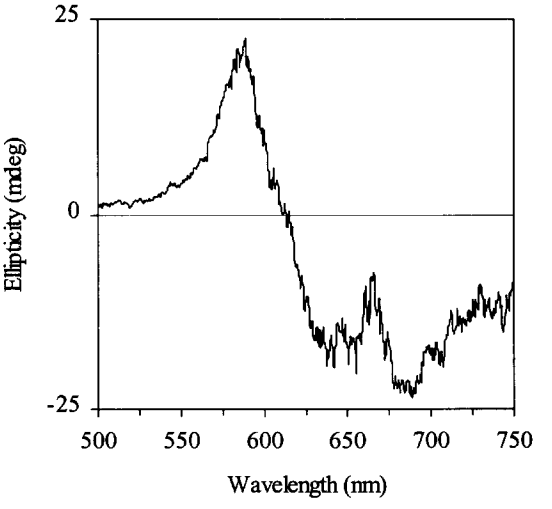

Figure 2. CD spectrum of $DMMB^+$ (25 μM) in the presence of native calf thymus DNA (0.14 mg/mL) in 50 mM phosphate buffer (pH 7).

For the investigation of the interaction of the $DMMB^+$ excited state with DNA we used covalently closed circular supercoiled ΦX174 phage RFI DNA (RFI), in combination with the DNA mobility shift assay on a 1% neutral agarose gel. In this assay, RFI (form I) runs fastest, the single-strand nicked DNA (form II) runs slowest, and the double-strand nicked DNA (linear DNA, form III) is intermediate between these two. Solutions (10 μL) of RFI (182 μM) and $DMMB^+$ (5 μM) in 50 mM phosphate buffer (pH 7) containing 0.5% DMSO (v/v) (P/D = 36.4) were irradiated[12] open to air at 12 °C for 0.5, 1, 2, 3 and 4 h. The samples were run on a 1% neutral agarose gel[13] and the data are presented in Figure 3. None of the controls, i.e. unirradiated and irradiated RFI for 4 h (lanes 1 and 2) nor unirradiated $DMMB^+$/RFI (lane 3), showed a change in the mobility of DNA. Note that the RFI is contaminated with a small amount of RFII (ca. 10–15% according to the information furnished by the supplier). RFI irradiated in the presence of $DMMB^+$ (lane 4) showed an increase of form II at the expense of RFI with the amount of nicking increasing proportionally with the absorbed light dose[14] (cf. lanes 4–8).

As noted above, $DMMB^+$ photosensitizes the formation of singlet oxygen with a quantum efficiency higher

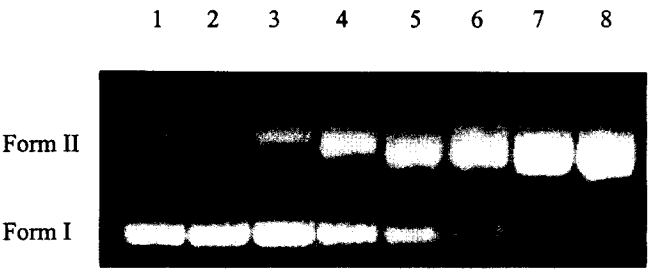

Figure 3. $DMMB^+$-photosensitized nicking of DNA at P/D = 36.4: lane 1,unirradiated RFI; lane 2, 4 h-irradiated RFI; lane 3, unirradiated $DMMB^+$/RFI; lanes 4–8, $DMMB^+$/RFI irradiated for 0.5, 1, 2, 3 and 4 h, respectively. All irradiations were at $\lambda > 520$ nm in air at 12 °C.

than that for MB^+.[6a,8] In addition, the association of $DMMB^+$ to DNA is ten times higher than is observed with MB^+.[6a] Singlet oxygen is a putative reactive oxygen species for biomolecules, including DNA, and one would expect enhanced photodamage of the biopolymer as a result of the increased level of association and quantum yield for singlet oxygen generation. We have therefore tested for the involvement of singlet oxygen in the $DMMB^+$-mediated nicking of DNA evident in Figure 3. Two classic diagnostic tests for the involvement of singlet oxygen in a reaction are the use of relatively selective singlet oxygen quenchers (examples include sodium azide and histidine), and the use of a stabilizing medium (such as D_2O) which increases the singlet oxygen lifetime.[15] Solutions (10 μL) of RFI (182 μM) and $DMMB^+$ (5 μM) in 50 mM phosphate buffer, prepared in H_2O or D_2O (pH/pD 7) (P/D = 36.4), with and without 20 mM of histidine (His), were irradiated ($\lambda > 520$ nm) under air at 14 °C for 45 min. The samples were analyzed on a 1% neutral agarose gel.[13] Four controls (i.e., unirradiated RFI, unirradiated $DMMB^+$/RFI in H_2O, unirradiated $DMMB^+$/RFI/His in H_2O, and RFI irradiated in H_2O in the absence of dye) were also run simultaneously. The gel results are presented in Figure 4 and the digitized video imaging data,[16] along with the mean number of single-strand breaks per plasmid molecule (*S*),[17] are presented in Table 2.

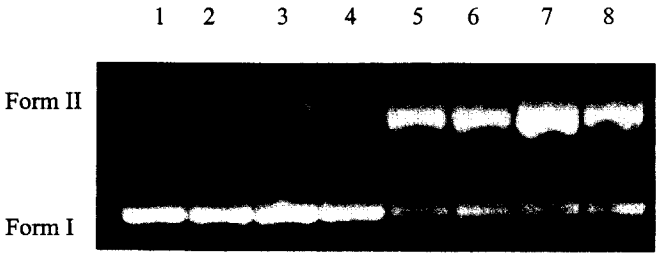

Figure 4. $DMMB^+$-photosensitized nicking of DNA at P/D = 36.4: lane 1, unirradiated RFI; lane 2, unirradiated $DMMB^+$/RFI/H_2O; lane 3, unirradiated $DMMB^+$/RFI/His/H_2O; lane 4, irradiated RFI/H_2O; lane 5, irradiated $DMMB^+$/RFI/H_2O; lane 6, irradiated $DMMB^+$/RFI/His/H_2O; lane 7, irradiated $DMMB^+$/RFI/D_2O; lane 8, irradiated $DMMB^+$/RFI/His/D_2O. All irradiations were at $\lambda > 520$ nm in air at 14 °C for 45 min.

Table 2. Data for digitized video imaging[a]

	1	2	3	4	5	6	7	8
Form I	92	91	90	90	42	46	30	46
Form II	8	9	10	10	58	54	70	54
S^b	0.1	0.1	0.1	0.1	0.9	0.8	1.2	0.8

[a]The net intensity of the digitized bands in Figure 4 (lanes 1–8) were used after subtracting the background fluorescence. The fluorescence intensity of RFII was divided by a factor of 1.66 to normalize the data to RFI that gives lower fluorescence when bound to DNA.[16] [b]*S* represents mean number of single-strand breaks per plasmid molecule.[17]

As was observed above (cf. Figure 3), none of the controls, unirradiated and irradiated RFI/H_2O (lanes 1 and 4), unirradiated $DMMB^+$/RFI/H_2O and $DMMB^+$/RFI/His/H_2O (lanes 2 and 3) showed a change in the DNA mobility. However, all the samples irradiated in the presence of $DMMB^+$ exhibited a decrease in RFI and a corresponding increase in RFII as a result of photonicking of the DNA (lanes 5–8). We note from the Table 2 that there is only a small (9%) level of quenching by His in H_2O (lane 5 vs. 6). D_2O markedly increased nicking (24% enhancement from lane 6 to lane 7), but the addition of His returns the amount of form II to the level seen in H_2O (cf. lanes 8 and lane 6). *These results indicate that singlet oxygen is not playing a major role in the nicking chemistry in H_2O*, though singlet oxygen *does* become a significant contributor to the nicking chemistry in D_2O. We conclude that type I (i.e., electron transfer) chemistry predominates in H_2O under these irradiation conditions, a proposal supported by our observation of the quenching of $DMMB^+$ fluorescence by DNA (see above).

In summary, we have shown that the photochemically potent phenothiazine dye, $DMMB^+$, creates single-strand breaks in supercoiled DNA upon excitation with visible light. Though the dye produces singlet oxygen more efficiently than does MB^+, this species does not appear to play a significant role in the dye's photonuclease activity. It is still unclear whether the same conclusion will valid for MB^+ itself.

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9. $DMMB^+$ of ca. 80% dye content was purchased from Aldrich (catalog #34,108-8), and purified on a 1.5 x 45 cm lipophilic Sephadex LH–20, bead size 25–100 μm (Sigma catalog #LH–20–100) slurry packed column in CH_3CN. The dye eluted with CH_3CN indicated > 94% purity by HPLC on a Varian Microsorb MV cyano column (5 μm, 100 Å, 4.6 x 250 mm) using 40% CH_3CN in 100 mM NaOAc (pH 4.5) at a flow–rate of 1 ml/min and monitoring the column effluent at 290 nm; 600 MHz ^1H NMR spectrum (CD_3OD): δ 2.55 (s, 6H, 2 x CH_3), 3.23 (s, 12H, 4 x NCH_3), 7.02 (d, J = 2.7 Hz, 2H, ArH), 7.16 ppm (d, J = 2.7 Hz, 2H, ArH); 125 MHz ^{13}C NMR (CD_3OD): δ 19.01, 41.30, 105.63, 119.08, 148.91, 150.64, 154.22, 155.45 ppm.
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12. The solutions were irradiated in small end-tapered tubes that were inserted in large Fisherbrand® borosilicate tubes. The irradiations were performed with a 450 W Hanovia medium pressure quartz mercury vapor lamp that emits from the far-UV into the infrared. The UVA, far-visible and infrared radiations were removed with a uranium yellow glass (cut-off < 330 nm), 2% potassium dichromate (cut-off < 520 nm) and circulating cooling water, respectively. Under this irradiation setup, $DMMB^+$ is essentially excited with the 578-nm mercury line.
13. Each sample was treated with 1/6th volume of bromophenol blue tracking dye (0.25% dye and 40% sucrose in water) and 10 μL was loaded on a 1% neutral agarose gel containing 2 μL of EtBr (10 mg/mL). The gel was run in 1X TBE buffer containing 3 μL of EtBr (10 mg/mL) at 76 V for ca. 4 h. The gel photograph was recorded on a Polaroid high-speed film type 57 using a Polaroid MP-3 Land Camera.
14. There was minimum degradation of a 0.14 mM aqueous solution of $DMMB^+$ when left in the dark at 4 °C for up to seven weeks. This is not the case for more dilute solutions (e.g., 5 μM) which, when left at room temperature, form a precipitate in a short period of time. However, some photodegradation of $DMMB^+$ in the absence of DNA was observed, as was evidenced from both UV-Vis and HPLC analyses. It has been shown that DNA protects intercalating phenothiazine dyes from photodegradation by quenching the dye singlet excited state, see reference 11a and Mohammad, T.; Morrison, H. *J. Chromatogr. B* **1997**, *704*, 265.
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16. A Kodak Digital Science DC40 Camera in combination with the 1D Analysis Software Version 1.6 was used for the quantification of the DNA bands. This equipment was available in the laboratory of Dr. D. Bergstrom with the assistance of Maneesh Pingle. The net intensity of the digitized bands was used after subtracting the background fluorescence. A correction factor of 1.66 was employed to normalize the data for RFII that gives higher EtBr fluorescence when bound to DNA, see (a) Ciulla, T. A.; van Camp, J. R; Rosenfeld, E.; Kochevar, I. E. *Photochem. Photobiol.* **1989** *49*, 293. (b) Hefetz, Y.; Dunn, D. A.; Deutsch, T. F.; Buckley, L.; Hillenkamp, F.; Kochevar, I. E. *J. Am. Chem. Soc.*, **1990**, *112*, 8528.
17. The mean number of single-strand breaks per plasmid molecule (*S*) were calculated from the Poisson distribution of the nicks using the equation $S = \ln f_I$ where f_I is the fraction of form I, see (a) Hertzberg, R. P.; Dervan, P. B. *Biochemistry* **1984**, *23*, 3934. (b) Vicendo, P.; Mouysset, S.; Paillous, N. *Photochem. Photobiol.* **1997**, *65*, 647. (c) Mitra, K.; Kim, W.; Daniels J. S.; Gates, K. S. *J. Am. Chem. Soc.* **1997**, *119*, 11691.