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# Fluorescence lifetime analysis of DNA intercalated ethidium bromide and quenching by free dye

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**Abstract**

The fluorescence characteristics of ethidium bromide (Eb) complexed to calf thymus DNA have been examined using fluorescence lifetime analysis for a range of DNA (effective nucleotide concentration) to Eb molar ratios. Control of both temperature and ion concentration is necessary for reproducible analyses. Eb complexed to double stranded DNA has a maximum fluorescence lifetime of 23 ns and is easily distinguishable from a fluorescence lifetime value of 1.67 ns corresponding to unbound Eb. In a solution of calf thymus DNA containing excess Eb a binding equilibrium is reached, and this corresponds to one Eb molecule for every five nucleotides. With increasing amounts of unbound Eb, the fluorescence lifetime of the DNA–Eb complex decreases with a concomitant drop in the steady state fluorescence intensity, without a change in the amount of Eb bound to DNA. It is concluded that unbound Eb, acting via a quenching mechanism, shortens the fluorescence lifetime of bound Eb and consequently decreases the overall fluorescence intensity. This means that a different approach is necessary: time-resolved fluorescence spectroscopy directly distinguishes between a decrease in fluorescence intensity due to quenching by an excess of unbound Eb from that due to a decrease in Eb binding to double-stranded DNA. These studies suggest that techniques which measure total steady state fluorescence intensity of bound Eb in order to infer relative amounts of double-stranded DNA must be interpreted with caution. For such assays to be valid it is essential that no unbound Eb be present; otherwise a variable correction factor is required to account for unbound Eb.

*Key words:* Time-resolved fluorescence; Ethidium bromide; DNA intercalation; Fluorescence quenching

## 1. Introduction

Time-resolved fluorescence spectroscopy is a sensitive technique for studying the conformational changes occurring within DNA both in chemical and cellular systems as it interacts with intercalating and/or groove binding drugs. There are fluorescent probes available with binding properties analogous to many anti-neoplastic agents used in chemotherapy; these therefore represent a useful model with which to study the binding interactions of such drug molecules with DNA. In the current study the interaction of ethidium bromide and DNA in solution has been measured with this technique. We have identified

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the need to take into account dynamic quenching of the fluorescence signal.

Fluorescence lifetime analysis (FLA) allows the quantitative resolution of discrete fluorescence lifetimes from a fluorescence decay curve, which may reflect different domains or molecular interactions of the probe within a particular target macromolecule. A summary of time-correlated single-photon counting is given by O'Connor and Phillips [1], and the various techniques used to collect a fluorescence decay distribution have been reviewed by Ware [2], Lakowicz [3], and Soini and Lövgren [4]. The system used in this study employs a pulse-fluorometry technique to collect the fluorescence decay distribution and a least-squares algorithm to deconvolute the decay components. An extensive review of the least-squares and other deconvolution algorithms has been compiled by Cundall and Dale [5].

Ethidium bromide (Eb), a phenanthridine drug, is a cationic molecule able to interact with polynucleotides. Both its fluorescence intensity and mono-exponential lifetime increase upon binding. This latter increase (from $< 2$ to $\approx 23$ ns) makes it a good candidate for monitoring DNA conformation with techniques such as FLA and rotational anisotropy analysis of the fluorescence depolarization. The use of Eb's fluorescence and binding properties to study the conformation of nucleic acids *in vitro* has been summarized by LePecq [6,7].

Eb binds to double-stranded polynucleotides primarily by intercalating between base pairs; this is thought to follow the adjacent excluded site binding model [8,9]. There is evidence that at high concentrations Eb may also associate with the outside of the DNA helix [10]. This secondary site binding only occurs at low ionic concentrations ($\leq 0.01$ M) and after binding at the primary site has been saturated [10–12]. To study conformational changes within DNA it is important to ensure that binding take place exclusively at the primary site [6].

The fluorescence of free Eb in solution is strongly quenched by the aqueous solvent and therefore unbound dye is only weakly fluorescent [12,13]. Upon intercalation, the Eb molecule enters a hydrophobic environment [10] in which the steric protection provided by the DNA base pairs prevents quenching by the aqueous solvent. The amount of exposure to solvent molecules determines the degree of quenching. The quenching is thought to occur by a proton transfer mechanism to water from the excited singlet state of free Eb [12].

In this paper FLA has been used to simultaneously measure the fluorescence lifetimes of both Eb free in solution and that intercalated into calf thymus DNA. The sensitivity of this kinetic technique resulted in the resolution of the lifetime component attributed to unbound or free Eb even when present at very low concentrations. The FLA results have been correlated with the equilibrium binding concentrations of Eb bound at the primary intercalation site as determined independently. The quenching of the fluorescence signal at or above maximum binding levels is discussed as a concern for biochemical or *in vitro* assays which infer relative amounts of double-stranded DNA by measuring steady state fluorescence intensity.

## 2. Materials and methods

Stock solutions of calf thymus (ct) DNA (Sigma type V sodium salt, highly polymerized) were prepared at 1 mg/ml in 0. 1 M Tris-HCl pH 7.5 (physiological pH) at two concentrations of NaCl, 0.05 M and 1 M, above and below the isotonic salt concentration (0.15 M) of serum. These solutions were stored at 5°C. Stock solutions of ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide, Sigma) were prepared at 1 mM in distilled water at the two salt concentrations and stored in the dark at 5°C. In each experiment, the final concentration of ctDNA was 50 μg/ml, or 143 μM (effective nucleotide concentration). Concentrations of Eb from 3 μM to 400 μM were used giving a DNA/Eb molar ratio ranging from 0.36 to 47.7.

To accurately determine the bound and free Eb concentrations at the various DNA/Eb ratios and salt concentrations, the spectrophotometric titration method described by Waring [14] and Angerer and Moudrianakis [11] was employed.

Concentrations of ctDNA ranging from 0 to 1000 μg/ml were added to 100 μM Eb. Absorbance measurements were made with a Hewlett Packard 8451A diode array Spectrophotometer using a 1 cm sample cuvette. From this absorption data the binding ratio $r$ (the number of bound Eb molecules per nucleotide) can be estimated.

The decay of fluorescence was measured with a PRA [1] fluorescence lifetime instrument (FLI) [15], which employs a least-squares deconvolution algorithm based in part on the least-squares method of Grinvald and Steinberg [16] to determine the goodness of fit of the decay data to a sum of exponentials given by,

$$F(t) = \sum_i \alpha_i \, e^{-t/\tau_i}, \qquad (1)$$

with $\alpha_i = i$th pre-exponential factor and $\tau_i = i$th decay time. The decay time constants $\tau_i$ are the mean lifetimes of the excited species. Estimates of uncertainty for $\alpha_i$ and $\tau_i$ are provided by the least-squares algorithm.

FLA also determines the fractional intensity of fluorescence due to each lifetime component in the decay curve as,

$$\%\text{int}_i = \frac{\tau_i \alpha_i}{\bar{\tau}} \times 100\%, \qquad (2)$$

where $\bar{\tau}$ = mean lifetime of the decay curve and is given by

$$\bar{\tau} = \sum_{i=1}^{n} (\tau_i \alpha_i). \qquad (3)$$

The $\%\text{int}_i$ values are indicative of the relative amounts of each of the fluorescence species present, but are also a function of the excitation and emission wavelengths selected since these will affect the relative excitation of and emission from the different species.

All fluorescence lifetime analyses were obtained with a free running $H_2$ discharge flashlamp operating at 6.0 kV at a pressure of −50 kPa and a frequency of 46 kHz. The decay profiles were collected over 512 channels of the multi-channel analyzer with a time-per-channel of 0.293 ns until 5000 counts were collected in the peak channel. The instrument response function (IRF) was obtained (fwhm 15 ns) with the use of a scattering solution (LUDOX Colloidal Silica, Du Pont) with one half of the data being collected prior to, and the other half after, sample collection. Following background subtraction the decay profiles were deconvolved from the IRF by computer analysis. In all cases an excellent fit of the data to either a single or a double exponential model (Eq. (1)) was obtained as indicated by the statistical tests (Reduced chi-squared $\leq 1.2$, runs test parameter $|\text{SGR}| < 1.96$ (95% confidence interval), Durbin Watson parameter $> 1.75$ for a double exponential, residuals and auto-correlation plots (inset) randomly distributed about the zero line) [16]. For all experiments the excitation and emission monochromators were set at 485 and 610 nm respectively, and unless otherwise stated the experiments were performed at room temperature (21–23°C). For temperatures of 2–4°C the 1 cm sample cuvettes were placed in a cooling enclosure attached to a circulating water bath.

The FLI was also utilized to obtain relative steady state fluorescence intensity values by summing the number of fluorescence events registered by the single photon counting instrument over the entire decay lifetime spectrum during a specific time interval.

The system was tested by measuring the fluorescence lifetimes of two reference compounds at room temperature, anthracene in cyclohexane ($\tau_{\text{meas}} = 4.17 \pm 0.02$ ns) and quinine sulfate in 0.1 N $H_2SO_4$ ($\tau_{\text{meas}} = 19.5 \pm 0.03$ ns), and the lifetimes were consistent with those tabulated by Lampert et al. [17].

## 3. Results

The FLA decay curve for Eb intercalated in ctDNA at a DNA/Eb ratio of 0.71 is shown in Fig. 1. Note the break in the curve which graphically illustrates a bi-exponential decay. The lower curve is the IRF for the flashlamp. Note the low chi-squared $< 1.1$ and that both the weighted residuals and the auto-correlation function C(I)

[1] Photochemical Research Assoc. Inc., Edinburgh Instruments, Riccarton, Currie, Edinburgh, UK, H14 4AL.

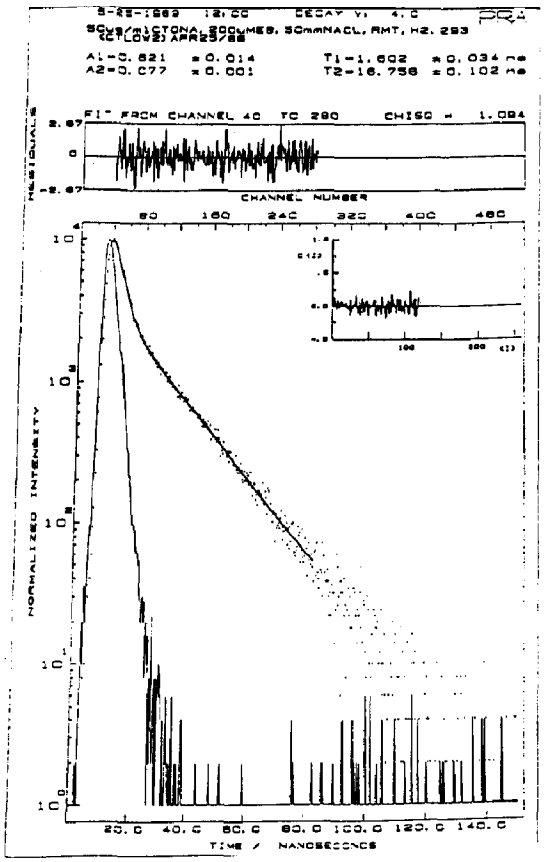


Fig. 1. Fluorescence decay curve (logarithm of normalized intensity versus time in ns) for ethidium bromide ($2\times10^{-4}$ M) bound to calf thymus DNA (50 μg/ml) in solution at room temperature containing 0.05 M NaCl. After deconvolution with the instrument response function (solid line curve of the lamp flash), a two-component fit of the points yields values for the pre-exponential factors $A_1$ (0.621) and $A_2$ (0.077), and the lifetimes $\tau_1$ (1.60 ns) and $\tau_2$ (16.78 ns). The weighted residuals (top trace) are randomly distributed about zero (±2.87), and the autocorrelation function $C(I)$ (±1.0) (inset), likewise.

plots (inset) are randomly distributed about the zero line, indicating the goodness of fit.

In the absence of DNA the fluorescence decay of unbound or free Eb was found to be mono-exponential and independent of the salt concentration, with an average lifetime, $\tau_f$, of $1.67 \pm 0.02$ ns (SEM). Measurements of $\tau_f$ were also made in a 0.05 M NaCl, 0.1 M buffered solution with a pH that varied from 5.0 to 10.5. Hepes buffer was used for the pH range of 5.0 to 8.5, and a glycine buffer was used for the pH range of 8.5 to 10.5. The $\tau_f$ was found to be constant within this pH range with an average value of $1.70 \pm 0.02$ ns (SEM). The fluorescence intensity of Eb in solution was found to be linear with concentration up to ≈ 100 μM after which a concentration dependent self-quenching occurred (data not shown).

The results of the FLA for Eb complexed to ctDNA for a range of DNA/Eb ratios are shown in Table 1. The lifetimes and percentages of both free and DNA-bound Eb are easily resolved and are measured simultaneously. The error values listed are the statistical uncertainties generated by the fitting algorithm. These values are listed to indicate that as $\%int_i$ decreases, the uncertainty in the lifetime value increases. These experiments were repeated at least five times and the repeatability is excellent (< 2% difference with $\%int_i$ greater than 10). The average values of $\tau_1$ for 0.05 M and 1 M NaCl at the various DNA/Eb ratios are $1.62 \pm 0.05$ and $1.62 \pm 0.07$, respectively. These values correlate well with the value for $\tau_f$, which was obtained in the absence of DNA. The fact that the $\tau_1$ values are slightly lower than $\tau_f$ may be due to the presence of the second lifetime component $\tau_2$ and the fact that these components are correlated or coupled [2].

Table 1
Fluorescence lifetime analysis of lifetime ($\tau_i$) [a] and intensity ($\%int_i$) [a] data for DNA–Eb complexes

| DNA/Eb (molar ratio) | $i$ [a] | $\tau_i$ | 0.05 M NaCl ± | 0.05 M NaCl $\%int_i$ | $\tau_i$ | 1 M NaCl ± | 1 M NaCl $\%int_i$ |
|---|---|---|---|---|---|---|---|
| 0.36 | 1 | 1.59 | 0.02 | 67.4 | 1.61 | 0.14 | 58.8 |
| 0.71 | | 1.58 | 0.04 | 43.4 | 1.70 | 0.03 | 44.3 |
| 1.4 | | 1.64 | 0.03 | 25.2 | 1.70 | 0.03 | 30.2 |
| 2.8 | | 1.68 | 0.15 | 9.4 | 1.58 | 0.06 | 18.7 |
| 5.7 | | 1.12 | 0.93 | 2.3 | 1.60 | 0.08 | 11.4 |
| 14.3 | | | – | | 1.54 | 0.14 | 7.7 |
| 47.7 | | | – | | 2.06 | 0.20 | 6.2 |
| 0.36 | 2 | 14.35 | 0.13 | 32.6 | 18.36 | 0.14 | 41.2 |
| 0.71 | | 16.54 | 0.10 | 56.6 | 19.21 | 0.12 | 55.7 |
| 1.4 | | 18.52 | 0.05 | 74.8 | 20.37 | 0.08 | 69.8 |
| 2.8 | | 20.04 | 0.05 | 90.6 | 21.50 | 0.06 | 81.3 |
| 5.7 | | 21.20 | 0.05 | 97.7 | 22.23 | 0.06 | 88.6 |
| 14.3 | | 22.45 | 0.04 | 100.0 | 22.91 | 0.06 | 92.3 |
| 47.7 | | 23.10 | 0.04 | 100.0 | 23.10 | 0.05 | 93.8 |

[a] $i$ is the number of components in the calculated decay curve (component 1 represents free Eb and component 2 is DNA-bound Eb)

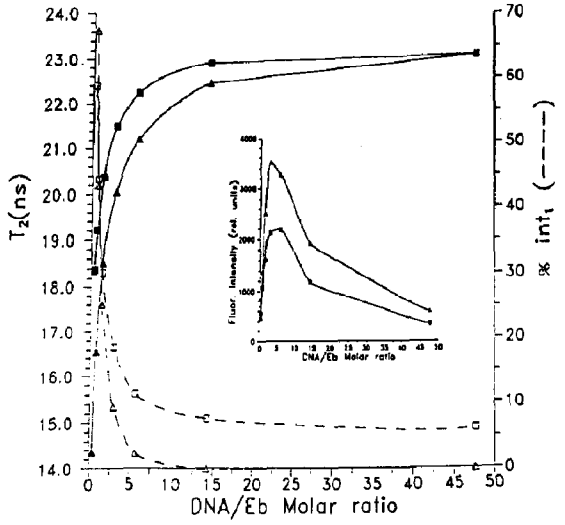


Fig. 2. Fluorescence lifetime ($\tau_2$) and fractional fluorescence intensity (%$int_1$) of the DNA–Eb complex as a function of the DNA/Eb molar ratio for 1 M NaCl (■ □) and 0.05 M NaCl (▲ △), respectively. Total steady state fluorescence intensity is also shown (inset).

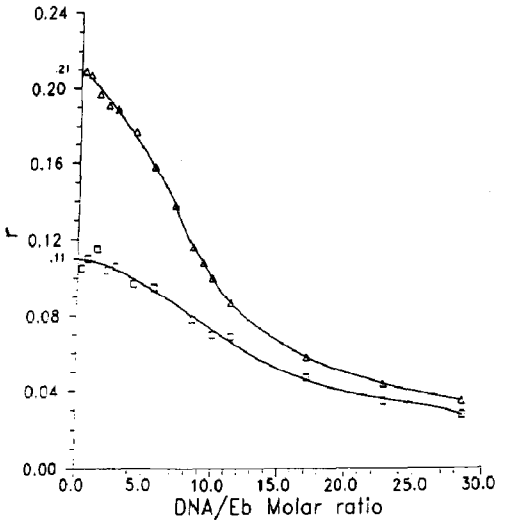


Fig. 4. The ratio of the number of bound Eb molecules per nucleotide ($r$) as a function of the DNA/Eb molar ratio for 1 M NaCl (□) and 0.05 M NaCl (△).

As the amount of added Eb increases (DNA/Eb ratio decreases), %$int_1$, increases, with a corresponding decrease in %$int_2$. As %$int_1$ becomes significant (DNA/Eb < 5.7, Table 1) the value of $\tau_2$ decreases dramatically (Fig. 2). Notice that the dramatic drop in $\tau_2$ at high Eb concentration (low DNA/Eb ratio) is also accompanied by a dramatic increase in %$int_1$ and a significant decrease in steady state fluorescence intensity (Fig. 2 inset).

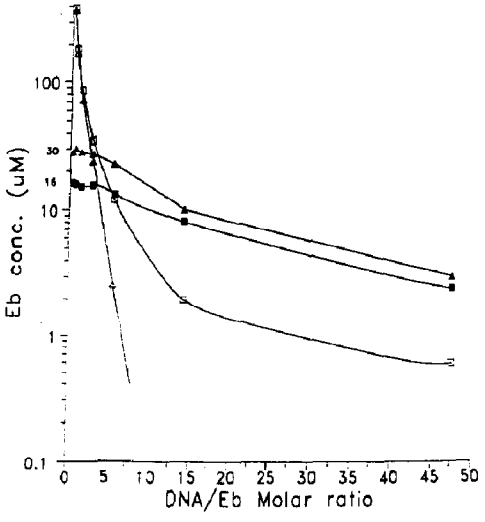


Fig. 3. The concentrations of bound (filled symbols) and free (open symbols) Eb determined spectrophotometrically as a function of DNA/Eb molar ratio for 1 M NaCl (■ □) and 0.05 M NaCl (▲ △), respectively.

The respective concentrations of Eb, determined spectrophotometrically, are shown in Fig. 3, which relates the concentration of free and bound Eb to the DNA/Eb molar ratio. The ordinate was plotted as a log scale to allow both the free and bound concentration curves to be seen clearly on the same graph. Unlike at 0.05 M NaCl, with 1 M NaCl at a DNA/Eb ratio > 5.7 there is always some free Eb present. This was also seen in the FLA results shown in Table 1.

The binding ratio $r$ is shown in Fig. 4 as a function of the DNA/Eb ratio. As the DNA/Eb ratio approaches zero $r$ approximates $n$, the number of binding sites per nucleotide [10]. Thus from Fig. 4, $n = 0.21 \pm 0.01$ for 0.05 M NaCl and $n = 0.11 \pm 0.01$ for 1 M NaCl. The 1.9-fold greater number of binding sites for 0.05 M NaCl is consistent with the observed steady state fluorescence intensity which is almost 2-fold greater at 0.05 M NaCl than at 1 M NaCl (Fig. 2 inset).

## 4. Discussion

The value of $\tau_f$ reported here is in agreement with that reported previously (1.8 ns [12], 1.7 ns [13], 1.8 ns [18]). The fluorescence lifetime of Eb bound to DNA, $\tau_b$, has also been measured previously at various DNA/Eb ratios, salt concentrations and temperatures. Our plateau value for $\tau_b$ of 23.1 ns is in good agreement with these values (22.5 ns [12], 24.2 ns [13], 22.5 ns [18], 24 ns [19]). It can be shown that the relative quantum increase (RQI) in fluorescence that occurs when a free Eb molecule intercalates within DNA is equal to $\tau_b$ divided by $\tau_f$ [16]. At a DNA/Eb ratio of 47.7, with a value of 1.67 ns for $\tau_f$, the RQI for our data is 13.8 which is consistent with values reported previously (13.7 [11], 12.5 [12], 14.2 [13]).

Burns [20] reported that $\tau_b$ has no temperature dependence above room temperature. At a temperature of 2°C, a temperature necessary for *in vitro* studies, the lifetime profile for $\tau_b$ at the various DNA/Eb ratios was consistently higher than at room temperature (data not shown) with an increase of 2 ns at the higher DNA/Eb ratios. This was anticipated since the fluorescence lifetime should increase with viscosity [20]. The dependence of lifetime values on temperature necessitates a constant temperature be maintained during the data collection period.

The dependence of the number of bound Eb molecules on the concentration of salt in solution as shown (Fig. 2, inset; Fig. 4) is also in agreement with previous studies. As the salt concentration decreases the number of allowable binding sites and the electrostatic DNA/Eb interaction increases, consistent with the hypothesis that the DNA helix takes on a more collapsed configuration as a result of the deactivation and screening of DNA phosphate groups in the presence of a high, competing salt concentration [21] and therefore allowing less Eb to intercalate.

Detailed Scatchard analyses by LePecq and Paoletti [10] revealed that at 1 M NaCl, $r$ approaches 0.2 in a linear fashion, and at very low salt concentrations, $10^{-4}$ M EDTA for example, there was a break in the Scatchard curve yielding an $r_1$ of 0.2 and an $r_2$ extending to 0.7. Angerer and Moudrianakis [11] also showed Scatchard analyses at 0.01 M NaCl, yielding $r_1 = 0.25$, $r_2 = 0.28$ and at 0.001 M NaCl yielding $r_1 = 0.31$ and $r_2 = 0.35$. Binding up to an $r$ value of 0.2 has been attributed to intercalation at the primary site while binding for $r > 0.2$ has been attributed to binding at a secondary site, possibly on the outside of the DNA helix. For $r > 0.2$ a corresponding decrease in fluorescence intensity was observed and therefore it had been suggested that binding at the secondary site was quenching the fluorescence of Eb bound at the primary site. Both salt concentrations used in this study are higher than that required to exceed the threshold ($r_{max} \approx 0.2$) for the onset of secondary site binding (Fig. 4).

The dependence of $\tau_b$ on the DNA/Eb ratio at various salt concentrations was first reported by Burns [20] and was also mentioned by Olmsted and Kearns [12] and shown more recently by Atherton and Beaumont [13]. In the latter two cases the decrease in $\tau_b$ with decreasing DNA/Eb was attributed perhaps to quenching by Eb bound at the secondary site since in both cases, the salt concentration was $< 0.01$ M. However as pointed out in both papers there was no intermediate lifetime component seen which could be attributed to Eb bound at this site, and therefore this site must be either non-fluorescent or have a fluorescence lifetime indistinguishable from free Eb. At a salt concentration (0.1 M NaCl) where only binding at the primary site is occurring, a constant value of $\tau_b$ has been reported independent of the DNA/Eb ratio [12,17]. In contrast, our results agree with those observed by Burns [20], which show a decrease in $\tau_b$ as the DNA/Eb ratio decreases at salt concentrations $> 0.01$ M. An alternate explanation offered by Atherton [12] is that the DNA structure is altered as the concentration of bound Eb molecules increases, forcing apart the DNA strands and thus allowing more quenching by aqueous solvent molecules. As seen in Figs. 3 and 4 there is a gradual increase in the number of Eb molecules bound at the primary site as more Eb is added. As each Eb molecule binds, the base pair separation increases by 3.4 Å, and the helical structure unwinds slightly [22] and therefore as the DNA strands are forced apart the increased accessibil-

ity to solvent molecules may account for the gradual decrease in $\tau_b$ especially at 0.05 M NaCl where relatively more Eb binding occurs than at 1 M NaCl (Figs. 3 and 4). However a point is reached (DNA/Eb ≈ 5.7) where a rapid decrease in $\tau_b$ is not accompanied by a significant increase in the number of bound Eb molecules, especially at 1 M NaCl, but is accompanied by a dramatic increase in the concentration of and fluorescence signal from free unbound Eb. At this DNA/Eb ratio, an inflexion is also seen in the steady state fluorescence intensity which suddenly decreases. Since in this study there is no binding at the secondary site, these support the hypothesis that unbound Eb is quenching the fluorescence signal from Eb intercalated at the primary site. Indeed Burns [20] had suggested quenching by a non-radiative transfer of energy to free Eb since the absorption spectrum of free Eb overlaps the emission spectrum of bound Eb.

In this study FLA has resolved the DNA/Eb ratio at which two components ($\tau_f$ and $\tau_b$) appear in the fluorescence decay profile (Table 1 and Fig. 2), and has correlated this DNA/Eb ratio to the appearance of free Eb as measured spectrophotometrically (Fig. 3). Below this DNA/Eb ratio, the rapid decrease in steady state fluorescence intensity (Fig. 2 inset) and $\tau_b$ (Fig. 2) with no change in the concentration of bound Eb (Fig. 3) argues strongly in favour of a dynamic quenching mechanism in which excess unbound Eb quenches the steady state fluorescence intensity, and therefore decreases the time-resolved fluorescence lifetime of bound Eb.

These results suggest caution in using biochemical or *in vitro* assays which infer a change in the amount of double-stranded DNA by a change in the steady state fluorescence intensity of bound Eb. In a recent study by Rauko et al. [23] the activity of the anti-neoplastic agent cis-DDP was studied by monitoring the decrease in fluorescence intensity assuming that a decreased Eb intercalation implies an increase in DNA base damage. The therapeutically active cis-DDP resulted in a greater decrease in fluorescence than the inactive trans-DDP. If as the quantity of double-stranded DNA decreased, the displaced Eb contributed to the decrease in fluorescence by quenching the fluorescence of the bound Eb molecules, then the activity of the cis-DDP would be overestimated, but this effect is probably small. In another study [24] using an alkaline filter elution assay, the ability of cis-DDP to produce interstrand crosslinks in the DNA of human brain tumour cells was monitored by the increased ability of the crosslinked DNA to reanneal to its native double-stranded configuration after a heating and cooling cycle. The resultant quantity of double-stranded DNA was inferred by the steady state fluorescence intensity of bound Eb. The degree of crosslinking and therefore the efficacy of the anti-tumour agent would be underestimated if unbound Eb is present and quenching the fluorescence intensity.

In such assays it is important that no free unbound Eb be present throughout the entire concentration range of double-stranded DNA that may be present in the experiment. In some cases, FLA may provide a more accurate determination of the amount of double-stranded DNA.

## References

[1] D.V. O'Connor and D. Phillips, Time-correlated single photon counting, (Academic Press, New York, 1984).
[2] W.R. Ware, in: Nato ASI Series A, Time-resolved fluorescence spectroscopy in biochemistry and biology, eds. R.B. Cundall and R.A. Dale, (Plenum Press, New York, 1983) pp. 23–55.
[3] J.R. Lakowicz, in: Applications of fluorescence in the biomedical sciences, eds. D.L. Taylor , A.S. Waggoner, F. Lanni, R.F. Murphy and R.R. Birge (Liss, New York, 1986) pp. 29–65.
[4] E. Soini and T. Lövgren, CRC Crit. Rev. Anal. Chem. 18 (1987), 105–154.
[5] R.B. Cundall and R.E. Dale, in: Nato ASI Series A, Time-resolved fluorescence spectroscopy in biochemistry and biology, (eds. R.B. Cundall and R.E. Dale) (Plenum Press, New York, 1983) pp. 115–296.
[6] J.-B. LePecq, in: Fluorescence techniques in cell biology (eds. A.A. Thaer and M. Sernetz) (Springer, Berlin, 1973) pp. 301–309.
[7] J.-B. LePecq, in: Biochemical fluorescence: concepts, Vol. 2, (eds. R. Chen and H. Edelhoch) (Dekker, New York, 1976) pp. 711–736.
[8] D.M. Crothers, Biopolymers 6 (1968) 575–584.
[9] S.N. Rao and P.A. Kollman, Proc. Natl. Acad. Sci. USA, 84 (1987) 5735–5739.

[10] J.-B. LePecq and C. Paoletti, J. Mol. Biol. 27 (1967) 87–106.
[11] L.M. Angerer and E.N. Moudrianakis, J. Mol. Biol. 63 (1972) 505–521.
[12] J. Olmsted(III) and D.R. Kearns, Biochemistry 16 (1977) 3647–3654.
[13] S.J. Atherton and P.C. Beaumont, Photobiochem. Photobiophys. 8 (1984) 103–113.
[14] M.J. Waring, J. Mol. Biol. 13 (1965) 269–282.
[15] D.P. Heller and C.L. Greenstock, AECL-10127, Atomic Energy of Canada Ltd. (1991).
[16] A. Grinvald and I.Z. Steinberg, Anal. Biochem. 59 (1974) 583.
[17] R.A. Lampert, L.A. Chewter D. Phillips, D.V. O'Connor, A.J. Roberts and S.R. Meech, Anal. Chem. 55 (1983) 68–73.
[18] J.-L. Mergny, D. Collier et al., Nucl. Acids Res. 19 (1991) 1521–1526.
[19] Ph. Wahl, J. Paoletti and J.-B. LePecq, Proc. Natl. Acad. Sci. USA 65 (1970) 417–421.
[20] V.W. Burns, Arch. Biochem. Biophys. 133 (1969) 420–424.
[21] A. Rich, A. Nordheim and A.H.-J. Wang, Ann. Rev. Biochem. 53 (1984) 791–846.
[22] D. Genest and Ph. Wahl, in: Nato ASI Series A, Time-resolved fluorescence spectroscopy in biochemistry and biology, (R.B. Cundall and R.E. Dale) (Plenum Press, New York, 1983) 523–539.
[23] P. Rauko, K. Povazanova and V. Reichelova, Neoplasma 35 (1988) 161–167.
[24] R. Sriram and F. Ali-Osman, Anal. Biochem. 187 (1990) 345–348.